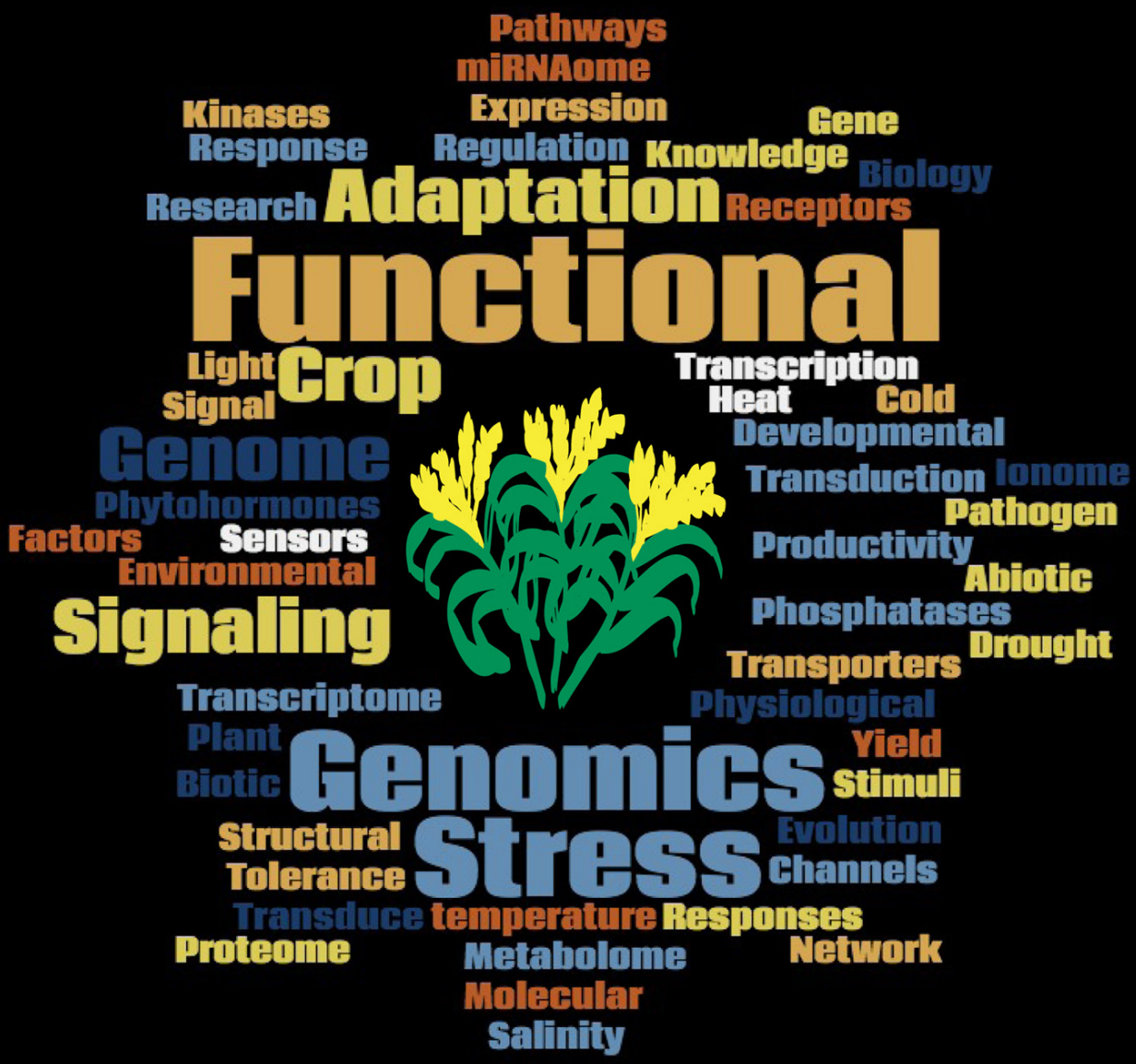
ABIOTIC STRESS SIGNALING IN PLANTS: FUNCTIONAL GENOMIC INTERVENTION

EDITED BY: Girdhar K. Pandey, Manoj Prasad, Amita Pandey and Maik Böhmer

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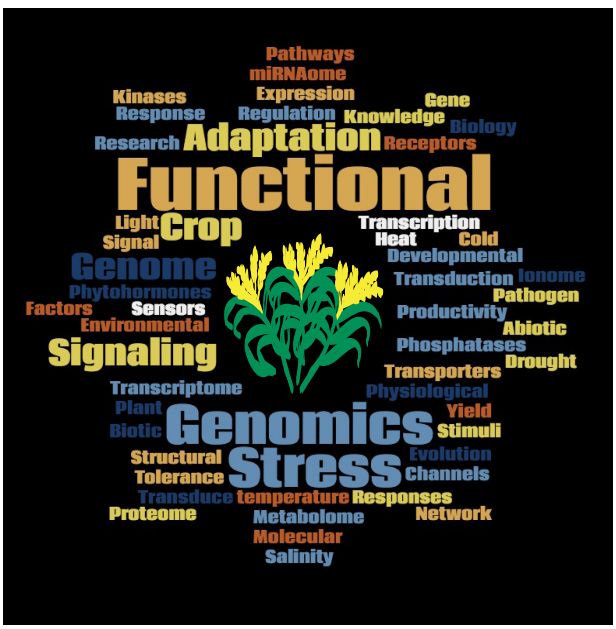
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ABIOTIC STRESS SIGNALING IN PLANTS: FUNCTIONAL GENOMIC INTERVENTION

Topic Editors:
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The cover image depicts the recent advent and use of functional genomics approaches to investigate the physiological, biochemical and molecular aspects of stress tolerance in plants. The strategies such as transcriptomics, metabolomics, ionomics and proteomics, have enabled the identification and characterization of genes associated with stress responses. Evidences originating from these studies will help to provide an insight into the stress responsive signaling network and may allow the alteration of this network to enhance crop productivity.

Abiotic stresses such as high temperature, low-temperature, drought and salinity limit crop productivity worldwide. Understanding plant responses to these stresses is essential for rational engineering of crop plants. In Arabidopsis, the signal transduction pathways for abiotic stresses, light, several phytohormones and pathogenesis have been elucidated. A significant portion of

plant genomes (Arabidopsis and rice were mostly studied) encodes for proteins involves in signaling such as receptors, sensors, kinases, phosphatases, transcription factors and transporters/channels. Despite decades of physiological and molecular effort, knowledge pertaining to how plants sense and transduce low and high temperature, low-water availability (drought), water-submergence, microgravity and salinity signals is still a major question for plant biologist. One major constraint hampering our understanding of these signal transduction processes in plants has been the lack or slow pace of application of molecular genomic and genetics knowledge in the form of gene function.

In the post-genomic era, one of the major challenges is investigation and understanding of multiple genes and gene families regulating a particular physiological and developmental aspect of plant life cycle. One of the important physiological processes is regulation of stress response, which leads to adaptation or adjustment in response to adverse stimuli. With the holistic understanding of the signaling pathways involving not only one gene family but multiple genes or gene families, plant biologist can lay a foundation for designing and generating future crops, which can withstand the higher degree of environmental stresses (especially abiotic stresses, which are the major cause of crop loss throughout the world) without losing crop yield and productivity.

Therefore, in this e-Book, we intend to incorporate the contribution from leading plant biologists to elucidate several aspects of stress signaling by functional genomics approaches.

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Editorial: Abiotic Stress Signaling in Plants: Functional Genomic Intervention

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Editorial on Research Topic

Abiotic Stress Signaling in Plants: Functional Genomic Intervention

The major challenge before the plant scientists is to develop strategies to increase the productivity of crop plants so as to feed the rapidly increasing global population. The increased anthropogenic activities have resulted in radical decrease of fertile land available for agricultural practices, and in addition, the impact of climate change, which poses environmental stress resulting in drastic decrease in crop productivity are major concerns for plant biologists.

The sessile nature of plants has naturally evolved sophisticated molecular mechanisms to sense and respond to stress conditions, which can result in stress tolerance or stress avoidance. Signal transduction networks form a larger proportion of this complex machinery, which enable the plants to sense the stress signals followed by transduction and finally generation of the response. A large number of signaling molecules have been identified and shown to be involved in perception and signal transduction pathways. Similar to animals, many of the signal transduction pathways are conserved in plants forming a plethora of proteins such as receptors, G-proteins, kinases, phosphatases, transcription factors, channels and transporters (Hirt and Shonozaki, 2003; Pareek et al., 2010; Pandey, 2012, 2013). Despite the high conservation in signaling pathways, plants have also evolved certain novel signaling mechanisms where several unique components have been identified that are not found in their animal counterpart.

The complex interplay of single and multiple signal transduction networks allows plants to respond to these stimuli. In addition, these networks are responsible for normal growth and development, and communication with the environment under particular growth condition. At the same time, these complex-signaling circuitries also enable plants to develop memory, "intelligence" and behavior despite the lack of a definite nervous system (Baluška et al., 2006; Pandey, 2013, 2015).

In the last decade, genetic and biochemical approaches have facilitated the identification of numerous signaling pathway components in Arabidopsis. In addition, generation of a linear as well as compound signaling response has been shown in response to a particular stress stimulus. The compound response leads to crosstalk within major plant signaling pathways and is often associated with the plant's unique ability to combat multiple environmental stress conditions (Yoshika and Shinozazaki, 2009; Pareek et al., 2010; Pandey, 2015).

Identification and characterization of functional units involved in signaling cascades has further enabled plant biologists to understand the flow of information in response to a particular stimulus. These efforts are further enabled with the availability of whole genome sequences of many model as well as crop plants. Besides, a dramatic progress and use of high-throughput approaches have instigated a deeper understanding of cellular response machinery.

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In the post-genomic era, a significant part of new experimental knowledge is provided by the progress in "Omics" based approaches such as transcriptomics, proteomics, metabolomics, interactomics and phenomics in several model organisms. These approaches have paved the foundation for "Functional Genomics", which aims at the identification of genes and delineation of their functions. For the first time, this has allowed the estimation and simulation of molecular network inside the cells. In addition, this affords a cell-wide view of the regulatory and metabolic processes involved in translation from the protein to metabolite level to mount a response or phenotype against specific stimulus (Pandey, 2015).

In an attempt to understand stress mediated signal transduction in plants, we have conceived this special issue on *"Abiotic stress signaling in plants: Functional Genomics intervention"* to collate and present the current state of knowledge in this field. With an overwhelming response from several leaders in the field of stress signaling, a total of 49 articles (opinion, hypothesis, mini review, review and original article) have been published in this special issue. All these articles are categorized in six different sections. The first section, "Genomics and functional genomics approaches" is comprised of five articles. This section mainly describes the application of different functional genomics approaches to understand the stress signaling pathways and components. The review article by Das et al. describes the implication of genomics and functional genomics methodologies in understanding the salt tolerance response in rice, with a greater emphasis on integration of "Omics" based approaches to generate future crops, which can tolerate high salt level in soil without compromising yield and productivity. Following this, Jain has elaborated the use of the genome-editing tool, CRISPR-Cas9, to generate abiotic stress tolerant crops as an opinion article. Budak et al. presents a review article on the role of genetic to functional genomics approaches in improving the drought tolerance trait in wheat. Usage of Omics-based approaches in understanding plant physiology under the purely anthropogenic stress conditions, space and microgravity, is elaborated in the review by Schüler et al. The improvement of crops by systematic data integration of whole life cycle of crop plants in the field condition is presented in the mini-review by Mochida et al. They discuss how the integration of different datasets generated for population genomics, chronological omic analyses, and computer-aided molecular network prediction, can result in a holistic understanding of crop phenology that can be studied in the field to prevent yield shortfall as a result of environmental fluctuations due to climate change.

The second section covers "Plant hormone and abiotic stress responses" and encompasses six articles that focus on the different aspects of regulation of stress-mediated responses by phytohormones. Phytohormones are crucial chemical messengers acting locally or systemically to regulate plant growth, development, and adaptation to environmental conditions. Three articles in this section elaborate the functional role of jasmonic acid (JA) and its signaling components in regulation of abiotic stress responses. The review article by Sharma and Laxmi describes the biosynthesis and signal transduction pathway and its role in regulation of temperature stress. Another review by Riemann et al. discusses the role of jasmonates hormonal network in salinity and drought stress responses. The research article by Singh et al. focuses on the elucidation of the role of JAZ repressor proteins under different mineral nutrient deficiencies in rice and chickpea using expression analysis. Simontacchi et al present a review article on the role of nitric oxide (NO) in regulating the abiotic stresses like low mineral nutrient supply, drought, salinity and high UV-B radiation responses. ABA is an extensively studied phytohormone, which regulate both abiotic as well as biotic stress responses in plants.

The research article by Kim et al. describes the development of transient gene expression for rice protoplasts (TGERP), which provides a powerful tool for functional genomic analysis of rice and other crop plants. In addition, the TGERP approach could unveil the complexities in different genes and gene networks involved in regulating stress signaling in plants. The genome-wide survey and expression analysis of AUX/IAA genes in chickpea and soybean by Singh and Jain accounts for the possible role of these genes in auxin signaling during development and abiotic stress conditions. The last article of this section by Singh et al. discusses the role of salicylic acid (SA) in reducing the toxicity caused by arsenic, mainly by inhibiting the translocation of arsenic from root to shoot in rice plants.

The third section, "Signal transduction components and abiotic stress responses," is comprised of 9 articles that emphasize different aspects of the signaling machinery and their components involved in abiotic stresses. Reactive oxygen species (ROS) act as crucial signaling molecules in programmed cell death and defense against pathogens in eukaryotic cells. Both biotic and abiotic stress signaling and responses are mediated by ROS; however, specificity is maintained in these two different major stresses. The review article by Jalmi and Sinha elaborates the differences and similarities of ROS mediated mitogen activated protein kinase (MAPK) signaling in response to abiotic and biotic stresses. The next three articles (two reviews and one research article) elaborate the role of calcium (Ca^{2+}) in abiotic stress signaling. Ca^{2+} is considered as the ubiquitous and magic bullet in cell signaling and regulating diverse biological responses in both plant and animal systems. The review articles by Zeng et al. and Virdi et al. comprehensively explain the role of the important Ca^{2+} sensors, calmodulin, and calmodulin-like proteins, in regulating diverse stress responses, especially abiotic factors in plants. Beside calmodulin, calcineurin B-like protein (CBL) is also one of the major Ca^{2+} sensors involve in regulating diverse stress physiology in plants. CBLs interact with a Ser/Thr protein kinase family called CBL-interacting protein kinases (CIPKs) and this CBL-CIPK module regulate diverse physiological processes, especially abiotic stress responses (Pandey et al., 2014; Sanyal et al., 2015).

The article by Meena et al. highlights the role of CIPK25 of chickpea in regulating the root growth and responses to salt and dehydration stress. The role of G-protein components also has been extensively studied in both animal and plant signaling processes including stress physiology. Despite high similarities in G-protein component mediated signaling processes in animal

and plants, they also show considerable variances in the signaling mechanism. Two articles by the same research group (Chakraborty et al. and Jangam et al.) emphasize the role of G-protein signaling components; GCR1 and GPA1 (Arabidopsis) and RGA1 (Rice D1) during multiple abiotic stresses by global transcriptomic approaches. Chandna et al. present the transcriptional differentiation of 14-3-3 proteins in *Brassica rapa* in response to stress and development. These 14-3-3 proteins are highly conserved protein present in all eukaryotes and involved in protein-protein interaction and hence regulate signal transduction processes. In an interesting article by Kaur et al. the role of methyl glyoxal (MG), a toxic compound produced as by-product of glycolysis is speculated to be a stress signal based on global gene expression profiling experiments in rice. The gene expression regulation of two-component system (TCS), which comprised of Histidine kinases (HKs), Histidine phosphotransfer protein (HPTs), and Response regulators (RRs) in abiotic stresses, tissue-specific manner and diurnal rhythm is presented by Singh et al. in both Arabidopsis and rice. The article by Jamsheer and Laxmi bring forward the expression analysis of FCS-like Zinc Finger (FLZ) genes in abiotic stress, sugars, and cellular energy level. FLZ proteins were found to interact with sucrose non-fermenting (SNF) related kinases (SnRK) and speculated to be involved in regulating the cellular energy and stress responses in plants.

In the fourth section, seven articles highlighting the role of transcription factors (TFs) in abiotic stress responses are compiled. The first article by Shao et al. addresses the role of NAC (NAC acronym is derived from three earliest characterized proteins with a particular NAC domain from petunia NAM, no apical meristem from *Arabidopsis,* ATAF1/2 and CUC2 cup-shaped cotyledon). These TFs are involved in regulating the plant development and stress responses including biotic and abiotic stresses.

Another review article by Singh and Laxmi describes the role of various TFs in regulating the drought responses in plants. Beside TFs, there are other factors, which either increase or decrease the rate of transcription, and the mediator complex is one such example. The review article by Samanta and Thakur describes in detail the function of the mediator complex in integration of diverse signaling pathway including stress responses in plants. Nguyen et al. has undertaken a differential gene expression analysis in drought sensitive and tolerant cultivars of chickpea and identified a subset of potential chickpea NAC transcription factors, which could be used for generation of drought tolerant chickpea with improved productivity under drought stress. The next two research articles by Yan et al. and Muthamilarasan et al. focus on the plant specific WRKY transcription factors from cotton, foxtail millet (*Setaria italica*) and green foxtail (*S. viridis*) in regulating stress responses in plants. Muthamilarasan et al. used global transcriptomic analysis to identify the potential WRKY transcription factors involved in abiotic stresses, and have pinpointed potential candidates for further functional characterization. Yan et al. identified the functional role of one of the WRKY members of cotton, which negatively regulates drought tolerance and positively regulates the resistance to *R. solani*.

The fifth section deals with "Gene expression analysis and response under stress conditions" and is comprised of 10 articles. After extensive research in the field of small non-coding RNA, it is well appreciated that these are key modulators of post-transcriptional gene silencing and are involved in regulating a diverse array of biological responses. The first four articles (Sablok et al.; Das et al.; Tripathi et al.; Ebrahimi et al.) present various aspects of small non-coding RNAs such as miRNA, siRNA and ta-siRNA in regulating biological processes ranging from germination and development to abiotic stress responses in plants. The detail transcriptomic profiling has been undertaken in Indian mustard (*Brassica juncea*), which is one of the major oil crops for the Indian population (Sinha et al.; Srivastava et al.).

The study by Sinha et al. present the *de novo* global gene expression analysis of cold-stressed Indian mustard siliques during pod filling to delineate the signaling network. The study by Srivastava et al. elaborates the identification of key candidate genes and regulatory network of arsenic-stressed rice seedlings by whole genome transcriptomic approach. A comparative root transcriptomic study in drought-response contrasting cultivars of soybean by Ha et al. identified differentially expressed genes encoding osmoprotectant biosynthesis-, detoxification-, or cell wall-related proteins, kinases, transcription factors and phosphatase 2C proteins. The genome-wide transcriptional and metabolic effects of low level xenobiotic stress on an important grass species, *Lolium perenne,* was studied by Serra et al. which revealed the involvement of multiple signaling cascades such as metabolic and phytohormone regulated pathways in xenobiotic stress. The comprehensive identification and expression analysis of membrane bound tetraspannin protein in rice was undertaken by Mani et al. Their detailed expression analysis hints to the possible involvement of tetraspanin proteins in cellular signaling and other biological processes in plant during different developmental stages and under different abiotic stresses. The last article of this section by Shukla et al. describes the role of natural variation in expression of regulatory and detoxification related genes under low phosphate and arsenic stress in Arabidopsis.

The sixth and last section entitled "Diverse aspect of abiotic stress responses" is comprised of 11 articles. These articles were organized with the aim of educating the readers on different proteins or processes related to diverse abiotic stresses. The first article by Khatri and Mudgil hypothesizes the functional role of N-MYC DOWN REGULATED-LIKE (NDL) proteins in abiotic stress responses involves regulation of microtubule organization. The review article by Jagadish et al. provides arguments for designing a target phenotype to mitigate abiotic stresses during pre- and post-anthesis in cereals with a focus on hormonal balances regulating stay-green phenotype versus remobilization. Another review article by Driedonks et al. elaborately describes the multi-level interaction of heat shock factors, heat shock proteins and redox system to impart acclimation or heat tolerance in crop plants.

In natural conditions, plants are constantly exposed to multiple abiotic stresses or a combination of biotic and abiotic stresses. This aspect is well elaborated by Pandey et al. where they

discuss the details of molecular and physiological mechanisms of plant's responses to multiple individual stresses and stress combinations that could lead to develop strategies to raise crop varieties with broad-spectrum stress tolerance. Cyclophillins are the proteins reported to bind to the immunosuppressant drug cyclosporin A in animal. Cyclophillin proteins are also known to exist in plants as multigene families and many of these also bind to the immunosupressant drug. Lee et al. have functionally characterized one of these family members, OsCYP21-4 from rice, which is localized exclusively in Golgi bodies. OsCYP21-4 overexpression confers salt and oxidative stress tolerance in rice. Small heat shock proteins (sHSP) are a diverse group of proteins that are mostly expressing in the seeds of plants. Kaur et al. identified one of the sHSP proteins from rice, OsHSP18.2, which is differentially expressed in seed and aging tissues. OsHSP18.2 is involved in seed vigor, longevity, and improve seed germination under abiotic stress conditions. Zhang et al. identified an aquaporin gene from cotton plant termed as tonoplast intrinsic protein (TIP), GmTIP2;3. Subcellular localization shows that GmTIP2;3 is plasma membrane localized protein and its heterologous expression improves osmotic stress tolerance in yeast. The expression of proline accumulation and metabolism-related genes under salt stress was analyzed by Wang et al. in *Kosteletzkya virginica*. In their expression analysis, the role of *KvP5CS1*, the key gene involved in proline accumulation, was found to be significant in leaves under salt stress. The study by Sinha et al. revealed the identification of proteins containing the universal stress protein (USP) domain in pigeonpea in drought stress. The evolutionary conservation of arginine-rich tandem zinc-finger protein (RR-TZF), AtTZF1, 2 and 3 homolog regulation under salt was found in distant species such as Arabidopsis, durum wheat and lower plants such as *Chlamydomonas* and moss by D'Orso et al. The last article of this section by Sinha et al. validated the selection of most suitable housekeeping reference genes as endogenous controls in gene expression studies in pigeonpea under salt and heat stress conditions.

Overall, most of the articles in this special issue emphasized the genomics and functional genomics aspects of various signal transduction components, pathways, and gene regulatory networks and their potential in developing climate resilient crop plants by multiple approaches to tame the major challenge of crop loss due to abiotic stresses in the field. Indeed, not all the aspects of signal transduction components and pathways regulating the abiotic stress signaling and responses could be covered but an in-depth insight can be extrapolated for many of these signaling pathways. A detailed discussion of several different aspects of abiotic stress signaling also put forward a large number of unanswered questions such as how plants respond to multiple stress factors in the field and how specificity and overlap is maintained in the response? One of the foremost challenges to plant biologists is how plants can be engineered to tackle the problem of crop loss due to both biotic and abiotic stresses in the field condition. Indeed, the advanced multidisciplinary approaches of genomics and functional genomics, biochemistry and physiology hold the key to address these important questions in the near future.

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Understanding salinity responses and adopting 'omics-based' approaches to generate salinity tolerant cultivars of rice

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Soil salinity is one of the main constraints affecting production of rice worldwide, by reducing growth, pollen viability as well as yield of the plant. Therefore, detailed understanding of the response of rice towards soil salinity at the physiological and molecular level is a prerequisite for its effective management. Various approaches have been adopted by molecular biologists or breeders to understand the mechanism for salinity tolerance in plants and to develop salt tolerant rice cultivars. Genome wide analysis using 'omics-based' tools followed by identification and functional validation of individual genes is becoming one of the popular approaches to tackle this task. On the other hand, mutation breeding and insertional mutagenesis has also been exploited to obtain salinity tolerant crop plants. This review looks into various responses at cellular and whole plant level generated in rice plants toward salinity stress thus, evaluating the suitability of intervention of functional genomics to raise stress tolerant plants. We have tried to highlight the usefulness of the contemporary 'omics-based' approaches such as genomics, proteomics, transcriptomics and phenomics towards dissecting out the salinity tolerance trait in rice. In addition, we have highlighted the importance of integration of various 'omics' approaches to develop an understanding of the machinery involved in salinity response in rice and to move forward to develop salt tolerant cultivars of rice.

Keywords: genomics, *Oryza sativa*, proteomics, salinity, transcriptomics, yield

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Introduction

Today's agriculture faces a daunting task of ensuring food security to the increasing human population on this planet (FAO, 2009). A great proportion (more than 60%) of this population depends on rice (*Oryza sativa* L.) as their staple food. Rice contributes up to 20% of the calories consumed by human nutrition worldwide. Therefore, rice production must increase during the coming time in order to keep pace with increasing world population. Asia is known as the main rice producer in the world by yielding more than 650 million tons (90% of total rice yield worldwide) grown in 145 million ha land.

Rice is grown in a diverse range of environments characterized by various climates and soil-water conditions. However, adverse environmental conditions critically threaten rice production and causes significant yield loss in large areas of main productive sectors. Both abiotic and biotic stresses frequently prevent the attainment of optimum growth and yield of rice. These stresses include high salinity, drought, heat, and cold which have negative effect on the yield and vegetative production of rice, and cause a key risk to worldwide food safety (Pareek et al., 2010; Mantri et al., 2012).

Amongst the various environmental stress factors, salinity is the main hazardous factor limiting crop productivity. Rice has been grouped as salinity susceptible cereal at its young stage (Lutts et al., 1995) and confines its efficiency of production at mature stage (Todaka et al., 2012). To increase the grain yield of rice under salinity, it is imperative to first understand the basic molecular machineries of salt tolerance in this plant. Tolerance toward salinity is a quantitative attribute in plants, regulated by a host of genes (Chinnusamy et al., 2005). Since the last decade, numerous genes imparting salinity tolerance in plants (including rice) have been identified and characterized such as those involved in transcription regulation, signal transduction, ion transportation and metabolic homeostasis (Verma et al., 2007; Singh et al., 2008; Singla-Pareek et al., 2008; Kumari et al., 2009). In the present text, we present our current understanding about effects of soil salinity on rice crop and the approaches used to increase the tolerance of this crop toward salinity. Further, critical evaluation of progress made toward raising salinity tolerant rice using functional genomics tools is also presented.

Soil Salinity as an Obstacle in Plant Growth, Photosynthesis, and Grain Yield

Salinization is one of the severe soil degradation factors. Approximately 6.5% of world's total area and about 20 percent of the cultivated area is already affected by soil salinity (Hakim et al., 2014). Saline area is increasing due to various factors including natural reasons as well as human activities. As per Reynolds et al. (2001), accretion of salts in the soil surface is caused by different factors in different geological and climatic regions. Salinity is frequently accompanied by water logging and alkalinity, which apply their individual specific effects on plant development (Yeo, 1999). Crop plants show a spectrum of reactions toward salinity including reduced growth and yield. Plants responses toward salinity is the collective outcome of the intricate communications among various processes linked to plant morphology, biochemistry, and physiology.

In most of the plants, obvious signs of damage by salinity are growth inhibition, senescence and death through long-standing exposure. Inhibition in seedling/plant growth is the initial step that leads to other indications, even though, programmed cell death may also take place under severe salinity. Salinity induces abscisic acid synthesis which leads to stomatal closure, reduced photosynthesis and photoinhibition. An instant outcome of salinity on plant development is inhibition of cell growth through abscisic acid synthesis.

Overloaded sodium ions around the root exterior disturb uptake of potassium. Because of the identical chemical properties of Na^+ and K^+, Na^+ has a negative effect on K^+ uptake. Under Na^+ stress, it is essential for plants to activate and maintain high-affinity K^+ uptake machinary rather than low affinity K^+ uptake one in order to uphold sufficient K^+ concentration in the cell. Shortage of potassium inside the cell unavoidably leads to decrease in plant growth, as K^+ is the most abundant cellular cation which plays an important role in preserving membrane potential, enzyme activities and cell turgor (Xiong and Zhu, 2002). After entering the cytosol, Na^+ inhibits the activity of an array of enzymes/proteins (Xiong and Zhu, 2002). This inhibition is K^+ dependent in the cell: a high Na^+/K^+ ratio cause damage to the cell. Accretion of Na^+ in the apoplast slowly increases the osmotic gradient connecting the out- and inside of the cell. To attain balance, water from inside of the cell moves outward into the intracellular spaces which cause cellular dehydration and ultimately, cell death. Constant contact of root with high salinity gradually decreases leaf size (Munns and Tester, 2008).

The effect of soil salinity upon photosynthesis process at its vegetative as well as reproductive stage has been studied by many researchers (Yeo et al., 1985; Dionisio-Sese and Tobita, 2000; Senguttuvel et al., 2014). It has been established that photosynthesis and chlorophyll concentration are inversely correlated with level of salt stress (Senguttuvel et al., 2014). Furthermore, it has also been reported that particular concentration of sodium chloride in the leaf causes reduction in photosynthesis to its half without affecting the concentration of chlorophyll (Dionisio-Sese and Tobita, 2000). Tolerance of crops to abiotic stresses depends upon their chlorophyll stability index. Salinity did not have much effect on the chlorophyll contents of the tolerant cultivars because they contain high chlorophyll stability index (Mohan et al., 2000; Sikuku et al., 2010). Similarly, ratio of chlorophyll-a and -b in plants also decrease due to salt stress (Senguttuvel et al., 2014). Unlike salinity susceptible varieties, tolerant varieties always maintain chlorophyll a/b ratio under salt stress conditions. Chlorophyll fluorescence parameters have also been found to disturbed due to salinity. It has been observed that the tolerant cultivars maintain a high Fv/Fm ratio than the susceptible one (Senguttuvel et al., 2014).

In plants, the harvest index (amount of shoot mass and yield) can fluctuate from 0.2 to 0.5, depending on the harshness of salinity (Husain et al., 2003). A small concentration of salt do not decrease plant's reproductive yield (although the plant's vegetative biomass is decreased) which is revealed in harvest index that goes up with salt stress. It has been established that grain yield in many crops do not reduce until a threshold salinity level is reached ('bent stick' relationship; USDA-ARS, 2005). A survey in USA (USDA-ARS, 2005) has shown that the yield of rice starts to decrease at 30 mM NaCl whereas in wheat, 60–80 mM NaCl could result in decline of the grain yield. This study shows the genetic difference among species. For instance, huge genetic dissimilarity has been found in durum wheat and barley, developed by irrigation with altered salt levels (up to 250 mM; Royo and Abio, 2003). These experiments show a sigmoidal curve instead of a 'bent stick' association between the level of salinity

and the crop yield. It has been reported that salinity reduces the efficiency of yield by reducing the formation of tillers (Maas and Hoffman, 1977). For various soils, salinity and waterlogging are interlinked. In Pakistan, use of high salt containing irrigation water causes poor soil texture and poor permeation of water (Qureshi and Barrett-Lennard, 1998). Secondary salinity occurs in Australia, where water-table increases to two meters of the soil layer which is near to the root sector. Moreover, as the porosity of soil is around 10 percent, it needs a little (∼100 mm) rainfall for water-table to increase up to the exterior surface to cause salinity and waterlogging stresses simultaneously (Barrett-Lennard, 2002).

Salinity Response is Highly Complex and Determined by Developmental Stages of the Rice Plant

Salinity is one of the key obstructions of rice production worldwide. Rice is especially grouped under salt-sensitive crop (Shannon et al., 1998). There are two important factors (threshold and slope) enough for determining salinity tolerance. Threshold indicates highest permissible salt without reduction in yield and slope indicates percent of reduction in yield per unit rise in salt level ahead of the threshold. The threshold of rice is 3.0 dsm^{-1} and slope is 12% per dsm^{-1} (Maas and Hoffman, 1977). In addition, rice is also differentially affected by salt stress at various growth stages. Moreover, the adverse effect of salinity on development of rice plant has been found to be related to different growth stages of the plant, type of salt, concentration of salt, exposure period of salt, water regime, pH of soil, humidity, solar radiation and temperature (Akbar, 1986). It has been established that rice plant is comparatively tolerant to salt stress during seedling stage, as at this stage, the injury can be considerably overcome in the later phases of development (Akbar and Yabuno, 1974). Hence, seedling stage is the ideal stage to categorize the rice genotypes into various groups based on their tolerance toward salinity. The rice genotypes have been categorized into different groups from extremely tolerant (score 1) to extremely sensitive (score 9) (**Table 1**). Janaguiraman et al. (2003) have shown that salinity tolerant rice varieties have higher rate of germination, shoot length, root length, and vigor index.

Besides the seedling stage, flowering stage is another highly sensitive growth stage in the life cycle of crop plants which is affected by salinity stress (Singh et al., 2004). This stage is vital as it determines grain yield. Salinity stress at booting stage affects the pollen viability which results in poor fertilization and consequent reduction in the percentage of filled grains and hence, the total plant yield. In a recent study targeting to access the effect of salinity on the pollen viability and grain yield in rice using various genotypes of rice (**Table 1**) (Mohammadi-Nejad et al., 2010). It has been found that most of the rice varieties show reduced pollen viability under salinity but those which have severe reduction in the pollen viability, along with severe decrease in plant yield, were classified as the salinity-susceptible genotypes for flowering stage (Khatun and Flowers, 1995). Some landraces such as Kalarata, Cheriviruppu, Pokkali, and Bhirpala have been found to be comparatively tolerant at flowering stage due to better viability of pollens and higher grain yield (upto 49%) under salt stress. Similarly, some of the other rice varieties i.e., IR72046-B-R-7-3-1-2, IR4630-22-2-5-1-3, and CN499-160-13-6 have also been categorized as salt tolerant at flowering stage based on their pollen viability and grain yield. Two rice genotypes (IR66946-3R-178-1-1 and IR65858-4B-11-1-2) which show high pollen viability and less grain yield have been categorized as sensitive for the flowering stage.

Seedling-stage salt tolerance is independent of flowering/reproductive stage tolerance (Singh et al., 2004), and has been established by the behavior of CN499-160-13-6 genotype which is a confirmed susceptible genotype at the juvenile stage but tolerant at the flowering stage. This analysis by Mohammadi-Nejad et al. (2010) indicate that seedling and flowering stage salt tolerance is determined by altogether different set of genes in rice. Recently, another group of rice researcher has analyzed the dry mass of rice shoot and root along with the grain yield under various levels of salinity (Hakim

TABLE 1 | Phenotypic trends of various rice genotypes under control and salinity treatment (Source; Mohammadi-Nejad et al., 2010).

Rice variety	Score	Pollen viability		Grain yield (g)	
		Control	Salinity	Control	Salinity
IR65209-3B-6-3-1	1	46.7	14.6	1.4	1.0
IR65858-4B-11-1-2	1	48.1	48.0	4.4	0.8
IR69588-4R-P-11-3	1	36.6	18.8	2.0	0.5
IR72046-B-R-7-3-1-2	1	49.4	24.7	1.3	0.1
IR71832-3R-2-2-1	1	19.5	12.3	1.7	0.4
IR71899-2-1-1	1	34	14.9	2.6	1.1
IR71991-3R-2-6-1	1	27.8	22.1	3.6	1.4
IR71995-3R-1-2-2	1	38.2	15.7	3.8	2.6
IR74099-3R-3-3	1	52.3	19.7	1.9	0.8
IR74105-3R-2-1	1	54.9	19.5	0.6	0.2
IR70023-4B-R-12-3-1	1	81.2	46.2	2.7	1.9
Cheriviruppu	3	59.4	20.8	6.8	4.2
Kala Rata 1-24	1	69	48.6	3.1	1.5
Bhirpala	5	45.1	26.2	2.1	1.4
IR4630-22-2-5-1-3	5	60.1	45.6	1	0.7
Pokkali (Ac.108921)	1	67.7	29.6	5.5	5.2
IR66946-3R-178-1-1	1	73	47.4	2.7	0.5
IR64	3	46.6	8.2	0.9	0.9
IR65185-3B-8-3-2	5	16	19.7	2.7	0.6
IR72046-B-R-4-3-2-1-2B-1	3	48.8	18.2	2.2	0.1
IR72043-B-R-6-3-3-3	5	39.3	15.4	1.5	0.3
IR72046-B-R-8-3-1-3	5	47.4	17.2	3.8	0.8
IR75000-69-2-1	5	50.6	26.4	1.8	1.6
IR29	9	24.7	18.3	0.2	0.2
Mojang Kor	7	57.9	27.6	1	0.5
Bao thai	9	24.9	13.8	0.3	0.3
CN499-160-13-6	9	42.2	26.9	3.9	2.0
Karuna	9	36	33.4	0.6	0.2
TCA4	7	29.8	16.5	2.1	0.6
Kinandang Patong	7	56.4	27.4	1.2	0.5

et al., 2014). In their report, it has been shown that the level of salinity is inversely proportional to the rice grain yield (**Figure 1**, **Table 2**). It has also been observed that the dry mass of shoot and root in rice decreases with the increase in the level of salinity. The grain yield reduction in rice by salinity stress might be due to the modification in flexibility of the cell wall, and subsequent reduction in the turgor pressure effectiveness in cell growth (Hakim et al., 2014). However, it is evident that increased salt level in soil disturbs the photosynthesis, causes shrinkage of cell contents, reduces growth and differentiation of tissues, cause imbalance in nutrition, injury of membranes, and ultimately, affects the yield contributing characters (Mahmod et al., 2009; Nejad et al., 2010; Hakim et al., 2014).

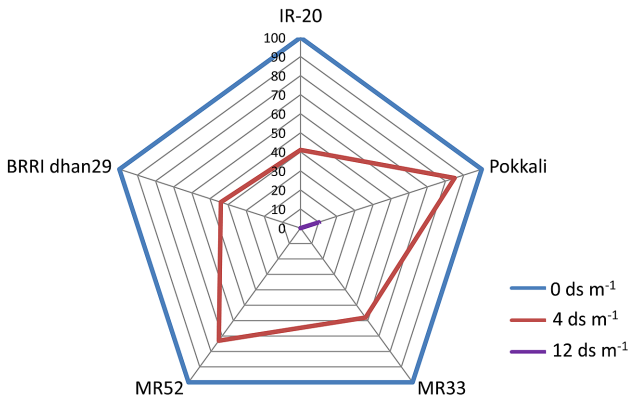


FIGURE 1 | Percentage decline in yield (g Hill^{-1}) of various germplasms of rice (IR-20, Pokkali, MR33, MR52, and BRRI dhan29) in response to salinity. Web digram was constructed taking yield of each genotype under non-stress condition (0 ds m^{-1}) as 100%. Note that Pokkali appears to be most tolerant genotype among the ones studied here, as it could give 10% yield even at 12 ds m^{-1}. (Source; Hakim et al., 2014).

TABLE 2 | Effect of salinity stress on plant growth as reported for different rice varieties (numbers in the bracket indicate the percentage relative to the control; Source; Hakim et al., 2014).

Rice variety	Salinity level (ds m^{-1})	Shoot Dry wt. (g)	Root Dry wt. (g)
IR-20	0	21.6	2.7
	4	16.4 (76)	1.56 (56)
	12	4.1 (19)	0.57 (20)
Pokkali	0	24.2	2.09
	4	21.2 (87)	1.60 (76)
	12	7.9 (32)	0.85 (41)
MR33	0	2.8	3.28
	4	15.9 (70)	2.28 (69)
	12	6.2 (27)	0.87 (26)
MR52	0	21.0	2.51
	4	16.9 (80)	1.90 (76)
	12	6.1 (29)	0.74 (29)
BRRI dhan29	0	2.9	2.87
	4	14.6 (64)	1.7 (62)
	12	3.9 (17)	0.52 (18)

Adaptive Mechanisms in Rice for Salinity Tolerance

Under salt stress conditions, rice plants exhibit various mechanisms to overcome the damage such as controlling the seedling vigor, reducing the intake of salt through roots, efficient intra cellular compartmentation and transport of salt.

Seedling Vigor

Salt stress leads to higher accumulation of Na^+ in shoots, mainly in mature leaves. Various reports have shown that limiting Na^+ accretion in shoot part under salt stress is linked to salinity tolerance of barley and wheat (Munns and James, 2003). In rice, it has also been verified that sodium ion accretion in shoot part is comparatively well linked with its growth under salt stress (Yeo et al., 1990). Rice varieties differ considerably in their rate of development with the most vigorous one being the conventional landraces and the shorter ones are the cultivated high yielding varieties. Naturally occurring salt tolerant varieties like Pokkali, Nona Bokora etc. belong to these conventional tall varieties. In spite of having comparable net transport of Na^+ ion through their roots as partially dwarf salt susceptible cultivars, the high vigor of land races permit them to tolerate growth decline by diluting the Na^+ content in rice cells.

Root Permeability and Selectivity

The lethal ions enter into the root along with water that travels from soil to the vascular part of the root by two routes, i.e., symplastic and apoplastic. In apoplastic pathway which is a non-energy driven pathway, water travels through intracellular regions to deliver the salt in xylem. In symplastic pathway, water enters in the roots through epidermal plasma membranes and then travels cell-to-cell through plasmodesmata until discharging to the xylem. Rice is a salinity susceptible crop and it has been revealed that a major quantity of sodium ion transported to the rice shoot parts at the time of salt stress is via apoplastic pathway (Krishnamurthy et al., 2009). Munns (1985) reported that, under 100 mM of sodium chloride stress, the transport rate of Na^+ ion toward shoots of salt tolerant barley is quite lower (only 20%) in relation to salt sensitive rice plants. This observation indicates that a major involvement of Na^+ bypass movement in salt stress-induced shoot causes sodium ion accretion in rice shoots.

Although water can passively move from roots through intercellular space, but there are morphological components called as suberin lamellae and Casparian band at the root endo- and exo-dermis, which restricts the apoplastic flow of ions and water to go inside the stele (Schreiber et al., 1999; Enstone et al., 2003). Casparian bands are formed by transverse and radial walls infusing the pores of primary cell wall with aromatic and lipophilic materials and suberin lamellae is deposited to the inside surface of cell walls (Ranathunge et al., 2004). Chemical nature of the root apoplastic barrier is crucial for their performance (Schreiber et al., 1999). It was observed that in roots, apoplastic barriers suberization was most common in salinity tolerant plants, which also has the least Na^+ accretion in the shoot parts (Krishnamurthy et al., 2009; Cai et al., 2011). Krishnamurthy et al.

(2009) have also revealed that in both susceptible and tolerant varieties, the expression of suberin biosynthetic genes was induced under salinity stress, which increased the reinforcement of these barriers in roots of rice. Though the mechanism of apoplastic movement of Na^+ has not been clear, Na^+ over-accretion through bypass movement in rice shoots is supposed to be the result of Na^+ reflexive flow into the xylem. Roots with weak barrier areas like lateral root originating sites and cell walls of root tip area were expected to be the possible entrance sites for Na^+ bypass movement (Yeo et al., 1987). Ranathunge et al. (2005) reported the disruption of the endodermal Casparian stripes, and ultimately crack through the fence in the exodermis at the time of lateral roots emergence at the pericycle region next to the phloem in the root of monocot. It was also shown that suberin lamella and casparian stripes in both endodermis and exodermis are not detectable at the apices of root (Ranathunge et al., 2003; Schreiber et al., 2005), signifying a weak fence at root apices of rice plants.

Symplastic movement of ions in root involves various ion selective channels/transporters present on the plasma membrane of the root cell which selectively allow the movement of ions inside the cell and maintain ionic balances under salinity. Plants have different defense machinery at the boundary of cell-xylem apoplast. A report has shown that Na^+ re-intake takes place from the xylem flow by adjacent tissues, and as a consequence, decreases flow of Na^+ into the shoot parts (Lacan and Durand, 1996). HKT is a Na^+/K^+ symporter found in the plant cell membrane which regulates transportation of Na^+ and K^+. Class 1 HKT transporter in rice removes excess Na^+ from xylem, thus protecting the photosynthetic leaf tissues from the toxic effect of Na^+ (Schroeder et al., 2013). This mechanism of salt tolerance has been depicted in **Figure 2A**.

Intracellular Compartmentation

Based on osmotic potential, plant can check Na^+ ion to go into the cell by energy driven process. K^+ and Na^+ are interceded by dissimilar transporters which have been verified by Garciadeblás et al. (2003). Cell ion homeostasis is maintained by the ion pumps like symporters, antiporters, and carrier proteins present on the membranes. In cereals, Na^+ exclusion systems were suggested to be composed of several transporters present on cell membrane like H^+-pump ATPases, Na^+/H^+ antiporter and the high-affinity uptake of K^+ ion (Jeschke, 1984). Salt Overly Sensitive or SOS pathway of homeostasis is an excellent example of ion management which is turned 'on' following the activation of the receptor in response to salinity and transcriptional induction of genes by signaling intermediate compounds (Sanders, 2000). Zhu et al. (1998) first reported three *sos* mutants of *Arabidopsis* which were hypersensitive to specific salt-NaCl. These three *sos1*, *sos2,* and *sos3* mutants exhibit altered phenotype with reference to Na^+ accretion. In SOS pathway, calcium binding protein SOS3 directly interacts and activates SOS2, a serine/threonine protein kinase (Liu and Zhu, 1998; Halfter et al., 2000). SOS3

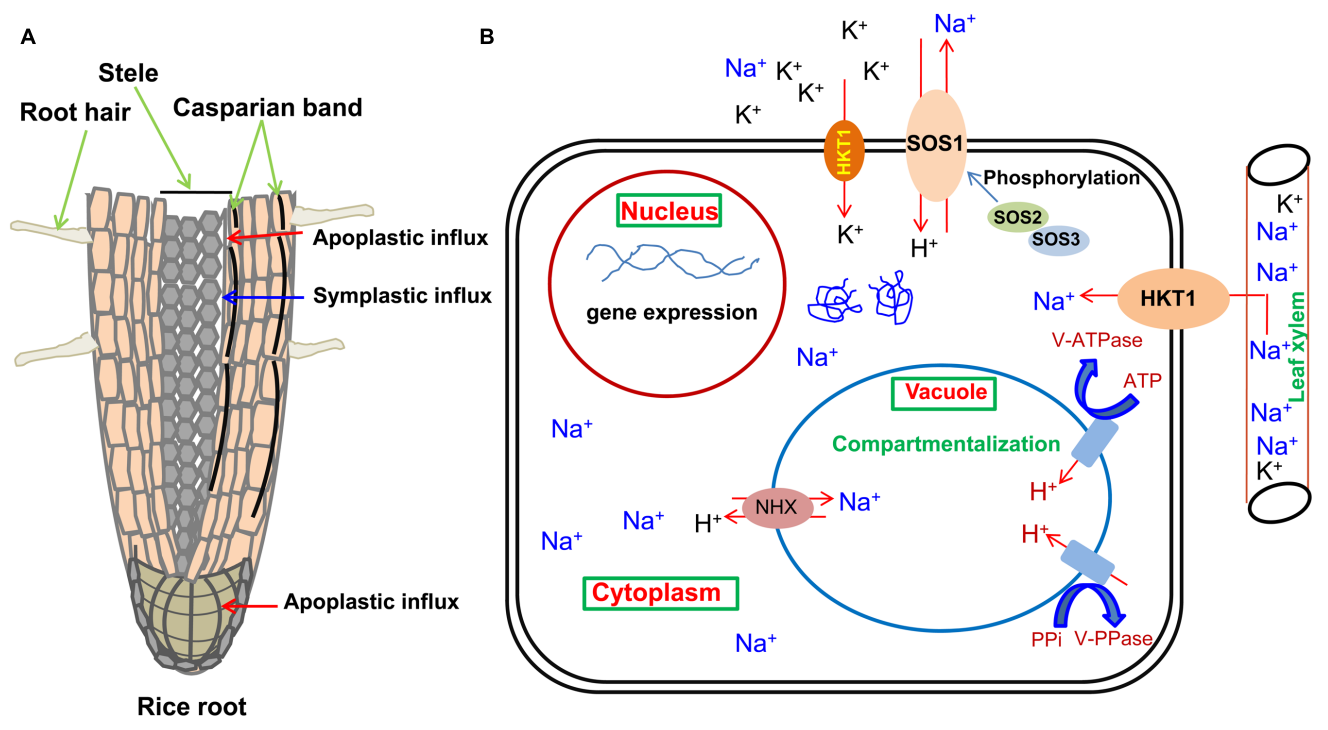


FIGURE 2 | Schematic representation of Na^+ influx in roots, its sequestration pathways and primary protective mechanisms as mediated by the transporters present on plasma membrane and tonoplast of the cell. (A) Influx of Na^+ through plant root. Red arrows represent probable Na^+ entry sites for the apoplastic bypass flow and blue arrow represents the path for symplastic movement. **(B)** Various transporters (NHX, HKT, SOS1) responsible for ion movement localized on the biological membranes have been shown for an individual cell of the plant. The energy providing (vacuolar H^+-ATPase or V-ATPase, vacuolar H^+-translocating pyrophosphatase or V-PPase) and activating molecules (SOS3, SOS2) are also shown.

recruits SOS2 on the cell membrane, where SOS2–SOS3 complex phosphorylates SOS1, a Na^+/H^+ antiporter on cell membrane, which extrudes Na^+ out of the cell (Quintero et al., 2002; Guo et al., 2004). Ma et al. (2012) have shown that under salinity stress in *Arabidopsis*, NADPH oxidases also work in ROS-mediated regulation of Na^+/K^+ balance.

When higher accumulation of Na^+ in cytosol occurs, Na^+ get sequested into the vacuole before it arrives to a toxic point for enzymatic reactions. This pumping action is regulated by vacuolar Na^+/H^+ antiporters (Blumwald, 2000). Increase in level of salt induces the Na^+/H^+ antiporter action but it amplifies more in salinity tolerant varieties than salinity susceptible ones (Staal et al., 1991). The Na^+/H^+ exchange in vacuole is determined through two separate proton pumps, i.e., vacuolar H^+-ATPase and vacuolar H^+-translocating pyrophosphatase (Blumwald, 1987). Manipulation in the levels of vacuolar transporter (NHX1) leads to improve salinity tolerance in rice, *Arabidopsis*, *Brassica* and Tomato (Apse et al., 1999; Zhang et al., 2001; Fukuda et al., 2004). Bassil et al. (2012) have reported one endosomal Na^+/H^+ antiporter (OsNHX5) and four vacuolar Na^+/H^+ antiporters (OsNHX1-4) in rice (**Figure 2B**).

Osmoprotectants

Most of the organisms including plants and bacteria accumulate certain organic solutes (such as sugars, proline etc.) due to osmotic stress. These compounds are called osmoprotectants because even when present in high concentrations they do not hinder with cellular enzymatic reactions (Johnson et al., 1968). These are found in cell cytoplasm and the inorganic ions like Cl^- and Na^+ are preferentially seized into the vacuole, consequently leading to the turgor preservation for the cell under osmotic pressure (Bohnert et al., 1995). The non-reducing sugar trehalose possesses a distinctive feature of reversible water storage ability to guard cellular molecules from dehydration stress. Garg et al. (2002) have reported that the trehalose biosynthesis and accumulation in transgenic rice can provide tolerance to salinity and drought stresses. Role of other osmoprotectants such as proline (Ahmed et al., 2010; Deivanai et al., 2011), glycine betaine (Makela et al., 2000; Ahmad et al., 2013), mannitol (Thomas et al., 1995) etc. in salt stress tolerance in plants has also been well documented.

Approaches for Improving Salinity Tolerance in Rice

Conventional Methods

Plant breeding methods have been adopted since long time to generate stress tolerant and high yielding rice varieties. Breeders have made genetic alterations in rice crops, at intergeneric, intraspecific and site-specific levels to generate salinity tolerant cultivars. It has been established that source(s) of salt tolerance are still to be explored within the cultivated germplasm of rice (Flowers et al., 1990). Nevertheless, there are evident signals that some conventional rice landraces and varieties (e.g., Pokkali, Bura Rata, and Nona Bokra) are better salinity tolerant than many prominent varieties. Pokkali has been popular as a gene donor in plant breeding programs to develop salinity tolerant cultivars. The better tolerance to salinity in Pokkali is generally credited to both its capacity to preserve low ratio of Na^+/K^+ in plants and its quicker expansion rate under salinity. Using IR29 and Pokkali a recombinant inbred population has been produced at the International Rice Research Institute, Philippines (Bonilla et al., 2002). A number of other salt-sensitive and salt-tolerant inbred lines have also been documented during screening for salt tolerance (Gregorio et al., 2002). Many salt tolerant cultivars of rice have been generated in various countries by breeding which includes CSR13, CSR10 and CSR27, IR2151, Pobbeli, PSBRc 84, PSBRc 48, PSBRc 50, PSBRc 86, PSBRc 88, and NSIC 106.

However, the fact is that the wild types or the landraces discussed here are connected with a host of innate difficulties of reduced agronomic characters like photo-sensitivity, tallness, low yield and poor grain quality. Hence, breeding for enhanced salt tolerance using these wild germplasm is a real challenge. Other problem with traditional plant breeding is reproductive difficulty where it is really problematic that if the gene is present in a wild counterpart of the crop, breeder faces trouble in introducing it to the domesticated variety. Therefore, keeping these in mind, several modern approaches have been adopted for production of salinity stress tolerant rice.

Omics-Based Approaches in the Modern Era

Plant molecular biology seeks to study biological and cellular processes like plant development, its genome organization, and communications with its surroundings. These multi-dimensional detailed studies require large-scale experimentation linking the whole genetic, functional and structural components. These large scale experimentations are known as 'omics.' Chief contributors of 'omics' include genomics, transcriptomics, proteomics, metabolomics, and phenomics. 'Omics' approaches are regularly used in various research disciplines of crop plants, including rice. These approaches have enhanced very fast during the last decade as the technologies advance. Following section describes how 'omics-based' approaches have helped in understanding and dissecting out the mechanism of salinity tolerance in rice and helped in generating several salt tolerant germplasms.

Genomics-Based Approach

Molecular marker resources and quantitative trait loci (QTL) mapping for rice salinity tolerance

Accessibility of the whole genome sequence of rice (Matsumoto et al., 2005) has contributed to the rapid development in the area of functional genomics of salinity tolerance in rice. This information further supported by development of a number of single nucleotide polymorphism (SNP) markers and simple sequence repeat (SSR) markers. Both SSR and SNP marker analysis have been successfully used to discover salt tolerant cultivars of rice (Dhar et al., 2011). In the recent past, development of next generation sequencing (NGS) has enabled the sequencing based genotyping way more efficient

(Ray and Satya, 2014). QTL studies for salt stress tolerance have been investigated by several researchers (Bonilla et al., 2002; Gregorio et al., 2002; Lin et al., 2004). Genetic maps of rice have been generated using recombinant inbreed lines developed from genetically distant varieties, such as indica and japonica rice as parents. Such combinations generate appreciably more polymorphism than that between the same subspecies. McCouch et al. (1988) published the first rice genetic map by restriction fragment length polymorphism technique; different fine maps have since been generated using various markers such as amplified SSR, random amplified polymorphic DNA and fragment length polymorphism (Kurata et al., 1994; Harushima et al., 1998). Moreover, the genomic tools [expressed sequence tags (ESTs) from salinity-stressed libraries, expression profiling by microarrays, whole genome sequence information, targeted or random mutation breeding, and complementation and promoter trapping approach] and methods that have become available offer chances to differentiate the salinity-tolerance-likned gene networks in more depth (Bohnert et al., 2001; Kumari et al., 2009; Soda et al., 2013). Seven QTL linked with salinity have been recognized for rice seedlings and mapped to different chromosomes (Prasad et al., 2000). Using F2 population obtained from a salinity tolerant mutant of rice (M-20) and the salinity susceptible wild variety (77–170A), a key gene for salinity tolerance has been mapped on chromosome 7 (Zhang et al., 1995). Koyama et al. (2001) showed the chromosomal location selectivity traits of an ion transport which are companionable with agronomic demands. Gregorio et al. (2002) have mapped a major *Saltol* QTL which is flanked by markers RM23 and RM140 on chromosome 1, using a population raised from a cross among Pokkali and IR29. More than 70% of the difference in salt uptake has been accounted by this QTL (Bonilla et al., 2002). Pokkali was the basis of positive alleles for this QTL, which accounted for decreased sodium and potassium ratio under salinity (Bonilla et al., 2002; Gregorio et al., 2002). Lin et al. (2004) have shown a QTL for increased shoot K^+ under salt stress in the similar position of chromosome 1. Mapping SKC1 on chromosome 1 was a breakthrough which preserves K^+ ion homeostasis in the salinity-tolerant cultivar (Nona Bokra) under salinity conditions (Ren et al., 2005).

Introduction of desired gene/genes into the rice genome for salinity tolerance – the 'reverse-genetic' approach

Plants react to salinity by limiting the intake of toxic ions like Na^+ and regulate their osmotic potential by producing compatible solutes (sugars, glycinebetaine, proline etc.) and partitioning toxic ions into the tonoplasts to maintain low Na^+ levels in the cytoplasm (Blumwald and Grover, 2006). Salinity tolerant transgenic rice plants were generated by getting ideas from the above observation (Kumar et al., 2013). Xu et al. (1996) produced transgenic rice by introduction and over-expression of late embryogenesis abundant (LEA) protein from barley. Their study demonstrated that the transgenic rice possessed a better growth rate under 200 mM of salinity and better recovery upon removal of stress. Similarly, genetically engineered rice has also been developed with the capacity to produce glycinebetaine by a gene (*codA*) encodes choline oxidase and it has been found to have better salt (150 mM NaCl) tolerance than the WT (Mohanty et al., 2002). Transgenic rice plants developed by over-expressing OsCDPK7 (a calcium-dependent protein kinase) gene were found to have the youngest leaf drooped 3 days after treatment with 200 mM sodium chloride in wild type plants, whereas transgenic plants showed better tolerance (Saijo et al., 2000). Several latest reports have shown a host of other genes related to antioxidants, transcription factors, signaling, ion homeotasis and transporters found to have key role in salinity tolerance (Garg et al., 2002; Blumwald and Grover, 2006; Zhao et al., 2006a; Jiang et al., 2012; Kumar et al., 2012; Kumari et al., 2013; Liu et al., 2014a; Rachmat et al., 2014).

Genome modification through mutation breeding for salinity tolerance in rice – the 'forward-genetics' approach

Although efforts to advance stress tolerance in plant by genetic manipulation have resulted in some significant achievements, mutation breeding technique has been accepted as a foremost strategy to obtain stress tolerant varieties as well as varieties with other desired traits (Wang et al., 2003; Flowers, 2004). Mutation breeding has a significant contribution toward production of high yielding and salt stress tolerant rice varieties (Cassells and Doyle, 2003; Parry et al., 2009; Das et al., 2014). There are many reports where mutation breeding has resulted in enhanced salinity tolerance in various rice cultivars. For example, rice seeds irradiated with carbon (C) or neon (Ne) ions have generated mutant variety with high salt tolerance (Hayashi et al., 2007). The Azolla-Anabaena symbiotic system provides green manure for flooded crops, mainly rice. Mutation breeding has produced Azolla variants tolerant to high salinity, toxic aluminum levels, and to herbicides (Novak and Brunner, 1992). Many such varieties of salinity tolerant mutant rice have been released in many countries all over the world so far and some of them have been listed in **Table 3**.

TABLE 3 | Salinity tolerant rice varieties produced through mutation breeding.

Crop variety	Mutation technique	References
Rice (6 B)	γ irradiation	Singh (2000)
Rice (A- 20)	γ irradiation	Singh (2000)
Rice (Atomita 2)	γ irradiation	Singh (2000)
Rice (Changwei 19)	γ irradiation	Singh (2000)
Rice (Emai No. 9)	γ irradiation	Singh (2000)
Rice (Fuxuan No. 1)	γ irradiation	Singh (2000)
Rice (Liaoyan 2)	γ irradiation	Singh (2000)
Rice (Mohan = CSR 4)	γ irradiation	Singh (2000)
Rice (Jiaxuan No. 1)	γ irradiation	Singh (2000)
Rice (Nipponbare)	γ irradiation	Hayashi et al. (2008)
Rice	γ irradiation	Jain et al. (1998), Jain and Suprasanna (2011)
Rice (Niab-irri-9)	γ irradiation	www.niab.org.pk/
Rice (Shua 92)	γ irradiation	Baloch et al. (2003)
Rice (Basmati 370)	γ irradiation	Saleem et al. (2005)

Transcriptomics Approach

Transcriptomics, also called as expression profiling, generally require a systematic and entire study of all the RNA transcripts that signifies the spatial and temporal gene expression of a cell, tissue of an organism under a certain biological circumstance (Thompson and Goggin, 2006). This technique leads to identification of a large number of differentially regulated transcripts due to cross talks and overlapping pathways under particular stress/environmental situations (Sahi et al., 2006; Walia et al., 2007). Microarrays have become one of the standard tools in molecular biology and have taken as commanding approach for the analysis of genome wide transcriptional response by studying the expression of all the expressed genes in a single experiment. The complete transcriptome at a given time point allow us to detect any stress-inducible genes which can suggest the specific biological processes and/or the regulation of transcriptional and translational machineries that are induced (Gracey and Cossins, 2003). In rice, EST based cDNA arrays and oligonucleotide microarrays have been used to understand the underlying biological meaning by studying and comparing the global gene expression patterns (Eyidogan and Öz, 2007). In the recent past, stress (including salinity)-inducible transcripts in rice were identified by using microarray technology (Wilson et al., 2007; Kumari et al., 2009; Plett et al., 2011; Garg et al., 2013). It is well documented that the mechanisms involved in salinity tolerance is complex and polygenic trait (Munns and Tester, 2008). Introduction of a single gene is least likely to improve the salt tolerance considerably. As an alternative, multiple genes involved in the key mechanism of the processes such as signaling, osmotic adjustment, ion homeostasis, vacuolar compartmentalisation of ions, restoration of enzymatic activity and oxygen free radical scavenging should be used (Bohnert et al., 2001). The transcription factors having a cascade effect that can regulate many other downstream genes may also prove vital in this regard. The main challenge is that it is not yet clear what are the genes that are needed to be studied and manipulated (Cuartero and Bolarin, 2010). Salt tolerance in rice is advantageous in this regard as rice is particularly salt sensitive at seedling and reproductive phase and a few QTLs having large effects are known to control the trait (Leung, 2008). The traits, though, have low heritability and are usually inherited quantitatively (Cuartero et al., 2006). The measurements of these traits in segregating populations are not always easy which demands careful coordination of environmental conditions over seasons and locations. The capability to evaluate the expression levels of whole genome in a single experiment by microarray technique allows biologists to see what are the genes induced or repressed under specific environmental extremes. The constraint is that in addition to the actual genes that control the stress response, it detects enormous number of related genes which might be involved in secondary or irrelevant downstream functions (Cuartero et al., 2006). Beside the challenge of recognizing the relevant target genes, the transcriptomic approach offers an efficient tool of identifying the gene(s) involved in specific stress tolerance mechanism.

Proteomics Approach

The study of a protein is the shortest and direct way to describe the role of the gene linked with the particular protein. Nevertheless, it must be noted that the proteome and genome of an organism do not always communicate to each other directly (Komatsu et al., 2009). Thus, the investigation done at the metabolome and proteome levels are evenly significant as the study of genomics. Proteomics study gives a platform to analyze complex biological functions which includes huge numbers of proteins as well as the interacting network of various proteins. Proteomics can serve as a main technique for exposing the molecular machineries that are concerned in interactions among the plant and diverse stresses including salt stress. Salinity stress induces the expression of various genes which are eventually reflected in the profile of the proteins. The function of salinity- and other stress-induced proteins has been extended by proteomic study in different tissue parts of rice (Fukuda et al., 2003; Chen et al., 2009; Lee et al., 2009; Liu and Bennett, 2011).

A scheme for direct recognition of proteins by differential display approach has been established and the proteins structure can be recognized by evaluation with the proteome database of rice or by Edman sequencing and mass spectrometry (MS). It has been shown that the present rice proteomic studies have so far concentrated on the recognition of polypeptides based on their available quantity upon subjecting to different stresses (Ma et al., 2013). The complex physiological response data of proteomics study were found to change over the severity of stress, therefore, making the data evaluation and integration analyses complicated. Henceforth, post-translational modification (PTM) could be a substitute to examine stress signaling functions. Many strategies have been created to distinguish PTM in plants. Particularly, the use of two dimensional PAGE method joined with the application of 5′-iodoacetamidofluorescein (5′IAF) or 2D-fluorescence difference gel electrophoresis (DIGE) allows the recognition of oxidized and reduced stress-linked proteins (Cuddihy et al., 2008; Fu et al., 2008). Several gel-free methods have also been identified for differential analysis of proteome. Instances are: multidimensional protein identification method that successfully recognizes individual protein components by eliminating band broadening for chromatographic recognition (Koller et al., 2002), isobaric tags for relative and absolute quantification and isotope-coded affinity tags (Griffin et al., 2001). These methods are considered as targeted techniques to recognize alteration in proteins by mass difference mean, among different proteomes.

Metabolomics Approach

Metabolites are the final product of cellular reactions which reflect the reaction of biological systems to environmental fluctuations (Royuela et al., 2000). The present movement in metabolomic analysis is to describe the cellular position at a specific stage by assessment of the whole metabolites in the cell (Hollywood et al., 2006). Metabolomics techniques complement proteomics and transcriptomics technique and show exact figures of the whole cellular course. A sequence of investigative method is accessible for the study of plant metabolome (Okazaki and

Saito, 2012), along with the application of modern and high throughput methods such as Fourier transform infrared (FT-IR; Johnson et al., 2003), ultra high-resolution fourier transform-ion cyclotron MS (Hirai et al., 2004), gas chromatography-MS (GC-MS; Kaplan et al., 2004), and nuclear magnetic resonance (NMR; Kim et al., 2010). Metabolomics came into view as an important tool for the study of environmental responses in plants (Bundy et al., 2009). Rice metabolomics studies have so far determined the types and the quality of metabolites which can endorse the germination of seed (Shu et al., 2008), the variation of metabolites among wild type and mutant plants (Wakasa et al., 2006), the metabolome profiling at various stages of development (Tarpley et al., 2005), and the examination of natural metabolite dissimilarity among different rice varieties (Kusano et al., 2007). Few studies have shown the metabolic impact of salinity on crop plants such as rice, tomato, grape vine, *Solanum lycopersicon* and *Arabidopsis* (Johnson et al., 2003; Gong et al., 2005; Zuther et al., 2007). Plants react to adverse situations by a sequential modification of their metabolism with transient, sustained, early reactive and late reactive metabolic changes. For instance, proline and raffinose gather to increased levels upon several days of exposure to salinity, cold, or drought, while central carbohydrate metabolism changes quickly in a time-dependent and complex way (Kaplan et al., 2004; Urano et al., 2009; Lugan et al., 2010). Some metabolic alterations are common to all the abiotic stress types, but others are particular. For instance, levels of sugars, sugar alcohols and amino acids usually amplify with response to different stresses. Remarkably, proline accumulates upon salinity, drought and cold stress but not upon heat stress (Kaplan et al., 2004; Gagneul et al., 2007; Kempa et al., 2008; Usadel et al., 2008; Urano et al., 2009; Lugan et al., 2010). In most of the studies, amount of TCA-cycle intermediates and organic acids got declined in glycophytes after salinity stress (Gong et al., 2005; Zuther et al., 2007; Gagneul et al., 2007), but got enhanced upon drought or temperature stress (Kaplan et al., 2004; Usadel et al., 2008; Urano et al., 2009). Usually, sugars are essential compatible solutes gathered in cells during stress response. Fumagalli et al. (2009) studied the metabolite profile of two different cultivars (Nipponbare and Arborio) of rice under salinity (150 mM) and showed enhanced sugar contents during salinity stress in both the cultivars. Their results also showed that salt stress altered the accumulation of various metabolites (glutamate, aspartate, proline, valine, lactate, alanine, malate etc.) in rice which have vital role in salt tolerance. They also suggested that NMR coupled with principal component analysis (PCA) is a commanding tool to characterize rice varieties under salinity or any other stress.

Phenomics Approach

Plant phenomics is advanced screening method which includes the study of plant phenotype, growth, and performance and eventually, identification of the required trait. Couple of screening methods for various morpho-physiological traits have been used to measure the tolerance to salinity in rice, including plant weight, Na^+ concentration, the ratio of Na^+/K^+ in shoot, leaf injury, survival rate of leaf following injury, leaf area and bypass movement in the root (Yeo et al., 1990; Asch et al., 2000; Zeng et al., 2003; Faiyue et al., 2012). However, most protocols that measure plant biomass are destructive, therefore making it difficult to measure active responses in plant growth in response to salt application and to collect seed from the individuals being measured. Current progress in image-based phenotyping have enabled the non-destructive assessment of plant responses to salinity over time and allow determination of shoot biomass measurements without having to harvest the whole plant (Rajendran et al., 2009; Berger et al., 2012; Hairmansis et al., 2014; Jansen et al., 2014). Upon salinity stress, growth of rice plants immediately slows due to stress, and plants produce fewer tillers (Munns and Tester, 2008; Rajendran et al., 2009; Horie et al., 2012). Over time, Cl^- and Na^+ accumulate to lethal concentrations in the plant, resulting in premature senescence of leaf and subsequent death (Munns and Tester, 2008; Munns et al., 2010; Horie et al., 2012). Notably, image-based phenotyping can differentiate among the effects of the osmotic and ionic components of salt stress in growing plants. It can be done by growth response measurement immediately after application of salt, before the increase in accumulation of toxic ions in the shoot. This permits for at least some analysis of salinity tolerance mechanisms (Rajendran et al., 2009; Sirault et al., 2009). A non-destructive image-based phenotyping method to analyze the responses of rice to different levels of salinity stress has been developed and revealed differences in the effects of salinity in two cultivars of rice, IR64 and Fatmawati (Hairmansis et al., 2014).

Automation of the phenotyping process in combination with automated plant handling and watering allows large numbers of plants to be screened efficiently with short handling. Entire populations of plant can be grown in soil media, emulating field conditions (at least for the earlier growth stages), hence permitting the transfer of knowledge from controlled environment to field growth conditions. Screening of 100s of mapping lines and/or rice accessions for bi-parental or association mapping studies can now be done relatively quickly for traits that require time course growth assessments. The use of these populations has the prospective to reveal the underlying genetic mechanisms of salinity tolerance in a forward genetics screen. As costs decrease, so the power of this approach will also increase, to allow more detailed characterization of rice genotypes (e.g., stomatal behavior) in response to salinity [e.g., by combination of infrared (IR), Red–Green–Blue (RGB) imaging and fluorescence techniques]. Use of non-destructive imaging technologies, in combination with measurements of tissue ion concentration, allows the differentiation between the osmotic and ionic components of salt stress in rice. This will allow the detection of new traits and sources of salinity tolerance genes that can be used to pyramid different salinity tolerance mechanisms into elite rice breeding lines.

Integration of 'Omics' Approach

'Omics' approaches seems to be overlapping and dependent on each other, and integration of all the 'omics' approaches is necessary to reach at an ultimate step i.e., raising of stress tolerant cultivars (**Figure 3**). Proteomic studies show vast overlapping in vital metabolism (e.g., Calvin cycle and carbohydrate metabolism) under salt stress. However, different metabolic pathways have been found to control and regulate

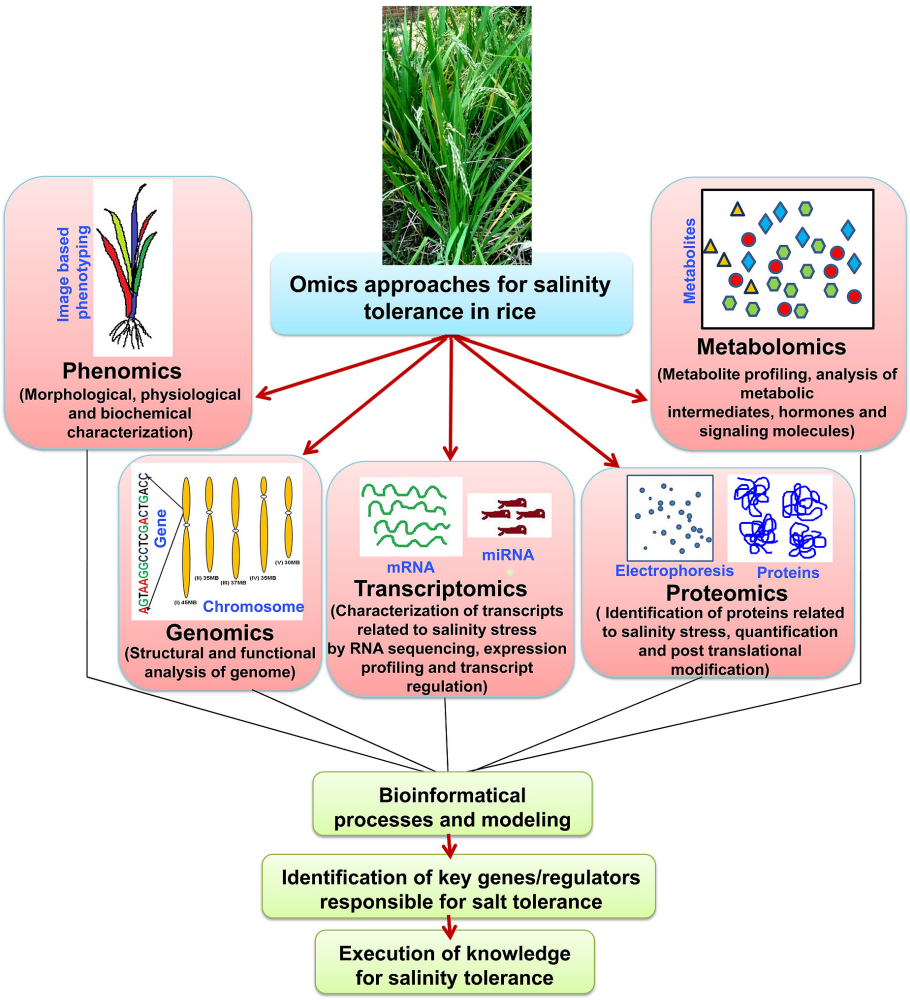


FIGURE 3 | 'Omics-based' approaches and their integration to develop salinity tolerant rice cultivars. Flow of information from various 'omics-based' platforms such as phenomics, genomics, transcriptomics, proteomics, and metabolomics needs to be integrated to understand complex traits such as salinity tolerance. Ultimately, the key genes/regulators responsible for salt tolerance need identification and validation using the tools of functional genomics.

under metabolomic level, mostly the biosynthesis of amino acid, photorespiration and citric acid pathway (Ma et al., 2013). This is probably due to the participation of downstream enzymatic reaction instead of cellular injury reactions, which encourage the pathways of complex metabolites. The proteomic approach presumes that the raise in quantity of protein amount is always escorted by biologically active compound, but in fact it may include the factors by posttranslational alterations, that may alter characteristics of the proteins. So, the function of the metabolite changing occured at the metabolomic point is not very much clear. Hence, it can be said that the growth of bioinformatics, in linking to the response at transcription level to either metabolomic or proteomic alterations, is yet to be done. The quick evolution in 'omics' research has led to more and more generation of data sets throughout all branches in life science studies. Different investigative applications, that are vital for the efficient incorporation of data resources, have been published in various databases. These huge datasets are found via four main stages: (a) data generation (b) data processing (c) data integration and the final step is (d) data analysis (Mochida and Shinozaki, 2011). The dispute in the incorporation of omics data investigation has been argued (Edwards and Batley, 2004). It was shown that the main trouble occurred from the partial and dissimilar form of information accessible on bioinformatics data sources. Hence, algorithmic methods have been planned as the solution for this kind of trouble (Ge et al., 2003). Currently, many servers have been established which allows the integration of high-throughput data and these servers also able to display the outcomes in a meaningful pathway of biology (Tuncbag et al., 2012).

The ultimate aim of integration of 'omics-based' approaches is to find out key stress responsive genes/proteins and introduction of those genes/proteins for generation of improved stress tolerant crop varieties. A list of transgenic salt tolerant rice cultivars generated by the introduction of key salt responsive genes/proteins has been listed in **Table 4**.

TABLE 4 | Transgenic rice cultivars developed by introduction of genes/proteins identified through 'omics-based' approach.

Source organism	Gene/Protein	Target rice cultivar	Reference
Oryza sativa	Glutamine synthetase	Kinuhikari	Hoshida et al. (2000)
Avena sativa	Arginine decarboxylase	TNG-67	Roy and Wu (2001)
Arthrobacter globiformis	Choline oxidase	Pusa Basmati-1	Mohanty et al. (2002)
Hordeum vulgare	LEA protein	Pusa Basmati 1	Rohila et al. (2002)
Atriplex gmelini	Vacuolar Na^+/H^+ antiporter	Kinuhikari	Ohta et al. (2002)
O. sativa	MAP kinase	Nipponbare	Xiong and Yang (2003)
Escherichia coli	Trehalose -6- phosphate synthase and Trehalose -6- phosphate phosphatase	Nakdong	Jang et al. (2003)
Vigna aconitifolia	Δ1-pyrroline-5-carboxylate synthetase	Kenfong	Su and Wu (2004)
O. sativa	Vacuolar Na^+/H^+ antiporter	Nipponbare	Fukuda et al. (2004)
Mouse	Calcineurin	Xiushui 04	Ma et al. (2005)
Arabidopsis thaliana	MYB transcription factor	TNG-67	Malik and Wu (2005)
Suaeda salsa	Catalase	Zhonghua No. 11	Zhao and Zhang (2006)
A. thaliana and *O. sativa*	DREB transcription factor	Kita-ake	Ito et al. (2006)
O. sativa	NAC transcription factor	Nipponbare	Hu et al. (2006)
Suaeda salsa	Vacuolar Na^+/H^+ antiporter	Zhonghua-11	Zhao et al. (2006b)
Schizosaccharo- myces pombe	Plasma membrane Na^+/H^+ antiporter	Zhonghua-11	Zhao et al. (2006a)
Arthrobacter pascens	Choline oxidase	TNG-67	Su et al. (2006)
Avicennia marina	SOD	Pusa Basmati-1	Prashanth et al. (2008)
E. coli	Catalase	Nipponbare	Nagamiya et al. (2007)
O. sativa	Glyoxalase II	Pusa Basmati-1	Singla-Pareek et al. (2008)
Pennisetum glaucum	Vacuolar Na^+/H^+ antiporter	Pusa Basmati-1	Verma et al. (2007)
O. sativa	Shaker potassium channel	Nipponbare	Obata et al. (2007)
E. coli	Catalase	Nipponbare	Motohashi et al. (2010)
O. sativa	*Ribosome-inactivating protein gene 18*	Nipponbare	Jiang et al. (2012)
O. sativa	Calmodulin-like gene	Pei'ai 64S	Xu et al. (2013)
O. sativa	Heat shock transcription factor *A7*	*Nipponbare*	Liu et al. (2013)
O. sativa	Vesicle trafficking gene	Zhonghua 11	Peng et al. (2014)
O. sativa	bZIP transcription factor	Zhonghua 11	Liu et al. (2014b)
Bermudagrass	DREB transcription factor	Jonghua 11	Chen et al. (2015)
Citrus tristeza virus	*Heat shock protein 70*	*Nipponbare*	Hoang et al. (2015)
A. thaliana	Bcl-2 associated gene product	*Nipponbare*	Hoang et al. (2015)
Baculovirus	*p35*	*Nipponbare*	Hoang et al. (2015)

Conclusion

The world population is increasing with fast pace supplemented with reducing cultivable land due to salinization of arable land naturally or by improper irrigation practices. Altogether, this leads to decrease in the production of salt sensitive cereal crop rice, a staple food grain of developing world. Presently, a lot of methods have been implicated for modifying the genetic makeup of rice plant to withstand high salinity and lesser yield compromise. Plant breeding and genetic engineering are two major adopted methods.

Plant breeding is an important tool for crop improvement to develop environmental stress tolerant crops and several salinity tolerant varieties for diverse rice plants were developed till the present date. Nevertheless, this technology has its own limitations on which it is based i.e., reproductive obstacle and fine genetic variations of rice. However, mutation breeding has come up as a robust tool to substitute the traditional breeding method. Genetic engineering and mutation breeding have effectively used the genetic alterations available for salinity tolerance in the wild counterparts as well as in other organisms for the generation of salt tolerant rice. Although, genome sequencing of rice plant was completed a decade back, but the function of a large group of genes is not known yet. Not only from rice, many genes of unidentified role (20–30% in each genome sequenced) from other plants can convey multiple stress tolerance and can be used for raising salinity tolerant transgenic rice. There is also lack of the integration of information from genomic, transcriptomic, proteomic, metabolomic as well phenomic studies, which is very important for the determination of key pathways or processes involved in complex trait like salinity tolerance. Additionally, even after significant development in the understanding of responses of plant stress, there is still a huge gap in our understanding of sensor and receptor in the signal transduction, signaling molecules and ion transporter. The use of "omics" tool along with genetic engineering and mutation breeding techniques have promising role in delineating gene response and gene function in plants under salinity stress.

Salt tolerance is a multigenic trait, which involves a complex of responses at metabolic, cellular, molecular, physiological and whole-plant levels. Till date, scientists around the world have developed a number of salt tolerant transgenic rice by altering genes involved in various salinity reaction mechanisms such as ion transport and balance, hormone metabolism, osmotic regulation, antioxidant metabolism, and stress signaling. In spite of successful raising of transgenic rice for salinity tolerance in plants, attainment has not been realized at field level yet. The future focus should be on in-depth study of intercellular and intracellular molecular interactions involved in salinity stress response and genetic engineering with key genes coding components of salt tolerance machinery in rice. Last but not the least, the salt tolerant transgenic rice should be in the hand of final user i.e., farmer.

Author Contributions

AP and SLS-P designed the concept of the manuscript. PD and KN performed the analysis of the topic, and drafted the figures, tables and manuscript. All authors read and approved the final manuscript.

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OPINION
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Function genomics of abiotic stress tolerance in plants: a CRISPR approach

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Keywords: abiotic stress, CRISPR-Cas9, genome editing, gene function, transcription

Introduction

Various abiotic stresses, such as drought, salinity, heat, flooding, ion toxicity and radiation are the major constraints to agricultural production. The understanding of molecular basis of plant response to these environmental conditions has been a major focus of research in the past decades. Several genes/pathways and regulatory networks involved in stress responses have been worked out employing various approaches. Quite a few of these components have been used for engineering abiotic stress tolerance in model and crop plants via classical biotechnological and/or breeding approaches. Success to generate stress-tolerant plants has been achieved to some extent, which has resulted in increased crop yield (Mickelbart et al., 2015). However, novel strategies are desirable to overcome the limitations of classical methods, such as lack of precision and requirement of substantial time to increase the crop production in the current climate change and ever increasing population scenario. The recent availability of genome editing tools provides ample opportunity to introduce targeted modifications in the genome efficiently to study the functional aspects of various components of the genome in diverse plants and offers potential avenues for production of abiotic stress-tolerant crop plants.

Genome editing tools provide a method for introducing targeted mutation, insertion/deletion (indel) and precise sequence modification using customized nucleases in a wide variety of organisms. Zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 (CRISPR-associated nuclease 9) are the most commonly used genome editing tools (Voytas, 2013; Mahfouz et al., 2014; Kumar and Jain, 2015). In general, these sequence-specific nucleases cause double-strand breaks (DSBs) at the target genomic locus/loci, which is/are repaired by the intracellular repair pathways; nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ leads to the introduction of indels and HDR can be used to introduce specific point mutations or insertion of desired sequences (such as tags or new domains) via recombination. Owing to the simplicity of programming, CRISPR-Cas9 system has opened a plethora of options for genome editing in various biological contexts. Here, we highlight the emerging applications and future avenues of CRISPR-Cas9 system to understand the biology of abiotic stress tolerance in plants.

CRISPR-Cas9 System: A RNA-Guided Nuclease for Genome Engineering

CRISPR-Cas9 system, derived from a prokaryotic RNA-guided defense system (Bhaya et al., 2011), has been most recently developed and is emerging as a method of choice for genome engineering (Harrison et al., 2014; Hsu et al., 2014; Sander and Joung, 2014). Several excellent reviews have

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described the discovery and mechanism of CRISPR-Cas9 system (Hsu et al., 2014; Sander and Joung, 2014; Kumar and Jain, 2015). The Cas9 nuclease-mediated cleavage is guided by a single guide RNA (sgRNA), which recognizes the target DNA via standard Watson-Crick base pairing (Sander and Joung, 2014; Kumar and Jain, 2015). The existence of a protospacer adjacent motif (PAM; NGG/NAG) site immediately 3′ of the target site is essential. The sgRNAs are of 20–22 nucleotides (nt) in length, which can be easily designed and synthesized as oligonucleotides. Thus, Cas9 nuclease can be targeted to any DNA sequence with 5′-$N_{(20-22)}$-NGG by changing the 20–22 nt guide sequence. Further, the modular nature of CRISPR-Cas9 system, small size of targeting sgRNA, and high efficiency provide additional advantages. The well-designed sgRNAs can provide high specificity with minimal/no off-target effects. Due to these advantages, CRISPR-Cas9 system is amenable to multiplexing, where mutations can be introduced into multiple genes/genomic loci simultaneously. The ease of targeting, high efficiency and possibility of multiplexed modifications with CRISPR-Cas9 system have opened up a broad range of applications in basic and applied research in plant biology.

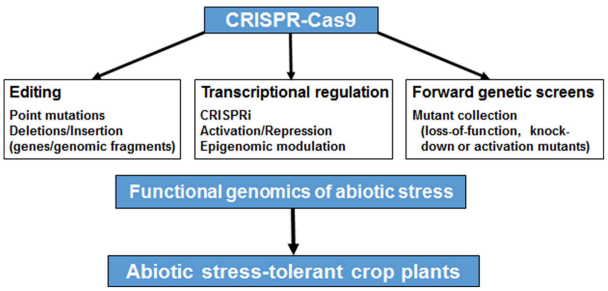


FIGURE 1 | An overview of the applications of CRISPR-Cas9 system in functional genomics of abiotic stress tolerance. CRISPR-Cas9 system can be used for genome editing (via introduction of point mutations, insertions or deletions), transcriptional regulation (via CRISPR interference, activation, repression or epigenetic modulation) or forward genetics screens (via generation of loss-of-function, knock-down or activation mutants using sgRNA libraries) for understanding the molecular basis of abiotic stress response, which can lead to generation of abiotic stress-tolerant crop plants.

Applications of CRISPR-Cas9 in Understanding Abiotic Stress Tolerance

The use of CRISPR-Cas9 system in engineering abiotic stress tolerance in plants has not been reported yet. CRISPR-Cas9 mediated genome engineering can enable manipulation of nearly any sequence in the genome (limited only by the availability of a PAM site) to reveal its function. CRISPR-Cas9 system has been successfully employed in bacteria, animals and plants for efficient genome editing (Feng et al., 2013; Jiang et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). Several web tools have been developed for designing optimized sgRNA(s) for the target genes/loci to avoid off-target effects (Hsu et al., 2013; Montague et al., 2014). Recently, a web tool CRISPR-P has been developed for designing sgRNAs in more than 20 plant species (Lei et al., 2014). The detailed protocols for targeted mutagenesis in model and crop plants via CRISPR-Cas9 have also been published (Belhaj et al., 2013; Shan et al., 2014). Further, some vectors and a toolkit have been developed for CRISPR-Cas9-mediated plant genome editing (Xing et al., 2014; Kumar and Jain, 2015). Some of these have been made available via Addgene (https://www.addgene.org/crispr/plant/), a non-profit plasmid repository. The availability of these information/resources provides a platform for use of CRISPR-Cas9 system in various applications (editing, transcriptional modulation and genetic screens) to dissect the molecular basis of abiotic stress response and generate stress-tolerant crop plants as outlined in **Figure 1**.

Abiotic stress is a complex trait, which is governed by multiple genes. There is a substantial interaction between components of several signaling, regulatory and metabolic pathways, which lead to abiotic stress response/adaptation (Nakashima et al., 2009; Hirayama and Shinozaki, 2010; Garg et al., 2014; Mickelbart et al., 2015). Further, plants have undergone whole genome duplication events and a large fraction of genes are represented by multi-gene families with functional redundancy. Many times knock-out of a single gene may not produce any/desired phenotype, thus making it difficult to reveal its function. Due to ease of design and high efficiency of sgRNAs, multiple genes can be targeted simultaneously using CRISPR-Cas9 system, which can overcome the problem posed by functional redundancy of genes. Multiplex genome editing has been successfully implemented in model and crop plants (Li et al., 2013; Mao et al., 2013; Zhou et al., 2014). Such approaches can allow deciphering the role of multiple and functionally redundant genes involved in the same biological process such as abiotic stress response. Another approach could be the pyramiding/stacking of multiple genes involved in a stress response pathway or regulatory network via HDR-mediated gene targeting. The genes involved in stress related gene regulatory network, signal transduction and metabolite production may be targeted via CRISPR-Cas9 technologies for production of stress-tolerant crop plants.

The availability of wild germplasm and genetic variations in crop plants is the key to crop improvement programs. However, the lack of enough natural germplasm, genetic diversity and mutant collections limit both basic and applied research, particularly in crop plants. The genome editing tools provide opportunity to overcome these limitations via creation of such variations in the genome of crop plants. Due to small size and ease of designing, sgRNA libraries targeting almost all the genes can be generated for any plant species, which can be used for generation of genome-scale point mutations and gene knock-outs. The availability of such collections can boost functional genomic studies in crop and non-model plants via large-scale genetic screens. SgRNA libraries can also enable the modification of non-coding genetic elements to facilitate the discovery of gene regulatory regions. Recent reports have demonstrated the potential of CRISPR-Cas9 system to perform robust negative and positive selection screens in human (Shalem et al., 2014; Wang et al., 2014). Although such resources have not been generated in plants as of now, these are expected in near future. The screening

of mutants with altered abiotic stress response in plants could enable gene function analyses and generation of stress-tolerant crop varieties.

The use of different versions of Cas9 proteins can further enhance the realm of CRISPR-Cas9 system. For example, dCas9 (catalytically inactive Cas9) can be used to disrupt gene function via CRISPR interference (CRISPRi) (Gilbert et al., 2013; Qi et al., 2013). Some effector domains such as KRAB/SID have been fused with dCas9 to enhance the transcriptional repression (Gilbert et al., 2013; Konermann et al., 2013). The use of paired Cas9 nickases can increase the specificity of mutagenesis substantially (Cho et al., 2014). The fusion of transcriptional activation domains, such as VP16/VP64 to dCas9 can activate the expression of target gene(s) and allow screening for gain-of-function phenotypes for abiotic stress tolerance. A few studies have reported strong activation effects of using multiple sgRNAs for a particular gene promoter (Mali et al., 2013; Perez-Pinera et al., 2013). The use of CRISPR-based synthetic transcriptional activator or repressor to modulate the transcription of target endogenous genes has been demonstrated in plants (Piatek et al., 2015). Tethering of dCas9 to epigenetic modifiers can help defining the role of methylation or different chromatin states in abiotic stress responses/adaptation. The same may be employed to regulate or fine-tune the stress-responsive gene expression. The reassembly of Cas9 protein can generate inducible CRISPR-Cas9 systems to enable spatially precise modifications. The use of light-inducible domains, CIB1 and CRY2, have been successfully demonstrated to construct TALENS (Konermann et al., 2013). The use of conditional (stress-inducible and/or tissue-specific) promoters to drive the expression of Cas9 and sgRNA can avoid undesired pleiotropic effects. A custom-designed zinc finger nuclease along with a heat-shock promoter have been used to induce mutations in an AP2/ERF family transcription factor gene, *ABA-INSENSITIVE 4*, involved in abiotic stress responses (Osakabe et al., 2010). A high frequency (up to 3%) of gene mutations resulting in the desired phenotypes was observed.

A recent study demonstrated the use of CRISPR-Cas9 system in genotyping naturally occurring variations, which allowed distinguishing homozygous biallelic mutants from wild-type (Kim et al., 2014). A few studies have demonstrated the production of homozygous transgenic plants in the first generation (Feng et al., 2013; Mao et al., 2013; Brooks et al., 2014; Zhang et al., 2014; Zhou et al., 2014), which presented the fastest possible method in a crop plant genome modification. Such approaches can reduce breeding or gene transformation time greatly for production of new varieties/transgenic plants with desired traits, such as abiotic stress tolerance. CRISPR technology is being seen as an advancement of plant breeding technologies. Non-transgenic approaches are also available for delivery of such nucleases to produce mutant plants (Marton et al., 2010). As a result, crop varieties produced using these technologies may qualify as non-GM and would have enormous impact on plant biotechnology and breeding.

Concluding Remarks

CRISPR-Cas9 system can greatly facilitate the study of gene/genome function and engineering abiotic stress tolerance in variety of plants. Several studies have demonstrated the robustness and versatility of CRISPR-Cas9 system in different biological contexts. Although the primary application of this tool has been the generation of gene knock-outs so far, harnessing other applications will be very important in the area of stress biology. CRISPR-Cas9 system has not been employed for studying abiotic stress response/adaptation pathways as of now. The development of novel regulatory module(s) from naturally existing components (genes, promoters, *cis*-regulatory elements, small RNAs and epigenetic modifications) can facilitate the engineering of signaling/regulatory and metabolic processes to modulate plant abiotic stress tolerance. Overall, the rapid pace of development and emerging applications of CRISPR-Cas9 system promise its immense contribution in understanding the gene regulatory networks underlying abiotic stress response/adaptation and crop improvement programs to develop stress-tolerant plants.

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REVIEW
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From Genetics to Functional Genomics: Improvement in Drought Signaling and Tolerance in Wheat

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Drought being a yield limiting factor has become a major threat to international food security. It is a complex trait and drought tolerance response is carried out by various genes, transcription factors (TFs), microRNAs (miRNAs), hormones, proteins, co-factors, ions, and metabolites. This complexity has limited the development of wheat cultivars for drought tolerance by classical breeding. However, attempts have been made to fill the lost genetic diversity by crossing wheat with wild wheat relatives. In recent years, several molecular markers including single nucleotide polymorphisms (SNPs) and quantitative trait loci (QTLs) associated with genes for drought signaling pathways have been reported. Screening of large wheat collections by marker assisted selection (MAS) and transformation of wheat with different genes/TFs has improved drought signaling pathways and tolerance. Several miRNAs also provide drought tolerance to wheat by regulating various TFs/genes. Emergence of OMICS techniques including transcriptomics, proteomics, metabolomics, and ionomics has helped to identify and characterize the genes, proteins, metabolites, and ions involved in drought signaling pathways. Together, all these efforts helped in understanding the complex drought tolerance mechanism. Here, we have reviewed the advances in wide hybridization, MAS, QTL mapping, miRNAs, transgenic technique, genome editing system, and above mentioned functional genomics tools for identification and utility of signaling molecules for improvement in wheat drought tolerance.

Keywords: wheat, ABA, drought, signaling, functional genomics, transcription factors, transcriptomics

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INTRODUCTION

Global warming has resulted in decreased precipitation and increased evaporation, causing more frequent drought spells worldwide. Drought reduces the plant yield up to 50% which is a great economic loss for the farming community (Akpinar et al., 2013). Consequently, development of drought tolerant wheat cultivars has become a serious challenge for the plant breeders to ensure the food security of the masses (Budak et al., 2013a). Drought is a multifaceted trait; plant responses to drought are affected by various factors including growth conditions, physiology, genotype, developmental stage, drought severity, and duration. Thus, drought tolerance mechanisms involve diverse gene expression patterns and as complex signaling pathways (Kantar et al., 2011a; Akpinar et al., 2012). Bread wheat is an important staple food worldwide, therefore efforts have been made to develop drought tolerant varieties (Budak et al., 2013b). Drought signaling pathways

involve crosstalk among various biomolecules which makes breeding for drought tolerance an uphill task (Akpinar et al., 2012). In recent years, genomics knowledge based on Next Generation Sequencing (NGS), gene editing systems (Shan et al., 2013), gene silencing (Yin et al., 2014), and over-expression methods (Saad et al., 2013) have increased our understanding about drought signaling pathways. At the transcriptome level, the RNA deep sequencing (Akpinar et al., 2015) and microarray analyses (Ergen et al., 2009) are employed to elucidate the differential expression of RNA transcripts involved in drought response. Moreover, microRNAs (miRNAs; Budak and Akpinar, 2015), hormones (Reddy et al., 2014), quantitative trait loci (QTLs; Barakat et al., 2015), metabolites (Xiao et al., 2012), transcription factors (TFs), and drought-related proteins (Lucas et al., 2011a; Alvarez et al., 2014) are key players in drought signaling. These factors regulate the gene expression in response to drought. TFs also interact with plant stress hormones, e.g., abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) in mediating drought response (Nakashima et al., 2014). To elucidate complex wheat drought signaling which will help in developing improved varieties, powerful tools are required for multiplexed or simultaneous detection of signaling molecules. Advances in functional genomics tools have provided us the opportunity to detect above mentioned molecules with ease, efficacy and accuracy thus opening a new era of crop improvement (Colmsee et al., 2012). In this review, we have summarized the advances in genetics, genomics, and functional genomics for identification of novel genes and their subsequent use in breeding programs for improved drought tolerance.

SIGNALING PATHWAYS IN WHEAT FOR DROUGHT TOLERANCE

Drought signaling is categorized into ABA-dependent and ABA-independent pathways as ABA is the first line of defense against drought. ABA-dependent signaling consists of two main gene clusters (regulons) regulated by ABA-responsive element-binding protein/ABA-binding factor (the AREB/ABF regulon) and the MYC/MYB regulon. Previous studies have shown that AP2/EREBP (ERF) TFs are engaged in both ABA-dependent and independent signaling pathways. Despite being two distinct and independent pathways, there is plausibly some crosstalk between both (Lata and Prasad, 2011). The AP2/ERF TFs family includes the ethylene-response factors, e.g., a TaERF promotes drought tolerance in wheat with increased proline and chlorophyll levels (Rong et al., 2014). The sucrose non-fermenting1-related protein kinase 2 family (SnRK2) consists of plant specific Ser/Thr kinases which are positive regulators of ABA signaling. SnRK2s were first reported to be involved in ABA signaling in wheat (PKABA1; Fujii and Zhu, 2012). The SnRK TFs are also involved in ABA independent pathway (Lata and Prasad, 2011). Although not specifically studied in wheat, the ABA-dependent pathways in rice and *Arabidopsis* have been extensively analyzed (Todaka et al., 2012). The ABA-independent regulons include the CBF/DREB (cold-binding factor/dehydration responsive element binding), NAC, and ZF-HD (zinc-finger homeodomain; Lata and Prasad, 2011). The transcriptional regulatory network based on DREBs is induced by dehydration in wheat. There are two known DREB regulons; DREB1/CBF and DREB2 (Edae et al., 2013). Above mentioned signaling pathways and their roles in drought tolerance have been extensively discussed in this review.

GENETICS BASED IMPROVEMENT IN DROUGHT SIGNALING

The selection of an appropriate breeding strategy to develop drought tolerant cultivars is the key step for a successful breeding program. Therefore, strategies which can transfer specific genes, exploit wild relatives of crops, identify and transfer genes with ease, require less time, and labor to develop cultivars are of great value (Hussain, 2015). The advances in genetics, genomics, and functional genomics have enabled researchers to combine one or more advantages of different strategies to develop drought tolerant wheat. Here, we have described the advances in these methods.

Classical Breeding for Improving Drought Tolerance in Wheat

Classical breeding dates back to 5,000–10,000 years when man domesticated selective plant species based on their better taste. Domestication was followed by selection of high yielding genotypes and cross-hybridization to recombine tolerance genes from different sources (Hussain, 2015). The presence of genetic variation in wheat is the key to identify the contrasting parents for classical breeding (Shelden and Roessner, 2013). Significant genetic variation in wheat for drought tolerance has been identified for selection of diverse parents. Cross-hybridization of wheat diploid progenitors produce drought tolerant synthetic hexaploid (SHs) wheat (see the section Introgression of Drought Tolerance Genes from Wild Species) with introgression of several novel drought tolerance genes (Zhang et al., 2005). Most of plant breeders selected the drought and other stress tolerant wheat varieties on the basis of higher yields and ignored the physiological mechanism behind it. Therefore, few cultivars having drought and abiotic stress tolerance have been developed in comparison to the ones improved for high yield (Hussain et al., 2015). Under drought, plant machinery shifts its focus to ABA production for downstream activation of signaling and tolerance mechanisms which lowers the grain filling and yield. Therefore, the balance between yield and drought tolerance needs to be investigated (Alvarez et al., 2014). ABA content has been used as selection index for screening wheat under drought and contrasting parents for its production have been crossed. Several QTLs and genes involved in signaling pathways have been identified in subsequent segregating populations (Iehisa et al., 2014; see the section QTL Mapping for Drought Signaling Genes in Wheat). Longer time, intensive labor requirements, transfer of non-desirable genes, limited resistance resources, genetic barriers and limited understanding of tolerance mechanisms are problems associated with classical breeding (Hussain, 2015).

Since breeding to date has not been focused on signaling pathways, there is a need to combine the available information collected by QTLs, MAS, and Omics tools with traditional traits for improved wheat drought tolerance.

Introgression of Drought Tolerance Genes from Wild Species

Significant loss of genetic diversity has occurred at three levels: (a) Species level (domestication), (b) Varietal level (green revolution), and (c) Gene level (breeding cycles). High yielding wheat developed through green revolution has less stress tolerance (Hussain, 2015). It is the time for plant breeders to look back and utilize this lost genetic diversity as some wild wheat relatives are potential sources of drought tolerance. For example, wild emmer wheat (*Triticum dicoccoides*) has inter and intra-varietal genetic diversity for water use efficiency (WUE), phenology, and contains several genes and QTLs for drought tolerance (Nevo and Chen, 2010). Gene expression studies in emmer wheat identified over 13,000 expressed sequence tags (ESTs) in response to drought (Ergen and Budak, 2009), and 33 outlier loci for drought tolerance were identified by single nucleotide polymorphisms (SNPs) markers (Ren et al., 2013). Transcriptomic analysis identified several genes and TFs involved in ethylene, IP3, and ABA dependent signaling pathways in wild emmer wheat (Ergen et al., 2009). SHs wheat constituted by crossing these wild relatives gained many novel QTLs and genes for ABA responsiveness and signaling (Iehisa et al., 2014). Role of *DREBs* in conferring drought tolerance to *T. dicoccoides* has also been established (Lucas et al., 2011b).

Introgression of drought tolerance to cultivated wheat from *Aegilops tauschii* was achieved by crossing it with durum wheat to make SHs wheat. DNA fingerprinting of SHs showed high genetic diversity with longer roots and higher soluble carbohydrates to resist water deficiency (Reynolds et al., 2007). D genome of *A. tauschii* contains several drought responsive genes potentiating the development of drought tolerant SHs wheat through crossing. *A. tauschii* and related SHs showed significant variation for ABA responsiveness when gene expression analyses were performed for ABA inducing *WABI5* and three downstream Cor/LEA protein coding genes (*Wrab18*, *Wrab17*, and *Wdhn13*) while the line with enhanced expression of *Wdhn13* showed salt and dehydration tolerance (Iehisa and Takumi, 2012). Proteomics approach has identified several proteins involved in ABA signaling (ABA 8′-hydroxylase, MPK6, dehydrin, 30S ribosomal protein S1, retrotransposon protein, a 70 kDa HSP) in the wild wheat relative, *Kengyilia thoroldiana* under drought. Proteins involved in antioxidative enzyme activity (thioredoxin peroxidase, ascorbate peroxidase, Cu/Zn superoxide dismutase) also showed increased expression levels (Yang et al., 2015). The value of wild wheat relatives as donors of drought genes has not only been established on morphological bases, but also validated with genomics (QTLs) and functional genomics (transcriptomics, proteomics, ESTs, SNPs) tools. Furthermore, their utility as drought gene donors has been confirmed in SHs. Therefore, we suggest that plant breeders should focus on wheat wild relatives to enhance the genetic diversity of wheat for drought tolerance.

Molecular Markers for Identification of Drought Signaling Genes

Selection in classical breeding is performed on the basis of morphological and economical traits which are highly influenced by the environment. Environmental influence on phenotypic expression creates confusion in selection of desirable traits. Discovery of DNA markers for economic and stress related crop traits have helped to select the desirable traits and parents with ease, efficacy and reliability in remarkably shorter time. Therefore markers, especially the SNPs have added more power to identify the genes linked to drought and other stresses (Budak et al., 2013b; Hussain, 2015). DNA markers for various genes involved in drought signaling have been reported, e.g., RAPD markers by using P21F/P21R and P25F/PR primers in A genome; and P18F/P18R primer in B genome mapped *DREB1* on 3A chromosome (Huseynova and Rustamova, 2010). In wheat, DREBs were tagged with five SNPs in A (P21F/P21R and P25F/PR primers), B (P18F/P18R primer), and D genome (P20F/P20R and P22F/PR primers). *DREB1* gene was tagged on chromosome 3A, 3B, and 3D. S646 and S770 SNPs were used and SNP S770 mapped *DREB-B1* between markers *Xfbb117* and *Xmwg818* on chromosome 3BL (Wei et al., 2009). SNPs identified the involvement of five signaling genes in yield and drought tolerance pathways. The *DREB1A* correlated with heading date, vegetation index and biomass, while flag leaf width, harvest index and leaf senescence were associated with *ERA1-B* and *ERA1-D* (enhanced response to ABA) genes. Other signaling genes, *1-FEH-A* and *1-FEH-B* (fructan-1-exohydrolase) were linked to yield and thousand kernel weight (Edae et al., 2013). Significant relationships between morpho-physiological traits and SNPs suggested key role of detected SNPs in drought tolerance.

High resolution melting (HRM) technology is the most powerful tool to identify the allelic variations. Use of HRM found that allelic variation in *DREB* TFs identified by SNPs led to variation in peptide sequences as well. The variation in peptide sequences was linked with differences in protein geometry and recognition of *cis*-elements involved in ABA signaling (Mondini et al., 2015). Two important TFs, i.e., *DREB1*, *WRKY1*, and a Na^+ transporter, *HKT-1* conferring drought and salt tolerance were also mapped by SNPs (Mondini et al., 2012). SNPs were used to map *TaSnRK2.8* gene which plays important role in carbohydrate metabolism, protein–protein interaction, and ABA signaling (Zhang et al., 2013). Chromosome locations and primers for these markers are given in **Table 1**. It can be concluded that MAS has a lot of promise to identify the signaling genes. Strong association between signaling genes and drought related physiological traits suggest that MAS should be focused to identify the signaling genes (Edae et al., 2013). This can help to improve the drought tolerance with less effort, time and resources and can speed up the breeding programs in future.

Transgenic Approaches for Improving Drought Signaling

Loss of tolerance genes by genetic erosion should be filled with such efficient and reliable methods that can transfer genes in

TABLE 1 | Molecular markers identified for wheat drought signaling genes.

Marker type	Primer	Chromosome location	Target drought signaling gene	Reference
SNP	P21F/P21R and P25F/PR	3A	*DREB1*	Wei et al., 2009
	P18F/P18R	3BL	*DREB1*	Wei et al., 2009
SNP S770		*Xfbb117-Xmwg818*		
SNP	P20F/P20R and P22F/PR	3D	*DREB1*	Wei et al., 2009
RAPD	P25F/PR	3A	*DREB1*	Huseynova and Rustamova, 2010
RAPD	P18F/P18R	3A	*DREB1*	Huseynova and Rustamova, 2010
SNP	DREB1a and DREB1b	U16709.1*	*DREB1*	Mondini et al., 2012
SNP	WRKY1	DQ323885.1*	*WRKY1*	Mondini et al., 2012
SNP	HKT-1	AF303376.1*	*HKT-1*	Mondini et al., 2012
SNP	P21	3A	*DREB1A*	Edae et al., 2013
SNP	ERA1B	3A, 3B, 3D	*ERA1-B*	Edae et al., 2013
SNP	ERA1D	–	*ERA1-D*	Edae et al., 2013
SNP	W12	6A	*1-FEH-A*	Edae et al., 2013
SNP	W32	–	*1-FEH-B*	Edae et al., 2013
SNP	M13	5A	*TaSnRK2.8*	Zhang et al., 2013
SNP	DREB2a and DREB2b	–	*DREB2*	Mondini et al., 2015
SNP	DREB3a and DREB3b	–	*DREB3*	Mondini et al., 2015

**Ref gene.*

a short time. Recombinant DNA technology has emerged as a powerful tool for the purpose. It provides the additional benefit of having no genetic barriers, thereby; so can transferring the genes from any wild relative, land race or other species (Hussain, 2015). Candidate genes for drought tolerance in wheat include TFs which regulate the signaling genes, genes encoding defense molecules [Reactive oxygen species (ROS), proline, JA, SA], and for production of defense proteins (Yang et al., 2010). Here, we have described the major achievements in wheat (see summary in **Table 2**).

Drought Signaling by Introducing *DREBs*

A soybean based DREB gene (*GmDREB*; Accession No. AF514908) was transformed to wheat by gene gun bombardment using ubiquitin and RD29A promoters, and transgenic plants with both promoters showed increased drought and salt tolerance (Shiqing et al., 2005). This increased tolerance of the crop was linked with to twofold higher proline production, stay green phenomenon under drought and survival and recovery on re-watering (SURV) after drought spell (Wang et al., 2006) suggesting a role of signaling pathway in downstream proline production. Transformation of wheat with a cotton originating DREB (*GhDREB*) improved drought, salt, and freezing tolerance due to higher production of soluble sugars and chlorophyll in leaves (Gao et al., 2009). Transgenic wheat with *DREB1A* was subjected to field screening on the basis of SURV and WUE. Although the event was selected in greenhouse, plants showed even higher yield under field drought (Pierre et al., 2012). However, there is dire need to find out activated genes or expressed proteins

TABLE 2 | Improvement in wheat drought signaling and tolerance by transgenic approaches.

Transformed gene	Improvement in signaling and tolerance	Reference
HVA1	Abscisic acid (ABA) signaling, produce Late embryogenesis abundant 3 (LEA3) for cell membrane integrity, higher biomass production and water use efficiency (WUE), drought, and salt tolerance	Sivamani et al., 2000
HVA1	ABA responsiveness, ABA signaling, higher WUE and relative water content (RWC), stable yield, drought tolerance	Bahieldin et al., 2005
GmDREB	ABA independent signaling (AIS), drought, and salt tolerance	Shiqing et al., 2005
GmDREB	AIS, 2-fold higher proline production, stay green, SURV, drought tolerance	Wang et al., 2006
GhDREB	AIS, higher soluble sugars and chlorophyll production, improved drought, salt, and freezing tolerance	Gao et al., 2009
DREB1A	Higher SURV, WUE, and yield under drought	Pierre et al., 2012
SNAC1	Activation of sucrose phosphate synthase, type 2C protein phosphatases and 1-phosphatidylinositol-3-phosphate-5-kinase genes for ABA signaling, high RWC, chlorophyll content and biomass, enhanced salinity and drought tolerance	Saad et al., 2013
Alfalfa aldose reductase	Antioxidant defense, ABA signaling, detoxification of aldehyde substrate, green biomass production, drought tolerance	Fehér-Juhász et al., 2014

by these TFs to fully understand their role in signaling pathways.

Drought Signaling by Introducing *HVA1* Gene

Various studies aimed to find the function of ABA in regulating the expression of drought tolerance genes. A number of such genes code for the proteins involved in stomata closure to check the transpiration against the cell dehydration. Late embryogenesis abundant 3 (LEA3) in barley is one of such protein encoded by *Hordeum vulgare* abundant protein 1 (*HVA1*) gene. This gene is activated by ABA treatment and several crops transformed with the *HVA1* gene showed improved drought and salt tolerance (Nguyen and Sticklen, 2013). *HVA1* gene was transformed to spring wheat by gene gun and its over-expression under maize ubiq1 promoter resulted in improved biomass production, WUE, drought, and salt tolerance due to activation of ABA signaling (Sivamani et al., 2000). Field evaluation of transgenic wheat with *HVA1* for six cropping seasons at four locations (USA and Egypt, both irrigated and rainfed area) showed stable yield, higher WUE and relative water content (RWC). These tolerance traits were directly correlated with the expression of *HVA1* gene in transgenic plants (Bahieldin et al., 2005). It is important to note that to date, the research station has not released it as a variety (Hayes and Xue, 2014).

Drought Signaling by Introducing Other Genes

Transformation of wheat with *SNAC1* gene under the control of ubiquitin promoter showed enhanced salinity and drought tolerance at seedling stage in lab conditions. Transgenic plants showed higher biomass, RWC, and chlorophyll content. Expression studies of *SNAC1* by qPCR showed over-expression of sucrose phosphate synthase, type 2C protein phosphatases and 1-phosphatidylinositol-3-phosphate-5-kinase genes, which are involved in ABA signaling (Saad et al., 2013). Transgenic wheat with cDNA of alfalfa aldose reductase recipient gene, involved in antioxidant defense and exhibited 1.5–4.3 times more detoxification of aldehyde substrate and 12, 26, and 41% increase in green biomass production in three separate transgenic lines, resulting in enhanced drought tolerance (Fehér-Juhász et al., 2014). Although the transfer of *DREBs*, *HVA1*, and *NAC* have resulted in enhanced drought tolerance by improving signaling pathways, there are no studies to date that show whole downstream signaling cascade improved in transgenic plants.

GENOMICS BASED IMPROVEMENT OF DROUGHT SIGNALING

Although a reference whole genome sequence has not been reported for wheat to date, efforts have been put to identify potential genomic regions carrying genes for drought signaling pathways. QTLs, miRNAs, and genome editing systems (e.g., CRISPR/Cas system) are major genomics based methods applied to discover and manipulate related genomic regions. The role of these approaches in characterizing genes involved in drought signaling in wheat is discussed below.

QTL Mapping for Drought Signaling Genes in Wheat

Drought tolerance is a complex and quantitative trait encoded by many genes, and thus, the identification of genomic regions carrying genes for drought signaling is important. Doubled haploids (DHs), F_2 populations (Ibrahim et al., 2012), recombinant inbred lines (RILS), near isogenic lines (NILS) are suitable populations for QTL mapping (Budak et al., 2013b). In wheat, the QTLs for yield and yield related traits in drought, and biomolecules involved in signaling pathways have been mapped. Inheritance of ABA accumulation and distribution in plants is not simple and several genes/QTLs are involved in it. A major QTL for ABA production was mapped on the long arm of the 5A chromosome between *Xpsr575* and *Xpsr426* loci (8 cM from *Xpsr426*) in single chromosome substitution line and their subsequent F_2 and DHs populations. Substitution lines were developed by from hybridization of low and high ABA producing Chinese Spring' and 'SQ1' genotypes (Quarrie et al., 1994). This QTL showed strong linkage with *Dhn1*/*Dhn2* locus depicting a direct association between ABA accumulation and early flowering based drought tolerance in wheat (Ibrahim et al., 2012). Nine QTLs were mapped in wheat in response to exogenously applied ABA, SA, JA, and ethylene, suggesting the presence of potential genes involved in signaling in these regions (Castro et al., 2008).

Several QTLs linked to ABA accumulation in leaves were mapped in an F_2 population developed from the cross of contrasting genotypes for ABA production. But one novel QTL was linked to both higher ABA content and smaller leaf size due to genetic linkage between the genome regions. The QTL location was a homoeolog of the major wheat gene *Vrn1* that code for number of tillers, ABA accumulation and leaf size (Quarrie et al., 1997). A major QTL for ABA production was mapped on chromosome 6D in an F_2 population developed from contrasting SHs. This QTL region has several genes for ABA responsiveness, seed dormancy, and regulation of LEA proteins that protect the cell machinery under dehydration stress (Iehisa et al., 2014). A major QTL for higher grain yield (21%), chlorophyll content and wider flag leaf on 7A chromosome was mapped in a DH wheat line by using psp3094 SSR. Exogenous ABA application activated this QTL suggesting that genes in this region might be involved in ABA signaling (Quarrie et al., 2007). Four homologs of *Arabidopsis* ABA signaling genes (*TmABF*, *TmVP1*, *TmERA1*, and *TmABI8*) were mapped in a wheat RILs population derived by crossing *T. boeoticum* and *T. monococcum*. The location of these QTLs was chromosome 3Am, 4Am, and 5Am (Nakamura et al., 2007).

Seven QTLs for ABA production in response to drought were identified on chromosomes 2A, 3A, 1B, 7B, and 5D in an F_4 population at 33% field capacity. The most important QTLs for ABA content were mapped on chromosomes 3B, 4A, and 5A on the marker location of *Wmc96*, *Trap9*, and *Barc164* (Barakat et al., 2015). In another study, five major QTLs for ABA responsiveness were identified on chromosomes 1B, 2A, 3A, 6D, and 7B in a wheat RILs population. A QTL located on chromosome 6D contributed 11.12% to variation for ABA against 5–8% contribution by other QTLs. Expression analysis

showed allelic differences in QTLs for three ABA responsive Cor/LEA protein coding genes, *Wrab15*, *Wdhn13*, and *Wrab17*. The expression of these genes was influenced by QTLs present on chromosomes 2A, 7B, and 6D in ABA treated seedlings (Kobayashi et al., 2010). In conclusion, several QTLs for ABA production and downstream signaling pathways have been mapped in wheat (**Table 3**) but most of studies have not focused further on this aspect. We recommend the use of functional genomics tools along with QTLs to identify the genes located in QTL regions.

miRNAs Involved in Drought Signaling and Tolerance

Extensive application of NGS platforms has greatly contributed in identification of 20–22 nt short non-coding RNAs called as miRNAs that play regulatory roles in many processes (Budak et al., 2015a). miRNAs bind to their target transcripts through complementary base pairing, and either direct the cleavage of the target or repress its translation, leading to the decreased expression of the target transcript. Thus, miRNAs can act both at the transcriptional or post-transcriptional levels. miRNA mediated gene-silencing mechanism regulates the expression of plant hormones, TFs, and other developmental/stress signaling pathways (Curaba et al., 2014). Gene silencing involved in plant stress regulation is also mediated by naturally occurring small RNAs (siRNAs; Lu et al., 2012), and complementary double-stranded RNA (dsRNA) generated from inverted repeat (IR) transgenes (Frizzi and Huang, 2010). Post transcriptional gene silencing is carried out by miRNAs and virus-derived small interfering RNAs (vsiRNAs) which helps in discovering gene functions and developing crops with improved stress tolerance (Feng et al., 2013). miRNAs are important regulators in plant drought signaling because their target genes have key roles in metabolism and signal transduction (Yin et al., 2014).

The miRNA gene transcripts '*MIR*' are spatially and temporally influenced by cellular signaling factors, particularly plant hormones such as ABA under stresses (Jung et al., 2015). Some conserved plant miRNAs such as miR159 (*Triticum*, French bean, cotton, maize), miR164 (*Triticum*, *Brachypodium*, sugarcane), miR172 (*Triticum*, *Arabidopsis*, *Brachypodium*, *Oryza*, cotton) and miR393 (*Triticum*, *Oryza*, *Medicago*, *Pinguicula*, *Arabidopsis*) control the expression of key TFs which regulate development and signaling pathways (Gupta et al., 2014). miRNAs are involved in various drought related cellular pathways, including auxin signaling, ABA response, antioxidant defense, osmoprotection, cell growth, respiration, and photosynthesis, e.g., miR169 shows bread wheat specific differential expression under drought (Ding et al., 2013). Several signaling genes (ARF, *MYB33*, *MYB101*, *TIR1*, *AGO1,*) and growth regulation factors (GRF) are targeted and regulated by drought responsive miRNAs and *DREBs* (Covarrubias and Reyes, 2010). Most of the miRNAs have their specific putative targets which lead to regulation of specific genes/TF involved in signaling/tolerance mechanisms. In such a study, various bread wheat based miRNAs and their targets (shown in parentheses) were identified as tae-miR159a,b (MYB3), tae-miR159c-5p (Dihydro-flavonoid reductase-like protein), tae-miR171f (sensor histidine kinase), tae-miR395i (ATP sulfurylase), tae-miR156k (SBP), tae-miR166l-5p (FAM10 family protein), tae-miR168b (dehydrogenase/reductase), tae-miR444c.1 (MADS-box TF), tae-miR1432 (mitochondrial phosphate transporter), tae-miR160a (ARF), tae-miR164b (NAC), tae-miR166h (HD-ZIP4), tae-miR169d (CCAAT-box TF), tae-miR319c (Acyl-CoA synthetase), tae-miR393b,i (TIR1), tae-miR396a,c,g (GRF), tae-miR444d (IF3), tae-miR827-5p (finger-like protein). The above mentioned miRNAs regulated the expression of their targeted TFs/genes thus playing key roles in drought tolerance mechanism (Ma et al., 2015).

Similarly, increased expression of miR156 in *T. dicoccoides* targets the SBP TFs and promoted flowering while miR398 targets copper superoxide dismutases, cytochrome C oxidase, and regulates ROS production under drought stress. Increased expression level of miR1432 targets calcium-binding EF which activates signal transduction pathways. Other important drought responsive miRNAs in *T. aestivum* and *T. dicoccoides* are miR396, miR528, miR6248 (Kantar et al., 2011b; Budak et al., 2015b), miR1435, miR5024, and miR7714 (Akpinar et al., 2015). On the other hand, miR166 exhibits decreased expression in *T. dicoccoides* under drought which targets HD-ZIP3 TF and

TABLE 3 | Quantitative trait loci (QTLs) mapped for drought signaling molecules in wheat.

Chromosome location	Function	Reference
MAPMAKER QTL; 5A	Located between Xpsr575-Xpsr426, ABA production	Quarrie et al., 1994
2A	ABA production, smaller leaf size, homoeolog of wheat gene *Vrn1* that controls number of tillers and ABA accumulation	Quarrie et al., 1997
7A	Activated on exogenous ABA application, involved in ABA signaling	Quarrie et al., 2007
3Am, 4Am, 5Am	QTLs for ABA signaling genes (*TmABF*, *TmVP1*, *TmERA1*, and *TmABI8)*	Nakamura et al., 2007
6A	Located at *Xgwm459* and *gwm334a region,* activated by exogenous ABA, salicylic acid (SA), jasmonic acid (JA), and ethylene application, contains signaling genes	Castro et al., 2008
1B, 2A, 3A, 6D, 7B	ABA responsiveness, regulate expression of ABA responsive LEA protein coding genes, i.e., *Wrab15*, *Wdhn13*, and *Wrab17*	Kobayashi et al., 2010
MAPMAKER QTL; 5A	Linked to *Dhn1/Dhn2* genes, early flowering based drought tolerance	Ibrahim et al., 2012
6D	ABA production and signaling, seed dormancy, regulation of LEA proteins	Iehisa et al., 2014
3A, 1B, 4A, 5A, 5D, 7B	Enhanced ABA production, located at marker locations of *Wmc96*, *Trap9*, and *Barc164*	Barakat et al., 2015

plays role in developmental while miR171 targets GRAS TF and is significant in abiotic stress responses (Kantar et al., 2011b). A summary of miRNAs involved in drought response and signaling is given in **Table 4**. For a further comprehensive reading on drought responsive miRNAs, we would recommend our reader to go through other articles from our group (Lucas et al., 2011b; Budak et al., 2015a,b). From above discussion, it is evident that miRNAs have a key role in regulating drought tolerance pathways and should be exploited for wheat improvement.

CRISPR/Cas Genome Editing System

In addition to ZEN and TALEN, an efficient bacterium based genome editing system called the Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR), with associated protein Cas (CRISPR/Cas system) has emerged. The CRISPR are loci with variable short spacers interspersed by short repeats, later transcribed into non-coding RNAs (ncRNA). This ncRNA then forms a complex with the Cas and guides the complex to slice complementary target DNA. The development of single guide RNAs (sgRNAs) which are fusions of essential parts of *trans*-activating crRNA (tracrRNA) and the sgRNA of CRISPR RNA (crRNAs) proved to be an essential improvement in adopting the CRISPR-Cas system for targeted editing of complex eukaryotic genomes (Jiang et al., 2014). Following *Arabidopsis*, the system has also been demonstrated in rice and other crop plants. In protoplasts of bread wheat cultivar Kenong199, an ortholog of the barley MLO protein, *TaMLO* gene was targeted and showed high INDEL frequencies of 26.5–38.0%. The number of unique sgRNA target candidates generated on average were 21 per cDNA of either A or D genomes. The mean mutagenesis frequency in protoplasts was 28.5% with the transformation efficiency of 70–80% (Shan et al., 2013). The ability of this system to delete large DNA segments stably is valuable in wheat genomics given its large genome size and complexity. Development of transgenic wheat cultivars with stable drought tolerance through targeted genome editing will potentially revolutionize crop breeding. To date, the use of this system in engineering abiotic/drought stress tolerance or signaling has not been reported; however, in the future, it may prove to be a valuable tool in discovering the functions of signaling pathway components.

FUNCTIONAL GENOMICS FOR DISCOVERING DROUGHT SIGNALING MOLECULES

In recent years, the use of functional genomics tools has considerably increased in elucidating abiotic stress tolerance in plants. These methods include transcriptomics, metabolomics,

TABLE 4 | Wheat miRNAs involved in drought stress signaling.

miRNAs	Differential expression under drought	Target genes related to signaling	Function	Reference
miR159a-5p	↑	MYB TF, GAMYB1 GAMYB2, genes for oligopeptide transport	Auxin Signaling, Oligopeptide transporter	Liu et al., 2013; Gupta et al., 2014
miR159a,b	↓	WRKY TF; MYB3; alkaline phosphatase, cytochrome P450, Mob1-like and TLD protein	Signaling	Ma et al., 2015
miR160a	↑	HSP 70; ARF; tetratrico peptide repeat (TPR)	Stress adaptation	Liu et al., 2015
miR164	↑	NAC domain TF, phytosulfokines, sHSPs 17; NAC TF; genes involved in MAPK signaling pathways	Signaling pathway, oxidative stress response	Gupta et al., 2014
miR166h	↑	Class III HD-ZIP protein 4	Stress response, phytohormones	Ma et al., 2015
miR167	↑	Dnaj heat shock n-terminal domain-containing protein	Auxin signaling pathway, developmental response	Liu et al., 2015
miR168	↑	Argonaute	Signal transduction, stress response	Gupta et al., 2014
miR169d	↓	CCAAT-box TF	ABA-responsive transcription, drought tolerance	Ma et al., 2015
miR171f	↓	Sensor histidine kinase	Stress response	Li et al., 2013
miR172	↑	Apetala2-like TF, ARF, helix-loop-helix DNA-binding protein	Signaling Pathway, stress response, development	Gupta et al., 2014
miR172a,b	↓	Floral homeotic protein, AP2 TFs	Flower development signaling pathway	
miR319	↓	MYB3, Acyl-CoA synthetase	Abiotic stress tolerance	Zhou et al., 2010
miR393	↑	bHLH TF, transport inhibitor response 1/auxin F-box	Signaling pathway, genes in auxin signaling, basal defense	Gupta et al., 2014
miR395i	↓	ATP sulfur lyasas and sulfur transporters	Abiotic stress	Sunkar et al., 2005
miR397	↑	Ice1 (inducer of CBF expression 1) TF, laccase	Response to water deprivation	Gupta et al., 2014
miR398	↑	COX, Superoxide dismutase (SOD) gene family	Respiration pathway	Kantar et al., 2011b
miR474	↓	PPR, protein kinase, kinesin, Leucine-rich repeat	Unknown	Kantar et al., 2011b
miR1029	↑	Apetala2-like TF, DREB TF	Signaling pathway abiotic stress	Gupta et al., 2014
miR1432	↑	Mitochondrial phoshphate transporter	–	Ma et al., 2015

↑, increased expression levels; ↓, decreased expression levels.

proteomics, and ionomics and are capable of discovering and characterizing the expression of genes or other molecules in drought with high accuracy and efficiency. These highly sensitive tools can perform the spatial analysis of tissues which helps to understand the drought tolerance mechanisms, see **Figure 1** (Ergen et al., 2009). We have summarized the advances in OMICS for discovery of drought signaling molecules below

Transcriptomics for Identification of Drought Signaling Pathways

Transcriptomics is the study of whole set of RNA transcripts (transcriptome) produced by an organism under certain conditions, like drought. Microarrays were used first for profiling transcripts in response to various stresses, but polyploidy has limited the number of studies in wheat. Microarray based analysis became more common after the commercial release of wheat Affymetrix Gene Chip® (Santa Clara, CA, USA) which contained over 55,000 probes for wheat transcripts (Nair et al., 2012). Ergen et al. (2009) identified several novel genes and TFs involved in ABA, ethylene, and IP3 dependent signaling by Affymetrix Gene Chip® based spatial profiling of root and leaf tissues in wild emmer wheat. In leaf tissue, glutamine-dependent asparagine synthetase, a putative ATP-binding protein, homeo-domain TF Hox22 homolog (linked to LEA3 proteins), carotenoid producing 9-*cis*-epoxycarotenoid dioxygenase, proline-rich protein precursor and many proteinase inhibitors of Bowman Birk type showed highest increased expression levels under drought. While, germin-like protein, protein degrading cysteine proteinase precursors, bZIP TFs, cytochrome P450, OsRR9 homolog (a signal receiver domain), *LOL1* protein, and G–C content rich motif binding MYB like TF RADIALIS exhibited decreased expression levels. In root tissues, several dehydrins, LEA/COR protein *WRAB1*, putative lipases, a Rab GTPase homolog, several cold regulated proteins, 12-oxophytodienoic acid reductase, a cold shock protein A-2, MYB-domain Hv1 TF, glutathione transferase (ROS scavenger), PRP homologs and *WCOR719* (actin depolymerization factor) showed the highest increased expression levels. In contrast, many HSPs, RmlC-type cupin domain, B12D proteins, two nodulin 93 proteins, RING-H2 finger protein and GTP-binding EF exhibited decreased expression levels (Ergen et al., 2009).

Microarray gene chip based transcriptomic analysis of *TAM111* and *TAM112* wheat genotypes identified 123 genes for production of ABA, JA, auxin, cytokinin, brassinosteroid, gibberellins, and ethylene, and signaling pathways involving these hormones. These transcripts showed differential expression at grain filling stage and transcripts for ABA biosynthesis exhibited increased expression levels in both genotypes. Two transcripts similar to *PDR12* (coding for ABA transporter), transcripts for auxin, LEAs, dehydrins, HSPs, aquaporins, and redox homeostasis showed decreased expression levels. Transcript analysis revealed a key role of ABA in regulation of transcripts

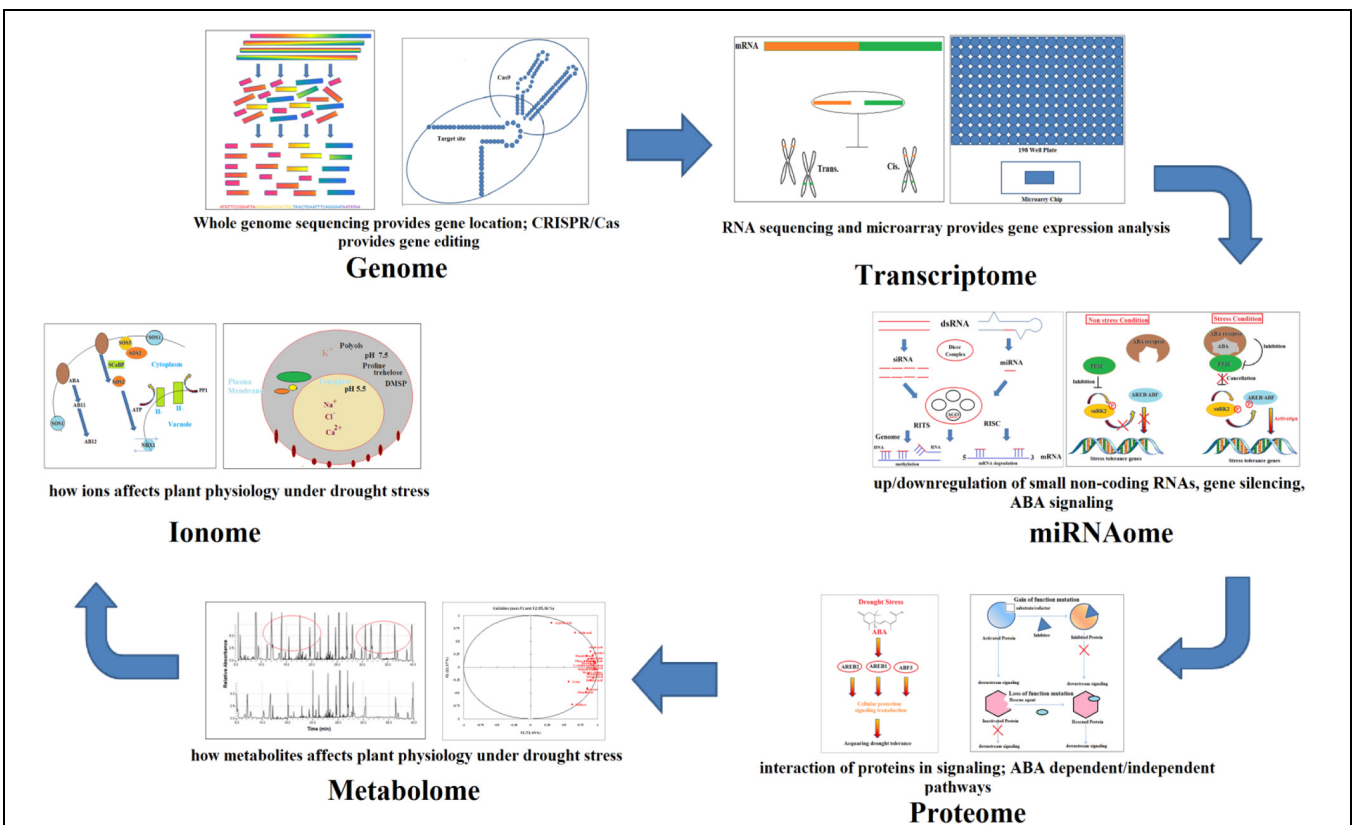


FIGURE 1 | Schematic diagram showing flow of information between functional genomics techniques for studying signaling pathways in plants.

and physiological changes linked with drought adaptation. Higher leaf ABA production showed strong association with higher yield and biomass under field drought which reduced the stomatal conductance and was linked to elevated transcript changes in flag leaf (Reddy et al., 2014). Low chip-to-chip variation, high reproducibility and probe density of Affymetrix Gene Chip® are its advantages but a major disadvantage of high relative cost has limited large scale studies. Therefore, there is dire need for more reliable and cost effective methods to study the complex drought signaling pathways.

Deep sequencing (RNA-Seq) based transcriptomics has made it a cost effective and powerful tool. There were unique transcripts found in *T. dicoccoides*, and *T. durum* under drought that they were engaged in drought signaling pathways (Akpinar et al., 2015). It can be concluded that RNA-Seq based transcriptomic analyses identified various drought signaling genes such as bZIP, TdNAC, and MYB genes which can be exploited in future studies.

Proteomics for Identification of Drought Signaling Pathways

Though transcriptomics provides the gene expression profiles, these profiles do not necessarily reflect protein levels as some transcripts may not be translated. Proteomics analysis provides clues into the actual fluctuations of the protein levels involved in signaling, regulatory, and enzymatic functions encoded by genome through transcripts. Advanced bioinformatics tools have helped to identify, characterize, and annotate novel proteins (Colmsee et al., 2012). The proteomic analysis of two wheat genotypes having contrasting drought tolerances showed that among differentially expressed proteins, 26% were involved in carbohydrate metabolism, 23% in detoxification and defense, and 17% in storage proteins. In drought, WD40 repeat protein, catalase isozyme 1, LEAs, Triticin precursor, sucrose synthase, and alpha amylase inhibitors exhibited increased expression levels in tolerant and decreased expression level in sensitive cultivar. On the other hand, ascorbate peroxidase, small and large subunit ADP glucose pyrophosphorylase and G-beta like protein showed decreased expression levels in sensitive cultivar (Jiang et al., 2012). Our group performed proteomics analysis using two wild emmer wheat varieties (TR39477 and TTD22), and one durum wheat cv. (Kızıltan). After 9 days of drought exposure, 75 differentially expressed proteins were detected with many being common to all three wheat genotypes, e.g., manganese superoxide dismutase (MnSOD), a glutathione transferase showed increased expression in the durum wheat (Budak et al., 2013a).

Alvarez et al. (2014) identified 1656 proteins and two unique peptides in wheat through proteomics analysis by using roots of from drought tolerant (Nesser) and sensitive (Opata) varieties for ABA-responsiveness. Important signaling proteins including monomeric G-proteins and their regulators, two lipoxygenases, K channel β subunits, a plasma membrane proton ATPase, calnexin, and an elicitor-induced protein showed an increased response to ABA in drought tolerant cultivar and *vice versa* in the sensitive. Though signaling protein 14-3-3 homologs exhibited increased expression in the drought tolerant cultivar, they remained unchanged in the sensitive one. Out of 151 ABA-responsive proteins, 100 showed increased expression levels but the rest showed decreased expression levels. An interesting finding was the abundance of multiple porin proteins and β-expansin precursor in the sensitive cultivar suggesting that the cell wall structure and membrane permeability might have influenced different adaptation to drought in both cultivars. Furthermore, six LEA proteins and several phosphatases were also identified in both cultivars (Alvarez et al., 2014).

Metabolomics for Identification of Drought Signaling Pathways

Metabolites are considered as signaling molecules as they are associated with physiological processes and are exported from each organelle to cytoplasm in the form of retrograde signals. Gas chromatography mass-spectrometry (GC–MS), liquid chromatography mass-spectrometry (LC–MS), capillary electrophoresis mass-spectrometry (CE–MS), and nuclear magnetic resonance (NMR) are the major analytical tools in metabolomics to detect, identify, and analyze small molecules. Initially, the analysis of metabolites was limited to a few compounds having major roles in drought tolerance. But, advances in these methods have enabled us to identify a wider range of metabolites produced under a specific condition. Metabolite profiling is a powerful tool to characterize genotype or phenotype of an organism for dissecting novel signaling pathways (Xiao et al., 2012). Plants accumulate compatible solutes to protect them from drought and oxidative stress for survival. GC–MS based metabolite profiling in moss *Physcomitrella patens* showed accumulation of compatible solutes in response to drought stress (Zhan et al., 2014). In wheat, levels of proline, tryptophan, and the branched chain amino acids leucine, isoleucine, and valine increased under drought in tolerant cultivars but organic acid levels decreased (Krugman et al., 2011). There are various examples where metabolites act as intracellular signals, e.g., in response to cytosolic sugar levels, trehalose 6-phosphate (T6P) enhances the redox transfer to AGPase, mediated by thioredoxin, mainly depending on metabolite balance between the chloroplast and cytosol. Metabolite profiling for elucidating signals in plants has been characterization in form of methyl-erythritol-cyclo-diphosphate (MECD; Xiao et al., 2012).

Ionomics for Identification of Drought Signaling Pathways

Ionomics is a high throughput analysis of the ion composition in an organism under a certain condition. It has immense applications in forward and reverse genetics, screening of mutants, finding mechanisms of ion uptake, compartmentalization, transport, and exclusion, thus helps to understand the mechanisms of drought and other abiotic stresses in plants (Shelden and Roessner, 2013). In wheat, ionomics studies under drought stress have not been reported yet. Together, ionomics and other genomics data can provide a complete picture of cellular changes against drought, enabling a thorough understanding of the underlying mechanisms

of tolerance (Colmsee et al., 2012). Furthermore, ionomics can help to identify novel genes coding for ions by utilizing phenotypic and genotypic data obtained from mapping populations. It can thus help in understanding the gene networks controlling the ion accumulation at different growth stages under drought stress (Satismruti et al., 2013). It should be noted that ionomics is a relatively new functional genomics tool with limited number of studies available, but spatial and highly sophisticated ion profiling will be a key in the future to understand the signaling pathways for drought tolerance.

SYSTEMS BIOLOGY APPROACHES TO DISCOVER SIGNALING PATHWAYS

System biology is a recent, fast growing and comprehensive analytical approach in life sciences to discover the control and

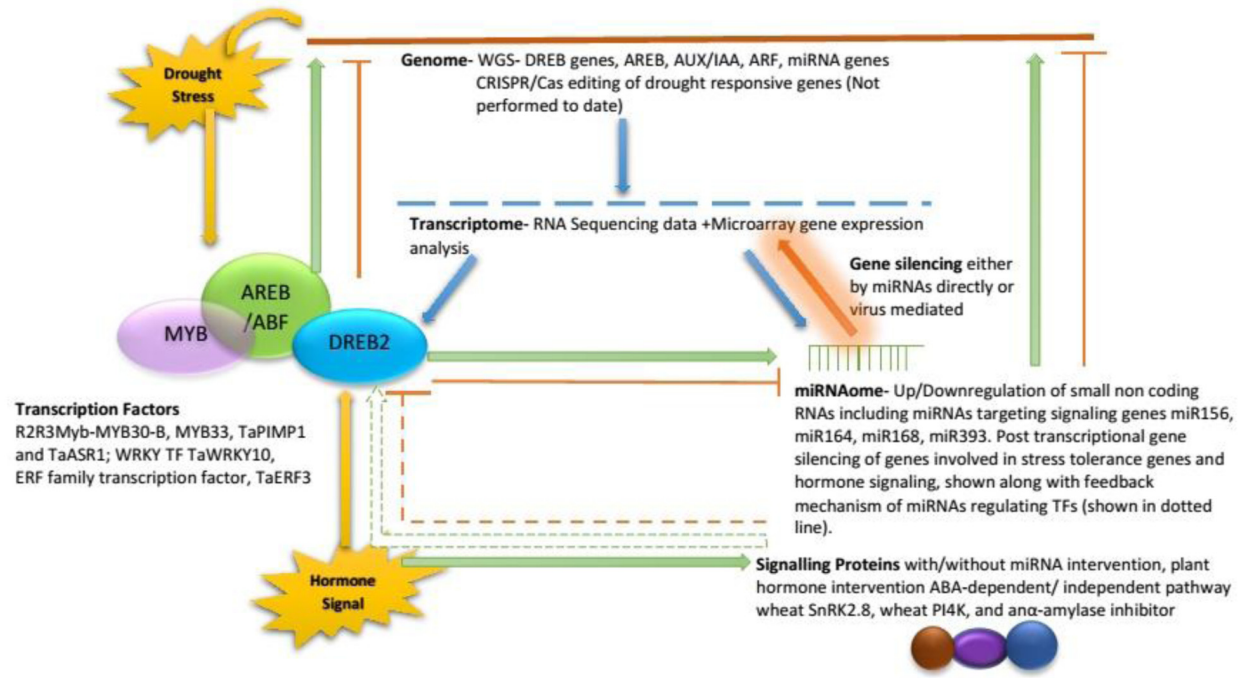


FIGURE 2 | Putative stress signaling pathway in wheat where functional genomics contribute.

TABLE 5 | Functional genomics studies for identification of drought signaling molecule in recent years.

Functional genomics tool	Drought signaling genes/molecules/mechanism identified	Reference
Transcriptomics	Hox22 TF linked to LEA3 proteins, ABA inducible LEA *WRAB1*, Dehydrins, Proline-rich protein precursor, Asparagine synthetase, 9-*cis*-epoxycarotenoid dioxygenase, Rab GTPase homolog, A-2 cold shock protein, MYB-domain Hv1 TF, glutathione transferase, *WCOR719*	Ergen et al., 2009
Transcriptomics	Genes for ABA, JA, auxin, cytokinin, brassinosteroid, gibberellins, and ethylene production and drought signaling based on these hormones. Transcripts for *PDR12*, auxin, LEAs, dehydrins, HSPs, aquaporins, redox homeostasis, and reduced stomatal conductance	Reddy et al., 2014
Proteomics	WD40 protein, catalase isozyme 1, LEA and alpha amylase inhibitors, ascorbate peroxidase, G-beta like protein, triticin precursor, sucrose synthase	Jiang et al., 2012
Proteomics	Ribulose-1,5-bisphosphate carboxylase large subunit, Osl_16800 protein, SORBIDRAFT_09g029170 protein, polyamine oxidase, Os02g0101500, Os03g0786100, Ferredoxin-NADP(H) oxidoreductase, Os03g0786100, Glutathione transferase, Mn superoxide dismutase, Cold regulated proteins	Budak et al., 2013a
Proteomics	Monomeric G-proteins and their regulators, lipoxygenases, K channel β subunits, plasma membrane proton ATPase, calnexin, an elicitor-induced protein, porin proteins, β-expansin precursor, LEAs, phosphatases	Alvarez et al., 2014
Proteomics	8′-hydroxylase, MPK6, dehydrin, 30S ribosomal protein S1, retrotransposon protein, 70 kDa HSP, thioredoxin peroxidase, ascorbate peroxidase, Cu/Zn superoxide dismutase	Yang et al., 2015
Metabolomics	Proline, tryptophan, leucine, isoleucine, and valine, organic acids	Krugman et al., 2011
Metabolomics	Trehalose 6-phosphate (T6P) promotes thioredoxin-mediated redox transfer to AGPase and MECD helped in elucidating signaling in plants	Xiao et al., 2012

regulation of intracellular biological systems, biochemical cycles and pathways in plants under different environmental stresses. Several computational studies have been involved, from few decades, to find the biochemical function of metabolites and small biomolecules responsible for biochemical pathways under drought stress (Gutiérrez et al., 2005). Most of the experimental methods are being used as the part of system biology including various target and untargeted metabolite analysis approaches used to identify the drought specific metabolites in different species of plants. GC–MS is one of the several approaches, was used to find metabolic compounds differentially expressed in tolerant (Excalibur and RAC875) and sensitive (Kukri) bread wheat cultivars under drought stress environment (Bowne et al., 2012). Another finding was aspartate-derived synthesis of few amino acids including lysine, methionine, and threonine in *Arabidopsis thaliana* done also explains the advantageous use of system biology approach, model based on measured kinetic parameters (Curien et al., 2009). Thus, system biology helps to understand the relationship and inter-connection among various types of bio-molecules involved in a certain tolerance mechanism.

CONCLUSION

Significant wheat yield losses due to drought lower farmers' income, food availability and ultimately affect the economy of various countries. Few drought tolerant wheat varieties have been developed as most of breeders have selected plants on the basis of morphological traits and ignored physiological basis of drought tolerance. Lost genetic variation for drought tolerance has been patched up by using wild wheat *T. dicoccoides* and *A. tauschii* in crossing and synthetic wheat showed improved drought tolerance. The improvement has been measured in terms of novel genes, QTLs, ESTs, and SNPs, e.g., three LEA protein coding genes (*Wrab18*, *Wrab17*, *Wdhn13*) involved in ABA signaling pathway were identified in SHs. Proteomics analysis identified ABA 8′-hydroxylase, MPK6, dehydrin, 30S ribosomal protein S1, and a 70 kDa HSP involved in ABA signaling in wild emmer wheat. In recent years, MAS have been used to select the plants which make the cultivar development process less time consuming. SNP markers have identified *DREB-B1*, *DREB1A*, *ERA1-B*, *ERA1-D*, *1-FEH-A*, *1-FEH-B*, *WRKY1*, *TaSnRK2.8*, and *HKT-1* genes in wheat. Many QTLs for ABA production, SA, JA, ethylene, and ABA based signaling, and QTL having genes for regulating other signaling genes (*TmABF*, *TmVP1*, *TmERA1* and *TmABI8*, *Wrab15*, *Wdhn13*, and *Wrab17)* have also been mapped. Transgenic approach provides the benefit of speedy gene transfer without any genetic barriers. Transformation of wheat with *GmDREB*, *GhDREB*, *DREB1A*, *HVA1*, *SNAC1*, and aldose reductase genes improved drought signaling and tolerance. Similarly, various miRNAs showed differential expression in drought and enhanced or silenced the expression of genes involved in drought signaling. However, the signaling genes, miRNAs, TF, etc., don't not express in isolation but interact with each other in signaling pathways (**Figure 2**).

In recent years, functional genomics has emerged as a power tool to identify the molecules involved in drought signaling pathways. Transcriptomics analyses identified various genes including Hox22, bZIP TF, dehydrins, *WRAB1*, *WCOR719*, *HSPs*, LEA, *TaWRKY17*, *TaWRKY16*, *TaWRKY24*, *TaWRKY19-C*, *TaWRKY59*, *TaWRKY82*, *TaWRKY61*, *TaWLIP19*, *TaWRKY10*, *TaNAC69*, and *TaMYB33* for drought signaling pathways. Proteomics and metabolomics have identified several proteins (Monomeric G-proteins, lipoxygenases, potassium channel β subunits, calnexin, LEAs, phosphatases) and metabolites (Proline, tryptophan, leucine, isoleucine, valine) involved in drought signaling as summarized in **Table 5**. In this way, highly efficient functional genomics tools have helped in identifying several important genes which can be exploited by breeders to develop drought tolerant wheat cultivars in futures. Similarly, genome-editing system CRSPR/Cas will be valuable in future for better understanding of drought tolerance mechanisms due to its ability to modify the genome. Study of miRNAs in future is also important in future as they are key regulators of signaling pathways.

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A Bird's-Eye View of Molecular Changes in Plant Gravitropism Using Omics Techniques

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During evolution, plants have developed mechanisms to adapt to a variety of environmental stresses, including drought, high salinity, changes in carbon dioxide levels and pathogens. Central signaling hubs and pathways that are regulated in response to these stimuli have been identified. In contrast to these well studied environmental stimuli, changes in transcript, protein and metabolite levels in response to a gravitational stimulus are less well understood. Amyloplasts, localized in statocytes of the root tip, in mesophyll cells of coleoptiles and in the elongation zone of the growing internodes comprise statoliths in higher plants. Deviations of the statocytes with respect to the earthly gravity vector lead to a displacement of statoliths relative to the cell due to their inertia and thus to gravity perception. Downstream signaling events, including the conversion from the biophysical signal of sedimentation of distinct heavy mass to a biochemical signal, however, remain elusive. More recently, technical advances, including clinostats, drop towers, parabolic flights, satellites, and the International Space Station, allowed researchers to study the effect of altered gravity conditions – real and simulated micro- as well as hypergravity on plants. This allows for a unique opportunity to study plant responses to a purely anthropogenic stress for which no evolutionary program exists. Furthermore, the requirement for plants as food and oxygen sources during prolonged manned space explorations led to an increased interest in the identification of genes involved in the adaptation of plants to microgravity. Transcriptomic, proteomic, phosphoproteomic, and metabolomic profiling strategies provide a sensitive high-throughput approach to identify biochemical alterations in response to changes with respect to the influence of the gravitational vector and thus the acting gravitational force on the transcript, protein and metabolite level. This review aims at summarizing recent experimental approaches and discusses major observations.

Keywords: gravity, plants, systems biology, proteomics, transcriptomics, metabolomics, spaceflight, microgravity

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PLANT RESPONSE TO DEVIATIONS FROM THE VERTICAL POSITION

Gravitropism is defined as the bending of a plant/organ along the direction of the gravity vector. Positive gravitropism describes growth toward the gravity vector, e.g., growth of the root into the soil. Negative gravitropism defines growth opposed to the gravity vector, e.g., growth of the shoot into the air (Frank, 1868). Gravitropic signaling and the role of auxin in gravitropism has recently

been reviewed (Lopez et al., 2014; Sato et al., 2015; Zadnikova et al., 2015). In this review we will only briefly discuss the current models of gravitropic responses and focus on the molecular changes measured by omics techniques.

In *Arabidopsis* roots, the root cap, which comprises of four tiers of columella cells and lateral root cap cells (Dolan et al., 1993), is known to be the site of gravity perception. Early decapping experiments showed a loss of the plant's gravitropic response. The ability to sense alterations of the gravity vector was recovered by regeneration of the root cap (Barlow, 1974). Particularly the inner cells of the second tier of columella cells contribute to root gravitropism as shown by laser ablation experiments (Blancaflor et al., 1998). The gravitropic response, on the other hand, takes place in the elongation zone of the root, physically separated from the site of perception.

In the shoot, mostly coleoptiles and pulvini of monocotyledons and hypocotyledons of dicotyledons were studied in respect to their gravitropic response (Sack, 1991). Genetic studies identified the endodermal cell layer in shoots as statocytes (Fukaki et al., 1998; MacCleery and Kiss, 1999).

Plant gravitropism can be divided into distinct phases: susception, perception, transduction, and response (curvature) (Perbal and Driss-Ecole, 2003; Limbach et al., 2005). Any alteration of the influence of the gravity vector is perceived with the help of specialized, starch-containing organelles, so called statoliths, in gravisensing cells (statocytes), which are sedimenting to the cell's new physiological bottom (Haberlandt, 1900; Caspar and Pickard, 1989; Kiss and Sack, 1989; Kuznetsov and Hasenstein, 1996; Kiss et al., 1997; Sack, 1997; Kiss, 2000; Morita, 2010). According to the starch-statolith hypothesis, sedimentation of the starch-filled amyloplasts triggers a signal transduction cascade leading to an asymmetric auxin transport and a curvature opposite of the gravitational vector. Additional models have been proposed, including the gravitational pressure hypothesis that is based on density differences and consequently the pressure exerted by the cytoplasm on the plasma membrane (Wayne et al., 1990; Wayne and Staves, 1996) or the tensegrity model, in which the membrane is outstretched on the cytoskeleton backbone of the cell and is in equilibrium between tensile and compressive forces (Ingber, 1997). The latter two models are in accordance with experimental data that still show a gravitropic response in starchless mutants (Caspar and Pickard, 1989). Statolith-dependent and -independent systems might also act in parallel (Perbal, 1999). In a refined model of the statolith hypothesis, it was suggested that not the pressure exerted by statolith sedimentation, but their interaction with membrane-bound receptors activates gravity perception (Braun and Limbach, 2006). In characean rhizoids, graviperception requires the contact of statoliths with membrane-bound receptor molecules rather than tension or pressure exerted by the weight of the statoliths (Limbach et al., 2005). Protein interactions between amyloplasts and membrane receptors might involve components of the TOC (TRANSLOCON OF OUTER MEMBRANE OF CHLOROPLASTS) complex (Stanga et al., 2009; Strohm et al., 2014). Experimental data so far, however, are controversial (Staves, 1997; Staves et al., 1997; Braun et al., 2002; Hou et al., 2003, 2004; Limbach et al., 2005; Valster and Blancaflor, 2008).

A common view is, that the biophysical signal of statolith sedimentation or of changes in cytoplasmic pressure is converted into a biochemical signal (Fasano et al., 2001; Plieth and Trewavas, 2002). Models have been put forward in which the sedimentation of statoliths leads to the activation of mechanosensitive ion channels at the plasmamembrane, endoplasmic reticulum, or at the tonoplast (Sievers et al., 1991; Yoder et al., 2001; Allen et al., 2003; Perbal and Driss-Ecole, 2003). In columella cells, the nucleus and the endoplasmic reticulum (ER) are localized at the proximal side of the root meristem and in the periphery of the cell. This ER, called nodal ER (Zheng and Staehelin, 2001), is thought to be a major reservoir for the second messenger Ca^{2+}. Statoliths may induce opening of mechanosensitive ion channels under contribution of the nodal ER and second messenger release (Leitz et al., 2009). Alternatively, statoliths may facilitate the opening of mechanosensitive ion channels under contribution of the cytoskeleton (Sievers et al., 1991; Volkmann and Baluska, 1999; Perbal and Driss-Ecole, 2003). The role of the cytoskeleton is still controversially discussed, but recently the general view is, that the actin cytoskeleton is a negative regulator of root gravitropism (Blancaflor, 2013) and might play a role in fine-tuning the gravitropic response.

Calcium elevations are considered a second messenger of early gravitropic signaling events (Sinclair and Trewavas, 1997; Chen et al., 1999; Chatterjee et al., 2000). The contribution of Ca^{2+} to gravitropism was mainly concluded from inhibitor studies (Belyavskaya, 1996), calcium binding proteins (Stinemetz et al., 1987; Heilmann et al., 2001) or cellular messengers known to be related to Ca^{2+} signaling (Perera et al., 1999). Recent studies on *Arabidopsis* seedlings expressing the luminescent Ca^{2+} reporter Aequorin demonstrated transient increases in $[Ca^{2+}]_{cyt}$ during the gravitropic response (Plieth and Trewavas, 2002; Toyota et al., 2008). Two waves of Ca^{2+} oscillations were observed, an initial transient Ca^{2+} increase after 3 s and a more sustained flux after 60 s (Toyota et al., 2008). Further second messengers, including Inositol 1,4,5- triphosphate (IP_3) (Perera et al., 1999, 2001a; Fasano et al., 2002), protons (Fasano et al., 2001), and reactive oxygen species (ROS; Joo et al., 2001) may also play a role in the gravitropic response. How these secondary messengers interact, their kinetics, and how they establish a response, remains unclear.

Signal transduction eventually leads to the relocalization of auxin carriers (Friml, 2003). Auxin is transported by influx carriers of the AUXIN RESISTANT 1/LIKE-AUX1 (AUX1/LAX) family and the efflux carriers of the PIN-FORMED (PIN) family (Friml, 2003). This transport is known as the "Chemiosmotic Hypothesis" (Kleine-Vehn and Friml, 2008) or Soda fountain model (Hasenstein and Evans, 1988). AUX1 and PIN1 contribute to auxin transport from vasculature into root tip through protophloem cells. In *Arabidopsis*, mutation in AUX1 results in severely agravitropic roots due to defects in auxin movements from the root apex to the distal elongation zone (Swarup et al., 2001). In the tip, PIN4 targets auxin to the center of the auxin maximum, which is located in the columella cells within the root cap. PIN3, PIN4, and PIN7 are localized in the columella cells. In this area, AUX1 ensures the uptake of auxin by columella cells, while the PIN proteins mediate efflux. PIN3 and PIN7 protein

distribution is dependent on the orientation of the root in the gravitational field. When the root is growing vertically, PIN3 and PIN7 are distributed symmetrically in the cell. If there is any deviation from the vertical into the horizontal position, PIN3 and PIN7 relocalize within a few minutes. The efflux carriers are then accumulating in the plasma membrane of the cell's new physiological bottom (Friml et al., 2002a,b; Friml, 2003; Kleine-Vehn et al., 2010). The relocalization of PIN3 and PIN7 are the first steps toward the establishments of a lateral auxin gradient upon gravistimulation (Blancaflor and Masson, 2003). While the total auxin flux in the root stays constant, auxin is redistributed from cells on the upper to the lower side of the root tip within 5 min after a gravitropic stimulus (Band et al., 2012), a timescale that is in accordance with statolith sedimentation and asymmetric changes in root pH and intracellular Ca^{2+}-concentration. *pin3pin7* mutants are more agravitropic than *pin3* and *pin7* single mutants, suggesting their functional redundancy. The corresponding auxin gradient is transported basipetally through epidermal and cortical cells of the root cap that express PIN2. This efflux carrier transports auxin to the elongation zones of the root, which leads to root curvature (Chen et al., 1999; Ottenschlager et al., 2003).

PIN-FORMED protein abundance and localization at the plasma membrane affects gravitropic response. PIN proteins undergo constitutive endocytotic recycling to different domains at the plasma membrane or via the prevacuolar compartment to the lytic vacuole for degradation (Abas et al., 2006; Kleine-Vehn and Friml, 2008). Modulation of vesicular trafficking affects PIN recycling and gravitropic response (Geldner et al., 2004; Paudyal et al., 2014). Recent results indicate that the polar auxin transport (PAT) mediated by PIN proteins can also be modulated by small secretory peptides called GOLVEN. A reduced concentration of those peptides impairs the formation of auxin gradients during tropic responses (Whitford et al., 2012). The GOLVEN signal specifically modulates PIN2 trafficking. Auxin as well as GOLVEN treatment increase PIN2 levels at the plasma membrane (Paciorek et al., 2005; Whitford et al., 2012). Furthermore, Gibberellic acid (GA) increases the level of PIN auxin transporters at the plasma membrane and promotes asymmetric auxin distribution during gravitropic curvature. A dilution and subsequent reduction in GA leads to an increased concentration of growth repressors of the DELLA protein family, which may reduce cell elongation rate (Band et al., 2012; Löfke et al., 2013).

Posttranslational modifications of PIN proteins additionally affect their role in gravitropism. The Ser/Thr kinase PINOID regulates PIN2-mediated basipetal auxin transport by regulating plasma membrane localization of PIN2 (Sukumar et al., 2009; Huang et al., 2010). PIN3 phosphorylation status can also affect root gravitropism (Ganguly et al., 2012). The D6 PROTEIN KINASE (D6PK) may regulate gravitropism via the phosphorylation status of PINs (Barbosa et al., 2014).

Despite the extensive regulation of PIN2, *pin2* mutants are actually not very agravitropic (Chen et al., 1998; Luschnig et al., 1998; Blakeslee et al., 2007). A triple mutant together with members of the p-glycoprotein (PGP) family of auxin efflux transporters, PGP1 and PGP19, however, is severely agravitropic (Blakeslee et al., 2007), indicating functional redundancy in basipetal auxin transport.

The auxin gradient promotes differential cell elongation on opposing sides of the stimulated organ. Auxin is known to promote or to inhibit plant growth in a dose-dependent manner. Growth mediated by auxin is based on the acid-growth theory (Grebe, 2005). Auxin is able to activate proton pumps which lead to the excretion of protons into the cell wall. This acidification may lead to a loosening of the cell wall, allowing the cell to grow and expand. After the growth phase, the cell wall regains stability (Grebe, 2005). The result of an auxin-induced differential cell elongation is a gravitropic curvature.

Auxin can rapidly mediate tropic response on a minute to hour timescale while maintaining stable developmental zonation in the root and then slowly influences size and location of these differentiation zones via the regulation of PLETHORA (PLT) transcription factors (Mahonen et al., 2014). GOLVEN peptides act via positive regulation of PLETHORA transcription factors (Whitford et al., 2012).

Finally, statoliths reposition in the columella cells when the root tip reaches 40°, which leads to the restoration of PIN3/PIN7 localization and symmetric auxin flow, about 100 min after a 90° gravitropic stimulus (Band et al., 2012; Sato et al., 2015). The latter phase of the root gravitropic bending response that can last up to 600 min, is likely orchestrated by newly synthesized target genes of auxin signaling (Band et al., 2012).

PLANTS' RESPONSE TO A MICROGRAVITY ENVIRONMENT

Gravity is a constant factor of life on earth. With the aim to achieve functional weightlessness, a status which is often described as simulated microgravity, different approaches are in use, such as clinostats, random positioning machines as well as magnets for magnetic levitation (Herranz et al., 2013a). The rotation devices are based on the assumption that biological systems need to be exposed to the influence of the gravity vector for a minimal period of time to allow them to adjust to it. If the influence of the gravity vector constantly changes its orientation, the object loses under appropriate simulation conditions its sense of direction and thus shows a behavior similar to the one seen under real microgravity conditions. Real microgravity can be achieved by drop towers, parabolic flights, sounding rockets, satellites, or space stations, like the ISS. Experiment time is limited. Therefore, very little is known of how altered gravity is perceived by the plant and how the system adapts to this new environmental situation. A role for calcium elevations in response to microgravity, as in the response to reorientation of the plant, is still controversial (Häder et al., 2006; Salmi et al., 2011; Hausmann et al., 2014). A transcellular calcium gradient in spores of *Ceratopteris richardii* is reduced within seconds in microgravity, indicating a fast regulation of calcium channels similar to the auxin transport in the root tip during gravity perception (Salmi et al., 2011). In *Arabidopsis* callus cultures, instead, an increase in calcium and

ROS was detected in response to microgravity (Hausmann et al., 2014).

Cellular responses that are affected by microgravity include the cell cycle, leading to decreased mitotic index and enhanced proliferation rate in meristematic root cells (Medina and Herranz, 2010). A second major target is the plant cell wall. In rice, a reduced thickness of the cell wall with increased extensibility and elongation in shoots and decreased elasticity in roots was observed (Hoson et al., 2002, 2003; Soga et al., 2003). Also changes in lignin levels in response to altered gravity forces have been observed in some plant species, e.g., mung beans, but not in others, e.g., pine and oat (Cowles et al., 1984). Changes in photosynthesis in response to microgravity, however, are controversial. While a reduction of the light harvesting apparatus and a higher chlorophyll a/chlorophyll b ratio was observed, direct measurements of photosynthetic activity revealed no changes in net photosynthesis, photosynthetic proton flux, and overall quantum yield (Stutte et al., 2006). The dependency of photosynthesis on gas exchange may be one reason for inconclusive results. The lack of convection in microgravity leads to reduced air flow resulting in altered gas exchange and accumulation of volatiles, e.g., ethylene (Porterfield, 2002), and is possibly leading to alterations in photosynthetic activity.

EXPRESSION CHANGES IN RESPONSE TO A DEVIATION FROM THE VERTICAL ORIENTATION

Complete sedimentation of the statoliths in the columella cells requires at least 5 min (Blancaflor et al., 1998; MacCleery and Kiss, 1999). The minimal gravitational stimulus that elicits a response, however, is estimated around 1 min (Blancaflor et al., 1998). Changes in secondary messenger concentration, e.g., IP3, pH, and Ca^{2+}, have been observed within this time frame. IP3 for example, is stimulated in gravitropic maize pulvini already after 10 s (Perera et al., 1999, 2001b). Observations of the early changes in gene expression may therefore help to identify missing components that translate the biophysical signal of statolith sedimentation into a biochemical signal (**Table 1**). Gravitational stimulation is generally achieved by one-time reorientation of plants in the gravity vector plane. In one of the earliest studies of *Arabidopsis thaliana* seedlings 39 genes showed an altered abundance after 15 min of gravitational stimulation, increasing to 132 genes after 30 min compared to constant 1 g conditions (Moseyko et al., 2002). Functional gene categories included response to oxidative stress, plant defense, heat shock, ethylene response, and calcium binding. Another study on gene expression changes, this time in root apices, identified gravity-specific gene regulations within 5–15 min of reorientation. A cluster of five genes was induced at least three fold by gravitropic stimulation even within 2 min of treatment (Kimbrough et al., 2004). The identified genes contained members of the auxin responsive family, genes that are induced very rapidly by the application of exogenous auxin (McClure and Guilfoyle, 1989). Taken together, these studies indicate that alterations of the influence of the gravitational vector is perceived as an abiotic stress signal when observed on the whole plant level, while in individual cells of plant organs involved in the gravitropic response, auxin signaling plays a major role in signal transmission.

The importance of auxin, also with respect to higher plants' shoot gravitropism, was underlined in at least three transcriptomic approaches. According to the Cholodny/Went hypothesis, an asymmetric auxin transport leads to a curvature in the direction of the gravitational vector (Went and Thimann, 1937). Auxin biosynthesis and signaling transcript levels changed after a deviation from the vertical position or in an already known agravitropic mutant (Esmon et al., 2006; Dong et al., 2013; Taniguchi et al., 2014). While no expression changes were observed after 10 min, at 30 min 30 genes changed in abundance, of which 19 transcripts were auxin responsive genes of the AUX/INDOLE-3-ACETIC ACID INDUCIBLE (IAA) and SMALL AUXIN UPREGULATED (SAUR) families (Taniguchi et al., 2014). Transcript analysis of plants of *Zea mays* wildtype and *zmla1* mutant, a homolog of the *Arabidopsis* LAZY1 gene and an agravitropic mutant regulating PAT, identified 931 alterations in transcript expression. GO annotation of the altered genes and localization studies suggested a function for LAZY1 in auxin signaling and translocation of auxin exporters (PIN proteins) (Dong et al., 2013). When the focus was set on changes in gene expression in opposing flanks of graviresponsive tissue, e.g., hypocotyl, shoot base or inflorescence stems, a role for auxin in these responses became particularly clear (Esmon et al., 2006; Hu et al., 2013; Taniguchi et al., 2014). Two hours of gravitropic and phototropic stimulation of *Brassica oleracea* identified eight genes with increased expression in elongating versus non-elongating hypocotyl flanks under both stimuli. All are members of auxin biosynthesis [GLYCOSIDE HYDROLASE 3.5 (GH3.5)], signaling (SAUR50), and response (EXPANSIN A1) (Esmon et al., 2006). In addition, all eight genes contain at least one consensus AUXIN RESPONSE FACTOR (ARF)-binding auxin response element and no auxin-induced expression was observed in an ARF7 mutant background.

Studies also show that gene regulations in response to gravitational or mechanical stimulations showed a great overlap. In whole *Arabidopsis* seedlings, 55 of the 141 identified genes, changed in abundance by gravitational stimulation, increased or decreased in transcript levels by mechanical stimulation (Moseyko et al., 2002). An even greater overlap between mechanical and gravitational stimulation was found in root apices, where 1730 genes were differentially regulated within 60 min of gravitropic or mechanical stimulation (Kimbrough et al., 2004). The alterations on the transcript level induced by both stimuli overlap by 96%. Many of the altered transcripts show increased or decreased levels in other abiotic and biotic stresses, too. They have functions as transcriptional regulators, in cell wall modification, as transporters, kinases, phosphatases, in hormone metabolism and in the cell cycle.

The first proteomic experiment to map changes in protein expression used 2D-GE and identified 16 alterations on the protein level in *A. thaliana* roots within 2 h of gravitational stimulation, some of them showing an altered abundance after 30 min (Kamada et al., 2005). Functional categories included

TABLE 1 | Studies that quantified differential expression of genes, proteins, and metabolites in response to a deviation from the vertical orientation.

Plant age[1]	Tissue	Replicates	Method	Duration	DEG/DEP/DEM	Growth hardware	Treatment	Reference
Transcriptomics								
3 weeks	*A. thaliana* seedlings	3	8 k array	15, 30 min	15 min: 39 30 min: 132	Petri dish, white light, transfer to dark 16 h prior to treatment	90° (dark)	Moseyko et al., 2002
1 week	*A. thaliana* root apices	2	ATH1	2, 5, 15, 30, 60 min	1730	Petri dish, dark	135° (dark)	Kimbrough et al., 2004
3 days	*B. oleracea* etiolated seedlings, upper and lower flanks of hypocotyl (1 cm)	3	ATH1	2 h	8	Pipette tips, dark	90° (dark)	Esmon et al., 2006
3 weeks	*O. sativa*, upper and lower flanks of shoot base	3	Rice Genome GeneChip	30 min, 6 h	30 min:167 6 h: 1202	Soil, white light, transfer to dark 16 h prior to treatment	90° (dark)	Hu et al., 2013
4–8 cm stem	*A. thaliana*, upper and lower flanks of inflorescence stems	1	*Arabidopsis* 2	10, 30 min	10 min: 0 30 min: 30	Soil, white light	90° (light)	Taniguchi et al., 2014
4 weeks	*Z. mays*, third aboveground node of shoot	2	RNAseq	–	931	Soil, white light	–	Dong et al., 2013
Proteomics								
1 week	*A. thaliana*, root apices	3	2D-GE	30 min, 3 h	16	Petri dish, white light	90° (light)	Kamada et al., 2005
8–10 cm stem	*A. thaliana*, top 4 cm of shoot	3	iTRAQ	2, 4 min	82	Soil, white light	90° (light; 4°C)	Schenck et al., 2013
Transcriptomics and Metabolomics								
1 week	*A. thaliana*, whole seedlings	1 n.a.	ATH1 GC-MS	24 h	Genes: 339 Metabolites: 15	Petri dish, white light	90° (dark)	Millar and Kiss, 2013

[1]*At start of treatment; DEG/DEP/DEM: differentially expressed genes, proteins, metabolites; n.a.: information not available; Rep: number of biological replicates.*

Ca^{2+} signaling, cytoskeleton stability, energy production, TCA cycle and chaperone function. Furthermore, a shift in the apparent molecular weight of proteins due to gravitropic responses was observed that may be caused by an altered glycosylation pattern. One of those proteins with a change in molecular weight is the 20S PROTEASOME β-SUBUNIT E1. According to the authors, the chaperone HEAT SHOCK COGNATE 70-2 and the proteasomal subunit may regulate dynamic processes involved in the response to changes of the gravitational vector.

In order to identify proteins involved in early signaling events, e.g., the conversion from the biophysical signal of sedimentation of amyloplasts to a biochemical stimulus, a study on *Arabidopsis* shoots focused on the early perception and signaling events of plants subjected to 2 and 4 min of a deviation from the vertical. To identify less-/agravitropic signaling mutants, the authors performed plant reorientations at 4°C, an approach known as the gravity-persistent signal (GPS) approach (Wyatt et al., 2002). GPS blocks the asymmetric auxin transport resulting in a lack of gravitropic curvature. If transferred back to room temperature the plant regains a bending phenotype. Using GPS treatment, 82 alterations on the protein level after gravitational stimulation were identified (Schenck et al., 2013). Thirty-five percent of the differentially expressed proteins were predicted to localize to chloroplasts or plastids, consistent with the hypothesis that gravity-sensing is related to these organelles (Kiss et al., 1989).

Promising candidates that were identified as being an important part of the perception of the gravitational vector and subsequent signaling are HEAT SHOCK PROTEIN 81-1 and GLUTATHIONE S-TRANSFERASE PHI 9 (GSTF9) and GSTF20. HSP81-1 is involved in abiotic stress signaling, induced by Ca^{2+} signaling and may interact with J-domain containing proteins like ALTERED RESPONSE TO GRAVITY 1 and ARG1-LIKE 2 that are already known to show reduced gravitropism (Sedbrook et al., 1999; Guan et al., 2003). GSTF9 and GSTF20 may regulate the synthesis of plant hormones or subsequent signaling (Chen et al., 2007; Schenck et al., 2013).

The first transcriptomic and metabolomic approach was a combined treatment of gravi- and photostimulation (blue or red light) of *Arabidopsis* seedlings (Millar and Kiss, 2013). Despite the current methodical limitations in quantification of

primary metabolites, the incorporation of metabolomic profiling into gravitational research was overdue, because this level is the last step prior to the physiological response. Gravity and light treatments led to shifts in amino acid pools (e.g., alanine, asparagine, glutamine, glycine, and isoleucine), decrease of sucrose and increase of hexoses, as well as decreased levels of secondary metabolites. Many of these altered primary metabolites are responsive to abiotic and biotic stresses, underlining the hypothesis of gravitropism and phototropism as exogenous stress stimuli of plants. As an example, changes in the pool of phenylalanine may lead to alterations in flavonoid biosynthesis. Those secondary metabolites are known to have an important function in the crosstalk between ethylene and auxin (Muday et al., 2012). On the transcript level, a 90° treatment for 24 h resulted in 339 alterations in gene abundance. Regulations on the transcript level could be correlated to changes on the metabolite level (Millar and Kiss, 2013). Increased levels of key enzymes of amino acid biosynthesis, THREONINE ALDOLASE 1 and GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1, explain the increase of corresponding metabolites. The altered abundance of carbohydrate metabolism enzymes explains a decrease in sucrose and increase in hexose sugars. Key enzymes of phenylpropanoid biosynthesis, e.g., CHALCONE SYNTHASE, are decreased in abundance.

Results of all studies indicate that changes of the influence of the gravitational vector lead to a general stress response in plants. Analyses on the proteomic and metabolomic level (Kamada et al., 2005; Millar and Kiss, 2013; Schenck et al., 2013) further support the hypothesis that an altered influence of the gravity vector is perceived as environmental stress (Millar and Kiss, 2013; Schenck et al., 2013), emphasizing the contribution of cytoskeleton, calcium signaling and chaperone function to plant's gravitational response. In tissue specific transcriptomic studies, roots or opposing tissue flanks of the shoot, the importance of the phytohormone auxin is underlined. Already after 2 min alterations in auxin responsive genes were observed (Kimbrough et al., 2004). Furthermore, genes of auxin biosynthesis, signaling and response are differentially regulated (Esmon et al., 2006; Dong et al., 2013; Taniguchi et al., 2014). These findings support the widely accepted Cholodny/Went theory, suggesting auxin as a driving factor of tropic responses. A lack of an auxin response in whole seedlings may be caused by a higher dilution of auxin biosynthesis, signaling and response genes in whole seedling RNA, because the action of the phytohormone is restricted to some cell layers in specialized tissues.

SIMULATED MICROGRAVITY AND SPACEFLIGHT

Plants are potential components of future life support systems for manned space travels. For this, plant responses to the space environment, and in particular to microgravity, have to be studied (**Table 2**). Transcriptomic and proteomic studies have been performed toward this goal. In contrast to reorientation experiments, spaceflight and real microgravity experiments pose additional challenges. A suitable hardware providing optimal culture and illumination conditions has to be developed and further spaceflight-related effects on plants have to be taken into account such as increased levels of radiation, poor exchange of gases due to the lack of convection, vibrations, and accelerations depending on the transport systems and operations onboard (Porterfield et al., 1997).

In order to avoid some of these additional environmental effects and allow a discrimination of pure microgravity-related effects, 1 g controls are essential. Under optimal conditions these are realized by the use of onboard 1 g reference centrifuges. It is, however, in most cases common to compare space flight samples to corresponding 1 g ground controls. Efforts have been made to replicate the growth conditions in space for the ground controls by using orbital environment simulators (OES) that replicate light, temperature and CO_2 conditions as recorded in space. Studies utilizing both controls show that more alterations in gene expression are observed comparing plants from space flight (SF) to ground controls (GC) than to on board 1 g flight controls (FC) (Correll et al., 2013; Fengler et al., 2015), suggesting that factors independent of gravity and operational-induced side effects have a profound effect on plant growth and development.

Another consideration is the age of plants that are transported into orbit. Dried seeds do not correspond to environmental changes during the transport phase. Callus cultures and plants that are transported as seedlings or fully grown, however, experience transient changes in accelerations, 1 g on ground, hypergravity during flight phase and microgravity in orbit, and other changes in growth conditions that are not only a result of the space environment.

Different experimental conditions therefore have a great effect on the responses of plants to the space environment. In leaves of wheat, for example, no changes in gene expression were detectable between ground control and space-grown plants (Stutte et al., 2006). Growth of *Arabidopsis* seedlings during space flight though, led to the alteration of 480 genes compared to ground controls (Paul et al., 2013). The latter study was also the first one to study responses in different plant organs separately, indicating that different organs display unique patterns of gene expression in response to spaceflight. From a total of 480 genes that were differentially expressed in leaves, hypocotyls, or roots, only 26 genes were uniformly regulated in all three organs, many of those being involved in cell wall remodeling.

Arabidopsis plants grown in a spaceflight environment are usually smaller than ground controls, possess smaller roots and fewer lateral roots (Paul et al., 2012a). The cell wall of *Arabidopsis* and rice plants is reduced in response to the spaceflight environment (Hoson et al., 2002, 2003; Soga et al., 2002). Furthermore, cell wall extensibility of shoots is increased, while the cell wall flexibility of roots is decreased, as compared to ground controls (Hoson et al., 2003). Transcriptomic and proteomic approaches showed transcripts and proteins associated with cell wall remodeling, root hair generation and cell expansion to be highly altered during spaceflight (Paul et al., 2012b, 2013; Correll et al., 2013; Mazars et al., 2014b; Fengler et al., 2015; Ferl et al., 2015; Kwon et al., 2015; Zhang et al., 2015) or during clinorotation and random positioning (Wang et al., 2006; Barjaktarović et al., 2009). Alterations in

TABLE 2 | Studies that quantified differential expression of genes, proteins, and metabolites in response to simulated microgravity and spaceflight.

Plant age[1]	Tissue	Replicates	Method	Duration	DEG/DEP /DEM	Growth hardware	Treatment	Reference
Transcriptomics								
1 week	*A. thaliana* shoots	1	21K	5 days	SF/GC: 182	PGF, Petri dish, white light	STS93 (light)	Paul et al., 2005
Spores	*C. richardii* spores	4	custom array	1,8,20 h	SF/GC: 365	BRIC, Petri dish, white light	STS93 (light)	Salmi and Roux, 2008
6 days	*A. thaliana* seedlings	n.a.	44K	1 h, 6 days	1 h: 412 6 days: 502	Petri dish, white light	3D clinostat (light)	Soh et al., 2011
Seeds	A. *thaliana etiolated* seedlings and callus culture	6–7	ATH1	13 days	SF/GC: 300	BRIC-LED, Petri dish, dark	STS131 (dark)	Paul et al., 2012b
Seeds	*A. thaliana* leaves, roots, hypocotyls	3	ATH1	12 days	SF/GC: 480	ABRS, Petri dish, white light	ISS (light)	Paul et al., 2013
4 days	*A. thaliana* seedlings	2–3	ATH1	2 days	SF/GC: 230 SF/FC: 27	EMCS, TROPI-2, white light	ISS, (blue, red/blue light)	Correll et al., 2013
Seeds	*B. rapa* var. *nipposinica*	1	RNAseq	27 days	SF/GC: 22.428	LADA, white light	ISS (light)	Sugimoto et al., 2014
Seeds	*A. thaliana* seedlings	3	ATH1	14 days	SF/GC: 192	BRIC, Petri dish, dark	STS131	Kwon et al., 2015
11 days	*A. thaliana* callus cultures	2	ATH1	5 days	SF/GC: 9 SF/FC: 0	SIMBOX, 1.2a plates	Shenzou-8	Fengler et al., 2015
Proteomics								
1 week	*A. thaliana* callus culture	3	2D-GE	8 h	18	Petri dish with solid medium	2D clinostat	Wang et al., 2006
1 week	*A. thaliana* callus culture	3	2D-GE	2 h	Clinorotation: 19 RPM: 8	Tubes and Petri dish	2D clinostat, RPM	Barjaktarović et al., 2007
1 week	*A. thaliana* callus culture	3	2D-GE Pro-Q	30 min	24	Petri dish	RPM	Barjaktarović et al., 2009
4 days	*A. thaliana* root	8	2D-GE	12 h	23	Culture flask	2D clinostat (dark)	Tan et al., 2011
1 week	*A. thaliana* callus culture	3	2D-GE	200 min	Levitation: 60 RPM: 1	Petri dish	RPM (dark) Diamagnetic Levitation (dark)	Herranz et al., 2013b
Seeds	*A. thaliana* seedlings		Label free	12 days	SF/GC: 149	EMCS, GENARA A	ISS (light)	Mazars et al., 2014b
3 days	*A. thaliana* callus culture	2	iTRAQ	14 days	45	SIMBOX	SHENZOU-8	Zhang et al., 2015
Seeds	*A. thaliana* leaves and roots	1–3	iTRAQ	12 days	Leaf: 256 Root: 358	ABRS, solid media plates	ISS	Ferl et al., 2015
8 days	*A. thaliana* roots	3	2D-GE	30 min	μg: 6 90°: 1	Petri dish, white light	2D clinostat (dark)	Schüler et al., 2015
Transcriptomics and Proteomics								
1 week	*A. thaliana* callus culture	2–3	2D-GE Pro-Q ATH1	20 s	PF/FC: 12 DEP PF/FC: 881 DEG	Culture flask with solid medium	Parabolic flight	Hausmann et al., 2014

[1]*At start of treatment; DEG/DEP/DEM, differentially expressed genes, proteins, metabolites; n.a., information not available; Rep, number of biological replicates; ABRS, advanced biological research system; BRIC, biological research in canisters; BRIC-LED, biological research in a canister–light-emitting diode; EMCS, European Modular Cultivation System; FC, 1 g inflight control; GC, 1 g ground control; PF, parabolic flight; PRO-Q, Pro-Q diamond phosphoprotein-stain; RPM, random positioning machine; SF, spaceflight; STS, space transportation system.*

gene expression that have a function in cell wall modification could be caused by spaceflight-induced changes in several hormone signaling pathways that are mediating growth and cell expansion. By using spaceflight 1 g controls and 1 g ground controls to elucidate the response to microgravity and exclude indirect effects by the spaceflight, 27 strictly graviresponsive transcripts were identified that were altered at least twofold in abundance, including genes of cell wall metabolism and actin cytoskeleton organization (Correll et al., 2013). By regulating the transport of cell wall components, the actin cytoskeleton is essential for a proper biosynthesis of the cell wall (Baluska et al., 2002). Also proteomic studies of *Arabidopsis* microsomes and callus cultures supported the involvement of cell wall modifications (Mazars et al., 2014b). A subsequent comparison of 1 g space control and 1 g ground controls of the same flight could furthermore show that cell wall modifying proteins are largely not altered on the protein level, suggesting that cell wall modifying enzymes are necessary for a response specifically to microgravity (Mazars et al., 2014a; Zhang et al., 2015). A comparison of different studies furthermore showed that regulation of protein activity occurs on multiple levels. REVERSIBLY GLYCOSYLATED POLYPEPTIDEs, involved in cell wall metabolism, did not only show a decreased abundance on the protein level (Wang et al., 2006; Schüler et al., 2015), but were also phosphorylated in response to clinorotation (Barjaktarović et al., 2009). Phosphorylation was suggested to affect the activity of the proteins and thereby cell wall metabolism.

In addition to changes in primary and lateral roots, *Arabidopsis* seedlings grown in space experience reduced root hair development (Kwon et al., 2015). This decrease may be due to reduced levels of peroxidases and cell wall modifying genes (Kwon et al., 2015). Out of 174 transcripts showing an altered abundance, 56 are enriched in root hairs and eight were shown to function in root hair development. Mutations in those peroxidases with a decreased abundance by spaceflight, led to a disruption of root hair formation in *Arabidopsis* (Kwon et al., 2015).

In other comparable spaceflight experiments, genes involved in production and response to ROS were altered (Paul et al., 2012b; Correll et al., 2013). A change in ROS levels is a secondary messenger of many abiotic and biotic stresses in plants (Apel and Hirt, 2004). ROS concentrations in the cell are kept in balance by an interplay of ROS production and scavenging via different enzymes and metabolites (Pitzschke and Hirt, 2006). Spaceflight/microgravity affects genes involved in ROS production and homeostasis by up- or downregulation. Increase in hydrogen peroxide levels and increased expression and phosphorylation of ROS scavengers, e.g., SUPEROXIDE DISMUTASE, CATALASE, GLUTATHIONE PEROXIDASE, THIOREDOXIN, and GLUTAREDOXIN, and marker genes were measured in short-term and long-term experiments (Hausmann et al., 2014; Sugimoto et al., 2014). Also in microgravity simulated by clinorotation, genes with antioxidant activity showed increased expression levels in response to short-term and long-term microgravity (Soh et al., 2011). In contrast, proteins of the response to oxidative stress were decreased in *Arabidopsis* calli and in *C. richardii* spores (Salmi and Roux, 2008; Zhang et al., 2015). This may indicate that different isoform of gene families are differentially regulated, as was directly observed in some studies (Barjaktarović et al., 2007; Fengler et al., 2015). ROS changes in plants might be a direct result of auxin signaling. In response to microgravity treatment of roots from wildtype and *pin2* mutant plants, a peroxidase was identified that showed altered levels in the wildtype, but not in the mutant (Tan et al., 2011).

In order to maintain ROS homeostasis for cell function and for signaling, proteins involved in this process are likely to be also posttranslationally regulated. Phosphoproteomic studies show a differential phosphorylation in response to 30 min of microgravity of proteins which are responsive to ROS (Barjaktarović et al., 2009). Taken together, these observations indicate that a complex regulation of antioxidant enzymes is necessary to maintain ROS homeostasis under microgravity, and that different levels of regulation are involved in this process.

Besides ROS, also transcripts involved in calcium signaling are altered under space conditions (Salmi and Roux, 2008; Soh et al., 2011; Paul et al., 2012b; Correll et al., 2013; Mazars et al., 2014b). Plants respond to a spaceflight environment with disruptions of calcium localization and signaling (Klymchuk et al., 2001; Nedukha et al., 2001; Salmi and Roux, 2008). Increase in calcium concentrations have been measured using genetically encoded reporters within 20 s of microgravity during parabolic flights (Hausmann et al., 2014). In addition, up to 25 calcium dependent genes were upregulated at the end of the 20 s microgravity phase. A proteomic approach focusing on microsomal membranes found further evidence for a link between calcium and auxin signaling in response to microgravity. PHOTOTROPIN 2 (PHOT2) is decreased in abundance after 12 days of space flight (Mazars et al., 2014b). PHOT2 is a blue light receptor and can trigger intracellular calcium increases. It was suggest that the calcium increase activates calcium sensors, such as TOUCH3 and PINOID BINDING PROTEIN 1 (PBP1) that interact with the AGC kinase PINOID (PID), a regulator of PAT (Mazars et al., 2014a). In the same experiment, TOUCH3 protein abundance was increased fourfold at the plasma membrane, indicating a calcium dependent regulation of PAT in response to microgravity. This hypothesis was further supported by decreased levels of CATION EXCHANGER 1, a protein of the vacuolar membrane that drives calcium influx from the cytosol to the vacuole, which leads to an increased cytosolic calcium concentration.

Taken together, these data point toward a similar response mechanism in microgravity as described for the reorientation of plants in the gravitational field. In microgravity, calcium is increased, thereby activating calcium binding proteins, leading to the activation of kinases, including CPK11 and PINOID. Calcium influx may directly trigger production of ROS, e.g., via the activation of calcium dependent protein kinases, or indirectly via changes in auxin transport within the plant.

Tissues involved in the perception of the gravitational stimulus, e.g., the root cap, express all proteins necessary for this signaling model. However, other cell types, e.g., undifferentiated cell cultures, are also able to detect a loss of the influence of gravity (microgravity) without specialized

gravisensing tissue (Martzivanou et al., 2006). In these cells, altered ROS production in response to changes in the influence of the gravitational field might be a result of general, but tissue-specific stress responses (Wang et al., 2006; Barjaktarović et al., 2007, 2009, Salmi and Roux, 2008; Herranz et al., 2013b; Fengler et al., 2015). Also in whole seedlings subjected to simulated microgravity or spaceflight, transcripts of stress related genes are oftentimes altered (Kwon et al., 2015). Short-term microgravity environments, e.g., parabolic flights, thereby induce similar transcript changes, i.e., cell wall, heat shock, response to hormones, which are also observed after long term spaceflight experiments. Transcripts of functionally related genes were regulated in different tissues. Expression of individual isoforms, however, was specific to different plant organs (Paul et al., 2013). A comparison of *Arabidopsis* callus cultures and seedlings grown in the same hardware showed two independent responses to spaceflight without similarities in transcript alterations (Paul et al., 2012b). In seedlings as well as callus cultures a response to abiotic and biotic stress was clearly detectable, but more pronounced in cell cultures (Paul et al., 2012b). The response to heat shock is the most prominent Gene Ontology (GO) in cell cultures followed by a general stress response (Paul et al., 2012b; Zupanska et al., 2013). Also in *Arabidopsis* shoots changes in HSP transcripts were observed (Paul et al., 2005). When plant responses to changes of the orientation with respect to the gravitational vector or to changes of intensity of the gravitational field were analyzed in the same study, HSP70-3 was the only protein with altered levels under both conditions (Schüler et al., 2015). Taking into account that the spaceflight environment includes several abiotic stresses, e.g., radiation, microgravity, or vibrations, over-expression of heat shock proteins may contribute to generalized tolerance for multiple alterations in environment conditions and may help to maintain cytoskeletal architecture, cell shaping, and protein remodeling (Swindell et al., 2007; Zupanska et al., 2013).

The notion that microgravity may constitute an abiotic stress, led some authors to screen for genes involved in Simulated Microgravity Stress (SMS; Soh et al., 2011). SMS-genes including WRKY transcription factors and phytohormone induced signaling transcripts were identified. On this gene list, some transcripts are known to be responsive to other biotic/abiotic stresses, too. This is supported by a proteomic approach that identified 18 altered proteins in *Arabidopsis* cell culture after 8 h of 3D clinorotation (Wang et al., 2006). Seven alterations are involved in stress responses (e.g., ALDEHYDE DEHYDROGENASE 2, GST, and CHITINASE). Also in seedlings, proteins, with an altered abundance, and being involved in general stress responses, were identified (Mazars et al., 2014b).

In summary, experimental approaches to impose changes of the gravitational field vary significantly in experimental setup and plant material. However, some common conclusions can be drawn from these studies. The plant cell wall and actin cytoskeleton are major targets for modifications. Earlier studies clearly show a spaceflight-induced cell wall thinning (Hoson et al., 2002, 2003). Root hair growth is also highly reduced in space (Kwon et al., 2015). The authors suggest a role of ROS in the alteration in growth. Results from further studies suggest that cell wall modifications of plants subjected to spaceflight may be directed by changes to the actin cytoskeleton (Correll et al., 2013; Mazars et al., 2014b).

Another response to simulated microgravity and spaceflight seems to be a general response to abiotic and biotic stresses. Especially cell cultures show a heat shock response. Those chaperones may help to maintain cytoskeletal architecture and cell shaping in a spaceflight environment (Zupanska et al., 2013). Furthermore, the biosynthesis and response to phytohormones and calcium signaling is altered under simulated microgravity and spaceflight conditions supporting the hypothesis that a highly reduced gravity environment resembles an abiotic and/or biotic stress in plant tissues (Salmi and Roux, 2008; Soh et al., 2011; Correll et al., 2013; Mazars et al., 2014b). Another stress marker is the change in cellular ROS levels. In some of the reviewed publications an increase of ROS scavengers (Sugimoto et al., 2014), an elevated level in hydrogen peroxide (Hausmann et al., 2014) and an alteration of genes with antioxidant activity (Soh et al., 2011) was shown if plants were subjected to spaceflight or simulated microgravity. In addition, dependency of some responses on the PIN2 auxin transporter and changes in calcium concentrations point towards a signaling mechanism in microgravity, involving calcium, ROS and auxin, that is comparable to the response to a reorientation in the gravitational field.

CONCLUSION/FUTURE PERSPECTIVES

The reviewed studies identified molecular components of plant responses to changes in the gravitational field or vector. It was previously known that calcium and auxin play a role in these processes. Omics profiling strategies allowed the identification of underlying genes and proteins with altered abundance by these messengers or that affect the function of these messengers. Especially alterations in the concentration of auxin biosynthesis (GH3.5) and auxin responsive (AUX/IAA, SAUR) genes have been observed. A comparison of these studies furthermore indicates that different plant organs and callus cultures respond differently to changes in the influence of gravity. More cell type specific studies are necessary to identify how different cell types respond and how these cell types interact to form plant responses. Since experimental time in space is limited, experiments in ground-based facilities (GBFs) will be extensively needed to compensate for space experiments and to provide the necessary number of replicates for robust results.

Hardware-specific differences between individual studies as well as differential operations of GBFs contribute to difficulties in understanding plant responses to changes in the influence of the gravitational field. Standardization of growth and experimental hardware has to be pursued for ground-based and flight facilities (Schüler et al., 2015). Validated operational parameters of simulation approaches and 1 g flight control will have to be an essential component in all space flight experiments. The

generation of more robust data would also benefit from scientific collaborations that perform the same experiments over multiple missions using standardized hardware. Sequential extraction protocols for RNA, proteins and metabolites would also reduce biological material and facilitate analyses across different levels of responses.

One of the main tasks for the future will be the integration of different datasets, covering various levels of cellular responses, ions, transcript changes, proteins, and hormones, into a common database to allow researchers to cross-analyse all results between different experimental conditions, tissues, and organisms. This will increase statistical power as compared to individual analyses with limited biological replicates. Larger datasets also allow for the development of mathematical models that are both descriptive and predictive and enable the generation of testable hypotheses. Complementary to broad omics techniques, reporter techniques with single cell resolution, e.g., genetically encoded hormone-, pH- and Ca^{2+}-reporters, should be used to study signaling events and signal transmission, e.g., from the columella cells to the elongation zone. Suitable hardware to study single cell response to changes in the influence of the gravitational vector is available in the form of microscopes with a vertical sample stage. Microscopes for cell specific studies under microgravity conditions have also been made available.

Next logical steps after the development of testable mathematical models are physiological tests of the coding genes by experiments with mutant lines and overexpression lines. These are necessary to further corroborate their function in gravitational responses. Limitation in experiments with transgenic plants in space and ground-based facilities can be overcome with the application of chemically mutagenized lines or newer techniques, e.g., CRISPR/Cas.

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Crop improvement using life cycle datasets acquired under field conditions

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Crops are exposed to various environmental stresses in the field throughout their life cycle. Modern plant science has provided remarkable insights into the molecular networks of plant stress responses in laboratory conditions, but the responses of different crops to environmental stresses in the field need to be elucidated. Recent advances in omics analytical techniques and information technology have enabled us to integrate data from a spectrum of physiological metrics of field crops. The interdisciplinary efforts of plant science and data science enable us to explore factors that affect crop productivity and identify stress tolerance-related genes and alleles. Here, we describe recent advances in technologies that are key components for data driven crop design, such as population genomics, chronological omics analyses, and computer-aided molecular network prediction. Integration of the outcomes from these technologies will accelerate our understanding of crop phenology under practical field situations and identify key characteristics to represent crop stress status. These elements would help us to genetically engineer "designed crops" to prevent yield shortfalls because of environmental fluctuations due to future climate change.

Keywords: population genomics, transcriptome, epigenome, crop phenology, machine learning

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Introduction

Abiotic stress conditions can have a negative effect on the productivity of agricultural systems. According to a recent report from the Intergovernmental Panel on Climate Change (IPCC), humanity is facing an increased risk of agricultural production shortfalls (https://www.ipcc.ch/report/ar5/). Modern plant science has achieved remarkable advances in elucidating the molecular systems associated with abiotic stress responses in plants under artificially controlled conditions inside the laboratory. This is especially true for the model plant species *Arabidopsis thaliana*, where functional genomic analyses after the completion of sequencing its genome have identified key genes involved in the regulatory network of abiotic stress responses (Hirayama and Shinozaki, 2010; Nakashima et al., 2014). However, several critical problems remain regarding the practical application of this laboratory-derived knowledge to molecular science based breeding of crops adapted to adverse environments. The next challenge in generating practical stress-tolerant crops that can withstand future climate changes requires an understanding of the responses of crops to multiple abiotic stresses under field growth conditions. Large fluctuations in multiple abiotic stress conditions and large heterogeneity between stress levels for different plant

genotypes and developmental stages are the chief causes of the complexity underlying variations in abiotic stress responses in crops under field conditions (Mittler and Blumwald, 2010).

The considerable recent advances in analytical technologies in omics-based research have provided crucial resources for investigating biological systems not only in model plants but also crop species (Mochida and Shinozaki, 2010). With large-scale transcriptome datasets, it will be feasible to perform correlated gene expression analyses to identify candidate genes involved in particular gene networks (Mochida et al., 2011; Obayashi et al., 2014). Metabolome analyses provide information on the accumulation patterns of metabolites in plants in various biological contexts, such as changes in environment, developmental stage, and genotype, and offer an efficient approach to revealing the metabolic systems underlying complex phenotypes (Tohge et al., 2011; Balmer et al., 2013; Fukushima and Kusano, 2013). Hormonomic analysis, which enables simultaneous profiling of phytohormones and their derivatives, also plays an important role in investigating phytohormone networks in different biological contexts (Kojima et al., 2009; Kanno et al., 2010). Integrated approaches using synergistic combinations of different omics systems, so called "*trans*-omics," are increasingly an effective means of investigating plant cellular systems in response to abiotic stresses (Dinakar and Bartels, 2013; Deshmukh et al., 2014). Furthermore, in the last decade, rapid progress in next-generation sequencing (NGS) technologies has enabled access to genome-scale sequence information from a wide range of organisms, even those with large and complex genome structures such as wheat and barley (Mochida and Shinozaki, 2011, 2013). Whole genome resequencing is a feasible NGS application for exploring genome-scale polymorphisms in natural variations, and to identify the association between genetic polymorphisms and phenotypic variations including those induced by stress. Another NGS application, RNA-seq, is highly scalable and can be used to rapidly acquire comprehensive transcriptome data in any species. The effective use of genome-scale datasets from various types of omics analyses rely on computer-aided approaches that have become increasingly important in studies to determine the responses of plant cellular systems to environmental changes. A broad range of bioinformatics techniques are essential to access large-scale omics datasets and to efficiently discover biologically significant information and then use this to answer specific questions on stress responses in plants. Systems approaches with mathematical modeling have recently received much attention for understanding biological phenomena under both controlled laboratory conditions and fluctuating field conditions.

With the currently available methods and resources for studying plant stress responses, it is expected that interdisciplinary efforts involving plant science and data science will enable exploration of factors that affect crop productivity and will aid discovery of genes and alleles associated with quantitative traits of stress tolerance in crops. It is essential not only to examine a snapshot of the cellular network under multiple stress conditions at a particular moment but also to monitor throughout the life cycle, since changes in physiological status over time might influence the eventual phenotype. The identification and estimation of the effects of parameters, based on an understanding of the genetics and physiology of responses to environmental changes of crops throughout their life cycle, are required to design a crop with the required performance of stress tolerances in the field condition. In this mini review, we provide an overview of recent advances in technologies that are key components for data driven crop design, such as crop population genomics, chronological *trans*-omics analysis, and computer-aided molecular network prediction (**Figure 1**).

Population Genomics in Crops

Genetic diversity in a crop population is a valuable resource for identifying alleles that can be exploited to improve crop productivity under a variety of adverse conditions (Huang and Han, 2014). Population-wide molecular phylogeographic analysis of a crop species can provide molecular evidence on its demographic history as a domesticated species (Saisho and Purugganan, 2007). Additionally, such analysis may identify relationships between biased geographic distributions and genetic differentiation, such as the particular genotype associated with a trait providing adaptation to a particular local environment. For example, in barley, a population-wide analysis of bio-geography and the degree of vernalization requirement showed a biased geographic distribution pattern of a quantitative growth habit trait (Saisho et al., 2011). As another example, a population-scale evolutionary analysis of *HvAACT1*, which encodes a citrate transporter involved in aluminum tolerance in barley and has a 1-kb insertion for Al-tolerance in the upstream region, only occurs in Al-tolerant cultivars in Japan, Korea, and China, suggesting adaptation to the acid soils of these areas (Fujii et al., 2012). These examples in barley demonstrate that population-scale exploration of the association between geographic distributions and genotypes could be an efficient strategy to identify alleles for locally adapted traits. The development of NGS has allowed high-throughput genotyping such as whole-genome re-sequencing, genotyping by sequencing (GBS), RNA-seq based genotyping, and exome sequencing, to rapidly generate genome-scale datasets on genetic polymorphism.

Whole genome re-sequencing analysis with information from a reference genome is a straightforward method to characterize genome-wide polymorphism patterns among accessions. Representative accessions, for example, elite lines in tomato, soybean, maize, and rice, have been investigated by whole genome re-sequencing, which has identified useful resources for further genetic studies in each crop (Lai et al., 2010; Arai-Kichise et al., 2011; Subbaiyan et al., 2012; Causse et al., 2013; Li et al., 2013). In some species with smaller genomes, the whole genome re-sequencing approach has been applied to population-wide analyses of genome-wide polymorphism patterns; this approach has been employed in poplar tree, tomato, common bean, and rice (Evans et al., 2014; Lin et al., 2014; Schmutz et al., 2014). Huang et al. (2012) carried out a whole-genome resequencing analysis in wild rice populations to generate a genome variation map, which also provided insights into the domestication history of domesticated rice. More recently, a core collection of 3000 rice accessions from 89 countries were re-sequenced and 18.9 million

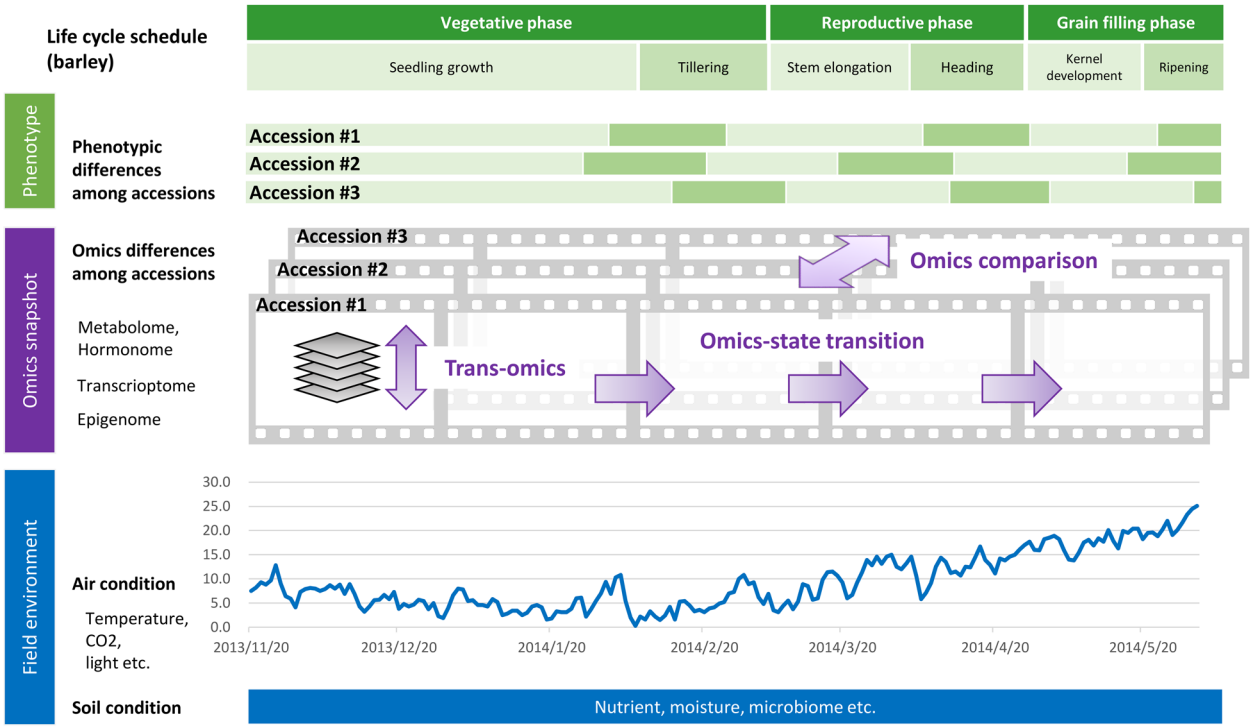


FIGURE 1 | Phenology datasets during the life cycle of a crop grown under field conditions. Time series omics data such as transcriptome, epigenome, and metabolome/hormonome data can be acquired as sequential "snapshots" throughout the crop life cycle. Parameters regarding field environment including air and soil conditions can be acquired as "streamed" datasets.

single nucleotide polymorphisms (SNPs) were found (Li et al., 2014a). A whole-genome resequencing dataset on a population wide scale can provide an important resource especially for understanding the demographic history of a domesticated species, and facilitate recognition of alleles associated with adaptive phenotypic variations, for example, tolerance of particular environments, by applying the resequencing dataset together with a dataset of the trait based on a genome-wide association study (GWAS).

Genotyping by sequencing or RNA-seq based genotyping are more affordable approaches than whole genome sequencing for genome-wide and population wide genotyping. GBS is a popular method that provides a rapid and robust approach for identifying sequences with a low level of representation in multiplex samples (Elshire et al., 2011; Poland et al., 2012). A number of genome-wide polymorphism datasets have been obtained from GBS analysis, for example, 2815 accessions of the USA national maize inbred seed bank using 681,257 SNPs (Romay et al., 2013), 971 worldwide accessions of sorghum with ∼265,000 SNPs (Morris et al., 2013), and 304 short-season soybean lines with >47,000 SNPs (Sonah et al., 2015); these have also been applied to GWAS analysis (so called GBS-GWAS analysis).

High-throughput genome-scale genotyping is a key technology to finding adaptive genes that might be of promise for improving crop productivity in particular environments. Careful analysis of associations between genome-wide patterns of polymorphism and phenotypic variations in adaptive traits holds great promise for elucidating crop species domestication histories at both the ecological and evolutionary levels (Huang and Han, 2014). Furthermore, such analyses enable the estimation of the genetic effects of candidate allelic combinations and quantification of heritability, which are critical parameters to production of reliable allelic combinations in the designed crop varieties.

Omics-Based Elucidation of Crop Phenology

Crops in the field are exposed to multiple environmental stimuli. Crop life cycle changes are often triggered by environmental signals, for example, temperature- and photoperiod-related cues for flowering, and timely initiation of these developmental changes is critical to final productivity. Therefore, understanding the physiological responses of crops to seasonal and short-term fluctuations in the environment is vital to estimation of their potential impact on the crop life cycle and eventual yield. For this purpose, omics-based long-term chronological profiling of crops under field conditions is an efficient strategy for characterizing phenological responses in gene regulatory networks. Such analyses provide insights into the regulation of gene functions in response to environmental fluctuations and are an aid for the identification of genes that are key mediators between environmental signals and crop productivity (Gibson, 2008).

Time-series transcriptome analysis during plant life cycles has become an efficient approach to infer phenological responses under variable environmental conditions. Richards et al. (2012) performed a time-series transcriptome analysis in *A. thaliana* shoots in the field from seedling to reproductive stages and found enrichment of several co-expressed gene clusters that were induced by abiotic and biotic stresses. Several studies have investigated the dynamics of genome-scale gene expression patterns using transcriptome analysis of a life cycle sample series from cultivated rice plants grown under field conditions (Sato et al., 2011; Nagano et al., 2012; Matsuzaki et al., 2015). It was shown that mathematical modeling and prediction of genome-wide transcriptional changes under field conditions could be successfully carried out based on life cycle transcriptome datasets and meteorological datasets (Nagano et al., 2012). Similarly, the transcriptome in a single clone of a grapevine cultivar was recorded over three consecutive years in 11 vineyards and it was demonstrated that the additive effects of temperature and water availability particularly influenced grape quality (Dal Santo et al., 2013).

Gene expression in response to developmental and environmental signals are often regulated by epigenetic mechanisms through small RNAs, histone modifications and DNA methylation (Chinnusamy and Zhu, 2009; Kinoshita and Seki, 2014). Recent studies in plants have shown that epigenetic mechanisms are involved in some important biological processes such as genomic imprinting, defense responses to pathogens, acclimation to abiotic stresses, and vernalization responses (Ikeda, 2012; Kim et al., 2012; Woods et al., 2014; Liu et al., 2015). Furthermore, some of these epigenetic modifications are inherited through mitotic and meiotic cell divisions. The meiotically heritable epigenetic modifications are termed "epialleles" and can cause heritable phenotypic variation (Kalisz and Purugganan, 2004; Weigel and Colot, 2012). In epigenetic regulation of plant stress tolerance, non-heritable epigenetic modifications are involved in acclimation as a short-term stress resistance response. Mitotically and meiotically heritable epigenetic modifications function as a "stress memory" within and across generations, respectively (Chinnusamy and Zhu, 2009). A recent study of epigenetic recombinant inbred lines (epiRILs) of *A. thaliana* showed that variations in DNA methylation cause heritable variation of ecologically important plant traits, such as root allocation, drought tolerance and nutrient plasticity (Zhang et al., 2013). Plant epigenome data are therefore vital to the understanding of epigenetic and genetic regulation of phenotypic diversity (Schmitz et al., 2013). It is now recognized that epigenetic diversity in populations and epigenetic changes in response to environmental fluctuation are also considerable factors in adaptation and evolution and could be a resource for improvement of crop stress tolerance.

Phenome analyses provide datasets on a variety of phenotypes using mutants and/or natural variants. With large-scale loss-of-function or gain-of-function mutants, phenome analyses using artificially induced mutants have played an essential role in discovering genes involved in phenotypic changes and for determining their biological functions (Kuromori et al., 2006, 2009). Recent advances in technologies such as sensors, imaging, and internet communication have begun to provide various tools for high-throughput plant phenotyping under field conditions (Klukas et al., 2014; Li et al., 2014b; Fahlgren et al., 2015; Grosskinsky et al., 2015). Remote phenotyping of crops in the field is emerging as a feasible application for drones with multiple sensors, not only for trait analysis in genetics but also for precision agriculture (Liebisch et al., 2015). Hand-held devices that aid phenotyping can be an efficient tool to carry out high-throughput phenotypic data acquisition (Vankudavath et al., 2012). Integration of imaging and sensing technologies have provided tools for non-invasive approaches to monitor biometrics of growing crops (Busemeyer et al., 2013; Li et al., 2014b; Kjaer and Ottosen, 2015). High-throughput plant phenotyping approaches have been synergistically applied to genetics to accelerate gene discovery in crops. For example in rice, a high-throughput rice phenotyping facility (HRPF) makes it possible to monitor 15 traits during the rice growth period; and these data can be applied to GWAS (Yang et al., 2014). In addition to conventional phenotyping, quantitative molecular profiles from various high-throughput analytical techniques such as metabolomics could be used as a comprehensive dataset of molecular phenotypes.

Metabolome analysis provides a comprehensive molecular snapshot based on metabolites synthesized in biological reactions. It can be affected by various factors, such as genetic and epigenetic factors, developmental stages and organs, environmental stimuli and diseases. Therefore, it could be thought that the metabolome can represent chemical phenotypes reflecting the physiological state in an organism (Mochida et al., 2009; Sakurai et al., 2013). The combinatorial use of high-throughput metabolome profiling and GWAS has become an efficient strategy to reveal the genetic architecture of biochemical properties in plants (Adamski and Suhre, 2013; Wen et al., 2014; Matsuda et al., 2015). Metabolome profiling at different plant developmental stages can provide stage-dependent information on the physiological state of the plant in response to the environment during the lifecycle (Onda et al., 2015). Therefore, chronological metabolome analysis throughout the plant life cycle under field conditions will also be a vital strategy to describe the physiological state and to extract state factors associated with traits in crops.

Computer Aided Understanding of Biological Phenomena in Plants

As described above, recent advances in omics analytical technologies have produced a wealth of genome-scale datasets even from crops growing in field conditions. One of the important issues in bioinformatics is how to deal with such large and heterogeneous datasets, and to establish heuristic procedures to accelerate gene discovery (Mochida and Shinozaki, 2011). Information resources such as databases and computational tools are extremely important for effectively handling genome-scale datasets. Additionally, data storage for omics datasets must ensure persistence and retrieval functionalities for shared use (Mochida and Shinozaki, 2011).

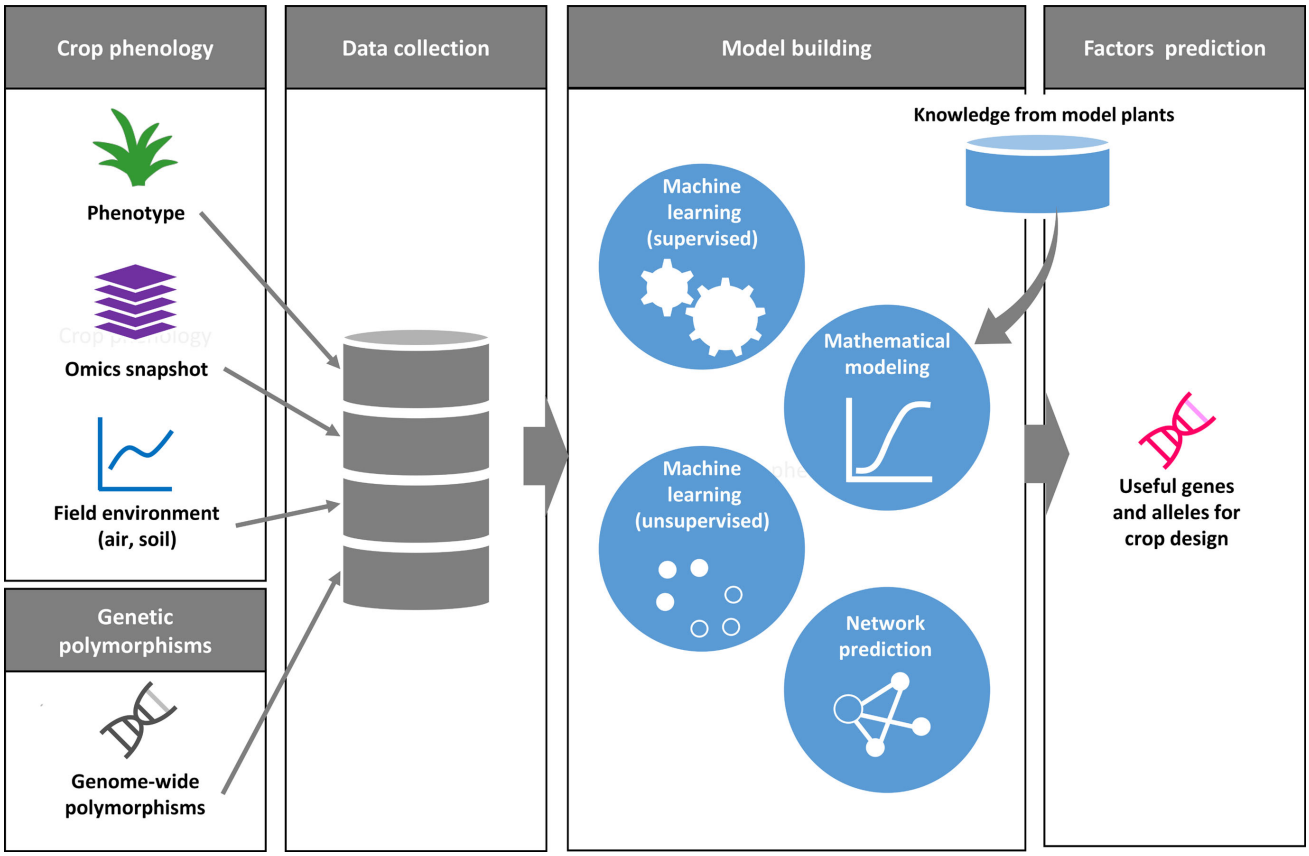


FIGURE 2 | A conceptual framework for phenology data driven crop design. Phenology datasets include plant phenotypes, omics snapshots with environmental data. A genome-wide polymorphism dataset is useful to find genetic association between accessions with traits as well as with physiological state. For model building, the collected data are applied to various types of computer-aided methods. Digitized datasets can also be assessed against data on well-characterized gene functions in model plants. Machine learning-based data clustering and network prediction may help us to identify candidate genes and alleles for crop improvement.

To gain a mechanistic understanding of biological systems, mathematical modeling and simulation approaches have been applied to the study of plant cellular metabolism, growth, developmental processes, and responses to the environment (Aikawa et al., 2010; De Vos et al., 2012; Katsuragi et al., 2013; Satake et al., 2013; Miyazaki et al., 2014). Mathematical modeling approaches are also used to understand a wide range of biological functions in growth, survival, and reproduction in plants, for example, in the circadian regulation of plant carbohydrate metabolism (Webb and Satake, 2015), phloem sucrose transport associated with rice grain yield (Seki et al., 2015), and silicon uptake in rice roots (Sakurai et al., 2015).

Machine learning is a field of computer science for the design of computational algorithms that automatically improve with experience. In the last two decades, this research field has dramatically advanced with the emergence of artificial intelligence and data science, and has been applied in various fields in science, technology and commerce (Jordan and Mitchell, 2015). Machine learning is also used in applications for the analysis of genome-scale datasets and other large-scale omics datasets in life science (Libbrecht and Noble, 2015). Learning methods in machine learning are usually classified into two primary categories of supervised and unsupervised learning. The supervised machine learning aims to produce an algorithm to predict output on unknown input via a training process using a dataset of known pairs of input and output. The unsupervised machine learning methods are used to extract structures and identify their features in a given dataset without examples for training. Computational modeling using machine learning has been performed recently with the aim of predicting gene networks based on large-scale transcriptome datasets in plants. For example, in *Arabidopsis*, supervised machine learning was used to build a network model of responses to stress conditions, to explore genes related to stress responses, and to predict molecular interactions (Ma et al., 2014; Nourani et al., 2015). Machine learning provides a data-driven approach to extract latent rules or patterns from a comprehensively collected dataset without any biased view on the biological phenomena of interest (**Figure 2**).

Conclusion and Future Perspectives

Cross-disciplinary research, including computer science, functional genomics, and crop phenology, will provide a unique opportunity to establish technologies for data-driven crop

design to prevent crop yield shortfall under changing future environments. It is expected that a synergy of life science and data science will allow us to perceive novel and latent values underlying the observed dataset by unbiased data-driven analyses. Unbiased illustration of physiological state dynamics of crops growing under field conditions could be an efficient strategy to figure out features of genetic factors but also "state factors" that determine eventual agronomical traits. Another issue for the data-driven approach is how we fill the gap between findings from model plants studied in laboratories and those from crops under field conditions to generalize our knowledge on plant systems including those in response to environmental changes. Complementary use of hypothesis-driven and data-driven approaches should be a practical way for further understanding of physiological responses to field environments with cross-referencing to knowledge from model plants that has been accumulated in laboratories. Therefore, platforms for computing and linking life science data will also play more significant roles in research on data-driven crop breeding.

Author Contributions

KM, DS, and TH conceived research and wrote the manuscript.

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frontiers in Plant Science

REVIEW
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Jasmonates: Emerging Players in Controlling Temperature Stress Tolerance

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The sedentary life of plants has forced them to live in an environment that is characterized by the presence of numerous challenges in terms of biotic and abiotic stresses. Phytohormones play essential roles in mediating plant physiology and alleviating various environmental perturbations. Jasmonates are a group of oxylipin compounds occurring ubiquitously in the plant kingdom that play pivotal roles in response to developmental and environmental cues. Jasmonates (JAs) have been shown to participate in unison with key factors of other signal transduction pathway, including those involved in response to abiotic stress. Recent findings have furnished large body of information suggesting the role of jasmonates in cold and heat stress. JAs have been shown to regulate C-repeat binding factor (CBF) pathway during cold stress. The interaction between the integrants of JA signaling and components of CBF pathway demonstrates a complex relationship between the two. JAs have also been shown to counteract chilling stress by inducing ROS avoidance enzymes. In addition, several lines of evidence suggest the positive regulation of thermotolerance by JA. The present review provides insights into biosynthesis, signal transduction pathway of jasmonic acid and their role in response to temperature stress.

Keywords: jasmonates, abiotic stresses, heat, cold, signaling

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INTRODUCTION

Adequate perception, amalgamation and transduction of signals are obligatory for the growth and development of an organism. Plant hormones are a group of structurally diverse signal molecules that organize all cellular processes, consequently ensuring an effectual developmental plan and rationalized use of resources. Plant hormones therefore act as middlemen in the transmittance of information from the environment to the organism. The study of plant hormones is centuries old when the 5 classical hormones auxin, cytokinin, ABA, GA, and ethylene were described. However, over the last decade many "non-traditional" plant growth regulators have been described. These include highly diverse group of oxidized compounds, collectively known as oxylipins. Oxylipins execute diverse functions ranging from developmental processes to stress responses in plants (Andersson et al., 2006). Plant oxylipins can be produced either enzymatically by LIPOXYGENASES (LOXs) or α-DIOXYGENASES (α-DOXsas) or non-enzymatically by autoxidation of polyunsaturated fatty acids (Göbel and Feussner, 2009). One of the well characterized examples of oxylipins is Jasmonates (JAs).

The history of jasmonates is very old and dates back to 1960 when Demole successfully characterized methyl jasmonate (MeJA) from jasmine flower *Jasminum grandiflorum*.

(Demole et al., 1962). Like many small esters, MeJA is volatile and has a sweet fragrance. Jasmonic acid (JA), on the other hand was isolated from fungal culture filtrate of *Lasiodiplodia theobromae* (Aldridge et al., 1971). JAs modulate many essential roles in plant development ranging from germination to vegetative growth to senescence. The role of JAs in dicotyledons such as tomato and *Arabidopsis* is well known, they are directly entailed in a number of physiological processes like stamen and trichome development, vegetative growth, cell cycle regulation, senescence, anthocyanin biosynthesis regulation, fruit ripening, cell cycle regulation (Parthier, 1991; Koda et al., 1992; Sembdner and Parthier, 1993; Creelman and Mullet, 1995, 1997; Koda, 1997; Wasternack and Hause, 2002; Browse, 2005; Wasternack, 2007; Balbi and Devoto, 2008; Pauwels et al., 2008; Zhang and Turner, 2008; Reinbothe et al., 2009; Yoshida et al., 2009). In addition, JAs activate plant defense mechanisms in response to insect-driven wounding, pathogen attack, and environmental stress, such as low temperature, salinity, heavy metal toxicity (Creelman and Mullet, 1997; Wasternack, 2007; Howe and Jander, 2008; Browse, 2009; Pauwels and Goossens, 2011). Studies in monocots have also confirmed the indispensible role of JAs in reproductive bud initiation and elongation, sex determination, leaf senescence and responses to the attack by pathogens and insects (Engelberth et al., 2004; Tani et al., 2008; Acosta et al., 2009; Yan et al., 2012).

BIOSYNTHESIS OF JASMONATES

JAs are biosynthesized by the sequential action of enzymes present in plastid, peroxisome and cytoplasm (Feussner and Wasternack, 2002). JA biosynthesis is initiated by the release of α-LINOLENIC ACID (α-LeA) (18:3) from chloroplast membranes by PHOSPHOLIPASE1 (PLA1) to generate JA substrate (Vick and Zimmerman, 1983). α-LeA liberation is followed by the incorporation of molecular oxygen by the lipoxygenase family enzyme, LINOLEATE OXYGEN OXIDOREDUCTASE (13-LOX) at carbon atom 13 of the substrate forming 13*S*-HYDROPEROXY-(9*Z*,11*E*,15)-OCTADECATRIENOIC ACID (13-HPOT). 13-HPOT undergoes dehydration by the ALLENE OXIDE SYNTHASE (AOS) to form cis (+)12-OXO-PHYTODIENOIC ACID (OPDA) (Turner et al., 2002; Devoto and Turner, 2003; Wasternack, 2007). Similarly, LOX, AOS and AOC together catalyze hexadecatrienonic acid (C16:3) to form dinor-OPDA (dnOPDA). OPDA and dnOPDA are further transported to peroxisome via transporter COMATOSE (CTS1) (Theodoulou et al., 2005), wherein, they are reduced to OXOPHYTOENIC ACID (OPC-8) and 12-OXOPHYTOENIC ACID (OPC-6), respectively by OPDA reductase 3 (OPR3). OPDA, dnOPDA, OPC8 and OPC6 are activated by the ACYL-COENZYME A SYNTHETASES to form CoA esters, so that the carboxylic acid side chains can be shorted by two or three rounds of β-oxidation by ACYL-COA OXIDASE (ACX), a MULTIFUNCTIONAL PROTEIN (MFP), and L-3-KETOACYL COA THIOLASE (KAT) (Schneider et al., 2005). Jasmonoyl-CoA, the final product of the β-oxidation reactions, is cleaved by THIOESTERASE (TE) to form cis-7-iso-jasmonic acid [(+)-7-iso-JA]. It is then catabolized further by JA CARBOXYL METHYLTRANSFERASE (JMT) to form volatile counterpart MeJA. MeJA ESTERASE (MJE) in turn converts MeJA back to JA. The reversible conversion between JA and Jasmonoyl-isoleucine (JA-Ile) is catalyzed by a JASMONATE AMINO ACID SYNTHETASE (JAR1).

METABOLIC FATE OF JA

There are plethora of jasmonate compounds. JA goes through several biochemical modifications (Sembdner and Parthier, 1993; Koch et al., 1997; Seo et al., 2001; Staswick and Tiryaki, 2004; Swiatek et al., 2004). JA, Cis Jasmone (CJ), MeJA and JA-Ile have some biological activity in plants (Wasternack, 2007; Fonseca et al., 2009a). CJ, a volatile counterpart of JA is biologically active and is released in response to herbivory and insect driven attack. Transcriptome analysis data of CJ treated *Arabidopsis* plants provided insights into a *COI1*-independent CJ signaling (Matthes et al., 2010).

Miersch and co-workers in 2008 reported the presence of high levels of 12-OH-JA, 12-HSO4-JA, and 12-O-Glc-JA in immature seeds and leaves of *Glycine max* and *Zea mays* (Miersch et al., 2008). The role of jasmonates (JAs) 12-OH-JA, 12-HSO4-JA, and 12-O-Glc-JA in sex determination has been studied in *Zea mays* (Acosta et al., 2009; Browse, 2009). JA, MeJA, and CJ are considered useful tools in anti-cancer therapy as they are known to induce cell death by mitochondria perturbation and subsequent release of cytochrome oxidase (Kim et al., 2004; Rotem et al., 2005). Michelet et al. (2012) reported the anti-aging potential of tetra-hydro-jasmonic acid in humans. Tetra-hydro-jasmonic acid is known to increase the synthesis of hyaluronic acid by increasing the expression of hyaluronase synthase 2 and hyaluronase synthase 3.

JASMONIC ACID PERCEPTION AND SIGNALING

JA signal perception and transduction involve numerous TFs, repressors and members of ubiquitin proteasomal pathway. The section gives information of different signaling components. The current model of JA signal transduction is given in **Figure 1**.

Bioactive Ligand

Fonseca and co-workers in 2009 provided evidences that (+)-7-iso-JA-L-Ile is the sole natural ligand of *A. thaliana* as revealed by detailed GC-MS and HPLC analyses. Also, experiments carried out by Thines et al. (2007) revealed that only JA-Ile out of MeJA, OPDA, and JA can promote *COI1*-JAZ binding, thus confirming JA-Ile to be the direct JA signaling ligand in plants.

SCF Complex

The ubiquitin-proteasome comprises of Skp1/Cullin/F-box (SCF). Earlier, researchers believed that screening *Arabidopsis* mutants insensitive to growth inhibition with bacterial coronatine, a structural and functional homolog of JA-Ile, would result in discovering JA receptor in plants (Feys et al., 1994; Fonseca et al., 2009b). Exhaustive genetic screens identified the allele of *coronatine insensitive 1 (coi1),* suggesting *COI1*

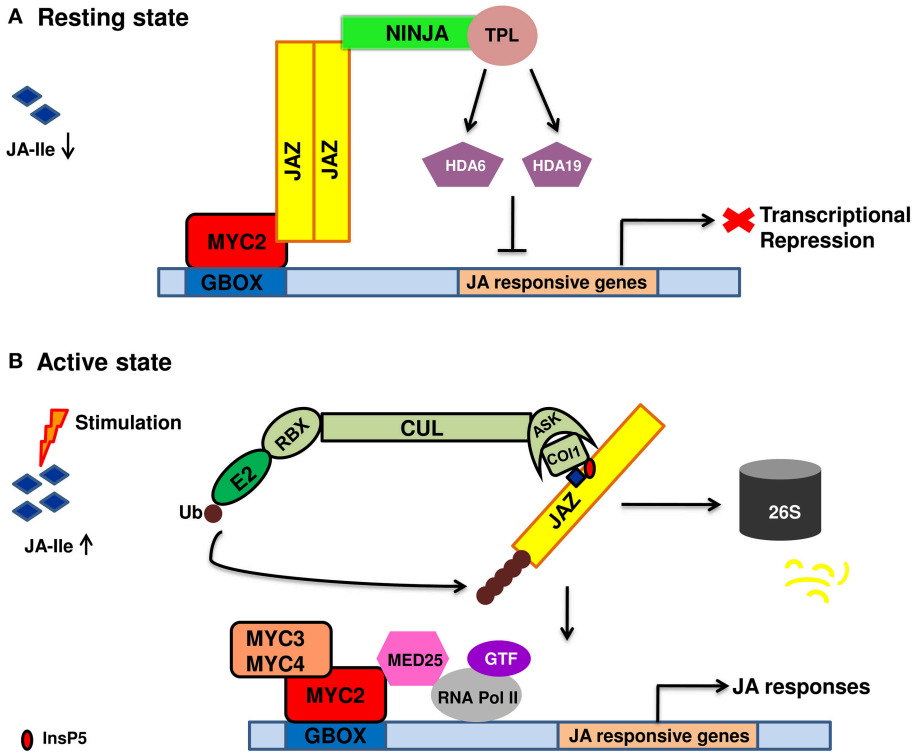


FIGURE 1 | Diagrammatic representation of jasmonic acid perception and signal transduction pathway. (A) In the absence of a stimuli, jasmonic acid is not synthesized. As a result, JA mediated gene expression is inhibited due to the binding of JAZ repressors to the transcriptional activator MYC2. JAZ proteins recruit TPL and adaptor protein NINJA. Together, JAZ-NINJA-TPL form an active transcriptional repression complex that inhibit jasmonate responses by converting an open complex to a closed complex by recruiting HDA6, HDA19. **(B)** Upon stimulation by pathogen/insect/wounding, JA is rapidly synthesized and readily epimerizes to JA-Ile. It then binds to *COI1*-JAZ-InsP5 co-receptor complex causing ubiquitination and proteasomal degradation of JAZ. This frees MYC2 and its homologs from repression which then bind to G-box element present downstream of JA-responsive genes upon homo/heterodimerization. This is followed by the recruitment of MED25 that in turn bring RNAPol II and general transcription factors hence, causing diverse jasmonate responses. JA, jasmonic acid; JA-Ile, Jasmonate–isoleucine; JAZ, Jasmonate ZIM domain; NINJA, Novel adaptor of JAZ; TPL, topless; *COI1*, coronatine insensitive; Cul, Cullin1; RBX1, ring box1; Ub, ubiquitin; ASK1, *Arabidopsis* skp1 homolog 1; InsP5, inositol pentakisphosphate; GTF, general transcription factor; HDA6, HDA19, histone deacetylase 6,19; MED25, mediator25; RNAPol II, RNA polymerase II.

functions in JA perception in plants. It was considered as the receptor from two lines of evidences- first, *coi1* mutant exhibits male sterility, defective responses to JA-treatment and wounding and susceptibility to necrotrophic pathogens and insects; secondly, *COI1* locus encodes an F-box protein that associates with its other counterparts SKP1, Cullin, and Rbx proteins to form an E3 ubiquitin ligase (Xie et al., 1998). *COI1* show approximately 33% sequence similarity with the auxin receptor TIR1 in amino acid sequence having leucine-rich-repeats and F-box motif (Yan et al., 2009).

JAZ Proteins

After the discovery of the receptor the most fascinating question was to find out the substrate for SCF^{COI1} E3 ubiquitin ligase complex. This substrate was anticipated to be the key negative regulator of JA signaling. In 2007, three independent research groups discovered a new family of protein in *Arabidopsis* called JASMONATE ZIM DOMAIN (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The JAZ proteins belong to the larger plant specific TIFY family, consisting of a core TIF[F/Y]XG motif within the ZN-FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) protein domain. *A. thaliana* consists of 12 JAZ proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2009) that are differentiated from other TIFY family proteins by the presence of C-terminally located Jas motif, $SLX_2FX_2KRX_2RX_5PY$ (Nishii et al., 2000; Vanholme et al., 2007; Yan et al., 2007). They contain N-terminal domain, a highly conserved C-terminal Jas domain that mediates the interaction with the *COI1* and several transcription factors, and the conserved protein- protein interaction domain, the ZIM (TIFY) domain that helps in JAZ dimerization and interaction with NINJA (Vanholme et al., 2007; Chung et al., 2009; Pauwels and Goossens, 2011; Wasternack and Hause, 2013). The Jas domain is exclusively required to repress downstream targets of JAZ proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007).

The initial clue about the role of JAZ proteins in JA-signaling came from the jasmonate-*insensitive 3* mutant (*jai3*), which is a mutant of *JAI3/JAZ3* gene. In *jai3-1* mutant, the JAZ3 protein

lacks the C-terminal portion which perturbs its binding and degradation via SCF^{COI1} complex. This resulted in accumulation of truncated JAI3/JAZ3 proteins in the mutant which blocked the JA-induced degradation of other JAZ proteins and hence dominant JA-insensitive phenotype (Chini et al., 2007).

Co-receptor Complex

The co-receptor complex is formed by the physical interaction of *COI1* with the Jas domain of JAZ proteins in the presence of JA-Ile (Yan et al., 2009; Sheard et al., 2010). More recently, the role of inositol pentakisphosphate (IP_5) as a cofactor in the formation of co-receptor complex has been substantiated (Sheard et al., 2010; Mosblech et al., 2011). JA, OPDA, MeJA, and JA-Ile were tested for affinity in *COI1 JAZ1* binding. Surprisingly, only JA-Ile functioned as ligand for *COI1*-JAZ interaction (Thines et al., 2007). Based on the information available hitherto, the true jasmonates receptor is a co-receptor complex, consisting of the SCF^{COI1} E3 ubiquitin ligase complex, JAZ degrons (*JAZ1* to *JAZ12*) and IP_5 (Sheard et al., 2010).

Co-repressors

Co-repressors are transcriptional regulators that inhibit transcription initiation. One such example is the group of Groucho/Tup1 corepressor family comprising of TOPLESS (TPL) and TPL-related proteins (TPRs). TPL and TPR mediate repression by recruiting histone deacetylases and demethylases that cause chromatin modification (Macrae and Long, 2011). TPL interacts with JAZ proteins via ETHYLENE RESPONSE FACTOR (ERF)-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif. Those JAZ proteins that do not have the repression motif recruit TPL through an adapter protein called NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). NINJA was first identified by Tandem affinity purification as an interactor of *JAZ1* (Pauwels et al., 2010).

JAZ Targets

The role of bHLH transcription factor MYC2 in mediating the transcriptional regulation of JA is well defined and thus has been considered the master regulator of many biological processes (Lorenzo et al., 2004; Dombrecht et al., 2007). The role of MYC2 in JA mediated responses is revealed by the study of its mutant *jasmonate-insensitive1* (*jin1*). Microarray analysis of wild type and the mutant *myc2*/*jin1* exposed the role of MYC2 in JA-dependent transcriptional regulation. MYC2 has twin function of an activator of JA-induced root growth inhibition, anthocyanin biosynthesis and oxidative stress tolerance and a repressor in mediating resistance to necrotrophic pathogens, insects and biosynthesis of tryptophan and indol glucosinolates (Lorenzo et al., 2004; Dombrecht et al., 2007). Besides MYC2, several other TFs control diverse JA response. These TFs are MYC3, MYC4, MYB, GL3, EGL3 AP, GL1 etc. (Cheng et al., 2011). MYC2 forms homo or heterodimers with its close homologs MYC3 and MYC4 to regulate the transcription of downstream targets (Fernández-Calvo et al., 2011) by binding to the G-box (5′-CAC GTG-3′) and G-box related hexamers (Abe et al., 1997; Boter et al., 2004; Yadav et al., 2005).

DISSECTING THE ROLE OF JASMONATES IN ABIOTIC STRESS

Out of 13 billion hectares of total land, only 1.6 billion is under farmland production accounting to only 12% of arable land (Syngenta, 2014)[1]. Agriculture must evolve in order to meet the demands of the increasing population. However, every year some part of the world suffers from drought, global increase in temperature, variable precipitation that eventually hampers the quality and quantity of crops. All these visible warning signs can have erratic production patterns all over the world. Plants encounter numerous challenges in terms of competition from other plants, organisms and because of the complex environment. All these provocations have made the plants tougher and more flexible. The morphological flexibility has given them the advantage to counteract, inhabit and endure biotic and abiotic challenges. Rapid changes in the plant biochemistry and physiology are mediated by the action of several phytohormones. By tradition cytokinin, auxins, brassinosteroids, and giberallins have always been associated to regulate developmental processes of plants, whereas, salicylic acid, JA and ethylene associate with plant defense and ABA regulates plant's response to abiotic stress. Now, it has been quite evident from many reports that all hormones affect multiple plant functions. Thus, one can say that hormones not only participate in plant developmental processes but also have a say in plant's response to abiotic stresses like drought, osmotic stress, chilling injury, heavy metal toxicity etc. These adversities have forced the plants to either employ avoidance as a mechanism in order to surmount the stress or choose defense over growth (Band et al., 2012; Murray et al., 2012; Petricka et al., 2012; Wasternack and Hause, 2013). Thus, stress activates signal transduction of hormones which may promote specific protective mechanisms.

Cold Stress

Among various environmental perturbations, cold stress or low temperature stress limits plant performance and geographical distribution. Cold stress can be categorized into chilling (0–15°C) and freezing that causes mayhem in tropical and subtropical plants by inducing chlorosis, necrosis, membrane damage, changes in cytoplasm viscosity, changes in enzyme activities (Ruelland and Zachowski, 2010) and ultimately death. All these physiological and biochemical changes elicit a cascade of events that cause changes in gene expression pattern and protein products and thus in due course induce plant species to adopt stratagems to tolerate low non-freezing temperatures and complete their life cycle, an experience known as cold acclimation response.

ICE-DREB1/CBF regulon plays an imperative role in cold response pathway in model plant *Arabidopsis thaliana* (Thomashow, 1999; Chinnusamy et al., 2007). *Inducer of CBF EXPRESSION 1 (ICE1)* is a MYC-type transcription factor that acts as a master regulator and controls *CBF/DREB1* pathway. In Arabidopsis, three *CBF/DREB1* are involved in the regulation of *COLD REGULATED (COR)* gene expression and tolerance to

[1] Syngenta (2014). Our industry 2014.

cold stress (Gilmour et al., 2000, 2004). In this pathway, *ICE1* positively regulates and activates *CBF/DREB1* genes that encode AP2/ ERF type TF family. CBFs, by binding to C-repeat *(CRT)* element induce COR genes leading to tolerance to cold stress (Thomashow, 2010).

Tropical and subtropical fruits like mango, avocado, papaya etc. exhibit symptoms due to chilling injuries such as browning discoloration and off-flavor in the fruit. Previous studies have specified the role of MeJA in alleviating chilling injury by inducing the production of cryo-protective agents, proteinase inhibitors, polyamines, ABA, lower activity of LOXs, and antioxidants (Wang and Buta, 1994; González-Aguilar et al., 2000; Cao et al., 2009; Zhao et al., 2013). Role of MeJA in response to freezing tolerance has also been studied in rice seedlings. It has been reported that rather than treating rice with MeJA during or after stress imposition, MeJA treatment before chilling remarkably enhances the survival ratio of chilled rice seedlings (Lee et al., 1997). Furthermore, it was observed that MeJA maintained the well-watered status of chilled plants by preventing stomatal opening and enhancing hydrolytic conductivity.

Du et al. (2013) reported an increase in the level of endogenous JA on exposure to cold stress. Microarray analysis of cold treated rice seedlings revealed upregulation of JA biosynthesis genes *OsDAD1, OsLOX2, OsAOC, OsAOS1, OsAOS2, OsOPR1,* and *OsOPR7*. Besides this, JA signaling genes such as *OsJAR1*, *OsbHLH148*, and *OsCOI1a* showed up regulation upon cold exposure. Furthermore, positive role of jasmonate in enhancing constitutive and cold acclimation–induced freezing tolerance of *Arabidopsis* has been reported by Hu et al. (2013). Treatment of WT *Arabidopsis* seedlings with exogenous methyl jasmonate improved the endurance and plant freezing tolerance. On the contrary, blocking JA biosynthesis and signaling pathway rendered the plant hypersensitive to freezing stress.

Of lately, role of JAZ repressors in controlling cold stress tolerance has also been investigated. Under normal growth conditions *Arabidopsis JAZ1* and *JAZ4* interact and suppress cold TFs *ICE1* and *ICE2*, thus quelling *ICE1-CBF/DREB1* pathway (Hu et al., 2013). Also, over expression of *JAZ1* or *JAZ4* repressed the cold-induced expression of *CBF/DREB1*, in that way contributing the transgenic plants sensitive to freezing. However, over expression of *ICE1* was able to salvage freezing sensitive phenotype of *coi1-1* mutant plants.

Recent studies have suggested the role of downstream transcription factors in the regulation of cold responses. Homologs of Arabidopsis MYC2 TF have been isolated and characterized in *Musa accuminata* (Peng et al., 2013; Zhao et al., 2013). *MaMYC2a* and *MaMYC2b* have been shown to be rapidly induced by MeJA treatment upon cold exposure. Expression profiles of CBF cold responsive pathway genes, including *MaCBF1, MaCBF2, MaCOR1, MaKIN2, MaRD2,* and *MaRD5* demonstrated the induction of CBF genes by MeJA upon cold stress. Also, they have been accounted to physically interact with *MaICE1*, therefore suggesting a potential cross talk between two signal transduction pathways.

Arabidopsis SENSITIVE TO FREEZING 6 (SFR6) controls cold regulated gene expression and is well known to act post-translationally on the CBF module (Knight et al., 1999, 2009; Boyce et al., 2003). Additionally, it is involved in regulating JA responses (Wathugala et al., 2012; Zhang et al., 2012). Very recently, SFR6 has been identified as the MEDIATOR16 of the plant mediator complex that is involved in recruiting RNA polymerase II to promoters carrying CRT/DREB motif (Hemsley et al., 2014).

Similar to JA, salicylic acid (SA) is a powerful tool in regulating cold stress tolerance. Exogenous application of SA on *H. vulgare* genotypes resulted in cold tolerance by enhancing antioxidant enzymes, ice nucleation activity and the patterns of apoplastic proteins (Mutlu et al., 2013). Accumulation of endogenous free SA and glucosyl SA has been reported during chilling in *Arabidopsis* shoots, wheat and grape berry (Scott et al., 2004; Wan et al., 2009; Kosová et al., 2012). However, it has been observed that concentration and duration of applied SA greatly influence its utility. High concentration and continual application of SA decreases the cold tolerance capacity of plants as observed in some *Arabidopsis* mutants, such as *CONSTITUTIVE EXPRESSER OF PATHOGENESIS-RELATED GENE1 (CPR1) AND ACCELERATED CELL DEATH6 (ACD6)*, in which SA is over-accumulated, exhibit a dwarf phenotype and freezing sensitivity (Scott et al., 2004; Miura et al., 2010).

Fung et al. (2004) reported an alleviation of chilling injury in freshly harvested green bell pepper (*Capsicum annuum*) by methyl SA (MeSA) and MeJA vapors. This reduction of chilling injury in the green bell pepper was related with an increase in the expression of the *ALTERNATIVE OXIDASE* (*AOX*) gene induced by MeSA and MeJA vapors. Feng et al. (2008) later reported that the expression of *AOX* was enhanced under chilling stress. It has been earlier reported that AOX expression increased in response to low temperature stresses in rice (Ito et al., 1997). All these observations suggest that AOX is involved in combating cold stress. Siboza et al. (2014) reported that combined treatment of MeJA and SA reduced ROS accumulation, lipid peroxidation and increased chilling tolerance in lemon during cold storage by increasing the synthesis of total phenolics and phenylalanine ammonia lyase (PAL) and inhibiting the activity of polyphenol oxidase (PPO) and peroxidase (POD). Induction of PAL activity has been considered a good marker of CI (Martínez-Téllez and Lafuente, 1993; Sanchez-Ballesta et al., 2000).

Findings by Miura and Ohta (2010) have indicated *ICE1* to be an essential integrator of SA signaling and cold response pathway. *ice1* mutant also showed an up-regulation of SA-inducible genes. Additionally, *CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 3 CAMTA3/AtSR1* has been shown to participate in enhancing cold tolerance by binding to the promoter of *CBF1* and *CBF2/DREB1C* (Doherty et al., 2009). Recently, Kim et al. (2013) extended the findings by showing the up-regulation of 15% cold inducible genes by *CAMTA* TFs. It has been established that *CAMTA3* behaves as a repressor of SA biosynthesis at warm temperature under non-stressed condition (Du et al., 2009). But, under cold conditions this repression is overcome as reported by Kim and co-workers in 2013, leading to an increase in the level of SA and up regulation of SA responsive

genes. However, the results indicate that SA does not contribute to freezing tolerance but genes induced by SA at low temperature increase the resistance to pathogen attack. The above findings raise the possibility that *CAMTA3* does not only cause changes in gene expression at low temperature but also plays a role in regulating genes involved in SA biosynthesis at low temperature.

Unlike the close interaction between components of JA signaling and cold regulated transcription factors and JA functioning as a crucial upstream signal to *ICE-CBF/DREB1* pathway (**Figure 2**), very few reports are available on the molecular mechanisms underlying SA-mediated improved plant tolerance to cold temperature. However, the above reports shed light upon the relatedness between cold signaling and SA signaling. Moreover, the participation of *ICE1* in both JA and SA signaling pathway, ROS avoidance mechanism employed both by SA and JA points out the possible crosstalk between JA and SA signal transduction pathway to fight cold stress.

Earlier work conducted by Wilen et al. (1993) pointed out synergism between ABA and JA in inducing freezing tolerance in bromegrass cell cultures. The role of JAs in imparting freezing stress tolerance via *CBF1/DREB1* pathway is well known. To further investigate their role in imparting freezing tolerance via *CBF/DREB1* independent pathway, Hu et al. (2013) carried out microarray and real time analysis in WT and *coi1* mutant plants upon cold treatment. They observed that many cold responsive genes that do not fall in *CBF1/DREB1* pathway were down-regulated in *coi1* mutant plants, thus indicating that jasmonate might play a role in imparting freezing tolerance via *CBF/DREB1* independent pathway. To further assess whether JAs affect all cold regulated expression of genes, Hu and co-workers carried out expression analysis of cold responsive genes falling in ABA pathway in WT and *coi1* plants and it was observed that the transcript level in *coi1* mutant plants changed similar to WT upon cold treatment. Hence, cold tolerance may be provided independently via ABA and JA signaling pathways.

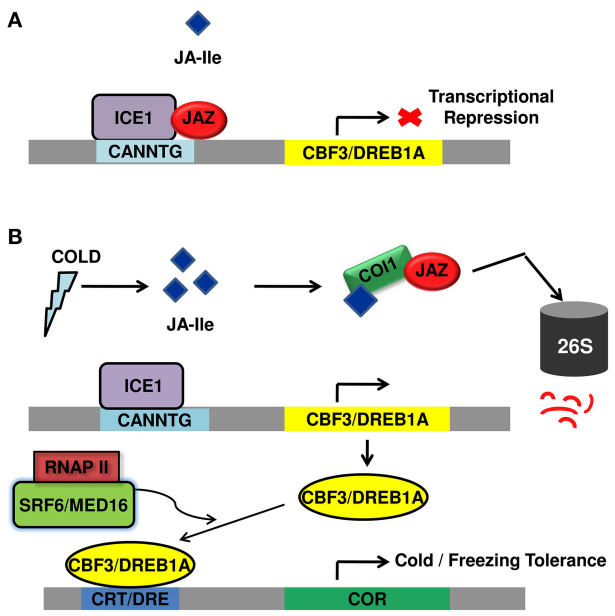


FIGURE 2 | Diagrammatic representation of regulation of cold stress tolerance by JA signal transduction pathway. (A) Under normal growth conditions, JAZ repressor proteins physically interact and suppress cold TF *ICE1*, thus repressing ICE/CBF-DREB1 pathway and rendering plants sensitive to freezing. **(B)** Upon cold induction, JA is synthesized that rapidly isomerizes to JA-Ile and lead to proteasomal degradation of JAZ. This frees *ICE1* that binds to CBF3 responsive element leading to its expression. The CBF proteins bind to CRT/DRE element causing the expression of COR genes that participate in cold/freezing tolerance. JAZ, jasmonate ZIM domain protein; TF, transcription factor; *ICE1*, inducer of CBF expression; *CBF-DREB1*, C repeat binding factor 1-dehydartion responsive element binding factor1B; COR, cold regulated; DRE, dehydration responsive element; CRT, C-repeat; SFR6/MED16, sensitive to freezing 6/Mediator 16; RNAPII, RNA polymerase II; *COI1*, coronatine insensitive.

Like jasmonates, ethylene (ET) has been demonstrated to modulate various abiotic stress responses. Based on findings from Arabidopsis, ET signaling pathway has been shown to negatively regulate freezing stress responses (Shi et al., 2012). EIN3, a positive regulator of ET signaling pathway transcriptionally represses the CBF pathway and thus acts antagonist to JA. Furthermore, Zhu and co-workers in 2011 revealed a crosstalk between JA and ET signaling pathways through the interaction between JAZ and their targets EIN3/EIL1 (Zhu et al., 2011). According to their model, ET is required for stabilization of EIN3/EIL1 and JA is needed for their release from JAZ degradation, as speculated by them. This dual regulation balances the detrimental effects on plant growth and development and establishes appropriate stress tolerance, as speculated by them. Thus, one can say that the CBF signaling pathway is regulated at multiple levels by JAs as it positively regulates freezing tolerance and at the same time represses the *CBF/DREB1* pathway via EIN3/EIL1.

The role of JAs and its interplay with other phytohormones to modulate cold stress responses has been demonstrated in this section. The interaction between different components for example JAZ with EIN3/EIL1 or JAZ with *ICE1*/*ICE2* mediates ET, JA, cold signal transduction pathways. Identifying the novel components involved in crosstalk between JA and other hormones in regulating CBF cold response pathway would help in dissecting the exact molecular mechanism.

Heat Stress

Temperature above the optimum for growth can be detrimental, causing injury or irreversible damage to plant growth and development. IPCC (2014) reports suggest an approximate increase of 4–5°C in the average temperature by the end of the twenty-first century. High temperature stress negatively influences plant processes and cellular machinery, thereby impairing cell homeostasis (Bokszczanin et al., 2013). Plants have the inherent ability to ameliorate the adverse effects of heat shock by the phenomenon of basal thermotolerance whereas, acquired thermotolerance is achieved when plants are pre-exposed to high, non-lethal temperature (Bokszczanin et al., 2013). In response to high temperature, plants synthesize HEAT SHOCK PROTEINS (HSPs) that prevent denaturation and assist refolding of damaged proteins (Boston et al., 1996).

The role of JA signaling in contributing to thermotolerance has been recently established in WT *Arabidopsis* (Clarke

et al., 2009). Exogenous application of low concentration of MeJA maintained cell viability in heat stressed plants as demonstrated by electrolyte leakage assays. Moreover, heating WT Arabidopsis led to the accumulation of several jasmonates including OPDA, MeJA, JA, and JA-Ile. But, no evidence was found that thermotolerance conferred by MeJA elicited HSP gene expression. Expression level of jasmonate inducible gene PDF1.2 was found to be high upon heat stress exposure. The final proof of the role of jasmonates in imparting thermotolerance was confirmed by mutant analysis wherein JA and SA signaling mutants *coi1-1, opr3, and jar1-1cpr5-1* were found to be sensitive to heat stress (Clarke et al., 2009). Thus, establishing the fact that both SA and JA provide basal thermotolerance.

SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1) protein operates as a cofactor of HEAT SHOCK PROTEIN 90 (HSP90) in both plants and mammals forming functional complexes and providing thermotolerance. Intensive genetic and biochemical screening confirmed the role of SGT1 in plant hormone signaling pathways that involve F-box proteins and ubiquitin ligases such as *COI1* of JA signaling. SGT1 maintains the steady state of *COI1* (Zhang et al., 2015). Reduced transcript levels of JA marker genes in HSP90 RNAi lines confirmed the crucial role played by HSP90 and HSP70 in JA-COR (coronatine) responses. Additionally, pre-treating WT *Arabidopsis* with HSP inhibitor attenuated COR (coronatine) triggered gene expression. Given the facts that HSP proteins have numerous substrates including transcription factors, E3 ligases, kinases and the above data suggested that *COI1* is a client protein of SGT1b–HSP70–HSP90 chaperone complexes. This widens the functional capacity of SGT1b–HSP70–HSP90 chaperone complexes in regulating JA responses.

WRKY super family consists of 74 members in *Arabidopsis thaliana* (Eulgem and Somssich, 2007) and is subdivided into three groups based on the number of WRKY domains and the features of their Zn finger like motifs (Eulgem et al., 2000). They are a group of regulatory proteins that participate in plant developmental processes but notably in a plethora of biotic and abiotic challenges. There are sufficient evidences that many WRKY genes participate in abiotic stresses, including heat stress. Several WRKY TFs have been revealed to impart thermotolerance like *AtWRKY25* (Zhu et al., 2009), *AtWRKY39* (Li et al., 2010), and *OsWRKY11* (Wu et al., 2009). Dang et al. (2013) showed that *CaWRKY40* was involved in heat stress and was transcriptionally induced by exogenous application of JA. Moreover, over expression lines of *CaWRKY40* derepressed JA biosynthesis *NtLOX1* by heat stress. Together, the findings suggest that JA mediates the expression of *CaWRKY40* leading to the expression of downstream thermotolerance-related genes.

JA and ET act as antagonists in regulating heat stress responses. Studies by Clarke et al. (2009) showed that despite being produced in response to heat stress, ET negatively regulates heat stress tolerance. They found out that *ein2* mutant displayed thermotolerance, hence suggesting that EIN2 mediated pathway negatively regulates thermotolerance. They also demonstrated that ET production was augmented by JA from studies carried out in WT and *opr3* mutant.

CONCLUDING REMARKS

The daunting issue of nutritional and food security has resulted in a quest among researchers to elucidate the action of phytohormones in stress related responses. Tolerance to (a)biotic stresses is a challenge of agro-economic impact. For this, a model eudicot, *Arabidopsis* has emerged as a quintessential system to study plant stress tolerance. However, crop plants are exposed to complex environmental perturbations in the field. Therefore, thorough research will be required to understand how crops respond to multiple abiotic stresses in order to develop new varieties that can withstand global climate changes.

The role of jasmonates in plant development is very well established (Wasternack and Hause, 2013). Nevertheless, a significant body of research suggests the role of jasmonic acid in plant responses to abiotic stresses. Growing sagacity of crosstalk between JAs and other hormones and with different components of abiotic signal transduction may allow us to dissect key factors involved in the crosstalk. Recent reports suggest interplay between cold regulated transcription factors and components of JA biosynthesis and signaling. The interaction of JAZ repressors with transcriptional activators *ICE1* and *ICE2* and falling upstream to the cold response pathway (Hu et al., 2013) as well as the induction of JA genes upon cold stimulus (Du et al., 2013; Hu et al., 2013) demonstrates the sharing of common signaling components and hence a closed interaction between the two signal transduction pathway. Additionally, several lines of evidences suggest the role of JAs in imparting thermotolerance. In addition, mutant analyses have also confirmed the role of JAs in heat tolerance responses. Modifying different upstream/downstream signaling components by genetic engineering can improve the adaptability of plants in response to temperature stress. However, there is still obscurity in the crosstalk among different signaling pathways. The application of forward and reverse genetic analysis in model plants along with the genomics and proteomics tools will help us in the discovery of new regulatory components and in dissecting the complex interactions between different signaling pathways to elucidate hormone action in stress related context.

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REVIEW
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Exploring Jasmonates in the Hormonal Network of Drought and Salinity Responses

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Present and future food security is a critical issue compounded by the consequences of climate change on agriculture. Stress perception and signal transduction in plants causes changes in gene or protein expression which lead to metabolic and physiological responses. Phytohormones play a central role in the integration of different upstream signals into different adaptive outputs such as changes in the activity of ion-channels, protein modifications, protein degradation, and gene expression. Phytohormone biosynthesis and signaling, and recently also phytohormone crosstalk have been investigated intensively, but the function of jasmonates under abiotic stress is still only partially understood. Although most aspects of jasmonate biosynthesis, crosstalk and signal transduction appear to be similar for biotic and abiotic stress, novel aspects have emerged that seem to be unique for the abiotic stress response. Here, we review the knowledge on the role of jasmonates under drought and salinity. The crosstalk of jasmonate biosynthesis and signal transduction pathways with those of abscisic acid (ABA) is particularly taken into account due to the well-established, central role of ABA under abiotic stress. Likewise, the accumulating evidence of crosstalk of jasmonate signaling with other phytohormones is considered as important element of an integrated phytohormonal response. Finally, protein post-translational modification, which can also occur without *de novo* transcription, is treated with respect to its implications for phytohormone biosynthesis, signaling and crosstalk. To breed climate-resilient crop varieties, integrated understanding of the molecular processes is required to modulate and tailor particular nodes of the network to positively affect stress tolerance.

Keywords: phytohormones, jasmonic acid, abscisic acid, abiotic stress, drought, salinity

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INTRODUCTION

During the last century, the Green Revolution led to food security for a rapidly growing global population through an impressive growth of productivity achieved by mineral fertilizers, chemical plant protection, and mechanization. However, the central factor driving the yield increase was genetics. For instance, the reduction of culm length by mutations in DELLA gibberellin-response factors (Peng et al., 1999) substantially reduced losses by lodging that, in rice, can reach up to 40% (Nishiyama, 1986). Although there is still potential for further increases in crop yield, it has also become clear that plant science of the new century must address additional targets: The land

amenable to agriculture is limited and crop production is further constrained by land use for biofuels, urbanization, and desertification. The situation is further accentuated by unpredictable patterns of climate change. The case of desperate farmers who, in expectation of high yields, spend their funds for seeds of high-yielding improved cultivars, and then witness crop failure due to altered rain, temperature and light regimes illustrates that crop breeding must integrate additional traits in addition to improved photosynthetic efficiency or optimal partitioning of assimilates to the culm, for example. Altered regimes of abiotic factors lead to alterations in biotic stress factors as a corollary. Crop resilience to biotic and abiotic stress conditions has therefore shifted into the focus of plant research worldwide (for review, see Passioura, 2002). During evolution, the immobile nature of plants has forced them to evolve unique and sophisticated mechanisms to tolerate abiotic stress. The natural variation in those mechanisms can be used to develop more tolerant crop plants. As prerequisite, we have to understand the underlying molecular, biochemical, and physiological aspects of stress tolerance.

Among the abiotic stress factors, the varied osmotic challenges posed by drought, salinity, and alkalinity together account for maximal yield losses in major crops. Water scarcity is probably the most serious constraint for crop quality and productivity among all environmental factors, compromising economic output and human food supply (Roche et al., 2009). Just salinity alone affects approximately 20% of the irrigated lands of the world. In addition, every year a large fraction of agricultural land is oversalted and becomes unusable (Yeo, 1999; Williams, 2001). The costs to agriculture caused by salinity are huge and are expected to increase as further regions are contaminated with salt (Ghassemi et al., 1995). For instance, deposition of toxic salt sediments and sea intrusion in tsunami-affected areas of the Maldives damaged 70% of agriculture land, destroyed some 370,000 fruit trees, and affected around 15,000 farmers, with estimated costs at around AU$ 6.5 million (FAO, 2005). Unfortunately, salinity is a man-made problem to some extent, caused by agricultural practices such as land clearing and the replacement of perennial vegetation with annual crops and irrigation schemes using salt-rich irrigation water or having insufficient drainage (Munns, 2002). The impact of drought in terms of yield, economy and negative effect on society is even more substantial (Kantar et al., 2011). An FAO report estimated that drought wrought irrevocable negative implications on two billion and killed 11 million people during the last century, more than any other hydro-meteorological hazard (FAO, 2013).

Although in general perceived as mere water scarcity, osmotic stress in reality represents a complex syndrome comprising at least three components that can occur either individually or in different combinations: water scarcity stress (drought), ionic stress (salinity), and nutrient-depletion stress (alkalinity). Adaptation to the three osmotic stresses requires cellular and physiological responses that must be at least partially different depending on the dominating stress component. For instance, drought can primarily affect cell turgidity causing growth arrest and stomatal closure, resulting in photosynthetic imbalance, and impaired redox homeostasis. Salinity can, in addition, impair ionic homeostasis. Thus, adaptation to salinity not only has to reinstall turgidity, but also has to reinstall the equilibrium between important ions such as sodium and potassium. Specific adaptive responses must be triggered by specific signaling cascades as well, involving specific molecular components. However, the number of molecular players that convey stress signals in plants is rather limited and many of these molecular players are shared between different stresses (Ismail et al., 2014b). One model to explain this specificity achieved by a limited set of mostly common factors conceptualizes particular spatiotemporal patterns (so called signatures) of these overlapping signals and the signaling pathways (Ismail et al., 2014b).

A proof of concept for this signatures model, is provided by stress induced calcium patterns. By means of aequorin-reporter plants, different stress factors were shown to produce different temporal signatures of calcium (Knight et al., 1991, reviewed in McAinsh and Hetherington, 1998). The fact that signatures differ between different stresses, does not prove *per se* that these signatures are causative for signal specificity. A functional proof requires manipulating the signatures, which is far from trivial. In case of calcium, this was successfully achieved in guard cells by rhythmic incubations with calcium-containing and calcium-free buffers, and this artificial calcium signature allowed rescuing the deficient stomatal closure in the *det3* mutant of *Arabidopsis* (Allen et al., 2000). Similar signatures seem to act also for other stress signals. For instance, the specificity of reactive oxygen species (ROS) as signals in the processing of drought and salinity stress seems to depend on their subcellular distribution (reviewed in Miller et al., 2010). Likewise, the interaction of jasmonate signaling with other signal chains converging at the proteasome can generate specific outputs (reviewed in Kazan and Manners, 2008). As common theme in these examples, the specificity of signaling seems to stem from specific combinations of fairly general primary signals. For instance, while drought, salt, and cold stress will all activate calcium influx, the "physiological meaning" of this influx is modulated by different, stress-quality specific second messengers to yield different responses (Xiong et al., 2002). This combinatorial model predicts that there exists something like a "grammar of stress signaling," and when we want to understand, how plants can discriminate the three components of osmotic stress and even specific combinations of them, we have to decipher this "grammar" in the first place.

As a proof of concept, temporal signatures have been dissected for salinity stress in grapevine cells (reviewed Ismail et al., 2014b). Input is either through a (mechanosensitive) calcium influx channel at the membrane, which often acts in concert through the membrane-located NADPH-oxidase Respiratory burst oxidase Homolog (RboH), generating apoplastic ROS. Transduction is conveyed by calcium-binding proteins (CDPKs, calcineurins), a MAP-Kinase cascade, and jasmonates [oxophytodienoic acid (OPDA), jasmonic acid (JA), JA-Isoleucine conjugate (JA-Ile), methyl jasmonate (MeJA)]. Depending on the relative temporal patterns of these upstream signals, the cellular responses were qualitatively different. In one case adaptive responses such as activation of enzymatic antioxidants, osmoprotectants or ion channels resulted in cellular adaptation, whereas in the other case, temporal shifts of early signaling events culminated in necrotic

death, which for a cell is a fatal outcome, but for a plant may be adaptive, because it provides a strategy to exclude noxious salt by abscission of a leaf that is thus sacrificed for the sake of the entire plant.

The synthesis, modification, and signaling of jasmonates has been reviewed comprehensively by Wasternack and Hause (2013), and these authors have also extensively treated the role of this pathway for the plant responses to different stress factors. The current review will therefore focus on the role of temporal jasmonate signatures for the adaptive response to osmotic challenges (drought and salinity stress). We address possible mechanisms ensuring specificity for these partially similar stress conditions that on the other hand require partially different responses. In particular, we show that dynamic feedback of jasmonate signaling upon jasmonate synthesis along with different ramifications in synthesis and modification of JA are relevant to constrain this potentially dangerous stress signal in a manner that is tuned with activation of other pathways, prominently abscisic acid (ABA) signaling.

THE PRIMARY CAUSE FOR SPECIFICITY MUST BE SEARCHED IN DIFFERENT CHANNELS OF PERCEPTION

Modulations in the phytohormonal levels and signaling status in response to abiotic stress have been intensively studied for decades, with often contradictive results, where upregulation of a given hormone was found to confer stress adaptation in one case, but was found to impair survival in a different case. These discrepancies show already that phytohormones apparently do not act as early transducers of stress signals, but rather seem to act as integrators of different upstream signals. Therefore, before we will deal jasmonates themselves, it is important to have a look on these upstream signals.

The actual input for *drought stress* signaling is certainly mechanical load of the membrane. The osmotically caused loss of turgidity will affect membrane tension, and this can be perceived through changes in the activity of mechanosensitive ion channels, a mechanism that was developed early in evolution and functions already in prokaryotic cells (reviewed in Kung, 2005). In plant cells, such mechanosensitive channels drive an influx of calcium. Contrarily, the calcium output caused by mechanical challenge of the membrane is also used to perceive a range of stress factors different from osmotic stress such as touch, gravity, wounding, or cold (reviewed in Nick, 2011). The molecular nature of these channels has remained elusive for decades. The recent discovery of the calcium channel OSCA1 from *Arabidopsis thaliana* that is gated by hyperosmotic stress (Yuan et al., 2014) might mean that a central player for the perception of osmotic challenge of the membrane has been identified. The influx of calcium can be transduced through calcium dependent kinases into activation of the NADPH oxidase RboH generating apoplastic singlet oxygen, such that calcium influx is followed (with some delay) by a transient oxidative burst (Dubiella et al., 2013). At the cellular level, adaptation is brought about by production of compatible osmolytes that will help to reinstall turgidity. Also, a well-known response is the synthesis of late-embryogenesis abundant (LEA) proteins that will prevent protein precipitation (Tunnacliffe and Wise, 2007). At the organismal level, rapid closure of stomata will reduce additional loss of water (for review Xoconostle-Cázares et al., 2011).

For *salinity stress*, the osmotically induced Ca influx is accompanied by a second factor, ionic stress. In fact, sodium ions can pass the plasma membrane by non-selective cation channels (NSCCs). A comparison of two grapevine cell lines that differ in salt tolerance revealed that efficient adaptation correlated with a more rapid uptake of sodium into the cytoplasm indicating that the concomitant increase of cytosolic calcium and sodium might act as a signal triggering salinity adaptation (Ismail et al., 2014a). The adaptive salt overly sensitive (SOS) module cannot only extrude sodium from the cytoplasm, but also links cytosolic sodium with calcium signaling (reviewed in Ismail et al., 2014b). Although some of the adaptive responses to salinity are shared with drought stress (reviewed in Hasegawa et al., 2000), such as induction of osmolytes, accumulation of LEA proteins, or stomatal closure, others are specific for salinity. For instance, sodium can be extruded by the SOS1 exporter (Munns and Tester, 2008), or it can be sequestered into the vacuole through the NHX1 transporter system (Munns and Tester, 2008), which allows to restore turgidity and thus to reinstall growth.

Alkalinity stress represents an accentuated version of salinity stress and is of vast agronomic impact with worldwide almost 1000 million hectares being affected (Rao et al., 2008). Although it is known that alkaline sodium stress has much harsher effects as compared to equimolar salinity at neutral pH (Wang et al., 2011), the molecular signals as well as the adaptive mechanisms are far from understood. In addition to osmotic challenge and ionic stress, alkalinity causes the precipitation of important nutrients including phosphates and metallic micronutrients, and also destroys the cellular structure of the roots (Li et al., 2009). Under physiological conditions, the apoplast is actively maintained at a slightly acidic pH of around 5.5 by proton ATPases localised in the plasma membrane and this activity is essential to sustain cell expansion growth (Haruta et al., 2010). Under alkaline conditions, this mechanism is interrupted. Moreover, the activity of osmotically induced calcium influx is expected to be impaired, because calcium enters the cell by cotransport with protons (which allows to conveniently monitoring this influx as transient alkalinization of the apoplast). As a second effect, the superoxide anions that are generated by the NADPH oxidase RboH to a certain extent even under normal conditions will not be dissipated due to the absence of protons as electron acceptors, leading to an accentuated stress-induced oxidative burst. Adaptation to alkalinity must involve mechanisms that transcend conventional salinity responses, a point that so far has not been appropriately considered in breeding programs (Bui, 2013). For instance, in addition to sequestering sodium in the vacuole, and quelling the accentuated oxidative burst, adaptation to alkalinity would also require powerful buffering of the apoplast, which might either be achieved through upregulation of proton ATPases or through secretion of organic acids.

The comparison of the three aspects of osmotic stress illustrates that each specific condition requires a specific adaptive response, which seems to be determined by specific equilibria

between different stress inputs. This adaptive response is costly, however. For instance, stomatal closure will reduce water loss by transpiration, but it will also reduce photosynthetic efficiency and lead to secondary photooxidative stress caused by unbuffered electron transport in the thylakoid (Pinheiro and Chaves, 2011). Similarly, the synthesis of polyamines binds precious bioavailable nitrogen (Alcázar et al., 2006). Therefore, these adaptive responses have to be carefully adjusted to growth and development. It is this adjustment, where phytohormonal signaling links with stress adaptation. The jasmonate signaling system seems to act as a hub, where different inputs are processed to yield an appropriate adaptive response.

The following sections investigate the role of jasmonates for drought and salinity signaling and attempt to dissect the interaction of jasmonate signaling with the signaling triggered by ABA. Jasmonate biosynthesis utilizes different metabolites with potentially different biological activity. A complex feedback regulation of this pathway allows for each node to function in either direction to process several inputs with ample ramifications into different outputs as required for a signaling hub (**Figure 1**). Such a system acts in a highly non-linear fashion, which means that even subtle modulations in the relative activities of individual components of this hub can result in a qualitatively different output. This also means that breeding of stress-tolerant crops might not require drastic genetic changes. Slight, but targeted shifts in the relative activities of jasmonate signaling components might be worth exploring. This is particularly interesting in view of the independent evolution of salinity tolerance in some of the 3000 grasses (Bennett et al., 2013), which may be suggestive of perturbances in a limited number of pathways, but at different nodes. Thus, there may be more than one road leading to Rome.

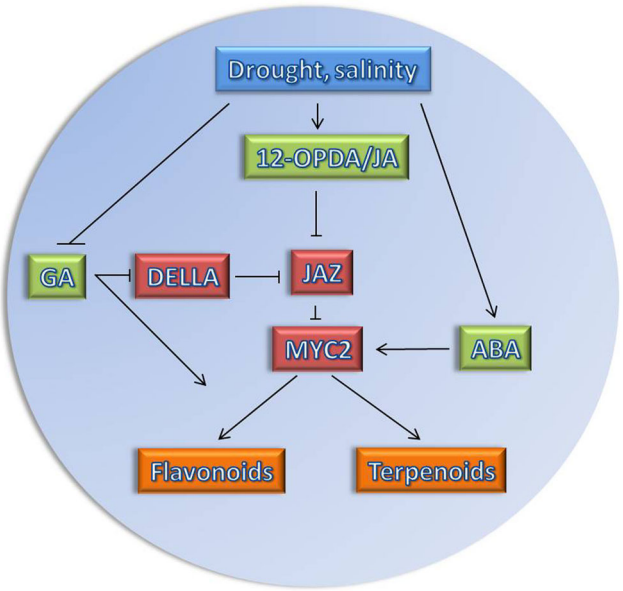


FIGURE 1 | Model of JA network in response to drought/salinity stress. Osmotic stress affects the biosynthesis of the plant hormones (highlighted in green), jasmonic acid (JA), 12-oxo-phytodienoic acid (12-OPDA), and abscisic acid (ABA) positively (Takeuchi et al., 2011; Hazman et al., 2015), while the biosynthesis of GA is repressed (Colebrook et al., 2014). Changes in hormonal levels affect several important key regulatory proteins (highlighted in red). The JAZ repressor is degraded when JA is produced, such that the transcription factor MYC2 will be activated. MYC2 could work as signaling hub (Kazan and Manners, 2013) as it is also positively regulated by ABA. Due to repression of GA biosynthesis, DELLA proteins, repressors of GA signaling, will accumulate. DELLA has been shown to interact physically with JAZ proteins and hence can relieve MYC2 from JAZ repression (Hou et al., 2010). MYC2 activates secondary plant metabolic pathways such as flavonoids and terpenoids (highlighted in orange) which are required for plant adaptation to stress conditions.

PLACING JASMONATES INTO THE DROUGHT AND SALINITY SIGNALING CASCADE

Both drought and salt stress are multidimensional in nature and affect plants at various levels of their organization (Yordanov et al., 2000). Therefore, the effects of stress are often observed at morpho-physiological, biochemical and molecular levels, such as growth inhibition (Bahrani et al., 2010), enhanced production of compatible organic solutes (Sánchez-Díaz et al., 2008; DaCosta and Huang, 2009), changes in the content of phytohormones (Perales et al., 2005; Seki et al., 2007; Huang et al., 2008; Dobra et al., 2010; Kohli et al., 2013), or altered expression of stress responsive-genes (Xiong and Yang, 2003; Yamaguchi-Shinozaki and Shinozaki, 2005; Huang et al., 2008). Changes tissue water status trigger some of these responses directly, while many others are brought about by plant hormone-dependent signaling (Chaves et al., 2003). The tolerance/adaptation response of plants to unfavorable environmental conditions strongly depends on chemical signals/secondary metabolites that are orchestrated by plant hormones in a complex balance between tolerance and growth (Sreenivasulu et al., 2012, **Figure 1**). It has been known previously that hormones do not function in discrete pathways, but rather influence each other at different levels (i.e., biosynthesis or signaling) to control environmental and developmental signaling pathways (Gray, 2004). This will create a signal transduction network that can integrate different inputs into a comprehensive output culminating in physiological adaptation of the plant to stress.

A key role in this hormonal network is played by the plant hormone ABA. Its function in the control of stomata closure and the responses to abiotic stress is well-established and has been intensively studied since decades (for review, see Mittler and Blumwald, 2015). Drought stress or high salinity cause accumulation of ABA in plants and extensive changes in gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA signaling triggered by receptors in the plasma-membrane as well as the cytoplasm, has been intensively studied in guard cells (for review, see Mittler and Blumwald, 2015). Subsequent signaling increases the concentration of cytosolic Ca^{2+} due to the activation of calcium channels in the endoplasmic reticulum, which further activates or inhibits ion channels in the plasma membrane. As a result of ion fluxes, water potential in the apoplast decreases and water flows out of the cell leading to a lower turgor of guard cells and closure of stomata. Due to this central function of ABA for the regulation of stomatal opening and closure and the control over other stress adaptive mechanisms, this hormone is very important

for the response to abiotic stress. However, usually changes in one hormonal pathway affects the pathways of other hormones and expectedly other hormones, especially those related to stress and growth responses, contribute to the overall response of the plant. One of these hormonal pathways currently attracting a lot of attention, is jasmonate signaling (JAs), conveyed by JA and its derivatives. JAs constitute a group of fatty acid-derived compounds that play prominent roles in coordinating inducible defense responses leading to increased tolerance to insect pests and necrotrophic pathogens (for review, see Wasternack and Hause, 2013). JAs are also required for specific steps of plant development like reproduction or photomorphogenesis (for review, see Svyatyna and Riemann, 2012). Biosynthesis, perception and action of JAs have been extensively studied. On the contrary, inactivation/removal mechanisms have remained elusive for a long time, but have been elucidated recently (Heitz et al., 2012; Aubert et al., 2015). In sharp contrast to most other plant hormones, JA must be activated by enzymatic coupling to isoleucine amino acid. The resulting JA-Ile functions as a ligand promoting assembly of a co-receptor complex between the F-box protein COI1 and so-called JA ZIM-domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007). JAZ proteins are transcriptional repressors that prevent the transcription of target genes under low JA-Ile levels, and are specifically ubiquitinated when JA-Ile accumulates under biotic stress. This is a signal leading to their proteolytic degradation, relieving active transcription of JA-responsive defense genes from repression (**Figure 2**). JA-Ile is therefore a master switch controlling various aspects of plant immunity/adaptation. Elements under JA control include the synthesis of digestive inhibitors targeting insects, volatile repellents, and many toxic or antimicrobial compounds that lower the performance of pests. Although role for jasmonates for the adaptation to salt stress has been suggested (Fujita et al., 2006), molecular mechanisms of the role of jasmonates for salt or drought stress-signaling are still mostly unclear. The following sections review what is known on how jasmonates contribute to the plant response toward these two intensively studied abiotic stresses, drought and high salinity.

JASMONATES AND DROUGHT STRESS

There is a steadily increasing body of evidence for the involvement of jasmonates in drought stress. Barley leaves exposed to simulated drought with sorbitol or mannitol exhibited increased endogenous contents of jasmonates, followed by the transcription of jasmonate-induced proteins (JIPs, Lehmann et al., 1995). A later study also showed that the contents of octadecanoids and JAs were enhanced by sorbitol treatment to a degree, sufficient to initiate JA-responsive gene expression (Kramell et al., 2000). In addition, endogenous JA content increased in maize root cells under drought stress (Xin et al., 1997), and this compound was also able to elicit betaine accumulation in pear leaves (Gao et al., 2004). In some studies, JA has been reported to improve drought tolerance but in others, it has been reported as a negative agent that causes notable reduction in growth and yield, hence the actual role of JA in drought stress remains controversial. Mostly, the observed responses depend on the type of plant and tissue in question, intensity and duration of drought stress and the dosage of JA applied (Lee et al., 1996; Kim et al., 2009). Therefore, a lot of the controversy might actually result from the fact that studies were done under different conditions, e.g., in various developmental stages, tissues, and with different stress regimes. This might be linked with the fact that also the degree of drought tolerance strongly depends on developmental stage in most plant species (Reddy et al., 2004; Rassaa et al., 2008).

Exogenous Jasmonates can Increase Drought Tolerance

Several reports suggest that exogenous application of jasmonates ameliorates the response of plants to drought stress. It has been reported that exogenous application of JA or MeJA increased the antioxidative capacity of plants under water stress (Bandurska et al., 2003). In the same context, other studies also showed that JAs play an important role in signaling drought-induced antioxidant responses, including ascorbate metabolism (Li et al., 1998; Ai et al., 2008). It has been observed that exogenous JA is effective in protecting plants from drought-induced oxidative damage as it enhances the activity of antioxidant enzymes (Nafie et al., 2011). It is also hypothesized that MeJA could ameliorate water stress tolerance in banana by regulating the growth, proliferation rate, proline accumulation, chlorophyll levels, tissue water status, oxidative stress, and membrane lipid peroxidation (Mahmood et al., 2012).

Another study was conducted by Anjum et al. (2011) in soybean (*Glycine max* L. Merrill) to explore the role of exogenous MeJA application in alleviating the adversities of drought stress. Soybean plants were grown under normal conditions until blooming and then were subjected to drought by withholding water followed by foliar application of MeJA. From the observed data, it was noticed that drought stress substantially lowered the yield and yield-related traits, whereas it accelerated the peroxidation of membrane lipids. Nonetheless, considerable increase in the activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and in proline, relative water contents (RWC) along with simultaneous decrease in membrane lipid peroxidation was observed when the drought stressed plants were treated by MeJA. These beneficial effects led to significant improvement in yield and harvest index under drought. Interestingly, MeJA application was also promotive under well-watered conditions. These results suggested that by modulating the peroxidation of membrane lipids and antioxidant activities, MeJA improved the drought tolerance of soybean.

Which Jasmonates Contribute to Drought Stress Signaling?

Jasmonates comprise a diverse group of JA derivatives (for review, see Wasternack and Hause, 2013), including its biosynthetic intermediate, 12-OPDA, which is capable of activating specific signaling events (Taki et al., 2005). Recent data indicate that 12-OPDA might be the jasmonate derivative which is mainly functional in the drought response.

De Domenico et al. (2012) measured the expression of key genes involved in oxylipin metabolism by quantitative PCR on

samples from stressed and non-stressed roots of a drought-tolerant and a drought-sensitive chickpea variety. In their study, they demonstrated that the drought tolerant variety reacts to drought with sustained and earlier activation of a specific lipoxygenase (*MtLOX1*), two hydroperoxide lyases (*MtHPL1* and *MtHPL2*), an allene oxide synthase (*MtAOS*), and an oxo-phytodienoate reductase (*MtOPR*). Over-expression of these genes correlated positively with the levels of major oxylipin metabolites from the allene oxide synthase (AOS) branch of the pathway, which finally leads to the synthesis of jasmonates. The roots of the tolerant variety accumulated higher levels of JA, its precursor OPDA and the active JA-Ile, suggesting a role of jasmonates for drought tolerance in chickpea.

Savchenko et al. (2014) identified that drought led to a block in the conversion of 12-OPDA to JA and further revealed that 12-OPDA was the functional convergence point of oxylipin and ABA biosynthesis pathways, to control stomatal aperture in plant-adaptive responses to drought stress. They used three *A. thaliana* ecotypes to demonstrate that wounding induced both 12-OPDA and JA levels, whereas drought induced only the precursor 12-OPDA. This implicated the AOS branch of the oxylipin pathway as a critical node. Levels of ABA were also mainly enhanced by drought and little by wounding. To explore more about the role of 12-OPDA in plant drought responses, they also generated a range of transgenic lines and exploited existing mutant plants that differ in their levels of stress-inducible 12-OPDA, but displayed similar ABA levels. The plants which were producing higher 12-OPDA levels exhibited enhanced drought tolerance and reduced stomatal aperture. Furthermore, on exogenous application of ABA and 12-OPDA, whether individually or combined, stomatal closure was promoted in the ABA and AOS biosynthetic mutants, albeit most effectively when combined. Using tomato (*Solanum lycopersicum*) and *Brassica napus*, they verified the potency of this combination in inducing stomatal closure in plants other than *Arabidopsis*. They concluded that drought was a stress signal that uncoupled the conversion of 12-OPDA to JA and also revealed 12-OPDA as a drought-responsive regulator of stomatal closure functioning most effectively together with ABA.

Jasmonates Contribute to Regulation of Stomatal Closure

Based on its accumulation during drought stress and its positive regulatory role in stomatal closure, JA has been proposed as important player for stomatal closure during drought stress (Gehring et al., 1997; Suhita et al., 2003, 2004). Soybean leaves under water stress showed a 15% loss of fresh weight and accumulated fivefold more JA within 2 h, but the level of JA declined to that of control plants by 4 h (Creelman and Mullet, 1995). MeJA-mediated stomatal closure has been related to cytoplasmic alkalinization in guard cells, production of ROS (via AtRboHD/F) and NO, and activation of K-efflux (Evans, 2003), as well as slow anion channels (Gehring et al., 1997; Suhita et al., 2003, 2004; Munemasa et al., 2007). All these effects are similar to those of ABA, thereby suggesting an overlapping use of signaling components for stomatal closure. This idea is also supported by observations made in the ABA hyposensitive *ost1* mutant, which turned out to be less sensitive to MeJA with respect to stomatal closure. Moreover, the MeJA insensitive mutant *jar1* displays reduced stomatal closure in response to ABA (Suhita et al., 2004).

Do ABA and JA Act Synergistically in Drought Stress Signaling?

Abscisic acid plays a key role in plant adaptation to adverse environmental conditions including drought stress. However, molecular, genetic and genomic analyses suggested that in addition to ABA-dependent pathways, ABA-independent regulatory systems are involved in stress-responsive gene expression (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997, 2000; Riera et al., 2005). Induction of ABA synthesis is one of the fastest phytohormonal responses of plants to abiotic stress, thereby triggering ABA-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006), causing stomatal closure, and hence reducing water loss via transpiration (Wilkinson and Davies, 2010), which will eventually restrict cellular growth. During the adaptive responses of plants to environmental stresses, the overlap between hormone-regulated gene expression profiles suggests the existence of a complex network with extensive interactions between the different hormone signaling pathways. In order to examine a crosstalk between ABA and JA signal transduction *Arabidopsis* ABA-insensitive (*ost1-2*) and MeJA-insensitive (*jar1-1*) mutants were studied for the participation of ABA and JAs in stomatal closing (Suhita et al., 2004). The authors investigated changes of cytoplasmic pH and ROS production in response to ABA or JA, and the mutants were used to assess the respective roles of the mutated genes in ABA or JA signaling pathways leading to stomatal closure. The modulation of Ca^{2+}ions was induced by both, ABA and JA. However, the primary actions of ABA and JA in the plasma membrane appear to be different: JA targets the Ca^{2+} channels whereas ABA activates effectors in the plasma membrane (e.g., phospholipase C and D). However, at the level of intracellular Ca^{2+}, both signal transduction pathways converge. Intracellular Ca^{2+} level is modulated to a much greater extent by JA than by ABA.

It is well established that JA biosynthesis is induced by stress conditions such as wounding and herbivory (Wasternack, 2007), but many JA-associated signaling genes are also regulated by drought stress (Huang et al., 2008). It has been shown that JA interacts with ABA-regulated stomatal closure by increasing Ca^{2+} influx, which activates a CDPK-dependent signal cascade. Treatment of turgescent, but excised *Arabidopsis* leaves with either ABA or MejA resulted in a reduction of stomatal aperture reduction within 10 min (Munemasa et al., 2007). Through the inhibition of ABA biosynthesis by chemical inhibitors or in ABA-deficient mutants, the MeJA-induced Ca^{2+} oscillations in guard cells are suppressed, and also stomatal closure is impaired (Hossain et al., 2011). Therefore, it has been postulated that MeJA-mediated regulation of stomatal closure interacts with ABA-mediated regulation of Ca^{2+} signal transduction pathways. Studies related to the interactions of ABA with MeJA in guard cells show that both hormones induce the formation of ROS and NO, and also that both are present at reduced concentrations in MeJA-insensitive plants (Munemasa et al., 2007).

The combined effect of ABA and JA for acclimation to stress in *Arabidopsis* may be mediated by an extensive genetic reprogramming to finally reach a new homeostasis (Harb et al., 2010). These authors suggested that endogenous JA together with high ABA level are sufficient to stimulate the preparatory response needed for drought acclimation (e.g., stomatal closure and cell wall modification) during the early stages of moderate drought (30% field capacity). Probably, JA is not required at high concentration under drought stress, and plant growth would be even negatively impacted by high concentrations. For example, the JA-insensitive *coi1* and *jin1*, mutants of *Arabidopsis* were found to be significantly resistant (or insensitive) to moderate drought stress. Biomass accumulation as compared to wild type under drought did not differ from the well-watered control. These results were in agreement with studies showing that the JA-mediated inhibition of seedling and root growth is suppressed in the *coi1* mutant (Xie et al., 1998). Harb et al. (2010) suggested that in the absence of JA signal perception, the developmental program for acclimation to stress, i.e., reduced growth is not switched on. Thus, the signaling pathways for plant growth under prolonged drought might converge on the down-regulation of JA biosynthesis to minimize its inhibitory effect on plant growth, thus establishing a new state of homeostasis by the acclimation process.

The proposed overlap between the JA and ABA stress signaling cascades (Fujita et al., 2006; Harb et al., 2010) has stimulated the search for transcription factors and kinases as promising candidates for common players in this interaction. For example, the transcription factor AtMYC2 plays a role in multiple hormone signaling pathways. From the genetic analysis of the jasmonate-insensitive *jin1* mutant, it was revealed that *JIN1* is allelic to *AtMYC2*, which was first identified as a transcriptional activator that is involved in the ABA mediated drought-stress signaling pathway (Abe et al., 2003). Downstream targets, such as *RD22*, a gene responsive to dessication and salt stress, is activated by both, AtMYC2 and R2R3MYB-type, transcription factors. Similarly, expression of *RD26* is induced by hydrogen peroxide, pathogen infections, and JA, as well as by drought, high salinity and ABA treatment (Fujita et al., 2004, 2006; Harb et al., 2010). In addition, protein phosphorylation and dephosphorylation by kinases and phosphatases, respectively, can significantly affect the regulation of morpho-physiology and gene expression associated with JA-dependent root growth. However, enzymes phosphorylating or dephosphorylating *AtMYC2* have not been identified yet (Kazan and Manners, 2008).

To specifically address the crosstalk of ABA and JA at the whole plant level, the tomato ABA-biosynthetic mutant *sitiens* was used. When the petioles of *sitiens* were incubated in JA, they did not show any indications of stomatal closure as assessed by gas-exchange measurements; however, when pre-incubated with ABA, petioles showed stomatal closure in response to JA (Herde et al., 1997). This suggested that in tomato, ABA was required for the JA-mediated stomatal regulation. In soybean, it was observed that exogenous application of MeJA did not affect endogenous ABA levels. However, water stressed barley seedlings that had been pre-treated with JA showed more than fourfold accumulation of ABA in comparison to the control. This clearly suggested a role for JA in ABA biosynthesis under water stress conditions (Bandurska et al., 2003). MeJA regulates numerous drought-responsive genes (Huang et al., 2008), many of which are also regulated by ABA with similar expression kinetics (Nemhauser et al., 2006; Huang et al., 2008). Overall, all these data support the concept of common signaling components for ABA and MeJA, including nitric oxide (NO; for review, see Daszkowska-Golec and Szarejko, 2013).

Involvement of Jasmonates in the Drought Response in Rice

Substantial information exists about the roles of phytohormones under drought in model plants such as *Arabidopsis*. A current challenge is to transfer this knowledge to other, economically more relevant, plant species. Two examples of investigations performed in rice are presented as case studies.

Seo et al. (2011) used a functional genomics approach that identified a basic helix-loop-helix domain gene (*OsbHLH148*) that conferred drought tolerance as a component of the jasmonate signaling module in rice. They found that *OsbHLH148* transcript levels were rapidly increased by treatment with MeJA or ABA, as well as by abiotic stresses including dehydration, high salinity, low temperature and wounding. Over-expression of *OsbHLH148* in rice conferred tolerance to drought stress. Expression profiling followed by DNA microarray and RNA gel-blot analyses of transgenic versus wild type rice identified genes that were up-regulated by over-expression of *OsbHLH148*. These genes included *OsDREB* and *OsJAZ*, genes involved in osmotic stress responses and jasmonate signaling, respectively. *OsJAZ1*, a rice ZIM domain protein, interacted with *OsbHLH148* in yeast two-hybrid and pull-down assays and it interacted with the putative OsCOI1 only in the presence of coronatine. Furthermore, the OsJAZ1 protein was degraded by rice and *Arabidopsis* extracts in the presence of coronatine, and its degradation was inhibited by MG132 which is a 26S proteasome inhibitor, suggesting 26S proteasome-mediated degradation of OsJAZ1 via the SCF^{OsCOI1} complex. These results suggested that OsJAZ1 was a transcriptional regulator of the OsbHLH148-related jasmonate signaling pathway leading to drought tolerance, suggesting that OsbHLH148, OsJAZ, and OsCOI1 constitute a signaling module in rice.

Another study by Kim et al. (2009) demonstrated that constitutive overexpression of the *Arabidopsis JASMONIC ACID CARBOXYL METHYLTRANSFERASE* gene (*AtJMT*) in rice increased the levels of MeJA by sixfold in young panicles in rice. Grain yield was greatly reduced due to lower number of spikelets and lower grain-filling rate as compared to non-transgenic (NT) controls. The number of spikelet organs, including the lemma/palea, lodicule, anther, and pistil were altered in these transgenic plants. The loss of grain yield and alteration in spikelet organ numbers were reproduced by treating NT plants with exogenous application of MeJA, thereby indicating that it was the increased levels of MeJA in the AtJMT transgenic rice panicles, that was responsible for the inhibition of spikelet development. Interestingly, in young NT panicles upon exposure to drought conditions, MeJA levels were increased by 19-fold resulting in a similar loss of grain yield. ABA levels were increased by 1.9- and 1.4-fold in the transgenic and drought-treated NT panicles

respectively. Increased levels of ABA in the AtJMT transgenic panicles grown in non-drought conditions suggests that it is MeJA, and not drought stress, which induces ABA biosynthesis under drought. A microarray strategy identified seven genes commonly regulated in the AtJMT transgenic and drought-treated NT panicles. Two of these genes, namely *OsJMT1* and *OsSDR* (for short-chain alcohol dehydrogenase), participate in rice in the biosynthesis of MeJA and ABA, respectively. Overall, these results suggested that plants produce MeJA during drought stress, which in turn stimulates the production of ABA, leading to a loss of grain yield.

The two examples above establish the importance of further studies exploring the role of JAs in combating drought stress in rice. Similar studies in other cereals may also be helpful in delineating the role of JA individually or in combination with other phytohormones toward understanding the plant response to drought and toward generating more resilient cereal plant varieties.

JASMONATES AND SALT STRESS: A RELATION NOT EASY TO BE FIGURED OUT

Salinity stress is at least as complex as drought stress. Initially, it mainly triggers three harmful effects, (i) osmotic stress (reduced water uptake),(ii) specific ion toxicity stress (mainly Na^+ ad Cl^-), and (iii) oxidative stress by uncontrolled production of ROS, including superoxide radicals (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). These ROS can cause oxidative damage to proteins, enzymes, DNA and RNA (Pessarakli, 2001; Sharma et al., 2012), but can also act as important stress signals. After their role in osmotic stress has been discussed above, now the role of jasmonates in the context of ion toxicity and oxidative stress will be addressed.

Several reports investigated the involvement of JA in salt stress. On the one hand, application of exogenous JAs diminished the damage by salinity in soybean (Yoon et al., 2009) and rice (Kang et al., 2005). On the other hand, the level of endogenous JAs increased under strong salt stress in rice roots (Moons et al., 1997) and in tomato (Pedranzani et al., 2007) suggesting that accumulation of JAs could also protect against salt stress. Nevertheless, it is not possible to draw a general connection between high levels of JA and adaptation. For example, a comparison of two grapevine cell lines differing in their salinity tolerance revealed that the accumulation of JA and JA-Ile was more pronounced in the sensitive *Vitis riparia* than in the salt-tolerant *Vitis rupestris* (Ismail et al., 2012, 2014a). Also, JA was induced after osmotic stress but not after salt stress in barley segments (Kramell et al., 2000) and rice seedlings (Takeuchi et al., 2011). Recent evidence suggests that alterations in the level of JAs affect salinity tolerance in rice. Kurotani et al. (2015) demonstrated that overexpression of *CYP94*, a gene encoding a catabolic enzyme inactivating JA-Ile, results in improved salt tolerance. Suppression of OsJAZ9, a repressor of JA signaling, produced higher sensitivity to JA and an increased sensitivity to salt (Wu et al., 2015). Conversely, rice mutants of the JA biosynthesis enzyme AOC, *hebiba*, and *cpm2*, showed an improved salt tolerance (Hazman et al., 2015). Whether it is the lack of JA or JA-Ile, or whether it is the absence of their precursor 12-OPDA which causes this tolerance, remains to be elucidated. The discrepancies with respect to the role of JAs for salt tolerance indicate that it may not be the presence or absence of JAs that decides the kind of response to salinity, but their timing and control, which may be more important (Ismail et al., 2014b).

Evidence for the Involvement of Jasmonates in the Uptake of Sodium Ions

In order to cope successfully with high salinity stress, it is necessary for plants to reduce the accumulation of sodium ions into the photosynthetic tissue. This holds especially true for glycophytes to which many important economic crops belong. Currently, it is not clear, whether and how extra- or intracellular sodium ions are sensed, there is no evidence for a receptor of sodium ions in *sensu stricto* (Zhu, 2007). Even though the molecular identity of Na^+ sensors has remained elusive, the plasma membrane Na^+/H^+ antiporter SOS1 might be a probable candidate (Shi et al., 2000). The transport activity of SOS1 is essential for sodium efflux from *Arabidopsis* cells but additionally, its long cytoplasmic tail can bind Na^+ and might therefore confer sodium sensing (Conde et al., 2011). The JA biosynthesis genes *ALLENE OXIDE SYNTHASE* (*AOS*) and *ALLENE OXIDE CYCLASE* (*AOC*) have been reported to be highly expressed in response to salinity in a SOS-dependent manner (Gong et al., 2001), suggesting a shared pathway. However, the exact link between jasmonates and SOS1 on the level of signaling and functional interactions is far from clear.

It is well known that the plasma membrane around the root hair epidermal cells is responsible for the influx of the largest portion of sodium ions into plant cells at the soil–plant interface. Several candidate genes have been reported as responsible for Na^+ uptake in plants including NSCCs, high-affinity K^+ transporters (HKTs), and low-affinity cation transporter (LCT1; Schachtman et al., 1997; Apse and Blumwald, 2007; Craig Plett and Møller, 2010). Additionally and equally important, controlling potassium supply during salinity stress is the main key for survival as the K^+/Na^+ ratio should be kept as high as possible for avoiding metabolic failure due to sodium toxicity (Kronzucker et al., 2013). Uptake of sodium ions into rice depends on jasmonates (Hazman et al., 2015), since the *aoc* mutants *hebiba* and *cpm2* accumulated significantly less sodium ions in their shoots, indicating that selective transporters, presumably located in the Casparian strip in the root, might be regulated by jasmonates. It is likely that this trait vary between species and even between cultivars within the same species, which might be one of the reasons, why salt tolerant and salt sensitive cultivars can accumulate different levels of JAs in response to salt stress.

JASMONATE CROSSTALK TO OTHER PHYTOHORMONES IN ABIOTIC STRESS SIGNALING

In the previous sections, JA crosstalk to ABA and the involvement of protein phosphorylation (e.g., of MYC2) and ubiquitination

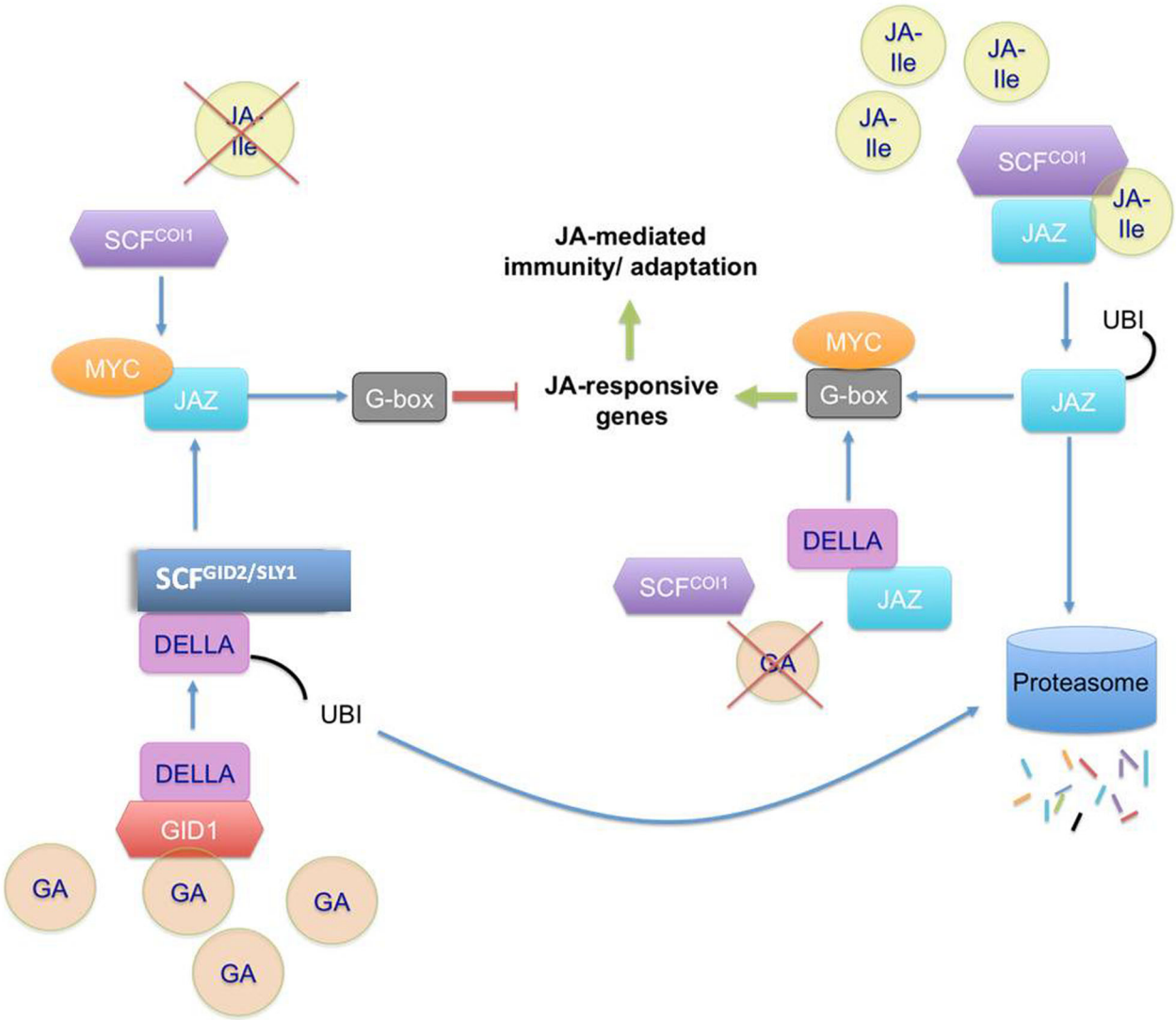


FIGURE 2 | Schematic representation of JA signaling activation and repression in the presence of JA and GA. JA-Ile mediates the activation of JA responsive genes by attaching to JAZ proteins and F-box COI1 complexes; which in turn become ubiquitinylated and are committed to the degradation pathway. MYC transcription factors 2, 3, and 4 are then free to attach to the G-box domain and activate the transcription of the JA-responsive genes. In the absence of JA-Ile, JAZ proteins bind to the transcription factors and restrict gene expression. A similar mechanism is observed in the presence/absence of GA. This pathway is mediated through DELLA proteins. In the presence of GA, the repression is activated. GA binds to the receptor GID1, then DELLA can bind to the hormone-receptor complex. Subsequently, DELLA binds to GID2/SLY1, the F-box protein in SCF-GID2/SLY1 which promotes DELLA ubiquitination. That leaves JAZ proteins free to bind to MYC transcription factors and the JA-responsive genes cannot be transcribed. On the other hand, the absence of GA means that DELLA are not sent to the degradation pathway and bind to JAZ. JAZ cannot bind to MYC transcription factors, which are available to bind the G-box and activate the transcription of JA-responsive genes.

(e.g., of JAZ repressors) as downstream effects of JA have been mentioned. Although the JA and ABA crosstalk for the regulation of stomatal opening is important, especially under drought, this seems to be not the only crosstalk of JA with other phytohormones or signaling molecules.

Gibberellic acid (GA), generally known as growth hormone, plays also a role in abiotic stress tolerance. The content of GAs may be involved in either growth suppression or promotion under a specific abiotic stress (Colebrook et al., 2014). Crosstalk between GA and JA can be mediated through the DELLA and JAZ proteins which directly interact with each other. This interaction would compete with the JAZ proteins binding to MYC2, a transcription factor activating JA responsive genes, whereby JAZ proteins negatively regulate the JA response. In the presence of JA, the JAZ proteins are committed to proteasome-mediated degradation, while in the presence of GA the DELLA proteins are recruited for proteasome-mediated degradation (**Figure 2**). Thus, in presence of GA, the JAZ proteins can again bind to MYC2, the alternate partner, and attenuate JA-responsive genes (Boter et al., 2004; Hou et al., 2010). Interestingly, a DELLA gene *RGL3* is transcriptionally upregulated by JA signaling, and the promotor of *RGL3* is a target of MYC2 (Wild et al., 2012). RGL3

physically interacts with JAZ1 and JAZ8, the latter being relatively resistant to JA-mediated degradation (Shyu et al., 2012; Wild et al., 2012). Thus, JA-mediated degradation of JAZ1 releases MYC2 to induce *RGL3*, which in turn binds the non-JA degradable JAZ8 enhancing the MYC2-dependent JA responses (Wild et al., 2012). Further, the GA content determines the extent of degradation of DELLA proteins, and thus the induction amplitude for the JA response, thus linking the two hormone signaling pathways. GA and ABA crosstalk follows a similar pattern under abiotic stress through another DELLA target (Ko et al., 2006; Zentella et al., 2007).

Interaction between auxins including the main natural auxin indole-3-acetic acid (IAA) and JA during plant growth and development have been described including phenomena such as cell elongation, abscission, and tendril coiling, but also wound responses (Saniewski et al., 2002). Recently Du et al. (2013) documented interactions between IAA and JA under drought in rice. Analysis of transcripts related to auxin and JA biosynthesis or signaling showed increased expression of auxin related genes under heat and cold, but a decrease under drought, which was paralleled by corresponding changes of IAA content. However, the content of JA and its associated genes increased under drought and cold but decreased under heat stress. Tiryaki and Staswick (2002) showed that the expression of JA-responsive genes was either repressed or induced by exogenous auxin, suggesting that JA- and auxin-triggered signaling can interact both antagonistically or synergistically. The underlying mechanisms as well as the biological context is far from understood, but one point of convergence might be the GH3 family of acyl acid amido synthetases which contribute to amino acid conjugation of both IAA and JA, which in case of JA generates the active signal, whereas it might be a mechanism of inactivation in case of IAA (Staswick et al., 2005; Khan and Stone, 2007). JA and auxin crosstalk during JA induced lateral root formation involving the ethylene response factor 109 (ERF109) was reported by Cai et al. (2014), and Jiang et al. (2014) recently reported that, in *Arabidopsis*, the WRKY57 acts as a node of convergence in JA and IAA-mediated signaling during JA-induced leaf senescence. *Arabidopsis* JAZ4/8 and IAA29, repressors of JA and IAA signaling, respectively, both competitively bind WRKY57, which is upregulated by IAA, but downregulated by JA. A rice AUX/IAA protein, OsIAA6 has been shown to correlate with drought tolerance in rice (Jung et al., 2015). Thus, JAZ, IAA, ARF, and WRKY genes are known to be act positively on drought tolerance, but the mechanisms of their interaction are yet to be elucidated.

Cytokinins (CK) as further important class of phytohormones driving cell division and meristem formation might also interact with JA signaling. The crosstalk between JA and auxin during meristem formation is well documented (Su et al., 2011), but, so far, there is not much evidence for interplay between JA and CK. However, the two hormonal pathways might be linked antagonistically (Sano et al., 1996; Naik et al., 2002; Stoynova-Bakalova et al., 2008). CK content *in vivo* and application of exogenous CK accelerate the JA-mediated stress response (Sano et al., 1996; Dervinis et al., 2010), while JA application induces the accumulation of CK ribosides (Dermastia et al., 1994). JA biosynthesis is activated in the roots during drought (Poltronieri et al., 2013), and repression of CK biosynthesis and signaling promotes the expansion of the root system, which should act positively on drought tolerance (Werner et al., 2010). Thus, JA and CK signaling/biosynthesis might mainly act in an antagonistic manner.

Similar to JA, also salicylic acid (SA) has been classically associated with biotic stress. However, a combined approach of proteomics and transcriptomics identified common proteins upregulated by JA and SA, associated with oxidative or abiotic stress responses (Proietti et al., 2013). On the other hand, SA can quell the induction of AOS in response to wounding, demonstrating a negative crosstalk from SA upon JA signaling (Harms et al., 1998). The role of SA under abiotic stresses including heat, salt and osmotic stress is well accepted and has been extensively reviewed (Horváth et al., 2007; Pal et al., 2013; Miura and Tada, 2014). The convergence between the JA and SA signaling in *Arabidopsis* was identified as the MAP Kinase 4 (AtMPK4), which negatively regulates the activation of SA- and the repression of JA-mediated defenses under biotic stress (Brodersen et al., 2006). Whether AtMPK4 exerts the same function under abiotic stress remains to be tested, but it is already known that AtMAPK4 is rapidly activated by abiotic stresses (Ichimura et al., 2000). In this regard the role of SA in stomatal closure is noteworthy (Miura and Tada, 2014).

Crosstalk between JA and ethylene is well known for defense against plant pests and pathogens. Once again, however, importance of such crosstalk between JA and ET in abiotic stress has been elaborated only recently (Kazan, 2015). The expression of the *Arabidopsis* ethylene response factor 1 (AtERF1) is activated by both JA and ET (Lorenzo et al., 2003). It has now been reported that synergistic activation of AtERF1 is required for drought and salinity tolerance (Cheng et al., 2013). Constitutive overexpression of AtERF1 additionally produced enhanced tolerance to heat as well. The set of genes upregulated in the AtERF1 overexpression plants can be assigned to heat, drought, salt and JA responses, respectively, but unlike for biotic stress, these genes are activated through binding of ERF1 to the dehydration response element (DRE) rather than to the GCC element (Cheng et al., 2013). For the salt stress response in tobacco, JA connects not only with ET, through a jasmonate responsive tomato ERF (*JERF1*), but also with ABA signaling (Zhang et al., 2004). Also a second tomato ERF (*SlERF.B.3*) is linked with the response to salt and cold stress (Klay et al., 2014).

The JA crosstalk to brassinosteroids has also been documented. Brassinosteroids were shown to negatively regulate the JA inhibition of root growth, the point of convergence being the F-Box protein coronatine insensitive 1 (COI1) required for JA response (Ren et al., 2009). Inhibition of JA induced accumulation of anthocyanins by brassinazole in *Arabidopsis* represents a second example for a negative impact of brassinosteroid signaling upon the JA pathway (Peng et al., 2011). In this interaction between JA and brassinosteroids the WD-repeat/Myb/bHLH transcriptional complexes were implicated (Qi et al., 2011). Concordance was shown between anthocyanins and drought tolerance in rice and *Arabidopsis* (Basu et al., 2010; Sperdouli and Moustakas, 2012), thus implicating a role for JA. Divi et al. (2010) reported on the

crosstalk between brassinosteroids, ET, SA, JA, and ABA using mutants in the respective phytohormone biosynthesis pathways. Recently the same authors studied the brassinosteroids-mediated stress tolerance and found distinct molecular signatures of ABA and JA (Divi et al., 2015).

Nitric oxide has emerged as a major signal affecting the JA, SA, and ET signaling in biotic stress response, and has meanwhile also been shown to act in abiotic stress responses as well (Wang et al., 2006; Song et al., 2008; Huang et al., 2009; Zhang et al., 2011). In this context, the role of NO in stomatal aperture has attracted most attention, because guard cells have emerged as convenient model system to dissect signaling cascades. The first tier of signaling is formed by pH, ROS, free Ca^{2+}, and phospholipid activating the next layer of signaling by complex interactions of these primary signals with ABA, ET, JA, and NO (Gayatri et al., 2013). By treatment with NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase (Xin et al., 2005), jasmonate-induced stomatal closure could be modulated indicating that NO acts downstream of JA. Like in most cases of signal crosstalk, specific roles, timing and convergence points of NO and JA signaling are still not characterized, which would be a precondition for strategies to promote plant tolerance to abiotic stress.

PROTEIN POST-TRANSLATIONAL MODIFICATION AND JASMONATES CROSSTALK

Post-translational modifications (PTM) regulate many proteins critical for JA biosynthesis and their signaling crosstalk with other phytohormones. Among the various possible protein modifications, protein phosphorylation, ubiquitination and SUMOylation have been in focus for these pathways. For example, PPS3 the potato homolog of *Arabidopsis* JAI3/JAZ3, is phosphorylated by StMPK1, the MPK6 homolog of *Arabidopsis* MAP kinase (Katou et al., 2005). Dephosphorylation of proteins involved in regulating JA content has also been shown (Schweighofer et al., 2007).

In JA signaling, an important role is played by the JAZ and the DELLA proteins, both of which are ubiquitinated in the presence of jasmonates and gibberellins, respectively, and undergo proteasome-mediated degradation. JAZ degradation leads to transcriptional activation and DELLA degradation leads to repression of the JA responsive genes by MYC2 (**Figure 2**). Recently, SUMOylation of DELLA was shown to sustain DELLA-mediated inhibition of growth under stress (Conti et al., 2014). SUMOylated DELLAs bind to the SUMO interacting motif of the GA receptor GID1 whose sequestration leads to an accumulation of non-SUMOylated DELLAs. It remains to be seen if in such conditions the SUMOylated or non-SUMOylated content of the DELLAs leads to their altered interaction with the JAZ proteins and influences the activity of the JA responsive genes.

The identification of *auxin-resistant1* (*axr1*) mutants with altered jasmonate responsive gene expression during screening for mutants resistant to MeJA- and auxin-mediated growth inhibition indicated JA-auxin crosstalk (Tiryaki and Staswick, 2002). *AXR1* encodes an enzyme that activates a second small protein related to ubiquitin (RUB1; Nedd8 in mammals). Therefore, both jasmonate and auxin signal transduction depends on small modifier proteins (Lorenzo and Solano, 2005).

Salicylic acid signaling is controlled by a SUMO E3 ligase (SIZ1) whereby *siz1* mutants of *Arabidopsis* accumulate SA (Lee et al., 2007). SIZ1 is also known to regulate drought responses without the involvement of DREB2A and ABA, with the expression of nearly 10% of the drought inducible genes being mediated by SIZ1 (Catala et al., 2007). These genes also include some for JA responses. Down-regulation of 11 genes of the brassinosteroid biosynthesis and signaling pathway was noted through the genome-wide expression analysis of the *siz1* mutants. This suggested an important role for protein SUMOylation in these pathways (Catala et al., 2007).

Interestingly, NO content is highly influenced by the SUMOylation of the nitrate reductases (NRs) NIA1 and NIA2, and SUMOylation substantially increases the activity of the two NRs (Park et al., 2011). Finally, SIZ1, the SUMO E3 ligase, is also involved in copper homeostasis putatively through regulating the metal transporters YELLOW STRIPE LIKE (*YSL1* and *YSL3*; Chen et al., 2011), which affects ethylene perception (Burkhead et al., 2009).

Apparently, SUMO conjugation-deconjugation to proteins plays an important role in abiotic stress (Castro et al., 2012; Raorane et al., 2013). This section implies that a number of effects of SUMO on abiotic stress may be mediated through affecting phytohormone biosynthesis and signaling crosstalk. When phytohormone crosstalk is so intricately linked that spatio-temporal signatures of active hormone content are the defining features, rather than overall content, PTM of the proteins involved in the various crosstalk routes becomes a regulatory feature that is likely as important as their transcription and translation *per se*. For a holistic understanding of JAs as emerging molecules of importance in abiotic stress tolerance, not only their crosstalk to other phytohormones but also the protein PTM aspects must be deeply explored.

CAN FINE-TUNING OF THE JASMONATE PATHWAY LEAD TO ABIOTIC STRESS TOLERANCE?

Plants can resist abiotic stresses through several distinct mechanisms, but the traits associated with resistance mechanisms are multigenic, often converging on genes shared by different stresses. Under stress conditions, the interactions happening between signaling pathways and their biological significance still remain unclear. As of now, these pathways are getting better resolved due to the evolution of new tools that allow the exploration of the physiological, genetic, and biochemical basis of such processes. The use of genomic, proteomic, and metabolomic approaches is gaining grounds not only in model plants such as *Arabidopsis* but also in crops such as rice. These investigative strategies will unravel new crosstalks between the different classes of stress hormones (for review, see, e.g., Kazan, 2015).

Keeping in view the immense losses caused by adverse environmental conditions like drought or salinity, there is an immediate need to develop new crop varieties with better adaptability or enhanced tolerance. Till date only a handful of labs

around the world have been able to show a direct relation between JA functioning as a stress hormone in salinity and drought tolerance. The examples discussed here present a substantial reason to suggest a central role of JA as a hormone for these stress responses. More research focus in this direction will help to explain various drought stress responses and provide powerful tools for improving drought tolerance in plants and to develop new drought tolerant varieties. However, it will be a big challenge to manipulate JA biosynthesis or signaling without giving rise to negative side effects commonly associated with reduced jasmonate function such as reduced fertility and enhanced sensitivity to pathogens. Finding the critical nodes in the phytohormone biosynthetic pathways, whose manipulation can be useful for stress tolerance without the associated penalties; will largely define the level of success in utilizing these pathways for breeding stress tolerant crop varieties.

AUTHOR CONTRIBUTIONS

MR, RD, MH, BM, AK, and PN drafted the manuscript. BM and MR designed the figures. MR, RD, AK, and PN revised the manuscript.

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JAZ Repressors: Potential Involvement in Nutrients Deficiency Response in Rice and Chickpea

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Jasmonates (JA) are well-known phytohormones which play important roles in plant development and defense against pathogens. Jasmonate ZIM domain (JAZ) proteins are plant-specific proteins and act as transcriptional repressors of JA-responsive genes. JA regulates both biotic and abiotic stress responses in plants; however, its role in nutrient deficiency responses is very elusive. Although, JA is well-known for root growth inhibition, little is known about behavior of *JAZ* genes in response to nutrient deficiencies, under which root architectural alteration is an important adaptation. Using protein sequence homology and a conserved-domains approach, here we identify 10 novel *JAZ* genes from the recently sequenced Chickpea genome, which is one of the most nutrient efficient crops. Both rice and chickpea *JAZ* genes express in tissue- and stimuli-specific manners. Many of which are preferentially expressed in root. Our analysis further showed differential expression of *JAZ* genes under macro (NPK) and micronutrients (Zn, Fe) deficiency in rice and chickpea roots. While both rice and chickpea *JAZ* genes showed a certain level of specificity toward type of nutrient deficiency, generally majority of them showed induction under K deficiency. Generally, *JAZ* genes showed an induction at early stages of stress and expression declined at later stages of macro-nutrient deficiency. Our results suggest that *JAZ* genes might play a role in early nutrient deficiency response both in monocot and dicot roots, and information generated here can be further used for understanding the possible roles of JA in root architectural alterations for nutrient deficiency adaptations.

Keywords: jasmonates, nutrient deficiency, root, gene expression, jas degron, TIFY

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INTRODUCTION

Jasmonates (JAs) form a family of oxylipin phytohormones, derived from oxidation of 18 and 16 carbon tri-unsaturated fatty acids (Wasternack and Kombrink, 2010). These phytohormones are known to regulate a wide-range of processes including spikelet development (Cai et al., 2014), senescence (He et al., 2002), root growth (Staswick et al., 1992), communication (both interplant and intra-plant for defense) (Okada et al., 2014) and defense responses against biotic stress (Feys et al., 1994) through degradation of JA signaling repressor proteins (JAZs) (Kazan and Manners, 2012). JA-Isoleucine (JA-Ile), a bioactive form of JA, binds to its receptor complex consisting of CORONATINE-INSENSITIVE1 (COI1), an F-box E3-ubiquitin ligase protein and JAZ repressor (Yan et al., 2009). This COI-JA-Ile complex interacts with JAZ proteins which contain at least two conserved regions, namely, TIFY and Jas at N and C terminal, respectively. The TIFY motif of

the JAZ proteins mediates homo- and heteromeric interactions (Chini et al., 2009; Pauwels and Goossens, 2011), whereas the Jas motif is necessary for interaction of JAZ proteins with COI1 in the presence of JA-Ile (Xie et al., 1998), leading to the degradation of JAZ repressors through 26S proteasomal pathway. The Jas motif also mediates interaction with MYC2 (a bHLH transcription factor regulating JA responsive genes) and facilitates inhibition of MYC2 activity (Chini et al., 2007). Therefore, in the absence of JA-Ile, a JAZ protein remains bound to MYC2, and inhibits the transcription of JA responsive genes. Interaction of JAZ with COI^{SCF} complex leads to the degradation of JAZ proteins resulting in release of MYC transcription factors and thus allowing JA responsive genes to be transcribed (Chini et al., 2007; Thines et al., 2007). A few JAZ proteins in Arabidopsis also have an EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) motif which allows direct binding of JAZ proteins to TOPLESS LIKE (TPL) without involvement of the Novel Interactor of JAZ (NINJA), an adapter protein. TIFY domains of a few JAZ proteins are also involved in the interaction with NINJA, which further recruits TPL through its EAR motif (Pauwels et al., 2010). Moreover, TPL recruits Histone Deacetylases (HDA6 & HDA19) which further suppress the gene expression via chromatin remodeling (Zhou et al., 2005; Wu et al., 2008).

Overexpression of *JAZ* without Jas motif (*JAZ1-*Δ*Jas*) resulted in male-sterile plants (Thines et al., 2007). Similarly, overexpression of the truncated splice variant of *AtJAZ10* (AtJAZ10.4) which was resistant to COI^{SCF}-mediated degradation, produced male-sterile plants (Chung and Howe, 2009). This observation was again validated by Cai et al. (2014) showing that OsJAZ1 could regulate the expression of E-class genes such as *OsMADS1*, *OsMADS7,* and *OsMADS8* which have roles in inflorescence and spikelet development, resulting in defected spikelet development in rice. It was further shown that Arabidopsis overexpressing *JAZ1-*Δ*Jas* has reduced host resistance to feeding by *S. exigua* larvae (Chung et al., 2008). Moreover, most of JA signaling genes like *AtJAZ1-10* were found to be upregulated on herbivore feeding. These results indicate a direct role of JAZ proteins in defense and plant development. In addition, recently, a few reports have also linked JA signaling with potassium (K) and Phosphorus (P) deficiency response (Chacón-López et al., 2011; Shankar et al., 2013, Takehisa et al., 2013; Wu et al., 2015).

Balanced mineral nutrients supply is critical for optimal growth and development of plants. Each mineral nutrient plays a critical role in the physiological and developmental aspects of plants (Marschner, 1995). Nutrient-deficiency responses are controlled by many factors including phytohormones. For example, cytokinins (CKs) negatively regulate the Pi (Phosphate) starvation response (Martín et al., 2000), abscisic acid (ABA) regulates both sulfur homeostasis and Pi-starvation responses (PSR) (Jiang and Zhang, 2001). While auxins seem to interact/regulate with signaling pathways for the homeostasis of many nutrients including nitrogen (N), phosphorus (P), sulfur (S), and potassium (K) (Franco-Zorrilla et al., 2004; Ticconi and Abel, 2004; Ashley et al., 2006; Kopriva, 2006; Zhang et al., 2007). Further, auxins regulate root hair and lateral root development under Pi deficiency to increase the root absorption area (López-Bucioet al., 2003). Cytokinins, on the other hand, regulate metabolic changes under nitrogen deficiency (Sakakibara, 2006). Therefore, phytohormones control both physiological and architectural adaptations for nutrient homeostasis.

JA is well-known for inhibiting root elongation (Staswick et al., 1992, Wasternack and Hause, 2013) and plays a key role in root meristem alteration under P deficiency (Chacón-López et al., 2011). Further, transcriptome analysis has revealed that many JA responsive genes including JA biosynthetic genes (*OsAOS1*, *OsLOX2,* and *OsLOX3*) and JAZ family genes (*OsJAZ2*, *-5*, and *-9*) are induced under K deficiency (Takehisa et al., 2013). As many nutritional deficiencies also modulate root system architecture (RSA) to enhance the acquisition of essential nutrients (Lynch, 2011), it becomes rational to study the behavior of JA signaling genes, especially *JAZ* repressors under both macro and micro nutrients deficiency. Previously, 12 JAZ proteins have been identified in *Arabidopsis thaliana* (Thines et al., 2007) while 15 in *Oryza sativa* (Ye et al., 2009), but there was no report for JAZ proteins in *Cicer arietinum,* a legume which is efficient in nutrient homeostasis (Schulze et al., 2006; Varshney et al., 2013). In this study, we have identified 10 JAZ proteins in the recently sequenced chickpea genome, examined their phylogenetic relationships and studied expression patterns of *JAZ* genes in rice and chickpea under macro (N, P, K) and micro (Fe, Zn) nutrients deficiency. Our results showed the structural and functional conservation of JAZ repressors in monocots and dicots, and their differential behavior under macro and micro mineral-deficiency suggested a potential role of JA in plant nutrient homeostasis.

MATERIAL AND METHODS

Identification of JAZ Proteins in Rice and Chickpea

Rice and Arabidopsis known JAZ proteins were obtained from previous studies (Vanholme et al., 2007; Ye et al., 2009). These protein sequences were then used as queries to search for potential JAZ proteins in other organisms, namely, *Physcomitrella patens*, *Brassica rapa*, *Linum usitatissimum*, *Zea mays*, *Medicago truncatula*, *Manihot esculenta*, *Populus trichocarpa*, *Ricinus communis*, *Solanum tuberosum,* and *Solanum lycopersicum* using BLASTP in their respective databases (http://phytozome.jgi.doe.gov/pz/portal.html). Protein sequences, so obtained, were scanned for the presence of TIFY and Jas domain using SMART (http://smart.embl-heidelberg.de/) and interpro (http://www.ebi.ac.uk/interpro/). Proteins with both TIFY and Jas domains were retained for further analysis. After removal of redundant hits, 165 unique proteins (Supplementary text 1) with both TIFY and Jas domain were aligned and a Hidden Markov Model (HMM) was generated using HMMER 3.0 (http://cryptogenomicon.org/2010/03/28/hmmer-3-0/). This HMM was then used for HMMER searches in the rice and chickpea protein databases. For chickpea, both desi (http://nipgr.res.in/CGAP/home.php) and kabuli

(http://www.icrisat.org/gt-bt/ICGGC/homepage.htm) genomes were searched ($p = e^{-50}$). All the protein sequences obtained were again searched for non-redundant hits and only unique hits were scanned for the presence of TIFY and Jas motifs. Final sequences were considered as potential JAZ proteins in rice and Chickpea.

Structural Analysis of CaJAZ and OsJAZ Proteins and Genes

The protein sequences were analyzed in SMART (http://smart.embl-heidelberg.de/) and aligned in ClustalX (http://www.ebi.ac.uk/Tools/msa/clustalo/) to confirm the presence of TIFY and Jas motifs. The TIFY (TIF(F/Y)XG) domain was extracted from SMART and INTERPRO databases while the Jas domain was identified manually from the CCT domain having $SLX_2FX_2KRX_2RX_5PY$ as the conserved amino acid motif. MEME (Multiple Expectation Maximization for Motif Elicitation, Bailey et al., 2009) was used to further identify the additional motifs in identified rice and chickpea JAZs. For nucleotide level investigation, *JAZ* genes were visualized in rice genome database and cDNA and genomic sequences were aligned manually. Chickpea *JAZ* information, like chromosomal location, genomic DNA sequences, exon and intron structures, protein sequences and coding sequences, were obtained from the CGAP database (http://nipgr.res.in/CGAP/home.php). Phylogenetic trees were generated for JAZ protein sequences of *Arabidopsis thaliana* (12), *Oryza sativa* (15), *Physcomitrella patens* (4), *Brassica rapa* (24), *Linum usitatissimum* (8), *Zea mays* (27), *Medicago truncatula* (9), *Manihot esculenta* (19), *Populus trichocarpa* (10), *Ricinus communis* (7), *Solanum tuberosum* (14), *Solanum lycopersicum* (6), and *Cicer aeriantum* (10). All protein sequences were aligned with ClustalX and an unrooted phylogenetic tree was generated using MEGA 6.06 (Molecular Evolutionary Genetics Analysis), with the neighbor-joining method. Phylogenetic trees were visualized using MEGA 6.06 software with bootstrap values from 1000 replicates at each branch (Tamura et al., 2007).

Promoter Sequence Analysis of *OsJAZ* and *CaJAZ* Genes

To identify the putative *cis*-acting elements in a promoter region, 2 kb region upstream of the start codon was scanned in the Plant *Cis*-acting Regulatory DNA Elements database (http://www.dna.affrc.go.jp/PLACE/) for both rice and chickpea *JAZ*s.

Ka/Ks Analysis of *OsJAZ* and *CaJAZ* Genes

For estimation of non-synonymous (Ka) and synonymous (Ks) substitution rates, the aligned amino acid sequences and their corresponding cDNA sequences of rice and chickpea *JAZ* genes conserved across the plant species, were analyzed using CODEML in the PAML interface tool of PAL2NAL (http://www.bork.embl.de/pal2nal).

Plant Growth Conditions and Different Nutrient Deficiency Treatments

Rice (*Oryza sativa* var PB1) seeds were surface-sterilized by 0.1% mercuric chloride for 10 min and, thereafter, washed five-times with sterile water and germinated on wet filter paper for 2 days in the dark at 37°C. Uniformly germinated rice seedlings were transferred to liquid culture medium (Yoshida et al., 1976) with the following composition: NH_4NO_3(1.40 mM), NaH_2PO_4 (0.32 mM), K_2SO_4 (0.51 mM), $CaCl_2.2H_2O$ (1 mM), $MgSO_4.7H_2O$ (1.7 mM), H_3BO_3 (19 μM), $ZnSO_4.7H_2O$ (0.15 μM), $CuSO_4.5H_2O$ (0.15 μM), $(NH4)_6MO_4O_2.4H_2O$ (0.015 μM), Citric Acid (70.75 μM), Na-Fe-EDTA (60 μM), and $MnCl_2.4H_2O$ (9.46 μM). Seedlings were transferred to complete media for the control and nutrient solution carrying lower concentration of NH_4NO_3(14 μM) for N deficiency (-N), NaH_2PO_4 (3.2 μM)for P deficiency (-P), K_2SO_4(5.1 μM) for K deficiency, $ZnSO_4.7H_2O$ (0.0015 μM) for Zn deficiency (-Zn), and Na-Fe-EDTA (0.6 μM) for Fe deficiency treatment. Seedlings were grown in a growth chamber maintained at 16 h photoperiod, 30/28°C day and night temperature, 280–300 μM photons/m^2/s photon density and ∼70% relative humidity.

Chickpea (var. ICC4958 desi) seeds were surface-sterilized with 70% ethanol for 2 min followed by 2% sodium hypochlorite carrying a drop of tween-20 treatment for 20 min. Thereafter, seeds were again surface-sterilized with 0.1% $HgCl_2$ for 1 min and washed with sterile water 5 times to remove the surfactants. Surface-sterilized seeds were soaked in water overnight and then transferred to wet germination paper for 2 days in dark. Uniformly, germinated seedlings were transferred to aerated liquid culture medium (1/4th Hoagland) with 696.9 μM $Ca(NO_3)_2$, 1.02 μM $MgSO_4.7H_2O$, 1.5 μM KNO_3, 459.9 μM H_3BO_4, 3.0 μM $CuSO_4$, 63.05 μM $MnCl_2.4H_2O$, 1.38 μM Na_2MoO_4, 7.9 μM $ZnSO_4$, 252.1 μM $NaH_2PO_4.2H_2O$, 57.8 μM Na-Fe-EDTA, 252.1 μM NH_4Cl for control. For N deficiency, seedlings were grown in Hoagland solution having KNO_3 and $CaNO_3$ replaced by equimolar concentration of K_2SO_4 and $CaCl_2$, respectively. For P deficiency, seedlings were grown in Hoagland solution having 2.52 μM NaH_2PO_4. For K deficiency, seedlings were placed in nutrient medium supplemented with 0.01 μM K_2SO_4 and KNO_3 was replaced by equimolar concentration of NH_4NO_3. For Zn and Fe deficiency seedlings were placed in nutrient solutions containing 0.07 μM $ZnSO_4$ and 0.57 μM Na-Fe-EDTA, respectively. Chickpea seedlings were raised in a chamber maintained at 12/12 h photoperiod, 23/18°C, 200–300 μM photons/m^2/s photon density and ∼70% relative humidity. Media were changed after every 2 days and pH was maintained everyday around 5.5 for both rice and chickpea.

In order to study the JA-inducible expression of chickpea *JAZ* genes, 12-days-old seedlings were treated with 100 μM Methyl-Jasmonate in liquid growth media for variable periods. The experiment was performed with three biological replicates.

Expression Analysis of *JAZ* Genes Under Different Nutrient Deficiencies in Rice and Chickpea Roots

Sample Collection, RNA Extraction, and cDNA Preparation

Root tissues were collected at 7 days (early response) and 15 days (late response) after stress treatment. Tissues were

frozen immediately in liquid nitrogen for further analysis. Sample collection was done during 2–3 p.m. every time to minimize the possible circadian effects. Experiments were repeated in three biological replicates. Total RNA from root was extracted using the TRIzol® method according to manufacturer's instruction, and further treated with DNAse to avoid genomic DNA contamination. cDNA was synthesized from 1 μg total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions.

Primer Designing and qRT-PCR

Primers for quantitative real-time PCR (qRT-PCR) were designed from coding region using PRIMER EXPRESS version 2.0 (PE Applied Biosystems™, USA) with default parameters. Each primer pair was checked for its specificity for its respective gene using BLAST tools of NCBI and TIGR databases. qRT-PCR was performed with cDNA using Fast SYBR® Green Master Mix to detect the quantity of double stranded product in Applied Bio systems 7500 Fast Real-Time PCR. Quantitative assays were performed in triplicates for each sample. The relative gene expression was calculated using the $^{\Delta\Delta Ct}$ method. *Ubiquitin5* (Os01g0328400) and Elongation Factor 1-alpha (AJ004960) were used as endogenous controls for rice and chickpea, respectively. A student's *t*-test was used for testing level of significance. Primer sequences for all the genes are listed in Table S1.

Tissue-specific expression patterns of CAJAZs were obtained from chickpea transcriptome database (CTDB; http://www.nipgr.res.in/ctdb.html) and the expression levels are provided as RPM (Reads per Million) values. While microarray data for *OsJAZs* was taken from rice expression database (http://www.ricearray.org/).

CaJAZ6 Cloning and Expression in Onion Epidermal Cells

The *CaJAZ6* sequence, obtained from chickpea database (http://nipgr.res.in/CGAP/home.php), was used for primer designing to amplify the ORF. Amplified sequence was confirmed using DNA sequencing for accuracy, and cloned in entry vector (pENTR™-DTOPO®). The ORF was then moved into the binary vector, pSITE3CA using LR reaction to produce YFP:CAJAZ6 fusion protein. DNA-coated gold particles were used for particle bombardment in onion epidermal cells as described (Giri et al., 2011). CaJAZ6 was visualized as YFP:CAJAZ6 fusion protein under fluorescence microscope (Nikon eclipse 80i).

RESULTS

JAZ Genes in Rice and Chickpea

Fifteen and twelve *JAZ* genes were reported earlier in rice and Arabidopsis, respectively (Thines et al., 2007; Ye et al., 2009). The Chickpea genome, a dicot like Arabidopsis, has been sequenced recently (Jain et al., 2013; Varshney et al., 2013). After comprehensive data mining in the rice genome for JAZ proteins, we did not find any new members of the family. Whereas, in chickpea, we identified 10 JAZ proteins using the protein blast and HMM searches. The identified chickpea JAZs were named according to their homology with Arabidopsis JAZs (**Table 1**; Figure S1). The chromosomal localization of *JAZs* was analyzed with Oryzabase for rice. Fifteen *JAZs* were located on six chromosomes. Five *OsJAZ* genes were present on chromosome 3, two each on chromosome 4 and 7, one on chromosome 8, two on chromosome 9, and three on chromosome 10 (Figure S2). Expansion of the rice *JAZ* gene family was also aided by tandem gene duplication as five genes are located in two duplicated blocks (*OsJAZ9-11* on chr 3; *OsJAZ12-13* on chr 10). Chromosomal positioning of *CaJAZ* genes showed that *CaJAZ10* was present on chromosome 2, *CaJAZ3b* on chromosome 6, *CaJAZ6* on chromosome 7 while three genes, *CaJAZ1b*, *CaJAZ12b,* and *CaJAZ3a* were present on chromosome 8 (**Table 1**). Four genes (*CaJAZ3c, 1a, 12a,* and *8*) were present on scaffolds (scaffold02277, 03027, 03745, and 06768, respectively).

Intron number varies from 0-6 in rice *JAZ* genes. Three *OsJAZ* genes, namely, *OsJAZ9, OsJAZ10,* and *OsJAZ13* are intron-less (Table S2). The intron later-theory correlates the increased intron

TABLE 1 | Structural and coding details of chickpea *JAZ* genes.

JAZ name	Protein ID	Chr.	Physical position		CTDB id	Domains/ motifs	CDS length	No. of exons	aa length	pI	Protein MW (Kda)	AL
CaJAZ10	Ca_01810.1	Ca_LG_2	12,357,154	12,363,824	TC11585	TIFY, Jas	901	8	300	6.73	32.94	1
CaJAZ3b	Ca_06739.1	Ca_LG_6	3,957,625	3,960,995	TC04335	TIFY, Jas	1021	7	346	8.71	36.421	3
CaJAZ6	Ca_07919.1	Ca_LG_7	8,049,102	8,052,009	TC17994	TIFY, Jas	625	5	208	9.78	23.2999	1
CaJAZ1b	Ca_08149.1	Ca_LG_8	3,475,024	3,476,860	TC10471	TIFY, Jas	676	5	225	9.35	24.48	1
CaJAZ12b	Ca_08181.1	Ca_LG_8	3,823,275	3,826,085	TC18764	TIFY, Jas	541	5	180	9.55	19.606	1
CaJAZ3a	Ca_08733.1	Ca_LG_8	10,385,422	10,391,976	TC05885	TIFY, Jas	1153	7	384	9.29	41.613	2
CaJAZ3c	Ca_17203.1	Scaffold02277	11,984	17,289	TC11159	TIFY, Jas	1009	7	336	9.21	35.339	3
CaJAZ1a	Ca_18081.1	Scaffold03027	3316	6294	TC11369	TIFY, Jas	769	5	256	9.11	27.732	1
CaJAZ12a	Ca_18578.1	Scaffold03745	8598	11,757	TC18345	TIFY, Jas	625	5	208	8.26	22.247	2
CaJAZ8	Ca_19748.1	Scaffold06768	4292	7024	-	TIFY, Jas	412	3	137	9.1	15.663	1

CDS, coding sequence; aa, amino acids; MW, molecular weight; AL, alternate splicing.

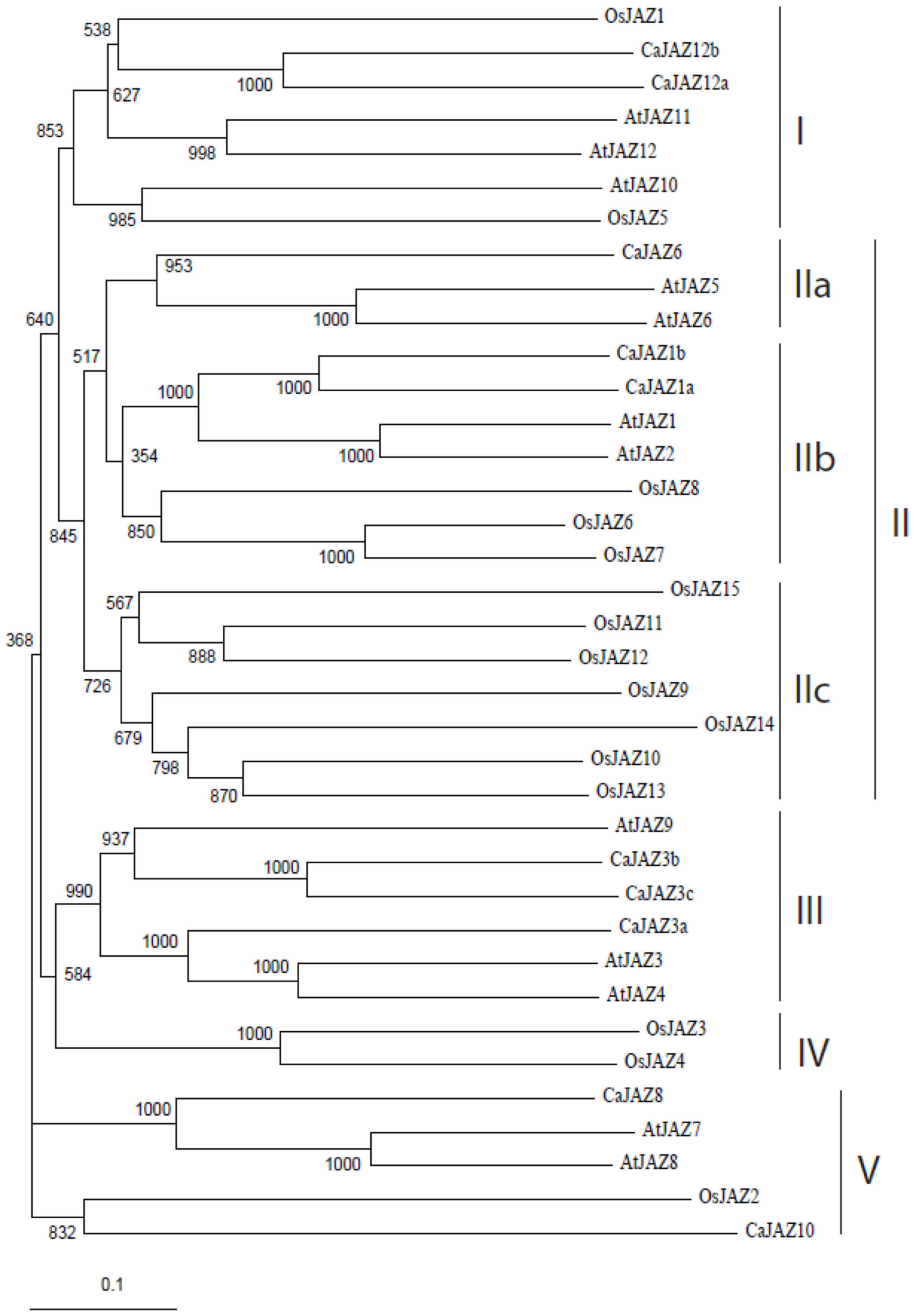


FIGURE 1 | Unrooted phylogenetic tree of rice, chickpea, and Arabidopsis JAZ proteins. Amino acid sequences of JAZ proteins were aligned in Clustal X and phylogenetic tree was constructed using NJ method. Scale bar represents amino acid substitution rate, bootstrap values are mentioned at each node.

numbers with complex regulation and therefore, a more recent origin of gene (Roy and Gilbert, 2006). In rice *JAZ* genes, either *OsJAZ9* or *OsJAZ10* is the founder member since they lack intron and are duplicated partners with high sequence identity. *OsJAZ3* and *OsJAZ4* with highest number of introns (6) might have evolved recently (Figure S3). Surprisingly, we didn't find any *JAZ* without introns in chickpea. The lowest number of introns was in *CaJAZ8* (2 introns) (**Table 1**; Figure S3).

Phylogenetic Relationship and Comparative Analysis of JAZ Proteins and Genes

To study the phylogeny of JAZ proteins, N-J tree of rice, Arabidopsis and chickpea JAZs was analyzed and the reliability was tested by bootstrap analysis for 1000 replicates. Rice, chickpea and Arabidopsis JAZs formed five well-defined clades (bootstrap value >50%). Clade 3 was formed exclusively by

chickpea and Arabidopsis proteins with bootstrap values greater than 93%. This clade comprised of AtJAZ3, 4, 9, and CaJAZ 3b, 3a, 3c revealing their homologous nature (**Figure 1**). Clade 1 also revealed the same result having chickpea and rice proteins in the same clade except OsJAZ1 and OsJAZ5 which showed homology with CaJAZ (12b and 12a) and AtJAZ (10 and 12), respectively. While Clade 2C contains exclusively OsJAZ proteins (which include OsJAZ9, 10, 11, 12, 13, 14, and 15) separating them from chickpea and Arabidopsis.

Protein Architecture of JAZ Repressors in Rice and Chickpea

Arabidopsis JAZs possess the TIFY and Jas conserved domains at the N- and C-terminus, respectively. These domains are essential for their repressor activity. Therefore, we scanned the newly identified CaJAZs and rice JAZs in the MEME web server for the presence of these domains. Two putative conserved motifs TIFY (acc. PF06200) and Jas domain (acc. PF09425) were detected in both CaJAZ and OsJAZ proteins (**Figure 2**, Table S3). Single TIFY and Jas motifs were present in every protein except OsJAZ14 which contained two TIFY motifs. It was also observed that all JAZ proteins have TIFY domain at their N-terminus while Jas domain were at C-termini (**Figure 2**).

The TIFY domain contains 28 amino acids with a conserved TIF(F/Y)XG as core motif (Bai et al., 2011). However, we noticed a few variations of this core sequence in rice and Chickpea JAZs (CaJAZ10,-1b, -12b, -1a, -8; OsJAZ5, -14, -15). Therefore, we aligned TIFY domains from 165 JAZ proteins (identified from diverse organisms) and found that the TIFY motif has variable amino acids in different proteins. Out of 165 proteins, TIFY variations were found in 46 proteins, while three proteins lack TIFY. The remaining 116 proteins contain TIFYXG as a conserved motif (**Figure 2C**, Figure S4). The secondary-structure prediction analysis in Arabidopsis showed that the TIFY domain usually forms a beta-beta-alpha motif. Besides TIFY, the Jas motif is important for the interactions of JAZs with both MYC2 and COI-SCF E3 ubiquitin ligase complex for repressor degradation. It forms the JAZ degron and is characterized by a highly conserved $SLX_2FX_2KRX_2RX_5PY$ consensus sequence and a conserved region of 5 amino acids (LPIAR as in AtJAZ1) at the N terminus (Sheard et al., 2010). The JAZ degron promotes JAZ-COI interaction (Shyu et al., 2012). It was further found that two basic amino acids (R, H, or K) in this region are very important for JA-Ile mediated COI-JAZ interaction; however, their absence does not affect the interaction of MYC2 with JAZ (Melotto et al., 2008). We found that these two basic amino acids are highly conserved in the loop region of Chickpea and rice Jas motifs (Figure S5).

cis-acting Elements in the Promoter Region of *JAZ* Genes

To gain the further insights on the regulatory mechanisms of *JAZ* genes, their putative promoter region was analyzed for identification of binding sites of transcription factors involved

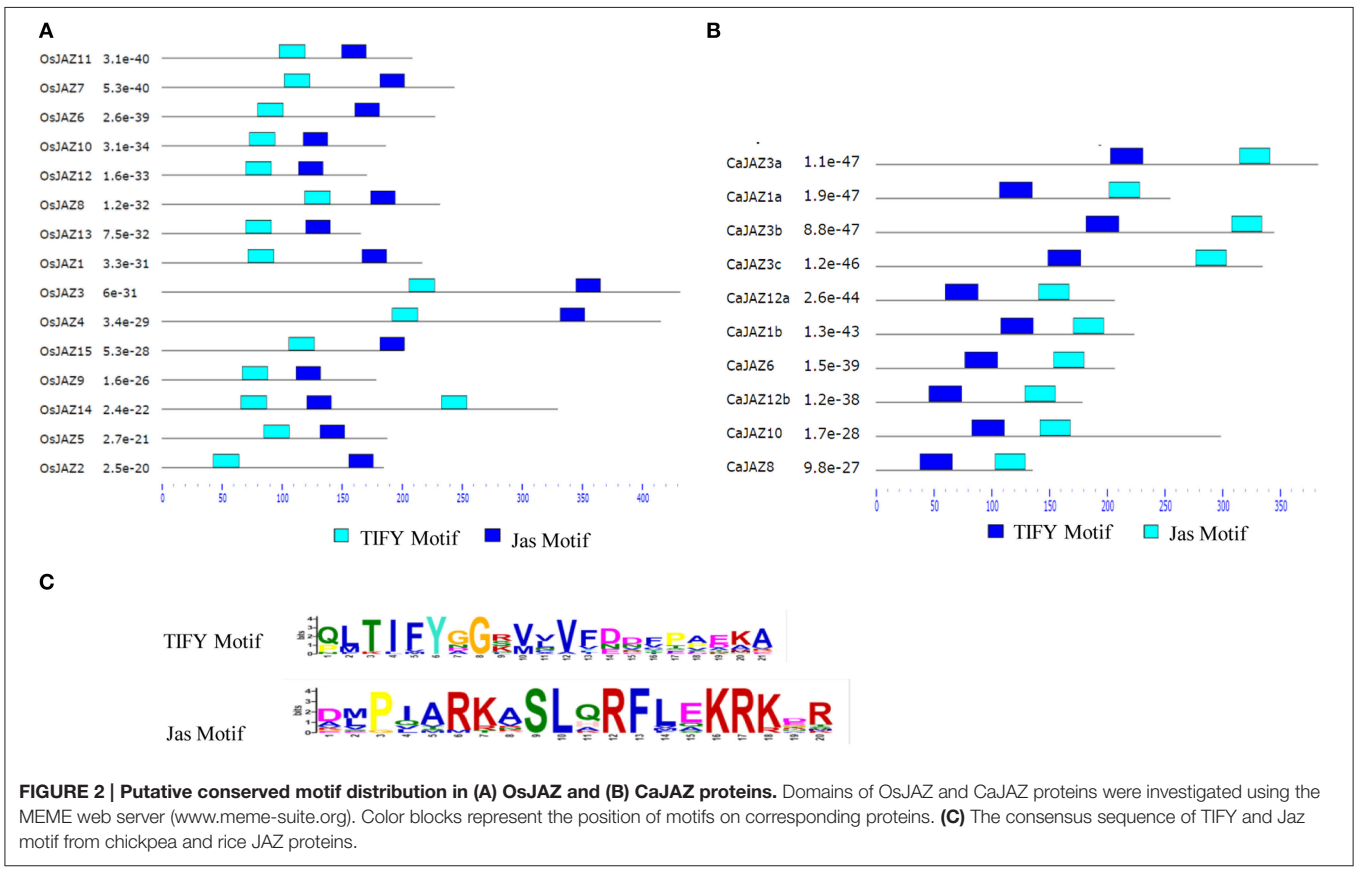


FIGURE 2 | Putative conserved motif distribution in (A) OsJAZ and (B) CaJAZ proteins. Domains of OsJAZ and CaJAZ proteins were investigated using the MEME web server (www.meme-suite.org). Color blocks represent the position of motifs on corresponding proteins. **(C)** The consensus sequence of TIFY and Jaz motif from chickpea and rice JAZ proteins.

in regulation of different nutrient stress responses. A variety of putative *cis*-elements were identified in rice *JAZ* genes. These include *P1BS,* a PHR1-binding sequence involved in regulation of PSR (phosphate starvation response) genes, IRO20S element which is an iron responsive element regulating iron responsive genes, GLMHVCHORD element, associated with nitrogen signaling and AMMORESIIUDCRNIA1 element which regulates the genes encoding nitrate reductase (Table S4). All nutrient deficiency related *cis*-acting elements were also present in *CaJAZ,* although variable in number, indicating a conservation of regulatory network for *JAZ* genes for nutrient deficiency between rice and chickpea (Table S5). Other than nutrient deficiency related *cis*-elements, both rice and chickpea JAZs genes upstream region also showed *cis*-elements related to development and environment stimuli (Tables S4, S5).

Validation of *CaJAZ* as JA-responsive/Signaling Genes

Both rice and Arabidopsis *JAZ*s showed transcriptional responses to JA-treatment (Chini et al., 2007; Ye et al., 2009). Our analysis on identified CaJAZs, showed presence of all conserved domains as reported in earlier known JAZs. Therefore, we analyzed the expression of *CaJAZ*s in response to JA for further confirmation. The expression levels of Jasmonate-associated genes (*CaJAZ*, *CaAOS1*, *CaCOI1,* and *CaMYC1* genes) were analyzed in response to JA treatment using qRT-PCR. As expected, all genes exhibited a differential expression pattern on treatment with Me-JA (**Figure 3**). We further confirmed the subcellular localization of YFP:CaJAZ6 fusion protein. As expected, YFP:CaJAZ6 localized to nucleus in onion epidermal cells (**Figure 4**), although some signal was also seen in membranes. This suggests that identified *JAZ* sequences in chickpea represent the true JAZ proteins and are involved in JA signaling.

Expression Profiling of *JAZs* Genes in Developmental Stages and Under Different Abiotic Stresses

Tissue-specific expression profiling of *CaJAZs* showed their expression in all tissues, namely, shoot, root, mature leaf, flower bud, and young pod (**Figure 4**; Figure S6), indicating their vital roles in growth and development as reported earlier in rice and Arabidopsis (Cai et al., 2014). *CaJAZ12b* expressed in all the tissues. While *CaJAZ1b* and *-6* showed preferential expression in shoot apical meristem. *CaJAZ3a* and *-10* did not

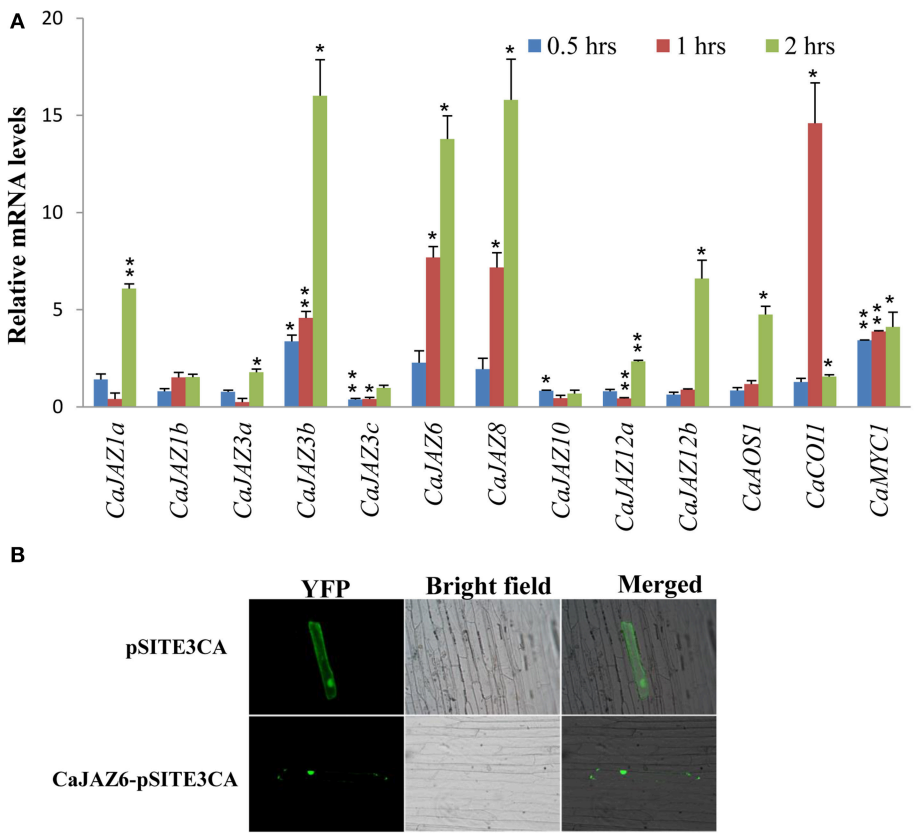


FIGURE 3 | Expression profile of *CaJAZs, CaAOS1, CaCOI1,* and *CaMYC1* genes in response to JA treatment (A) and subcellular localization of YFP:CaJAZ6 fusion protein (B). Expression was studied in methyl JA-treated root tissue using qRT-PCR. Chickpea *EF1-alpha* gene was used as endogenous control. Error bars indicate standard error of mean. Particle bombardment method was used for transforming the onion epidermal cells using DNA coated gold particles. Transformed cells were visualized under a florescence microscope. (*$p < 0.05$, **$p < 0.01$).

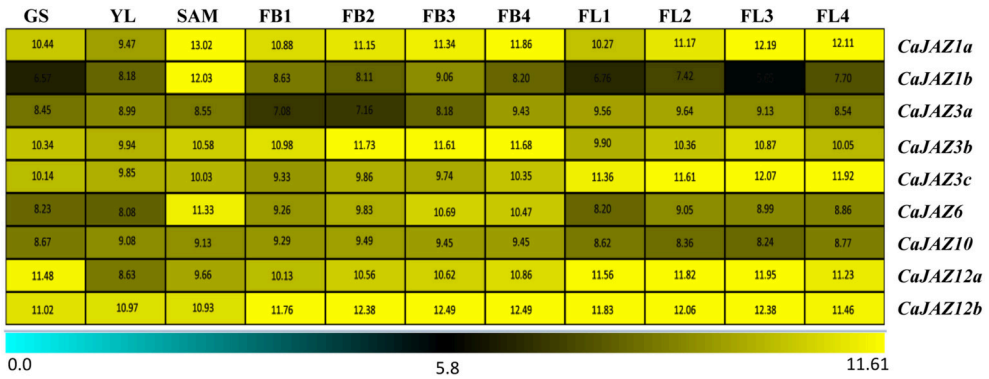


FIGURE 4 | Gene expression profile for *CaJAZ* genes in germinating seedling (GS), young leaf (YL), and 8 week stages of flower development [flower bud (4 mm; FB1), flower bud (6 mm; FB2), flower bud (8 mm; FB3), flower bud (8–10 mm; FB4), flower (unopened; FL1), flower (opened; FL2), flower (mature; FL3), and flower (drooped; FL4)]. Expression data was retrieved from CTDB (http://www.nipgr.res.in/ctdb.html). Scale bar is showing relative transcript levels. Numbers on boxes show RAM (reads per million) values.

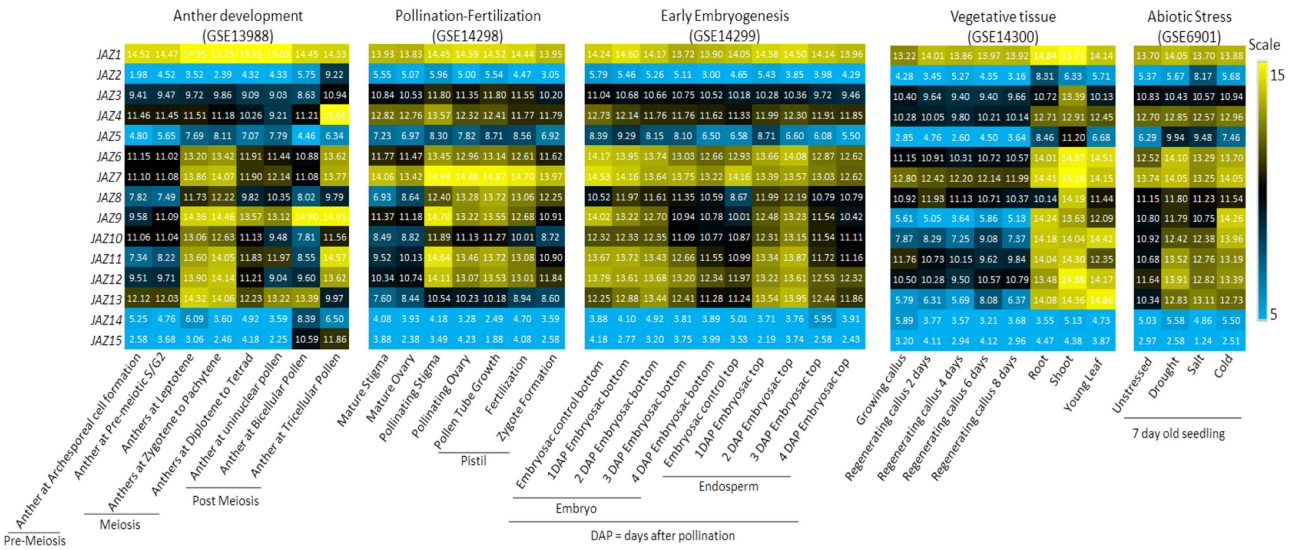


FIGURE 5 | Expression of rice JAZs in various developmental stages and in response to abiotic stress. *JAZ* expression is examined during reproductive development, vegetative tissues, and under abiotic stress conditions. Reproductive stages are further divided into anther development, pollination to fertilization of pistil and early embryogenesis stages. Heatmaps are made on average log expression values obtained from rice Affymetrix expression data (indicated in white font). Experiment IDs of the microarray-experiments used for analysis are indicated on top of the heatmaps. Gene names for respective expression are shown on the extreme left.

show high expression in any of the tissue. Their differential expression in different tissues indicates their possible roles in that particular tissue; however, some redundancy in the function still exists.

Microarray expression data of *OsJAZs* indicate that the majority of them are differentially expressed in different tissues at reproductive and vegetative stages (**Figure 5**). These genes also showed responsiveness to cold, salt and drought stresses. All *OsJAZs* appear to have lower expression in callus cells. *OsJAZ1*, *-3*, and *-4* are highly expressed in most tissues, and are little affected by drought, salt, and cold. *OsJAZ2*, *-14*, and *-15* appear to be specific to late meiosis, though the first of these is also elevated in salt stress. *OsJAZ5* is most expressed in shoots but is elevated in roots in drought and salt stress, while *OsJAZ8* is shoot-specific and its root transcription is unaffected by the 3 stresses (**Figure 5**). Their expression patterns indicate the possible roles of JA in plant development and also in response to environmental stresses. Interestingly, most of the *JAZs* were also expressed in roots. So far only *OsJAZ1* and *9* have been reported to play roles in root alteration and abiotic stress responses (Ye et al., 2009; Cai et al., 2014). Therefore, it would be interesting to delineate the different functions of remaining *JAZ* genes in plant growth and development as well as response to biotic and abiotic stresses.

Expression Profiles of *JAZ* Genes Under Nutrient Deficiency Response

Most of the nutrient deficiencies are sensed at root tips, which often lead to root-architecture modulation. Therefore, we studied the expression patterns of *JAZ* genes under selected macro and micro nutrients deficiency in root tissue. Root lengths of rice and chickpea were recorded after 15 days of growth under N, P, K, Fe, and Zn deficiency. We found significant decrease in root length under P and K deficiency and significant increase under N starvation in rice seedlings. However, chickpea root was significantly reduced under N, P, K, and Fe deficiency. Zn deficiency does not influence the root length of rice and chickpea (**Figure 6**). Expression patterns of *JAZs* were studies under these nutrients deficiency at early (7 days) and late (15 days) stages to get insights into their possible involvement in regulating the plant response.

N Deficiency

Transcript profiling of chickpea *JAZs* showed upregulation of *CaJAZ10* and *CaJAZ1a* and downregulation for *CaJAZ6* and *CaJAZ8* (**Figure 7**) in response to early N deficiency. *CaJAZ10* was upregulated throughout the N starvation while *CaJAZ8* was found to be downregulated (**Figure 7**). Thus, *CaJAZ10* and *CaJAZ8* are both early and late responsive genes under N deficiency. We also found *CaJAZ6*, *-12b*, *-3c*, and *-12a* being upregulated at 15 days only, confirming them as late N deficiency responsive genes. Moreover, *CaJAZ3b*, *-1b*, *-3a* were unchanged throughout the experimental duration (**Figure 7**). In rice, *OsJAZ1* was not induced under N deficiency, however; most of the other *OsJAZ* genes were upregulated at both 7 days and 15 days (**Figure 8**).

P Deficiency

P deficiency influenced the expression dynamics of most of chickpea and rice *JAZ* genes. *CaJAZ10, CaJAZ3b, CaJAZ12b, CaJAZ3c, CaJAZ1a, CaJAZ12a,* and *CaJAZ8* were upregulated under P deficiency at 7 days and none was downregulated. *CaJAZ3b* remained upregulated even after 15 days of low P stress while all other *CaJAZ* genes were significantly downregulated (**Figure 7**). Rice *JAZ* genes *OsJAZ13* and *OsJAZ14* were downregulated, *OsJAZ1* and *OsJAZ15* were non-responsive while all other *JAZ* genes were highly upregulated under P deficiency at 7 days of treatment (**Figure 8**). Interestingly, majority of them were also downregulated on 15th day of treatment.

K Deficiency

Under K deficiency, most of *CaJAZ* genes were late responsive in nature. *CaJAZ3b* was upregulated while *CaJAZ3a* was downregulated at early stage (**Figure 7**). On the other hand, 4 genes were upregulated at 15 days. It is noteworthy that *CaJAZ1a* remains upregulated throughout the K deficiency. Many other genes also showed a trend of differential expression but it was statistically non-significant. However, most of rice *JAZ* genes were upregulated under K deficiency. Interestingly, rice genes followed a common pattern (Up- at 7 days followed by downregulation at 15 days; **Figure 8**). Noticeably, five genes *OsJAZ3*, *-4*, *-5*, *-8*, and *-15* were nonresponsive at 15 days of K deficiency, however; these genes were upregulated at 7 days of K deficiency. Therefore, *OsJAZ3, OsJAZ4, OsJAZ5, OsJAZ12,* and *OsJAZ15* were exclusively early responsive.

Fe Deficiency

The expression analysis of chickpea *JAZs* showed that majority of genes (*CaJAZ3b*, *-6*, *-3c*, *-1a*, *-12a*, and *-8*) were downregulated at 7 days of Fe deficiency. Only *JAZ3a* was upregulated after 7 days and remained so even after 15 days (**Figure 7**). Besides *CaJAZ3a*, three more genes, namely, *CaJAZ6*, *-1b*, *-1a* also showed transcript induction at 15 days of deficiency, indicates their role in late response. Further, *CaJAZ3c* and *-8* remained downregulated throughout the experiment. Expression analysis of *OsJAZ* genes showed largely either downregulation or non-responsiveness at both the stages of Fe deficiency. Only *OsJAZ15* showed upregulation at 15 days stage. Although, a few genes (*OsJAZ5*, *-8*, *-9*, and *-12*) showed marginal upregulation at 7 days, they were again significantly downregulated at the late stage of stress. Further, most of the rice *JAZ*

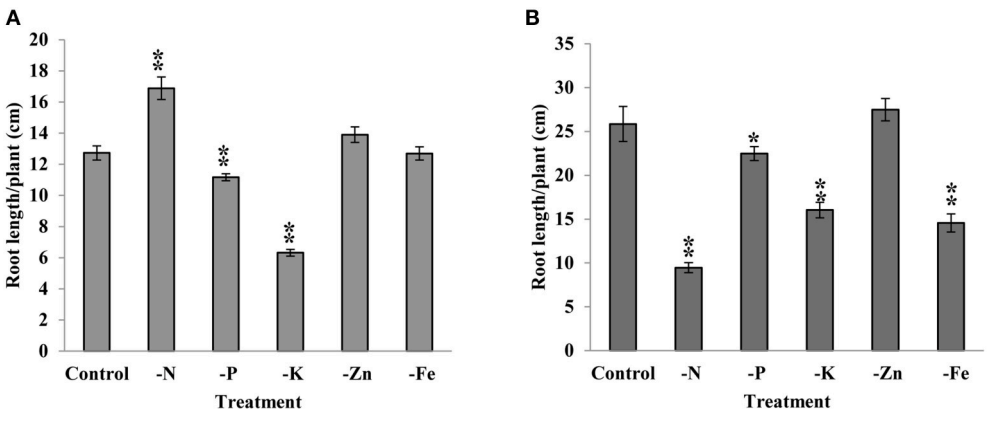


FIGURE 6 | Root length of rice (A) and Chickpea (B) under different nutrient stresses recorded after 15 days of treatment. Length of longest root was used for measurement. Each bar represents the average of 20 plants/treatment with standard error. "*" indicates *p*-values.

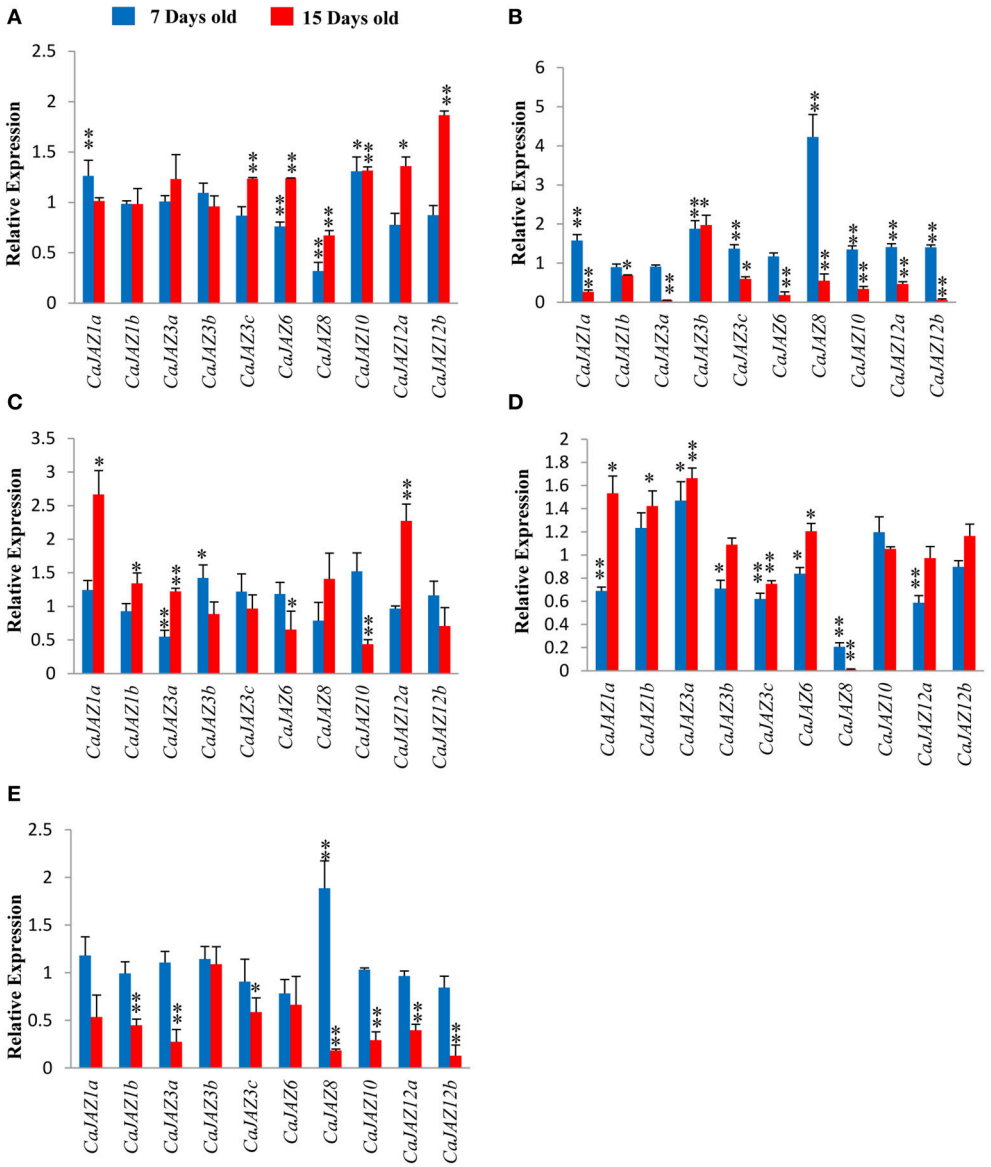


FIGURE 7 | Relative expression profile of *CaJAZ* genes under (A) N, (B) P, (C) K, (D) Fe, and (E) Zn deficiency after 7 (early response) and 15 days (late response) of the respective stress treatment in chickpea root. qRT -PCR was used for quantitation of gene expression. Each bar shows the average of three biological replicates. Relative mRNA levels in treated plants were calculated using unstressed plants as control. *EF1-alpha* gene was taken as endogenous control. (*$p < 0.05$, **$p < 0.01$).

genes were downregulated at 15 days of Fe deficiency except three which were either unchanged (*OsJAZ2*, *-8*) or upregulated (*OsJAZ15*) after 15 days of Fe deficiency (**Figure 8**).

Zn Deficiency

Expression analysis of Chickpea *JAZ* genes showed that all but one gene were unaffected at the 7th day of Zn deficiency, whereas *CaJAZ10*, *-1b*, *-12b*, *-3a*, *-3c*, *-12a*, and *-8* were downregulated at 15th days. We found only one gene (*CaJAZ8*) being upregulated at 7 days of Zn deficiency. Downregulation of most genes at 15 days of treatment indicated their involvement in late response (**Figure 7**). Expression analysis of most rice *JAZ* genes showed a high level of concordance with chickpea expression pattern under Zn deficiency. The majority of the genes were either downregulated or non-responsive at 7 days of Zn deficiency. Only *OsJAZ5* and *13* were upregulated at 7 days (**Figure 8**). Similarly, all rice *JAZs* were found downregulated in response to Zn deficiency at 15 days. Moreover, Zn deficiency has severely affected *OsJAZ2* (75% downregulation) and *OsJAZ13* (95% downregulation) which are the most downregulated genes in rice.

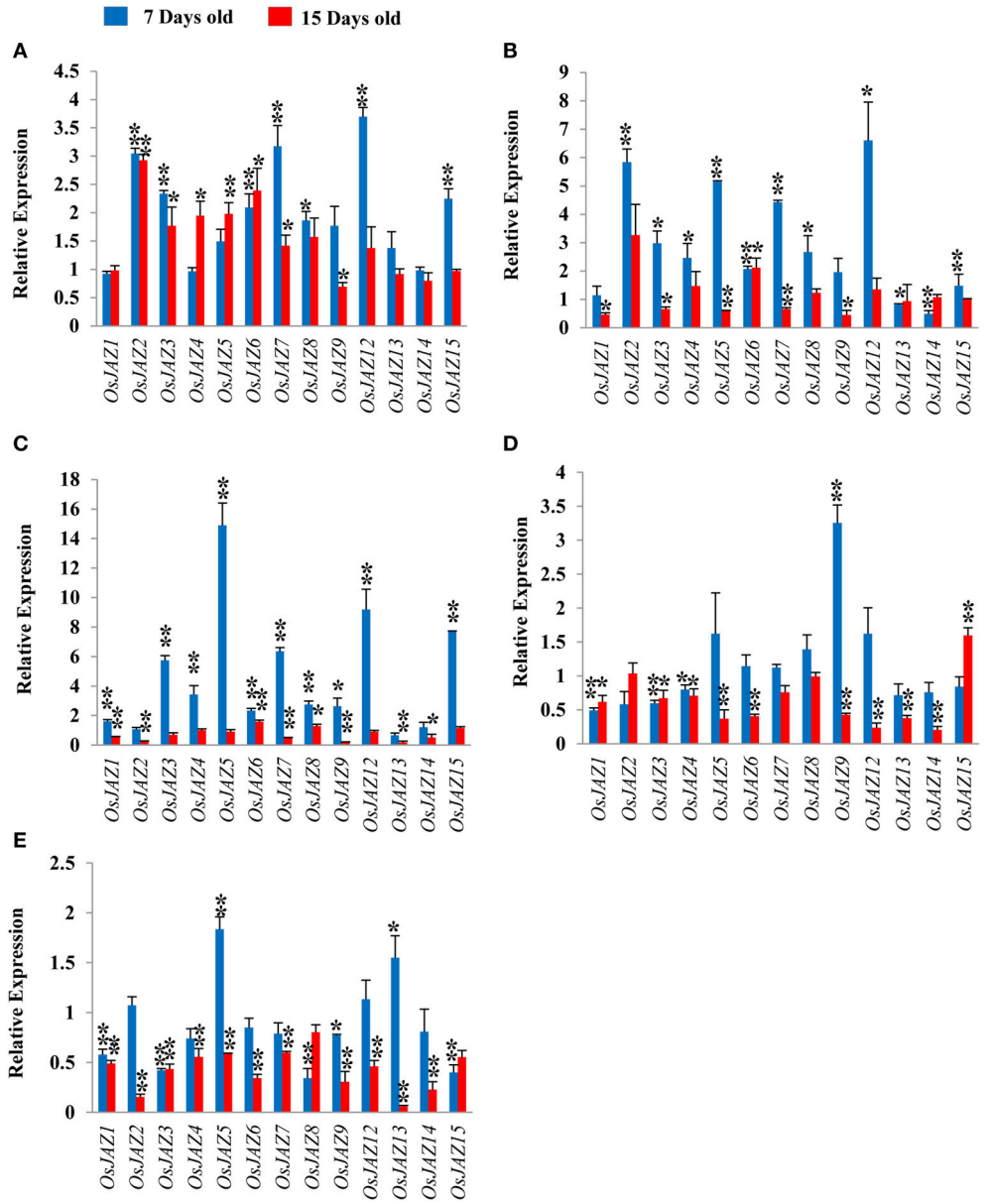


FIGURE 8 | Relative expression profile of *OsJAZ* genes under (A) N, (B) P, (C) K, (D) Fe, and (E) Zn deficiency after 7 (early response) and 15 days (late response) of respective stress treatment in rice root. qRT-PCR was used for quantitation of gene expression. Each bar shows the average of three biological replicates. Relative mRNA levels in treated plants were calculated using unstressed plants as control. *Ubiquitin5* gene was taken as endogenous control. (*$p < 0.05$, **$p < 0.01$).

DISCUSSION

Plant adaptations to nutrient deficiency largely involve root architectural and physiological adjustments. We found significant decreases in rice root length under P and K deficiency and significant increases under N starvation. However, chickpea root length was significantly reduced under N, P, K, and Fe deficiency. It is noteworthy here that root elongation/reduction under nutrient deficiency is also dependent on genotype (Fageria et al., 1988). Noticeably, nutritional deficiencies also induce the biosynthesis of oxylipins and glucosinolates, as reported under K deficiency (Troufflard et al., 2010). These compounds are known precursors of JA; indicating roles for JA-mediated signaling in nutritional deficiency responses in plants. Further, most of the JA biosynthetic genes were found downregulated in a transcriptome study of the *lpi* (low phosphate insensitive) mutant (Chacón-López et al., 2011) in Arabidopsis. Application of Me-JA (Methyl Jasmonate) resulted in negative regulation of the expression of *FRO2* (Ferric Reduction Oxidase2), *IRT1* (Iron Regulated Transporter1) and *FIT* (Fer-like Iron deficiency

induced Transcription factor) genes (Maurer et al., 2011), revealing a JA role in Fe deficiency responses. These studies indicate a role of JA in nutrient deficiency response in root. JAZs are repressor of JA signaling and also have nutrient responsive *cis*-element in their putative promoter regions. Therefore, we studied JAZ-gene behavior in the roots of rice and Chickpea as they serve the primary site for local sensing of nutrient availability in the surrounding environment.

JAZ Proteins in Rice and Chickpea

Rice and Arabidopsis contain 15 and 12 *JAZ* genes, respectively (Thines et al., 2007; Ye et al., 2009). While chickpea genome size is ∼5 times bigger than Arabidopsis, the number of *JAZ* genes (10) identified is less, probably because the genome sequence is incomplete (Jain et al., 2013; Varshney et al., 2013). Although, JAZ varied in the composition of intron/exons, one intriguing feature of rice *JAZs* was the presence of long intergenic regions (LIR) implying a complex regulation of these JA-responsive genes (Figure S7). Their diverse expression patterns in development/stimuli-specific manners further support this notion. Out of three tandemly duplicated genes (*OsJAZ9-11*), *-9*, and *-10* lack introns but show opposite gene orientation (Jiang et al., 2013). *JAZ11* shared the gene orientation with *JAZ10* but had two introns. Further, *OsJAZ10* did not differentially express under nutrient deficiency. A similar discrepancy was observed for *JAZ13* and *-14*. This indicates an architectural and functional divergence in tandemly duplicated JAZs.

Almost all of the JAZs identified here contain conserved TIFY and Jas domain at their N- and C-terminal ends. The amino acid composition in the motifs is also largely conserved. TIFY domains mediate homo and hetero-dimerization interactions within JAZ proteins. It also mediates interaction between JAZ proteins and MYC transcription factors (Bai et al., 2011). The Jas motif is essential for JA-mediated receptor-repressor complex degradation for activation of JA signaling. The presence of these highly similar domains and architecture indicates the conserved nature of JAZ proteins in monocots and dicots, as reported in other gene families (Giri et al., 2013). Further, active JA is perceived by the JAZ-COI co-receptor complex and an alpha helix formed by the Jas degron may provide a low affinity anchor for JAZ proteins to dock on COI to form a JAZ-COI co-receptor complex. The substitution mutation of F (Phenylalanine) by A (alanine) in Jas degron disrupts JAZ1-COI1 interaction (Sheard et al., 2010). We found few JAZs with slight variations at Jas domain while OsJAZ14 encodes a truncated Jas domain. The multiple alignment of the Jas motif from various organisms (Supplementary text 1) also showed variable amino acid sequences. A comprehensive activity analysis of such JAZs would confirm the effects of these variations on JA-signaling. Further, the EAR motif which is involved in the regulation of JA-responsive genes via chromatin remodeling (Zhou et al., 2005; Wu et al., 2008; Berr et al., 2010) was also present in three rice JAZs (OsJAZ2, OsJAZ8, and OsJAZ13) and one chickpea (CaJAZ8). This further indicates the similar roles for rice and Chickpea JAZs in complex cellular signaling, mediated by JA.

Molecular phylogenetic analysis revealed that chickpea proteins are closer to Arabidopsis than that of rice. This could be due to the fact that both Arabidopsis and chickpea are dicots while rice is a monocot (Lee et al., 2011). In a phylogenetic tree of all 165 JAZ proteins from different organisms, rice, and chickpea JAZs were randomly distributed in different clades (Figure S8). While it shows the conservation of JAZ protein in diverse plants but a species level specification is not visible. The estimation of Ka/Ks substitution rates of rice and chickpea *JAZ* genes conserved across 11 other monocot and dicot species revealed that a larger fraction (∼76%) of such genes contained Ka/Ks <1.0, indicating a negative/purifying selection pressure (**Table 2**). The remaining conserved genes had Ka/Ks > 1.0 and thus are under positive selection pressure. This is in good concordance with the substitution ratio of non-synonymous to synonymous SNPs (Ka/Ks < 1.0) documented earlier in multiple plant species (Parida et al., 2012; Victoria et al., 2012; Varshney et al., 2013). The Ka/Ks was lowest in the *JAZ* gene-pairs conserved between *C. arietinum* and *M. truncatula* (0.29), followed by *O. sativa* and *Z. mays* (0.35) genes and highest between *C. arietinum* vs. *P. patens* (1.60). Collectively, the Ka/Ks estimates implicate the evolutionary closeness and divergence among 13 plant species based on rice and chickpea *JAZ* gene family, which is consistent with a number of previous studies (Lee et al., 2011; Zeng et al., 2014).

All *CaJAZs* exhibited a differential expression on Me-JA treatment. Three genes, namely, *CaJAZ3b*, *-6*, and *-8* also showed a similar expression maxima with *CaCOI1*. However, induction of JAZs by JA alone does not confirm their roles in JA signaling as JAZs with truncated JAs domain are also induced by JA (Ye et al., 2009). Nuclear localization of CaJAZ6, as also reported for rice and Arabidopsis JAZs, and the presence of highly conserved domains further confirmed the true nature of CaJAZ and their role in JA signaling.

A variety of potential *cis*-elements are present in 2 kb upstream region of *JAZ* genes. Few of them are known to be involved in nutrient deficiency responsive gene expression. Presence of these nutrient responsive motifs also encouraged us to explore

TABLE 2 | Ka/Ks measured in rice and chickpea *JAZ* genes conserved among 11 other plant species.

Plant species	Ka/Ks	
	Oryza sativa	***Cicer arietinum***
Ricinus communis	1.1	0.77
Zea mays	0.35	0.98
Solanum tuberosum	1.21	0.73
Solanum lycopersicum	1.24	0.71
Populus trichocarpa	1.31	1.35
Physcomitrella patens	1.54	1.6
Medicago truncatula	0.93	0.29
Manihot esculenta	1.15	0.81
Linum usitatissimum	1.1	0.75
Cicer arietinum	0.95	1
Brassica rapa	1.08	0.7
Arabidopsis thaliana	0.89	0.65
Oryza sativa	1	0.95

the differential expression analysis of *JAZ* repressors under different nutrient stresses. *P1BS* element associated with low P responsive genes (Sobkowiak et al., 2012) was detected in six genes (*OsJAZ1*, *-2*, *-4*, *-6*, *-11*, and *-14*) promoter sequences. Noticeably, *OsJAZ2*, *-4*, and *-6* (having 4 copies of *P1BS* elements) showed significant induction under low P while *OsJAZ11* having only one copy did not express under low P. Similarly, role of copy number was also observed for CaJAZs under P stress. Further, the presence of AMMORESIIUDCRNIA1 and GLMHVCHORD elements, related to nitrogen signaling (Loppes and Radoux, 2001) in *OsJAZ2* and *OsJAZ3,* corroborates their higher upregulation under N deficiency in rice. Interestingly, all *OsJAZ* containing IRO20S elements (Ogo et al., 2006) were significantly downregulated after 15 days of Fe deficiency. Moreover, JA application is known to downregulate the Fe deficiency responsive genes. Therefore, JAZs may be involved in this process. However, the intricate connections between Fe deficiency responsive marker genes and JA signaling machinery need to be established via interaction studies between JAZ repressors and IRO2 genes (a bHLH transcription factor) through ChIP-PCR or EMSA assay to further understand Fe homeostasis in rice.

Expression of Rice and Chickpea *JAZs* Under Mineral Nutrient Deficiency

Both rice and chickpea *JAZs* showed significant differential expression under five selected nutrient's deficiencies, and a few common trends also emerged between them (Table S6). They are induced early, and suppressed at a later stage of P deficiency. Similarly, rice JAZs followed an initial up- and later down regulation pattern under K deficiency. *CaJAZs* on the other hand, showed a late upregulation. Interestingly, a P and Fe interaction was observed, JAZs that were induced by P were largely suppressed under Fe deficiency. While N deficiency induced their expression at both early and late stages, they were commonly suppressed under Zn deficiency. This high degree of similarity between rice and chickpea JAZs in terms of expression indicates their functional conservation between monocot and dicot. This further strengthens their emerging role in regulation of nutrient deficiency response in plants (Wu et al., 2015). Interestingly, tandemly duplicated rice *JAZs* followed different expression under these conditions; indicating on sub- or neo-functionalization as observed for other gene families (Vij et al., 2008).

Soil N deficiency is one of the severe problems to derail the overall plant growth and yield (Hirel et al., 2007; Kraiser et al., 2011). Among the various adaptations, RSA alteration through decrease in primary root length and increase in lateral root elongation has been observed (López-Bucioet al., 2003; Gruber et al., 2013). The decreased primary root length in chickpea here reflects the behavior reported in Arabidopsis under N deficiency (Gruber et al., 2013). However, increased root length of rice under N deficiency is also in agreement with an earlier report (Zhang et al., 2015). Further, N deficiency also induces the accumulation of JA in maize seedlings (Schmelz et al., 2003). In a recent study, JA biosynthetic genes like *OPR1*, *LOX5,* and *AOS* were differentially expressed under N deficient conditions in barley (Comadira et al., 2015). In our experiments, *OsJAZs* were also upregulated under N starvation irrespective of stress duration. The upregulation of *OsJAZ* genes pertains to the repression of JA signaling which in turn can increase the root elongation for N uptake. Chickpea has a different mechanism of N homeostasis which might be causing its different root behavior. However, functional validation of this hypothesis would delineate the molecular mechanisms of root elongation through JAZ repressors under N deficiency in rice. Our co-expression analysis revealed that *CaJAZ12b* and *CaJAZ12a* co-express under N, P, and Zn deficiency which strongly correlates to their high degree of sequence similarity and noticeably their branch length is very close to each other in the phylogenetic tree. Similarly in rice, two groups of *OsJAZs* (*OsJAZ3*, *-4*, and *OsJAZ6*, *-7*) having high sequence similarity showed similar expression patterns under N, K, and Fe deficiency and are nearest neighbors, phylogenetically.

Under P deficiency we found a significant decrease in primary root length of chickpea which corresponds to the Arabidopsis phenotype (Pandey et al., 2013). P deficiency inhibits the primary root growth, increases lateral root length and enhances root hair length and density (Lynch, 2011; Niu et al., 2012). These adaptations increase the root surface area to enhance the P acquisition. JA signaling through its downstream components is also known to influence the RSA (Troufflard et al., 2010). In our expression analysis most of the *CaJAZ* and *OsJAZ* genes were upregulated at 7 days of low P stress; however, as the stress was prolonged for 15 days, expression of most of JAZs were subsided. This may be due to the fact that root undergoes rapid local sensing under P deficiency as it comes in contact with low P containing medium (Svistoonoff et al., 2007). *JAZ* genes are also induced transiently and degraded quickly while conveying the signal to downstream genes (Thines et al., 2007). These expression patterns of *JAZs* were also evident in N and K macronutrient deficiency in rice, indicating a potential role for them in low P signaling.

K in soil combines with silicates and other molecules to form insoluble compounds and become unavailable to plants (Gruber et al., 2013; Meena et al., 2014). Root growth inhibition is a well-known response to both K deficiency and JA application (Troufflard et al., 2010). It has been reported that many JA biosynthetic genes like Lipoxygenase (*LOX*), Allene Oxide Synthase (*AOS*), and Allene Oxide Cyclase (*AOC*) were upregulated under K deficiency (Armengaud et al., 2004; Troufflard et al., 2010; Shankar et al., 2013; Takehisa et al., 2013), suggesting an active role of JA in root growth development and architecture modulation under K deficiency. We also found similar root growth inhibition of chickpea and rice seedlings under K deficiency. However, any role of JAZ repressors remains largely elusive. In earlier transcriptome studies of rice root under K deficiency, *OsJAZ9* was downregulated while *OsJAZ12* and *-13* were found to be upregulated (Takehisa et al., 2013). We also found similar behavior of *OsJAZ9* and *OsJAZ12* in our experiments. It is noteworthy that most *OsJAZs* were upregulated after 7 days of all three macronutrient (N, P, K) deficiency. However, the induction of *OsJAZs* was highest under K deficiency. This indicates that they are early responsive and suppress the JA signaling to alter the RSA of the rice for

increasing nutrient uptake. Further, *OsJAZ6* is upregulated at an approximately constant level under NPK deficiency throughout the experiment. This gene is an interesting candidate for N, P, and K starvation studies involving the JA machinery. Chickpea on the other hand, showed mixed expression patterns and therefore, needs to be investigated further.

Being an important constituent of the electron transport system, iron (Fe) deficiency affects the plant growth and yield (Kobayashi and Nishizawa, 2012; Maathuis and Diatloff, 2013). We found a significant reduction in primary root length of chickpea under Fe deficiency, which is in agreement with earlier observations in Arabidopsis (Gruber et al., 2013). It has been further reported that P deficiency enhances Fe availability in root and shoot (Zheng et al., 2009; Rai et al., 2015). Interestingly, we also noticed that while JAZs were induced by low P they were largely suppressed under Fe deficiency. However, the actual role of JAZs remains to be investigated in this interaction. Most of the rice *JAZ* genes are either down regulated or nonresponsive under Fe deficiency. Noticeably, it has been reported that JA application suppresses the Fe deficiency responsive marker genes *IRT1*, *FRO2,* and *FIT* in Arabidopsis (Maurer et al., 2011). This behavior of *OsJAZs* indicates that Fe deficiency negatively affects the regulation of JA signaling. In our expression analysis most of the rice and chickpea *JAZ* genes were down regulated at 15 days of Zn deficiency. Incidentally, JA accumulation under Zn deficiency has been reported in *Sorghum bicolor* (Li et al., 2013). Therefore, we tested the expression level of JA biosynthetic genes *OsAOS2* and *CaAOS1* under Zn deficiency (Figure S9). While *CaAOS1* was upregulated at both 7 days and 15 days of Zn deficiency, *OsAOS1* showed marginal upregulation at 7 days but downregulated at 15 days stage. Although it supports our hypothesis to a large extent, quantification of JA in rice and Chickpea under Zn deficiency would reveal the role of JAZs. Nevertheless, these observations collectively indicate the probable roles of JAZ repressors in nutrient signaling in chickpea and rice.

OsJAZ2 showed high sequence similarity with CaJAZ10. Interestingly, these two genes followed almost identical expression patterns under all nutrient deficiencies. This makes OsJAZ2 a true homolog of CaZAJ10. However, OsJAZ1 lies in the same clade with CaJAZ12b and 12a but they did not follow similar expression trends suggesting a divergent regulation. Both rice and chickpea *JAZs* also showed diverse organ- and tissue-specific expressions. Many of them are also induced by cold, salt and drought stress. OsJAZ9 and AtMYC2 have already been shown to regulate the salinity and drought stress tolerance (Abe et al., 2003; Seo et al., 2011; Wu et al., 2015). Therefore, it would be interesting to investigate the role of JAZs in molecular coordination between JA and these abiotic stresses.

CONCLUSIONS

We have identified 10 *JAZ* repressor genes from the newly sequenced chickpea genome and investigated their roles in mineral nutrient deficiency response. Early induction of *JAZs* in root indicates their signaling roles leading to the adaptation to nutrient deficiency, probably via RSA alterations. Our findings add to the emerging roles of JAs in plant nutrient deficiency response via JAZ repressors, and provide a novel resource to study its applications in crop improvement.

AUTHOR CONTRIBUTIONS

AS and BP conducted the experiments and wrote the manuscript. JG conceived the idea, designed the project, analyzed data, and wrote the manuscript. PD did microarray based expression analysis and helped in manuscript writing. LN helped in conducting various experiments. SP contributed to concept and helped in manuscript writing.

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SUPPLEMENTARY MATERIAL

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frontiers in Plant Science

REVIEW
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Plant Survival in a Changing Environment: The Role of Nitric Oxide in Plant Responses to Abiotic Stress

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Nitric oxide in plants may originate endogenously or come from surrounding atmosphere and soil. Interestingly, this gaseous free radical is far from having a constant level and varies greatly among tissues depending on a given plant's ontogeny and environmental fluctuations. Proper plant growth, vegetative development, and reproduction require the integration of plant hormonal activity with the antioxidant network, as well as the maintenance of concentration of reactive oxygen and nitrogen species within a narrow range. Plants are frequently faced with abiotic stress conditions such as low nutrient availability, salinity, drought, high ultraviolet (UV) radiation and extreme temperatures, which can influence developmental processes and lead to growth restriction making adaptive responses the plant's priority. The ability of plants to respond and survive under environmental-stress conditions involves sensing and signaling events where nitric oxide becomes a critical component mediating hormonal actions, interacting with reactive oxygen species, and modulating gene expression and protein activity. This review focuses on the current knowledge of the role of nitric oxide in adaptive plant responses to some specific abiotic stress conditions, particularly low mineral nutrient supply, drought, salinity and high UV-B radiation.

Keywords: drought, mineral nutrition, nitric oxide, salinity, ultraviolet radiation, UV-B

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INTRODUCTION

Plants are sessile organisms that are by necessity confined to the precise site in which the seed germinates. From its germination, until new seed production begins, plants live in a heterogeneous and fluctuating environment. Along their evolution, plants have developed exquisite mechanisms to cope with the multiple stress conditions that affect them during their life cycle. Although stresses

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; COP1, constitutively photomorphogenic1; cPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorescein diacetate; DAF-2 DA, 4,5-diaminofluorescein diacetate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; GA, gibberellin; GID, gibberellins receptor; GSNO, *S*-nitrosoglutathione; HY5, elongated hypocotyl5; L-NAME, N_{ω}-Nitro-L-arginine methyl ester; L-NMMA, L-NG-monomethylarginine; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PLP, pyridoxal 5′-phosphate; ROS, reactive oxygen species; sGC, soluble guanylatecyclase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase; UVR8, UV resistance locus 8.

are multiple, corresponding plant responses usually involve common components and signaling pathways. Recent research has unveiled nitric oxide (NO) as one critical component in several plant acclimation responses to both biotic and abiotic stress conditions. NO was recognized in the late 1970s as a small molecule actually produced by plants (Klepper, 1979). Since then the corpus of information has rapidly become impressive starting with the identification of NO function as a bioactive molecule in mammals (Furchgott and Zawadzki, 1980). In such a context, this review will focus on plants under several specific abiotic stress conditions, namely low-nutrient supply, drought, salinity and high ultraviolet (UV) radiation.

SOURCES AND FATE OF NITRIC OXIDE

Pioneering work on physiological effects of NO in plants (Leshem and Haramaty, 1996; Noritake et al., 1996) demonstrated that NO acts as a novel key player in not only plant growth but stress adaptation and senescence control as well. Substantiated experimental evidence clearly shows the free radical molecule NO acts to mediate biochemical processes related to a broad spectrum of physiological events that determines plant performance under a wide range of conditions (**Figure 1**).

The molecular targets of NO and related compounds include mitochondrial and chloroplastic proteins and complexes (Mannick, 2001; Abat and Deswal, 2009; Palmieri et al., 2010); broad distributed low and high molecular weight thiols, iron-containing proteins, amine-containing compounds such as nucleic acids, and phenolic groups such as tyrosine (Joshi et al., 1999); lipid radicals localized in membranes and lipoproteins (Rubbo, 2000); the TIR1 auxin receptor (Terrile et al., 2012), outward-rectifying K^+ channels in guard cells (Sokolovski and Blatt, 2004); as well as free radicals such as superoxide anion (Patel et al., 1999). These elements lead us to the notion that specific sites of generation, or fast synthesis and delivery mechanisms, are required to achieve proper NO levels close to the target molecules.

The question then arises how the required levels of NO are specifically reached. In mammals, local NO levels increase rapidly under stimuli such as blood pressure, oxygen consumption, or infections (Moncada and Erusalimsky, 2002) and the source of those NO bursts relies on the activity of a family of enzymes that employ L-arginine (arg), O_2, and NADPH, requiring tetrahydrobiopterin as cofactor. Nitric oxide synthases enzymes (NOSs) function in signal transduction cascades linking temporal changes in Ca^{2+} level to NO production, which in turn acts as an activator of sGC. The constitutive enzymes are designated nNOS and eNOS, after the cell types in which they were originally found (neurons and endothelial cells), while the inducible form (iNOS) is typically synthesized in response to inflammatory or proinflammatory mediators (Stuehr, 1999). Encoded by three different genes, NOSs isoforms differ in localization, regulation, and catalytic properties. In photosynthetic organisms a form of NOS (OtNOS), which shares 42% similarity to human NOSs, has been described from the marine unicellular microalgae *Ostreococcus tauri*, belonging to the oceanic picoplankton (Foresi et al., 2010). However, in higher plants the source of NO is far from offering a clear picture and many potential sites have been proposed for its synthesis (Mur et al., 2013). Analysis of fully sequenced plant genome reveals no homology with known NOSs, nevertheless it cannot be discarded that a new form may have evolved in higher plants (Correa-Aragunde et al., 2013). This is puzzling since plants show an arg-dependent NO synthesis (Flores et al., 2008) and when treated with fungal elicitors respond with a strong NO burst within minutes, much in the same way as an animal host responds to infection (Foissner et al., 2000; Laxalt et al., 2007).

Plastids seem to be related with NO generation. Early reports have shown NO production in chloroplasts as a consequence of abiotic stress imposition (Gould et al., 2003). Pineapple plants (*Ananas comosus*) exposed to water-stress showed an increase in NO production, observed in the chloroplast-containing cells of the mesophyll, whereas no NO generation was detected in the chlorophyll-less cells of the hydrenchyma or in the vascular tissues (Freschi et al., 2010). NO also accumulates in the chloroplasts of *Arabidopsis* cells after Fe treatment, where it acts downstream of Fe to promote an increase of AtFer1 (*Arabidopsis* ferritin 1) mRNA level (Arnaud et al., 2006), and supporting the hypothesis of an arg-dependent synthesis, this increase is blocked by L-NMMA, which is analogous to arg inhibiting mammalian NOSs. Tewari et al. (2013) also described endogenous NO and peroxynitrite ($ONOO^-$) generation in chloroplasts from *Brassica napus* (Tewari et al., 2013). A valuable tool for the study of NO synthesis relies on mutant *Arabidopsis* plants with defective NO accumulation, among them *atnoa1* (defective in NOA1/RIF1 protein) which exhibits reduced NO levels (He et al., 2004; Tewari et al., 2013). In this mutant, the NO level was restored upon the application of sucrose, suggesting that the relationship between NO synthesis and NOA1/RIF1 protein is indirect (Van Ree et al., 2011). NOA1/RIF1 might bind plastidial ribosomes and be required for their normal function and proper protein synthesis in plastids (Gas et al., 2009). The defective NO production in this loss-of-function mutant is then an indirect effect of interfering normal plastid functions, supporting the notion that plastids play an important role in regulating NO levels in plant cells (Gas et al., 2009). Furthermore, *in vitro* studies employing isolated chloroplasts, showed that these organelles were able to produce NO when supplemented with adequate substrates (Jasid et al., 2006). *In planta*, the functionality of chloroplasts positively impacts NO content, where a decline in photosynthetic capacity due to the presence of herbicides or phenological changes, profoundly affected NO level in cotyledons (Galatro et al., 2013).

Nitric oxide synthesis can take place in other cellular compartments besides chloroplasts, like mitochondria (Gupta et al., 2005), peroxisomes (Corpas et al., 2009), cytosol (Rockel, 2002), and plasma membranes (Stöhr et al., 2001). In opposition to the multiplicity of mechanisms and sites suggested for NO synthesis, only two substrates are usually pointed out by researchers: arg and nitrite. Nitrite might generate NO upon reduction through the activities of cytosolic NR (Yamasaki et al.,1999); membrane-bound nitrite reductase (Ni:NOR; Stöhr et al., 2001); xanthine oxidoreductase (Corpas et al., 1997;

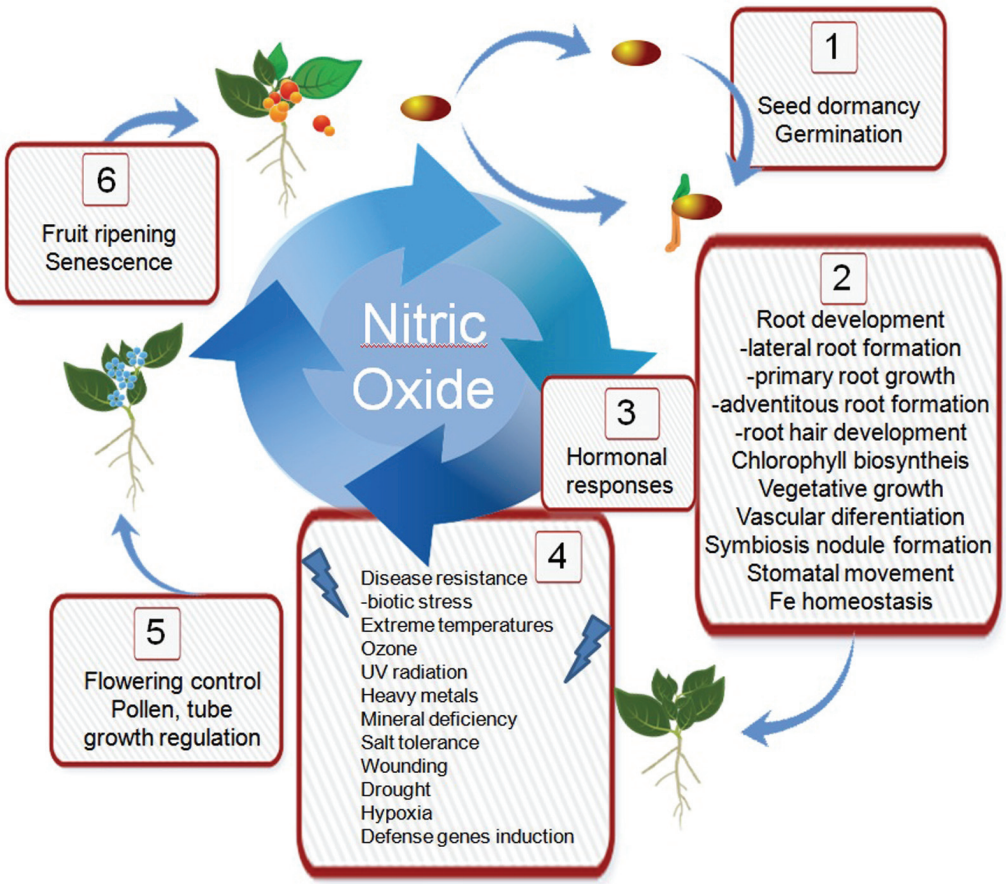


FIGURE 1 | Nitric oxide participates in morphogenesis and development of plants through the interaction with hormones, reactive oxygen species, calcium, and protein post-translational modifications. (1) Dormancy and germination. NO efficiently breaks the dormancy and/or promote germination of several orthodox seeds and could play a pivotal role in sensing environmental conditions appropriate for seed germination (Kopyra and Gwóźdź, 2003; Krasuska et al., 2015). Ethylene and NO, counteract ABA action in seeds improving dormancy release and germination (Arc et al., 2013). Nitrite is the substrate for NO synthesis under hypoxia, and is proposed an auto-regulatory mechanism since NO reversible inhibits seed O_2 consumption (Borisjuk et al., 2007). **(2)** NO regulates lateral root formation (Correa-Aragunde et al., 2008), primary root growth (Fernández-Marcos et al., 2011), adventitious roots formation (Pagnussat et al., 2004) and root hair development (Lombardo et al., 2006). NO affects chlorophyll level (Liu and Guo, 2013), vegetative growth (Beligni and Lamattina, 2001; Lozano-Juste and León, 2011), symbiosis nodule formation (Hichri et al., 2015), stomatal movement (García-Mata and Lamattina, 2001; Chen et al., 2013b), and Fe homeostasis (Buet and Simontacchi, 2015). **(3)** Interaction between NO and hormonal responses has been reviewed in Sanz et al. (2015). **(4)** For description of NO participation in selected abiotic stress conditions see the text. NO is also an important component of the mechanism coordinating and regulating Ca^{2+} and ROS signaling in plant immunity (Trapet et al., 2015). **(5)** Internal and external signals such as photoperiod, vernalization, gibberellins, and the circadian clock induce reproductive development. NO treatment delayed floral transition in *Arabidopsis* (He et al., 2004), however, NO level increase in apical meristem during vegetative/reproductive transition in wheat (Kolbert et al., 2011), and three NO donors induce floral transition in *Lemna aequinoctialis* (Khurana et al., 2011). **(6)** Fruit ripening and senescence. NO fumigation suppressed respiration and ethylene production and delay in ripening of commercial fruits (Singh et al., 2009; Manjunatha et al., 2010). NO acts antagonistically to ethylene in ripening and senescence, NO delays senescence in cotyledons, cut flowers, and leaves (Procházková and Wilhelmová, 2011).

Wang et al., 2010); the interaction with plastidial carotenoids (Cooney et al., 1994); and, under hypoxic or anoxic conditions, mitochondrial electron transport become an important site for nitrite reduction (Planchet et al., 2005; Stoimenova et al., 2007). In isolated chloroplasts the nitrite-dependent NO generation was reduced in the presence of DCMU suggesting photosynthetic electron transport plays a role (Jasid et al., 2006). An attractive alternative hypothesis for NO synthesis is related to non-enzymatic pathways. Under acidic conditions, the protonation of nitrite to yield NO is favored and the presence of ascorbic acid or phenolic compounds accelerates this conversion, where the apoplast fulfills these requirements (Bethke et al., 2004). Interestingly, changes in apoplastic pH go along with many physiological processes in plants, such as development, growth, leaf movement, gas exchange, and pathogen defense (Amtmann, 1999). In the same sense, increases in the activity of plasma membrane H^+-ATPase plays an important role in the plant response to nutrient and environmental stresses, as observed under phosphorous deficiency (Shen et al., 2006), salt stress (Vitart et al., 2001), and changes in nitrogen supply (Młodzińska et al., 2015). In roots, ascorbic acid was twofold increased as a consequence of Fe deficiency (Zaharieva and Abadía,

2003), and increases in ascorbate synthesis and recycling was observed under Fe deficiency in algae (Urzica et al., 2012). A decrease in apoplastic pH or an increase in reductants, under different physiological or stress conditions, suggests that a non-enzymatic pathway for NO synthesis could be operative in these conditions.

Once NO is formed, there must be mechanisms to decrease its concentration. The presence of target molecules, including superoxide radical, thiols, and Fe-containing molecules, helps to maintain or reduce the levels of NO. In addition, there are specific scavenging mechanisms. Non-symbiotic hemoglobins constitute an important factor in controlling NO levels in plant cells and tissues (Hebelstrup et al., 2013). Physiological relevance of hemoglobins were deduced following the observation of morphogenic events stimulated by NO which are often repressed by increased hemoglobin levels and vice versa (Hebelstrup et al., 2013). During hypoxia NO increases, and stress-induced non-symbiotic hemoglobins are thought to modulate the NO level through its transformation in nitrates (Dordas et al., 2003; Perazzolli et al., 2004). The enzyme nitrosoglutathione reductase (GSNOR) does not act directly on NO but on nitrosoglutathione (GSNO), which serves as both a reservoir and a NO donor (Feechan et al., 2005).

The function of NO in stress tolerance has been approached by means of pharmacological experiments where altering NO levels with donors and scavengers was achieved, by employing mutants (e.g., decreased endogenous NO levels in *nia1nia2*, *Atnoa1* and increased in *nox1/cue1*), or developing transgenic plants, expressing a rat *nNOS* under the control of a constitutive promoter in *Arabidopsis* and tobacco plants (Chun et al., 2012; Shi et al., 2012), and more recently *Arabidopsis* transformed with Ot*NOS* gene under the control of a stress-inducible promoter (Foresi et al., 2015). These transgenic plants exhibited higher NO synthesis and displayed enhanced tolerance to a range of biotic and abiotic stresses. In particular, Ot*NOS* transgenic lines showed higher germination rate as compared to wild type *Arabidopsis*, a better performance under methyl viologen exposure, as well as under NaCl and drought stresses as below considered (Foresi et al., 2015).

NO AND PLANT ACCLIMATION TO LOW MINERAL NUTRIENT SUPPLY

An important limitation for plant productivity in most agricultural systems is the low availability of some mineral nutrients in the soil, which provides a heterogeneous environment that suffers important temporal and spatial variations (Shen et al., 2011; Yu et al., 2014). Plants possess several strategies to extract mineral nutrients from that complex system, under low-nutrient availability conditions, which include major changes in the pattern of root growth, the increased activity of transport systems with capacity to acquire nutrients from very diluted solutions, as well as the release of compounds that either contribute to increase nutrient availability in the neighborhood of roots or modify its chemical form thus favoring the accumulation by roots. All these strategies converge in determining an increased efficiency of nutrient acquisition, which is agronomical meaning (White and Brown, 2010; Andrews and Lea, 2013). In addition, plants can enhance their capacity to generate biomass by each unit of nutrient already acquired thus increasing the efficiency of utilization (Rose and Wissuwa, 2012; Santa-María et al., 2015). Current evidence indicates that NO contributes to modulate some of these mentioned processes, for specific elements, arguing for a pivotal role of this small molecule in determining the efficiency of acquisition and utilization of several macro and micronutrients. In this section, we will consider some examples of this statement for the three major nutrients usually applied as fertilizers, namely nitrogen, phosphorus, and potassium, while a minor note added for zinc and iron. Although, intra and extra-cellular interactions between plants and microorganisms in soils are out of the scope of this review, it should be mentioned that the pattern of root growth as well as the accumulation of major nutrients can be strongly influenced by the interaction of roots with soil living microorganisms through processes that frequently involve NO (di Palma et al., 2011, and references therein) which could further contribute to determine acquisition efficiency.

Nitrogen

The two chemical forms of nitrogen preferred by plant roots, present in non-fertilized soils, are nitrate and ammonium. The possibility that nitrogen nutrition and NO are interconnected arises primarily, but not uniquely, from the observation that a route for the generation of NO in plants involves the enzyme NR (Yamasaki et al., 1999). The activity of this enzyme constitutes the first step in the assimilation of nitrogen by plants in soils where the dominant chemical form of this element is nitrate. Noticeably, multiple environmental factors influence, to a variable degree, the expression of NR coding genes as well as the activity of the encoded enzymes (Yanagisawa, 2014). One of those factors is the availability of nitrate in the media encountered by roots during their development (Crawford, 1995). In turn, nitrate nutrition influences the generation of NO in plant tissues (Vanin et al., 2004; Zhao et al., 2007a; Manoli et al., 2014), while effects of NO on NR activity have been also documented (Jin et al., 2009; Rosales et al., 2011; Sanz-Luque et al., 2013). Recent findings suggest that assimilation of nitrate, that clearly influences NO and NO-reservoirs (typically *S*-nitrosothiols), could be feedback regulated by NO-dependent post-transcriptional mechanisms at the points of uptake and nitrate reduction (Frungillo et al., 2014). Therefore, a reciprocal influence between NO and nitrate nutrition appears to be self-evident. In this context several lines of evidence indicate that NO could exert a strong effect on plant acclimation responses to conditions of variable nitrogen availability which could influence nitrogen acquisition efficiency via modulation of root growth. It is well known that modifications of root system architecture to nitrate supply involve both local and systemic effects (Zhang, 1998; Yu et al., 2014). Localized effects of nitrate involve the stimulation of lateral root development in nitrate rich patches, while systemic effects involve inhibition of lateral root growth as

well as changes in carbon partitioning between shoots and roots and, in some cases, a negative effect on primary root growth. Both kinds of responses have been extensively studied, however, contradictory reports have been offered regarding the effect of nitrate supply on primary root growth as recently highlighted by Trevisan et al. (2014) for maize and *Arabidopsis*. Inhibition, stimulation or no effect of nitrate supply on primary root growth have been observed following uniform nitrate treatments indicating that a fine control of root plastic responses is involved. In this context, attempts to decipher the role of NO should be ascribed to the pattern of root growth observed under each precise growing condition. In this regard it has been observed a rapid increase of NO accumulation in maize roots exposed to 1 mM nitrate, which essentially involves the root transition zone, this increase was suppressed when tungstate (inhibitor of NR) or cPTIO (NO scavenger) were added to the medium (Manoli et al., 2014). In this case, nitrate-stimulated primary root growth was suppressed by tungstate and cPTIO but not by the arg analog, L-NAME. These results argue for a stimulatory effect of NO accumulation on primary root growth induced by 1 mM nitrate. An earlier work, reported that both NO accumulation and maize root elongation were reduced in the presence of 10 mM nitrate, thus suggesting the possibility that decreased primary root growth at high nitrate levels is causally related to a reduced NO accumulation (Zhao et al., 2007a). The contradictory effects exerted by external nitrate supply can be, at least in part, conciliated if this anion acts in a dual mode on primary root growth as proposed by Trevisan et al. (2014). In such a case nitrate may stimulate NO accumulation at relatively low external nitrate concentrations (1 mM) and decrease it at high external nitrate concentrations (10 mM). Therefore, the final outcome in terms of primary root growth will be dependent on the levels of NO in a root-responsive zone. A recent study on the transcriptome and proteome of maize plants suggests that the root apex transition zone could play a major role in nitrate sensing (Trevisan et al., 2015). Interestingly, that work suggests an important role of non-symbiotic hemoglobins in the control of NO levels in that zone.

For some plants like rice, nitrogen is mainly incorporated in the form of ammonium. A recent work with two rice cultivars grown either only in the presence of ammonium or in a combination of ammonium and nitrate (i.e., partial nitrate nutrition) but maintaining an equal provision of nitrogen, has illustrated the potential relevance of NO in determining varietal differences in the pattern of root growth and nitrogen accumulation (Sun et al., 2015). The cultivar Nanguan displays a higher yield under conditions of increasing nitrogen supply than the Elio one, the former being highly responsive to partial nitrate nutrition (Duan et al., 2007). Under conditions of partial nitrate nutrition Nanguan plants display a higher accumulation of nitrogen than when grown solely in the presence of ammonium, while Elio does not shown such a positive response. In this context, (Sun et al., 2015) under conditions of partial nitrate nutrition, Nanguan plants display both increased lateral root density and enhanced NO accumulation relative to plants grown only in the presence of ammonium. Furthermore the uptake of nitrogen per unit of root weight increased for Nanguan but not for Elio under conditions of partial nitrate nutrition, a pattern that correlates with enhanced accumulation of transcripts coding for putative ammonium and nitrate transporters. Interestingly, with the addition of an NO donor, SNP to an ammonium medium, lateral root formation is promoted, nitrogen uptake and expression of those transporters in Elio are increased, while the opposite effects were observed when cPTIO was added under conditions of partial nitrate nutrition (Sun et al., 2015). These results open the possibility to influence the pattern of root growth as well as the uptake capacity under specific conditions of nitrogen supply, by manipulating elements involved in NO signaling. The extent to what these findings can be extrapolated in regards to other higher plants or any other photosynthetic organisms needs to be carefully assessed. It has been shown in the green alga *Chlamydomonas reinhardtii,* that addition of nitrate alone or high ammonium concentrations in the presence of nitrate, increases the endogenous levels of NO. In this case, the presence of ammonium in the medium correlated to a decreased expression of genes coding for both ammonium and nitrate transporters through a mechanism that likely involves sGC (De Montaigu et al., 2010). In turn, both ammonium and nitrate depletion from low external concentrations are reversibly inhibited by the presence of NO donors in the uptake medium, suggesting the possibility that NO exerts a direct post-translational modification over the transporters involved, in addition to a probable effect exerted at the transcriptional level (Sanz-Luque et al., 2013).

Phosphorus

Evidence for the involvement of NO in plant responses to low phosphorus (P) supply has been obtained for white lupin, *Lupinus albus*. In this, as well as in some other dicot and monocot plant species, P-deficiency triggers the development of cluster roots, which is accompanied by the release of low molecular weight substances, including organic acids and protons (Lambers et al., 2011; Niu et al., 2013). These responses help plants to increase P-acquisition through intensive soil P-mining. In this context, it has been shown that white lupin roots exposed to P-deficiency display enhanced NO accumulation in the primary and lateral root tips, correlated to the formation of cluster roots and increased citrate exudation (Wang et al., 2010). In turn, the addition of the NO-donors SNP and GSNO leads to proliferation of cluster roots; while the NO scavenger cPTIO abolishes this process (Wang et al., 2010; Meng et al., 2012). Transcriptomic analysis performed at different stages of cluster root development have indicated that NO production in mature cluster-root correlates with an enhanced accumulation of xanthine oxido-reductase coding transcripts (Wang et al., 2010, 2014), suggesting this enzyme is related to NO production. Studies with the xanthine oxido-reductase inhibitor allopurinol provide further support to this idea (Wang et al., 2010). Since xanthine oxido-reductase activity is involved in purine degradation (Werner and Witte, 2011), and it is known that nucleic acids constitute a major pool of P in plants suffering from P-deficiency (Veneklaas et al., 2012), the activity of that enzyme could influence the pool of P available within the tissues

while providing a possible route for the increased accumulation of NO, which could further stimulate citrate exudation induced by P-deprivation (Wang et al., 2014). This scheme shows possible signaling pathways consistent with findings from a growth simulation model (Wissuwa, 2003). That is, increased P-utilization at the root level, in this case via handling a P reservoir into a pathway that also involves NO formation, could result in enhanced P-acquisition and corresponding plant performance under conditions of P-scarcity.

The control of plant responses to low P-supply involves the concerted action of several signaling and response mechanisms. Among them, a pivotal role has been proposed for the GA-GID-DELLA module (Jiang et al., 2007). DELLA proteins, whose primary action is to restrict plant growth, can bind to GA once this hormone interacts with GID, leading to subsequent DELLAs degradation at the proteasome and thus favoring growth (Harberd et al., 2009). DELLAs are known to be involved in plant responses to several stress conditions, among them, some derived from low nutrient supply (Jiang et al., 2007; Moriconi et al., 2012). It has been also shown that in some processes NO and GAs frequently exert opposite effects, suggesting a possible link between them. Providing experimental support to this possibility it has been observed that in some plant responses to light, the action of NO involves DELLAs (Lozano-Juste and León, 2011). A primary effect of P-deprivation in *Arabidopsis*, but certainly not in all plant species (Niu et al., 2013), is the restriction of primary root growth (Williamson, 2001; López-Bucio et al., 2002). It has been also observed that DELLAs exert a restriction on primary root growth (Jiang et al., 2007) and that NO exerts a similar effect (Fernández-Marcos et al., 2011) in a DELLAs partially dependent mode (Fernández-Marcos et al., 2012). Therefore the possibility that NO and DELLAs interact under conditions of low P-supply has emerged. A recent work with *Arabidopsis* (Wu et al., 2014) offered evidence that NO and GAs actually exert opposite effects on primary root growth under conditions of low P-supply. Addition of cPTIO as well as L-NMMA, a putative inhibitor of NOS enzymes, revert the root growth inhibition that takes place at low external P-concentrations. Moreover, under conditions of adequate P-supply, addition of SNP led to stabilization of RGA, one of the five DELLA proteins present in *Arabidopsis* (Wu et al., 2014). This data suggest the possibility that some changes in the *Arabidopsis* root system architecture under conditions of low P-supply requires the interaction between NO and the GA-GID-DELLAs module. Consistent with this claim, it has been recently shown that *Arabidopsis* plants exposed to low P-supply, display enhanced NO production (Royo et al., 2015). In addition, the possibility that an NO burst could be induced by other soil organisms, or by their exudates, should be taken in consideration. Accumulation of P and other elements by plants largely depends on the establishment of mutualistic associations between plant roots and fungal partners, particularly in plant species that do not form cluster-roots (Lambers et al., 2011; Niu et al., 2013). In this context, two recent relevant observations need to be mentioned: (a) that in the mutualistic association between *Medicago truncatula* and *Gigaspora margarita*, the exudates from the fungal partner may induce a rapid NO accumulation in plant roots (Calcagno et al., 2012), and (b) that in the interaction between *Medicago truncatula* and *Glomus versiforme*, the formation of the arbuscule depends on DELLA proteins (Floss et al., 2013). Determining whether or not both phenomena occur for a specific pair of partners and if they are causally related, should be considered a priority in research on the role of NO in determining P-acquisition efficiency.

Potassium

The possibility that NO contributes to modulate potassium (K^+) accumulation by plants has been recently advanced. According to our modern understanding of the classic model of Epstein et al. (1963) the inward flux of K^+ to roots occurs through both selective and non-selective pathways. The selective pathway, operative at low or moderate external K^+-concentrations, involves the activity of at least two transport entities: the inward rectifier K^+-channel AKT1 and HAK1-like transporters (Pyo et al., 2010; Rubio et al., 2010), whose relative contributions to K^+-accumulation depend on the external supply of K^+ as well as on the whole ionic environment encountered by roots during their development (Spalding, 1999; Santa-Maria et al., 2000). It has been observed that the addition of the NO-donor SNP, increases the content of K^+ under conditions of exposure to high salt concentrations in the halophytic plant *Kandelia obovata*, and that this increase correlates with an increase of AKT1 transcripts abundance (Chen et al., 2013a). However, in plants grown in the absence of salt stress, the addition of SNP does not lead to improved K^+-nutrition in this plant species. In the search for *Arabidopsis thaliana* NO-hypersensitive mutants grown under "normal conditions", Xia et al. (2014) recently identified the *sno1* mutant, which is allelic to the formerly identified gene *sos4*. Interestingly *sno1* plants grown in an otherwise non-stressed environment are hypersensitive to SNP and SNAP, displaying low K^+-content and a high content of PLP, an active form of vitamin B6. The authors reported that PLP also becomes enhanced in the *nox1* mutant. Since *nox1* plants display enhanced endogenous accumulation of NO, the above finding connects modulation of vitamin B6 accumulation with endogenous NO levels. Furthermore, inward K^+-currents as determined in *Xenopus oocytes* expressing AKT1 were modulated by PLP, but not by other vitamers, thus providing a direct link between PLP and K^+-transport through AKT1. These findings suggest a possible mode by which NO modulate K^+-accumulation in *Arabidopsis* roots. According to it, some external signals that lead to enhanced NO accumulation could indirectly result, via *SNO1* (*SOS4*), in a reduction of AKT1 activity. This modulation could be potentially relevant in terms of the efficiency of K^+-utilization by plants since AKT1 channels operate over a wide range of external K^+-concentrations. At high external concentrations of K^+ (Kronzucker et al., 2006), as well as of other nutrients like P and NH_4^+ (Cogliatti and Santa-Maria, 1990; Britto et al., 2001), a high ratio between efflux and influx takes place, that can be interpreted in terms of futile ion cycling (Britto et al., 2001; Kronzucker et al., 2006) which could exert an important impact in terms of energy expenditure. Therefore,

NO-control of K^+-inward flux through AKT1 at relatively high K^+-concentrations could help to redirect carbon resources to other specific pathways. Certainly, this strategy may be not relevant when the stress condition is the low availability of K^+. To our knowledge, it remains unknown whether NO is differentially accumulated under conditions of potassium deprivation. Moreover, the possibility that NO could modulate the contribution of HAK1-like transporters to K^+-uptake, which usually constitutes the main pathway for K^+-influx at very low external potassium concentrations, needs to be directly assessed.

Let us to add two notes on the findings commented above. Firstly, the regulation of a K^+-channel by NO has been primarily observed in the context of plant responses to drought. In this regard, it has been shown that NO deactivates guard cell inward K^+-currents through a process that involves $Ca_2{}^+$ signaling (García-Mata et al., 2003). Secondly, while PLP influences AKT1 inward currents, it does not affect currents mediated by another Shaker-like K^+-channel, KAT1, indicating that PLP may be not a general modulator of Shaker-like K^+-channels (Xia et al., 2014). On the other hand, the link between NO and PLP evidenced in that work, could be particularly relevant since vitamin B6 may play a role under several stress conditions.

Zinc and Iron

Besides the role of NO in the accumulation of major nutrients, early studies indicated that this reactive nitrogen species participates in the control of the homeostasis of transition metals, particularly Fe (Graziano et al., 2002). More recently, the possible role of NO in the modulation of Zn capture has been documented both in plants exposed to excessive amounts of this transition element (Xu et al., 2010) as well as under conditions of adequate and deficient Zn-supply (Buet et al., 2014). Addition of GSNO to wheat plants deprived of Zn led to accelerated leaf senescence and decreased Zn allocation to shoots. Interestingly, the presence of GSNO in the growth medium acts as a repressor of the enhancement of Zn-net uptake capacity that takes place during Zn-deprivation, which has been linked with the pattern of Zn-translocation to shoots (Buet et al., 2014). In spite of the obvious interest of this data, no clear assessment on the influence of Zn supply on endogenous NO production was obtained through the use of the sensitive fluorescent probe DAF-FM DA. On the other hand, in the Zn-hyperaccumulator plant *Solanum nigrum*, NO accumulation occurs during the course of exposure to high Zn-levels and correlates with enhanced Zn accumulation, which was severely impaired in the presence of L-NAME or cPTIO (Xu et al., 2010). More studies are necessary to make a clear assessment regarding the role of NO on Zn nutrition. On the other hand, literature on the role of NO on Fe uptake, distribution and utilization become extensive since the pioneering work of Graziano et al. (2002), describing a recovery from chlorotic phenotype in maize and tomato plants without changes in total Fe content as a consequence of NO exposure. This observation suggests a possible role of NO in determining Fe utilization efficiency.

Redox changes between two oxidation states make Fe essential for living organisms, for which inevitably poses an oxidative risk. While low Fe availability impairs growth and photosynthesis, excess Fe accumulation can catalyze ROS generation through Fenton's reaction, leading to oxidative damage. Thus, maintaining Fe homeostasis in plants is vital, for which NO turned out to be an important factor through its interaction with hormones, glutathione, ferritin, frataxin, and Fe-compounds (for a review, see Buet and Simontacchi, 2015).

Nitric oxide increases in the root epidermis of tomato plants under Fe scarcity, being this production essential for the observed Fe-deficiency induced responses (Graziano and Lamattina, 2007; García et al., 2010). NO can affect Fe uptake from the soil solution through the modulation of root architecture (Pagnussat et al., 2002) and the up-regulation of genes involved in Fe incorporation (Graziano and Lamattina, 2007). On the other hand, internal Fe availability and delivery in plants might be influenced by the presence of low-molecular weight complexes containing Fe and NO, these nitrosyl iron complexes are paramagnetic species that can be detected employing electron paramagnetic resonance techniques (Simontacchi et al., 2012). Sorghum seeds (*Sorghum bicolor*) germinated in the presence of an NO donor showed increased NO levels which paralleled with high fresh weight of embryonic axes, a decrease in oxidative stress indexes (lipid and protein oxidation) and a high content of nitrated proteins (Jasid et al., 2008). Mono- and di-nitrosyl iron complexes have been observed in sorghum, soybean, and wheat embryos incubated in the presence of a variety of NO donors (SNP, DETA-NONOate, and GSNO), as well as in *Hibiscus rosa-sinensis* after the addition of exogenous nitrite (Vanin et al., 2004; Simontacchi et al., 2012). After NO exposure in sorghum embryonic axes, total Fe remained constant, while the Fe fraction that can be easily interchanged (labile iron pool) increased. Mono- and di-nitrosyl iron complexes have a direct impact on the labile iron pool (Simontacchi et al., 2012), and as a consequence they might act to improve Fe availability in tissues. Moreover, nitrosyl iron complexes are likely to serve both as tissue-storage forms of NO and as a chelated form of Fe that is potentially resistant to participate in oxidative stress-induced damage (Lu and Koppenol, 2005). The presence of complexes between Fe and NO might contribute to the improvement of Fe availability as a part of the complex network of plant Fe homeostasis, other aspects include modulation of gene expression and the morphological responses mediated by hormones.

Noticeably the cluster roots above mentioned in the context of low P-supply are also formed under conditions of Fe deficiency (Watt, 1999). Interestingly, it has been proposed that NO is a shared molecule for the formation of cluster roots induced by P and/or Fe deficiency (Meng et al., 2012). This suggests the relevance of NO in the confluence of signaling mechanisms involved in plant responses to these two nutrient starvation conditions.

ROLE OF NO IN OVERCOMING WATER DEFICIT

Productivity of crops is dramatically affected by naturally occurring long-term or severe drought. Plants can overcome

temporary water deficit, when the rate of transpiration exceeds water uptake, through stomatal closure. Under long-term drought conditions, morphological adaptive responses including inhibited leaf expansion, leaf abscission, and changes in root architecture become operational. The susceptibility of individual plants to drought stress depends both upon the plant structural and physiological adaptations to water limitation, and the duration and intensity of soil and atmospheric water deficits (Zeppel et al., 2015).

Drought stress, often exacerbated by high solar irradiance and high air temperature, inhibits photosynthesis and enhances production of ROS, leading to photooxidative stress (Miller et al., 2010). When photosynthesis is severely constrained by drought, secondary metabolites might have the potential to improve the functional roles of antioxidant enzymes. On a daily basis, plants orchestrate individual components of their antioxidant machinery, complemented by isoprenoid and phenylpropanoid biosynthesis, to prevent irreversible oxidative damage caused by combined environmental stresses (Tattini et al., 2015). In this regard, under other abiotic stress NO is able to enhance secondary metabolites biosynthesis through activation of phenylalanine ammonia-lyase (PAL) enzyme (Hao et al., 2009; Kovácik et al., 2009).

It has been observed that mild water deficit leads to enhanced NO synthesis in cucumber roots, and that exogenous NO (pretreatment with 100 μM SNP and GSNO) was able to counteract membrane damage and lipid peroxidation in water-stressed plants (Arasimowicz-Jelonek et al., 2009). NO donor (SNP 200 μM) has also been proven to exert a protective effect in wheat seedlings exposed to polyethylene glycol-induced drought stress, observed as enhanced growth, high relative water content and less oxidative damage (Tian and Lei, 2006). The ability of exogenous NO to promote adaptive responses to cope with water deficit conditions might be related with its direct action as antioxidant, the effects on root morphology and a role in stomatal closure (Shao et al., 2010). Furthermore the role of NO under water shortage has been clearly demonstrated employing transgenic plants. The expression of proteins that increase NO level in plants leads to a better performance under long-term drought conditions in terms of biomass, plant height and less damage to membranes. In addition transgenic plants (expressing mammalian NOS) accumulated significantly higher levels of proline and sucrose as compared to wt (Shi et al., 2014). Transgenic *Arabidopsis* lines expressing the OtNOS protein survived longer periods without watering, compared to plants with the empty vector, and detached leaves from Ot*NOS* lines exhibited a phenotype of reduced water loss under drought (Foresi et al., 2015). On the other hand Lozano-Juste and León (2010), found that mutant plants (*nia1nia2noa1-2*) impaired in the activity of AtNOA1 and NR enzymes exhibited low levels of NO as well as a markedly different phenotype, displaying hypersensitivity to ABA. These plants, when exposed to long term water deficit, were more resistant to dehydration than wild-type plants, showing lower transpiration rates. The observed reduced water losses in NO-deficient plants may be due to the hypersensitivity to ABA through an effect on stomatal closure. This process was essentially mediated by a mechanism independent of *de novo* NO biosynthesis (Lozano-Juste and León, 2010).

Under water deficit stress, stomatal conductance is reduced as part of the systemic response triggered by a signal that originates in the root system. One of the components of such a response is the production or redistribution of ABA leading to stomatal closure. As it compiled in **Table 1**, experimental data showed that exogenous NO induces stomatal closure, and conversely removal of endogenous NO by scavengers inhibits stomatal closure in response to ABA; exogenous ABA enhance NO generation, and ABA-induced stomatal closure is reduced in mutants impaired in NO generation (Neill et al., 2008).

In guard cells, NO action relies in promoting specifically intracellular Ca^{2+} release, thus regulating Ca^{2+}-sensitive K^+ and Cl^- channels at the plasma membrane (García-Mata et al., 2003). Particularly in *Vicia faba* stomatal guard cells, NO regulates inward-rectifying K^+ channels ($I_{K,in}$) through its action on Ca^{2+} release from intracellular Ca^{2+} stores. Depending on its concentration, NO can inactivate the outward-rectifying K^+ channel ($I_{K,out}$) probably by post-translational modification (Sokolovski and Blatt, 2004). The effect of NO in guard cells is likely mediated via a Ca^{2+}-dependent rather than a Ca^{2+}-independent ABA signaling pathway. A role for NO in the fine tuning of the stomatal movement of turgid leaves that occurs in response to environmental fluctuations has been also suggested (Ribeiro et al., 2009). Recently, hydrogen sulfide has been reported as a new component of the ABA-dependent signaling network in stomatal guard cells, which acts promoting NO production (Scuffi et al., 2014).

One of the most complex plant adaptations tending to efficient water conservation is the Crassulacean acid metabolism, allowing plants uptake CO_2 at night when the rate of transpiration is low in environments characterized by seasonal or intermittent restrictions in water supply. A progression from C3 to CAM metabolism occurs along plant ontogeny in those species that are constitutive CAM (e.g., *Ananas comosus*), while facultative CAM are able to perform either C3 or CAM photosynthesis depending on the environmental conditions (e.g., *Mesembryanthemum crystallinum*; Cushman, 2001). In the latter, environmental factors such as light intensity, temperature, salinity, photoperiod, and especially water availability have long been recognized to affect the magnitude of CAM expression, understood as an increase in the activities of the enzymes phospho*enol*pyruvate carboxylase, malate dehydrogenase, and phospho*enol*pyruvate carboxykinase, as well as nocturnal accumulation of malate (Freschi et al., 2010). In young pineapple plants an increase in the leaf content of ABA preceded the up regulation of CAM enzymes, moreover ABA was able to modulate CAM expression in the absence of water deficit and also trigger an increase of NO localized in chloroplasts (Freschi et al., 2010). Removal of NO from the tissues either by adding NO scavenger or by inhibiting NO production significantly impaired ABA-induced up-regulation of CAM, indicating that NO likely acts as a key downstream component in the ABA-dependent signaling pathway. In plants under water deficit gas-phase chemiluminescence analyses and fluorescence microscopy (employing DAF2-DA) revealed increased levels of NO emission,

TABLE 1 | Nitric oxide, stomatal movements, and plant water status.

Plant material	Treatment	NO level in guard cells*	Observations	Reference
Vicia faba, Salpichroa organifolia and *Tradescantia sp.* leaves	SNP/SNAP	nd	Increased stomatal closure.	García-Mata and Lamattina, 2001
	SNP + cPTIO SNAP + cPTIO	nd	Inhibition of stomatal closure.	
Triticum aestivum seedlings and detached leaves	SNP	nd	Enhanced relative water content. Decreased transpiration rate.	
Vicia faba epidermal strips	ABA + cPTIO	↓	Inhibition of ABA-dependent stomatal closure.	García-Mata and Lamattina, 2002
	SNP/ABA	↑	Increased stomatal closure.	
	SNAP/SNP	nd	Inactivation of $I_{K,in}$ in guard cells.	García-Mata et al., 2003
	UV-B	↑	Increased stomatal closure.	He et al., 2005
	UV-B + cPTIO UV-B + L-NAME	↓	Inhibition of UVB-dependent stomatal closure.	
Pisum sativum epidermal peels	ABA/SNP/GSNO	↑	Induction of stomatal closure.	Neill et al., 2002
Arabidopsis thaliana epidermal fragments	ABA/nitrite	↑	Stimulation of NO synthesis, followed by stomatal closure.	Desikan et al., 2002
	ABA/H_2O_2	↑	Stimulate stomatal closure.	Bright et al., 2006
	H_2O_2 + PTIO	↓	Inhibition of stomatal closure.	
Arabidopsis thaliana	Dehydration stress + SNP/nitrite	↑	Under water shortage the effect of NO-dependent stomatal closure was not observed.	Ribeiro et al., 2009
Arabidopsis prt6 (altered proteolysis of transcriptional regulators)	SNAP/SNP	↑	Plants are defective in NO perception mechanism, and guard cells are insensitive to NO-induced closure.	Gibbs et al., 2014
Arabidopsis triple *nia1nia2noa1-2* mutant (impaired activity of proteins AtNOA and NR)	Dehydration stress	**	Enhanced resistance to dehydration, lower transpiration rate and stomatal conductance as compared to WT.	Lozano-Juste and León, 2010
	ABA	**	Stomata were more sensitive to ABA-induced closure as compared to WT.	

**NO level in guard cells was evaluated through fluorescence microscopy.*
***nd:** non determined.*
Fluorescence associated with NO level increased (↑) or decreased (↓) after treatment as compared to control (without treatment).
***Mutant nia1nia2noa1-2 plants exhibited lower NO in all tissues, including guard cells, as compared to WT plants.*
*SNAP, SNP, GSNO are NO donors, cPTIO/PTIO are NO scavengers, **l**-NAME inhibits animal NOS.*

as it was previously mentioned localized in chloroplast, which temporally preceded the stress-induced CAM metabolism. In turn, unstressed pineapple plants that were daily exposed to NO donors SNP, NOC9, and gaseous NO during 15 days exhibited increases in the activities of the enzymes required for CAM metabolism as well as in the D-malate concentration (Freschi et al., 2010).

PLANTS RESPONSES TO DEAL WITH UV-B: NO AS PROTAGONIST

The primary energy source for plants is sunlight; however, part of this radiation is in the UV range. The UV region of the sun electromagnetic spectrum is usually subdivided into UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (200–280 nm). It is known that, short wave UV-C radiation is completely absorbed by atmospheric gases, UV-B radiation is partially absorbed by stratospheric ozone and only a very small proportion is transmitted to the surface, while UV-A is hardly absorbed by ozone (Frohnmeyer and Staiger, 2003). Although UV-B is a relatively minor component of sunlight, it has high energy and thus can exert detrimental effects on plants. In addition, morphological, physiological, biochemical, and molecular effects in plants may occur as reviewed by Kataria et al. (2014).

It has long been described that chloroplasts are very sensitive to UV-B radiation. Light-induced damage is targeted mainly to photosystem II (PSII), with inactivation of electron transport and oxidative damage of the reaction center, particularly to the D1 protein (Aro et al., 1993). UV-B exposure of isolated soybean chloroplasts enhanced lipid peroxidation as assessed by measuring the content of thiobarbituric acid reactive substances (TBARS) and the carbon-centred radical generation by electronic paramagnetic resonance (EPR; Galatro et al., 2001). Carbonyl groups content, an index of protein damage, was also increased in the chloroplasts after UV-B treatment (Galatro et al., 2001). On the other hand, exposure of isolated chloroplasts to GSNO, as NO donor, led to a decrease in the generation rate of chloroplastic lipid radicals, as well as in the content of carbonyl groups in proteins as compared to control chloroplasts (Jasid et al., 2006). A protective effect of NO against oxidative stress under UV-B radiation has been described. UV-B treatment increased ion leakage, H_2O_2 content, and thylakoid membrane protein oxidation in bean (*Phaseolus vulgaris*) leaves (Shi et al., 2005).

Also, maximum efficiency of PSII photochemistry (F_v/F_m) and the quantum yield of PSII (Φ PSII) decreased under UV. SNP, employed as NO donor, could prevent ion leakage increase and chlorophyll loss, alleviating UV-B induced photo damage. As well, thylakoid membrane carbonyl groups and H_2O_2 were decreased by NO exposure (Shi et al., 2005). Thus, NO could exert a protective role against protein oxidation under stress conditions as it was previously reported in relation to lipid oxidation (Radi, 1998). As chloroplasts can produce NO (Jasid et al., 2006; Galatro et al., 2013; Tewari et al., 2013), and NO can alleviate the oxidative effects of UV-B radiation, this source of NO could be operative under UV-B radiation. In broad beans, exposure to UV-B induced an NO generation in cytosol and chloroplasts in guard cells from epidermal strips (He et al., 2005). This NO generation was evaluated employing DAF-2 DA and laser scanning microscope, fluorescence being particularly intense in chloroplasts after UV-B exposure. Conversely, as previously described, if UV-B radiation exposure exceeds the capacity of NO to protect from damage to PSII and inactivation of electron transport occurs, it would also compromise the capacity of chloroplasts to produce NO under this environmental stress condition, enhancing UV-B damage.

The adverse effects of UV-B on plants involve oxidative stress. As an example of this, in soybean chloroplasts, ascorbic acid and thiols were increased when plants were exposed to a high dose of UV-B (60 kJ m^{-2}; Galatro et al., 2001). Shi et al. (2005) described that SOD, APX, and CAT activities increased under UV-B radiation, and that SNP treatment led to a further enhancement. NO can induce specific isoforms of antioxidant enzymes in soybean leaves subjected to enhanced UV-B radiation (Santa-Cruz et al., 2014). Both transcripts levels and the activities of SOD, CAT, and APX have been found to be significantly induced by the treatment with SNP alone. UV-B radiation produced a significant decrease in transcripts levels of antioxidant enzymes related to hydrogen peroxide scavenging, APX, and CAT. However, irradiation of SNP-pretreated plants prevented CAT and APX down-regulation caused by UV-B radiation, but did not further enhance SOD transcripts levels respect to SNP alone (Santa-Cruz et al., 2014). Hemeoxygenase (HO) has antioxidant properties and is up-regulated by ROS in UV-B-irradiated plants (Yannarelli et al., 2006). Santa-Cruz et al. (2010) proposed that NO is implicated in the signaling pathway leading to HO-1 isoenzyme up-regulation and, together with ROS, modulates the activity of this enzyme under UV-B radiation. A certain balance between NO and ROS seem to be required to trigger the full response. Experiments performed in soybean plants treated with SNP in the absence of UV-B showed NO itself could up-regulate HO-1 mRNA expression, although to a lesser extent. Taking into account that HO is a chloroplast-localized enzyme, HO could play a key role in protecting the chloroplast against UV-B-induced oxidative stress. Heme catabolism through HO produces biliverdin that together with ascorbic acid, play a role in controlling H_2O_2 levels in the chloroplast (Santa-Cruz et al., 2010).

UV RESISTANCE LOCUS8 is a UV-B-specific signal transduction component that plays a vital role in mediating plant responses to UV-B. UVR8 controls the expression of the transcription factor HY5 (ELONGATEDHYPOCOTYL5), important in the regulation of seedling photomorphogenesis and UV-protection (Brown et al., 2005). Some studies unveiled that UVR8 is a plant UV photoreceptor protein that regulates gene expression involved in the prevention and repair of UV-B damage by exposure of plants to low UV-B, leading to photosynthetic acclimation (Rizzini et al., 2011; Singh et al., 2014). UVR8 mediates several photomorphogenic responses to UV-B, as the suppression of hypocotyl elongation, stomatal differentiation, stomatal closure, and the synthesis of UV protective flavonoids and anthocyanins (Tossi et al., 2014). Interestingly, some of these responses are also mediated by NO. In response to UV-B, *Arabidopsis* plants increase NO and H_2O_2 levels, however, in *uvr8-1* null mutants stomata remains opened without changes in NO and H_2O_2, conversely GSNO treatment induced stomatal closure even in mutant plants (Tossi et al., 2014). Recently, Hayes et al. (2014) linked the inhibition of stem elongation reported by UV-B radiation with DELLAs protein stabilization.

Abscisic acid is a plant hormone that regulates many developmental and growth processes in plants, as well as signaling mechanisms associated with responses to environmental stresses (Tuteja, 2007). It has been suggested that UV-B triggers an increase in ABA concentration, being an early ABA-mediated response involved in signaling pathways to counteract UV-B in maize leaves (Tossi et al., 2009). The increase in ABA concentration is followed by H_2O_2 generation and an enhancement of NO production through, at least in part, a NOS_{like} activity. Moreover, in guard cells, the NO necessary for stomatal movements in response to UV-B, seems to be generated by the activity of NR, being part of a multifaceted pathway also mediated by ABA, UVR8, COP1, HY5, NADPH oxidase, and H_2O_2 (Tossi et al., 2014).

On the other hand, it is known that flavonoids and anthocyanins are important actors in protecting plants from UV-B effects. It has been reported that the up regulation of chalcone synthase gene (*Chs*), an enzyme involved in flavonoid synthesis, by UV-B was reduced by NOS inhibitors or NO scavengers (Mackerness et al., 2001), supporting a role for NO in flavonoid increase under UV-B. In this context, Tossi et al. (2011) proposed an interesting model to explain plant responses to increased UV-B involving ROS, NO, and flavonoids. According to it, UV-B radiation increases both ROS and NO. Then, NO reduces ROS levels and up regulates the expression of several genes involved in flavonoid and anthocyanin synthesis (as the maize transcription factor ZmP and MYB12, its *Arabidopsis* functional homolog; as well as their target genes *Chs*, and *Chi* –chalcone isomerase-). Thus, synthesis of some flavonoids and anthocyanins are increased being able to absorb UV-B and also scavenge ROS. It is known that NO is involved not only in accumulation, but also in localization of flavonoids under UV-B treatment (Tossi et al., 2012). In UV-B stressed maize seedlings NO and flavonoids are systemically induced, being flavonoid accumulation dependent on the NO activation of biosynthetic genes (*Chs* and *Chi*; Tossi et al., 2012).

Ethylene production in plants is stimulated under several developmental processes and under stress conditions, including

UV-B radiation. NO and ROS have also been implicated in UV-B induced ethylene production in maize seedlings (Wang et al., 2006). NO generation, through an increased arg-dependent activity, seems to play an important role in UV-B responses, acting in the same direction or synergistically with ROS to induce ethylene synthesis. However, further experiments are needed to know the mechanism involved in ethylene accumulation (Wang et al., 2006).

Nitric oxide is widely accepted as participating toward the growth and development of the plant, and as a response to several stress conditions as UV-B radiation. It is now clear that NO is a key factor to cope with increased levels of UV-B in plants. Through several responses that involve signaling pathways implicated in antioxidant enzymes induction, flavonoid and anthocyanin synthesis, and hormonal responses, NO can diminish UV-B impact by reducing oxidative stress in plants. Although the knowledge for NO functions in plants has been largely improved, some signaling events are still matter of active research and remain an issue to be fully elucidated.

NO: A CRITICAL COMPONENT IN PLANT RESPONSES TO SALT STRESS

Salinity is along with drought one of the most extended adverse conditions affecting plant growth and development. Salt stress disturbs plant growth through both toxic and osmotic components (Munns, 1993), which in turn could result in oxidative stress and death. Importantly, not all plants respond in a similar way to those components because of the presence of a panoply of tolerance strategies that help to overcome the stress through different acclimation mechanisms. Mechanisms commonly used by plants to cope with salinity involve handling ionic relations, accumulation of osmo compatible organic solutes, and modulation of enzymatic and non-enzymatic components of the antioxidant machinery as well as controlling the execution of a cell death program. Evidence for the potential involvement of NO in plant responses to the salinity occasioned by high NaCl concentrations was obtained more than 10 years ago. It was shown that exposure of rice plants to a relatively low concentration of the NO donor SNP led to a better performance to the subsequent exposure of plants to 100 mM NaCl (Uchida et al., 2002). A similar observation was made soon after in *Lupinus luteus* (Kopyra and Gwóźdź, 2003) as well as in maize (Zhang et al., 2006). In the last case it was observed that the protective action exerted by plant pre-treatment with SNP was reverted in the presence of an NO scavenger; while the addition of ferrocyanide, a SNP analog which do not generates NO, do not produce protection. The protective effect exerted by exogenous addition of NO was associated with maintenance of a high relative water content and chlorophyll, while ion leakage was maintained low. Moreover, a clear effect on Na^+ and K^+ accumulation was also observed. These results suggested that NO protects plants, at least during a relatively short period of exposure to NaCl, by helping to control water status, maintaining ionic homeostasis and reducing damage imposed during early phases of salt stress response. Providing evidence that endogenous NO could be actually involved in plant responses to salinity, an enhancement of endogenous NO accumulation has been observed in several plant species exposed to saline stress (Zhang et al., 2006; Valderrama et al., 2007; David et al., 2010; Monreal et al., 2013; Manai et al., 2014). Moreover, *Atnoa* mutant plants that display reduced NO level show a higher sensitivity to NaCl stress (Zhao et al., 2007b). Conversely, expression of the Ot*NOS* gene from the algae *Ostreococcus tauri* under the control of a stress-responsive promoter has shown to confer *Arabidopsis* plants enhanced accumulation of NO in roots and leaves when exposed to 100 mM NaCl, which was associated with improved capacity to resist that high salt concentration (Foresi et al., 2015).

Effects of NO over ion accumulation during the course of salt stress have been repeatedly observed (i.e., Zhao et al., 2004, 2007b; Zhang et al., 2006; Zheng et al., 2009; Chen et al., 2010, 2013a; Shi et al., 2012). A common observation to most of these findings is that an enhancement of NO is accompanied by exclusion of Na^+ and retention of K^+, leading to improved K^+/Na^+ ratios. K^+/Na^+ ratio, particularly in leaves, has been frequently considered as a pivotal component of salt resistance. However, it should be noted that it is a complex trait that depends on the activity of multiple transport systems, that belong to several families of transporters, which mediate K^+ and Na^+ transport at different plant points, as well as on the capacity to maintain the membrane potential at the plasma-membrane and at the tonoplast at adequate values; being it primarily related with H^+-ATPases activity. A stimulating effect of NO on the activity of Na^+/H^+ antiporters operating either at the tonoplast or at the plasma membrane has been unveiled (Zhang et al., 2006; Chen et al., 2010). The activity of these transporters leads, depending on their precise site of action, to Na^+ exclusion into the vacuoles or to the external medium, and alleviates the potentially deleterious effect of a high Na^+ concentration in the cytoplasm. Activities of these Na^+/H^+ antiporters require, as above mentioned, an appropriate H^+ gradient. It has been observed that increased NO accumulation is usually companied by an enhancement of proton pump activities at both the plasma membrane and the tonoplast and/or an enhancement of transcripts coding for them (Zhang et al., 2006; Chen et al., 2010). On the other hand, K^+ nutrition is known to be a key component of the tolerance to multiple stress conditions, among them salinity, in the cell walled eukaryotic organisms so far studied (Cakmak, 2005; Mangano et al., 2008; Shabala and Pottosin, 2014). Moreover in those organisms, as well as in animals, decay of K^+ concentration constitutes a critical step in the execution of cell death programs that take place during the response to stress conditions (Demidchik et al., 2010; Lauff and Santa-María, 2010). In this context, the control of H^+-transport activity by NO may help to avoid the membrane depolarization that takes place during the massive flux of Na^+ and therefore contributes to ensure an adequate inward flux of K^+ as well as to reduce K^+ loss from cells. The above mentioned positive effect of NO on the expression of AKT1 at high salt concentrations (Chen et al., 2013a) could constitute an additional NO-dependent strategy used by plants exposed to high NaCl concentrations to keeping K^+ in the cytoplasm within values high enough to avoid cell death. Cell death generated by salinity is usually preceded by oxidative damage. In such a context, it should be noted that

the addition of NO to plants suffering from salt stress results in reduced oxidative damage as indicated by a reduction of lipid peroxidation and/or hydrogen peroxide content (Zheng et al., 2009; Wang et al., 2011; Chen et al., 2014). In addition it has been shown that NO exerts a differential modulation of the antioxidant response under conditions of salinity (Hasanuzzaman et al., 2011; Wang et al., 2011; Zeng et al., 2011; Chen et al., 2014).

These findings suggest that NO plays an important, even not yet fully understood, role on plant responses that help them to cope with salt stress. Noticeably, NO could participate in plant responses to this adverse condition in other, less obvious, ways. As an example of this statement it has been recently observed that the activity of the enzyme phospho*enol*pyruvate carboxylase-kinase, which regulates the activity of phospho*enol*pyruvate carboxylase in C4 plants and becomes enhanced under salinity conditions, is likely dependent on NO accumulation (Monreal et al., 2013).

MECHANISMS UNDERLYING BIOLOGICAL EFFECTS OF NO

The "chemical biology" of NO describes its reaction with specific biological molecules and provides a framework to understand its participation in apparently unconnected events (Wink and Mitchell, 1998). $NO^{\cdot}$ is a paramagnetic molecule with an unpaired π^* electron, which can easily diffuse across membranes. Upon oxidation, nitrosonium anion NO^+ is formed, which participates in nitrosation reactions when added to an amine, thiol, or hydroxyl aromatic group. The addition of a second electron in the 2p-π orbital of $NO^{\cdot}$ produces nitroxyl anion (NO^-). Under cellular conditions, interconversion of $NO^{\cdot}$, NO^+, and NO^- can take place (Hughes, 1999). Reaction with metal centers, thiols, oxygen molecule, and free radicals constitutes the way through which NO modulates plant responses.

Reaction with Metals

Nitric oxide readily forms coordination complexes with transition metals, in the case of Fe named nitrosyl iron complexes, which can be thought as NO^+ carriers. Potentially toxic forms of iron are able to catalyze the formation of hydroxyl radical ($HO^{\cdot}$) through Fenton's reaction. The protective effects awarded to NO (Jasid et al., 2008) could be related with the ability of NO to inhibit Fenton chemistry binding ferrous iron and thus preventing oxidative stress (Kanner et al., 1991; Lu and Koppenol, 2005).

Nitric oxide also reacts with hemoproteins in the ferrous, ferric and ferryl forms. Direct reaction of NO leading to nitrosyl Fe formation is the clue for enzyme activation or inactivation (e.g., sGC and catalase; Wink and Mitchell, 1998). In a typical and deeply studied metal-nitrosylation reaction NO activates sGC. This enzyme contains an iron-heme component essential to the reaction mechanism. The binding of NO to the heme triggers an increase in sGC activity, and guanosine 3′, 5′, monophosphate (cGMP) production leading to biological responses in animals such as vasodilation and neurotransmission among others. Research performed in plants showed that NO induces a transient increase in cGMP levels (Durner et al., 1998; Neill et al., 2003), and inhibitors of sGC block the NO-induced activation of phenylalanine ammonia-lyase (Durner et al., 1998).

Nitric oxide-dependent transcriptional changes accompanying root branching were observed in sunflower seedlings (Corti Monzón et al., 2014). NO-mediated gene regulation could be related with the regulatory effects on Zn-finger transcription factors through metal nitrosylation, as it was described in human cells (Schäfer et al., 2000), or in *Escherichia coli* where the regulatory domain of the transcriptional activator NorR forms a mononitrosyl-iron complex, enabling the activation of transcription by RNA polymerase (D'Autréaux et al., 2005).

Reaction with other Free Radicals

Reaction between NO and superoxide anion (O_2^-), is diffusion limited ($k \approx 7\times10^9$ M^{-1} s^{-1}), and constitutes an exception because NO does not usually react very fast (Henry and Guissani, 1999). In plants, the simultaneous generation of O_2^- and NO has a synergistic function in defense responses (Asai et al., 2008). This reaction establishes a link between reactive oxygen and nitrogen species metabolism (Wink and Mitchell, 1998), and leads to the formation of peroxynitrite ($ONOO^-$), a potentially toxic powerful oxidant, which reacts with major classes of macromolecules. NO is a potent inhibitor of the propagation phase of lipid peroxidation, acting as peroxyl radical ($LOO^{\cdot}$) scavenger (Hogg and Kalyanaraman, 1999). Lipid peroxidation is a deleterious component in oxidative imbalance produced during the course of most if not all abiotic stresses, and the protective effect of NO may be related with this reaction taken in consideration its accumulation in lipophilic environments (Patel et al., 1999). Nitrolipids (nitro fatty acids) formed by interaction of unsaturated lipids and NO-derived species have been detected in animals and plants, and has been proposed as a function of mediation in signal transduction (Rubbo and Radi, 2008; Fazzari et al., 2014).

Reaction with Sulfhydryl Groups

NO^+ is a strong electrophilic species and reacts toward most biological R-SH (Gaston, 1999), leading to the formation of *S*-nitrosothiols (SNO). SNO in general and nitrosoglutathione (GSNO) in particular are considered NO^+ reservoirs and carriers, which can be found at high concentrations in biological systems. GSNO, the major cellular reservoir of NO, is transformed in oxidized glutathione and ammonium by the activity of GSNOR. Interestingly, the activity of GSNOR is in turn inhibited in the presence of excessive NO through *S*-nitrosylation mechanisms. Thus, high NO level prevents GSNO degradation with probable impact on further nitrate uptake and reduction (Frungillo et al., 2014). Alterations in glutathione pools, the major plant thiol, could have important implications in cellular redox status with impact in cell signaling (Vivancos et al., 2010). Electrophilic reaction of NO^+ with cysteinyl sulfhydryl moieties (*S*-nitrosylation) is considered a cell signaling mechanism with important functional involvement in various plant physiological processes. An updated compilation of proteins regulated through *S*-nitrosylation is presented in Lamotte et al. (2014). Of great

importance in NO cross-talk with hormones in determining root architecture, and thus important in several plant stress responses, are the regulatory effects of NO mediated by reversible Cys-nitrosylation that have been described in the auxin receptor TIR1 (Terrile et al., 2012). This post-translational protein modification leads to an enhanced receptor-hormone interaction and increased auxin-dependent gene expression (Terrile et al., 2012). In addition, evidence has been offered for increased NO levels and the occurrence of differential *S*-nitrosylation of some proteins following salt stress (Fares et al., 2011; Tanou et al., 2012; Camejo et al., 2013). Besides NO levels, protein nitrosylation has been related with an over accumulation of GSNO, as in cases of low GSNOR activity or impaired activity of thioredoxin-h5 (TRXh5; Kneeshaw et al., 2014). Plant TRXh5 exhibit a potent and selective protein-SNO reductase activity which is determinant for salicilic acid-dependent plant immune signaling (Kneeshaw et al., 2014).

Regulation of the activity of transcription factors is a key mechanism through which NO is able to affect physiological processes. In animals, NO favors the binding of a transcription factor (CREB), that regulates the expression of several genes involved in neuron survival, through *S*-nitrosylation of nuclear proteins (Contestabile, 2008). Other nuclear factor-κB (NF-κB) binding activity is regulated through *S*-nitrosylation at Cys-62 residue (Contestabile, 2008). Recently, a general mechanism for NO sensing in plants has been proposed based on targeted proteolysis of plant-specific transcriptional regulators (Gibbs et al., 2014). The group VII ethylene response factors (VII ERF transcription factors) act as sensors of NO via the N-end rule proteolysis pathway, regulating NO-mediated processes during plant transcriptional response to hypoxia, seed germination and regulation of ABA sensitivity among others. Evidence suggests that in the presence of NO, these proteins are destabilized via the N-end rule pathway, likely through interaction with cysteine, and are stabilized in the absence of NO, providing a general homeostatic mechanism for perception and transduction of NO (Gibbs et al., 2014).

Reaction with Protein Tyrosines

Nitration of aromatic groups involves the addition of a nitro group (NO_2^+). The nitration of tyrosine residues in proteins may interfere with tyrosine phosphorylation, a generalized means of controlling enzymatic activity. Furthermore, nitration of free tyrosine and protein tyrosine residues is often used as an index of peroxynitrite (an NO derived species) presence in tissues. However, yield of nitration reactions of peroxynitrite is influenced by CO_2 concentrations (Santos et al., 2000), this effect being studied *in vitro* as well as in animal systems. Thus, nitration events may be influenced in different plant cell types according to reactive nitrogen species formation and CO_2 levels. In plants specific protein nitration has not been extensively studied as *S*-nitrosylation. In chloroplasts, tyrosine nitration sites have been identified in PSI, PSII, cytochrome b6/f and ATP synthase complex (Galetskiy et al., 2011), and enhanced protein nitration accompanied NO increase in salt stressed pea plants (Camejo et al., 2013). The identification of potential nitrated proteins *in vivo* is under explored, as are functional studies of the impact of this post-translational modification in protein activity. Targets of nitration were identified in sunflower hypocotyls (Chaki et al., 2009), in *Arabidopsis* under non-stressed conditions (Lozano-Juste et al., 2011) and after hypersensitive response (Cecconi et al., 2009). Nitroproteomic analysis was performed in roots and leaves of citrus plants exposed to salt stress. Photosynthesis-related proteins were the main group modified in leaves and disease/defense related proteins were the group affected in roots (Tanou et al., 2012), a total of 88 and 86 proteins underwent tyrosine nitration in leaves and roots, respectively. Activity of glutamine synthetase, a key enzyme for root nodule metabolism is subjected to inactivation by means of tyrosine nitration (Melo et al., 2011). Finally, an extensive analysis was performed in leghemoglobins where specific tyrosines were identified as nitration sites in bean and soybean nodules (Sainz et al., 2015).

CONCLUDING REMARKS

Nitric oxide acts to prevent oxidative damage which likely helps to maintain photosynthetic capacity as well as other major metabolic processes; it interacts with plant hormones thus helping to modulate root architecture in several ways as well as stomatal movement; NO sets an internal ionic environment that helps to maintain basic cellular functions; and determines changes in gene expression patterns as well as protein activities, proving to alleviate abiotic stress impact. NO levels can be modulated by means of exogenous synthetic NO donors, genetic manipulation as well as through the interaction with microorganisms (mycorrhizas, plant-growth promoting bacteria) and/or through changes in endogenous synthesis and scavenger mechanisms.

In general, different stress conditions still require NO, as well as ROS signaling, in order to elaborate the appropriate responses. Although knowledge on NO-mediated responses to abiotic stresses has been frequently, but not always, well documented, the precise pathways involved in NO signaling for each specific stress condition are just starting to emerge.

A further knowledge on the sources of NO generation in plants, the endogenous and exogenous factors that can affect NO levels in plant cells, as well as the multiple signaling pathways implied in physiological and morphological stress responses could help to develop strategies to improve plant growth and development under unfavorable conditions. This situation becomes especially important for agronomic cultures where mineral nutrient efficiency and environmental stress resistance are important factors that help combat human nutrition problems.

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Functional characterization and reconstitution of ABA signaling components using transient gene expression in rice protoplasts

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The core components of ABA-dependent gene expression signaling have been identified in *Arabidopsis* and rice. This signaling pathway consists of four major components; group A OsbZIPs, SAPKs, subclass A OsPP2Cs and OsPYL/RCARs in rice. These might be able to make thousands of combinations through interaction networks resulting in diverse signaling responses. We tried to characterize those gene functions using transient gene expression for rice protoplasts (TGERP) because it is instantaneous and convenient system. Firstly, in order to monitor the ABA signaling output, we developed reporter system named pRab16A-fLUC which consists of *Rab16A* promoter of rice and luciferase gene. It responses more rapidly and sensitively to ABA than pABRC3-fLUC that consists of ABRC3 of *HVA1* promoter in TGERP. We screened the reporter responses for over-expression of each signaling components from group A OsbZIPs to OsPYL/RCARs with or without ABA in TGERP. OsbZIP46 induced reporter most strongly among OsbZIPs tested in the presence of ABA. SAPKs could activate the OsbZIP46 even in the ABA independence. Subclass A OsPP2C6 and -8 almost completely inhibited the OsbZIP46 activity in the different degree through the SAPK9. Lastly, OsPYL/RCAR2 and -5 rescued the OsbZIP46 activity in the presence of SAPK9 and OsPP2C6 dependent on ABA concentration and expression level. By using TGERP, we could characterize successfully the effects of ABA dependent gene expression signaling components in rice. In conclusion, TGERP represents very useful technology to study systemic functional genomics in rice or other monocots.

Keywords: ABA, rice protoplast, reconstitution of signaling, transient expression, dual luciferase assay

Introduction

Living organisms use receptors to recognize factors in the extracellular environment such as light, water, and pathogens. Receptors trigger biochemical events that transduce the signal to the inside of cell. In response to such signals, metabolism and gene expression within cells are altered. Plants are sessile organisms that cannot move away from adverse environments toward favorable environments. Thus, plants are exposed to much more diverse environmental conditions compared to animals and have to respond and adapt to adverse environments to survive. Therefore, it might

be supposed that plants have more complex signal transduction systems than animals. Indeed, several unique molecular systems to transduce signals are present in plants (Trewavas, 2002).

One of the biggest challenges looming for agricultural research and policy is how to feed the estimated nine billion people on the planet in 30 years, especially in the phase of global warming (Gregory and George, 2011; Springer and Duchin, 2014). Accordingly, one of the major concerns of crop research scientists is to increase crop productivity in adverse environments. For this, it is necessary to understand the molecular mechanisms underlying signal transduction related to abiotic stress and crop productivity. Thus, methodologies to monitor signal sensing, transduction and output are required. Among several types of signal outputs, gene expression and protein synthesis alteration are the most suitable outputs which monitor the effects of signaling. Reporter systems for gene expression consist of promoters of genes regulated by target signals and reporter genes such as *chloramphenicol acetyltransferase* (CAT), *beta-glucuronidase* (β-GUS), *beta-galactosidase* (β-GAL), *luciferase* (LUC), and *green fluorescent protein* (GFP), (Peach and Velten, 1992; Cormack et al., 1998; Hammerling et al., 1998; Blazquez, 2007). Recently LUC has been most often used as a transcription monitoring reporter in plants and animals because LUC has a short turnover time and high sensitivity (Yoo et al., 2007).

It takes much time and effort to develop whole-plant transcriptional assay systems. Thus, transient gene expression systems are often used to study signaling in plants. For these purposes, agro-infiltration and transient gene expression systems in protoplasts are most commonly applied in dicot plants (Sainsbury and Lomonosoff, 2014). Transient gene expression in protoplasts also has been performed in monocots such as rice and maize. However, the efficiency of protoplast isolation and transformation was low and large amounts of protoplasts were required because of low-sensitivity of reporter systems. Recently efficient protoplast isolation methods were reported and successfully used for cell biology studies such as subcellular localization and cellular interaction analyses, including BiFC, in rice (Chen et al., 2006; Zhang et al., 2011). These advances suggest that rice protoplasts might be useful to monitor gene expression and identify gene functions in signaling pathways (Sheen, 2001).

ABA plays important roles in abiotic stress tolerance of plants. Recently ABA signaling components that regulate ABA-dependent gene expression were identified from receptors to transcription factors in *Arabidopsis* and rice (Park et al., 2009; Umezawa et al., 2009; Kim et al., 2012; Soon et al., 2012). When the ABA concentration in the cell goes up, ABA receptors PYL/RCAR bind ABA and interact with subclass A PP2Cs, which normally suppress SnRK2. As a result, SnRK2 activates bZIP transcription factors by phosphorylation and ABA-dependent gene expression is activated in *Arabidopsis* (Xiang et al., 2008; Kulik et al., 2011). In rice, the orthologs of these signaling components have been identified by bioinformatics (Kim et al., 2012; He et al., 2014). Rice contains 10 OsPYL/RCARs, 9 subclass A PP2Cs, 10 SAPK (Stress/ABA-activated protein kinases) and 10 group A bZIP transcription factors (Kim et al., 2012). The ABA signaling pathway of *Arabidopsis* was successfully reconstituted via transient expression in *Arabidopsis* mesophyll cell protoplasts (Fujii et al., 2009). However, in rice the functions of few ABA signaling components genes have been confirmed and the signaling pathway has not been reconstituted yet.

In this study, we developed the system of a transient gene expression for rice protoplasts (TGERP), reconstituted the ABA signaling components using TGERP and characterized the effects of components in ABA signaling through monitoring gene expression based on the LUC reporter. This system is suitable for high-throughput analysis because it generates data rapidly, quantitatively and inexpensively. Thus, it represents valuable technology for functional genomics approaches in the post-genomic era of rice.

Materials and Methods

Plant Material and Growth Conditions

Rice (*Oryza sativa* cv. Dongjin) dehulled seeds were sterilized with 70% ethanol for 1 min followed by 50% sodium hypochlorite of for 40 min and thoroughly washed 5–6 times with sterile distilled water. To isolate protoplasts, these seeds were grown on 1/2 Murashige and Skoog (MS) medium and initially kept under dark conditions for 8–10 days to induce long stems before being placed under long-day conditions (16 h light and 8 h dark) for 1–2 days at 28°C.

Rice Protoplast Isolation and Transfection

Rice protoplast isolation methods were reported by several research groups (Chen et al., 2006; Zhang et al., 2011; Kim et al., 2012). We modified those methods and optimized them as follows. A bundle of rice seedlings (about 36 seedlings) were chopped into 0.5–1 mm strips using a surgical blade. Chopped seedlings were quickly transferred to freshly prepared enzyme solution (1.5% cellulose R-10, 0.75% macerozyme R-10, 0.6 M mannitol, 10 mM MES at pH 5.7, 0.1% BSA, 3.4 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 50 μL mL^{-1} ampicillin) and soaked for 3–4 h in the dark with gentle shaking (50 rpm). After enzymatic digestion, the enzyme solution containing protoplasts was diluted with three volumes of W5 solution (0.1% glucose, 0.9% NaCl, 2 mM MES, 0.08% KCl, and 125 mM $CaCl_2$ at pH 5.65) before filtration to remove undigested stem tissues. Diluted protoplasts were filtered through 145-μm mesh into 50-mL conical tubes. The protoplasts were collected by centrifugation at 100 *g* for 10 min at 28°C. After washing, collected protoplasts were re-suspended in 4 mL W5 solution, and then re-suspended protoplasts were floated on 5 mL 22% sucrose to separate burst protoplasts. After centrifugation, intact protoplasts were collected from the green layer between the sucrose and the W5. After intact protoplasts were washed one more time with W5 solution, the protoplasts were re-suspended in MaMg solution (600 mM mannitol, 15 mM $MgCl_2$, and 5 mM MES at pH 5.65). For transfections, 300 μL protoplasts were mixed with plasmid constructs and 330 μL PEG solution [400 mM mannitol, 100 mM $Ca(NO_3)_2$, and 40% PEG-6000]. The mixture was incubated for 30 min at 28℃. After incubation, W5 solution was added stepwise to dilute the PEG solution. Protoplasts were collected by centrifugation at 100 g for 10 min at 28°C. Supernatants were

removed and the protoplasts were re-suspended in W5 solution and incubated.

Subcellular Localization and Bimolecular Fluorescence Complementation Assay

For subcellular localization analysis, the sequences encoding OsbZIPs, SAPKs, OsPP2Cs, and OsPYL/RCARs were amplified by PCR with specific primers, and PCR products were inserted into the pENTR/D/TOPO vector (Invitrogen, USA). The products were recombined into pMDC43 or pMDC83 vectors using LR Clonase (Invitrogen, USA). The TOPO cloning and LR reactions were carried out according to the manufacturer's instructions (Invitrogen, USA). The plasmids (10 μg) were introduced into rice seedling protoplasts by PEG-mediated transfection. GFP fluorescence was observed and images were captured with an Axioplan fluorescence microscope (AxioImager M1, Carl Zeiss, Jena, Germany).

For BiFC assays, coding sequences for SAPK9, OsPYL/RCAR2, and OsPYL/RCAR5 were cloned into the pVYCE vector resulting in fusion with the C-terminus of the yellow fluorescent protein (YFP). Coding sequences for OsPP2C6 and OsPP2C8 were cloned into pVYNE vector, resulting in fusion with the N-terminus of the YFP sequence (Waadt et al., 2008). Rice protoplasts were transfected with plasmid combinations (15 μg each) of fluorescent protein fragments by PEG-mediated transfection. Reconstituted YFP fluorescence was observed and images were captured with an Axioplan fluorescence microscope (AxioImager M1, Carl Zeiss, Jena, Germany) at 16–24 h incubation. In both experiments, 1–5 $\times$ 10^6 cells mL^{-1} protoplasts were used.

Construction of Reporter Vector for Dual-Luciferase Assays

To construct an ABA-responsive reporter plasmid vector consisting of the *Rab16A* promoter fused with firefly luciferase (fLUC), we amplified the *Rab16A* (Loc_Os11g26790) promoter region including 91 bp of 5′UTR by PCR from *Oryza sativa* cv. Dongjin genomic DNA with specific primers (Rab16A–F, 5′-CTGAGAGAGGATGACCCT TGTCACC-3′; Rab16A-R, 5′-TTTGGCGTCTTCCATCCTGCTTAAGCTAAAGCTGA-3′), and the fLUC gene including the Nos terminator region was amplified by PCR from the pABRC3-fLUC reporter plasmid with specific primers (fLUC-F, 5′-TTTAGC TTAAG CA GGATGGAAGACGCCAAAAACATAAAGAAAGGCCCGC-3′; NosT-R, 5′-GATCTAGT AACATAGATGACACCGCGCGCG-3′). These two PCR products were re-amplified using Rab16A-F and NosT-R primers. The final PCR products were cloned into pCRTM8/GW/TOPO vector (Invitrogen, USA), and the resulting reporter vector was named as pRab16A-fLUC (**Supplementary Figure S1**).

Dual-Luciferase Assays

For dual-luciferase assays, coding sequences for OsbZIPs and OsPP2Cs were cloned into the transient expression vector pGEM-UbiHA, which contains the maize *ubiquitin* promoter and sequence encoding a 3XHA tag. Coding sequences for SAPKs and OsPYL/RCARs were cloned into the transient expression vector pGEM-UbiFlag resulting in fusion with the flag tag. The resulting effector plasmids were used for rice protoplast transfection. After transfection, transfected protoplast cells were divided into two samples and incubated in W5 solution with or without ABA. After incubation, the protoplasts were harvested, frozen in liquid nitrogen and stored at –80°C. The frozen protoplasts were re-suspended in 100 μL Passive lysis buffer (Promega, USA). Reporter activities were measured in 10 μL lysate using a dual luciferase assay system according to the manufacturer's instructions (Promega, USA). The pRab16A-fLUC and pABRC3-fLUC constructs were used as ABA-responsive reporters (8 μg plasmid per transfection). pAtUBQ-rLUC (*Renilla* luciferase) was added to each sample as an internal control (1 μg per transfection; **Supplementary Figure S1**). OsbZIP-HA, SAPK-Flag, OsPP2C-HA, and OsPYL/RCAR-Flag effector plasmids were used at 10 μg per transfection. The relative luciferase activity [fLUC/(*Renilla* luciferase/*Renilla* luciferase average)] was calculated to normalize values after each assay.

Results

Rab16A Promoter Fused to Luciferase is Suitable as a Gene Expression Reporter for ABA Signaling in TGERP

The first step to investigate ABA-dependent gene expression regulation using TGERP is to establish ABA-responsive reporter systems. Accordingly, we constructed a reporter vector consisting of *Rab16A* promoter fused with fLUC. The reason for using *Rab16A* promoter is that it has been known as a representative ABA-responsive marker gene in rice (Mundy and Chua, 1988; Mundy et al., 1990; Nakagawa et al., 1996; Miyoshi et al., 1999; Xu et al., 2006; Lu et al., 2009; Kim et al., 2012; Joo et al., 2014). We examined the ABA-responsive induction of fLUC using pRab16A-fLUC and pABRC3-fLUC, which has been used as a control compared to pRab16A. Both pABRC3-fLUC and pRab16A-fLUC were individually transfected with pAtUBQ-rLUC as internal control and transiently over-expressed for 2, 4, and 16 h in the presence of 0, 5, 10, and 20 μM ABA in rice protoplasts. As shown in **Figure 1A**, pABRC3-fLUC expression was not induced under any concentration of ABA at 2 and 4 h. However, as ABA concentration increased from 5 to 20 μM, pABRC3-fLUC expression at 16 h was induced 36, 50, and 63%, respectively. By contrast, pRab16A-fLUC expression was induced under ABA treatments beginning at 2 h (**Figure 1B**). Thus, ABA treatments led to rapid and significant induction of pRab16A-fLUC expression compared to the pABRC3-fLUC expression under the same conditions. In particular, the increasing rate of pRab16A-fLUC induction by addition of 5 μM ABA, that is 4.4-, 7.2- and 6.5-fold at 2, 4, and 16 h, respectively, was more efficient compared to that of pRab16A-fLUC induction by addition of 10 and 20 μM ABA suggesting that 5 μM ABA was sufficient to induce pRab16A-fLUC expression (**Figure 1B**). When we compared the increase of fLUC expression at each time under different ABA concentrations, the relative rate of fLUC induction

was very similar at 4 and 16 h. Induction rates were 7.2-, 8.6-, and 10-fold at 4 h, and 6.5-, 8.6-, and 10-fold at 16 h under 5, 10, 20 μM ABA conditions, respectively (**Figure 1B**). However, at 2 h, fLUC was induced 4.4-, 5-, and 6.9-fold (**Figure 1B**). These data suggest that the proper time to monitor the ABA-mediated regulation of gene expression using pRab16A-fLUC is 4 h after ABA treatment in TGERP. Overall, these results indicate that *Rab16A* promoter fused to fLUC can be used as a reporter system for ABA-dependent gene expression due to rapid and significant response to ABA in TGERP.

Group A OsbZIPs Differentially Induce the Rab16A Promoter in TGERP Depending on ABA Concentration

Group A OsbZIPs are major transcription factors regulating ABA-dependent gene expression (Lu et al., 2009; Amir Hossain et al., 2010; Yang et al., 2011; Joo et al., 2014). In case of TRAB1 (OsbZIP66), it induced more expression of luciferase reporter gene through ABRC of *Osem* promoter in the presence of ABA using rice cultured-cell protoplasts (Hobo et al., 1999). Accordingly, we examined whether OsbZIP12, -23, and -46, already functionally characterized could induce the pRab16A-fLUC reporter in an ABA-dependent manner in TGERP as in whole-plant systems (Xiang et al., 2008; Amir Hossain et al., 2010; Yang et al., 2011; Tang et al., 2012; Joo et al., 2014; Park et al., 2015). First, we monitored how rapid OsbZIPs could be synthesized in TGERP. Reporter constructs representing genes from three different subclasses of group A OsbZIPs, namely OsbZIP12:GFP, OsbZIP23:GFP, and OsbZIP46:GFP, were transfected into protoplasts, and GFP fluorescence was observed at 2, 4, and 16 h. GFP fluorescence started to appear in the nucleus from 2 h and fluorescence intensity was strongly enhanced after 2 h until 16 h (**Figures 2A–C**). At 4 h, OsbZIP protein synthesis seemed to be at an exponential stage and it appeared that this was sufficient time to allow expression of protein in TGERP (**Figure 2B**).

When we examined the effects of OsbZIPs in terms of pRab16A-fLUC expression, OsbZIP12, -23, and -46 all enhanced the activities of the *Rab16A* promoter, both time and ABA-concentration dependently as shown in **Figures 2D–F**. However, the *trans*-activation activity among the three OsbZIPs was quite different. Representatively at 4 h OsbZIP12 induced the luciferase 5.4-, 5.7-, and 6.1-fold, OsbZIP23 induced luciferase 4.5-, 5.4-,

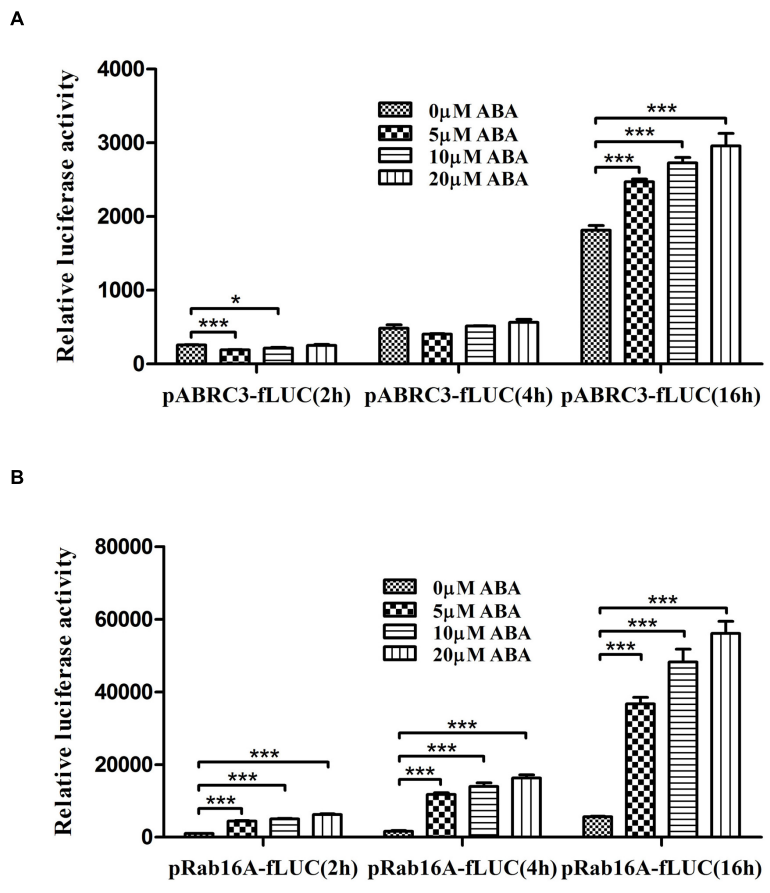


FIGURE 1 | *Rab16A* promoter responds rapidly and significantly to ABA in rice protoplasts. Dual luciferase assay driven by ABA-responsive promoters. **(A)** pABRC3-fLUC reporter. **(B)** pRab16A-fLUC reporter. The mean value of relative luciferase activity for three independent experiments is shown, and error bars indicate SD; analysis of variance (ANOVA) with Tukey's test, $^{*}P < 0.05$, $^{***}P < 0.001$.

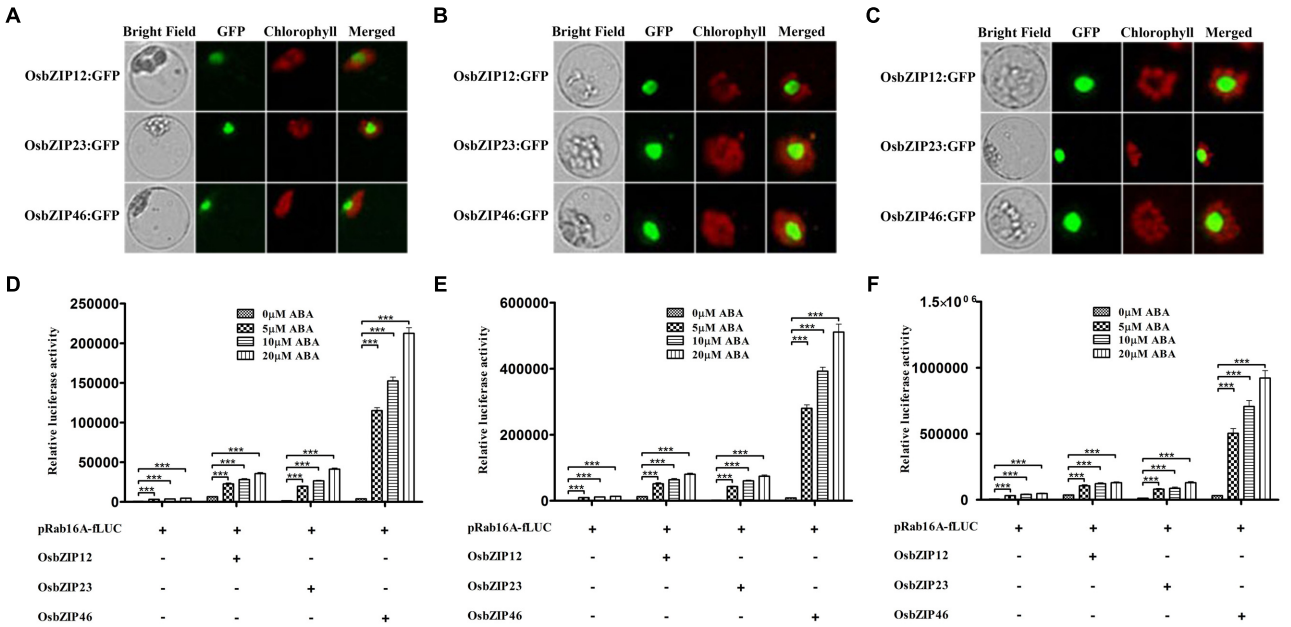


FIGURE 2 | OsbZIPs have differential *trans*-activation activity for *Rab16A* promoter in rice protoplasts. (A) Expression analysis of OsbZIP12:GFP, OsbZIP23:GFP, and OsbZIP46:GFP in rice protoplasts. After transfection, protoplasts were incubated for **(A)** 2 h, **(B)** 4 h, and **(C)** 16 h. GFP signals of OsbZIP12:GFP, OsbZIP23:GFP, and OsbZIP46:GFP were detected after 2 h incubation and gradually increased. OsbZIP:GFPs were used at 10 μg per transfection. Exposure time of GFP fluorescence was 200 ms. Chlorophyll auto-fluorescence is in red to distinguish it from GFP (green) fluorescence. **(D–F)** Dual luciferase assay after 2 h **(D)**, 4 h **(E)**, and 16 h **(F)** incubations. His-tagged OsbZIP12, OsbZIP23, and OsbZIP46 were transfected with pRab16A-fLUC reporter plasmid and pAtUBQ-rLUC plasmid as an internal control into rice protoplasts by PEG transfection. After transfection, protoplasts were incubated for 2, 4, and 16 h in the presence of 0, 5, 10, and 20 μM ABA under light. The mean value of relative luciferase activity for three independent experiments is shown, and error bars indicate SD; ANOVA with Tukey's test, $^{***}P < 0.001$.

and 5.6-fold and OsbZIP46 induced 29-, 34.8-, and 38.7-fold in 5, 10, and 20 μM ABA concentration, respectively (**Figure 2E**). These results indicate that OsbZIP46 has the strongest *trans*-activity in response to ABA among the three OsbZIPs for the *Rab16A* promoter. In conclusion, 5 μM ABA concentration and 4 h ABA treatment seems to be appropriate conditions to monitor ABA-dependent gene expression using pRab16A-fLUC reporter and OsbZIPs in TGERP.

Over-Expression of SAPK2 can Increase *Trans*-Activity of OsbZIP46 Independent of ABA in Rice Protoplasts

SAPKs can be classified into three different subclasses in terms of ABA-dependent kinase activity (Kobayashi et al., 2004; Kulik et al., 2011). SAPK2, -6, and -9 belonging to each subclass (I, II, and III, respectively) has been shown to bind OsbZIP46 directly and SAPK2 and -6 can phosphorylate OsbZIP46 without ABA in *in vitro* phosphorylation assay (Tang et al., 2012). However, transcriptional activity of OsbZIP46 enhanced directly by these SAPKs has not been confirmed yet. Thus we examined whether SAPK2, -6, and -9 could activate the OsbZIP46 in TGERP. Firstly, we confirmed whether the protein synthesis of SAPK2, -6, and -9 is enough at 2 and 4 h. GFP:SAPK2, GFP:SAPK6, and GFP:SAPK9 started to show much weaker GFP fluorescence after 2 h incubation than OsbZIP (**Figure 3A**) and GFP signal was significantly enhanced at 4 h for all SAPKs (**Figure 3B**). It seems that the expression of SAPKs required more induction time as compared to OsbZIPs. To examine the effects of SAPKs through the OsbZIP46 in the presence or absence of ABA, SAPK2, -6, and -9 were co-transfected with OsbZIP46, respectively. After 2 h incubations, fLUC expression was up to 48% greater with over-expression of SAPK2, whereas the over-expression of SAPK6 and -9 decreased fLUC expression without ABA (**Figure 3C**). After 4 h incubations, over-expression of SAPK2, -6, and -9 enhanced the fLUC expression by 3.1-, 1.7-, and 1.5-fold without ABA, respectively (**Figure 3D**). With 5 μM ABA at 2 h, over-expression of SAPK2 and -9 increased fLUC expression about 1.3-fold (**Figure 3C**). With 5 μM ABA at 4 h, over-expression of SAPK2, -6, and -9 increase fLUC expression about 1.3-, 1-, and 1.2-fold, respectively, but one-way ANOVA showed no significant effects (**Figure 3D**). Taken together, in the absence of ABA, SAPK2 can activate OsbZIP46 most significantly among three different subfamilies of SAPKs.

OsPP2C6 and -8 can Suppress the *Trans*-Activity of OsbZIP46 Completely through Inactivation of SAPK9 but Show Differential Characteristics in TGERP

Some SnRK2s, *Arabidopsis* orthologs of rice SAPKs, have previously been shown to bind directly to several group A PP2Cs and are inactivated by PP2C-mediated dephosphorylation in *Arabidopsis* (Umezawa et al., 2009; Vlad et al., 2009; Soon et al.,

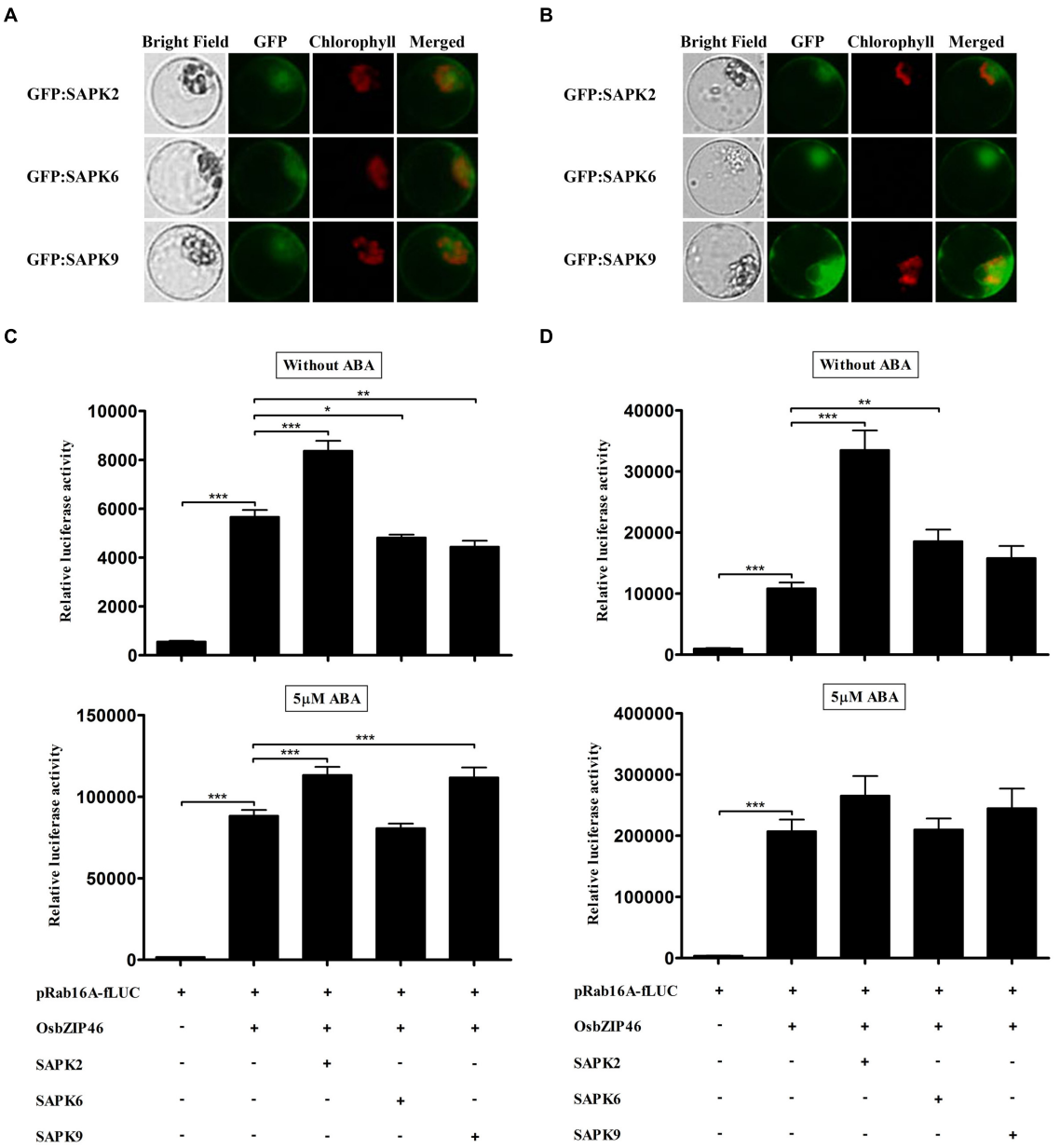


FIGURE 3 | Over-expression of SAPKs cannot increase *trans*-activation activity of OsbZIP46 significantly in wild-type rice protoplasts in the presence of ABA. (A,B) Expression analysis of GFP:SAPK2, GFP:SAPK6, and GFP:SAPK9 in rice protoplasts. After transfection, protoplasts were incubated for **(A)** 2 h and **(B)** 4 h. GFP signal of GFP:SAPK2, GFP:SAPK6, and GFP:SAPK9 was detected after 2 h incubation and gradually increased. GFP:SAPKs were used at 10 μg per transfection. Exposure time of GFP fluorescence was 600 ms. Chlorophyll autofluorescence is in red to distinguish it from GFP (green) fluorescence. **(C,D)** Dual luciferase assay after 2 h **(C)** and 4 h **(D)** incubations. Flag-tagged SAPK2, -6, and -9 were transfected with HA-tagged OsbZIP46, pRab16A-fLUC reporter plasmid and pArUBQ-rLUC plasmid as an internal control. After transfection, protoplasts were incubated for 2 and 4 h in the presence of 0 and 5 μM ABA under light. The mean value of relative luciferase activity for three independent experiments is shown, and error bars indicate SD; ANOVA with Tukey's test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2012; Xie et al., 2012). Therefore, we examined whether SAPK9 interacts with subclass A PP2Cs of rice using BiFC experiments in rice protoplasts (**Figures 4A,B**). Interestingly, SAPK9 interacted with two OsPP2Cs showing different subcellular localizations of complexes; the complex between OsPP2C8 and SAPK9 was localized in nucleus and the complex of OsPP2C6 and SAPK9 was observed in cytosol and nucleus, depending on the subcellular localization of the OsPP2Cs (**Figures 4A–D**). Yeast two hybrid assay also showed SAPK9 interacted with OsPP2C6 and OsPP2C8 (data not shown). The OsPP2Cs proteins showed much stronger expression than SAPKs in terms of GFP fluorescence (**Figures 4C,D**). We also monitored fLUC

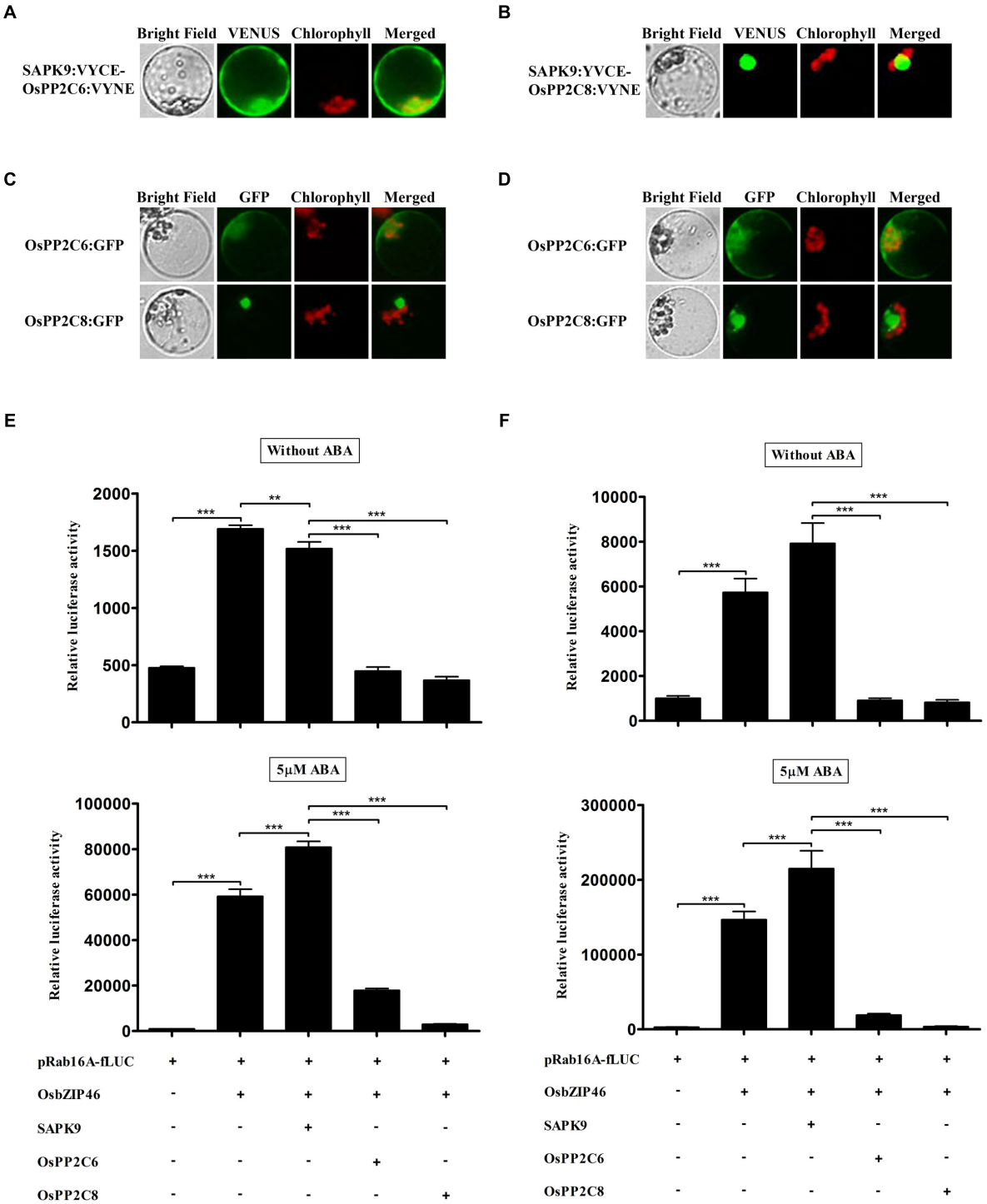


FIGURE 4 | OsPP2Cs completely suppress the *trans*-activity of OsbZIP46 in rice protoplasts. (A,B) Interactions of SAPK9 with OsPP2C6 and OsPP2C8 in rice protoplasts. The interaction of SAPK9 with OsPP2C6 and OsPP2C8 was detected by BiFC analysis. SAPK9 interacts with OsPP2C6 in both the nucleus and cytosol and with OsPP2C8 in the nucleus. **(C,D)** Expression analysis of OsPP2C6:GFP and OsPP2C8:GFP in rice protoplasts. After transfection, protoplasts were incubated for **(C)** 2 h and **(D)** 4 h. GFP signal of OsPP2C6:GFP and OsPP2C8:GFP was detected after 2 h incubation and gradually increased. GFP:OsPP2Cs were used at 10 μg per transfection. Exposure time of GFP fluorescence was 400 ms. Chlorophyll autofluorescence is in red to distinguish it from GFP (green) fluorescence. **(E,F)** Dual luciferase assay after 2 h **(E)** and 4 h **(F)** incubations. HA-tagged OsPP2C6 and -8 were transfected with HA-tagged OsbZIP46, Flag-tagged SAPK9, pRab16A-fLUC reporter plasmid and pAtUBQ-rLUC plasmid as an internal control. After transfection, protoplasts were incubated for 2 and 4 h in the presence of 0 and 5 μM ABA under light. The mean value of relative luciferase activity for three independent experiments is shown, and error bars indicate SD; ANOVA with Tukey's test, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression in dual luciferase assays to characterize the effects of OsPP2Cs on ABA-dependent gene expression. After 2 h incubation, over-expressed OsPP2C6 and OsPP2C8 decreased the fLUC expression to about 70 and 76%, as compared to the fLUC expression without OsPP2C in the absence of ABA. In the 5 μM ABA condition, the expression was decreased to about 78 and 96%, respectively (**Figure 4E**). At 4 h, the effects on luciferase activity of OsPP2C6 and OsPP2C8 were similar to those at 2 h, but inhibition activity was stronger. OsPP2C6 and OsPP2C8 decreased the fLUC expression to about 89 and 90% in the absence of ABA and about 91 and 98% in the presence of 5 μM ABA at 4 h (**Figure 4F**). Overall, our results showed that OsPP2C6 and -8 have similar inhibition activity of the fLUC expression in absence of ABA, but OsPP2C8 has stronger inhibition activity than OsPP2C6 in the presence of ABA.

OsPYL/RCARs have Differential Activities for ABA-Dependent Suppression of OsPP2Cs in TGERP

In ABA-dependent gene expression signaling, ABA receptors, PYL/RCARs interact with subclass A PP2Cs and inhibit its activity in *Arabidopsis* and rice (Park et al., 2009; Hao et al., 2011; Mosqunaa et al., 2011; Antoni et al., 2012; Kim et al., 2012; Zhao et al., 2013; He et al., 2014). These PYL/RCARs can be classified into dimer and monomer receptors, which have different characteristics (Hao et al., 2011; Okamoto et al., 2013; He et al., 2014). We found that both OsPYL/RCAR2 (the dimer form ABA receptor) and OsPYL/RCAR5 (the monomer form) could interact with OsPP2C6 in BiFC analysis (**Figures 5A,B**). Both ABA receptors were expressed in cytosol and nucleus, and the proteins were expressed enough to monitor at 4 h (**Figures 5C,D**). To examine the ABA receptor activity, we co-transfected all of the ABA signaling components from OsbZIP transcription factor to ABA receptor and reconstituted ABA signaling in rice protoplasts. As shown in **Figures 5E,F**, over-expression of OsPYL/RCAR2 and OsPYL/RCAR5 did not significantly change fLUC expression in the absence of ABA, at 2 and 4 h. However, in the presence of 5 μM ABA, over-expression of OsPYL/RCAR5 led to significant induction of fLUC, which increased threefold at 2 h and fivefold at 4 h. By contrast, OsPYL/RCAR2 did not induce fLUC at 2 or 4 h in the presence of 5 μM ABA (**Figures 5E,F**). In addition, when more DNA of OsPYL/RCAR2 and -5 was transfected, fLUC expression was increased in the presence of ABA. These results show that OsPYL/RCAR2 and OsPYL/RCAR5 have similar interaction partners but have different signaling effects dependent on ABA concentration.

Discussion

After the complete sequencing of the rice genome, re-sequencing of rice cultivars and genomic resources have given blue print of rice genome for researchers and breeders (International Rice Genome Sequencing Project, 2005; Xu et al., 2012). Also bioinformatics analysis tools and database development to compare the genomes, transcriptomes, proteomes and metabolomes have opened the era of systems biology (Sato et al., 2011). However, functionally identified genes remain relatively few and molecular mechanisms of signaling pathways identified systematically are also lacking in rice. The slow progress in functional genomics of rice compared to other -omics such as structural genomics, proteomics, and metabolomics are related to the time-consuming and laborious genetic analysis methods for the whole plant. Thus, transient functional identification systems are required for high-throughput analysis in rice and other crops for functional and systematic research in the new functional genomics era.

Several research groups have reported successful rice protoplast isolation from stem and sheath of young green seedlings, different from the green leaves used in *Arabidopsis* and maize (Chen et al., 2006, 2015; Zhang et al., 2011). These differences are related to different leaf anatomy; rice has very thin leaves with very few mesophyll cells. Protoplasts have been used as protein expression systems in various species. In tobacco and soybean, for instance, CAT activity was detected 30 min and 6 h after transfection, respectively (Grosset et al., 1990). In our TGERP, all proteins were detectable by 2 h after transfection based on GFP fluorescence, although the expression intensity was quite different among proteins. The proteins accumulated proportionally to incubation times from 2 to 16 h. Thus, signaling effects of genes could be monitored between 4 and 16 h after transfection in our TGERP.

In *Arabidopsis*, there are several different reporter systems that consist of promoter or *cis*-elements responsive to the signal of interest and reporter genes such as LUC, GUS, and GFP (Sheen, 2001; Yoo et al., 2007). The *RD29B* promoter was used as a reporter for reconstitution of ABA signaling in *Arabidopsis* (Fujii et al., 2009). The *RD29B* promoter contains the ABRE and is known to respond specifically to ABA in *Arabidopsis* mesophyll protoplasts (Nakashima et al., 2006; Msanne et al., 2011). However, when we used pABRC3-fLUC reporter system, which contains a synthetic promoter (ABRC3) consisting of an ABRE, Coupling element 3 (CE3) and 35S minimal promoter (**Supplementary Figure S1**), it was not very responsive to ABA in our TGERP. By contrast, pRab16A-fLUC reporter system showed rapid and strong responsiveness to ABA by itself as well as by OsbZIP like RD29B promoter in *Arabidopsis*. This result suggests that *cis*-elements other than ABRE might play roles in ABA responsiveness in the *Rab16A* promoter.

In *Arabidopsis* protoplasts, SnRK2.6 activates ABF2 (abscisic acid response elements binding factor 2) and induces the expression of luciferase fused with *RD29B* promoter more than fivefold under ABA treatment (Fujii et al., 2009). However, OsbZIP46 was not significantly activated by SAPKs under ABA treatment conditions in our TGERP. This finding suggests that there might be sufficient endogenous SAPKs to activate over-expressed OsbZIPs fully in wild-type rice protoplasts treated with ABA. Kobayashi et al. (2004) reported that SAPK2 is rapidly activated by osmotic stress whereas SAPK9 is tightly regulated by ABA. In our TGERP, SAPK2 could activate OsbZIP46 in the absence of ABA. In contrast to SAPK2, SAPK9 could not activate OsbZIP46 in the absence of ABA. These findings suggest that SAPK2 might be activated by

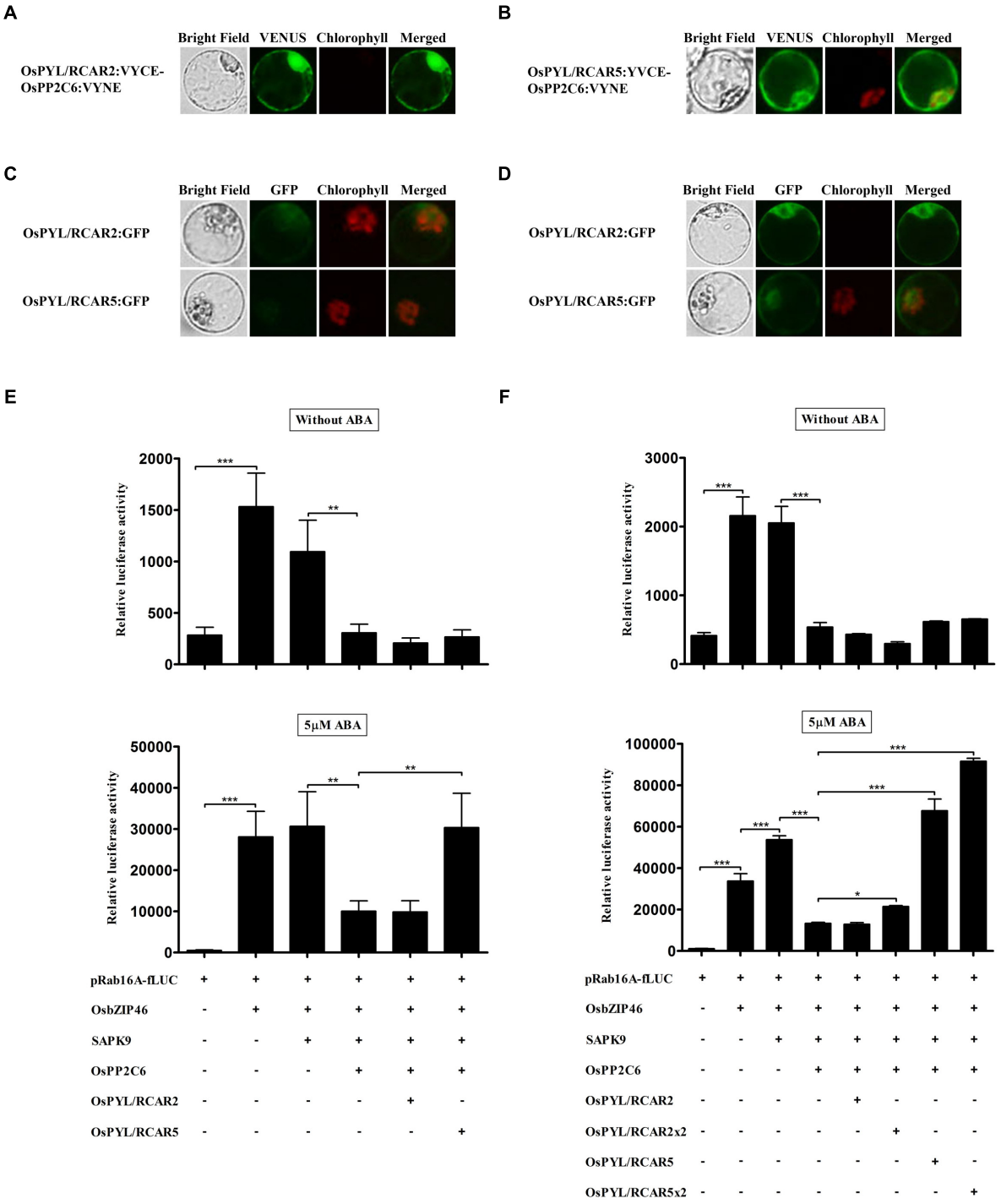


FIGURE 5 | OsPYL/RCARs differentially increase ABA-dependent signaling outputs in rice protoplasts. (A,B) Interactions of OsPP2C6 with OsPYL/RCAR2 and OsPYL/RCAR5 in rice protoplasts. The interaction of OsPP2C6 with OsPYL/RCAR2 and OsPYL/RCAR5 was detected by BiFC analysis. OsPP2C6 interacts with OsPYL/RCAR2 and OsPYL/RCAR5 in both the nucleus and cytosol. **(C,D)** Expression analysis of OsPYL/RCAR2:GFP and OsPYL/RCAR5:GFP in rice protoplasts. After transfection, protoplasts were incubated for **(C)** 2 h and **(D)** 4 h. GFP signal of OsPYL/RCAR2:GFP and OsPYL/RCAR5:GFP was detected very weakly after 2 h incubation and gradually increased. OsPYL/RCAR:GFP were used at 10 μg per transfection. Exposure time of GFP fluorescence was 600 ms. Chlorophyll autofluorescence is in red to distinguish it from GFP (green) fluorescence. **(E,F)** Dual luciferase assay after 2 h **(E)** and 4 h **(F)** incubations. Flag-tagged OsPYL/RCAR2 and -5 were transfected with HA-tagged OsbZIP46, Flag-tagged SAPK9, HA-tagged OsPP2C6, pRab16A-fLUC reporter plasmid and pAtUBQ-rLUC plasmid as an internal control. After transfection, protoplasts were incubated for 2 and 4 h in the presence of 0 and 5 μM ABA under light. The mean value of relative luciferase activity for three independent experiments is shown, and error bars indicate SD; ANOVA with Tukey's test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

osmotic stress in rice protoplasts and that SAPK9 might be more tightly regulated by ABA compared to SAPK2. Thus, our TGERP showed signaling effects of SAPKs similar to those in plants in the absence of ABA.

He et al. (2014) classified rice ABA receptors into monomer and dimer forms. It was previously reported that dimer-form receptors could not suppress the activity of OsPP2Cs ABA-independently *in vitro* but monomer-form receptors could suppress the activity of OsPP2Cs ABA-independently according to the concentrations of OsPYLs and OsPP2Cs *in vitro*. In our experiments BiFC results showed ABA independent interaction between OsPYL/RCAR2 and 2 with OsPP2C2 respectively. And we also confirmed that OsPP2C6 interacted with OsPYL/RCAR2 in the absence of ABA (data not shown). However, we found that dimer-form OsPYL/RCAR2 and monomer-form OsPYL/RCAR5 both failed to suppress OsPP2C6 in the absence of ABA. Even in the presence of ABA, the dimer-form OsPYL/RCAR2 was required in higher concentrations than OsPYL/RCAR5 to suppress OsPP2C6 activity and moreover the suppressor activity of OsPYL/RCAR2 was quite low as compared to OsPYL/RCAR5 that completely suppressed OsPP2C6 activity in the presence of 5 μM ABA. Thus, we can monitor the different ABA sensitivities among receptors *in vivo* experiments in terms of suppression of OsPP2C by OsPYL/RCARs. Such kinds of differences imply that OsPYL/RCARs might participate differentially in ABA signaling pathway depending on the cellular ABA concentrations. In summary, we successfully developed a monitoring system for ABA signaling in rice protoplasts and reconstituted the signaling components to demonstrate similar signaling characteristics as previously reported for whole plants. Thus, we showed that transient gene expression systems in rice protoplasts are suitable not only for functional analysis of single genes but also for characterization of signaling pathways or gene networks. This system therefore represents very useful technology to study functional genomics in rice or other monocots.

Author Contributions

NK, S-JM, and B-GK designed the research. NK and E-HC cloned constructs. NK carried out dual luciferase assay. S-JM and EK performed the BiFC and GFP analysis. MM and J-AK setup the rice protoplast PEG transformation methods. IY, M-OB, S-DY revised the manuscript. NK, S-JM, and B-GK analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00614

FIGURE S1 | Schematic diagram of constructs used in this study. (A,B) ABA-responsive reporter vectors used in TGERP. Nucleotide sequences of the *HVA1* promoter fragment of barley **(A)** and full length *Rab16A* promoter of rice **(B)** containing ACGT (red) and non-ACGT (blue) core sequences. ABA-responsive elements are underlined. **(C)** Internal vector used in the transient assays.

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Genome-wide survey and comprehensive expression profiling of Aux/IAA gene family in chickpea and soybean

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Auxin plays a central role in many aspects of plant growth and development. *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) genes cooperate with several other components in the perception and signaling of plant hormone auxin. An investigation of chickpea and soybean genomes revealed 22 and 63 putative *Aux/IAA* genes, respectively. These genes were classified into six subfamilies on the basis of phylogenetic analysis. Among 63 soybean *Aux/IAA* genes, 57 (90.5%) were found to be duplicated via whole genome duplication (WGD)/segmental events. Transposed duplication played a significant role in tandem arrangements between the members of different subfamilies. Analysis of Ka/Ks ratio of duplicated *Aux/IAA* genes revealed purifying selection pressure with restricted functional divergence. Promoter sequence analysis revealed several *cis*-regulatory elements related to auxin, abscisic acid, desiccation, salt, seed, and endosperm, indicating their role in development and stress responses. Expression analysis of chickpea and soybean *Aux/IAA* genes in various tissues and stages of development demonstrated tissue/stage specific differential expression. In soybean, at least 16 paralog pairs, duplicated via WGD/segmental events, showed almost indistinguishable expression pattern, but eight pairs exhibited significantly diverse expression patterns. Under abiotic stress conditions, such as desiccation, salinity and/or cold, many *Aux/IAA* genes of chickpea and soybean revealed differential expression. qRT-PCR analysis confirmed the differential expression patterns of selected *Aux/IAA* genes in chickpea. The analyses presented here provide insights on putative roles of chickpea and soybean *Aux/IAA* genes and will facilitate elucidation of their precise functions during development and abiotic stress responses.

Keywords: gene family, *Aux/IAA*, chickpea, soybean, gene duplication, transposed duplication, gene expression, abiotic stress

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INTRODUCTION

Auxin regulates cell division and elongation to drive plant growth and development (Woodward and Bartel, 2005). Perception of auxin and control of auxin-regulated gene expression is mediated by proteins belonging to three families including, receptors (F-box proteins), repressors [Auxin/Indole-3-Acetic Acids (Aux/IAAs)] and transcription activators auxin response factors (ARFs). The transmission of auxin signal depends upon interactions between components of these

protein families. Under low concentration of auxin, formation of an ARF-Aux/IAA hetero-dimer results in the repression of target ARF transcription factors (Tiwari et al., 2001; Guilfoyle and Hagen, 2007). When auxin concentration is high, a co-receptor complex consisting of an F-box protein from the transport inhibitor response1 (TIR1)/auxin signaling F-box protein (AFB) family and an Aux/IAA protein, binds auxin (Dharmasiri et al., 2005; Tan et al., 2007). The F-box protein, being a component of a Skp1–Cullin–F-box (SCF) E3 ubiquitin ligase (Gray et al., 2001; Kepinski and Leyser, 2005), polyubiquitinylates and targets the Aux/IAA proteins for degradation (Maraschin et al., 2009). This degradation event relieves ARF transcription factor repression, thus allowing auxin-regulated gene transcription (Reed, 2001; Tiwari et al., 2001).

Aux/IAA proteins contain four conserved sequence motifs, among which motif I, an amino-terminal leucine repeat motif (LxLxLx) functions as transcriptional repressor of downstream auxin-regulated genes (Tiwari et al., 2001, 2004). Motif II, a TIR1/AFB recognition sequence with the conserved degron-sequence, GWPPV, is responsible for the stability of Aux/IAA proteins (Tiwari et al., 2004). Its interaction with the F-box protein, TIR1, leads to rapid degradation of Aux/IAA proteins (Dharmasiri et al., 2005). Motif III contains a βαα region and motif IV represents an acidic region (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Motifs III and IV of Aux/IAA proteins enable homo- and/or hetero-dimerization with other Aux/IAA or ARF proteins and control the expression of downstream auxin-responsive genes (Kim et al., 1997; Ulmasov et al., 1997; Remington et al., 2004; Overvoorde et al., 2005). Although the presence of four conserved motifs is characteristic of Aux/IAA family, some members do not have one or more of these motifs and are called non-canonical members (Reed, 2001; Jain et al., 2006; Wang et al., 2010a; Audran-Delalande et al., 2012). Particularly, some members lack conserved motif II and are incapable of being recognized by TIR1/AFB proteins, indicating that these Aux/IAA proteins may be involved in other auxin-regulated biological processes (Jain et al., 2006; Kalluri et al., 2007; Sato and Yamamoto, 2008; Wang et al., 2010a,b; Audran-Delalande et al., 2012).

Many *Aux/IAA* genes have been characterized on the basis of mutant analysis in *Arabidopsis,* which demonstrated the important functions of Aux/IAA family genes in various developmental processes. For example, functional loss of IAA1/AXR5, a substrate of SCF^{TIR1}, showed auxin-related growth defects and auxin-insensitive phenotype (Yang et al., 2004). Loss-of-function mutant, *iaa3/shy2,* affects auxin homeostasis and formation of lateral roots (Uberti-Manassero et al., 2012). The mutants, *iaa7/axr2*, *iaa17/axr3*, *iaa19/msg2,* and *iaa28* showed reduction in lateral root numbers (Tatematsu et al., 2004; Okushima et al., 2007; Uehara et al., 2008; Rinaldi et al., 2012), whereas *iaa14/slr* mutant blocked lateral root formation entirely (Fukaki et al., 2002). A gain-of-function mutant, *iaa16,* showed hampered plant growth and decreased response to auxin (Rinaldi et al., 2012). In rice, over-expression of *OsIAA1* led to inhibition of root elongation and shoot growth (Song et al., 2009) and a gain-of-function in *OsIAA11* resulted in the loss of lateral roots (Zhu et al., 2012). *OsIAA23* was found to be involved in post embryonic maintenance of quiescent center in rice (Jun et al., 2011).

The members of Aux/IAA gene family have been identified in several plant species, including *Arabidopsis* (Liscum and Reed, 2002), rice (Jain et al., 2006), *Populus* (Kalluri et al., 2007), maize (Wang et al., 2010b), tomato (Wu et al., 2012), *Vitis vinifera* (Cakir et al., 2013), and *Medicago* (Shen et al., 2014). However, a genome-wide analysis of Aux/IAA gene family in chickpea and soybean (for which genome sequences are available) is lacking as of now. Chickpea and soybean are very important legume crops, which serve as major source of proteins and carbohydrate. Considering diverse role of Aux/IAA family members in other plants, it is important to explore this gene family in chickpea and soybean. In this study, we identified *Aux/IAA* genes in chickpea and soybean genomes. We analyzed their sequence characteristics, genomic organization, *cis*-regulatory elements, and performed evolutionary duplication analysis. Furthermore, we analyzed spatio-temporal differential expression between Aux/IAA paralogs in various tissues/stages of development and under abiotic stress conditions. These data would facilitate future studies on elucidating the exact biological functions of *Aux/IAA* genes in legumes.

MATERIALS AND METHODS

Identification of *Aux/IAA* Genes

Kabuli and desi chickpea genome annotations were downloaded from Legume Information System[1] (LIS; Varshney et al., 2013) and Chickpea Genome Analysis Project[2] (CGAP2; Parween et al., 2015), respectively. Soybean genome annotation was downloaded from Phytozome (v10, www.phytozome.net). Chickpea and soybean proteomes were searched to identify Aux/IAA proteins via HMMER and Basic Local Alignment Search Tool (BLASTP) algorithms using the published *Arabidopsis* Aux/IAA protein sequences as query. All obtained protein sequences were examined for the presence of Aux/IAA (PF02309) domain using the Hidden Markov Model of Pfam[3] and SMART[4] tools. Physiochemical parameters of each gene were calculated using ExPASy compute pI/Mw tool[5]. Information regarding cDNA sequences, genomic sequences and ORF lengths were obtained from the GFF file available at the respective genome project webpages.

Gene Structure, Phylogenetic Analysis, and Motif Prediction

Analysis of exon/intron organization of the *Aux/IAA* genes was performed with Gene Structure Display Server[6] (GSDS). Multiple sequence alignments of the full-length protein sequences from chickpea, soybean, and *Arabidopsis* were performed with MAFFT

[1]http://cicar.comparative-legumes.org/
[2]http://nipgr.res.in/CGAP2/home.php
[3]http://pfam.sanger.ac.uk/search
[4]http://smart.embl-heidelberg.de
[5]http://web.expasy.org/compute_pi/
[6]http://gsds.cbi.pku.edu.cn

using default parameters and phylogenetic tree was constructed by UPGMA method using CLC Genomics Workbench (v4.7.2). Bootstrap analysis was performed using 1,000 replicates and the tree was viewed using FigTree (v1.3.1). Motif organization of chickpea and soybean Aux/IAA proteins was investigated by MEME web server[7].

Chromosomal Location and Gene Duplication

Information about the chromosome location was obtained from the GFF file and details of the segmentally duplicated regions in the soybean genome were retrieved using the SyMAP database (Soderlund et al., 2006). Synteny analysis for *GmIAA* genes was performed using Plant Genome Duplication Database[8] (PGDD). The genes and segmental duplicated regions were mapped to the soybean chromosomes using the Circos tool (Krzywinski et al., 2009). On the basis of *K*s value obtained for each gene pairs from PGDD, divergence time was calculated to investigate evolution of soybean *Aux/IAA* genes. The divergence time (T) was calculated as $T = \text{Ks}/(2 \times 6.1 \times 10^{-9}) \times 10^{-6}$ Mya, based on a rate of 6.1×10^{-9} substitutions per site per year. For Ks value less than 0.3, divergence time was after the *Glycine* whole genome duplication (WGD) event, when Ks value was more than 1.3, divergence time was after the gamma WGT (whole genome triplication) event, and if Ks value was between 0.3 and 1.3, divergence time was after legume WGD event and before the *Glycine* WGD event. To determine the significance or contribution of the transposed duplication in *Aux/IAA* gene evolution, Soytedb[9] was investigated to find out nearest transposable elements around *Aux/IAA* genes.

Expression Profiling Using RNA-seq and Microarray Data

For expression profiling in chickpea, we used the RNA-seq data of 17 different tissues, namely germinating seedling (GS), root (R), shoot (S), stem (ST), mature leaves (ML), young leaves (YL), shoot apical meristem (SAM), flower bud stages (FB1-4), flower stages (FL1-5), and young pod (YP) from previous studies (Jain et al., 2013; Singh et al., 2013). High quality filtered reads were mapped to the genome sequence of kabuli chickpea (Varshney et al., 2013) using TopHat (v2.0.6). Cufflinks tool was used to estimate the transcript abundance of genes in the form of fragments per kilobase of transcript per million reads (FPKM) in different tissues as described previously (Garg et al., 2015).

The expression of 63 *GmIAA* genes was investigated based on the RNA-seq data from 19 tissues available at Gene Expression Omnibus (GEO) database, including three samples from soybean seed compartments, GloEP (globular stage embryo proper; GSM721717), EmSCP (early maturation seed coat parenchyma; GSM721719), and GloS (globular stage suspensor; GSM721718); 10 other tissues samples, Gs (globular stage seed; GSM721725), Hs (heart stage seed; GSM721726), Cs (cotyledon stage seed; GSM721727), Es (early maturation stage seed; GSM721728), Ds (dry seed; GSM721729), R (root; GSM721731), ST (stem; GSM721732), L (trifoliate leave; GSM721730), FB (floral bud; GSM721733), and WS (whole seedling 6 days after imbibition; GSM721734); three cotyledon development samples, CoM (mid-maturation cotyledon; GSM721277), CoL (late maturation cotyledon; GSM721278), and CoS (seedling cotyledon; GSM721280); and three early maturation seed parts, EcoEm (early maturation embryonic cotyledon; GSM1213-856), EmEA (early maturation embryonic axis; GSM1213857), and EmSC (early maturation seed coat; GSM1213855). For the expression analysis of *GmIAA* genes, the RPKM method was employed to correct for biases in total gene size and normalize for total reads obtained in each tissue library (Mortazavi et al., 2008; Nagalakshmi et al., 2008). Heatmaps of normalized expression values of *Aux/IAA* genes of chickpea and soybean were generated using R package pheatmap.

For abiotic stress response analysis of chickpea *Aux/IAA* genes, we used raw RNA-seq data from root and shoot under desiccation, salt and cold stresses from our previous study (Garg et al., 2015). Read mapping and differential gene expression analysis was performed as described (Garg et al., 2015) using the kabuli chickpea genome as reference. The microarray data of soybean under salt and drought stresses were downloaded from the GEO database from accession numbers GSE41125 and GSE40627, respectively. Probe sets corresponding to the *GmIAA* genes were identified from the file GeneModels_AffyProbe.txt[10].

Plant Materials, RNA Isolation and Quantitative PCR Analysis

Chickpea (*Cicer arietinum* L. genotype ICC4958) seeds were grown in field and culture room for collection of various tissue samples. From field grown plants, mature leaf (ML), young leaf (YL), flower buds (FB1-FB4; where FB1, FB2, FB3, and FB4 were 4, 6, 8, and 8–10 mm size flower buds, respectively), flowers (FL1–FL5; where FL1 was young flower with closed petals, FL2 was flower with partially opened petals, FL3 was mature flower with fully opened petals, FL4 was mature flower with opened and faded petals and FL5 was drooped flower with senescing petals), young pods (YP) were harvested as described (Singh et al., 2013). Root (R), shoot (S), and GSs were harvested as described (Garg et al., 2010; Singh et al., 2013). Abiotic stress treatments (desiccation, salinity, and cold) were given and root and shoot tissue were harvested as described (Garg et al., 2010, 2015). Total RNA was isolated, quality, and quantity were checked as described (Singh et al., 2015). Gene-specific primers for selected *CaIAA* genes were designed using the Primer Express (v3.0) software (Applied Biosystems, Foster City, CA, USA) (Supplementary Table S1). Specificity of each primer pair was determined via BLAST search. Quantitative PCR reactions for at least two biological replicates each with three technical replicates were performed employing 7500 fast real-time PCR system (Applied Biosystems) as previously described (Garg et al., 2010). *Elongation factor-1 alpha* (*EF-1α*) was used as a reference gene for normalization of gene

[7]http://meme-suite.org/

[8]http://chibba.agtec.uga.edu/duplication

[9]http://www.soybase.org/soytedb

[10]http://www.seedgenenetwork.net/media

expression levels (Garg et al., 2010). Statistical significance of the differential expression patterns was determined using the Student's *t*-test. Genes with ≥ 2-fold expression change (in at least one tissue/condition/time point) with $P \leq 0.05$ were regarded as differentially expressed.

Promoter Sequence Analysis

Genomic co-ordinates of coding sequences were determined using GFF files obtained from chickpea and soybean genome annotation projects. The regions of 1,000 bp upstream from start codon were extracted from the genome sequences. *Cis*-regulatory elements on both strands of promoter sequences were scanned using NewPLACE web server[11].

RESULTS AND DISCUSSION

Identification of *Aux/IAA* Genes in Chickpea and Soybean

In order to identify the members of *Aux/IAA* gene family in chickpea (kabuli) and soybean genome, BLASTP and HMM profile searches were performed against their respective proteomes. The *Aux/IAA* gene family members identified via these two searches were combined and a non-redundant list was obtained for chickpea and soybean. For further confirmation and identification of the conserved Aux/IAA domains, all candidate proteins were subjected to domain analysis using Pfam and SMART databases. A total of 22 and 63 Aux/*IAA* genes in kabuli chickpea and soybean genome, respectively, were confirmed. Further the analysis of recent version of desi chickpea genome (CGAP2) identified 21 Aux/IAA family members. BLASTP analysis showed the presence of all these 21 *Aux/IAA* genes in the kabuli chickpea genome. Due to identification of higher number of genes, all further analyses were performed on *Aux/IAA* genes from kabuli chickpea. Chickpea and soybean genes were numbered according to their location on the chromosomes (Supplementary Table S2). Various information of *CaIAA* and *GmIAA* genes, including gene name, gene identifier, chromosome location, mRNA length, features of deduced protein sequences, and their gene, CDS, protein and promoter sequences are given in Supplementary Table S2.

The number of *CaIAA* members (22) identified in chickpea are less as compared to *Arabidopsis* (29; Liscum and Reed, 2002) and rice (31; Jain et al., 2006), but higher than its very close relative *Medicago* (17; Shen et al., 2014). Lesser number of *Aux/IAA* genes in chickpea and *Medicago* may be due to some evolutionary constraints. However, the number of *Aux/IAA* members in soybean (63) is much higher as compared to other plants. Soybean possesses 9.2-fold larger genome size (∼1,150 Mbp) and 1.75-fold higher gene count (∼46,400) than *Arabidopsis* (Cannon and Shoemaker, 2012). Given the noticeable differences in genome size and estimated gene count between soybean and *Arabidopsis*, the *Aux/IAA* genes in soybean seem to be highly expanded. The presence of twice as many of these genes in soybean versus *Arabidopsis* may be mainly due to the recent polyploidy and segmental duplication events in soybean evolutionary history (Schmutz et al., 2010). The sizes of the CaIAA proteins varied markedly ranging from 112 (CaIAA16) to 362 (CaIAA2) amino acids. Similarly, sizes of GmIAA proteins also varied from 53 (GmIAA6) to 367 (GmIAA48) amino acids. Furthermore, predicted isoelectric points varied from 4.64 (CaIAA19) to 9.63 (CaIAA1) in chickpea and 5.24 (GmIAA42) to 9.27 (GmIAA25) in soybean, suggesting that different CaIAA and GmIAA proteins might function in different microenvironments.

Phylogenetic Relationship, Gene Structure and Sequence Similarity

To examine the phylogenetic relationship among the Aux/IAA proteins of chickpea, soybean, and *Arabidopsis*, a rooted tree was constructed using alignments of their full-length amino-acid sequences (**Figure 1**). Phylogenetic distribution indicated that Aux/IAA proteins can be classified into two major groups, A and B (**Figure 1**) similar to *Arabidopsis* and rice (Remington et al., 2004; Jain et al., 2006), which are further subdivided into four and two subgroups, respectively. Similar groupings have been reported in other plant species too (Cakir et al., 2013; Gan et al., 2013). The group A (A1–A4) consisted of 12 members of CaIAA and 41 GmIAA proteins, structuring 25 sister pairs (five pairs of GmIAA-CaIAA, 16 pairs of GmIAA-GmIAA and four pairs of AtIAA-AtIAA proteins). Group B (B1–B2) included 10 CaIAA and 22 GmIAA proteins, which formed 15 sister pairs (11 pairs of GmIAA–GmIAA, four pairs of AtIAA–AtIAA). Phylogenetic tree topology revealed that sister pairs located at the terminal nodes show high similarity and were assigned as paralog or ortholog pairs (**Figure 1**, Supplementary Figure S1). The sequence similarity within chickpea and soybean Aux/IAA proteins ranged from 9 to 80.3 and 6.5 to 93.9%, respectively (Supplementary Figure S1). All paralog pairs of soybean determined through phylogenetic analysis were found to be duplicated via WGD events (**Figure 1**, Supplementary Table S3), except GmIAA6 and 7 (tandemly duplicated). Furthermore, higher sequence similarity was observed between paralog pairs, suggesting that these genes evolved via genome duplication event and may perform similar functions. Interestingly, phylogenetic analysis predicted four homologs of AtIAA16 in the soybean genome (GmIAA14, 36, 59, and 63). In *Populus*, four orthologs of AtIAA16 have also been found, but were absent in rice, indicating their specific function in dicots. Moreover, diversity of gene structure (exon-intron organization) is also a possible explanation for the evolution of multigene families. The exon-intron organization in the coding sequences of each *Aux/IAA* genes of chickpea and soybean were compared. As expected, in most of the sister-pairs, similar exon-intron organization was observed. This conservation of exon-intron organization between subfamilies and the dissimilarity within subfamilies supported the results of phylogenetic and genome duplication analysis.

The established model for auxin signal transduction represents auxin-mediated degradation of these short-lived

[11] https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=ja&pj=640&action=page&page=newplace

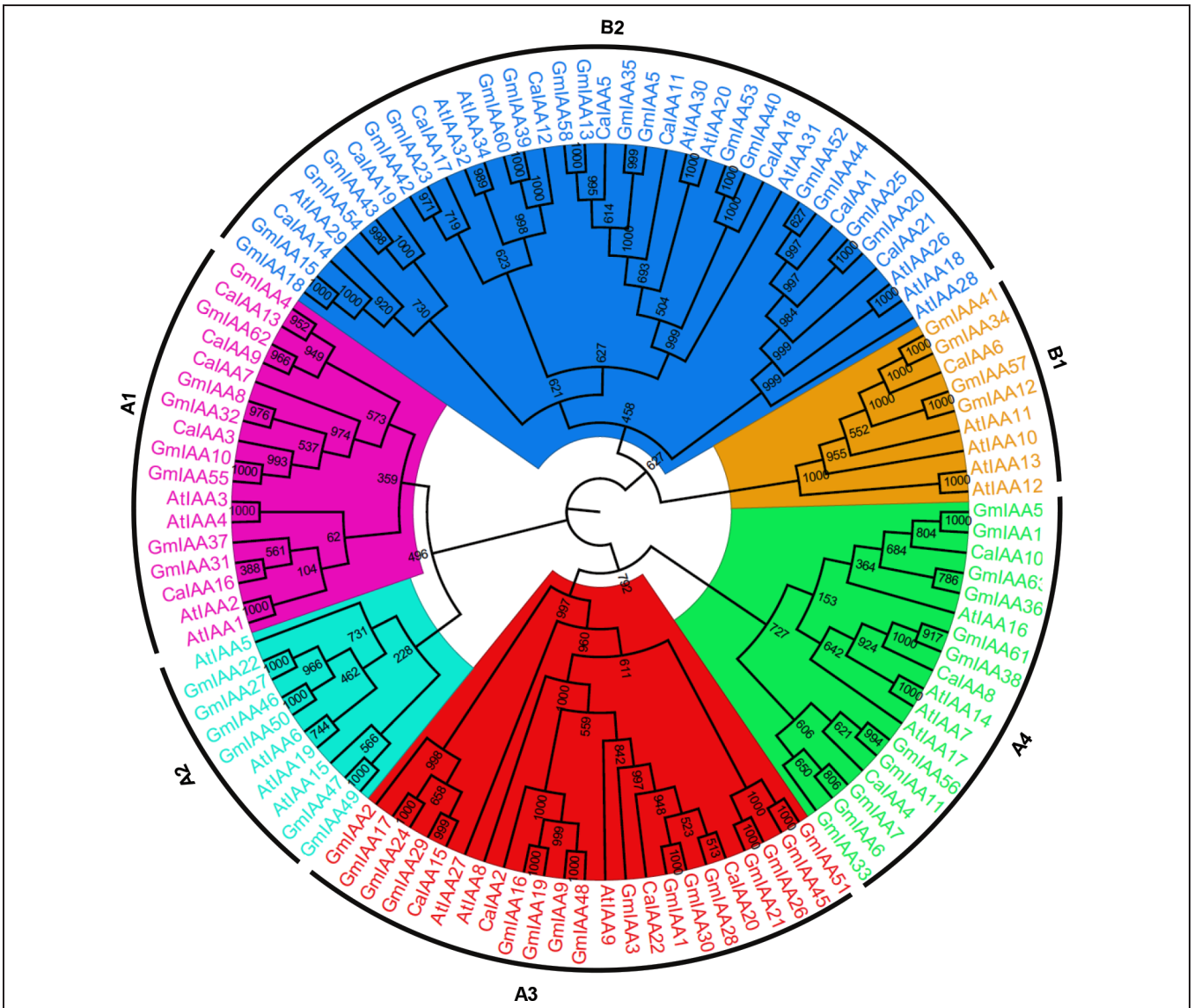


FIGURE 1 | Phylogenetic relationship among *Aux/IAA* genes from chickpea, soybean, and *Arabidopsis*. The deduced full-length amino acid sequences of chickpea (CaIAA), soybean (GmIAA), and *Arabidopsis* (AtIAA) genes were aligned by MAFFT and the phylogenetic tree was constructed by CLC Genomics Workbench using the UPGMA method. The members of each Aux/IAA subfamily are shown in different colors. The numbers on the nodes represent bootstrap values from 1000 replicates.

proteins that have four characteristic conserved domains. Conspicuously, the chickpea genome represents six such non-canonical Aux/IAA proteins (CaIAA5, 11, 12, 16, 17, and 19) that do not have conserved domain II, which is crucial for protein degradation, whereas 13 (GmIAA5, 6, 13, 23, 31, 35, 37, 39, 40, 42, 53, 58, and 60) such proteins were found in the soybean genome (**Figure 2**). These non-canonical proteins were found to be long-lived as compared to the canonical Aux/IAA proteins (Dreher et al., 2006). In tomato, such non-canonical Aux/IAA proteins were found to have expression pattern restricted to narrow development stages (Audran-Delalande et al., 2012), suggesting that these proteins may have a very specific function during development in plants for mediating auxin responses.

Chromosomal Location and Duplication

The chromosomal distribution of 22 *CaIAA* genes revealed their location on all the eight linkage groups (Supplementary Table S2). Eight *CaIAAs* were present on chromosome 4, five on chromosome 7, three on chromosome 3, two on chromosome 6, and one on chromosome 1, 2, 5, and 8 each. In soybean, 63 *GmIAA* genes were located on 16 of 20 chromosomes, except for chromosomes 11, 12, 16, and 18 (**Figure 3**, Supplementary Table S2). Out of 63 *GmIAA* genes, nine genes were present on chromosome 10, eight on chromosome 13, six on chromosome 2, five on chromosome 3, 8, and 19 each, four on chromosome 7, 15, and 20 each, three on chromosome 1, and two on chromosome 4, 6, 9, and 17 each. Chromosomes 5 and 14 harbored only



FIGURE 2 | Gene structure and motif organization of Aux/IAA family members in chickpea and soybean. *Left panel* illustrates the exon–intron organization of *Aux/IAA* genes in chickpea and soybean. The exons and introns are represented by boxes and lines, respectively. *Right panel* shows motif organization in chickpea and soybean Aux/IAA proteins. Motifs of Aux/IAA proteins were investigated by MEME web server. Six motifs representing four domains I, II, III, and IV of Aux/IAA proteins are displayed at the bottom.

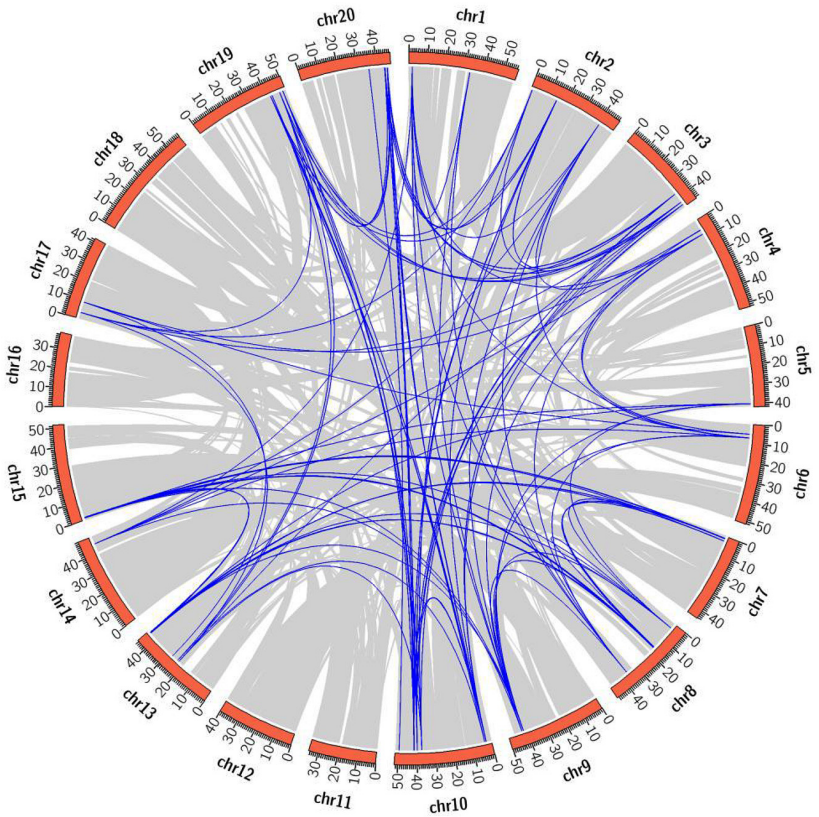


FIGURE 3 | Mapping of segmentally duplicated *GmIAA* genes on soybean chromosomes. Gray ribbons indicate collinear relationships among the blocks in whole genome and blue ribbons show *GmIAA* paralogs. The soybean chromosomes are arranged in a red circle and the size of each arc show the size of respective chromosome (Mb).

one *GmIAA* gene each. The chromosomal location of *Aux/IAA* genes of chickpea and soybean showed tandemly located gene clusters. The gene cluster in chickpea included *CaIAA3* and *4* on chromosome 3. For soybean, eight such clusters were observed, including *GmIAA6*, *7* and *8* on chromosome 2, *GmIAA10,* and *11* on chromosome 3, *GmIAA32* and *33*, *GmIAA37* and *38* on chromosome 10, *GmIAA46* and *47* on chromosome 13, *GmIAA49* and *50* on chromosome 15, *GmIAA55* and *56* on chromosome 19, and *GmIAA61* and *62* on chromosome 20.

Soybean genome has undergone one WGT and two WGD events (legume WGD and *Glycine* WGD), and about 75% genes have multiple paralogs (Schmutz et al., 2010; Severin et al., 2011). Among paralog genes, ∼50% displayed expression subfunctionalization (Roulin et al., 2012) that may cause phenotypic variation in polyploids (Buggs et al., 2010). Besides WGD, tandem duplication generates consecutive gene copies in the genome through unequal chromosomal crossing over (Freeling, 2009) and may contribute in the expansion of gene families (Cannon et al., 2004). Dispersed duplicates (not tandemly or segmentally duplicated) arises via either DNA or RNA based transposition mechanisms (Ganko et al., 2007; Cusack and Wolfe, 2007; Freeling, 2009) and may play an important role in altering gene function and creating new genes (Woodhouse et al., 2010; Wang et al., 2011). To find the potential relationship between putative paralog pairs of *Aux/IAA* genes of soybean and tandem/segmental duplications, we performed duplication analysis using PGDD. Within the identified duplicated *GmIAAs*, a larger fraction of them (57, 90.47%) were duplicated through WGD/segmental events, and only *GmIAA6* and *7* were tandemly duplicated (**Figure 3**, Supplementary Table S3). In the syntenic block, some genes from different subfamilies showed the tandem relationship. For example, paralog gene pairs, *GmIAA6/33*, *GmIAA10/55*, *GmIAA37/62,* and *GmIAA46/50* displayed tandem relationship with *GmIAA8/32*, *GmIAA11/56*, *GmIAA38/61,* and *GmIAA47/49*, respectively (**Figure 3**, Supplementary Tables S2 and S3). Presence of transposable elements in the flanking regions of these genes, suggested that they were tandemly arranged due to transposed duplication events (Supplementary Table S4). In addition to WGD events, some other gene duplication events were also found in few *Aux/IAA* genes of soybean. In A1 subfamily, six paralog genes (*GmIAA8/32*, *10/55,* and *37/62*) resulted from the common ancestor sites experiencing three WGD events, while a dispersed gene (*GmIAA4*) through the transposed duplication located among two transposable elements (Supplementary Tables S3 and S4). In A4 subfamily, ten paralog genes (*GmIAA6/33*, *11/56*, *14/59*, *36/63,* and *38/61*) were resultant from common ancestor sites, which

experienced three WGD events, while *GmIAA7* was found to have tandem connection with *GmIAA6* (Supplementary Tables S3 and S4). The *GmIAA* genes grouped into B1 and B2 subfamilies exhibited transposed duplication, which were flanked by two transposable elements each (Supplementary Tables S3 and S4).

Furthermore, to investigate whether Darwinian positive selection is involved in the divergence of *GmIAA* genes after duplication and to trace the dates of the duplication blocks, the substitution rate ratios (Ka/Ks) of all paralog pairs were extracted from PGDD database. *Ks* values were used for calculating approximate dates of duplication events. The segmental duplications of the *GmIAA* genes in soybean were assumed to originate from 2.35 Mya (million years ago, $Ks = 0.03$) to 327 Mya ($Ks = 4.26$), with a mean value of 17.6 Mya ($Ks = 0.23$, Supplementary Table S5). Previous studies have shown that the soybean genome has undergone two rounds of WGD, including an ancient duplication prior to the divergence of papilionoid (58–60 Mya) and a *Glycine*-specific duplication that has been estimated to have occurred ∼13 Mya (Schmutz et al., 2010). Most of the WGD/segmental duplications of the *GmIAA* genes seem to have occurred around 13 Mya when *Glycine*-specific duplication occurred (Supplementary Table S5). According to the ratio of non-synonymous to synonymous substitutions (Ka/Ks), the history of selection acting on coding sequences can be measured (Li et al., 1981). A pair of sequences will have Ka/Ks <1, if one sequence has been under purifying selection, but the other has been drifting neutrally, while Ka/Ks = 1, if both the sequences are drifting neutrally and rarely, while Ka/Ks >1 at specific sites, when they were under positive selection (Juretic et al., 2005). Ka/Ks for all *GmIAA* duplicated pairs were less than 1 (Supplementary Table S3), which suggests that all gene pairs have evolved mainly under the influence of purifying selection pressure with limited functional divergence after segmental duplications.

Cis-regulatory Elements in Promoters of *CaIAAs* and *GmIAAs*

The analysis of *cis*-regulatory elements in the promoter sequences is an important aspect in understanding the gene function and regulation. We searched 1 kb promoter region of all the *CaIAA* and *GmIAA* genes to determine putative *cis*-regulatory elements involved in their transcriptional regulation using NewPLACE database. Many *cis*-regulatory elements identified in the promoters were found to be related to auxin, ABA, SA, sugar, light, drought, salt, and cold responses indicating that these genes are linked to phytohormone signals, and/or abiotic stresses (Supplementary Table S6). Previous studies suggest that light is involved in regulation of Aux/IAA protein activity. For example, phytochrome A (phyA) interacts with Aux/IAA proteins, as revealed by yeast two-hybrid analysis (Soh et al., 1999). Moreover, oat phyA was able to phosphorylate IAA1, SHY2/IAA3, IAA9, AXR3/IAA17, and PS-IAA in vitro (Abel et al., 1994; Soh et al., 1999). The domain II mutants, *axr2-1*, *axr3-1*, *shy2-1*, and *shy2-2*, develop leaves in dark (Kim et al., 1998; Reed et al., 1998; Nagpal et al., 2000). Presence of auxin, ABA and cytokinin responsive *cis*-regulatory elements in the promoters of *CaIAA* and *GmIAA* is also consistent with previous reports, such as the interactions of auxin with other phytohormones (cytokinin or ABA) to regulate many aspects of plant growth and development (Paponov et al., 2008). Many *CaIAA* and *GmIAA* genes were found to harbor AuxRE motif in their promoter, which is important for binding of ARFs and transcriptional activation of *Aux/IAA* genes (Tiwari et al., 2001, 2003). Interestingly, promoter sequences of those *CaIAA* and *GmIAA* genes, which lack AuxRE motif, were found to harbor sugar-responsive motif (SREATMSD), suggesting that there is an association between sugar and auxin responses. Both sugar and auxin are essential for plants and control similar processes. In *Arabidopsis*, these two signaling pathways were found to interact with each other (Mishra et al., 2009). Bioactive GAs (gibberellic acid) influence nearly all aspects of plant growth and development from germination to hypocotyl elongation, stem growth, circadian rhythm, and reproductive organ and seed development (Lovegrove and Hooley, 2000; Hartweck and Olszewski, 2006). The presence of gibberellic acid response element (GARE) in many of the *Aux/IAA* genes (Supplementary Table S6), indicate their role in such processes. In addition, *cis*-regulatory elements known for regulation of endosperm, embryo, cotyledon, seed storage proteins related responses were also predicted in the *CaIAA* and *GmIAA* gene promoters (Supplementary Table S6), suggesting their role in seed development. Circadian element, which is involved in circadian control, was abundantly found in the promoter region of chickpea and soybean *Aux/IAA* genes (Supplementary Table S6), potentially indicating that they may have a distinct diurnal expression pattern. In *Arabidopsis* and rice, a class of element defined by the core motif '(a/g)CCGAC' named as dehydration responsive element/C-repeat (DRE/CRT) was reported for drought, low temperature, and salt inducible expression (Yamaguchi-Shinozaki and Shinozaki, 1994; Meier et al., 2008). Interestingly, we also found this motif in many of the *CaIAA* and *GmIAA* gene promoters (Supplementary Table S6), indicating their role under abiotic stress conditions. Overall, the promoter analysis demonstrated the presence of a variety of *cis*-regulatory elements in the upstream regions of chickpea and soybean *Aux/IAA* genes. These results provide further support for the various functional roles of *Aux/IAA* genes in a wide range of developmental processes and abiotic stress responses.

Differential Expression of Chickpea and Soybean Aux/*IAA* Genes during Development

To know the putative function of *Aux/IAA* genes in chickpea and soybean during development, we analyzed their expression profiles in different vegetative and reproductive tissues, using available RNA-seq data sets (Jain et al., 2013; Singh et al., 2013). Chickpea RNA-seq data included eight tissues/organs, such as GS, root (R), shoot (S), stem (ST), mature leaf (ML), young leaf (YL), SAM, young pod (YP) and nine stages of flower development (FB1-4 and FL1-5). Many of *CaIAAs* illustrated a distinct tissue-specific expression pattern

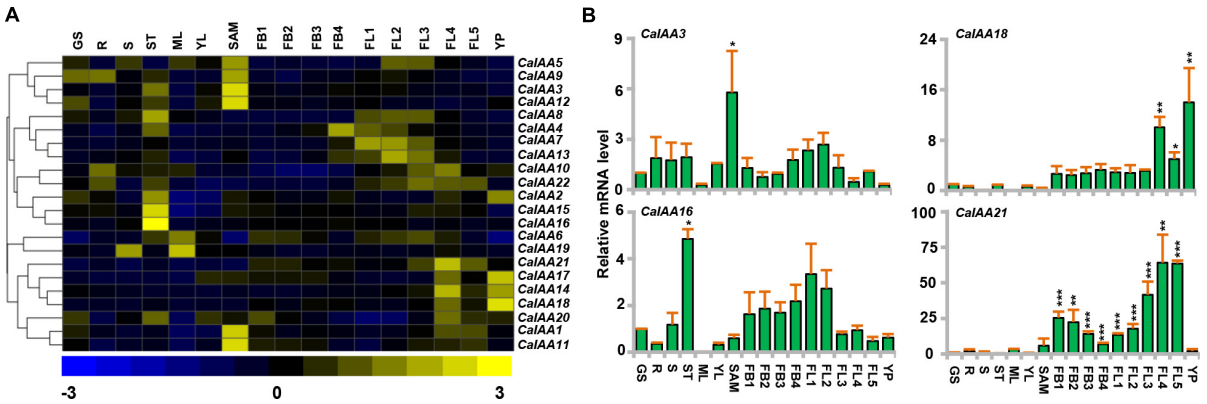


FIGURE 4 | Expression profiles of chickpea *Aux/IAA* genes. (A) The normalized RNA-seq expression data was used to construct heatmap. Hierarchical clustering was conducted in R using the pheatmap package with a dissimilarity metrics based on Euclidean distance with complete linkage algorithm. Color key at the bottom represents row wise *Z*-score. **(B)** qRT–PCR analysis of *CaIAA* genes in various tissue/stages of development. Expression of germinating seedling (GS) was taken as a reference to determine relative mRNA level in other tissues for each gene. Error bars indicate SE of mean. R (root), S (shoot), ST (stem) ML (mature leaf), SAM (shoot apical meristem), FB1-4 (stages of flower bud), FL1-5 (stages of flower), YP (young pod). Data points marked with asterisk (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$) indicates statistically significant difference between control (GS) and other tissues.

(**Figures 4A,B**). For example, *CaIAA15* and *16* revealed specific expression in stem, indicating their role in stem development (**Figures 4A,B**; Supplementary Table S7). It has been found that mutation in *Aux/IAA* genes affect stem elongation (Reed, 2001). Furthermore, *CaIAA1, 3, 11,* and *12* showed higher expression in SAM, among which *CaIAA3* was validated through qRT-PCR (**Figures 4A,B**; Supplementary Table S7), implying their involvement in SAM maintenance. In rice, *OsIAA23* was found to be involved in postembryonic maintenance of quiescent center (Jun et al., 2011). *CaIAA4, 7, 10, 13,* and *21* revealed higher transcript accumulation during stages of flower development (**Figures 4A,B**; Supplementary Table S7), suggesting their possible role in flower development. In rice, *OsIAA4* and *26* were found to be up-regulated in panicle (Jain and Khurana, 2009), while *MtIAA9* in *Medicago,* showed higher expression level in flower (Shen et al., 2014). *CaIAA14, 17* and *18* revealed distinctly higher expression in stages of flower development and young pod (YP). In tomato, *SlIAA9* was shown to be involved in fruit development (Wang et al., 2005). The expression profile of at least four *CaIAA* (*CaIAA3, 16, 18,* and *21*) genes was studied by qRT-PCR to validate the RNA-seq results. The expression patterns obtained via qRT-PCR were found to be well correlated with that of RNA-seq (**Figures 4A,B**).

For soybean, normalized RNA-seq data from 19 tissues were used, which included various tissues/organs, seed compartments, and stages of seed development. *GmIAA* genes showed specific and overlapping expression patterns in various tissues/organs and stages of development analyzed (**Figure 5**, Supplementary Table S7), indicating they might execute specific functions or work redundantly. *GmIAA60* was specifically expressed in WS (whole seedling), while its paralog *GmIAA39* expressed specifically in Ds (dry seed), indicating their functional divergence. *GmIAA15* has higher expression in root (**Figure 5**, Supplementary Table S7) and presence of root-responsive *cis*-regulatory element in its promoter (Supplementary Table S6), suggested its role in root development. In *Arabidopsis*, many *IAA* genes (*AtIAA1*, *AtIAA18,* and *AtIAA28*) were also found to have role in root development (Rogg et al., 2001; Yang et al., 2004; Uehara et al., 2008). In addition, paralogs *GmIAA45* and *51* showed higher transcript accumulation in shoot (**Figure 5**, Supplementary Table S7), signifying their functional conservation with role in shoot development, which is further revealed by presence of shoot responsive *cis*-regulatory element in their promoter sequences (Supplementary Table S6). However, *GmIAA6*, *31,* and *33* exhibited higher transcript levels in floral bud (FB) (**Figure 5**, Supplementary Table S7) and detection of pollen specific *cis*-regulatory elements in their promoter sequences suggests their putative role in development of FBs. Many *GmIAA* genes were detected with increased transcript accumulation at various stages/organs of seed development too. For instance, *GmIAA49* exhibiting specific expression in Gs (globular stage seed), *GmIAA13, 35, 43, 54,* and *56* in GloEP (globular stage embryo proper), *GmIAA5* in GloS (globular stage suspensor), *GmIAA22* and *GmIAA23* in Cs (cotyledon stage seed), *GmIAA59* in EmSCP (early maturation seed coat parenchyma), *GmIAA62* in EmEA (early maturation embryonic axis), and *GmIAA42* in CoL (late maturation cotyledon) (**Figure 5**, Supplementary Table S7). In addition, many seed related *cis*-regulatory elements were detected in their promoter sequences, such as S000143, S000353, S000449, S000148, S000421, S000292, S000144, S000419, S000420, S000100, S000102, and S000377 (Supplementary Table S6), that further added support for their putative role in seed development. In *Arabidopsis*, two Aux/IAA proteins have also been reported for their involvement in seed development (Hamann et al., 1999; Ploense et al., 2009). In the gain-of-function mutant, *iaa18*, PIN1 was asymmetrically expressed with stronger expression at only one side of the embryo and caused aberrant cotyledon outgrowth in the

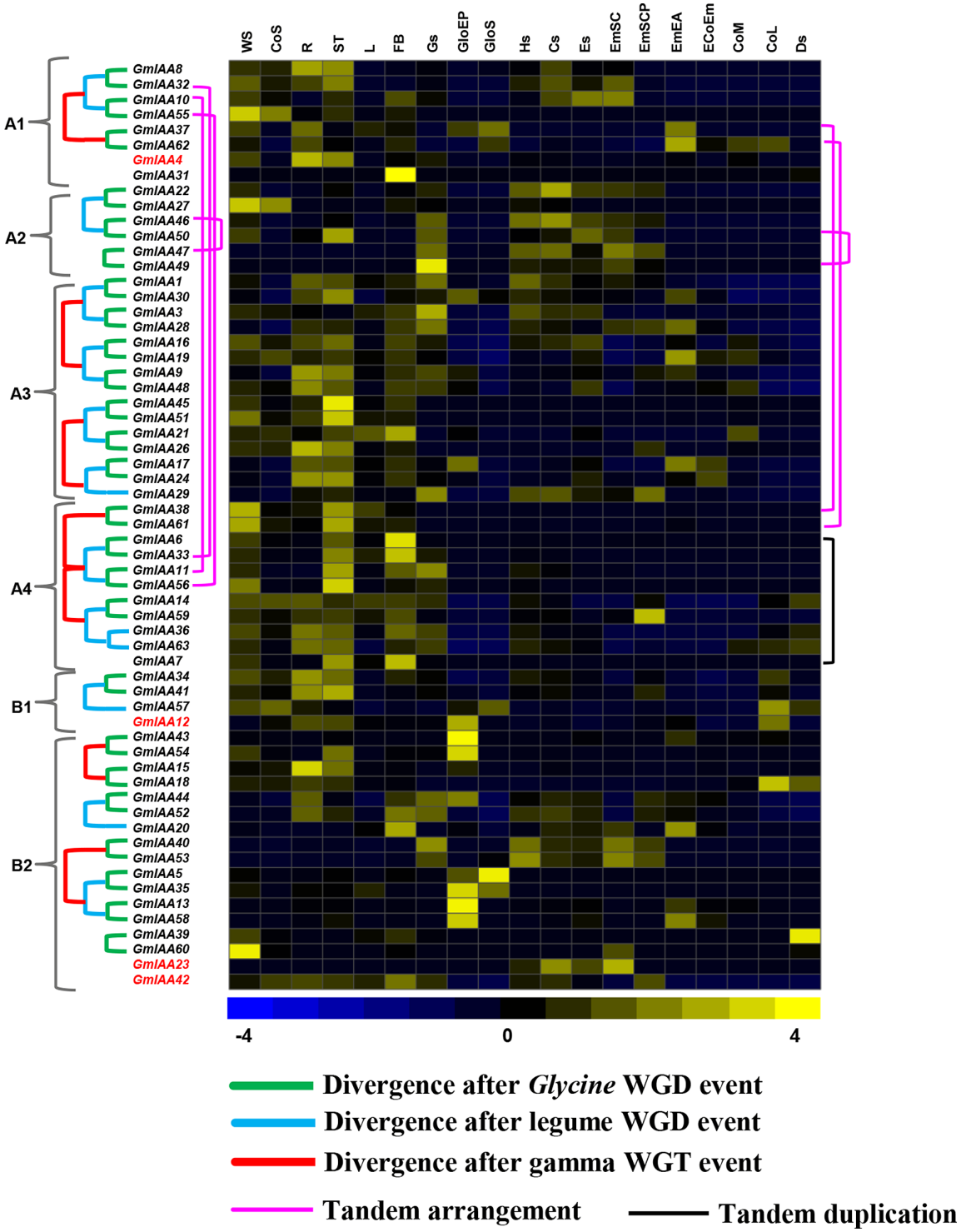


FIGURE 5 | Expression profiles and the evolutionary patterns of soybean *Aux/IAA* genes. The normalized RNA-seq expression data of 19 tissues was used to construct heatmap. Three samples from soybean seed compartments: GloEP (globular stage embryo proper), EmSCP (early maturation seed coat parenchyma), and GloS (globular stage suspensor); 10 other tissues samples: Gs (globular stage seed), Hs (heart stage seed), Cs (cotyledon stage seed), Es (early maturation stage seed), Ds (dry Seed), R (root), ST (stem), L (trifoliate leave), FB (floral bud), and WS (whole seedling 6 days after imbibition); three cotyledon development samples: CoM (mid-maturation cotyledon), CoL (late maturation cotyledon), and CoS (seedling cotyledon); three early maturation seed parts: EcoEm (early maturation embryonic cotyledon), EmEA (early maturation embryonic axis), and EmSC (early maturation seed coat). The lines show the syntenic blocks containing the corresponding *GmIAA* genes, which experienced the WGD events. Gene names in red show dispersed duplicates. Color key at the bottom represents row wise *Z*-score.

embryos (Ploense et al., 2009). Another, gain-of-function mutant, *iaa12/bdl,* also showed cotyledonary defects (Hamann et al., 1999). Most of *GmIAA* genes showed relative low expression levels in soybean CoS (seedling cotyledon), L (trifoliate leave), Hs (heart stage seed), Es (early maturation stage seed), EcoEM (early maturation embryonic cotyledon), and CoM (mid-maturation cotyledon) tissues (**Figure 5**, Supplementary Table S7).

On the whole, the tissue-preferential expression exhibited by several *Aux/IAA* genes in chickpea and soybean is indicative of their involvement in biology of specific plant tissues and developmental processes. It would be interesting to further validate their functions in transgenics.

Overlapping and Differential Expression Patterns of Duplicated *GmIAA* Genes

Duplicated genes possibly lead to subfunctionalization (separation of original function), neofunctionalization (gain of novel function), or nonfunctionalization (loss of original function) based on their evolutionary fates (Prince and Pickett, 2002). Therefore, we also examined the functional redundancy of duplicated *GmIAA* genes. In soybean, 50% of the paralogs from the recent WGD event were found to be differentially expressed and thus might have undergone functional divergence. Among *GmIAAs*, 16 paralog pairs (*GmIAA1/30, GmIAA3/28, GmIAA6/33, GmIAA8/32, GmIAA9/48, GmIAA411/56, GmIAA13/58, GmIAA17/24, GmIAA34/41, GmIAA36/63, GmIAA37/62, GmIAA38/61, GmIAA40/53, GmIAA43/54, GmIAA44/52,* and *GmIAA45/51*) representing segmental duplications shared almost indistinguishable expression patterns (**Figure 5**, Supplementary Table S7). On the contrary, the expression patterns of another eight paralogs (*GmIAA10/55, GmIAA14/59, GmIAA15/18, GmIAA16/19, GmIAA21/26, GmIAA22/27, GmIAA39/60,* and *GmIAA46/50)* diversified significantly (**Figure 5**). Interestingly, paralogs from much earlier duplication events (legume WGD and gamma WGT) have more diverged expression patterns. For example, three paralog gene pairs of B2 subfamily, *GmIAA5/35, GmIAA13/58,* and *GmIAA40/53* diverged into two clades after gamma WGT event. After experiencing WGT, *GmIAA40/53* formed one paralog pair, and other was composed of *GmIAA5/35* and *GmIAA13/58* (**Figure 5**). The former paralog gene pair was expressed during the stages of seed development, but the two latter paralog gene pairs detached after the legume WGD event, were highly expressed in the GloEP (**Figure 5**, Supplementary Table S7). Other paralog genes from different divergence events also showed similar expression divergence (**Figure 5**). Besides gamma WGT, legume and *Glycine* WGD also contributed to the expression divergence of paralog *GmIAA* genes. For instance, paralog genes, *GmIAA6/33* exhibited higher expression in stem and FB, whereas *GmIAA11/56* revealed higher expression only in stem (**Figure 5**, Supplementary Table S7). Similarly, paralog genes *GmIAA22/27*, separated from *Glycine* WGD showed distinctively higher expression in Cs

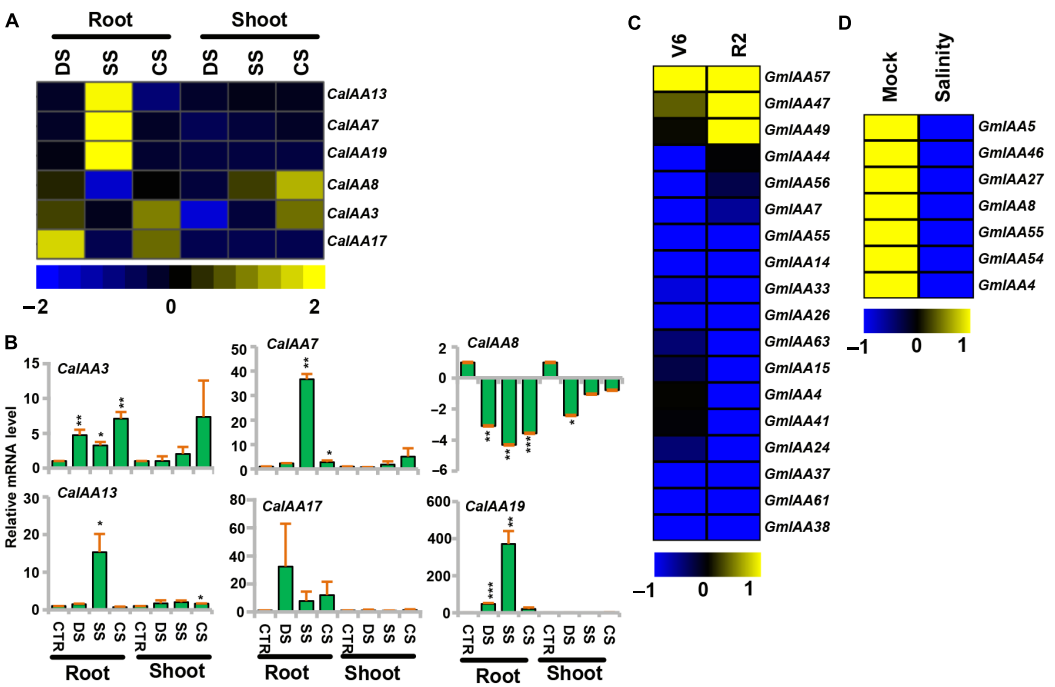


FIGURE 6 | Expression profiles of *CaIAA* and *GmIAA* genes under abiotic stress conditions. (A) Heatmap shows differential expression of *CaIAA* genes based on RNA-seq data. **(B)** qRT–PCR analysis of *CaIAA* genes under various stress treatments. Root and shoot control (CTR) was taken as a reference to determine relative mRNA level under stress conditions. Error bars indicate standard error of mean. Data points marked with asterisk (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$) indicate statistically significant difference between control and stress treatments. **(C,D)** Differential expression of *GmIAA* genes in response to drought and salinity stress conditions. Color scale shows $\log_2$ fold change relative to control sample. DS (desiccation), SS (salinity), CS (cold stress), V6 (vegetative stage leaves), R2 (reproductive stage leaves).

(cotyledon stage seed) and CoS (seedling cotyledon), respectively (**Figure 5**, Supplementary Table S7). These results indicate that gamma, legume and *Glycine* WGT events contributed significantly in functional diversity of *GmIAA* gene paralogs.

Altogether, we can speculate that *GmIAAs* have been retained by significant subfunctionalization in soybean during the course of evolution. Meanwhile, it is interesting to note that most of the paralog genes with similar expression profiles belong to the same subfamily and grouped as sister pairs in the phylogenetic tree (**Figures 1** and **5**). For example, two paralogs, *GmIAA13/58* and *GmIAA5/35* in the same subfamily formed sister pairs and displayed similar expression patterns (**Figure 5**). The similar expression pattern of genes from same subfamily of phylogenetic tree indicates that most of these genes may have evolved coordinately in coding and regulatory (promoter) regions, leading to their functional redundancy. Such functional redundancy has been reported in *Aux/IAA* family in *Arabidopsis* too (Overvoorde et al., 2005).

Differential Expression Patterns of *Aux/IAA* Genes under Abiotic Stress

Plants are frequently exposed to environmental stresses, like desiccation, salinity, and cold during their life cycle, which affect their growth and development. Several reports highlighted that the auxin-responsive genes were also engaged in various stress responses (Ghanashyam and Jain, 2009; Jain and Khurana, 2009; Wang et al., 2010a; Kumar et al., 2012; Cakir et al., 2013). To gain more insights into the role of chickpea and soybean *Aux/IAA* genes in abiotic stress tolerance, we analyzed their expression profiles under desiccation, salinity, and cold stresses using RNA-seq data for chickpea (Garg et al., 2015) and microarray data for soybean. Many of the chickpea and soybean *Aux/IAA* genes showed induction under desiccation, salinity and/or cold stresses (**Figure 6**). For instance, transcript level of *CaIAA3* was induced significantly under desiccation in root, whereas it was induced in both root and shoot under cold (**Figures 6A,B**, Supplementary Table S8) and its promoter sequence harbors desiccation and cold responsive *cis*-regulatory element (S000407; Supplementary Table S6), indicating its role in desiccation and cold stress. *CaIAA7* showed induction under salinity in root and in shoot under cold stress, while *CaIAA13* was up-regulated in root under salinity (**Figures 6A,B**, Supplementary Table S8). Transcript level of *CaIAA17* was found to be markedly induced in root under desiccation and cold stresses (**Figures 6A,B**, Supplementary Table S8), indicating its role in desiccation and cold stress responses. However, *CaIAA19* illustrated enhanced expression in root under both desiccation and salt stresses (**Figures 6A,B**, Supplementary Table S8), signifying its role in root under abiotic stresses. In rice, *OsIAA9* and *OsIAA20* have been found to be induced under both desiccation and salinity stress conditions (Jain and Khurana, 2009). Further, putative salt stress-related *cis*-element (S000453) was found in promoters of *CaIAA3*, *7*, *13,* and *19* (Supplementary Table S6), which has been demonstrated to be responsible for salt stress response (Park et al., 2004). In response to desiccation and salt stresses, the transcript level of *CaIAA8* was suppressed in shoot and root (**Figures 6A,B**, Supplementary Table S8), respectively, indicating that the function of this gene is related to desiccation and salt stresses. Many *SbIAA* genes of *Sorghum bicolor* have been found down-regulated under drought conditions (Wang et al., 2010a). All the differentially expressed *CaIAAs* were analyzed through qRT-PCR also and expression patterns obtained from qRT-PCR and RNA-seq were correlated well (**Figures 6A,B**).

In soybean, *GmIAA57* revealed distinctly higher transcript accumulation in vegetative stage (V6) leaves under drought stress, while paralogous pair *GmIAA47* and *49* showed noticeably increased accumulation of transcripts in reproductive stage (R2) leaves under drought stress (**Figure 6C**, Supplementary Table S8). Their promoter sequences showed presence of desiccation responsive *cis*-regulatory elements (S000174, S000413; Supplementary Table S6), indicating their function in drought stress responses. In response to salt stress, the transcript levels of *GmIAA4*, *5*, *8*, *27*, *46*, *54,* and *55* were decreased in seedling (**Figure 6D**, Supplementary Table S8), demonstrating the function of these genes related to salt stress. Although the salt stress-related *cis*-element (S000453) was found in the promoters of *GmIAA4*, *5*, *8*, *27*, *46*, *54,* and *55* (Supplementary Table S4), which is reported to induce the transcript level under salt stress (Park et al., 2004), their expression levels were significantly down-regulated under salt stress (**Figure 6D**, Supplementary Table S8). This might indicate that some unidentified *cis*-regulated elements may play an important role in regulating the expression of these *GmIAAs* during stress responses in soybean. Moreover, consistent with our result, many *OsIAA* genes (*OsIAA7*, *8*, *12*, *14*, *17*, *21*, *25,* and *31*) have also been reported to be suppressed in rice under salt stress (Song et al., 2009). The present study clearly revealed that the many of the *Aux/IAA* genes from chickpea and soybean were expressed at significantly higher levels under drought, cold, and salt treatments. It will be interesting to further investigate them to understand their role in abiotic stresses response/signaling.

CONCLUSION

In this study, we have performed a comprehensive analysis of *Aux/IAA* genes in chickpea and soybean and provided insights on the evolution of this gene family. The comprehensive expression profiling indicated that members of *Aux/IAA* gene family are involved in many plant responses during development and abiotic stress conditions. Particularly, *CaIAA1*, *3*, *4*, *11*, *12*, *13*, *15*, *17*, *18,* and *21* in chickpea and *GmIAA6*, *13*, *22*, *23*, *31*, *33*, *35*, *39*, *42*, *43*, *45*, *51*, *54*, *56*, *60,* and *62* in soybean were found to have role in various aspect of development, including root, stem, flower bud, flower, and seed development. Further, *CaIAA3*, *7*, 8, *13,* and *17* in chickpea and *GmIAA4*, *5*, *8*, *27*, *46*, *47*, *54,* and *55* in soybean revealed their putative function in abiotic stress responses. The presence of important *cis*-regulatory elements related to various development processes and abiotic stress responses in the promoter of these genes also provided insights into their putative function. These genes are important candidates for further functional characterization. Our analysis suggested that the duplicated *Aux/IAA* genes may perform specific function due to their subfunctionalization. Overall, information reported here

for the *CaIAAs* and *GmIAAs* genes should facilitate further investigations related to their functions in plant development and stress responses.

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Salicylic acid modulates arsenic toxicity by reducing its root to shoot translocation in rice (*Oryza sativa* L.)

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Arsenic (As) is posing serious health concerns in South East Asia where rice, an efficient accumulator of As, is prominent crop. Salicylic acid (SA) is an important signaling molecule and plays a crucial role in resistance against biotic and abiotic stress in plants. In present study, ameliorative effect of SA against arsenate (As^V) toxicity has been investigated in rice (*Oryza sativa* L.). Arsenate stress hampered the plant growth in terms of root, shoots length, and biomass as well as it enhanced the level of H_2O_2 and MDA in dose dependent manner in shoot. Exogenous application of SA, reverted the growth, and oxidative stress caused by As^V and significantly decreased As translocation to the shoots. Level of As in shoot was positively correlated with the expression of *OsLsi2*, efflux transporter responsible for root to shoot translocation of As in the form of arsenite (As^{III}). SA also overcame As^V induced oxidative stress and modulated the activities of antioxidant enzymes in a differential manner in shoots. As treatment hampered the translocation of Fe in the shoot which was compensated by the SA treatment. The level of Fe in root and shoot was positively correlated with the transcript level of transporters responsible for the accumulation of Fe, *OsNRAMP5*, and *OsFRDL1*, in the root and shoot, respectively. Co-application of SA was more effective than pre-treatment for reducing As accumulation as well as imposed toxicity.

Keywords: arsenate, salicylic acid, rice seedlings, antioxidants, Fe transporters

Introduction

Arsenic (As) is posing a serious health concern in South East Asia especially in Bangladesh and West Bengal in India. Long term As exposure leads to skin lesions and various types of cancers (Kumar et al., 2015). Safe level of As in drinking water is10 $\mu g\ l^{-1}$, as recommended by World Health Organization in 1993, while the level of As in ground water has been reported up to 3200 $\mu g\ l^{-1}$ in West Bengal and Bangladesh that is enough to show the severity of problem (McCarty et al., 2011). Arable land can be contaminated through irrigation by As rich water. More than 90% production of rice comes from South East Asia that is heavily contaminated by As, thus significant amount of As also accumulates in various parts of rice which serves as a major entry route for As in to food chain. Presence of As in grains also hampers the nutritional value of rice in terms of trace nutrients and amino acids (Kumar et al., 2014a).

Arsenic is non-essential element for plant and present in environment both in inorganic as well as organic forms. Arsenate (As^V) and arsenite (As^{III}) are predominant inorganic forms. As toxicity symptoms in plants range from inhibition of root growth, photosynthesis to death of plant (Mishra et al., 2014; Kumar et al., 2015). Arsenate shows structural analogy with phosphate so it is mainly transported through high affinity phosphate transporters (Tripathi et al., 2007). In paddy field, As^{III} is the predominant chemical species of As due to anaerobic growing conditions (Takahashi et al., 2004). Further, most of the As taken up by the plants is also reduced and stored as As^{III} (Pickering et al., 2000; Mishra et al., 2013). Arsenite is transported through aquaporin channels. Two major As^{III} transporters, Lsi1 and Lsi2 have been reported in rice. Lsi1 is localized at the distal side of both exodermis and endodermis cells of rice roots and mediates the influx of As^{III}. Lsi2 is localized at the proximal side of both exodermis and endodermis cells and plays an important role in As^{III} transport to the shoots and ultimately to the rice grains (Ma et al., 2008). Arsenate can replace phosphate from many biochemical reactions leading to disruption of energy flow while As^{III} interferes with functioning of proteins and enzymes through thiol interaction (Finnegan and Chen, 2012). As is a redox active metalloid and induces the generation of reactive oxygen species (ROS) leading to lipid peroxidation, disruption of cellular redox state, and associated toxicity (Finnegan and Chen, 2012). In rice As mediated redox imbalance has been shown to the major factor causing toxicity (Srivastava et al., 2014). To cope up with ROS production plants are equipped with various antioxidant enzymes and molecules (GSH, Ascorbate). GSH also serves as substrate for phytochelatins (PCs), the metal and metalloids chelating ligands, therefore, reduces free As inside cell (Kumar et al., 2014b).

Salicylic acid and its derivative (acetylsalicylic acid) have been used for therapeutic purpose since more than a century. SA is synthesized by two pathways, the isochorismate pathway and the phenylalanine ammonia-lyase pathway (Vlot et al., 2009). SA is an important signaling molecule and its role in protection against various biotic and abiotic stresses has been well studied in plants (Yuan and Lin, 2008; Vlot et al., 2009). Upon pathogen attack endogenous level of SA gets enhanced and binds to catalase (CAT) that leads to enhanced level of H_2O_2. The H_2O_2 serves as secondary messenger to induce the expression of pathogen related proteins and ultimately initiates systemic acquired resistance (Vlot et al., 2009). SA has been reported to provide protection against heavy metal stress such as, against mercury in *Medicago sativa* (Zhou et al., 2009), cadmium stress in barley, rice and soybean (Metwally et al., 2003; Guo et al., 2007; Noriega et al., 2012), and against nickel stress in mustard (Yusuf et al., 2012). Guo et al., (2007) hypothesized that enhanced level of H_2O_2 by SA serves as secondary messenger to improve plant defense against abiotic stress. SA is reported to abate the chlorosis under iron deficient conditions and also promotes iron (Fe) uptake and translocation in *Arachis hypogaea* (Kong et al., 2014) and enhanced mineral nutrient uptake including Fe in maize (Gunes et al., 2007).

Iron acquisition mechanism in various plants is divided in two main categories: Strategy I in non-graminaceous plants and Strategy II in graminaceous plants (Römheld and Marschner, 1986). The two main processes in the Strategy I are the reduction of ferric chelates (Fe^{+3}-chelate) at the root surface and the absorption of the generated ferrous (Fe^{+2}) ions across the root plasma membrane (Kobayashi and Nishizawa, 2012). Rice belongs to family graminae which uses strategy II for Fe uptake where the plant roots secretes mugenic acid (MA) that forms Fe^{+3}-MA complex and is taken up by root cells by YSL transporters (Kobayashi and Nishizawa, 2012). There are several Fe transporters in which OsFRDL1, OsYSL2, and OsNRAMP5 follow strategy II and uptake only chelated Fe^{+3}, but rice also has a unique transporter OsIRT1 which enables the plant to directly uptake the Fe^{+2} from soil beyond the strategy II (Ishimaru et al., 2006). The key regulator of Fe transporters is OsIRO2, strongly induced under iron deficient conditions (Ogo et al., 2007). OsFRDL1 is expressed in rice root pericycle and encodes citrate effluxer that is required for efficient Fe translocation (Yokosho et al., 2009) and OsYSL2 is responsible for long distance transport of chelated Fe^{+3} to sink tissues (Ishimaru et al., 2010). Along with the Fe^{+3}, OsNRAMP5 also contributes to Mn^{+2} and Cd^{+2} transport in rice (Ishimaru et al., 2012).

This study is hypothesized to investigate positive impact of SA on As^V tolerance in rice. We analyzed changes in As accumulation, oxidative stress, antioxidant enzymes activities, As^{III}, and Fe transporters in As^V exposed plants under co-application, and pre-treatment of SA.

Materials and Methods

Growth Conditions and Experimental Design

Seeds of *Oryza sativa* cv. Pant4 collected from Masina Research Centre, Pvt. Ltd., Bihar (India), were surface sterilized using 10% H_2O_2 for 30 s and washed with Milli Q water. Seeds were germinated on moist pre-sterilized blotting sheets in a tray, placed in seed germinator for 4 days at 25°C, relative humidity was 65%. After 7 days, 50 uniform size seedlings were selected and placed in 150 ml beakers, covered with black sheet, containing 100 ml of 100% Hewitt nutrient medium, prepared in Milli-Q water (pH 6.8–7.0) and grown for another 10 days under light intensity 210 $\mu M\ cm^{-2}s^{-1}$ (16/8 h; day/night). 10 days old plants were provided As^V (25 and 50 μM) using the salt Na_2HAsO_4 and SA (100 μM) in the nutrient medium and grown for 7 days. Plants treated by 25 and 50 μM As^V, 100 μM SA for 7 days abbreviated as As^V25, As^V50 and SA, respectively. Plants treated with As^V25, As^V50 supplemented with SA abbreviated as SA + $As^V25$and SA + As^V50. For Pre-treatment of SA, plants were grown in 100 μM SA for 3 days and then transferred to Hewitt solution containing As^V25, $As^V50$for 7 days and they are abbreviated as SA Pre+$As^V25$and SA Pre + As^V50. Plants grown in As^V deprived medium termed as SA Pre and plants grown only in Hewitt solution served as control.

Estimation of Chlorophyll and Carotenoids

Fresh leaves (0.1 g) were crushed in 5 ml of 80% acetone and centrifuged at 10,000 × *g* for 10 min. The supernatant was

used for estimation of chlorophyll by Arnon (1949) method and carotenoids by Duxbury and Yentsch (1956) method.

Estimation Hydrogen Peroxide and MDA

Fresh leaves (0.5 g) were crushed in 5 ml of 0.1% trichloroacetic acid and centrifuged at 10,000 × *g* for 10 min. The supernatant was used for estimation of MDA and H_2O_2. MDA and H_2O_2 by Heath and Packer (1968) and Velikova et al. (2000), respectively.

Assay of Antioxidant Enzymes

Fresh leaves (0.3 g) were ground in liquid N_2 using a mortar, and homogenized in 3 ml of buffer containing 50 mM potassium phosphate buffer (pH 7.8) and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 8000 × *g* at 4°C for 15 min. and supernatant was used for ascorbate peroxidase (APX), guaiacol peroxidase (GPX), CAT, superoxide dismutase (SOD), and Nitrate reductase (NR) activity, and nitrite and soluble protein concentration.

The activity of SOD (EC 1.15.1.1) was measured by Beauchamp and Fridovich (1971), APX (EC 1.11.1.11) by Nakano and Asada (1981), GPX (EC 1.11.1.7) by Kato and Shimizu (1987), CAT (EC 1.11.1.6) by Scandalios et al. (1983), NR (EC 1.7.99.4), and nitrite by Hageman and Reed (1980).

Estimation of Non-Protein Thiolic Metabolites and Ascorbic Acid

The level of GSH and GSSG was measured by following the protocol of Hissin and Hilf (1976). Plant material (500 mg) was frozen in liquid nitrogen and homogenized in 0.1 M sodium phosphate buffer (pH 8.0) containing 25% meta-phosphoric acid. The homogenate was centrifuged at 20,000 × *g* for 20 min at 4°C. Total glutathione (GSSG and GSH) content was determined fluorometrically in the supernatant after 15 min incubation with *o*-phthaldialdehyde (OPT). Fluorescence intensity was recorded at 420 nm after excitation at 350 nm on a Hitachi F 7000 fluorescence spectrophotometer.

Non-protein thiol (NPT) content was measured by following the method of Ellman (1959). The concentration of PCs was calculated as PCs = NPT – (GSH + GSSG; Duan et al., 2011).

For estimation of ascorbic acid (Asc), fresh leaves (0.5 g) were crushed in 5 ml of 0.1% trichloroacetic acid and homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was used for estimation of Asc by Shukla et al. (1979).

Element Estimation

The elements (As and Fe) content was determined following Mallick et al. (2012). Briefly, plant tissues were washed three times with Milli Q water and plants separated in root and shoot and oven dried at 70°C. Dried plant tissues (root 300 and shoot 500 mg) were digested in HNO_3: HCl (3:1). Digested samples were filtered through Whatman filter paper 42 and volume was made to 10 ml by Milli-Q water. As and Fe were estimated by using AAS (GBC Avanta S, USA) fitted with a hydride generator (MDS 2000) using NaH_2BO_4+NaOH (3 M) and HCl (3 M). The values were presented in μg per gram dry weight (μg g^{-1}dw).

Endogenous Salicylic Acid Estimation

Presence of SA in shoot samples were analyzed by HPLC (Dionex Ultimate 3000) using UV detector at 210 nm by following the method of Pan et al. (2010). The mobile phase was programmed with linear gradient of A (0.1% of formic acid in methanol) and B (0.1% of formic acid in water) as 0–20 min; 30–100% A, 20–22 min; 100% A and then 22–25 min; 100–30% of A. Flow rate was maintained at 0.3 ml min^{-1}. Retention time for SA was recorded at 22.4 min.

Gene Expression Analysis Using Quantitative RT-PCR

Approximately 5 μg, RNase free DNase-treated, total RNA isolated from roots of rice plants was reverse-transcribed using SuperScriptII (Fermentas, USA), following the manufacturer's recommendation. The synthesized cDNA was diluted 1:5 in DEPC water and subjected to quantitative RT-PCR (qRT-PCR) analysis. The qRT-PCR was performed using an ABI 7500 instrument (ABI Biosystems, USA) using primers listed in Supplementary Table S1. Each qPCR reaction contained 5 μl of SYBR Green Supermix (ABI Biosystems, USA), 1 μl of the diluted cDNA reaction mixture (corresponding to 5 ng of starting amount of RNA) and 10 pM of each primer in a total reaction volume of 10 μl. The qPCR reactions were performed under following conditions: 10 min at 95°C and 40 cycles of the one step thermal cycling of 3 s at 95°C and 30 s at 60°C in a 96-well reaction plate. Actin gene was used as an internal control to estimate the relative transcript levels of the target gene. Specificity of amplicons generated in qPCR reactions was verified by melt curve analysis. Each qPCR reaction was performed in triplicate (technical replicates) for each biological replicate (three for each treatment). Relative gene expression was calculated using $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001).

Statistical Analysis and Analytical Quality Control

The whole experiment was set up in the randomized block design. The data were subjected to Duncan's Multiple Range Test (DMRT) for the analysis of significant difference between the treatments. Analytical data quality of the elements, was ensured through repeated analysis ($n = 6$) of Standard Reference Material. Standard Certified reference material (CRM 028-050) used for the accuracy of the AAS procured from Resource Technology Corporation, USA (Lot no. IH 028), and the values obtained varied between −3.97 to 22.86% error between ten measurements. The blanks were run all the time to eliminate the background noise.

Results

Morphology and Photosynthetic Pigments

Arsenate had deleterious impact on plant growth. A dose dependent decrease of 6 and 17% at 25 μM and 26 and 31% at 50 μM As^V was observed in root and shoot, respectively, than control. SA alone treatment enhanced the root and shoot length by 39

and 19%, respectively, than control. Co-application of SA and As^V50 enhanced the root and shoot growth significantly (58 and 36%, respectively) than 50 μM As^V alone treated plants. SA pre-treated plants also experienced less toxicity during exposure to As^V. Arsenate induced reduction in biomass was also significantly recovered by SA supplementation. Under As^V stress total chlorophyll was reduced significantly in dose dependent manner with maximum approximately 22% reduction at 50 μM As^V than control while carotenoid content was increased significantly in As^V50 treatment than control. SA co-application with As^V, reverted chlorophyll loss caused by As^V stress (**Table 1**).

Element Content in Root and Shoot

The rice plants accumulated significant amount of As in upon exposure to As^V in dose dependant manner. In all treatments more than 90% of As was confined in to the roots. SA co-application to As^V treated plants had no significant impact on As accumulation in root. However, the shoot As was reduced significantly, i.e., around 30% reduction in both SA + As^V25 and SA + As^V50 than As^V alone treated plants was observed. SA pre-treated plants also accumulated 16 and 17% less As in shoot upon exposure to 25 and 50 μM As^V, respectively, (**Table 2**).

Arsenate treatment significantly enhanced total Fe accumulation in comparison to control plants. However, the most of the accumulated Fe was localized in the roots. The translocation of Fe to shoot was reduced drastically (6% of total accumulation at As^V50) in As^V treated plants which was 33% lower than control shoot. Co-application as well as pre-treatment of SA reduced the total Fe accumulation in comparison to As^V alone, however, its translocation to shoots increased significantly, i.e., 30 and 45% increased at SA + As^V25 and SA + As^V50 than As^V alone treated shoots and the level of Fe in shoots were comparable to control (**Table 2**).

Oxidative Stress and Antioxidants

Salicylic acid alone treatment had no significant impact on MDA content in rice shoot while H_2O_2 content was enhanced by 28% than control. Arsenate treatment enhanced the MDA content by ca. three- and fourfolds at 25 and 50 μM As^V exposed plants, respectively, than control. Similar trend was observed in H_2O_2 content. Pre-treatments as well as co-application of SA and As^V has reduced the level of MDA and H_2O_2than As^V alone treated plants, although co-application was more effective than pre-treatment (**Figures 1A,B**).

Salicylic acid treatment also moderated As^V induced antioxidant activities. SOD activity got enhanced ca. two- and threefolds, respectively, in As^V25 and As^V50 treated plants in shoot than control. Co-application of SA and As^V significantly reduced SOD activity which was about 42 and 55% than As^V25 and As^V50, respectively. SA pre-treatment to As^V exposed plants showed 32 and 50% less SOD activity the respective As^V treatments (**Figure 1C**).

TABLE 1 | Effect on shoot, root lengths (cm), fresh-weight (mg), total chlorophyll (mg g^{-1}fw), and carotenoid content (mg g^{-1}fw) *Oryza sativa* after 7 days of treatment with different combinations of Arsenate (As^V) and Salicylic acid (SA).

	Root length	Shoot length	Biomass	Total chl	Carotenoids
Control	$3.88^{cd} \pm 0.26$	$26.3^{de} \pm 1.8$	$274.24^{d} \pm 0.01$	$2.29^{de} \pm 0.06$	$0.15^{ab} \pm 0.008$
SA	$5.40^{g} \pm 0.36$	$31.3^{f} \pm 2.5$	$345.51^{f} \pm 0.0$	$2.44^{f} \pm 0.08$	$0.15^{bc} \pm 0.008$
As^V25	$3.63^{bc} \pm 0.29$	$21.8^{bc} \pm 1.4$	$226.84^{ab} \pm 0.07$	$1.94^{b} \pm 0.02$	$0.18^{ef} \pm 0.004$
As^V50	$2.85^{a} \pm 0.19$	$18.0^{a} \pm 1.2$	$218.94^{a} \pm 0.07$	$1.79^{a} \pm 0.07$	$0.18^{f} \pm 0.006$
SA + As^V25	$4.88^{f} \pm 0.33$	$26.3^{de} \pm 1.7$	$266.79^{cd} \pm 0.06$	$2.31^{e} \pm 0.01$	$0.14^{a} \pm 0.004$
SA + As^V50	$4.50e^{f} \pm 0.30$	$24.5^{cd} \pm 1.9$	$248.73^{bcd} \pm 0.01$	$2.17^{c} \pm 0.02$	$0.16^{cd} \pm 0.001$
SA Pre	$4.28^{de} \pm 0.29$	$28.1^{e} \pm 1.9$	$312.84^{e} \pm 0.016$	$2.48^{f} \pm 0.05$	$0.16^{cd} \pm 0.001$
SA Pre +A s^V25	$3.52^{bc} \pm 0.28$	$24.3^{cd} \pm 1.6$	$253.36^{bcd} \pm 0.08$	$2.22^{cde} \pm 0.05$	$0.17^{de} \pm 0.007$
SA Pre + As^V50	$3.33^{ab} \pm 0.26$	$20.6^{ab} \pm 1.4$	$239.91^{abc} \pm 0.01$	$2.19^{cd} \pm 0.05$	$0.17^{de} \pm 0.008$

Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are means of three replicates ±SD.

TABLE 2 | Accumulation (μg g^{-1}dw) of Arsenic (As) and Fe in the root and shoot of *Oryza sativa* after 7 days of treatment with different combinations of As^V and SA.

	As root	As shoot	Fe root	Fe shoot
Control	–	–	$364.2^{a} \pm 18.1$	$60.2^{cde} \pm 5.1$
SA	–	–	$403.3^{a} \pm 30.0$	$68.3^{e} \pm 7.1$
As^V25	$317.82^{a} \pm 66.2$	$28.84^{bc} \pm 2.7$	$566.6^{bc} \pm 49.2$	$48.4^{ab} \pm 5.8$
As^V50	$454.26^{b} \pm 22.2$	$38.00^{d} \pm 4.1$	$632.5^{c} \pm 61.3$	$40.2^{a} \pm 3.3$
SA + As^V25	$290.83^{a} \pm 42.9$	$19.96^{a} \pm 2.3$	$522.4^{b} \pm 63.1$	$62.8^{de} \pm 3.9$
SA + As^V50	$426.74^{b} \pm 56.9$	$26.55^{bc} \pm 2.9$	$528.8^{b} \pm 41.1$	$58.3^{cde} \pm 4.4$
SA Pre	–	–	$409.2^{a} \pm 17.7$	$64.4^{de} \pm 4.6$
SA Pre + As^V25	$302.56^{a} \pm 46.9$	$24.22^{ab} \pm 2.7$	$486.6^{b} \pm 38.4$	$55.8^{bcd} \pm 5.8$
SA Pre + As^V50	$432.32^{b} \pm 53.3$	$31.19^{c} \pm 1.8$	$551.4^{b} \pm 43.6$	$52.3^{bc} \pm 6.6$

Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are means of four replicates ±SD.

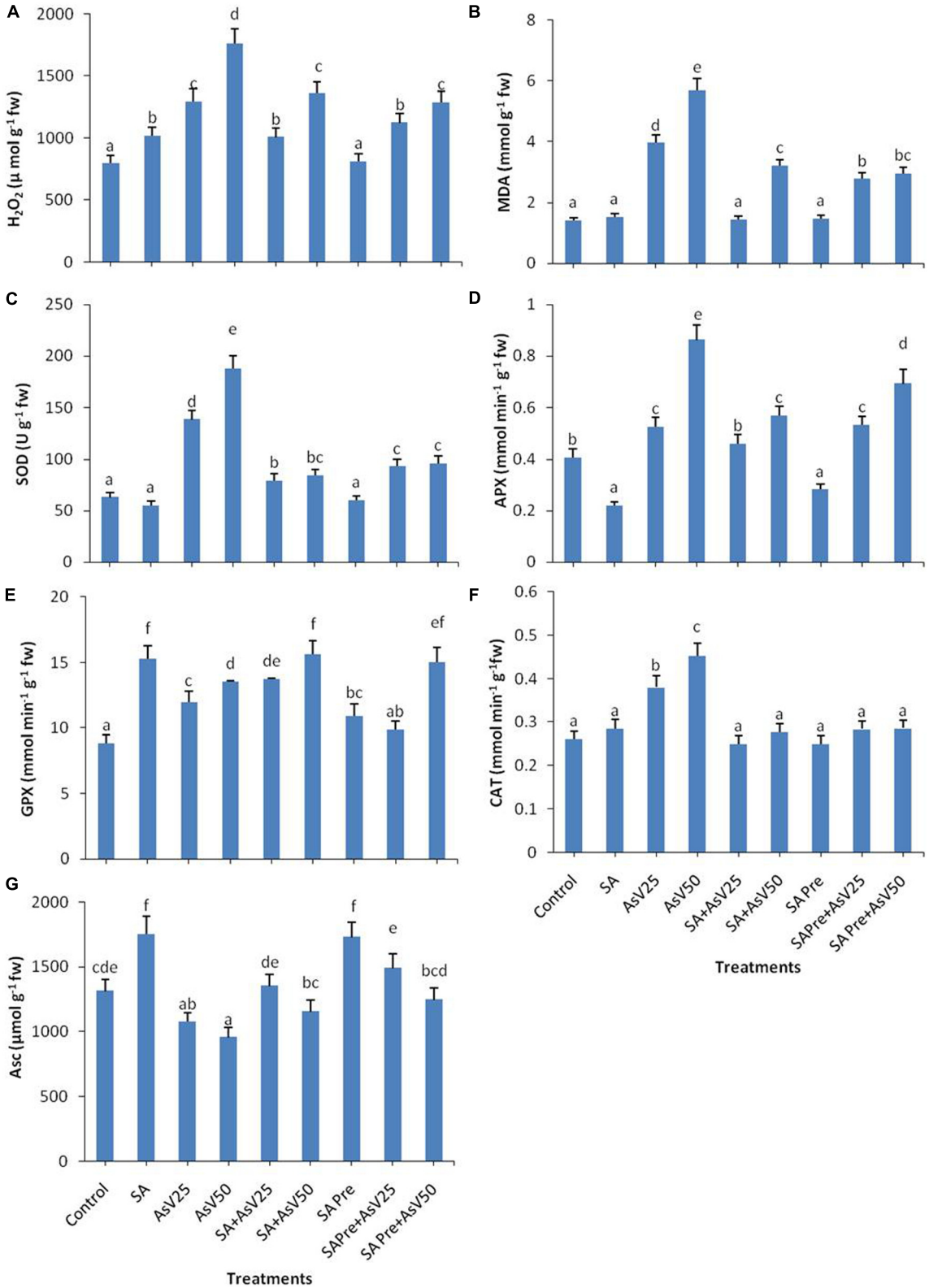


FIGURE 1 | Effect on (A) H_2O_2, (B) MDA, (C) SOD, (D) APX, (E) GPX, (F) CAT, and (G) Ascorbate in shoot of the *Oryza sativa* after 7 days of treatment with different combinations of Arsenate (As^V) and Salicylic acid (SA). Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are mean of three replicates ±SD.

Salicylic acid alone treatment reduced the APX activity to approximately half while 50 μM As^V treatment approximately doubled the APX activity than control. Co-application of SA and As^V50 reduced APX activity by 34% also SA pre-treatment (SA Pre + As^V50) reduced APX activity by 20% than As^V50 treated plants (**Figure 1D**). SA alone treatment enhanced the GPX activity by ca. twofold, furthermore As^V treatment also enhanced the activity significantly than control. Co-application of SA and As^V also enhanced GPX activity than corresponding alone As^V treated plants (**Figure 1E**). Under As^V stress, CAT activity was enhanced 45 and 72% at 25 and 50 μM, respectively, than control. Co-application or pre-treatment of SA and As^V, reduced the CAT activity than As^V alone exposed plants (**Figure 1F**). SA alone treatment has enhanced the Asc content by 33% while exposure to 50 μAs^V reduced the Asc level by upto 27% than control. Co-application of SA and As^V further enhanced the Asc content significantly than corresponding As^V alone treated plants. SA pre-treatment also enhanced the level of Asc upon As^V exposure in all treatments than corresponding As^V exposed plants (**Figure 1G**).

Nitrate Reductase, Nitrite, and Endogenous Level of SA

Nitrate reductase activity was significantly enhanced by SA as well as As^V treated plants in comparison to control. Co-application of SA with lower As^V (25 μM) has enhanced the NR activity significantly while with higher As^V (50 μM) NR activity was reduced significantly than As alone treatments. SA pre-treatment to As^V exposed plants has no significant impact on NR activity than corresponding alone As^V exposed plants (**Figure 2A**).

The level of nitrite was almost doubled in SA alone treated plants, while a dose dependent decrease in nitrite level was observed under As^V stress plants than control. Co-application of SA and As^V enhanced the nitrite level in comparison to As^V alone exposed plants. SA pre-treated plants had almost double nitrite than control. However, when SA pre-treated plants were exposed to As^V the levels of nitrite was lower than control and were comparable to As^V alone treated plants (**Figure 2B**). There was no significant change in level of endogenous level of SA in shoot in all treatments except for SA alone treated plants where endogenous level of SA was enhanced significantly than control (**Figure 2C**).

Non-Protein Thiol Metabolism

The level of total non-protein thiol (NPT) did not show any significant change in response to the treatments in comparison to control except for SA alone treated plant (**Figure 3A**). SA treatment has enhanced the GSH level by 25% while As^V stress has reduced the GSH content in dose dependent manner than control. Co-application of SA and As^V enhanced GSH content 7

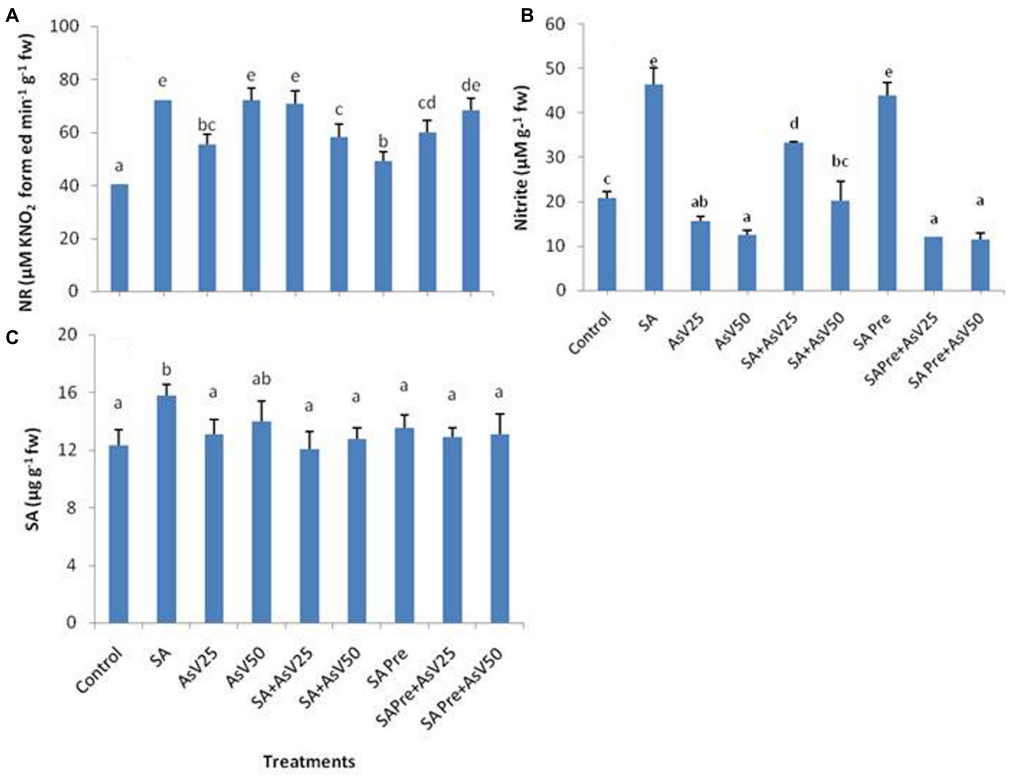


FIGURE 2 | Effect on (A) Nitrate reductase, (B) Nitrite and (C) Endogenous level of SA in shoot of the *Oryza sativa* after 7 days of treatment with different combinations of As^V and SA. Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are means of three replicates ±SD.

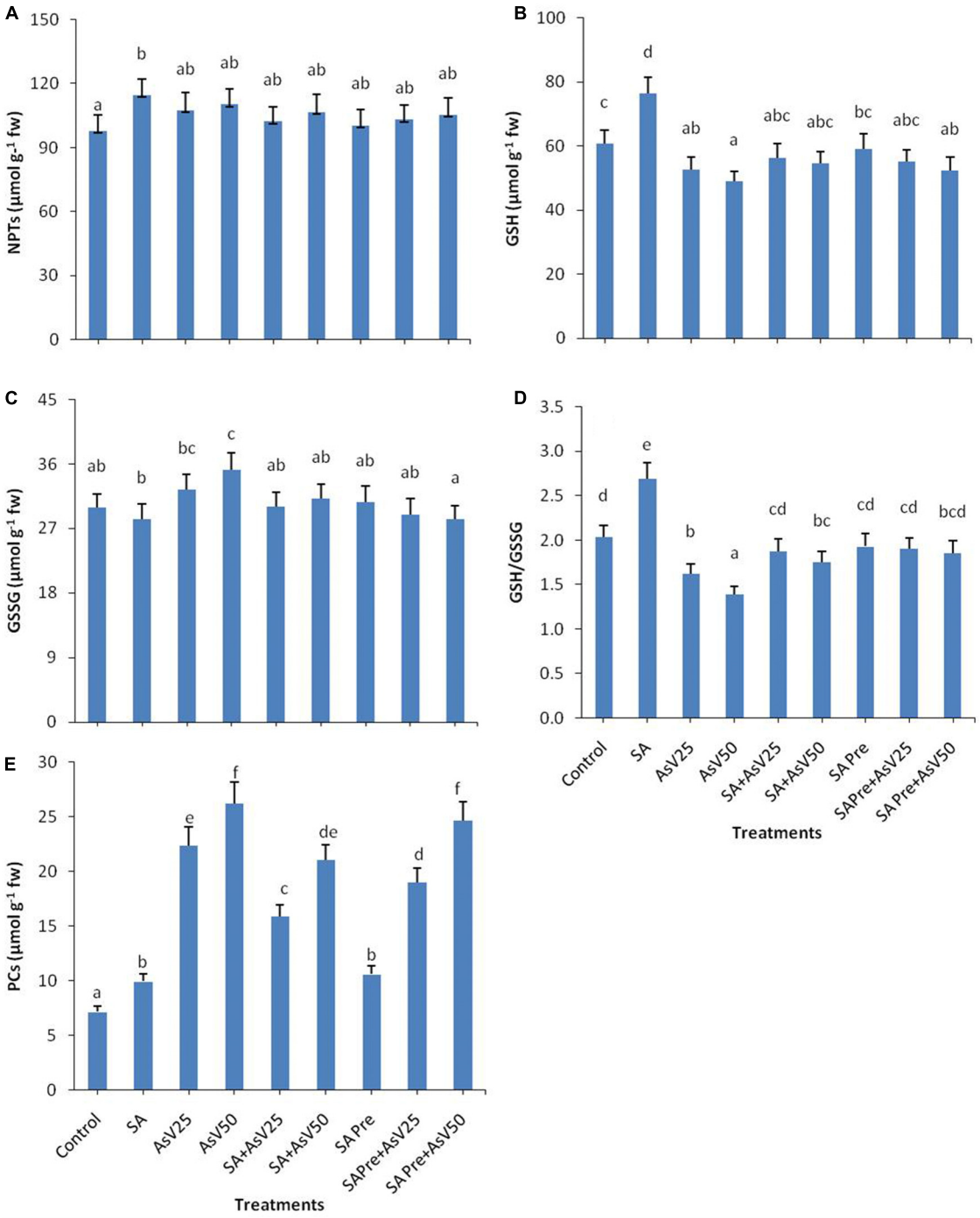


FIGURE 3 | Effect on (A) NPTs, (B) GSH, (C) GSSG, (D) ratio of GSH/GSSG, and (E) Phytochelatins (PCs) in shoot of the *Oryza sativa* after 7 days of treatment with different combinations of As^V and SA. Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are means of three replicates ±SD.

and 11% than corresponding As^V alone treated plants though the levels were not statistically significant different than control. Pre-treatment of SA with As^V had no significant impact on GSH level than corresponding As^V alone treated plants (**Figure 3B**). Alone SA treatment had no significant impact on GSSG level while As^V50 has significantly enhanced GSSG content than control (**Figure 3C**). Ratio of GSH/GSSG was enhanced by 32%in SA treatment plants while in As^V treated plant the ratio was reduced by 20 and 31% in dose dependent manner than control. Co-application of SA and As^V has enhanced the GSH/GSSG ratio

than their corresponding As^V alone treated plants. SA pre-treated As^V exposed plants also showed enhanced GSH/GSSG ratio than As^V alone treated plants (**Figure 3D**).

Both SA alone and As^V alone treatments enhanced the level of PCs to 1.4 and up to 3.5-fold (at As^V50), respectively, as compared to control. Co-application of SA and As^V reduced the PCs accumulation by 29 and 19% than corresponding alone As^V treated plants though the values were still significantly higher than controls. Similar effects were observed in SA pre-treated plants both with and without As^V (**Figure 3E**).

Arsenite and Iron Transporters

Salicylic acid alone treatment enhanced the expression level of *OsLsi1* to ca. threefold than control. Arsenate alone treatment also enhanced *OsLsi1* expression significantly in comparison to control though the levels were far lower than SA alone. Co-application of SA and As^V enhanced *OsLsi1* expression around twofolds than As^V alone treated roots. Pre-treatment of SA, with or without As^V, had no significant impact on *OsLsi1*expressionin comparison to control or respective As^V alone treatments (**Figure 4A**).

Arsenate exposure enhanced the *OsLsi2* expression level to ca. five- and sevenfold in dose dependent manner than control. Co-application of SA and As^V as well SA pre-treatment to As^V exposed plants lowered the expression of *OsLsi2* in comparison to As^V alone treatments, however, the levels were still significantly higher than control roots (**Figure 4B**).

The expression of *OsNRAMP5* was enhanced more about threefold in As^V treated plant roots, however, SA alone treatment also increased the expression of *OsNRAMP5* by about 2.5-folds. Co-application of SA and As^V has reduced the expression level by 20% and 31% in comparison to respective As^V alone exposed plants. SA pre-treatment slightly reduced the expression level of *OsNRAMP5* in comparison to As^V alone treatment which was significant at 50 μM As^V.

OsIRO2 expression level enhanced 13-fold in SA treated plants and As^V25 and As^V50 have enhanced ca. eight and ca. sixfold than control. Co-application of SA and As^V further enhanced expression level and that was comparable to SA treated plants. SA pre-treatment to As^V50 stressed plants significantly increased the expression level of *OsIRO2* than As^V50alone treated plants.

OsFRDL1 expression level was decreased under As^V stress in dose dependent manner than control. Co-application of SA and As^V enhanced the expression level significantly than corresponding As^V alone treated plants. In SA pre-treated plants exposed to As^V50 the expression level of *OsFRDL1* approximately doubled than As^V50 alone treated plants.

OsYSL2 expression level was enhanced to ca. 16-fold in SA alone treated plants and As^V alone treatment enhanced the expression by about 10- and 13-fold in dose dependent manner than control. Co-application of SA and As^V reduced the expression level than SA alone as well as corresponding As^V alone treated plants. SA pre-treatment also enhanced the expression level 13-fold than control. SA pre-treatment to As^V stressed plants sharply declined the expression level than corresponding alone As^V treated plants (**Figures 4C–F**).

Discussions

Salicylic acid serves as an important signaling molecule in plant system which has been shown to play role in against heavy metal toxicity (as detailed in introduction). The present experiment was designed to investigate the ameliorative effect of SA during As toxicity. The co-application and pre-treatment of SA with As was used to investigate persistence of signaling aspects of SA.

Arsenic is well known to adversely affect the plant growth and development upon its accumulation (Kumar et al., 2015). In present study as well a significant amount of As was accumulated by the rice plant that hampered the plant growth severely. Application of SA, either co- or pre- treatment with As^V has significantly reduced the total accumulation of As (Root + shoot) with more reduction in the shoot. Though, the co-application of SA was more effective in reducing As accumulation than pre-treatment of SA. Thus, SA treatment has negatively impacted the root to shoot translocation of As. This might be due to SA-mediated down regulation of root to shoot As transporters. In present study OsLsi2, transporter responsible for root to shoot As^{III} transport in rice (Ma et al., 2008), has been found to be down regulated at mRNA level. Since As^{III} is the dominant form inside the plant (Pickering et al., 2000; Mishra et al., 2013) and also probably the main As species translocated to the shoots. Thus, down regulation of *OsLsi2* would negatively affect the As accumulation. In the present study As accumulation was positively correlation with *OsLsi2* expression level ($R = 0.87$). Down regulation of *OsLsi2*was resulted in lower As accumulation in rice shoots in response to thiourea supplementation with As (Srivastava et al., 2014). OsLsi1 is primarily responsible for As^{III} transport to root from extracellular medium, was not found correlated with root uptake of Asin present study. This might be due to fact that in present experiment plants were treated with As^V which is transported by the phosphate transporters (Tripathi et al., 2007). Alternatively, SA has been reported to activate ATP-binding cassette (ABC) transporters in soybean (Eichhorn et al., 2006). The ABC transporters are responsible for vacuolar sequestration of As(III)-PC complexes (Song et al., 2010). Therefore, it might be possible that most of the accumulated As in SA treated rice plants were sequestered in root vacuoles in the form of As(III)-PC, as a result less As could be transported to the shoot. Further, SA pre-treatment has been reported to enhance PCs synthesis in maize root (Szalai et al., 2013). Although SA mediated resistance against heavy metal viz., Cd, and Mn, has reported in previous studies (Metwally et al., 2003: Shi and Zhu, 2008) no reduction in the level of accumulation was observed. Since less accumulation of metalloid in shoot might also affect its level in grain which would have great implications with respect to human toxicity through food chain As contamination.

In present study SA treatment has enhanced the plant growth in terms of root, shoot length and biomass. Co-application of SA and As^V, partially restored the plant growth in As^V exposed plants. Growth stimulating effects of SA has been previously reported in soybean (Gutiérrez-Coronado et al., 1998), wheat (Shakirova et al., 2003), and maize (Gunes et al.,

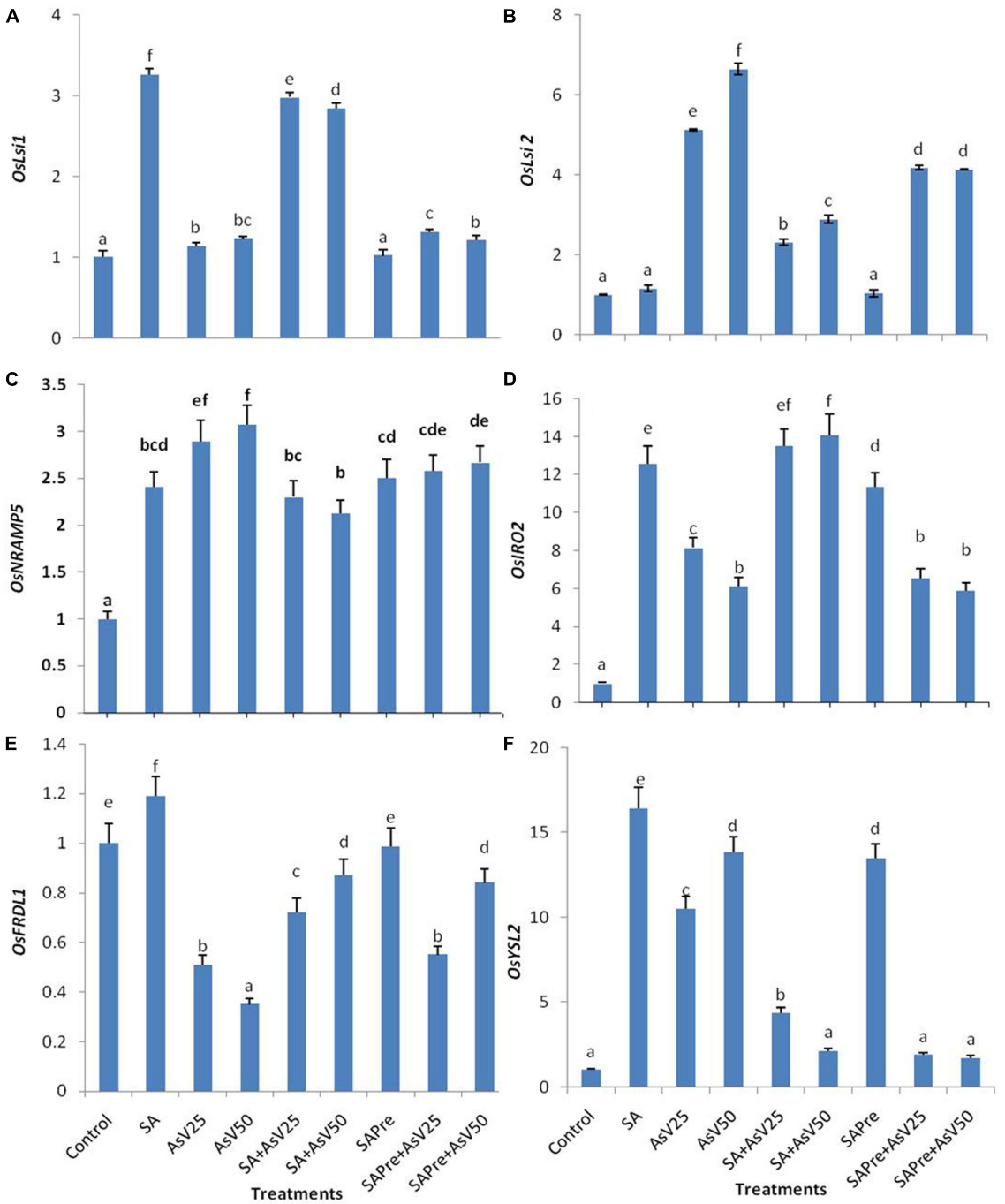


FIGURE 4 | Relative expression of transcript level of iron transporters (A) *OsLsi1,* (B) *OsLsi2,* (C) *OsNRAMP5*, (D) *OsIRO2*, (E) *OsFRDL1,* and (F) *OsYSL2* in roots of *Oryza sativa* after 7 days of treatment with different combinations of As^V and SA. Values marked with same alphabets are not significantly different (DMRT, $p < 0.05$). All the values are means of three replicates ±SD.

2007). This growth restoration by SA could be auxin mediated or due to lowering of As accumulation in shoot. In SA treated wheat seedlings, higher level of auxin has been reported (Shakirova et al., 2003). SA inducible transcription factors (OBP1, OBP2, and OBP3) were found to be responsive to auxin (Kang and Singh, 2000). Pre-treatment of SA also reverted the As^V mediated inhibition of plant growth. In present study, a marked reduction in chlorophyll content was observed in As^V treated plants. Similar results were previously reported by Rahman et al. (2007) in rice and by Mishra et al. (2014) in *Ceratophyllum*. SA supplementation to As^V treated plants reverted As^V induced chlorosis. Similar reversion of

chlorosis was observed in maize under salinity stress (Khodary, 2004). In present study As^V also reduced the Fe content in shoot that may also be responsible for As^V mediated chlorosis while SA has enhanced the iron content in shoot and reverted the chlorosis. Previously Kong et al. (2014) also reported the increased uptake of Fe in *Arachis hypogaea* by foliar application of SA. SA induces the nitric oxide (NO) synthesis in plants (Zottini et al., 2007) that is reported to enhance the bioavailability of Fe (Graziano and Lamattina, 2007). In present study NR activity and nitrite level was also enhanced by SA treatment, indicating enhanced level of NO that also supports above mentioned hypothesis of SA mediated enhancement of NO leading to enhanced Fe availability and increase in photosynthetic pigments.

OsFRDL1, responsible for Fe efflux into xylem (Inoue et al., 2004), was down regulated in As^V stressed plants and this decrease was concomitant with reduced Fe accumulation in shoot. OsYSL2, responsible for long distance transport of Fe (Ishimaru et al., 2010), was enhanced in both SA, and As^V treated plants, however, no increase in Fe accumulation in shoot was observed. OsNRAMP5 is involved in uptake of Fe in root (Ishimaru et al., 2012). The expression of OsNRAMP5 was enhanced in both SA and As^V treated plants and an increase in Fe accumulation in root was observed as well.

Inside the cell As induces ROS synthesis that leads to oxidative stress (Finnegan and Chen, 2012). In present study oxidative stress is indicated by enhanced level of H_2O_2 and MDA content in As^V treated plant as reported earlier in rice (Tripathi et al., 2012). Disturbed redox homeostasis in response to As^V has been reported as the main factor for hampered growth of rice seedlings (Srivastava et al., 2014). SA treatment reduced the level of As^V induced H_2O_2 and MDA which indicates that SA protected the plant against As^V mediated oxidative stress. Similar protective effects of SA has been observed against Cd stress in rice (Guo et al., 2007) and barley (Metwally et al., 2003) and against As stress in *Arabidopsis* (Odjegba, 2012). SA mediated responses are associated with H_2O_2 accumulation (Drazic and Mihailovic, 2005). At moderate level, H_2O_2 serves as a secondary messenger for activation of stress resistance mechanism in plants (Noctor and Foyer, 1998). In present study SA application activated a slight accumulation of H_2O_2.

Ascorbate and glutathione (GSH/GSSG) are two important antioxidants. They are redox buffering agents in the apoplast and protect the plasma membrane from oxidation (Noctor and Foyer, 1998). The ratio of GSH/ GSSG is an important marker for oxidative stress (Tausz et al., 2004). The reduced GSH/ GSSG ratio in present study showed disturbed redox balance upon As^V exposure. Application of SA has enhanced the GSH/ GSSG ratio. Enhanced GSH/ GSSG ratio in response to SA has also been reported in cucumber seedlings (Shi and Zhu, 2008). The enhanced As^V tolerance upon thiourea application was suggested to be associated with TU ability to maintain plant redox homeostasis through improved GSH/GSSG ratio (Srivastava et al., 2014). Ascorbate is an effective scavenger for free radicals (Hasanuzzaman and Fujita, 2013). Co-application of SA and As^V also enhanced the level of Asc thus improving the redox balance under As^V stress. Similar results were observed in Alfa during mercury stress (Zhou et al., 2009). The increase in the level of GSH might be due to the fact that gene encoding glutathione-dependent formaldehyde dehydrogenase /GSNO reductase was activated by SA in *Arabidopsis* (Dıaz et al., 2003).

In present study, under As^V stress the activity of antioxidant enzymes APX, GPX, SOD, and CAT were enhanced in dose dependent manner. These enzymes consume the H_2O_2 as substrate so with the enhancement of H_2O_2 concentration, activity of these enzymes also enhanced. SA has high affinity to CAT and APX thereby inhibits their activities (Vlot et al., 2009; Manohar et al., 2015). In the present study SA supplementation to As^V stressed plants reduced the APX and CAT activity than As^V alone treated plants. SA is believed to inhibit CAT by the chelation of heme Fe and by causing conformational changes (Rüffer et al., 1995). However, Durner and Klessig (1996) suggested that SA-mediated inhibition of CAT probably results from peroxidative reactions.

Guaiacol peroxidase exists in various isoenzyme forms in rice and has varied functions in plant metabolism but H_2O_2 serves as necessary substrate for their activity. Arsenate stress enhanced the GPX activity in dose dependent manner. SA enhanced the activity of GPX as previously reported in *Medicago sativa* (Zhou et al., 2009) and in rice (Guo et al., 2007).

In present study, no significant change was observed in endogenous level of SA under As^V stress that is in contrast to previous studies which reported enhancement in endogenous level of SA under abiotic stress (Miura and Tada, 2014). Since rice shoot has high level of endogenous SA among all the plants tested for SA (Silverman et al., 1995), therefore, under abiotic stress endogenous level of SA may not show any significant change. Similar results were found under biotic stress when little or no change was observed in endogenous level of SA, during bacterial or fungal infection (Silverman et al., 1995; Yang et al., 2004).

Conclusion

Taken together it is evident from present work that SA has reduced the As^V induced oxidative stress and effectively modulated the enzymatic and non-enzymatic antioxidants. SA also played a role in enhancing Asc, GSH, and PCs in plants subjected to As^V stress. SA reduced the As accumulation in shoot and also overcame the As induced Fe deficiency in shoot so the elaborated study of SA signaling may be helpful in developing the As resistant crops.

Author Contributions

RT, PT, VP, DC, Shekhar Mallick designed experiments and reviewed manuscript. AS, GD performed experimental work and prepared figures. MT Operated Thermocycler. Seema Mishra, SD

reviewed manuscript. All authors have read and approved the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00340/abstract

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ROS mediated MAPK signaling in abiotic and biotic stress- striking similarities and differences

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Plants encounter a number of environmental stresses throughout their life cycles, most of which activate mitogen activated protein kinase (MAPK) pathway. The MAPKs show crosstalks at several points but the activation and the final response is known to be specific for particular stimuli that in-turn activates specific set of downstream targets. Interestingly, reactive oxygen species (ROS) is an important and common messenger produced in various environmental stresses and is known to activate many of the MAPKs. ROS activates a similar MAPK in different environmental stimuli, showing different downstream targets with different and specific responses. In animals and yeast, the mechanism behind the specific activation of MAPK by different concentration and species of ROS is elaborated, but in plants this aspect is still unclear. This review mainly focuses on the aspect of specificity of ROS mediated MAPK activation. Attempts have been made to review the involvement of ROS in abiotic stress mediated MAPK signaling and how it differentiates with that of biotic stress.

Keywords: abiotic/biotic stress, MAPKs, protein tyrosine phosphatases, RBOH, ROS, signaling crosstalks, Sty1

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Introduction

Plants show complex signaling network to transduce any external stimuli to the inside of the cell for an appropriate cellular arrangement giving rise to a particular response. The response is such that it helps the plant to cop up with environmental stresses that it experiences throughout its life. To exhibit a particular response, it is important for the plant to perceive the stimulus and transmit it into the nucleus of the plant cell. The perception is specifically done by cell wall receptors which then by several mechanisms activate internal signaling components. One of the most important changes that occur upon perception of external stimuli is change in redox state. Plants come across two types of stresses, abiotic and biotic. Change in redox state is a common outcome of both the stresses. This change in redox state occurs due to the production and accumulation of reactive oxygen species (ROS) in two powerhouses of plants, i.e., chloroplast and mitochondria (Apel and Hirt, 2004; Mittler et al., 2004). ROS are important secondary messengers that are poised at the core of signaling pathway in plants maintaining normal metabolic fluxes and different cellular functions (**Figure 1**). Besides chloroplast and mitochondria these are mainly produced by cell wall NADPH oxidases, peroxidases, while they are scavenged by numerous scavenging enzymes (Apel and Hirt, 2004; Nurnberger et al., 2004). The level of ROS determines whether it will be defensive or destructive molecule and its level is maintained through coordination between ROS production and turnover (Mittler et al., 2004; Miller et al., 2007). Function of ROS is also governed by its site of production, site of action and duration of action. When environmental stress becomes detrimental to the plant, it activates genetically controlled process called programmed

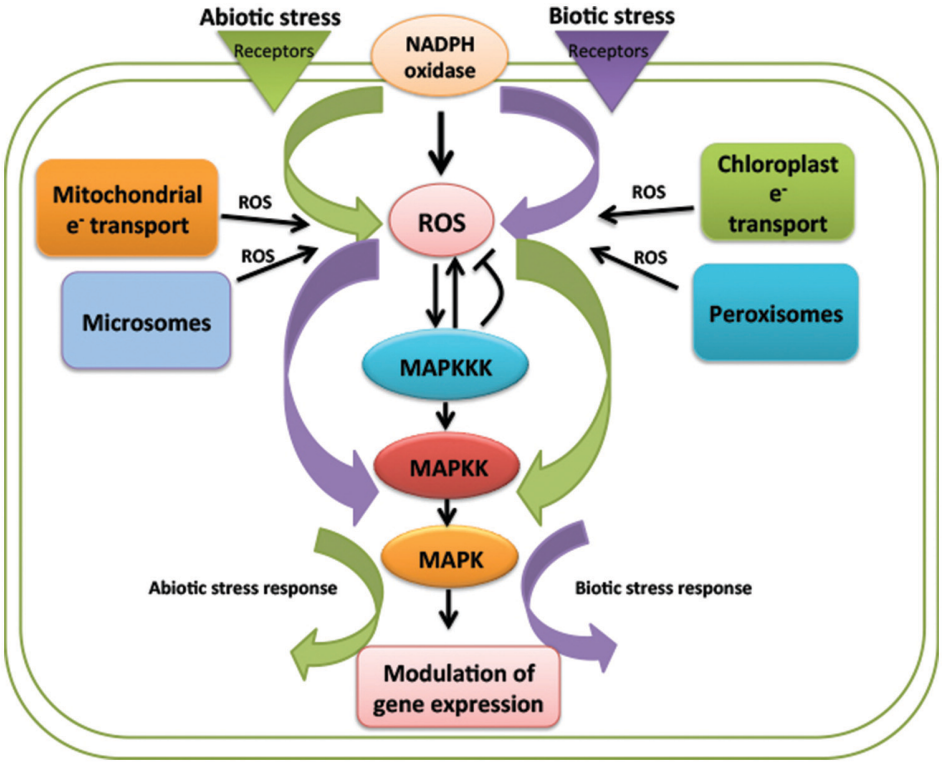


FIGURE 1 | Schematic representation of reactive oxygen species (ROS) regulation of mitogen activated protein kinases (MAPK) signaling pathway in biotic and abiotc stresses. ROS is a common messenger produced in response to both the stress response, acting either up- or downstream of MAPK cascade. Despite being a common regulator of MAPKs signaling the response shown by plant is different in both the stresses. Purple and green color represents biotic and abiotc stress, respectively.

cell death to specifically eliminate damaged tissues. In this process plants produce excess of ROS which helps in destroying stressed and damaged tissue. Signal transdcution pathways regulates the level of ROS production thereby protecting the plants from adverse effect of ROS (Bowler and Fluhr, 2000; Mittler et al., 2004).

One of the most important signaling cascades working in transmitting stress related stimuli is mitogen activated protein kinase (MAPK) cascade. MAPKs are highly conserved signaling pathway, play major role in signal transduction of diverse stress responses even in combination of many stresses. MAPK cascade consist of three tier components MAPKKKs, MAPKKs, and MAPKs carrying out phosphorylation reaction from upstream receptor to downstream target (Hamel et al., 2006). MAPKs are not only known to be activated by perception of ligand but are also activated by these ROS molecules. These phosphorylation cascades are found to work either upstream or downstream of ROS (Asai et al., 2002; **Figure 1**). MKK4-MPK3/6 module is known to play role in ROS production by acting upstream of NADPH oxidase and other way round H_2O_2 produced is known to activate MPK3 and MPK6 (Kovtun et al., 2000).

The manner in which plants respond to environmental stress depends on the type of stress and the outcome shown is mainly specific to particular stress. Some of the mechanisms like ROS production are common factor or outcome of both abiotic and biotic stresses whereas other mechanisms like activation of signal transduction networks, downstream activation of transcription factors and gene modulation becomes specific for specific stimuli (**Figure 1**). Question lies behind the specific activation of signaling cascade by upstream secondary messengers like ROS.

Environmental stresses encountered by plants are known to activate MAPK pathway. The MAPK activation is mostly specific but at times crosstalks are also reported in this signaling pathway (Sinha et al., 2011). Interestingly ROS which is produced by various environmental stresses is known to activate MAPKs giving a specific response. But the mechanism behind the specific activation of MAPK cascade by ROS is still unclear. This review mainly focuses on the aspect of specificity of ROS mediated MAPK activation. Attempts have been made to review the involvement of ROS in abiotic stress mediated MAPK signaling and how it delineates from that of biotic stress. In this review an update is provided on ROS regulated MAPK signaling and how it is differentially regulated by ROS produced in response to abiotic and biotic environmental stresses.

ROS Production and its Turnover

Reactive oxygen species is being continuously produced in cell during normal cellular processes by aerobic respiration in

chloroplast, mitochondria, peroxisomes, etc., ROS produced is counteracted by scavenging enzymes to maintain its level. Apart from its production from normal metabolic activities, majority of apoplastic ROS is produced by NADPH oxidase, (called as respiratory burst oxidase RBO in mammals) as first studied in mammalian ROS production. Cell wall peroxidases, germin like oxalate oxidases and amino oxidase also are involved in ROS production (Doke, 1983; Apel and Hirt, 2004; Nurnberger et al., 2004). NADPH oxidases in plants are named as Respiratory Burst Oxidase Homologs (RBOH) after their mammalian analogs. The first studied NADPH oxidase gene in plant was rice *OsrbohA* (Groom et al., 1996). Plants show different isoforms of *Rboh* genes. There exist ten *Rboh* genes in *Arabidopsis* from *AtrbohA–AtrbohJ* (Torres et al., 1998). *Rboh* genes were first identified to generate ROS in response to biotic stress. Study on mutant and antisense lines of *Rboh* genes *AtrbohD* and *AtrbohF*, gave the proof of production of oxidative burst by RBOH in pathogen infection (Torres et al., 2002). ROS generated by RBOH also impose their role in abiotic signaling and same genes are involved in ROS production in this signaling. The same Rboh isoform is able to carry out different ROS dependent function in response to different stimuli and in different cellular context (**Table 1**). The difference in outcome might exist due to complex interaction between different Rboh isoforms and with other signaling components (Foreman et al., 2003; Kwak et al., 2003; Kubo et al., 2005; Miller et al., 2009; Müller et al., 2009).

Plant response to a particular environmental stress also depends on level of ROS which is maintained by a balance between its production and turnover. This balance of ROS level is required for performing its dual role of acting as a defensive molecule in signaling pathway or a destructive molecule. There are total 152 genes involved in regulating ROS production and turnover (Mittler et al., 2004). Different antioxidants like ascorbate, tocopherol, glutathione, etc., play an important role in maintaining ROS level. Major enzymes involved in maintaining ROS homeostasis are ascorbate peroxidase (APX1), catalase (CAT1 & 2), thylakoid aperoxidase (tAPX), mitochondrial oxidase (AOX) and Cu-Zn- superoxide dismutase 2 (CSD2). Studies on mutants lacking these enzymes have revealed a strong link between biological processes, stress responses and ROS (Rizhsky et al., 2002; Pnueli et al., 2003; Miller et al., 2007).

MAPKs Cascade Activation and ROS Generation- What Comes First?

Sensing of ROS by plant cell is done either by receptors, ROS sensitive transcription factors like heat shock factors, NPR1 or by ROS mediated inhibition of phosphatase (Mittler et al., 2004; Miller and Mittler, 2006). Once the ROS are sensed it turns on signal transduction pathway further causing differential gene expression. It can activate signal transduction pathway within the cytoplasm of cell or in the organelles where it is being produced. ROS are considered to activate signal transduction pathways in linear fashion but at times it can also work at different levels in a particular pathway. It is also likely that ROS mediated signaling pathway can act on ROS production to maintain its homeostasis in case if the ROS levels are high.

Upon perception of variety of stress stimuli MAPK cascades are activated. MAPK ultimately phosphorylate and activate several downstream targets like transcription factor, other kinases, phosphatases, and cytoskeleton associated proteins (Hamel et al., 2006; Rodriguez et al., 2010; Sinha et al., 2011). During environmental stimuli MAPKs acts on RBOH thus regulating its activity and ROS production (Asai et al., 2008). Two MAPK cascades NPK1-MEK1-NTF6 and MEK2-SIPK, known till now are found to regulate RBOH mediated oxidative burst and ROS produced is involved in mediating disease resistance (Asai et al., 2008). Recent study reported that MEKK1-MKK5-MPK6 mediates salt induced expression of iron superoxide dismutase gene further inducing ROS production (Xing et al., 2015). These studies suggest that ROS acts downstream of MAPK pathway. However, ROS an important messenger produced in various stress responses are well known to exert their effect on MAPKs, thus acting upstream of MAPKs. Upon pathogen attack ROS being produced activates *Arabidopsis* MPK3, MPK4, and MPK6. MAPK Cascade working in *Arabidopsis* in response to pathogen attack downstream of ROS is MEKK1-MKK4/5-MPK3/6 (Asai et al., 2002). Another MAPK cascade MEKK1-MKK2-MPK4/6 is known to work downstream of ROS participating in both abiotic and biotic stress signaling (Teige et al., 2004; Pitzschke et al., 2009; Furuya et al., 2014). MAPK cascades activated by ROS in

TABLE 1 | Involvement of different *Rboh* genes isoforms in different environmental stresses and plant development.

Rboh genes	Function	Reference
AtrbohA	–	–
AtrbohB	Role in post seed ripening	Müller et al., 2009
AtrbohC	Root hair formation; mechanosensing	Foreman et al., 2003; Monshausen et al., 2009.
AtrbohD	Systemic signaling in response to diverse stimuli like pest attack, mechanical wounding, heat, cold, high light intensity and salinity. Accumulation of reactive oxygen species (ROS) in plant defense; ROS dependent ABA signaling in *Arabidopsis*.	Torres et al., 2002; Kwak et al., 2003; Miller et al., 2009; Pogány et al., 2009.
AtrbohE	Differentially expressed during differentiation of mesophyll cells to tracheary elements.	Kubo et al., 2005
AtrbohF	Accumulation of ROS in plant defense; ROS dependent ABA signaling in *Arabidopsis*.	Miller et al., 2009
AtrbohG	–	–
AtrbohH	Expressed in pollen and involved in pollen tube formation.	Potocký et al., 2007
AtrbohI	–	–
AtrbohJ	Expressed in pollen and involved in pollen tube formation.	Potocký et al., 2007

particular stimuli are also known to regulate ROS production by feedback mechanism. Some studies suggest MAPK cascades to exert positive feedback regulation on ROS production. A study in maize revealed that ABA activates 46 KDa MAPK which acts downstream of H_2O_2 and further positively regulate RBOH for H_2O_2 production (Lin et al., 2009). Another cascade positively regulating ROS production is OXI1-MPK6 which is itself activated by ROS. OXI1 (Oxidative signal-induced kinase 1) is a serine/threonine MAPKKK (Asai et al., 2008). MEKK1-MKK4-MPK3/6 is known to act upstream of NADPH oxidase stimulating ROS production in pathogen attack and H_2O_2 produced is in turn known to activate MPK3 and MPK6 (Kovtun et al., 2000). Besides positive regulation of ROS production, MAPK cascade, NDPK2-MPK3/6 is known to negatively regulate ROS production, further giving tolerance against cold, salt, and oxidative stress (Moon et al., 2003). From these data it is clear that both ROS and MAPKs regulate each other's activities but the mechanisms of their connections and basis of positive and negative feedback regulation still remains elusive.

ROS Mediated Signaling Crosstalks among Various Environmental Stresses

Mitogen activated protein kinases are important regulators of diverse cellular processes and stress responses. As an important player they show crosstalks at several points in signaling pathways in response to abiotic and biotic stresses that include ROS signaling. It is always noted that a single MAPK cascade is involved in two or more different stress responses. Also an upstream MAPK activated by a response can activate different downstream targets (Andreasson and Ellis, 2010). ROS is a common factor produced in abiotic as well as biotic stress and there are still not enough reports to clear how ROS activated MAPKs behave differently in different stress response (**Figure 2**). Below are some examples of ROS mediated activation of MAPK signaling cascades in abiotic and other environmental stresses.

In *Arabidopsis*, a MAPKKK, MEKK1 is activated upon abiotic factors like salt, cold, wound, and drought and biotic factors like bacterial and fungal elicitors (Asai et al., 2002; Teige et al., 2004; Pitzschke et al., 2009; Furuya et al., 2014; Xing et al., 2015). It is known that ROS which is being produced in these stimuli causes the activation of MEKK1. In abiotic stimuli MEKK1 activates MKK2-MPK4/6 module while in biotic stress it activates MKK4/5-MPK3/6-VIP1/ACS6 module (Asai et al., 2002; Meng and Zhang, 2013) (**Figure 2A**). Later, MEKK1-MKK1/2-MPK4 module acting upstream of MKS1/WRKY33 was also known to work in mediating pathogen related cues (Huang et al., 2000; Kong et al., 2012; **Figure 2A**). MEKK1 acting upstream of WRKY53 also showed role in plant senescence (Miao et al., 2008). ROS produced during different environmental stresses like ozone, heavy metal, biotic stress, and ABA treatment causes activation of MPK3 and MPK6 further mediating different responses (Droillard et al., 2002; Lu et al., 2002; Ahlfors et al., 2004; Liu and Zhang, 2004; Yoo et al., 2008). OXI1 is known to have different targets and show diversified activities which might suggest the crosstalk of OXI1 with other signaling pathways (Howden et al., 2011). MPK3 and MPK6 acting downstream of OXI1 mediates two different biological responses, stimulating resistance toward fungal pathogen and also play role in root development (Rentel et al., 2004; Hirt et al., 2011; Howden et al., 2011) (**Figure 2B**). Apart from OXI1, ANP1, and NDPK2 acts upstream of MPK3 and MPK6 and thus imparting tolerance to abiotic stresses like heat, cold, and salt stress (Kovtun et al., 2000).

Besides occurance of these signaling crosstalks in model plant *Arabidopsis*, it is also observed in crop plant rice. H_2O_2 is known to activate MPK3 and MPK6 in rice and gets activated by upstream kinase MKK6. This cascade show involvement in giving resistance to fungal pathogen as well as show tolerance to abiotic stresses, like heavy metal, salt, cold, and UV rays (Ding et al., 2009; Rao et al., 2010; Kumar and Sinha, 2013; Sheikh et al., 2013; Singh and Jwa, 2013; Wankhede et al., 2013a,b) (**Figure 2C**). The question that naturally comes to mind is what decides a same pathway to act in two different processes.

Above examples on ROS mediated crosstalks among MAPKs suggest that ROS produced in different environmental stresses mediates activation of similar MAPKs but the interaction within MAPKs and the final response toward these stresses becomes fundamentally different. At first point the differences comes from the ability of MAPKs to interact with different downstream targets. In this the scaffolding proteins also play a major role. But it also seems like ROS imparts an important role as messengers encoding total information for activating different responses.

ROS – a Key Player in Stress Signaling but What Determines its Specificity?

The manner in which plant responds to any environmental stress depends on the type of stress and the outcome shown is mainly specific to particular stress. ROS is a common factor to both abiotic and biotic stress. Whereas, other mechanisms like activation of components in signal transduction, transcription factors becomes specific for a stress. In above mentioned studies, we saw ROS mediated activation of MAPK cascades in both biotic and abiotic stresses. The cues from different ROS molecules activating different pathways can be integrated or can activate a specific response to a single ROS molecule. MAPK pathways show convergence at several points in signaling even though activated by single messenger produced in different stresses. Beside an important role of scaffolding proteins, different ROS species also play an important role in making this difference (Torres et al., 2002; Kwak et al., 2003; Yoshioka, 2004; Miller et al., 2009). Reports suggests that the specificity of response in each stress can be due to identity of ROS species produced by different Rboh isoforms, their level, site of production and action, diffusibility and half life (Bhattacharjee, 2010; Tripathy and Oelmüller, 2012).

Plant show 10 isoforms of *RBOH* genes involved in producing different species of ROS and thus behaving differentially in various environmental cues (**Table 1**). RBOH in plants has FAD and NADPH binding motifs at C-terminal and unlike that of mammalian homolog has two Ca^{+2} binding motifs and

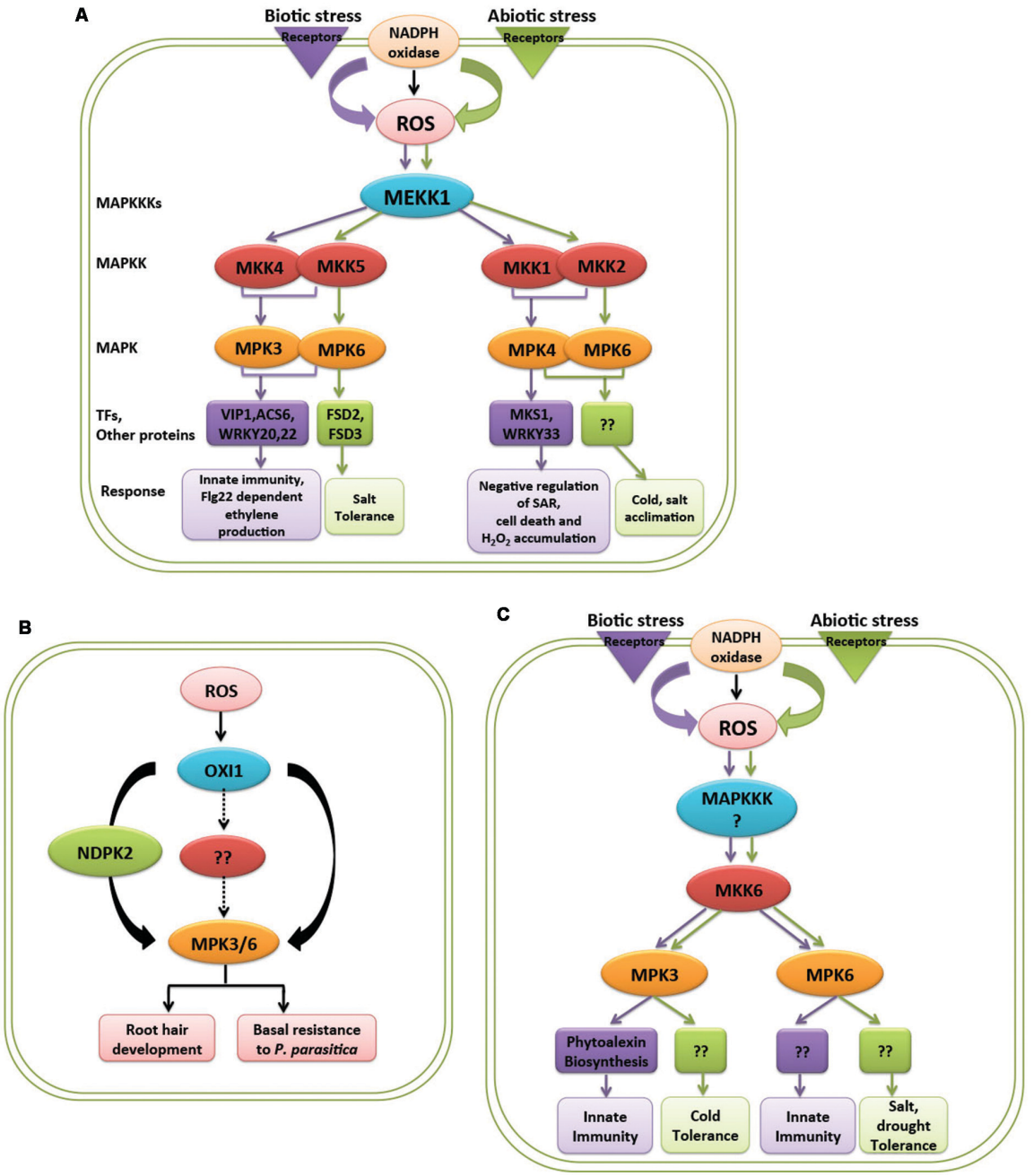


FIGURE 2 | Response of MAPK cascades activated by ROS in biotic and abiotic stresses. (A) MEKK1 is a common MAPKKK activated by ROS produced in both biotic and abiotic stress, activates different downstream components of MAPK cascade in *Arabidopsis*. **(B)** ROS activated OXI1 mediates two different responses by activating MPK3 and MPK6 in *Arabidopsis*. **(C)** ROS generated by biotic and abiotic stress mediates different responses through activation of MKK6 and MPK3/MPK6 in rice. Purple and green color represents biotic and abiotc stress, respectively.

phosphorylation target sites at N-terminal region. It is with the help of these motifs the activity of RBOH is regulated (Oda et al., 2008). The mechanism of its regulation includes phosphorylation by various signaling molecules like CDPKs, MAPKs, etc., (Lin et al., 2009). Regulation of RBOH dependent ROS production is also done with the help of amino acid residues motifs present in it. Phosphatidic acid (PA) is one of the main factors necessary for abscisic acid induced ROS production in stomatal cells. PA binding motifs present in RBOHD, i.e., Arginine residues at 149, 150, 156, and 157 are required for ROS production and closure of stomata (Zhang et al., 2009). Whereas RBOHF which is also involved in ABA dependent stomatal closure is regulated by phosphorylation of Serine 13 and Serine 174 by OPEN STOMATA 1 (OST1) (Sirichandra et al., 2009). OsRac1

involved in pathogen defense positively regulates RBOHB activity by binding to N-terminal region of RBOHB containing EF hand motifs. OsRac1 has two different forms having role in two different processes, one involved in ROS production and other in suppression of defense responses (Wong et al., 2007). This shows first step where RBOH induced ROS production is regulated in which different amino acid residues and motifs are involved in ROS production in response to different environmental responses. In addition, different RBOH homologs either single or in combination work in different stimuli giving a specific response.

Perception of different ROS species by different mechanism is still not well known and this can also explain the specific activation of downstream signal transduction by ROS in different environmental stimuli. Different locations of ROS production, different perception mechanisms and therefore different targets talks about the specificity in its response. The study on different mechanisms of action, half life and migration of different ROS species has already been carried out. The properties of most important ROS species produced in plant stress are given in **Table 2**.

The question behind the specific activation of downstream signaling components by ROS, differentially in abiotic and biotic stresses giving a specific response against a particular stress is still an enigma. The mechanism behind the specificity of MAPK activation by ROS is still elusive in plants, however, their yeast and mammalian counterparts have provided few mechanisms behind this aspect. Yeast MAPK Sty1 (Spc1, Phh1) orthologs of mammalian p38 and JNK families of MAPK play an important role in cell cycle progression and is activated in response to numerous stresses like heat, oxidative, UV, osmotic stress, and nutrient limitation (Degols and Russell, 1997). ATF transcription factor is among key substrate of Sty1 kinase. In oxidative stress conditions Sty1 not only increases phosphorylation of Atf1 but also increases its mRNA stability. Sty1 induces expression of subsets of genes in response to specific stimuli and different sets of genes are being induced by Sty1 in different concentrations of same stimuli. Low levels of H_2O_2 activates Sty1 to induce AP1 (activator protein 1) like transcription factor, whereas higher levels of H_2O_2 activates Sty1 to induce Atf1 transcription factor (Chen et al., 2008) (**Figure 3**). The difference in the downstream activation of Sty1 substrates even in response to same type of stimuli is due to H_2O_2 induced reversible oxidation of Cysteine residues of Sty1. Day and Veal (2010), suggested that oxidation of two Sty1 MAPKKK Cysteine residues Cys-153 and Cys-158 by H_2O_2 are essential for specific transcriptional activation of Atf1 transcription factor. These residues are important for hydrogen peroxide-induced gene expression and Atf1 mediated oxidative stress resistance but not for other functions of Sty1 (Day and Veal, 2010) (**Figure 3**).

Apart from the direct regulation of MAPKs by ROS, they also exert their effect indirectly through the activities of protein phosphatases and other kinases. Phosphatases are important regulators in MAPK signaling maintaining the activity of MAPKs at various points. Based on the MAPK phosphorylation sites, i.e., serine, threonine and tyrosine, phosphatases present are tyrosine phosphatases and serine/threonine phosphatases. Work carried out by scientific groups on protein tyrosine phosphatases (PTP) have suggested that reduced cysteine residue in the catalytic domain is essential for catalytic activity in plants (Gupta et al., 1998; Xu et al., 1998). A study revealed redox dependent regulation of PTP in oxidative stress. This study suggested that cysteine residues are oxidized by H_2O_2 in order to make PTP inactive and thus ultimately regulating MAPK signaling pathway (Blanchetot et al., 2002; Gupta and Luan, 2003). Another study in mice has put forward the possible mechanism in which age associated formation of ROS activates p38 MAPK pathway. Activation of p38 MAPK is done by ROS induced oxidation of thioredoxin and its release from the complex of ASK1 (apoptosis stimulating kinase 1). Reduced thioredoxin bound to ASK1 inhibits its activity to further activate p38 MAPK. The balance of free and bound ASK1 regulates the level of p38 MAPK components and their activity. This study suggests ROS mediated activation of p38 MAPK through unbound ASK1 and oxidation of thioredoxin (Hsieh and Papaconstantinou, 2006).

This exemplify that different types of ROS and different levels of ROS can react with different amino acid residues in protein and can give rise to different modified products, thus possibly explaining how ROS species can induce different sets of responses via the similar signaling pathway.

TABLE 2 | Properties and mode of action of ROS species on proteins.

ROS species	Source	Migration distance	Half life	Reaction with proteins residues	Reference
Hydrogen peroxide (H_2O_2)	NADPH oxidases and cell wall peroxidise (membrane), Chloroplast, Mitochondria, peroxisomes	1 μm	1 ms	Cystein residues	Miller et al., 2009; Bhattacharjee, 2010
Superoxide (O_2^-)	Membrane, chloroplast, Mitochondria	30 nm	1–4 μs	Fe-centers	Miller et al., 2009; Bhattacharjee, 2010
Hydroxyl radical (OH^-)	Membrane, chloroplast, Mitochondria	1 nm	1 μs	Not known	Miller et al., 2009; Bhattacharjee, 2010
Singlet oxygen (O_2)	Membrane, chloroplast, Mitochondria	30 nm	1–4 μs	Tryptophan, Histidine, Tyrosine, Methionine, Cysteine	Miller et al., 2009

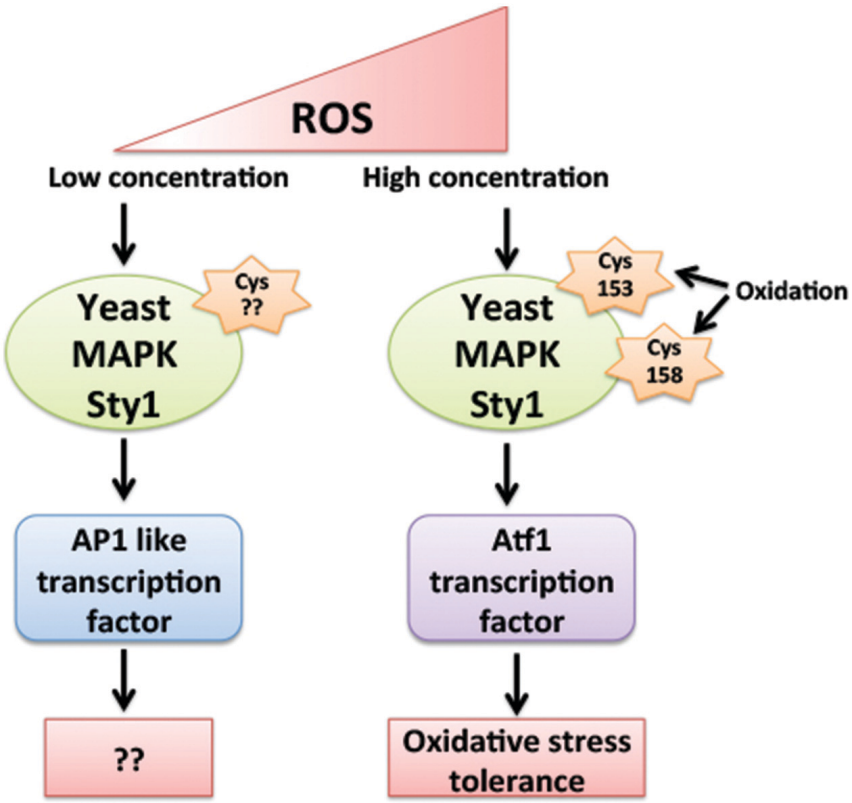


FIGURE 3 | Regulation of Yeast MAPK Sty1 (orthologs of mammalian p38 and JNK) by different levels of ROS. Sty1 induces the expression of two different transcription factors depending on its activation by different levels of H_2O_2. Low levels of H_2O_2 activates Sty1 to induce AP1 (activator protein 1) like transcription factor, whereas higher levels of H_2O_2 activates Sty1 to induce Atf1 transcription factor. The difference in the activation of Sty1 substrates is due to ROS induced oxidation of different Cysteine residues of Sty1. Oxidation of Cys-153 and Cys-158 by H_2O_2 is essential for specific induction of Atf1 transcription factor.

Controversies about ROS Dependent MAPK Activation

Earlier studies in *Arabidopsis* suggested MPK3 and MPK6 to work upstream of AtRBOH-D ROS production and H_2O_2 produced was in turn known to activate MPK3 and MPK6 (Kovtun et al., 2000; Asai et al., 2008). However, a recent report suggests that AtRBOH-D dependent ROS production and MPK3/MPK6 activation are two independent events in plant immunity. It was studied using *Atrboh* mutant that flg22 triggered ROS production was blocked whereas MPK3/MPK6 activation did not get affected. It was also reported that pretreatment with SA enhance ROS production independently of MPK3/6 activation (Xu et al., 2014).

Conclusion

Mitogen activated protein kinases are important regulators of diverse cellular processes and stress responses, showing crosstalks at several points in signaling. Single MAPK cascade is involved in two or more different stress responses. Also an upstream MAPK activated by multiple responses show different downstream targets and thus different response. ROS is a common messenger produced in abiotic as well as biotic stress activating MAPK pathways and it is still not clear in plant how ROS activated MAPKs behave differently in different stress response. Also ROS and MAPKs show feedback loop to regulate each other's activities but the mechanisms and basis of positive and negative feedback regulation still remains elusive.

On the basis of the information available in the literature, it becomes clear that the ROS by itself has ability to regulate the downstream signaling pathway components and to impart a specific response toward a particular stress. It can activate a similar MAPK cascade in different stresses and can exert different responses accordingly. It is understood that the regulatory mechanisms of MAPKs by ROS are more elaborated in yeast and mammals, whereas in plants better understanding of the regulatory functions of ROS and MAPK cascades is required.

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REVIEW
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Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses

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Transient changes in intracellular Ca^{2+} concentration have been well recognized to act as cell signals coupling various environmental stimuli to appropriate physiological responses with accuracy and specificity in plants. Calmodulin (CaM) and calmodulin-like proteins (CMLs) are major Ca^{2+} sensors, playing critical roles in interpreting encrypted Ca^{2+} signals. Ca^{2+}-loaded CaM/CMLs interact and regulate a broad spectrum of target proteins such as channels/pumps/antiporters for various ions, transcription factors, protein kinases, protein phosphatases, metabolic enzymes, and proteins with unknown biochemical functions. Many of the target proteins of CaM/CMLs directly or indirectly regulate plant responses to environmental stresses. Basic information about stimulus-induced Ca^{2+} signal and overview of Ca^{2+} signal perception and transduction are briefly discussed in the beginning of this review. How CaM/CMLs are involved in regulating plant responses to abiotic stresses are emphasized in this review. Exciting progress has been made in the past several years, such as the elucidation of Ca^{2+}/CaM-mediated regulation of AtSR1/CAMTA3 and plant responses to chilling and freezing stresses, Ca^{2+}/CaM-mediated regulation of CAT3, MAPK8 and MKP1 in homeostasis control of reactive oxygen species signals, discovery of CaM7 as a DNA-binding transcription factor regulating plant response to light signals. However, many key questions in Ca^{2+}/CaM-mediated signaling warrant further investigation. Ca^{2+}/CaM-mediated regulation of most of the known target proteins is presumed based on their interaction. The downstream targets of CMLs are mostly unknown, and how specificity of Ca^{2+} signaling could be realized through the actions of CaM/CMLs and their target proteins is largely unknown. Future breakthroughs in Ca^{2+}/CaM-mediated signaling will not only improve our understanding of how plants respond to environmental stresses, but also provide the knowledge base to improve stress-tolerance of crops.

Keywords: calcium signal, calmodulin, calmodulin-like protein, calmodulin-binding protein, signal transduction, abiotic stress

Introduction

As sessile organisms, plants encounter various types of environmental stresses, which are generally classified into biotic stresses such as insect and pathogen attacks, and abiotic stresses such as unfavorable temperature, lack of or excessive amounts of water, salinity, heavy metal toxicity, chemical toxicity, and nutrient deficiency. On the other hand, the process of industrialization

inevitably brings many detrimental effects to the environment. Poorly controlled release of wastes from industrial processes and human life not only adds various toxic chemicals to our water and soil but also release harmful gasses into the atmosphere. Obviously, human activities are creating environmental challenges, making sustained crop production difficult. Classic agricultural technologies such as irrigation, applications of fertilizer, insecticides, fungicides, and chemical phytoprotectants have helped to improve crop yield, but the effects are limited, the costs are high and the impacts on the ecosystems and human health are undesirable and dangerous. Understanding how plants perceive and respond to various environmental stresses provides the necessary platform to create crop varieties which could fit better into the challenging environments, and has become one of the most important tasks for plant scientists around the world.

Calcium is one of the most abundant elements on earth. Ca^{2+} concentration outside the plasma membrane is usually at millimolar level. Since Ca^{2+} can form insoluble compounds with phosphate derivatives and complex with macromolecules, high levels of cytosolic Ca^{2+} are toxic to cells. Ca^{2+} concentration in the cytoplasm and nucleus is usually maintained at 50–100 nM under resting conditions (Reddy, 2001; Yang and Poovaiah, 2003). Ca^{2+} gradient across the plasma membrane as well as inner membrane system are involved in cell signaling process controlled by stimulus responsive Ca^{2+} permeable channels, Ca^{2+} pumps and Ca^{2+}/H^{+} exchangers (Reddy, 2001; Kudla et al., 2010). Accumulating evidence reveals that various external stimuli such as gravity, light, cold, heat, drought, water-logging (hypoxia), salt, wind, touch, wounding, and pathogen attacks can quickly induce elevations in cytosolic Ca^{2+} concentration (Poovaiah and Reddy, 1993; Evans et al., 2001; Reddy, 2001; Snedden and Fromm, 2001; Zhu, 2001). Signal-induced nuclear Ca^{2+} changes have also been documented (Van Der Luit et al., 1999; Pauly et al., 2000), but they are not as well studied as cytosolic Ca^{2+} transients (Reddy et al., 2011). The excessive amount of Ca^{2+} in cytoplasm is quickly moved out of the cell or pumped back into the endogenous Ca^{2+} reservoirs such as vacuole and endoplasmic reticulum (ER) through the involvement of Ca^{2+} pumps and Ca^{2+}/H^{+} exchangers distributed on the plasma membrane and inner membrane system (Reddy, 2001; Kudla et al., 2010). Interestingly, the transient changes of intracellular Ca^{2+} concentration triggered by various stimuli differ from each other in terms of amplitude, duration, frequency, and spatial distribution inside the cell; and these stimulus-specific Ca^{2+} transients are named calcium signatures by Webb et al. (1996). Stimulus-specific signals are decoded by downstream effector proteins to generate specific or overlapping responses (Poovaiah et al., 2013). These effectors include Ca^{2+} sensor proteins which are represented by three major types in plants, namely calmodulin (CaM)/CaM-like (CML) proteins, calcium-dependent protein kinases (CDPKs) and calcineurin B like (CBL) proteins (Luan, 2009). In this review, our primary focus will be limited to CaM/CMLs and their important roles in plant abiotic stress signaling and responses.

CaMs AND CMLs

Calmodulin is a ubiquitous Ca^{2+}-binding protein which exists in all eukaryotes (Snedden and Fromm, 1998; Yang and Poovaiah, 2003; McCormack et al., 2005; Kim et al., 2009; Du et al., 2011). It is a small acidic protein composed of two pairs of EF-hands located at both the N- and C-terminus. In *Arabidopsis*, seven genes encode four CaM isoforms (CaM1/4; CaM2/3/5; CaM6; CaM7), which differ only in one to five amino acid residues (McCormack and Braam, 2003; McCormack et al., 2005). It has been reported that different CaM isoforms differ in binding and regulating downstream effectors (Lee et al., 2000; Yoo et al., 2005). The slight differences in their structural features may have considerable impacts on their binding to targets (Yamniuk and Vogel, 2005).

In addition to canonical CaM, there are 50 genes coding for CaM-like proteins in the *Arabidopsis* genome which are made of varying number of EF hands and share at least 16% of overall sequence identity with canonical CaM (McCormack and Braam, 2003). Similarly, five *CaM* and 32 *CML* genes, respectively are reported in the rice genome (Boonburapong and Buaboocha, 2007). Despite having four EF hands, most CMLs show low (less than 50%) overall similarity to CaMs (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007; Perochon et al., 2011). Several *Arabidopsis* CMLs, including CML37, 38, 39, and 42 displayed an electrophoretic mobility shift in the presence of Ca^{2+}, indicating that, like CaMs, CMLs also act as Ca^{2+} sensors (Vanderbeld and Snedden, 2007; Dobney et al., 2009). Besides EF-hands, CaMs and CMLs do not carry any known functional domain, and hence usually have no enzymatic or biochemical functions. So far the only exception is CaM7 from *Arabidopsis* which was reported to specifically bind Z-/G-box in a Ca^{2+}-dependent manner and act as a transcription factor to regulate light-responsive gene expression and light morphogenesis (Kushwaha et al., 2008). Therefore, identifying CaM/CML targets and understanding the impacts of CaM/CML-binding on their functional behaviors are the major challenges in deciphering the functional significance of CaM/CMLs at molecular, biochemical, and physiological levels.

It is well-documented that Ca^{2+}-binding-induced conformational changes in CaMs and CMLs usually increase their binding affinity to downstream targets through hydrophobic and electrostatic interactions (Snedden and Fromm, 1998; Hoeflich and Ikura, 2002). A stretch of 16–35 amino acids in the target proteins called CaM-binding domain (CaMBD) is usually necessary and sufficient for its interaction with CaM (Rhoads and Friedberg, 1997; Hoeflich and Ikura, 2002). In some cases, CaM interacts with its target proteins in a Ca^{2+}-independent manner, and this kind of interaction requires that the target proteins carry an IQ motif, a stretch of amino acids fitting a conserved pattern of IQXXX(R/K)GXXXR where I could be replaced with "FLV" and "X" represents any amino acid residue (Hoeflich and Ikura, 2002; Yamniuk and Vogel, 2004). CMLs could follow similar models to interact with their targets; however, this assumption requires experimental verification. Usually, CaMBDs are not conserved in their primary structure, however, most of the Ca^{2+}-dependent CaMBD peptides share a conserved secondary

structure, a basic amphipathic helix with hydrophobic residues arranged on one side and positively charged residues arranged on the other side (Snedden and Fromm, 2001; Du and Poovaiah, 2004). Hence, most CaM and CML target proteins have to be identified empirically.

Targets of CaMs and CMLs

As mentioned above, the interactions between CaM/CMLs and target proteins are usually Ca^{2+}-dependent; regular strategies used for detection of protein–protein interaction including yeast-two-hybrid and coimmunoprecipitation are not effective and fruitful in identifying CaM/CML-binding proteins. The majority of the CaM-binding proteins (CBPs) from plants were identified by screening cDNA expression libraries with labeled CaM as probes (usually ^{35}S-labled; Fromm and Chua, 1992; Reddy et al., 1993; Yang and Poovaiah, 2000b). Another effective approach to identify CBPs is utilizing protein microarray (Popescu et al., 2007); however, false positive identification is still a major concern and making protein chips with adequate coverage is currently a challenge. Accumulated results indicated that CaM bind to a variety of CBPs in plants, which include kinases, phosphatases, transcription factors, receptors, metabolic enzymes, ion channels and pumps, and cytoskeletal proteins (Snedden and Fromm, 2001; Bouche et al., 2005; Kim et al., 2009; Du et al., 2011; Reddy et al., 2011). Hence, it is reasonable to conclude that, in most cases, CaMs and CMLs act as multifunctional regulatory proteins, and their functional significance is materialized through the actions of their downstream target proteins. CBPs with well-defined CaM-binding domain, CaM-binding property and involved in plant responses to abiotic stresses are listed in **Table 1**.

Ca^{2+}/CAM and ROS Crosstalk in Plant Response to Stresses

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (·OH) are usually produced in various physiological processes and serve as a class of second messengers (Van Breusegem et al., 2001; Apel and Hirt, 2004). While controlled production of ROS is essential to signal appropriate actions to protect plants from various environmental stresses, excessive accumulation of ROS causes damages to plant cells. Oxidative stress is defined as disruption of the cellular redox balance, which could be triggered by a wide range of biotic and abiotic stimuli (Rentel and Knight, 2004). Because of its long half-life and excellent permeability, H_2O_2 is broadly accepted as the major form of ROS in plant cells. It is well known that H_2O_2 can trigger increases in cytosolic Ca^{2+} by activating the Ca^{2+}-permeable channels (Price et al., 1994; Rentel and Knight, 2004). On the other hand, H_2O_2 production during oxidative burst is also dependent on continuous Ca^{2+} influx, which activates not only the NADPH oxidase, an EF-hand containing enzyme on the plasma membrane (Xing et al., 1997), but also the CaM-binding NAD kinase (NADK), which supplies NADP cofactor for ROS production through NADPH oxidase (Harding et al., 1997; Karita et al., 2004; Turner et al., 2004).

In addition, early studies from heat-stressed maize seedling suggested that ROS homeostasis and the entire antioxidant system including catalase, superoxide dismutase (SOD) and ascorbate peroxidase, could be regulated by Ca^{2+} influx and intracellular CaM (Gong et al., 1997a). Later plant catalases, a class of H_2O_2 scavenger enzymes catalyzing its degradation to water and oxygen was found to bind CaM in a Ca^{2+}-dependent manner (Yang and Poovaiah, 2002b). The activity of the *Arabidopsis* CAT3 is stimulated by Ca^{2+}/CaM rather than Ca^{2+} or CaM alone, but catalases from other organisms such as *Aspergillus niger*, human and bovine, do not interact with CaM (Yang and Poovaiah, 2002b). A peroxidase from *Euphorbia latex*, was also reported to be a CBP activated by Ca^{2+}/CaM (Medda et al., 2003; Mura et al., 2005). Evidence also suggests that another class of ROS-scavenging enzyme SOD could be regulated by CaM in maize, although the specific *SOD* gene has not been cloned (Gong and Li, 1995). The critical role of Ca^{2+}/CaM in balancing ROS actions was further supported by the observation that the oxidative damage caused by heat stress in *Arabidopsis* seedlings is exacerbated by pretreatment with CaM inhibitors (Larkindale and Knight, 2002).

In addition to these direct regulations on ROS homeostasis, Ca^{2+}/CaM-mediated signaling is also well known to regulate ROS-related signal transduction at various stages. Maize *CAP1* encoding a novel form of CaM-regulated Ca^{2+}-ATPase was shown to be induced only during early anoxia, indicating its possible role in oxygen-deprived maize cells (Subbaiah and Sachs, 2000). CaM may also participate indirectly in regulating ROS content through the CaM-regulated λ-aminobutyrate (GABA) synthesis and the GABA shunt metabolic pathway (Bouche et al., 2003). Recently, it was demonstrated that ZmCCaMK and OsDMI3 (also called OsCCaMK) from maize (*Zea mays*) and rice (*Oryza sativa*), respectively, play a critical role in ABA-induced antioxidant actions (Ma et al., 2012; Shi et al., 2012), suggesting a role for CCaMK in plant oxidative stress response. *Arabidopsis* MPK8 was found to be activated by CaM and activated MPK8 suppresses wound-induced ROS accumulation via transcriptional control of *RbohD* expression, revealing a novel mechanism for CaM-mediated signaling to fine-tune ROS homeostasis under wounding stress (Takahashi et al., 2011). Interestingly, some genes encoding CaM-binding transcription factors (*CAMTAs*) and co-factor (*AtBTs*) are responsive to H_2O_2, suggesting that CaM-mediated signaling could directly regulate gene expression in plant responses to oxidative cues (Yang and Poovaiah, 2002a; Du and Poovaiah, 2004; Wang et al., 2015).

CaM/CML-Mediated Regulation of Abiotic Stress Signaling

Heat Stress

Prolonged high temperature is usually lethal to all organisms; fluctuations in temperature above optimal level, usually called heat shock (HS), impose major stress affecting plant growth and

TABLE 1 | Involvement of calmodulins (CaMs), CaM-like proteins (CMLs), and CaM-binding proteins (CBPs) in plant responses to diverse abiotic stresses.

Protein category	Protein name	Plant species	Stress	Reference
CaM	AtCaM3	*Arabidopsis thaliana*	Heat	Liu et al. (2005), Zhang et al. (2009), Xuan et al. (2010)
	AtCaM7	*A. thaliana*	Heat	Liu et al. (2005)
	OsCaM1-1	*Oryza sativa*	Heat	Wu et al. (2012)
	GmCaM4/5	*Glycine max*	Salt	Park et al. (2004)
	ZmCaM	*Zea mays*	Heat	Gong et al. (1997a,b)
	MBCaM1	*Vigna radiata*	Salt	Botella and Arteca (1994)
	NpCaM1	*Nicotiana plumbaginifolia*	Cold	Van Der Luit et al. (1999)
	TaCaM1-2	*Triticum aestivum*	Heat	Liu et al. (2003)
	RsCaM	*Raphanus sativus*	Heavy metal	Rivetta et al. (1997)
CML	AtCML8	*A. thaliana*	Salt	Park et al. (2010)
	AtCML9	*A. thaliana*	ABA, drought, salt	Magnan et al. (2008)
	AtCML12/TCH3	*A. thaliana*	Heat	Braam (1992)
	AtCML18/CaM15	*A. thaliana*	Salt	Yamaguchi et al. (2005)
	AtCML24/TCH2	*A. thaliana*	Heat, cold, H_2O_2, ABA, drought, ion stress	Braam (1992), Polisensky and Braam (1996), Delk et al. (2005)
	AtCML37/38/39	*A. thaliana*	Salt, ABA	Vanderbeld and Snedden (2007)
	AtCML42	*A. thaliana*	Drought, ABA	Vadassery et al. (2012)
	OsMSR2	*O. sativa*	Cold, drought, heat, salt, ABA	Xu et al. (2011)
Protein phosphatase	AtPP7	*A. thaliana*	Heat	Kutuzov et al. (2001), Liu et al. (2007)
Protein kinase	AtCBK3/CRK1	*A. thaliana*	Heat	Wang et al. (2004), Liu et al. (2008)
	AtCRCK1	*A. thaliana*	Cold, salt, ABA, H_2O_2	Yang et al. (2004)
	AtCRLK1	*A. thaliana*	Cold	Yang et al. (2010a,b)
	OsCCaMK/DMI3	*O. sativa*	ABA, H_2O_2, dehydration, oxidative stress	Shi et al. (2012)
	ZmCCaMK	*Z. mays*	ABA, oxidative stress	Ma et al. (2012)
	TaCCaMK	*Triticum aestivum*	ABA, salt	Yang et al. (2011)
	PsCCaMK	*Pisum sativum*	Cold, salt	Pandey et al. (2002)
	PvNADK	*Phaseolus vulgaris*	Cold	Ruiz et al. (2002)
	NtNADK	*N. tabacum*	Oxidative stress, pathogen,	Harding et al. (1997), Karita et al. (2004)
Transcription factor and co-factor	AtSR1/CAMTA3	*A. thaliana*	Cold	Doherty et al. (2009), Du et al. (2009), Kim et al. (2013)
	AtSR2/CAMTA1	*A. thaliana*	Drought, cold, salt, heat	Doherty et al. (2009), Kim et al. (2013)
	AtSR4/CAMTA2	*A. thaliana*	Cold	Kim et al. (2013)
	SlSR1L	*Solanum lycopersicum*	Drought	Li et al. (2014)
	AtMYB2	*A. thaliana*	Salt, drought	Abe et al. (2003), Yoo et al. (2005)
	AtABF2/AREB1	*A. thaliana*	ABA, drought	Popescu et al. (2007), Yoshida et al. (2010)
	AtCBP60g	*A. thaliana*	ABA, drought	Wan et al. (2012)
	AtGTL1	*A. thaliana*	Dehydration	Yoo et al. (2010)
	AtGT2L	*A. thaliana*	Cold, salt, ABA	Xi et al. (2012)
	PtGTL1	*Populus tremula*	Drought	Weng et al. (2012)
	AtBT1-5	*A. thaliana*	Salt, cold, H_2O_2, SA	Du and Poovaiah (2004)
	AtBT2	*A. thaliana*	Cold, ABA, H_2O_2	Mandadi et al. (2009)
Ion transportor	AtCNGC1	*A. thaliana*	Heavy metal	Sunkar et al. (2000)
	AtNHX1	*A. thaliana*	Salt	Apse et al. (1999), Yamaguchi et al. (2005)
	AtACA4	*A. thaliana*	Salt	Boursiac et al. (2010)
	SCA1	*Glycine max*	Salt	Chung et al. (2000)
	NtCBP4	*N. tabacum*	Heavy metal	Arazi et al. (1999), Sunkar et al. (2000)
	ZmCAP1	*Z. mays*	Anoxia	Subbaiah and Sachs (2000)
	MCamb1/2	*Physcomitrella patens*	Cold, osmotic stress, ABA	Takezawa and Minami (2004)

(Continued)

TABLE 1 | Continued

Protein category	Protein name	Plant species	Stress	Reference
Metabolic enzyme	AtCAT3	*A. thaliana*	Oxidative stress	Yang and Poovaiah (2002b)
	ZmSOD	*Z. mays*	Oxidative stress	Gong et al. (1997a)
	OsGAD	*O. sativa*	Anoxia	Aurisano et al. (1995)
	ELP/EcPeroxidase	*Euphorbia characias*	H_2O_2	Medda et al. (2003), Mura et al. (2005)
	BiGly-I	*Brassica juncea*	Salt, dehydration, heavy metal	Deswal and Sopory (1999), Veena et al. (1999), Singla-Pareek et al. (2003)
Unclassified	AtCaMBP25	*A. thaliana*	Osmotic stress	Perruc et al. (2004)
	AtIQM1	*A. thaliana*	ABA, dehydration	Zhou et al. (2012)
	ZmHsp70	*Z. mays*	Heat	Sun et al. (2000)
	DgHsp70	*Orchardgrass*	Heat	Cha et al. (2012)
	pTCB48	*N. tabacum*	Heat	Lu et al. (1995)

productivity. Almost all organisms including plants synthesize HS proteins (HSPs), a class of chaperons to assure normal function of various client proteins under adversely high temperature conditions. It was observed long ago that HS induced a quick and strong increase in cytosolic Ca^{2+} in tobacco (Gong et al., 1998). Expression of CaM in the maize coleoptiles was found to be remarkably induced during HS and was affected by Ca^{2+} level, suggesting that Ca^{2+} and CaM may be involved in the acquisition of HS-induced thermotolerance (Gong et al., 1997b). Liu et al. (2003) observed an increase in intracellular Ca^{2+} within one min after wheat was subjected to 37°C HS. Expression of CaM mRNA and protein was both induced by HS in the presence of Ca^{2+}, and expression of *HSP26* and *HSP70* was stimulated by exogenous application of Ca^{2+}. HS-induced expression of *CaM* was 10 min earlier than that of *HSP*s, and both were suspended by pharmacological reagents which interfere with Ca^{2+} signaling. These results indicate that Ca^{2+} and CaM are directly involved in HS signaling (Liu et al., 2003). The Ca^{2+}/CaM signaling system was also proposed to be involved in the induction of *HSP* genes in *Arabidopsis* (Liu et al., 2005). Using molecular and genetic tools, Zhang et al. (2009) found that *Arabidopsis* AtCaM3 was involved in the Ca^{2+}/CaM-mediated HS signal transduction pathway. *atcam3* loss-of-function mutant showed a pronounced decrease in thermotolerance after 50 min of incubation at 45°C. The compromised thermotolerance of *atcam3* mutant could be rescued by functional complementation with 35S promoter driven AtCaM3, and overexpression of *AtCaM3* in wild-type (WT) background increased thermotolerance of the transgenic plants. Furthermore, the DNA-binding activity of HS transcription factors and the expression of tested HS genes at both mRNA and protein levels were shown to be down-regulated in *atcam3* null mutant and up-regulated in its overexpressing lines upon HS treatment (Zhang et al., 2009). A role for CaM in HS signaling was also demonstrated in rice (Wu and Jinn, 2012; Wu et al., 2012). HS was reported to induce biphasic cytosolic Ca^{2+} transients, and this signature feature was found to be reflected in the HS-induced expression of *OsCaM1-1*. OsCaM1-1 was observed to localize to the nucleus and overexpression of *OsCaM1-1* in *Arabidopsis* resulted in enhanced thermotolerance which coincided with elevated expression of HS-responsive *AtCBK3*, *AtPP7*, *AtHSF,* and *AtHSP* at a non-inducing temperature. Nitric oxide (NO) level in plants was found to be elevated by high temperatures (Gould et al., 2003), and exogenous application of NO donor provides effective protection to plants under heat stress (Uchida et al., 2002; Song et al., 2006). However, for a long time it was unknown how NO is involved in protecting plants from damage by HS. Recently, *Arabidopsis* CaM3 was reported to act as a downstream factor of NO in activation of HS transcription factors, accumulation of HSPs and establishment of thermotolerance (Xuan et al., 2010).

Calmodulin-binding proteins have also been shown to play a crucial role in mediating plant responses to heat stress. *pTCB48* encoding a CBP was isolated by screening a cDNA expression library constructed from tobacco cell cultures subjected to HS, and its expression was strongly induced by HS treatment, suggesting a role in HS response (Lu et al., 1995). Maize cytosolic Hsp70 was identified to bind CaM in the presence of Ca^{2+} and could inhibit the activity of CaM-dependent NADK in a concentration-dependent manner, but its possible function in HS response has not been elucidated (Sun et al., 2000). Recently, DgHsp70, a homolog of cytosolic Hsp70 from orchardgrass (*Dactylis glomerata*) was also found to bind AtCaM2 in the presence of Ca^{2+}, and the binding of Ca^{2+}/CaM decreased the ATPase and foldase activities of this chaperon protein (Cha et al., 2012). PP7 is a ser/thr protein phosphatase which interacts with CaM in a Ca^{2+}-dependent manner (Kutuzov et al., 2001). *Arabidopsis AtPP7* was induced by HS and its knockout mutant is impaired in thermotolerance, while the overexpression of *AtPP7* results in increased thermotolerance and increased expression of *AtHSP70* and *AtHSP101* following HS treatment (Liu et al., 2007). Interestingly, AtPP7 was also found to interact with HS transcription factor AtHSF1 implying that AtPP7 could also regulate the expression of HSP genes via AtHSF1 (Liu et al., 2007). However, the mechanistic detail by which AtPP7 dephosphorylates and regulates downstream substrates such as AtHSF1 is not clear. *Arabidopsis* AtCRK1 (CDPK-related protein kinase 1, also called AtCBK3) was identified as a Ca^{2+}-dependent CBP (Wang et al., 2004). Liu et al. (2008) found that AtCBK3 activates HSFs which further regulate HS gene expression by binding to HS elements.

Cold Stress

Ca^{2+} has been recognized as a vital second messenger coupling cold stress to specific plant responses (Knight et al., 1991; Dodd et al., 2010). Researchers found that cold shock and wind initiate Ca^{2+} transients in both cytosol and nucleus in transgenic tobacco (*Nicotiana plumbaginifolia*) seedlings expressing aequorin, and the expression of *NpCaM-1* is induced by both cold shock and wind but mediated by distinct Ca^{2+} signaling pathways operating predominantly in the cytoplasm or in the nucleus (Van Der Luit et al., 1999). Transgenic *Arabidopsis* plants overexpressing *CaM3* showed decreased levels of *COR* (cold regulated) transcripts suggesting CaM may function as a negative regulator of cold-induced gene expression (Townley and Knight, 2002). Genes encoding CMLs, such as *AtCML24/TCH2* and *OsMSR2* (*O. sativa* Multi-Stress-Responsive gene2, a novel CML gene), were also found to be induced by cold treatment and thus, likely participate in transducing cold-induced Ca^{2+} signals (Polisensky and Braam, 1996; Delk et al., 2005; Xu et al., 2011).

In addition, as downstream effectors of Ca^{2+}/CaM-mediated signaling, CBPs are also known to be involved in plant responses to cold stress. Ca^{2+}/CaM-regulated receptor-like kinase CRLK1, which is mainly localized in the plasma membrane, was found to be involved in cold tolerance (Yang et al., 2010b). CRLK1 carries two CaM-binding sites in both N- and C-termini with affinities for Ca^{2+}/CaM of 25 and 160 nM, respectively (Yang et al., 2010b). *crlk1* knockout mutant plants grow and behave like WT plants under regular conditions, but are more sensitive to chilling and freezing treatments than WT plants (Yang et al., 2010b). In addition, cold response genes such as *CBF1*, *RD29A*, *COR15a,* and *KIN1* showed delayed responses to cold in *crlk1,* suggesting a positive role for CRLK1 in regulating cold tolerance. MEKK1, which is a member of the MAP kinase kinase kinase family, was shown to interact with CRLK1 both *in vitro* and *in planta* (Yang et al., 2010a). Knockout mutation of CRLK1 abolished the cold-triggered MAP kinase activities, and altered cold-induced expression of genes involved in MAP kinase signaling (Yang et al., 2010a). Therefore, Ca^{2+}/CaM-regulated CRLK1 may modulate cold acclimation through MAP kinase cascade in plants. Other CaM-binding kinases are also suggested to be involved in cold acclimation. The expression of *PsCCaMK* in pea (*Pisum sativa*) roots was found to be up-regulated by low temperature or salinity stress (Pandey et al., 2002), and the activity of the Ca^{2+}/CaM-dependent NADK was found to be increased by cold shock (Ruiz et al., 2002).

AtSRs/CAMTAs belong to one of the best characterized classes of CaM-binding transcription factors in plants and animals (Reddy et al., 2000; Yang and Poovaiah, 2000a, 2002a; Bouche et al., 2002; Choi et al., 2005; Du et al., 2011; Reddy et al., 2011). In an attempt to understand transcriptional control of CBF2 (a critical regulator of cold acclimation), Doherty et al. (2009) compared the promoter sequence of three CBFs and found seven conserved DNA motifs CM1 to 7 in their promoters. CM2 is a typical AtSR1/CAMTA3 recognition motif, and importantly, the expression of *CBF2* was found to be positively regulated by AtSR1/CAMTA3. Although *camta3* knockout mutant had no phenotypic change under cold stress, *camta1/camta3* double mutant was found to have reduced freezing tolerance (Doherty et al., 2009). Recently, AtCAMTA1, AtCAMTA2, and AtCAMTA3 were shown to participate in cold tolerance by cooperatively inducing *CBF* genes and repressing SA biosynthesis (Kim et al., 2013). These results filled a long-standing knowledge gap between cold induced Ca^{2+} transients and cold-regulated gene expression.

Several *Arabidopsis* MADS box transcription factors were identified as putative CBPs by a high throughput proteomics approach (Popescu et al., 2007). Expression of some of these MADS box genes, including *AGL3*, *AGL8*, *AGL15*, and *AGL32*, was reported to be suppressed by cold stress (Hannah et al., 2005), implying a role for Ca^{2+} signal to regulate cold responses through the MADS proteins, however, whether and how Ca^{2+}/CaM regulates MADS box transcription factors remain to be addressed. The expression of *CBF2* is down-regulated in transgenic *Arabidopsis* plants constitutively expressing *AGL15*, in comparison to WT plants (Hill et al., 2008). GT factors are plant-specific transcription factors sharing a conserved trihelix DNA-binding domain that specifically interacts with GT *cis*-elements (Wang et al., 2014). Recently, AtGT2L, a classic member of the GT-2 subfamily, was identified to encode a Ca^{2+}-dependent CBP and it is responsive to cold, salt and ABA treatments (Xi et al., 2012). Furthermore, overexpression of *AtGT2L* resulted in elevated expression levels of cold- and salt-specific marker genes *RD29A* and *ERD10*, both in basal and chilling- or salt-treated conditions. These results indicated that Ca^{2+}/CaM-binding AtGT2L is involved in plant responses to cold and salt stresses (Xi et al., 2012).

Salt and Drought Stress

High salinity and drought are the major environmental stresses frequently experienced by plants, and both impose osmotic stress on plant cells. Osmotic stress induces a series of responses at the molecular and cellular levels and a primary event is an increase in the cytosolic Ca^{2+} concentration and subsequent transduction of Ca^{2+} signals that promotes appropriate cellular responses in an effort to mitigate potential damages (Xiong and Zhu, 2002; Zhu, 2002). In addition to the well documented salt-overly-sensitive (SOS) pathway (Chinnusamy et al., 2004; Mahajan et al., 2008), CaM-mediated signaling is also actively involved in plant response to osmotic stress (Bouche et al., 2005). Overexpression of a salt-induced CaM gene from soybean, *GmCaM4*, in *Arabidopsis* confers salt stress tolerance through the up-regulation of DNA-binding activity of a MYB transcription factor MYB2. Interestingly, MYB2 was also reported to interact with CaM in a Ca^{2+}-dependent manner and regulate salt and dehydration responsive genes (Abe et al., 2003; Yoo et al., 2005). *AtCML8*, an ortholog of *GmCaM4*, was also found to be induced by salt treatment (Park et al., 2010). Another similar CML protein AtCML9 was found to be involved in osmotic stress tolerance through ABA-mediated pathways (Magnan et al., 2008). *AtCML9* was readily induced by abiotic stress and ABA; knock-out mutant *atcml9* showed a hypersensitive response to ABA during seed germination and seedling growth stages, and exhibited enhanced tolerance to salt and dehydration stresses. Furthermore, expression of several stress and ABA-responsive

genes including *RAB18*, *RD29A,* and *RD20* was altered in *atcml9*. The rice CML gene *OsMSR2* was also suggested to be involved in ABA-mediated salt and drought tolerance (Xu et al., 2011). As the most abundant vacuolar Na^+-proton exchanger in *Arabidopsis*, Na^+/H^+ exchanger 1 (AtNHX1) regulates various cellular activities such as maintaining pH, ion homeostasis, and protein trafficking. Yamaguchi et al. (2005) found that AtCaM15 (also called AtCML18) is localized in the vacuolar lumen and interacts with the C-terminus of AtNHX1. The interaction between AtCaM15 and AtNHX1 is affected by both Ca^{2+} and pH, and the binding of AtCaM15 to AtNHX1 alters the Na^+/K^+ selectivity of the exchanger by decreasing its Na^+/H^+ exchange speed. The interaction between AtCaM15 and AtNHX1 suggests the presence of Ca^{2+}-pH-dependent signaling components in the vacuole, which are involved in mediating plant responses to salt stress. In addition to the above mentioned CaM/CMLs, *CML37*, *CML38,* and *CML39* are also responsive to various stimuli, including salt, drought, and ABA (Vanderbeld and Snedden, 2007), but whether they are also involved in osmotic stress tolerance remains to be identified.

A few CaMBPs are involved in the signaling pathways triggered by salt, drought or osmotic stresses. Wheat (*Triticum aestivum*) *TaCCaMK* was down-regulated by ABA, salt and PEG treatments, and overexpression of *TaCCaMK* reduces ABA sensitivity of *Arabidopsis*, indicating that TaCCaMK is a negative regulator of ABA-mediated signaling (Yang et al., 2011). *Arabidopsis AtACA4* encoding a CaM-regulated Ca^{2+}-ATPase was found to be localized to small vacuoles, which is similar to PMC1, the yeast vacuolar Ca^{2+}-ATPase, and AtACA4 confers tolerance against osmotic stresses imposed by high NaCl, KCl, and mannitol, when expressed in the yeast K616 strain lacking Ca^{2+} transporter PMC1 (Geisler et al., 2000). A CaM-regulated Ca^{2+}-ATPase gene from soybean, *SCA1*, was found to be induced by salt stress (Chung et al., 2000). Methylglyoxal (MG), a byproduct of carbohydrate and lipid metabolism and a potent mutagenic chemical known to arrest growth, reacts with DNA and protein and increases sister chromatid exchange; and glyoxalase enzymes, including glyoxalase I (gly-I) and glyoxalase II (gly-II), catalyze the detoxification of MG with the involvement of glutathione (GSH; Thornalley, 1990). Glyoxalase I from *Brassica juncea* (BjGly-I) was reported to be a Ca^{2+}/CBP, and its enzymatic function is significantly stimulated by Ca^{2+}/CaM binding (Deswal and Sopory, 1999). The expression of *BjGly-I* is induced by salt, dehydration and heavy metal stresses; ectopic expression of *BjGly-I* in tobacco conferred remarkable tolerance to exogenous MG and high salt stress (Veena et al., 1999). AtCaMBP25 was identified to be a CaM-binding nuclear protein and is induced by dehydration, low temperature or high salinity. Overexpression of *AtCaMBP25* compromised the tolerance of transgenic plants to osmotic stress, and silencing *AtCaMBP25 via* antisense approach increased plant tolerance to osmotic stress. These results suggested that AtCaMBP25 functions as a negative regulator in plant tolerance to osmotic stress, revealing a connection coupling Ca^{2+} signals to plant responses to osmotic stresses (Perruc et al., 2004).

Ca^{2+}/CaM-regulated transcription factors are also involved in plant response to salt and drought stresses. A few *CAMTA* genes from *Arabidopsis* and soybean are up-regulated by salt and dehydration treatments (Yang and Poovaiah, 2002a; Galon et al., 2010; Wang et al., 2015). *Arabidopsis* CAMTA1 is involved in drought stress response (Pandey et al., 2013). Knockout mutant *camta1* was shown to be more sensitive to drought stress, and expression of many drought responsive genes was affected in this mutant. Similar to AtCAMTA1, tomato CAMTA homolog SlSR1L was also positively involved in drought stress tolerance (Li et al., 2014). In addition to regulating salicylic acid (SA)-induced defense response and systemic acquired resistance (Wang et al., 2009; Zhang et al., 2010), AtCBP60g, a CaM-binding transcription factor from *Arabidopsis* was found to positively regulate drought stress response (Wan et al., 2012). Transgenic plants overexpressing *CBP60g* displayed hypersensitivity to ABA and enhanced tolerance to drought stress. AtGTL1 (GT-2 LIKE1), a CaM-binding member of the GTL transcription factor family, was found to be a negative regulator of drought tolerance (Yoo et al., 2010). *AtGTL1* expression was down-regulated by dehydration stress, and loss-of-function mutant *gtl1* showed better survival under drought stress by reducing transpiration, due to lower stomata density on the abaxial surface and higher expression of *SDD1*, which is a negative regulator of stomatal development and is repressed by AtGTL1 (Yoo et al., 2010). Similarly, PtaGTL1 identified from *Populus tremula* × *P. alba* could bind to CaM and regulate water use efficiency and drought tolerance (Weng et al., 2012). Another transcription factor AtABF2/AREB1, which was identified as CBP through protein microarray analysis (Popescu et al., 2007), was also found to be up-regulated by ABA, dehydration, and salinity stresses (Yoshida et al., 2010). Single and multiple mutants of ABF2, 3, and 4 showed varying degrees of reduced survival rate under drought conditions, implying functional redundancy among these three ABFs and Ca^{2+}/CaM could regulate drought tolerance through ABF2/AREB1 (Yoshida et al., 2010).

Heavy Metal Stress

Elevated concentration of both essential (e.g., Cu and Zn) and non-essential (e.g., Cd, Hg, Pb, and Ni) heavy metals in the soil can cause toxicity and inhibit plant growth. It was reported that Ca^{2+}/CaM is involved in radish (*Raphanus sativus* L.) responses to Cd^{2+} toxicity during the early phases of seed germination (Rivetta et al., 1997). Ca^{2+} added in the medium could partially reverse the Cd^{2+}-induced growth inhibition of the germinating embryo, and this coincides with decreased Cd^{2+} uptake. An equilibrium dialysis study revealed that Cd^{2+} compete with Ca^{2+} for CaM-binding, hence Cd^{2+} could significantly reduce the binding of Ca^{2+}/CaM to its target proteins. Apparently, supplementation of Ca^{2+} in the medium counteracts the toxicity of Cd^{2+} by restoring the Ca^{2+}-dependent interaction between CaM and its targets during the radish seed germination. A tobacco (*N. tabacum*) cyclic nucleotide gated ion channel (CNGC) called NtCBP4 was identified to be a CBP through protein–protein interaction-based library screening, and shown to be localized to plasma membrane. Transgenic tobacco plants with elevated expression of *NtCBP4* displayed tolerance to Ni^{2+} and hypersensitivity to Pb^{2+}, and consistently showed decreased Ni^{2+} and increased Pb^{2+} accumulation, suggesting

that NtCBP4 is involved in heavy metal uptake across the plant plasma membrane (Arazi et al., 1999). However, transgenic plants expressing a truncated version of NtCBP4 lacking the C-terminal stretch covering the CaMBD and part of the putative cyclic nucleotide-binding domain showed improved tolerance to Pb^{2+} and lower accumulation of Pb^{2+}, and loss-of-function mutation of AtCNGC1, a homolog of NtCBP4 in *Arabidopsis*, also resulted in Pb^{2+} tolerance. These results suggested that CaM-binding is required for the normal function of both AtCNGC1 and NtCBP4 for the transport of heavy metals (Sunkar et al., 2000).

Conclusion and Perspectives

Ca^{2+} is a critical second messenger coupling diverse stimuli to various physiological responses in plants. CaM, as well as CML, is one of the most extensively studied Ca^{2+} sensors, which mediate interpretation of Ca^{2+} signals in all aspects of plant life, especially in responses to environmental stresses, through interaction with and regulation of various downstream target proteins. **Figure 1** depicts an overview of generation and interpretation of Ca^{2+} signals which are regulated by CaM/CMLs during plant responses to abiotic stresses. One of the most actively regulated class of target proteins are calcium permeable channels, pumps, and antiporters which are actively involved in the generation of intracellular Ca^{2+} transients. This indicates that the preciseness and accuracy of Ca^{2+} signal itself is closely monitored by CaM-mediated regulation. Although more than 50 proteins from different plant species have been identified as CBPs with well-defined CaM-binding properties (**Table 1**), the CaM-mediated regulations of these target proteins are frequently presumptive including SAURs, PCBP, AtBTs, and WRKYIIds (Yang and Poovaiah, 2000a; Reddy et al., 2002; Du and Poovaiah, 2004; Park et al., 2005). Only a few cases of CaM-mediated regulation *in planta* are supported with empirical evidences, such as GAD, CCaMK, AtCAT3, MLO, DWF1, AtSRs/CaMTAs, CRLK1, and CBP60g (Snedden et al., 1996; Kim et al., 2002; Yang and Poovaiah, 2002b; Du and Poovaiah, 2005; Gleason et al., 2006; Tirichine et al., 2006; Du et al., 2009; Wang et al., 2009; Yang et al., 2010b). Hence, more emphasis should be placed on studying the CaM-mediated regulation of target proteins to further improve our understanding of CaM-mediated signaling. Currently, most of the CBPs are targets of canonic CaMs which count for only 10% of the CaM/CML family. The targets of most of the CMLs are not reported yet, let alone the CML-mediated regulation of downstream targets and associated signal transduction. Identification of novel target proteins of

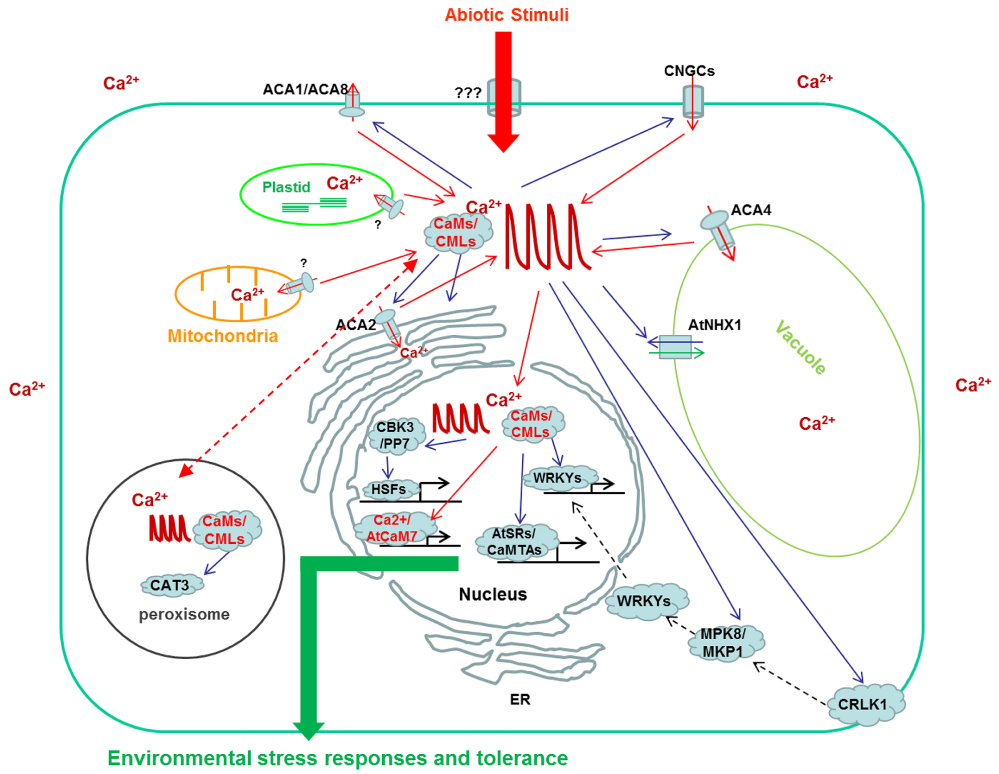


FIGURE 1 | Schematic representation of Ca^{2+} transients and their modification and interpretation by CaM/CMLs as well as their target proteins in plant cells under abiotic stresses. This model is not exhaustive and only includes the actions of a limited number of CaM/CMLs and target proteins; CaMs/CMLs/CBPs involved in biotic stresses and Ca^{2+} signal interpretation by other sensors such as CBLs and CDPKs are not included. Actions modifing Ca^{2+} transients or CaM/CMLs are presented by red arrows and actions regulated by Ca^{2+}/CaMs or Ca^{2+}/CMLs are presented by blue arrows. The dashed arrows imply multiple regulations extended to nucleus.

CaMs and CMLs especially those interact with CMLs deserve special attentions. Environmental cues are known to trigger stimulus specific Ca^{2+} transients. In an effort to explain how the simple Ca^{2+} ion could act as a messenger to couple various environmental stimuli to appropriate physiological responses with astonishing accuracy, Webb et al. (1996) proposed the theory of "Ca^{2+} signature" which hypothesized that stimulus-triggered increases in intracellular Ca^{2+} concentration vary in terms of duration, frequency, amplitude, and spatial distribution, and these carry specific information when they are interpreted into different physiological responses. An obvious support for this hypothesis is that the different Ca^{2+} spikes triggered by Nod factor from rhizobia and Myc factor from arbuscular mycorrhizal fungi could be interpreted through the action of the same Ca^{2+}, Ca^{2+}/CaM dependent protein kinase, CCaMK, into different physiological responses to support the establishment of root nodulation symbiosis or arbuscular mycorrhization (Kosuta et al., 2008). Although exciting progress on how Ca^{2+} signals are interpreted into various physiological responses has been made in the last decade, what we know so far may be very limited in scope when one considers the complicated Ca^{2+} signaling network. Many issues such as specificity, preference and flexibility of interaction between various CaM/CMLs and target proteins *in planta* are barely understood. The dynamics of Ca^{2+}/CaM mediated regulation, the mechanistic details by which a particular effector detects a difference in Ca^{2+} signature and initiates distinct signaling pathways, are basically unknown. Progress in addressing these issues will help in understanding the most amazing properties, the versatility, efficiency and accuracy of Ca^{2+}-mediated signaling in plant responses to environmental stresses.

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REVIEW
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Abiotic stress responses in plants: roles of calmodulin-regulated proteins

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Intracellular changes in calcium ions (Ca^{2+}) in response to different biotic and abiotic stimuli are detected by various sensor proteins in the plant cell. Calmodulin (CaM) is one of the most extensively studied Ca^{2+}-sensing proteins and has been shown to be involved in transduction of Ca^{2+} signals. After interacting with Ca^{2+}, CaM undergoes conformational change and influences the activities of a diverse range of CaM-binding proteins. A number of CaM-binding proteins have also been implicated in stress responses in plants, highlighting the central role played by CaM in adaptation to adverse environmental conditions. Stress adaptation in plants is a highly complex and multigenic response. Identification and characterization of CaM-modulated proteins in relation to different abiotic stresses could, therefore, prove to be essential for a deeper understanding of the molecular mechanisms involved in abiotic stress tolerance in plants. Various studies have revealed involvement of CaM in regulation of metal ions uptake, generation of reactive oxygen species and modulation of transcription factors such as CAMTA3, GTL1, and WRKY39. Activities of several kinases and phosphatases have also been shown to be modulated by CaM, thus providing further versatility to stress-associated signal transduction pathways. The results obtained from contemporary studies are consistent with the proposed role of CaM as an integrator of different stress signaling pathways, which allows plants to maintain homeostasis between different cellular processes. In this review, we have attempted to present the current state of understanding of the role of CaM in modulating different stress-regulated proteins and its implications in augmenting abiotic stress tolerance in plants.

Keywords: abiotic stress, Ca^{2+}, calmodulin, calmodulin-binding proteins, plants

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Introduction

Plants, being sessile, have evolved various biochemical and metabolic processes to sense developmental, hormonal, and environmental changes under normal and stress conditions. Of the various secondary messengers, such as hydrophobic- (diacylglycerol, phosphatidylinositol, etc.) and hydrophilic molecules (Ca^{2+}, cAMP, cGMP, IP3, etc.), and gases [nitric oxide (NO), carbon monoxide, etc.] in eukaryotes, the role of Ca^{2+} has been studied most extensively (Xiong et al., 2002). Different environmental, hormonal and developmental stimuli induce transient fluctuations in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels, the frequency and amplitude of which vary according to the strength of the signal (McCormack et al., 2005). These changes in Ca^{2+} signatures are decoded

by an array of Ca^{2+}-binding proteins such as (i) calcium modulating protein or calmodulin (CaM), (ii) CaM-like (CML) and other EF-hand containing Ca^{2+}-binding proteins, (iii) Ca^{2+}-dependent protein kinases (CDPKs), and (iv) calcineurin B-like proteins (Bouché et al., 2005 and references therein). Due to the differences in the number of EF-hand motifs, different Ca^{2+}-relay sensors (CaM/CMLs) show variability in their affinity to Ca^{2+} since EF-hand motifs bind to Ca^{2+} cooperatively (Babu et al., 1988; McCormack et al., 2005).

CaM, the most well-characterized Ca^{2+} sensor, is an evolutionarily conserved, acidic, heat stable, and multifunctional protein consisting of two globular domains, each with two Ca^{2+}-binding EF-hand motifs (Babu et al., 1988; Rhoads and Friedberg, 1997). Although CaM lacks its own catalytic activity, binding to or chelating of Ca^{2+} causes conformational changes in the globular domains leading to interaction with the target proteins (Snedden and Fromm, 2001; McCormack et al., 2005). Using CaM-binding transcription activators (CAMTA) for mathematical modeling, Liu et al. (2015) have recently demonstrated that interaction of Ca^{2+}-CaM with the target proteins results in non-linear amplification of the Ca^{2+} signals, thereby allowing greater versatility to cells in deciphering different Ca^{2+} signatures for changes in gene expression. Contrary to humans, that contain only a single CaM protein (Fischer et al., 1988), multiple forms of this protein are reported in plants (McCormack et al., 2005; Al-Quraan et al., 2010). A total of seven genes encoding four different CaMs (CaM1/CaM4, CaM2/CaM3/CaM5, CaM6, and CaM7), that share a minimum 97% identity at the primary sequence level, are observed in *Arabidopsis thaliana* (Bender and Snedden, 2013; Zhu et al., 2015 and references therein), whereas, 10 cDNAs encoding three CaM proteins have been predicted in wheat (*Triticum aestivum*; Yang et al., 1996). Similarly, rice (*Oryza sativa*) genome contains five true genes encoding two sets of CaM proteins (Boonburapong and Buaboocha, 2007). The OsCaM1 encoded by *OsCaM1-1, OsCaM1-2,* and *OsCaM1-3* in rice differs by two amino acid residues from the CaM encoded by *OsCaM2* and *OsCaM3*. Multiple genes for CaM i.e., four, six, and seven have been reported in potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*), respectively (Zhao et al., 2013). Molecular evolution of different CaMs and CMLs in plants has been reviewed extensively in a recent study (Zhu et al., 2015). Though primarily cytosolic, CaM is also localized in peroxisomes, plastids, mitochondria, the extracellular matrix, and nuclei (Jarrett et al., 1982; Roberts et al., 1983; Dauwalder et al., 1986; Ma et al., 1999; van Der Luit et al., 1999; Reddy et al., 2002; Yang and Poovaiah, 2002a), signifying versatility in its roles. Besides CaM, plants also contain CML proteins that show 16 to 75% amino acid identity with the former and are not reported in animals (McCormack et al., 2005). *Arabidopsis* genome is predicted to encode 50 CMLs that show variable number of EF hands ranging from 1 (CML1) to 6 (CML12) (McCormack et al., 2005). Recent studies have demonstrated specific roles for some of the CMLs in developmental, hormonal and stress responses (Bender and Snedden, 2013). The proteins that bind to CaM do not show conservation in their primary amino acid sequence. However, the different CaM-binding proteins (CaMBPs) are characterized by the presence of amphiphilic α-helical domains that interact with CaM through both hydrophobic and strong electrostatic interactions (O'Neil and DeGrado, 1990). Recent studies employing proteome microarray have revealed that of the 1133 *Arabidopsis* proteins tested, ∼25% showed interaction with one or the other isoform of CaM/CML (Popescu et al., 2007). However, interaction of most of these proteins with CaM/CML *in vivo* awaits validation by parallel strategies such as bimolecular fluorescence complementation (BiFC) or fluorescence resonance energy transfer (FRET) assays. The targets of CaM comprise a disparate group of proteins such as metabolic enzymes, kinases, transcription factors, etc., and have been reviewed extensively (Bouché et al., 2005; Reddy et al., 2011; Das et al., 2014). Since adverse environmental conditions pose major challenge to sustainable crop productivity, therefore, our discussion in this study will focus primarily on regulation by CaM of proteins that are implicated in abiotic stress response of plants.

Expression of CaM and its Post-translational Modification

The expression of genes encoding different CaM proteins in plants is affected differentially by phytohormones and environmental stresses, as was observed for *MBCaM1* and *MBCaM2* in *Vigna radiate* (Botella and Arteca, 1994). This study revealed that exogenous application of IAA and exposure to salt stress resulted in upregulation of *MBCaM1*, whereas expression of *MBCaM2* was not affected significantly. Though expression of both *AtCaM3* and *AtCaM7* was enhanced by heat shock in *A. thaliana,* the increase in transcript level of the former was observed earlier (Liu et al., 2005). Differential regulation of different *CaM* genes by diverse abiotic stress conditions can be attributed to differences in the upstream regulatory elements (Park et al., 2009; Jung et al., 2010; Chinpongpanich et al., 2012) that may enable the plants to respond in a stimulus-specific manner (Al-Quraan et al., 2010).

Post-translational methylation at Lysine-115 (L-115) is a prominent feature in most CaMs (Klee and Vanaman, 1982). L-115 methylation of CaM has been implicated in protection against adenosine triphosphate (ATP)-ubiquitin-dependent proteolysis that affects its intracellular levels (Gregori et al., 1985). Activity of target proteins can be affected differently by the CaM methylation status. This is also evident from earlier studies where activities of glutamate decarboxylase (GAD; Yun and Oh, 1998) and myosin light chain kinase in plants (Roberts et al., 1984) were shown to be independent of CaM methylation but activation of NAD kinase was adversely affected (Roberts et al., 1986; Harding et al., 1997). Role of methylation of CaM in regulating different cellular processes was further

Abbreviations: ABA, abscisic acid; Ca^{2+}, calcium; CaM, calmodulin; CaM-KMT, CaM N-methyletransferase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; HS, heat shock; IAA, indole-3-acetic acid; IP3: inositol 1,4,5-trisphosphate; IP6, myo-inositol hexaphosphate; NLSs, nuclear-localization signals; PA, phosphatidic acid; TFs, transcription factors; ROS, reactive oxygen species.

validated by overexpression of a synthetic gene encoding VU-3 CaM in transgenic tobacco plants. VU-3 CaM cannot undergo methylation due to substitution of L-115 with Arg. These transgenic plants showed impaired growth and development due to hyperactivation of a CaM-dependent NAD kinase, and consequent increase in NADPH and reactive oxygen species (ROS) levels (Harding et al., 1997). These studies provided evidence that CaM-methylation is critical for regulation of different developmental pathways in plants.

Methylation of CaM is catalyzed by CaM N-methyletransferase (CaM-KMT), a highly conserved protein among eukaryotes (Magnani et al., 2010). Expression of *CaM-KMT* gene is differentially modulated by phytohormones and abiotic stresses. Contrary to cytokinin (kinetin) that down-regulated *CaM-KMT*, the expression of this gene was shown to increase in response to auxin, salt-, and water stress (Banerjee et al., 2013), implying its role in hormone and stress signaling pathways. Overexpression of *CaM-KMT* and its suppression in *Arabidopsis*, resulting in hyper- and hypomethylation, respectively, were associated with attenuated and enhanced root length phenotype, respectively (Banerjee et al., 2013). Furthermore, the hypomethylated CaM lines also exhibited greater tolerance to abscisic acid (ABA), cold-, and salt stress, suggesting that methylated form of CaM might be interacting specifically with effector proteins of different stress signaling pathways. This conclusion was also supported by the observations that as compared to unmethylated CaM, the methylated CaM showed specifically higher binding affinity with proteins such as germin-like proteins (GLP9 and GLP10), cytochrome P450 20A1 (CP20A), and N-xylose isomerase (Banerjee et al., 2013). CaM provides further versatility in fine tuning of cellular responses to different stimuli through alteration in its subcellular localization after post-translational modification, as observed for CaM53 in petunia (*Petunia hybrida;* Rodriguez-Concepcion et al., 1999). Plants contain several CMLs (Bender and Snedden, 2013) but studies on the regulation of these proteins by methylation have not been carried out yet. Methylation of CMLs, if demonstrated, may provide further flexibility in the regulation of different developmental and stress signaling pathways in plants.

Role of CaM in Maintenance of Ca^{2+} Homeostasis

In comparison to cell wall and organelles, where Ca^{2+} is in millimolar concentration, Ca^{2+} in the cytosol is maintained at relatively lower levels (100–200 nm) because higher concentration of this ion is toxic for phosphate-based energy systems (Bush, 1995; Reddy, 2001; Clapham, 2007). Intracellular levels of Ca^{2+} in cytoplasm and endomembrane system are regulated through control of influx and efflux mechanisms. Though, passive influx of Ca^{2+} into cytosol takes place through Ca^{2+}-channels (Sanders et al., 1999), its efflux is an active process that is mediated by Ca^{2+}/H^{+} antiporters and Ca^{2+} pumps. Energy for this process is provided by ATP hydrolysis and proton motive force (Bush, 1995). Two types of Ca^{2+}-ATPases, IIA and IIB, have been reported in plants and animals (Axelson and Palmgren, 1998). The endoplasmic reticulum (ER)-type Ca^{2+}-ATPases are known as type IIA Ca^{2+} pumps and activity of these pumps is not regulated by CaM (Chung et al., 2000). On the contrary, the activity of type IIB Ca^{2+} pumps is stimulated by CaM (Malmström et al., 1997; Harper et al., 1998), and these proteins are localized to tonoplast (Malmström et al., 1997), plasma membrane (PM; Bonza et al., 2000), chloroplast inner membrane (Huang et al., 1993), and ER (Hong et al., 1999).

Expression of Ca^{2+}-ATPase encoding genes is differentially modulated under different stress conditions. Exposure to high salt (NaCl) concentrations and fungal elicitor was observed to enhance the expression of soybean Ca^{2+}-ATPase encoding *SCA1* gene, whereas, addition of mannitol and KCl had no apparent effect (Chung et al., 2000). The SCA1 protein consists of two CaM-binding domains (CaMBDs) at amino acid residues 1–40 and 52–71, respectively, in the N-terminus region and binds to CaM in a Ca^{2+}-dependent manner. The ATPase activity of SCA1 is stimulated following its interaction with Ca^{2+}-CaM. The N-terminus domain is autoinhibitory for ATPase activity of SCA1 since deletion of this region resulted in activity that was similar to native protein assayed in the presence of Ca^{2+}-CaM. Though Chung et al. (2000) did not study whether stress-induced increase in mRNA transcripts of *SCA1* was accompanied by an increase in the corresponding protein, it is likely that enhanced levels of this enzyme may be contributing to lowering of Ca^{2+} to the basal levels so as to prevent Ca^{2+} toxicity (Clapham, 2007). It appears that regulation of Ca^{2+} levels through Ca^{2+}-pumps, which are modulated by CaM, appears to be a part of feedback mechanism that maintains Ca^{2+} homeostasis in the cell. Since canonical and divergent CaM isoforms are reported to bind differentially to one of the *Arabidopsis* Ca^{2+}-ATPases (AtACA8; Luoni et al., 2006), systematic studies on the kinetics of stress-induced changes in $[Ca^{2+}]_{cyt}$, and expression of different Ca^{2+}-ATPases and CaM isoforms are required to elucidate the mechanism of this feedback regulation.

Regulation of Glutamate Decarboxylase by CaM

γ-Amino butyric acid (GABA) is an ubiquitously found non-protein amino acid. Characterized as a neurotransmission inhibitor of central nervous system in animals (Mody et al., 1994; Hampe et al., 2001), the role of this molecule is still a matter of speculation in plants. GABA is produced as a result of glutamate decarboxylation, catalyzed by GAD. The basic structure of animal and plant GADs is conserved (Ueno, 2000) and these proteins show 75–86% similarity at the protein sequence level (Gallego et al., 1995; Johanson et al., 1997; Turano and Fang, 1998; Yun and Oh, 1998; Akama et al., 2001; Bouché et al., 2004; Oh et al., 2005; Lee et al., 2010). The genes encoding GADs in plants are present in multiple copies, with five copies each predicted in the genomes of rice as well as *Arabidopsis* (Akama and Takaiwa, 2007). Expression of different GAD isoforms in plants is regulated in a tissue-dependent manner and is induced by different abiotic stresses such as heat-, osmotic-,

and oxidative stress (Zik et al., 1998; Lee et al., 2010). These observations indicate that GADs play important roles in growth and development, and stress responses in plants. Contrary to animals and *Escherichia coli* (Ueno, 2000), GAD proteins in yeast (Coleman et al., 2001) and plants (Baum et al., 1993; Gallego et al., 1995; Turano and Fang, 1998; Zik et al., 1998; Lee et al., 2010) are characterized by the presence of a CaMBD in the proximal C-terminal region. Of the two GAD proteins in rice, OsGAD1 shows the presence of an authentic CaMBD (Akama et al., 2001), suggesting that the CaM-binding property could be isoform-dependent. The maximum activity of plant GADs is observed under acidic conditions and is independent of Ca^{2+}-CaM. However, at neutral pH, Ca^{2+}-CaM becomes an obligatory requirement for the activity of these proteins (Snedden et al., 1995). Stress-induced increase in GABA levels, attributed to enhanced acidification and increase in Ca^{2+} levels, leads to Ca^{2+}-CaM-induced dimerization of C-terminus domains that results in activation of GADs (Arazi et al., 1995; Snedden et al., 1995; Baum et al., 1996). Overexpression of petunia *GAD* gene, that encoded a protein lacking the CaM-binding domain, in transgenic tobacco resulted in severe morphological abnormalities indicating the role of glutamate and GABA in plant growth and development (Baum et al., 1993, 1996). Constitutive overexpression of the truncated *OsGAD2,* that encoded a protein lacking the C-terminus region, resulted in 40-fold increase in its activity. This experiment is the first evidence of Ca^{2+}-CaM-independent activation of GAD enzyme in plants (Akama and Takaiwa, 2007). It is evident that C-terminal domain of OsGAD2 protein is involved in autoinhibition. Although a GAD protein lacking the CaMBD (OsGAD2) has only been reported in rice, these studies suggest that regulation of GADs through Ca^{2+}-CaM-dependent and Ca^{2+}-CaM-independent pathways may provide greater versatility to plants in their response to different environmental conditions. Genome level surveys in different plant species might shed more light on the presence and roles of CaMBD-lacking GADs in Ca^{2+}/CaM signaling pathways.

Implications of CaM in Regulation of Plant Responses to Heavy Metals and Xenobiotic Compounds

Intensive industrialization and agriculture processes result in the release of toxic heavy metals such as nickel, cobalt, cadmium, copper, lead, chromium, and mercury which degrade the ecosystem and are consistently threatening agricultural production, particularly in the developing countries. Toxic levels of heavy metals in plants adversely affect the protein and enzyme structures through interaction with sulfhydryl groups and disrupt the integrity of PM, thereby, inhibiting different metabolic processes such as photosynthesis and respiration (Ovečka and Takáč, 2014; Emamverdian et al., 2015). Uptake of heavy metal ions in plants is mediated through channel proteins that are localized to PM. These proteins consist of transmembrane domains and a putative cyclic nucleotide monophosphate domain that overlaps with CaMBD at C-terminus (Köhler et al., 1999). Overexpression of an 81 kDa PM-localized protein, NtCBP4, in tobacco conferred higher levels of tolerance to Ni^{2+} due to reduced uptake of this metal ion (Arazi et al., 1999). NtCBP4 protein is homologous to *Arabidopsis* cyclic nucleotide gated channel protein CNGC1 and binds to CaM. The transgenic plants, however, showed hypersensitivity to Pb^{2+} that was attributed to its over accumulation. Subsequent studies revealed that deletion of CaM- and cyclic nucleotide binding domains resulted in abrogation of Pb^{2+}-hypersensitivity, primarily due to attenuation in the uptake of this ion by transgenic plants (**Table 1**; Sunkar et al., 2000). These studies demonstrate that alteration in CaM-binding property of the channel proteins is a potentially viable strategy for engineering tolerance to toxic metals in crop plants, hence, needs to be explored further.

Ca^{2+}-CaM pathway is also implicated in the regulation of apyrases in plants. These proteins hydrolyze nucleoside di- and triphosphates. Hydrolysis of these nucleosides in animals has been demonstrated to be essential for neurotransmission (Todorov et al., 1997) and in prevention of thrombosis (Marcus et al., 1997). In plants, activity of a pea (*Pisum sativum*) apyrase, PsNTP9, localized to nucleus and also present extracellularly (Thomas et al., 2000), was shown to be enhanced by Ca^{2+}-CaM (Chen and Roux, 1986). Subsequent studies revealed that different isoforms of apyrases in *Arabidopsis* bind differentially to CaM (Steinebrunner et al., 2000). Of the two different apyrases AtAPY1 and ATAPY2 (*A. thaliana* apyrase 1 and 2) in *Arabidopsis*, only the former showed interaction with CaM. The differential regulation of different isoforms by Ca^{2+}-CaM may imply distinct role of these proteins in plants that warrants further elucidation. Overexpression of pea apyrase, besides resulting in enhanced growth and phosphate transport in transgenic plants (Thomas et al., 2000), is also reported to confer higher level of tolerance to different herbicides (Windsor et al., 2003) and toxic concentrations of cyclohexane and plant growth regulators (Thomas et al., 2000; **Table 1**). How these physiological attributes are modulated through Ca^{2+}-CaM pathway remains unknown and need to be investigated experimentally by overexpressing apyrases that lack different domains.

Exposure to plants to high salt concentration is associated with accumulation of cytotoxic compound methylglyoxal, that is produced as a byproduct of glycolysis metabolism (Yadav et al., 2005). Methylglyoxal, present in low concentration (μM) in all organisms studied (Richard, 1993), is detoxified by the enzymes glyoxalase I (GlyI) and glyoxalase II (GlyII; Mannervik and Ridderström, 1993). Expression of *GlyI* and *GlyII* genes in plants is enhanced under different abiotic stress conditions (Kaur et al., 2014a,b and references therein). Overexpression of *Gly* genes from wheat (*TaGlyI*), *Beta vulgaris* (*BvM14-GlyI*), *Brassica juncea* (*GlyI*) and rice (*GlyII)* has been reported to confer enhanced tolerance to salt-, metal-, osmotic- and oxidative stress in transgenic plants (Singla-Pareek et al., 2003, 2006; Lin et al., 2010; Alvarez Viveros et al., 2013; Wu et al., 2013), implying the role of these genes in stress adaptation.

Deswal and Sopory (1999) demonstrated that activity of GlyI enzyme was stimulated by CaM, Ca^{2+} and Mg^{2+} ions, with CaM-induced increase being additive when both the ions were

TABLE 1 | Functional characterization of genes encoding different calmodulin (CaM)-binding proteins by transgenic analysis.

S. No.	CaM-binding protein	Source of gene	Transgenic host	Approach	Effect on stress tolerance	References
CaM-BINDING TRANSCRIPTION FACTORS						
1	CaM-binding transcription activator (CAMTA3)/*A. thaliana* signal responsive 1 (AtSR1)	*Arabidopsis thaliana*	*A. thaliana*	T-DNA insertion mutants	Increase in tolerance to CS	Doherty et al., 2009; Du et al., 2009
2	60 kDa CaM-binding protein (CBP60g)	*A. thaliana*	*A. thaliana*	Constitutive overexpression	Enhancement in DS tolerance	Wan et al., 2012
3	CaM-binding trihelix transcription factor (AtGTL1)	*A. thaliana*	*A. thaliana*	Loss-of-function mutation	Improved DS tolerance	Yoo et al., 2010
4	CaM-binding-GTL1 (PtaGTL1)	*Populus tremula x Populus alb*a	*A. thaliana*	Constitutive overexpression	Decline in DS tolerance	Weng et al., 2012
5	25 kDa CaM-binding protein (AtCAMBP25)	*A. thaliana*	*A. thaliana*	Constitutive expression	Decrease in OS tolerance	Perruc et al., 2004
6	CaM-binding WRKY39 (AtWRKY39)	*A. thaliana*	*A. thaliana*	*wrky39* mutant	Impaired HS tolerance	Li et al., 2010
				Constitutive overexpression	Increase in HS tolerance	Li et al., 2010
CaM-BINDING KINASES, PHOSPHATASES AND OTHER ENZYMES						
7	Ca^{2+}-dependent CaM receptor-like kinase (GsCBRLK)	*Glycine soja*	*A. thaliana*	Constitutive overexpression	Enhanced tolerance to CS, SS and OS	Yang et al., 2010a
8	Ca^{2+}/CaM-regulated receptor-like kinase (AtCRLK1)	*A. thaliana*	*A. thaliana*	T-DNA knock-out mutation	Increase in CS tolerance	Yang et al., 2010b
9	CaM-binding protein kinase 3 (AtCBK3)	*A. thaliana*	*A. thaliana*	Constitutive overexpression	Enhanced tolerance to HS	Liu et al., 2008
10	CaM-binding Ser/Thr phosphatase (AtPP7)	*A. thaliana*	*A. thaliana*	Constitutive overexpression	Increase in HS tolerance	Liu et al., 2007
11	CaM-binding mitogen-activated protein kinase phosphatase (NtMKP1)	*N. tabacum*	*N. tabacum*	Constitutive overexpression	Reduction in wound-induced activation of genes	Yamakawa et al., 2004
12	CaM-binding MKP1 (OsMKP1)	*Oryza sativa*	*O. sativa*	*Retrotransposon Tos17*-mediated loss-of-function mutation	Constitutive activation of wound response	Katou et al., 2007
13	Full length CaM-binding protein/cyclic nucleotide gated channel (NtCBP4)	*Nicotiana tabacum*	*N. tabacum*	Constitutive overexpression	Enhanced tolerance to Ni^{2+} but hypersensitivity to Pb^{2+}	Arazi et al., 1999
	Truncated NtCBP4 lacking CaMBD	*N. tabacum*	*N. tabacum*	Constitutive overexpression	Abrogation of Pb^{2+}-hypersensitivity	Arazi et al., 1999
14	Apyrase (PsNTP9)	*Pisum sativam*	*A. thaliana*	Constitutive overexpression	Increased tolerance to cyclohexane and N^6-[2-isopentyl]adenine (2iP)	Windsor et al., 2003

CaMBD, CaM-binding domain; CS, cold stress; DS, drought stress; HS, heat stress; MAPK, mitogen-activated protein kinase; OS, osmotic stress.

present together. However, binding of CaM with GlyI protein *in vivo,* and regulation of glyoxalase pathway by Ca^{2+}-CaM needs to be demonstrated by using BiFC/FRET assays, mutants and pharmacological approaches. Recent studies have revealed up to 11 different *Gly* genes in rice and *A. thaliana* (Kaur et al., 2013). Since no information is available on CaMBD(s) in members of GlyI and GlyII families, we carried out *in silico* analysis of these proteins from rice and *Arabidopsis* using online tool (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/browse.html). This study revealed the presence of up to three different putative CaMBDs in several members of GlyI and GlyII protein families in *Arabidopsis* and rice (Table S1). AtGlyI-13.5 and OsGlyI-1 proteins also showed the presence of an unclassified CaMBD and an IQ motif, respectively. Different *GlyI* genes in rice show differential inducibility under different abiotic stress conditions (Mustafiz et al., 2011). Therefore, specificity of CaM for different Gly proteins need to be investigated for understanding the role of Ca^{2+}/CaM pathway in the regulation of these enzymes.

Regulation of Reactive Oxygen Species by CaM

Production of ROS is one of the major determinants of stress-induced damage to the cells (Mittler, 2002). Hydrogen peroxide (H_2O_2), one of the ROS, is a secondary signal molecule that constitutes an important component of signal transduction pathways that enable the plants to respond to changes in external stimuli (Del Río, 2015). H_2O_2 in plants is generated during photorespiration, mitochondrial electron transport and β-oxidation of fatty acids (Scandalios et al., 1997), and its intracellular levels are stringently controlled for maintaining homeostasis in the cell. Catalase degrades H_2O_2 into water and

O_2, and is one of the critical antioxidant enzymes that protect the cells against ROS-induced damage under stressful conditions. As compared to single isoform in animals, plants contain multiple isoforms of catalases (McClung, 1997; Scandalios et al., 1997). Contrary to bacteria, bovine, human or fungi, plants also contain catalases that are activated after binding to Ca^{2+}-CaM (Yang and Poovaiah, 2002b). The CaMBD of *Arabidopsis* catalase, AtCAT3, is located in the C-terminus amino acid residues 415–451 and is autoinhibitory for its enzyme activity (Yang and Poovaiah, 2002b). It has been postulated that binding of Ca^{2+}-CaM to this domain relieves autoinhibition and makes these proteins catalytically active in response to Ca^{2+} spike. For studying variability in CaMBDs of different catalases, we carried out multiple sequence alignment that revealed high homology in this region [73.0 and 70.3% identity of AtCAT3 CaMBD with rice and sorghum (*Sorghum bicolor*) homologs, respectively] (Figure S1; Table S2), suggesting functional conservation.

H_2O_2 is also reported to activate Ca^{2+}-channels (Pei et al., 2000), thereby, inducing an increase in the $[Ca^{2+}]_{cyt}$ levels (Price et al., 1994). Paradoxically, the production of H_2O_2 from NADPH is catalyzed by NADPH oxidase that requires continuous influx of Ca^{2+} for its activity (Keller et al., 1998). Furthermore, the activity of NAD kinase that generates the substrate (NADPH) for NADPH oxidase from NAD^+ is also modulated through Ca^{2+}-CaM (Harding et al., 1997). These observations indicate toward an intricate mechanism of feedback regulation of different enzymes through Ca^{2+} and CaM that may enable the plants to maintain H_2O_2 homeostasis, thus offering protection against stress-induced damage.

CaM is involved in Modulation of Transcription Factors

Precise regulation of gene expression is imperative for successful completion of life cycle of an organism and its capability to withstand adverse environmental conditions. Expression of genes is regulated by a battery of transcription factors (TFs) and approximately two- and three-thousand genes encoding these proteins have been identified in human and *A. thaliana*, respectively (The *Arabidopsis* Genome Initiative, 2000). Activity of several TFs is modulated by Ca^{2+} (Bouché et al., 2002), either by direct binding, as for DREAM proteins (Carrión et al., 1999) or through Ca^{2+}-CaM. Modulation of TFs by CaM can either be through direct interaction, as observed for helix-loop-helix TFs (Corneliussen et al., 1994) or through the control of kinase-mediated phosphorylation (Corcoran and Means, 2001). The proteins belonging to CaM-binding transcription activator (CAMTA) family of TFs in plants are conserved at structural and sequence levels, suggesting their essential role in the cell (Choi et al., 2005). The expression of different *CAMTA* genes is induced in response to different environmental cues such as heat stress, light, hormone, and pathogen attack (Yang and Poovaiah, 2002a), implying their role in stress response. Multiple isoforms of CAMTA proteins are reported in plants. Six genes (*AtCAMTA1-6*) encoding different proteins have been identified in *Arabidopsis* (Bouché et al., 2002; Finkler et al., 2007), rice (Choi et al., 2005), and sorghum (this study; Table S3). CAMTA proteins are characterized by the presence of specific domains that are responsible for nuclear localization (a bipartite signal in the N-terminal), non-sequence-specific DNA-binding domain (TIG domain), protein-protein interaction domain (ankyrin domain) and IQ domain in the C-terminus (Gly^{872}–Arg^{889}; Bouché et al., 2002).

A rice CaM-binding TF, OsCBT, that shows 44.1% amino acid identity with *Arabidopsis* AtCAMTA5 (Table S3) was demonstrated to possess two CaMBDs, CaMBD1 (amino acid residues 764–777) and CaMBD2 (amino acid residues 825–845; Figure S2; Table S4; Choi et al., 2005). CaMBD1, consisting of an IQ-motif, binds to CaM in a Ca^{2+}-independent manner and constitutes the Ca^{2+}-dependent CaM-dissociation domain. On the contrary, interaction of CaMBD2 with CaM is Ca^{2+}-dependent. Therefore, the presence of these domains enables OsCBT to interact with CaM in absence as well as presence of Ca^{2+}. OsCBT is localized to nucleus due to the presence of two different nuclear localization signals (NLSs) at both N-terminus (amino acid residues 73–90) and C-terminus (amino acid residues 837–840; Choi et al., 2005). Studies by these authors further revealed that coexpression of *OsCaM* and *OsCBT* genes resulted in inhibition of transcriptional activity of the latter, thereby providing evidence for the role of CaM in regulation of gene expression.

In order to understand the significance of CaM-regulation of CAMTA proteins in plants, we retrieved amino acid sequences of these proteins from *Arabidopsis*, rice, and sorghum genome databases and carried out multiple sequence alignment analysis of CAMBD1 and CAMBD2 (Figure S2; Table S5). This analysis revealed that Ca^{2+}-dependent CaMBD2 is conserved among these proteins, whereas CaMBD1 is observed only in AtCAMTA5 and 6 in *Arabidopsis,* and in a single isoform in sorghum (acc. no. XP_002462876.1; Figure S2). It is evident that in contrast to CaMBD1, CaMBD2 appears to be evolutionarily conserved in plant taxa. Conservation of Ca^{2+}-dependent CaMBD (CaMBD2) in plants may be the result of an adaptive strategy to regulate the cellular responses under conditions that lead to an alteration in Ca^{2+} signal. On the contrary, the presence of Ca^{2+}-dependent CaM-dissociation domain in an isoform-specific manner suggests that different members of CAMTA proteins family may be playing distinct roles in Ca^{2+}/CaM pathway, thereby providing versatility to plants in responding to different environmental conditions. We also speculate that since OsCBT shows 98.7% identity to another CAMTA protein in rice (acc. no. LOC_Os07G30774.1; Table S3), the two may be polymorphic variants of the same protein that needs to be validated by analyzing additional genotypes. Understanding the functional significance of OsCBT polymorphism may reveal novel insights into the role of these proteins in Ca^{2+}/CaM signaling pathway.

The C-repeat binding factors or CBF transcription factors, induced rapidly under cold stress, play an important role in acquisition of tolerance to cold stress through regulation of ∼100 other genes (collectively designated as CBF-regulon) that are implicated in cold acclimation (Maruyama et al., 2004). Imposition of cold stress also results in the elevation of $[Ca^{2+}]_{cyt}$ levels due to its release from vacuolar as well as

extracellular stores (Knight et al., 1996). One of the CAMTA proteins, CAMTA3, in *Arabidopsis* (also termed as *A. thaliana* signal responsive 1 or AtSR1) was demonstrated to regulate expression of CBF2 by binding to a conserved DNA-binding motif CM2 or CG-element (vCGCGb) present in the *ZAT12* promoter region of the latter (Doherty et al., 2009). These studies established a link between cold-induced increase in intracellular Ca^{2+}, expression of CBF-regulon, and cold tolerance (**Table 1**). The role of CAMTA TFs in cold stress tolerance of plants is further supported by their localization to nucleus (Bouché et al., 2002) and by the presence of at least one CAMTA3-interacting CG-element in the promotor regions of 30 different genes induced in early stage of cold stress (Doherty et al., 2009). CAMTA3 regulates rapid and transient general stress response by binding to a *cis*-acting element RSRE (rapid stress-response element; CGCGTT; Benn et al., 2014). Though CAMTA protein is constitutively present in the nucleus and binds to DNA even in the absence of CaM (Bouché et al., 2002; Yang and Poovaiah, 2002a), the RSRE-mediated expression of genes is observed only under stress. It has been proposed that RSRE-mediated expression under stress by CAMTA3 may be induced after binding with Ca^{2+}-CaM that facilitates interaction of this protein with other TFs, resulting in transcription of stress-responsive genes (Bjornson et al., 2014).

Besides cold stress adaptation, CAMTA3 is also involved in repression of plant immunity since the *Arabidopsis* plants mutated in *CAMTA3 (atsr1)* showed enhanced resistance to virulent strain of *Pseudomonas syringae*. The *atsr1* plants depicted early induction of *PR1* (*PATHOGENESIS RELATED 1*) gene, after 6 h inoculation of *P. syringae* as compared with 24 h in wild type plants (Du et al., 2009). Repression of defense response by *AtSR1* is attributed to the suppression of *EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1)* gene that encodes a positive regulator of salicylic acid synthesis. Du et al. (2009) provided evidence that regulation of *EDS1* by AtSR1 protein is through its interaction with CG-element present in the promotor of the former. Ca^{2+} signaling is crucial for stimulating the production of ROS and NO that lead to the induction of hypersensitive response (Guo et al., 2003; Lecourieux et al., 2006; Ali et al., 2007). However, the uncontrolled defense response also affects the growth of plants adversely (Gurr and Rushton, 2005). By acting as a suppressor of immune response, *AtSR1* may be playing a central role in fine tuning the defense response of plants. Suppression of immune response by AtSR1 is regulated through Ca^{2+}-CaM since deletion of CaMBD from this protein results in failure to suppress plant immunity (Du et al., 2009). The negative regulation of immune response by Ca^{2+}-CaM-AtSR1 complex is proposed to be released through AtSR1-Interacting-Protein-1 (SR1-1P1) that binds to AtSR1 and facilitates ubiquitination and degradation of the latter upon pathogen challenge (Zhang et al., 2014). Though AtCaMTA3 is acting as a positive regulator of cold stress tolerance and also as a suppressor of immune response in plants, the underlying mechanism(s) responsible for cross-talk between the two pathways are not understood (**Figure 1**). Therefore, interaction between the pathogen-induced hyperresponse and cold acclimation needs to be elucidated by studying the kinetics of *SR1-1P1* and *AtSR1* induction under combined exposure. These studies may lead to an understanding of the different mechanisms that allow plants to maintain a balance between the two responses (**Figure 1**).

One of the CAMTA proteins in *Arabidopsis*, CAMTA1, has also been demonstrated to play a role in auxin response (Galon et al., 2010) and drought adaptation (Pandey et al., 2013; **Figure 1**). Exogenous application of auxin results in enhanced levels of $[Ca^{2+}]_{cyt}$ and induces TFs such as MYB77 that binds to auxin response factor, thereby regulating growth and development processes in plants (Shin et al., 2007; Galon et al., 2010). Galon et al. (2010) observed that expression of several genes (∼17), that are otherwise induced by auxin signaling pathways, was upregulated in plants mutated in *CAMTA1*. On the contrary, expression of genes that are involved in flavonoid biosynthesis and sulfur metabolism pathways was down regulated in *CAMTA1* mutants. Expression of *CAMTA1* is reported to enhance under different stress conditions (Yang and Poovaiah, 2002a). Therefore, suppression of auxin response could be a crucial factor in maintaining homoeostasis, as inhibition of growth and development under stress may enable the plants to divert resources toward stress adaptation. Although intracellular Ca^{2+} levels are enhanced in response to both auxin and stress conditions (Galon et al., 2010), the role of Ca^{2+}/CaM in *CAMTA1*-induced inhibition of auxin response has not been demonstrated. Therefore, studies employing mutants, Ca^{2+}-channel blockers and CaM-antagonists are required to provide further insights into the role of Ca^{2+}/CaM signaling pathway in CAMTA1-mediated auxin response.

Plants contain another large family of TFs that are characterized by the presence of NAC domain (No Apical Meristem in Petunia; *A*TAF1, ATAF2, and Cup-Shaped Cotyledon in *Arabidopsis*), with 100–150 members reported in rice (Nuruzzaman et al., 2010) and foxtail millet (*Setaria italica*; Puranik et al., 2013). *NAC* genes have been reported to play a crucial role in stress adaptation since overexpression of one of the genes of this family, *SNAC1* (*STRESS-RESPONSIVE NAC 1*), in rice imparted drought tolerance without any associated yield penalty (Hu et al., 2006). NAC proteins are characterized by the presence of a conserved N-terminus DNA-binding domain and a C-terminus variable region that is implicated in activation (Tran et al., 2004) as well as repression (Kim et al., 2007) of the transcription. The transcription repressor activity of an *Arabidopsis* NAC protein (CBNAC) was enhanced after Ca^{2+}-dependent interaction with CaM (Kim et al., 2007; **Figure 1**). Conserved domain analysis of different NAC proteins of *Arabidopsis*, carried out in the present study (data not shown), revealed that CaMBD (comprising of amino acid residues 471–512 in CBNAC) is not conserved among different members of this family. The selective presence of CaMBD among NAC proteins suggests that different members of this family are regulated differentially by CaM and perform distinct regulatory functions. Experimental analysis is required to determine the CaM-binding property of different NAC proteins so that the role of these proteins can be established in Ca^{2+}/CaM signal transduction pathway.

Ca^{2+}/CaM pathway is also implicated in the regulation of MYB TFs. These proteins regulate several aspects of growth and

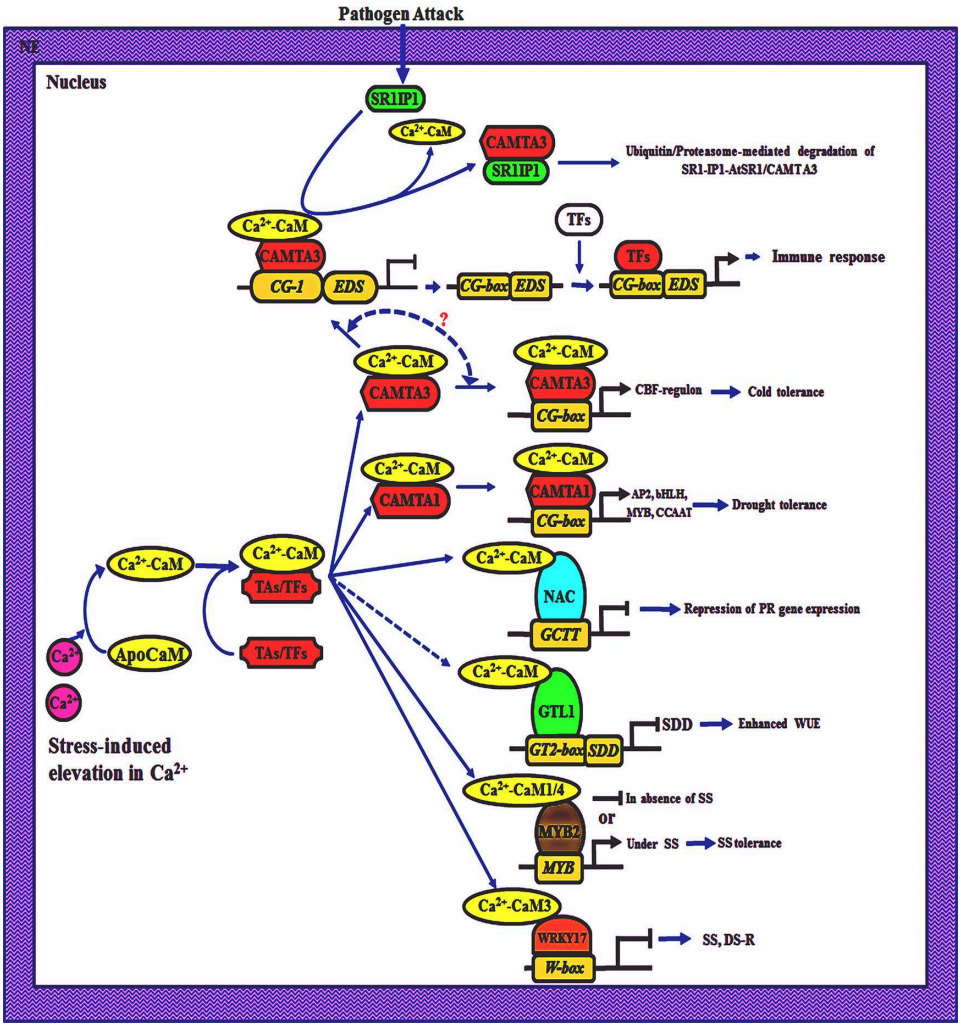


FIGURE 1 | Schematic representation of Ca^{2+}/calmodulin (CaM)-mediated transcriptional regulation in plants. Plants respond to different hormonal, developmental and environmental cues through transient fluctuations in cytosolic Ca^{2+} which is detected by CaM, leading to conformational changes in the latter. The Ca^{2+}-loaded CaM (Ca^{2+}-CaM) interacts with transcriptional activators (TAs) such as CaM-binding transcriptional activators (CAMTA) or transcription factors (TFs) such as NAC, MYB, WRKY, GTL1, resulting in repression (–⊣) or induction (→) of the downstream genes. NE, nuclear envelop; SR1IP1, SR1-Interacting-Protein-1; EDS1, Enhanced Disease Susceptibility 1; SDD1, Stomatal Density and Distribution 1; SS, Salt stress; DR, drought stress-regulon; WUE, water use efficiency; PR, Pathogenesis-Related genes.

development such as cell cycle, morphogenesis, and secondary metabolism in plants (Kranz et al., 1998). DNA-binding activity of one of the MYB proteins, AtMYB2, that controls expression of dehydration- and salt stress-responsive genes in *Arabidopsis* was reported to enhance after interaction with a salt stress-induced isoform of *G. max* CaM (GmCaM4; Yoo et al., 2005). These authors also observed that Ca^{2+}-dependent interaction of AtMYB2 with GmCaM4 resulted in transcriptional activation of genes of proline biosynthetic pathway leading to an increase in proline accumulation. On the contrary, the activity of AtMYB2 was inhibited by another CaM isoform, GmCAM1, thus, implying differential role of CaM isoforms in regulation of these TFs. Site-directed mutagenesis, resulting in substitution of Lys^{69} to Arg^{69} in CaMBD (amino acid residues 63–82) of AtMYB2 protein, abrogated the binding with GmCaM1. On the contrary, the interaction of AtMYB2 with GmCaM4 was inhibited only when both L^{69} and I^{78} were replaced with Arg (Yoo et al., 2005). These two isoforms have been proposed to play specific roles in plants. The constitutive expression of GmCaM1 maintains the transcription of AtMYB2-regulated genes at basal levels under control conditions. On the other hand, the salt stress-induced accumulation of GmCaM4 protein leads to enhanced expression of genes such as *DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1, DEHYDRATION-RESPONSIVE PROTEIN RD22,* and *ALCOHOL DEHYDROGENASE 1* that encode protective proteins, leading to stress tolerance (Yoo et al., 2005). Role of *GmCaM4* in stress adaptation was also supported by the studies which demonstrated that overexpression of this

gene resulted in a concomitant increase in stress tolerance of transgenic *Arabidopsis* plants, whereas overexpression of *GmCaM1* had no significant effect. Identification of other members of MYB TF family that are regulated by Ca^{2+}-CaM will further enhance our understanding of the role of these proteins in signal transduction pathways responsible for stress adaptation in plants.

The WRKY transcription factors, comprising of 74 members in *Arabidopsis*, are characterized by the presence of DNA-binding or WRKY domain at N-terminus and C2H-C/H zinc motif (Eulgem et al., 2000). The WRKY domain consists of amino acid residues WRKYGOK, and based on the number of these domains and zinc motifs these TFs are grouped as G1, G2 ($G2_{a+b}$; $G2_c$; $G2_d$) and G3 (Eulgem et al., 2000). One of the group $G2_d$ members in *Arabidopsis*, AtWRKY7, is induced by pathogen attack and salicylic acid, and depicts interaction with CaM only in the presence of Ca^{2+} (Park et al., 2005). The CaMBD (^{72}VAVNSFKKVISLLGRSR^{88}) present in the C-terminus of AtWRKY7 is distinct from the classical CAMBDs described until now and is conserved in several group $G2_d$ members (WRKY11, 15, 17, 21, 39, and 74; Park et al., 2005). Exposure to pathogens and salicylic acid also results in Ca^{2+} spike (Du et al., 2009). Therefore, the possibility that AtWRKY7 may be regulated through Ca^{2+}-CaM pathway cannot be ruled out and needs to be demonstrated experimentally. Until recently, the WRKY TFs were implicated in the modulation of immune response, and growth and development of plants (Rushton et al., 2010). Recent studies have shown that these proteins also play an important role in abiotic stress adaptation (Tripathi et al., 2014 and references therein). A WRKY gene, *GhWRKY17*, in cotton has been implicated in drought and salt tolerance through regulation of ROS production and ABA signaling pathway. *In silico* analysis of amino acid sequences revealed that AtWRKY17 is 42.7% identical to GhWRKY17 but CaMBDs of these proteins share 78% identity, signifying functional conservation (Yan et al., 2014). However, the role of Ca^{2+}-CaM pathway in regulation of activity of the GhWRKY17 and implications thereof on stress tolerance of plants are still a matter of conjecture and warrant further investigations (**Figure 1**).

Another plant-specific family of TFs that bind to CaM consists of CBP60 proteins (Bouché et al., 2005). One of the members of this family, CBP60g, in *Arabidopsis* showed Ca^{2+}-dependent interaction with CaM (Wang et al., 2009b). Though CBP60g, localized to nucleus, is implicated in defense signaling, recent studies have shown a role of this protein in abiotic stress tolerance also (**Table 1**). Wan et al. (2012) observed that CBP60g-overexpressing transgenic *Arabidopsis* plants, besides exhibiting higher levels of salicylic acid and enhanced resistance to *P. syringae*, also demonstrated increased tolerance to drought stress. These authors also reported higher sensitivity of the transgenic lines to ABA. However, the molecular mechanism(s) responsible for CBP60g-induced drought tolerance in the transgenic plants and the role of Ca^{2+}/CaM in its regulation are not known and need to be explored further. Apart from that, whether the ABA-hypersensitivity of transgenic plants was due to an increase in endogenous ABA or because of changes in the expression of proteins of ABA signaling pathway also needs to be addressed for deciphering the molecular basis of these observations. To conclude, these studies suggest that regulation of different TFs through Ca^{2+}/CaM pathway is a crucial aspect of abiotic stress response in plants and unraveling of these mechanisms may lead to novel strategies for developing stress tolerance in different crops.

One of the most desirable features for drought stress adaptation in plants is to maximize water use efficiency (WUE; Nobel, 1999). Of the several variables affecting WUE, stomatal aperture and density, which regulate the rate of transpiration (Chaerle et al., 2005), are affected by several environmental factors such as light, temperature, CO_2, and water availability. The family of trihelix transcription factors or GT factors, comprising of 30 and 31 members in *Arabidopsis* and rice, respectively (Riechmann et al., 2000; Wang et al., 2014b), is characterized by the presence of highly conserved trihelix domain and binds specifically to the GT-elements (Dehesh et al., 1992; Zhou, 1999). The GT factors, divided into five different groups viz., SIP1, SH4, GTγ, GT-1, and GT-2 in *Arabidopsis*, are implicated in the regulation of different developmental processes and responses to abiotic and biotic stresses (Wang et al., 2014b). Recent studies have demonstrated that one of the members of this family, *GTL1* (*GT-2 LIKE 1*), acts as a negative regulator of WUE since loss of function mutation in this gene in *Arabidopsis* resulted in enhanced tolerance to water stress (**Table 1**; Yoo et al., 2010). On the contrary, overexpression of its poplar (*Populus tremula* × *Populus alb*a) homolog *PtaGTL1* suppressed the drought tolerance (Weng et al., 2012; **Table 1**). The negative regulation of WUE by GTL1 protein is mediated through suppression of *SDD1* (*STOMATAL DENSITY AND DISTRIBUTION 1*) following its binding to GT2-box (GGAAT) in the promoter of the latter (**Figure 1**). *SDD1* encodes a subtilisin-like protease and represses the stomatal development through activation of genes encoding ER (ERECTA) and TMM (Too Many Mouths) proteins (von Groll et al., 2002; Shpak et al., 2005), resulting in a decrease in stomatal density and consequently enhancement in WUE. PtaGTL1 has been recently demonstrated to interact with CaM in a Ca^{2+}-dependent manner through the C-terminus amino acid residues 528–551 and 555–575 (Weng et al., 2012), suggesting a role for Ca^{2+}-CaM pathway in the regulation of this protein. However, effect of Ca^{2+}-CaM on interaction of GTL1 protein with *SDD1* promoter, imperative for understanding the regulation of GTL1 through Ca^{2+}-CaM pathway under stress conditions, has not been demonstrated *in vitro* or *in vivo* and awaits validation. GT-1 *cis*-element (GAAAAA) that binds to *Arabidopsis* GT-1-like transcription factor, AtGT-3b, is also observed in the promoter region of a soybean CaM gene, *SCaM-4* (Park et al., 2004). These authors observed that there was a concomitant and rapid increase in mRNA transcripts of *SCaM-4* and *AtGT-3b* after pathogen attack and NaCl treatment, the induction of former being attributed to the presence of GT-1 *cis*-element. Regulation of *SCaM-4* expression through interaction between *GT-1 cis*-element and GTL-1 transcription factor (Park et al., 2004) points toward a complex feedback loop that may allow precise control over Ca^{2+}-CaM-mediated stress response(s). However, *in silico* and empirical analysis of promoter regions of different *CaM* genes

for the presence of GT-1 elements and their regulation by GTL TFs in different plant species is required to validate this speculation.

Negative regulation of stress tolerance is also observed for a plant-specific gene encoding a 25 kDa protein, AtCAMBP25, in *Arabidopsis* that shows low affinity interaction with CaM in the presence of Ca^{2+} (Perruc et al., 2004). Interaction of AtCAMBP25 with CaM appears to be isoform-specific since it showed binding to typical CaM, AtCaM1, but not to the less conserved form, AtCaM8. AtCAMBP25 is localized to nucleus but its role in transcription is yet to be demonstrated. Although mRNA transcripts corresponding to *AtCAMBP25* showed rapid accumulation under different stress conditions, constitutive expression of this gene in transgenic lines, however, resulted in hypersensitivity to salt- and osmotic stress (Perruc et al., 2004). By virtue of being a negative regulator, it is likely that *AtCAMBP25* is involved in the maintenance of homeostasis under salt- and osmotic stress conditions (**Table 1**). Also, the isoform-specific interaction of CaM with this protein may enable the plants to fine tune their response in a stress-specific manner.

Implications of Ca^{2+}-CaM in Signal Transduction through Modulation of Stress-regulated Kinases

Transduction of signal through phosphorylation and dephosphorylation, mediated by kinases and phosphatases, respectively, allows the cells to respond and adapt to the environmental changes (Charpenteau et al., 2004). CaM regulates the activity of several kinases in plants, and genes for these proteins have been cloned and characterized in several plant species (Zhang and Lu, 2003 and references therein). The characteristic features of different CaM-binding kinases (CBKs) include the presence of CaMBD, variable N- and C-terminal domains, and a protein kinase catalytic domain (Zhang and Lu, 2003). Expression of several CBK genes is modulated differentially by different stressors and phytohormones, suggesting their role in abiotic stress response in plants. Hua et al. (2004) reported significant increase in mRNA transcript levels of *NtCBK2* in tobacco in response to salt stress and gibberellic acid, whereas, auxin, ABA, heat-, cold-, and osmotic stress had no significant effect. These authors attributed the salt stress-induced increase in the expression of *NtCBK2* to a decrease in osmotic potential, which appears unlikely since expression of this gene was unaltered by polyethylene glycol in the same study. Therefore, it is likely that *NtCBK2* plays a role in signal transduction specifically under salt stress.

The substrate phosphorylation activity of NtCBK2, and autophosphorylation activity of *Arabidopsis* CBK, AtCBK1, were enhanced several folds after binding to Ca^{2+}-CaM (Hua et al., 2003; Xie et al., 2003; Zhang and Lu, 2003; Ma et al., 2004). On the contrary, the autophosphorylation activity of lily (*Lilium longiflorum*) and tobacco CBKs was downregulated by CaM (Liu et al., 1998; Sathyanarayanan et al., 2001). It is evident that CaM acts as both negative and positive regulator of CBK activity in plants. Further, it was observed that CaM had no effect on autophosphorylation activity of maize (*Zea mays*) CBK (ZmCaMK) but its substrate phosphorylation activity showed obligated requirement for Ca^{2+}-CaM (Pandey and Sopory, 1998, 2001) suggesting that different activities of the same protein can also be affected differently by CaM. The auto- and substrate phosphorylation activities of rice CBK (OsCBK), despite its higher affinity for CaM, are not regulated through Ca^{2+}-CaM (Zhang et al., 2002), indicating that CaM might also be involved in modulation of these proteins through other mechanisms that are yet to be identified. Species-dependent differential regulation of CBKs by CaM signifies diversity in the perception of signals and their transduction in response to different stimuli in plant taxa.

A large family of kinases belonging to receptor-like serine/threonine kinases (RLKs), with at least 600 RLK homologs predicted in *Arabidopsis* (Hardie, 1999), is also reported in plants. Majority (75%) of the RLKs are localized to PM with the remaining (25%) present in the cytoplasm. The cytoplasmic- and PM-localized RLKs also differ in their domain architecture, with the former containing only the kinase domain (Yang et al., 2004), whereas the latter also show the presence of additional extracellular ligand-binding- and membrane spanning domains (Torii, 2000). These proteins enable the plants to respond to external cues through signal transduction by recognition of extracellular signals, followed by autophosphorylation on the cytoplasmic kinase domain (Stone and Walker, 1995). Members of both PM- and cytoplasm-localized RLK families are reported to interact with CaM and have been implicated in stress adaptation response. The transcript level of *CRCK1* that encodes CaM-binding receptor-like cytoplasmic kinase (CRCK1), a cytoplasmic RLK in *Arabidopsis*, was upregulated by salt- and cold stress, ABA, and H_2O_2 treatments (Yang et al., 2004). The substrate- and autophosphorylation activities of CRCK1 were enhanced following Ca^{2+}-dependent interaction with CaM through amino acid residues 160–183. The kinase activities of PM-localized RLKs of *Glycine soja* (GsCBLRK; Yang et al., 2010b) and *Arabidopsis* CRLK1 (Yang et al., 2010a), implicated in salt- and cold stress tolerance, respectively, are also regulated through Ca^{2+}-dependent interaction with CaM (**Table 1**). Compared with a single CaMBD in GsCBLRK, two CaMBDs (CaMBD1 at N-terminus amino acid residues 30–49 and CaMBD2 at C-terminus amino acid residues 369–390) are observed in CRLK1, suggesting that besides kinase activity, CaM may also be regulating other functions of this protein. This speculation is also supported by the fact that autophosphorylation activity of another PM-localized RLK, AtCaMRLK, is not affected by its interaction with Ca^{2+}-CaM (Charpenteau et al., 2004). Though dynamics of interaction of GsCBLRK and CRLK1 with CaM has not been investigated, it is likely that difference in the number of CAMBDs in these two proteins results in differential affinity with CaM that may be critical for regulating their activities differentially. Variability in regulation of different activities of plant CBKs by CaM signify evolutionary divergence and is possibly the result of myriad adaptive processes operating under diverse environmental conditions that enable the plants to respond in a stimulus-specific manner.

Growth and development in plants, and their responses to stressful conditions are also modulated through mitogen-activated protein kinases (MAPKs), a different class of kinases that have been reviewed earlier (Pedley and Martin, 2005). The MAPKs are activated and inactivated through phosphorylation and dephosphorylation by MAPK kinase (MEK) and MAPK phosphatases (MKPs), respectively (Katou et al., 2007). Regulation of MKPs through CaM is a feature unique to plants and constitutes an important regulatory point in signal transduction (Katou et al., 2007). Genes coding for MKPs have been cloned and characterized from diverse plant species such as rice (*OsMKP1*; Katou et al., 2007), *Arabidopsis* (*AtMKP1*; Lee et al., 2008), wheat (*TMKP1*; Ghorbel et al., 2015), and tobacco (*NtMKP1*; Yamakawa et al., 2004; Figure S3). These proteins have been demonstrated to interact with CaM in a Ca^{2+}-dependent manner. Despite high similarity in their amino acid sequences, these proteins show variability in the number of CaMBDs and their affinities toward CaM. Only a single putative CaMBD is observed in NtMKP1 and OsMKP1, compared with two in AtMKP1 (CaMBD1 at amino acid residues 445–469 and CaMBD2 at amino acid residues 669–692) and TMKP1 (CaMBD1 at amino acid residues 398–449 and CaMBD2 at amino acid residues 618–669; Lee et al., 2008; Ghorbel et al., 2015). In AtMKP1, the interaction of CaM was reported to be stronger with CaMBD2 as compared to CaMBD1. *In silico* analysis of different MKP1 proteins revealed that as compared to 40.7–59.8% in dicots, identity among monocots ranged between 68.6 and 80.5%, suggesting higher level of conservation in the latter (Table S6). The CAMBD2 in one of the maize MKP1 isoforms (ZmMKP1.2) showed less conservation as compared to CAMBD1 (Table S7). Recent studies demonstrated that deletion of the two CaMBDs (amino acid residues 398–449 and 618–669) from TMKP1, an ortholog of AtMKP1, resulted in a 4-fold increase in phosphatase activity of this protein (Ghorbel et al., 2015), suggesting an autoinhibitory role of these domains. Contrary to AtMKP1, which showed enhanced phosphatase activity following interaction with Ca^{2+}-CaM (Lee et al., 2008), the effect of Ca^{2+}-CaM on activity of TMKP1 was cofactor-dependent. The phosphatase activity of TMKP1 was regulated positively by Ca^{2+}-CaM in the presence of Mn^{2+} or Mg^{2+}, whereas interaction with Ca^{2+}-CaM in the absence of these metal ions abrogated the enzyme activity (Ghorbel et al., 2015). However, Ca^{2+}-CaM had no significant effect on phosphatase activity of the protein that lacked C-terminus amino acid residues. The divergence in CaMBDs and their differential affinity toward Ca^{2+}-CaM may allow the plants to respond in a signal-specific manner, suggesting distinct regulatory functions of these proteins in different species. The MKPs are also proposed to act as negative regulators of defense response, as overexpression of NtMKP1 was reported to result in the suppression of kinase activity of several MAPKs that are induced in defense and wound responses (Yamakawa et al., 2004; **Table 1**). These observations indicate that Ca^{2+}-CaM-regulated MKPs may constitute a crucial link between Ca^{2+} signaling and MAPK signaling pathways, enabling the plants to maintain homeostasis under biotic and abiotic stress conditions.

Regulation of Heat Shock Response through Ca^{2+}/CaM Signal Transduction Pathway

Global warming is projected to result in an increase in average temperature (Angilletta, 2009), implying that heat stress may become one of the major limiting factors for crop productivity. Recent studies suggest that yields of rice decline by 10 percent for every 1°C increase over mean minimum temperature during the growing season (Peng et al., 2004). Imposition of heat stress results in oxidative damage to cell wall, protein misfolding and denaturation or aggregation at cellular levels (Wang et al., 2004). Understanding the heat stress response in plants is, therefore, imperative for developing crops that are tolerant to high temperature stress. The plants, in general, respond to heat stress by selective repression and induction of genes. Synthesis of heat shock proteins (HSPs) is one of the protective strategies that enable the plants to cope with heat stress. The HSPs act as chaperones, prevent aggregation and recycle the aggregated proteins (Wang et al., 2004; Yamada et al., 2007). Plant HSPs are categorized into different categories viz., small HSPs or sHSPs (12–40 kDa), HSP60 (chaperonin), HSP70, HSP90 and HSP100 (Wang et al., 2004 and references therein). The promoter regions of HSP genes consist of heat shock elements (HSEs; 5′-AGAAnnTTCT-3′), that are recognized by heat shock factors (HSFs) which regulate the expression of these genes (Baniwal et al., 2004; Gao et al., 2008). As compared to other eukaryotes, the number of genes encoding HSFs in plants is substantially higher, with 21 and 25 genes reported in *Arabidopsis* and rice, respectively (Nover et al., 2001; Wang et al., 2009a).

Imposition of heat stress leads to elevation in $[Ca^{2+}]_{cyt}$, and Ca^{2+}/CaM signal transduction pathway plays a crucial role in regulating the response of plants to thermal stress (Gong et al., 1998; Liu et al., 2003; Wu et al., 2012). Changes in PM fluidity in response to heat shock result in transduction of signal through cytoskeleton, Ca^{2+} signatures and CDPKs, followed by an activation of mitogen-activated protein kinases (MAPKs; Sangwan et al., 2002). Ca^{2+} has also been implicated in increased DNA-binding activity of HSFs through direct interaction (Mosser et al., 1990; Li et al., 2004). These studies, therefore, point toward involvement of Ca^{2+} in multiple regulatory pathways for controlling the heat shock response in plants. The role of Ca^{2+} in heat shock response has been further validated by the use of Ca^{2+}-channel blockers, which provided evidence in favor of heat stress-induced influx of Ca^{2+} from the apoplast (Bush, 1995; Liu et al., 2003; Wu et al., 2012).

In the absence of heat stress, HSPs in the cell are maintained at basal levels through repression of transcription of genes. Heat stress-induced increase in $[Ca^{2+}]_{cyt}$ in plants, reported to occur within 4 and 7 min in wheat and rice, respectively, precedes the induction of *CaM* genes (Liu et al., 2003; Wu et al., 2012), followed by transcriptional activation of genes that encode different HSPs. These studies, therefore, point toward the role of Ca^{2+}/CaM pathway in the regulation of HSPs. Wu et al. (2012) recently reported that heat stress-induced increase

in $[Ca^{2+}]_{cyt}$, and expression of *OsCaM1-1* and *OsHSP17* in rice was abrogated by CaM antagonists, chlorpromazine and trifluoperazine. Although, this aspect needs to be validated independently, these findings suggest that Ca^{2+} homeostasis and expression of *CaM* genes may be under the control of feedback regulation (**Figure 2**).

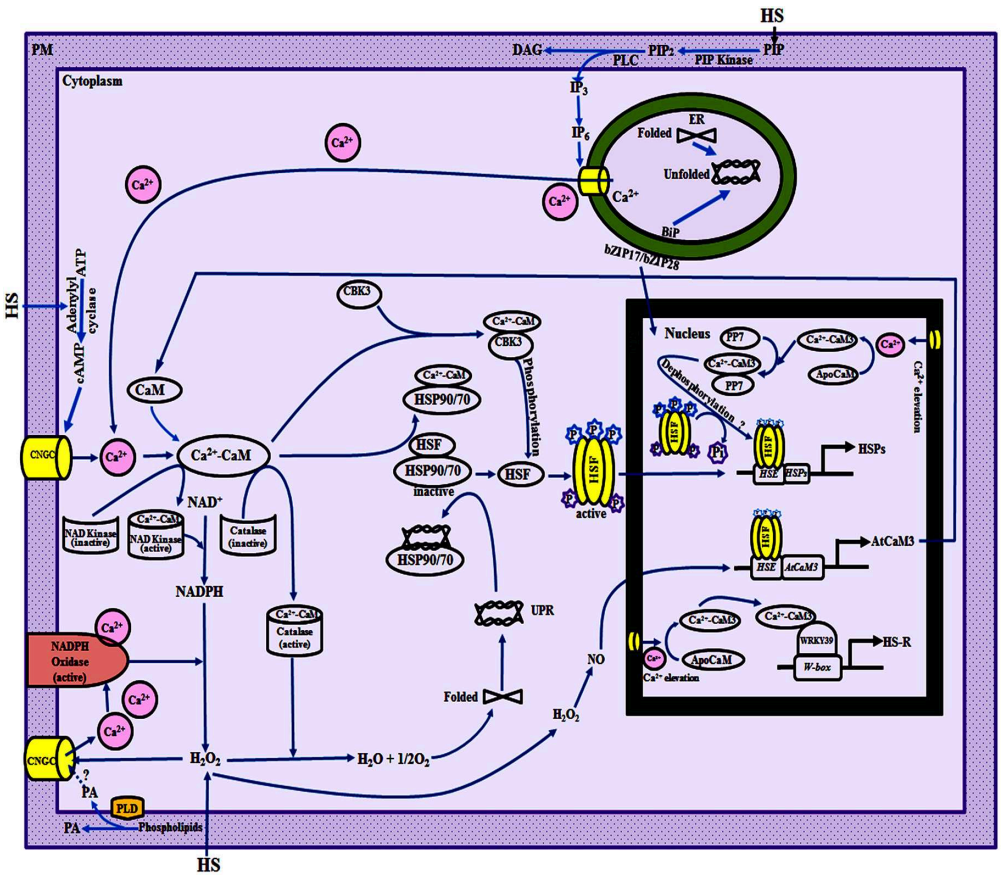


FIGURE 2 | A model illustrating the role of Ca^{2+}/calmodulin (CaM) in regulation of heat shock response and H_2O_2 homeostasis in plants. Changes in plasma membrane (PM) fluidity due to heat stress result in activation of phospholipase D (PLD) and phosphatidylinositol-4-phosphate 5-kinase (PIP kinase), and consequently the accumulation of various lipid signaling molecules such as phosphatidic acid (PA) and phosphatidylinositol-4, 5-bisphosphate (PIP_2; Mishkind et al., 2009). Thermal stress also activates phospholipase C which converts PIP_2 into diacyl glycerol (DAG) and D-myo-inositol-1,4,5-trisphosphate (IP_3). IP_3 may be phosphorylated and converted into IP_6 that interacts with endoplasmic reticulum (ER)-localized Ca^{2+}-channels thus resulting in release of Ca^{2+} from intracellular stores (Mishkind et al., 2009; Mittler et al., 2012). Rapid influx of extracellular Ca^{2+} in the cell can also occur due to temperature-induced activation of the PM-localized cyclic nucleotide gated channels (CNGCs) which are non-selective inward cation channels. The CNGC may be activated by heat stress-induced rapid burst in H_2O_2 levels (Pei et al., 2000) by cyclic adenosine monophosphate (cAMP) that is produced by heat stress-activated adenylyl cyclase (Köhler et al., 1999) and/or by PA (Mittler et al., 2012). The thermal stress-induced increase in $[Ca^{2+}]_{cyt}$ leads to conversion of ApoCaM to Ca^{2+}-CaM. The expression of HSP genes is repressed in the absence of heat stress which is proposed to be due to interaction of heat shock factors (HSFs) with HSP90 (Zou et al., 1998) and/or HSP70 (Sun et al., 2000). The Ca^{2+}-CaM and the denatured proteins, produced as a result of unfolded protein response (UPR) due to reactive oxygen species (ROS)-mediated oxidation, bind to HSP70/HSP90, thereby releasing the HSFs (Yamada and Nishimura, 2008; Virdi et al., 2009, 2011). H_2O_2 acts upstream of NO which regulates the expression of *AtCaM3* through modulation of the binding of HSF to the heat shock elements (HSEs; Wang et al., 2014a). The temperature-induced increase in NO leads to enhanced levels of *AtCaM3*, that then binds to Ca^{2+} and activates the protein kinase AtCBK3 (*Arabidopsis thaliana* Ca^{2+}-CaM-binding Kinase 3), resulting in phosphorylation and trimerization of HSFs which are then translocated to the nucleus (Queitsch et al., 2000). The interaction of activated HSFs with HSEs leads to synthesis of HSPs. Dephosphorylation of the HSFs at selected amino acid residues by a nuclear-localized Ca^{2+}-CaM-binding phosphatase (PP7) may lead to continuous activation of heat shock-regulon (HSR) but the precise mechanism is still not understood. The heat shock response through Ca^{2+}-CaM-mediated regulation of WRKY transcription factors appears to be independent of HSF-mediated pathway. The WRKY39, after binding to Ca^{2+}-CaM, interacts with the W-box elements present in the upstream promotor regions of different genes involved in thermotolerance. The ER also plays a critical role in thermal adaptation through UPR-induced release of ER membrane-tethered transcription factors such as bZIP17/bZIP28/bZIP60, which after release are translocated to the nucleus and activate the transcription of genes encoding ER-chaperones and brassinosteroid-signaling pathway related genes (Che et al., 2010; Deng et al., 2011). The intracellular levels of H_2O_2 under stress also appear to be maintained through Ca^{2+}-CaM (Wang et al., 2014a). The H_2O_2-induced activation of CNGCs (Pei et al., 2000) results in an increase in the $[Ca^{2+}]_{cyt}$ (Price et al., 1994) that further activates NADPH oxidase that converts NADPH to H_2O_2 (Keller et al., 1998). The conversion of NAD^+ to NADPH is catalyzed by NAD kinase that is also modulated through Ca^{2+}-CaM (Harding et al., 1997). These observations, therefore, suggest the presence of an intricate feedback regulation through Ca^{2+}/CaM pathway which allows the plants to maintain H_2O_2 homeostasis. BiP, binding immunoglobulin protein; ER, endoplasmic reticulum; HS, heat stress; NE, nuclear envelop; PM, plasma membrane.

Recent studies in *Arabidopsis* have provided evidence that H_2O_2 acts upstream of NO (Wang et al., 2014a). NO regulates the expression of *AtCaM3* and functions upstream to Ca^{2+}-CaM in the signal transduction pathway under heat stress (Xuan et al., 2010). The heat stress-induced increase in NO leads to enhanced levels of AtCaM3 protein that then binds to protein kinase AtCBK3 in the presence of Ca^{2+} (Liu et al., 2008), leading to phosphorylation of HSFs (**Figure 2**). The phosphorylated HSFs, after interaction with HSEs, activate the expression of HSP genes resulting in thermotolerance in plants (Queitsch et al., 2000). In further support for the role of *AtCBK3* in heat stress adaptation, Liu et al. (2008) observed that *cbk3* mutants of *Arabidopsis* exhibited reduced thermotolerance and overexpression of *AtCBK3* rescued the mutant plants (**Table 1**). Another likely mechanism by which CaM regulates heat shock response in plants is through regulation of protein phosphatases. The protein phosphatase AtPP7 in *Arabidopsis* exhibits Ca^{2+}-dependent binding to CaM, and mutation in *AtPP7* resulted in decreased thermotolerance (Kutuzov et al., 2001; Liu et al., 2007; **Table 1**). Compared with cytosolic localization of AtCBK3 protein (Liu et al., 2008), the nuclear localization of AtPP7 (Liu et al., 2007) suggests that the latter might be activating the HSFs through its phosphatase activity as HSFs are reported to show enhanced transcriptional activity after dephosphorylation at some sites (Høj and Jakobsen, 1994).

Heat shock response in plants also appears to be regulated by CaM through interaction with HSPs such as HSP70 (Sun et al., 2000) and HSP90 (Virdi et al., 2009, 2011). The HSP90 (Zou et al., 1998) and HSP70 (Sun et al., 2000) have been proposed to act as repressors of HSFs in the absence of heat stress. It has been proposed that denatured proteins, produced as a result of unfolded protein response under heat stress, bind to HSP70/HSP90, thereby releasing the HSF that undergoes trimerization, phosphorylation, and nuclear localization leading to the transcription of genes encoding HSPs (Reindl et al., 1997; Yamada and Nishimura, 2008). Studies in our lab demonstrated that sorghum HSP90, SbHSP90, binds to CaM in a Ca^{2+}-dependent manner (Virdi et al., 2009). It was also observed that the steady state levels of SbHSP90 are regulated through Ca^{2+}/CaM pathway since the heat stress-induced increase in HSP90 levels was abrogated in the presence of exogenous Ca^{2+}-channel blockers and CaM-antagonists (Virdi et al., 2011). On the basis of these observations, we proposed that in addition to denatured proteins, as suggested by Yamada and Nishimura (2008), dissociation of HSP90-HSF complex might also involve interaction of Ca^{2+}-CaM with HSP90, thereby releasing the HSF to activate the transcription of HSP genes (Virdi et al., 2011). The role of HSP90 as a natural inhibitor of HSF is also supported by studies in *Arabidopsis* (Yamada et al., 2007) and sorghum (Virdi et al., 2011) where expression of *HSP* genes was shown to be enhanced by exogenous application of a specific inhibitor of HSP90, geldanamycin, even in the absence of heat shock. Further evidence for validation of the proposed model was also provided by recent studies that reported enhanced expression of *AtCBK3, AtPP7, AtHSF,* and *AtHSP* genes in absence of heat stress in the transgenic *Arabidopsis* that overexpressed *OsCaM1-1* (Wu et al., 2012). On the contrary, overexpression of *AtHSP90.3* lowered the expression of *AtHsfA1d, AtHsfA7a, AtHsfB1, AtHsp101,* and *AtHsp17*, leading to impaired thermotolerance of transgenic *Arabidopsis* (Xu et al., 2010). Therefore, regulation of heat shock response in plants by CaM through multiple pathways may ensure redundancy and enable them to cope up with thermal stress (**Figure 2**).

In addition to HSPs, plants are also reported to express heat-specific isoforms of FK506-binding proteins, FKBPs, which show peptidyl prolyl *cis-trans* isomerase activity (Schreiber, 1991). In plants, the multi-domain FKBPs, such as FKBP62, FKBP73, and FKBP77, are characterized by the presence of FKBP domains at N-termini, and CaM-binding and tetratricopeptide repeat domains at C-termini. Though wFKBP73 and wFKBP77 in wheat have been implicated in assembly of functional glucocorticoid complex with p23 and HSP90 (Owens-Grillo et al., 1996; Pratt and Toft, 1997), the implication of CaM in regulation of these proteins is still a matter of speculation and awaits validation. One of the FKBPs in *Arabidopsis*, AtFKBP62 or ROF1, is heat-inducible and forms a complex with HSP90, which is then translocated from cytosol to nucleus following exposure to thermal stress (Meiri and Breiman, 2009). The heat shock-induced localization of ROF1-HSP90 in the nucleus takes place as a result of interaction of transcription factor HSFA2 with HSP90 in this complex, leading to synthesis of sHSPs and development of thermotolerance. The role of CaM, if any, in the AtFKBP62-mediated stabilization of HSFA2 and its localization to nucleus, is not understood yet and needs further investigations.

Concluding Remarks and Future Directions

Modulation of intracellular Ca^{2+} levels in response to unfavorable conditions is one of the important components of signaling pathways that allow plants to adapt to changing environmental conditions. Of the various Ca^{2+} sensors, CaM is one of the most well-characterized proteins that decodes Ca^{2+} signals and regulates activities of diverse proteins. Approximately, 3% of the proteome in *Arabidopsis* has been reported to be involved in Ca^{2+} signaling (Reddy et al., 2011). In recent years, considerable progress has been made in our understanding of the role of CaM in regulating different cellular processes in plants. These studies have resulted in the identification and characterization of various proteins that are regulated by CaM. CaM not only acts as a sensor of Ca^{2+} but also regulates its intracellular levels by modulating the activity of Ca^{2+}-ATPases. Detailed investigations into the specificity of different CaM isoforms for different Ca^{2+} pumps may reveal the role of these interactions in generating stimulus-specific signals. In addition to Ca^{2+} transport, CaM also affects uptake of heavy metal ions through modulation of channel proteins such as NtCBP4. Engineering of these proteins offers a promising alternative for developing transgenic plants that are able to tolerate toxic levels of heavy metal ions. Further, how CaM regulates activities of apyrases and Gly proteins, that confer tolerance to xenobiotic compounds, is still a matter of speculation. Understanding the physiological implications of interaction of CaM with apyrases and Gly enzymes may unravel

strategies for enhancing stress tolerance in crops by transgenic technologies (**Table 1**).

Production of ROS under stress conditions is one of the important factors that cause damage to cellular components. Relieving autoinhibition of catalase by Ca^{2+}-CaM, and regulation of NADPH oxidase and NAD kinase through Ca^{2+}/CaM signaling pathway are indicative of complex feedback mechanisms that may allow the cells to maintain H_2O_2 homeostasis. Since ROS are also involved in biotic stress response, studies are required to determine the role of Ca^{+2}-CaM in regulating cross-talk between biotic- and abiotic stress pathways that enable the cells to maintain appropriate levels of ROS. Regulation of TFs by Ca^{+2}-CaM appears to be a crucial factor in stress adaptation response of plants. CaM-regulated TFs are involved in both activation and suppression of stress responses. For instance, CAMTA3 acts as a suppressor of plant immunity, whereas GTL1 functions as a negative regulator of drought tolerance (**Table 1**). Furthermore, a given TF can also affect two processes differently. For example, CAMTA3 acts as a negative regulator of immune response as well as a positive regulator of the cold stress response (**Table 1**). It appears that the stress-specific functions of a TF are dependent upon the effector proteins that bind to the promoter regions of these genes. Identification of upstream DNA elements and regulatory proteins that bind to these regions will provide further insights into the molecular mechanisms which determine multiple functionality of Ca^{2+}-CaM-regulated TFs. Studies are also needed to understand the significance of different domains that facilitate interaction of TFs, such as NAC, with CaM in Ca^{2+}-dependent and Ca^{2+}-independent manner. This will provide insights into the mechanisms that regulate the interaction of CaM with these proteins, and may also allow engineering of specific domains for improving stress tolerance of crop plants. As of now, very few stress-regulated CaM-binding TFs have been identified but a growing body of data suggests that many more such proteins could exist (**Table 1**). Therefore, there is a need to carry out systematic analysis for identification and characterization of CaM-regulated TFs. Cross-talk between different regulatory pathways also needs to be investigated for developing a comprehensive view of stress response in plants.

Regulation of several processes such as maintenance of Ca^{2+} and H_2O_2 homeostasis, and heat shock response by CaM appears to be mediated through complex feedback mechanisms involving interaction of several different pathways. It is evident that CaM is acting as a hub for integration of different signal transduction pathways that enable cells to respond to different stimuli and maintain homeostasis. System biology approach would, therefore, be helpful in developing a deeper understanding of these mechanisms. Another aspect that needs to be explored further is to understand the cross-talk between regulation of different stress-modulated CaMBPs and phytohormones. With advances in protein microarray, transcriptome, and genome sequencing technologies, the number of stress-regulated CaMBPs that are identified in economically important crops is expected to increase further. Analysis of these proteins by using functional genomics approaches will, therefore, be an important factor in unraveling novel candidate genes for improving stress tolerance of crops by biotechnological interventions.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00809

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Expression of chickpea CIPK25 enhances root growth and tolerance to dehydration and salt stress in transgenic tobacco

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Calcium signaling plays an important role in adaptation and developmental processes in plants and animals. A class of calcium sensors, known as Calcineurin B-like (CBL) proteins sense specific temporal changes in cytosolic Ca^{2+} concentration and regulate activities of a group of ser/thr protein kinases called CBL-interacting protein kinases (CIPKs). Although a number of CIPKs have been shown to play crucial roles in the regulation of stress signaling, no study on the function of CIPK25 or its orthologs has been reported so far. In the present study, an ortholog of Arabidopsis CIPK25 was cloned from chickpea (*Cicer arietinum*). CaCIPK25 gene expression in chickpea increased upon salt, dehydration, and different hormonal treatments. CaCIPK25 gene showed differential tissue-specific expression. 5′-upstream activation sequence (5′-UAS) of the gene and its different truncated versions were fused to a reporter gene and studied in Arabidopsis to identify promoter regions directing its tissue-specific expression. Replacement of a conserved threonine residue with an aspartic acid at its catalytic site increased the kinase activity of CaCIPK25 by 2.5-fold. Transgenic tobacco plants overexpressing full-length and the high active versions of CaCIPK25 displayed a differential germination period and longer root length in comparison to the control plants. Expression of CaCIPK25 and its high active form differentially increased salt and water-deficit tolerance demonstrated by improved growth and reduced leaf chlorosis suggesting that the kinase activity of CaCIPK25 was required for these functions. Expressions of the abiotic stress marker genes were enhanced in the CaCIPK25-expressing tobacco plants. Our results suggested that CaCIPK25 functions in root development and abiotic stress tolerance.

Keywords: ***Cicer arietinum*, CIPK25, root, expression, salinity, dehydration**

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Introduction

Dehydration and salinity are the major abiotic stresses that account for the major loss of crop yield. As it is difficult to physically remove salt from the soil, improving crop tolerance to high salt becomes a critical task. Identification of molecular components conferring salt tolerance in plants can provide genetic markers or candidate genes for achieving this goal. Salinity causes injury to plant by ionic toxicity and osmotic stress that can also be induced by dehydration. These environmental stresses adversely affect photosynthesis, metabolism, and growth. Plants develop

a variety of mechanisms to protect themselves from these environmental stresses. The major factors of plant responses to dehydration and salinity stress include perception and transduction of stress signals via signaling cascade and activation of stress-responsive genes. Calcium has been widely regarded as a ubiquitous second messenger of physiologically and environmentally induced signaling pathways in plants (Trewavas and Malhó, 1998). Internal and external stimuli such as developmental, light, and stresses elicit specific temporal changes in cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$. The kinetics and magnitude of calcium ion concentration, i.e., "calcium signature" or $[Ca^{2+}]_{cyt}$ differs between different signals and possibly contributes to the specific response. These calcium signatures are decoded and transmitted by an array of Ca^{2+}-binding proteins that relay this information into downstream responses. Calcineurin B-like (CBL) proteins are one of these protein families that functions by interacting with and activating CBL-interacting kinases (CIPKs) and, thereby, amplify and specify the signaling pathways.

CIPKs were first reported in Arabidopsis and grouped into sucrose non-fermented-1 (SNF-1) family kinases. SOS2 gene was identified in a genetic screening of salt overly sensitive (sos) mutants as a sucrose non-fermenting 1-like enzyme and subsequently, referred to as CIPK24 within the CIPK family (Liu et al., 2000; Kolukisaoglu et al., 2004). Genome-wide analysis has identified 26 CIPKs in Arabidopsis (Kolukisaoglu et al., 2004; Lyzenga et al., 2013), 33 CIPKs in rice (Kolukisaoglu et al., 2004; Piao et al., 2010), 27 CIPKs in poplar (Yu et al., 2007), and 43 CIPKs in maize (Chen et al., 2011). Extensive research in this area in the past few years led to the functional characterization of many CIPK genes from Arabidopsis and several other species and revealed their potential roles in abiotic stress tolerance. AtCIPK24/SOS2 was shown to be activated by interacting with AtCBL4/SOS3 and provided tolerance against salinity by phosphorylating and activating plasma membrane located Na^+/H^+ antiporter/SOS1 and, thereby, enhancing salt detoxification through Na^+-extrusion into extracellular space (Liu and Zhu, 1997, 1998; Qiu et al., 2002). AtCIPK24/AtSOS2 was also found to interact with nucleoside triphosphate kinase 2 (NDPK2) as well as AtCAT2/AtCAT3, which were involved in reactive oxygen species (ROS) signaling and scavenging (Verslues et al., 2007) suggesting that AtCIPK24/AtSOS2 was a crucial regulator in the salt stress signaling network and was able to mediate both Na^+ homeostasis and the oxidative stress response. AtCIPK23 and AtCIPK6 were reported to have a crucial role in K^+ homeostasis. AtCBL1/AtCBl9-AtCIPK23 complex can directly activate the plasma membrane localized potassium channel AtAKT1, enhancing K^+ uptake under low-K^+ conditions (Xu et al., 2006). AtCBL4-AtCIPK6 complex was shown to modulate the activity of the K^+-channel AKT2 by relocating it from endoplasmic reticulum membrane to the plasma membrane. Apart from AtCIPK23, its closest homolog AtCIPK9 also interacted with AtCBL3 to regulate K^+ homeostasis under low K^+ conditions (Liu et al., 2013). Apart from abiotic stress, CIPKs were also reported to participate in signaling related to development. AtCIPK19 loss-of-function mutant was impaired in pollen tube growth and polarity (Zhou et al., 2015). AtCIPK3, −9, −23, and −26 were shown to function downstream to CBL2 and −3 in maintaining magnesium homeostasis (Tang et al., 2015).

CIPKs from other plant species also displayed similar function as their orthologs in Arabidopsis. CIPK6 genes of chickpea (*Cicer arientinum*) and *Brassica napus* were shown to be involved in plant response to abiotic stress and abscisic acid signaling (Tripathi et al., 2009; Chen et al., 2012). Overexpression or suppression of SOS2 ortholog of apple MdSOS2 enhanced or reduced, respectively, the salinity tolerance in transgenic apple callus (Hu et al., 2012). Rice CIPK31 was found to be involved in germination and seedling growth under abiotic stress conditions in rice (Piao et al., 2010). Heterologous expression of cotton CIPK6 (GhCIPK6) in Arabidopsis significantly enhanced tolerance to multiple abiotic stresses (He et al., 2013). Expression of CIPK21 of maize (ZmCIPK21) enhanced salt tolerance in Arabidopsis (Chen et al., 2014). Previously, we reported a screening for drought-induced expression tag sequences (ESTs) of chickpea (Boominathan et al., 2004) and identified a putative CIPK-encoding EST. Deduced amino acid sequence of the full-length clone showed significant homology with Arabidopsis CIPK25 and, therefore, was named as CaCIPK25 (*C. arietinum* CIPK25). So far, no report on the characterization of CIPK25 of any plant is available in the literature. Here, we report a study on CaCIPK25 and showed that its expression in tobacco enhanced tolerance to salt and water-deficit stress. Further, replacement of a conserved threonine residue with aspartic acid in the kinase domain increased autokinase activity of CaCIPK25 and subsequently, stress-tolerance of the transgenic plants. We explored the tissue-specific expression of CaCIPK25 by fusing its 5′-upstream activation sequence (5′UAS) with a reporter gene. Different deletion constructs of 5′UAS were used to delineate the sequences responsible for expression in specific tissues.

Methods and Materials

Plant Materials, Growth Conditions, and Treatments

Seeds of chickpea (*Cicer arietinum* L.) cv. BGD72 were surface sterilized and imbibed in water for overnight. Pre-soaked seeds were germinated in pots containing the agropeat: vermiculite (3:1) and grown in the growth chamber at 25°C ± 2°C and 40% relative humidity for 6 days in 10-h light conditions. For tissue specific expression analysis, root, stem, matured leaves, and flower of 90-day-old chickpea plants were used. For different treatments, 6 days-old pot-grown seedlings were used. The 6 days-old seedlings in pots were exposed to 4°C for cold stress. For salt or dehydration (20% polyethylene glycol 8000 w/v) stress, the roots of the seedlings were dipped in 250 mM sodium chloride or PEG solutions, respectively, for specified periods. For abscisic acid (ABA, 100 μM), salicylic acid (SA, 15 μM), and methyl jasmonate (MeJA, 100 μM) treatments, the solutions were sprayed on leaves and the roots were dipped in these solutions as well for the specified periods. The control samples were treated with water similarly. For auxin 5 μM IAA (indole acetic acid) and cytokinin (5 μM BAP) treatments, roots of the seedlings were

dipped into the auxin and cytokinin solutions. Total RNA from the whole seedlings was used for gene expression.

Construct Preparation, Transgenic Plant Development, and Staining

Full length coding sequence (CDSs) was cloned by 5′ and 3′-RACE (rapid amplification of cDNA ends) using the primers mentioned in Supplementary Table 1 following previously described method (Tripathi et al., 2009). CDS of full-length and point-mutated CaCIPK25 were cloned in binary vector pBI121 for overexpression in *Nicotiana tabacum* var. Xanthi (tobacco). 2.3 kb long 5′-upstream activation sequence (UAS) along with 5′ untranslated region (UTR) of CaCIPK25 was retrieved from the chickpea genome sequence (Jain et al., 2013). Full-length and truncated 5′UAS along with the 5′UTR of CaCIPK25 were amplified by PCR using the primers mentioned in the Supplementary Table 1 and were cloned in pBI101 to drive expression of β-glucuronidase (GUS) and introduced in *Arabidopsis*. *In silico* analysis of the 5′-UAS was done using the tool PLACE (Higo et al., 1999). Transgenic *Arabidopsis* lines were made by floral dip method as described before (Tripathi et al., 2009). T_0 seeds were screened for kanamycin (50 mg/l) resistance to identify independent transgenic lines. T_3/T_4 homozygous transgenic seeds were used for experiments. The *Agrobacterium*-mediated transformation of tobacco leaf explants was performed with *A. tumifaciens* gv 3101 as described earlier (Gelvin and Schilperoort, 1994). For salt tolerance experiments, seedlings grown on ½-strength Murashige-Skoog (MS) medium with 1.5% sucrose for 8 days were transferred to the same medium with or without 250 mM sodium chloride and kept for 10 days and returned to growth medium for recovery. Histochemical GUS staining was done by vacuum infiltration of GUS-staining solution composed of 50 mM sodium phosphate buffer pH 7.0, 2 mM EDTA, 0.12% Triton X-100, 0.4 mM potassium ferrocyanide, 0.4 mM potassium ferricyanide, 1.0 mM 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexyl ammonium salt (X-Gluc) (Sigma-Aldrich, MO, USA) for 5–15 min and then incubated at dark from 2 to 12 h depending on tissue type. The stained tissues were cleared from chlorophyll by incubating in 70% ethanol at 65°C for 1 h and visualized by stereomicroscope.

RNA Isolation and Expression Analysis

Total RNA was extracted from different tissue samples using Trizol (Invitrogen, CA, USA). Eight-day-old seedlings of tobacco were used for RNA isolation. First strand cDNA was synthesized by High Fidelity cDNA synthesis kit (Roche Diagnostics GmbH, Germany) and oligo dT primer at 50°C for 30 min. Quantitative real-time PCR (qRT-PCR) experiments and calculations were performed using three technical and three biological replicates following the methods described before (Meena et al., 2015). Briefly, the reaction was performed in 10 µl reaction volume with 225 nM of each of the forward and reverse primers (Supplementary Table 2) and 2X Power SYBR Green PCR master mix (Applied Biosystems, CA, USA) using Vii A 7 Real-Time PCR System (Applied Biosystems, CA, USA). Chickpea Elongation factor 1-α (EF-1α) (GenBank: AJ004960.1) and tobacco actin (GenBank: BAD27408) genes were used as internal controls. Calculations were done using delta-delta Ct method. Paired students *t*-test was conducted to determine statistical significance of the results.

Site-directed Mutagenesis, Bacterial Expression, and Kinase Assay

Site-directed mutagenesis was done by replacing threonine (T) with aspartic acid (D) at 171th position in CaCIPK25. Mutagenesis reactions were carried out on double-stranded plasmid DNA using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) following parameters: 95°C for 30 s; 16 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 7 min using primers CaCIPK25T/D171F and CaCIPK25T/D171R. The bacteria-derived template (double stranded plasmid DNA used as a template for PCR reaction) was digested with methylation-specific restriction enzyme Dpn I at 37°C for 6 h and the digested PCR product was transformed into *Escherichia coli* DH5α cells. The mutation and the fidelity of the rest of the construct were confirmed by DNA sequencing. For the bacterial expression, coding sequence of CaCIPK25 was cloned into pGEX4T-2 to produce glutathione-S-transferase (GST)-fused protein. The construct was introduced into *E. coli* BL21 (DE3)-codon plus. Protein expression was induced by 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The bacterial pellet was suspended in lysis buffer (10 mM phosphate buffer pH7.0, 140 mM NaCl, 2.7 mM KCl, 0.5 mg lysozyme/gm of pellet) and was incubated for 1 h at 40°C. This cell suspension was sonicated 3 times with 20 s pulse and was centrifuged at 13,000 rpm at 4°C for 15 min to collect the supernatant. The GST-CaCIPK25 was affinity-purified using GSH-sepharose beads (GE Healthcare, USA). Kinase activity was measured as the incorporation of radioactivity from γ^{32}P-ATP into the CaCIPK25 and myelin basic protein (MBP). The purified recombinant GST-CaCIPK25 protein (0.5 µg) and MBP (0.5 µg) were incubated in the kinase buffer (10 µCi γ^{32}P-ATP, 20 mM Tris-HCl (pH 8.0), 5 mM $MnCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, and 1 mM DTT) for 30 min at 30°C. The reaction was stopped by addition of 4 X SDS-sample buffer. Reaction samples were boiled for 5 min. to denature proteins and separated by 10% SDS-PAGE electrophoresis and viewed by autoradiography.

Results

Cloning and Sequence Analysis of CaCIPK25

A previously reported screening to identify dehydration-inducible chickpea expression sequence tags (ESTs) (Boominathan et al., 2004) yielded an EST (GenBank: CD051323) with high expression under dehydration. 5′ and 3′ RACE (Rapid amplification of cDNA ends) resulted in a cDNA clone of 1727 base pair (bp) in length. Deduced protein sequence of the clone displayed highest homology (67% identity and 81% similarity) with CIPK25 of Arabidopsis and annotated as chickpea CIPK25 by NCBI (National Center for Biotechnology Information), hence, referred to as CaCIPK25 (*C. arietinum* CIPK25, NCBI: XP_004498818). The protein sequence showed 88% identity with a *Medicago truncatula* CBL-interacting protein kinase (XP_003588823.1) and 74% identity with CIPK25 of

Theobroma cacao (XP_007011728.1). In addition to the 1257 bp long protein-coding sequence (CDS), the cDNA clone also possessed a 5′-untranslated region (5′UTR) of 174 bases in length and a 3′-untranslated region (3′UTR) of 295 bases in length (Supplementary Text 1). Sequencing of the genomic DNA clone and comparison with the genome sequence showed that the gene was intronless and was located on linkage group 4. The deduced protein sequence was 418 amino acids in length with estimated molecular mass 47.55 kDa. CaCIPK25 was quite shorter in size than its *Arabidopsis* ortholog, which is of 488 aa, however, like other CIPKs, possessed an N-terminal SNF-1-related serine/threonine protein kinase domain (12–266 aa) and a C-terminal regulatory domain (295–409 aa) with a CBL-interacting NAF/FISL module. The activation loop (DFG…APE) and the threonine residue (Thr171), substitution of which by aspartic acid in SOS2 resulted in constitutive kinase activity, were conserved in CaCIPK25 (**Figure 1**).

Stress-mediated and Tissue-specific Expression of CaCIPK25

Expression profiling of *CaCIPK25* by qRT-PCR in chickpea, showed its highest expression in flower was about 2.5-fold, while the expressions in stem and leaf were 0.5− and 0.3−folds, respectively, of its expression level in root. Similar tissue-specific expression profile of the corresponding transcript (TC05858) was reported using RNASeq data (Garg et al., 2011) (**Figure 2A**). Upon treatment with 250 mM of sodium chloride, *CaCIPK25* transcript level increased at 1 h by 2.8-fold, reached at 5-fold by the 6th h and then declined to 3.9-fold at the 24th h. *CaCIPK25* expression steadily increased by PEG treatment from 4-fold increase after 1 h to 48-fold increase after 24 h of treatment. Exposure to low-temperature did not affect its expression significantly. Both the ABA and auxin treatments enhanced *CaCIPK25* expression, however, with different time kinetics. The expression quickly increased to 4.4-fold within 1 h of IAA treatment and then slowly declined to 1.8-fold at 24th h, whereas, transcript level increased slowly to 4.1-fold after 12 h of treatment with ABA and decreased to 3.9-fold after 24th h. Auxin and cytokinin function antagonistically and synergistically in root development. Accordingly, BAP treatment reduced *CaCIPK25* expression by more than 2-fold after 6 and 24 h of treatment. Treatment with methyl jasmonate (MeJA) and salicylic acid (SA) resulted in similar expression profiles with a slow increase in transcript level to about 4-fold after 12 h and then decrease to about 2-fold after 24 h (**Figure 2B**). 2.2 kb upstream activation sequence (5′UAS/promoter) together with the 5′UTR (Supplementary Text 1) of CaCIPK25 gene was cloned and fused with the reporter gene β-glucuronidase (*GUS*) (pCaCIPK25-GUS) and introduced in Arabidopsis to monitor tissue-specific expression of the gene at different growth stages. pCaCIPK25-*GUS* displayed a strong expression all over the radicle just after germination. However, with growth, the primary and the lateral roots, except the root tips and lateral root initials comprising of meristem cells, showed strong expression of the gene. This differential GUS staining in root indicated that *CaCIPK25* promoter activity is suppressed in tissues with high auxin concentration. The cotyledons, mostly the veins, showed moderate GUS expression. However, the true leaves showed a low GUS staining. The stem did not show any detectable GUS expression. As expected, the strongest expression was observed

```
CaCIPK25 ------------------------------MEELKANILFGKYEMGRVLGKGTFAKVYYG 30
AtCIPK25 MGSKLKLYPLLNHSSVFHPDSRYQSAPTMEEEQQQLRVLFAKYEMGRLLGKGTFGKVYYG 60
                                        *: : .:**.******:******.*****
CaCIPK25 KEIETGEGVAIKVIDKNKVKKEGMMEQIKREISVMRLVKHPNIVNLKEVMATKTKILFVM 90
AtCIPK25 KEITTGESVAIKIINKDQVKREGMMEQIKREISIMRLVRHPNIVELKEVMATKTKIFFIM 120
         *** ***.****:*:*:::**:***********:****:*****:***********:*:*
CaCIPK25 EYIRGGELFAKVEKGKLKEETARKYFQQLISAVEFCHSRGVAHRDLKPENLLLDEHGDLK 150
AtCIPK25 EYVKGGELFSKIVKGKLKEDSARKYFQQLISAVDFCHSRGVSHRDLKPENLLVDENGDLK 180
         **::*****:*: ******:.************:*******:**********:**:****
CaCIPK25 ISDFGLSALPEQLRQDGLLHTQCGTPAYVAPEVVRKRGYNGFKADTWSCGVILYALLAGF 210
AtCIPK25 VSDFGLSALPEQILQDGLLHTQCGTPAYVAPEVLRKKGYDGAKGDIWSCGIILYVLLAGF 240
         :***********: ********************:**:**:* *.* ****:***.*****
CaCIPK25 LPFQHENLITMYNKVFKEEYRFPPWFSPESKRLISKILVADPERRITISSIINDTWFKKG 270
AtCIPK25 LPFQDENLMKMYRKIFKSEFEYPPWFSPESKRLISKLLVVDPNKRISIPAIMRTPWFRKN 300
         ****.***:.**.*:**.*:.:***************:**.**::**:*.:*:. .**:*.
CaCIPK25 LST----------INSNDDLESEMVMINS-----SPKFFNAFEFISSMSSGFDLSGLF 313
AtCIPK25 INSPIEFKIDELEIQNVEDETPTTTATTATTTTTPVSPKFFNAFEFISSMSSGFDLSSLF 360
         :.:           * :*:  :  .   :      ********************.**
CaCIPK25 EEKKKRGSVFTSKCSVSEIVSKIESAAKSLRFKVGKVKEFKLKLQGMMEGRKGKLAVTAE 373
AtCIPK25 ESKRKLRSMFTSRWSASEIMGKLEGIGKEMNMKVKRTKDFKVKLFGKTEGRKGQIAVTAE 420
         *.*:*  *:***: *.***:.*:*. .*.:.:** :.*:**:** *  *****::*****
CaCIPK25 IYEVAPELAVVEFSKCSGDTFEYVKFFEDDVRPALKDIVWSWQGE--------------- 418
AtCIPK25 VFEVAPEVAVVELCKSAGDTLEYNRLYEEHVRPALEEIVWSWHGDNHNNNIVKSNGNYVS 480
         ::*****:****:.*.:***:** :::*:.*****::*****:*:
CaCIPK25 --------
AtCIPK25 DENSGSDC 488
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FIGURE 1 | Protein sequence alignment of AtCIPK25 and its ortholog CaCIPK25 in chickpea using ClustalW. The activation loop is shown in shaded region with the conserved threonine residue marked with an arrow. The NAF/FISL module responsible for interaction with CBL is shown in box.

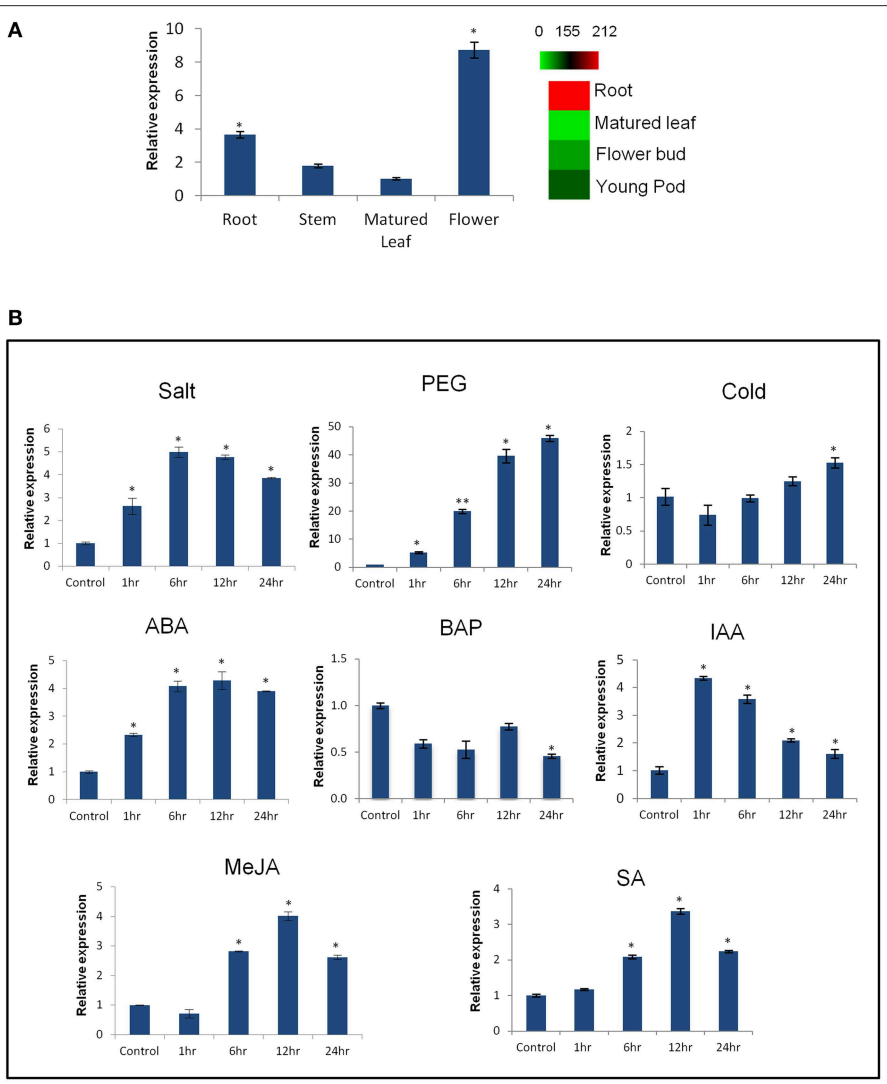


FIGURE 2 | Expression analysis of *CaCIPK25* gene in different tissues and under different treatments. (A) Fold expression of CaCIPK25 gene, keeping its expression level in matured leaf as reference, in different tissues as assessed by qRT-PCR. Digital expression profile of the corresponding transcript using RNASeq data is shown by a heatmap. **(B)** Fold expression change of *CaCIPK25* gene in whole chickpea seedlings upon treatment with various stresses and hormones as mentioned. Chickpea *EF1α* gene was used as internal control. Values in the Y-axis describe fold-changes in expression. X-axis describes time periods. Standard deviations were derived from three biological replicates. *, ** indicate statistically significant difference ($p < 0.05$) and ($p < 0.01$), respectively, from the control sample.

in flower, specifically, in the petals, anthers, and stigma. The promoter activity in flower was dependent on growth stage as GUS activity was not visible in the immature flowers (**Figure 3**).

Analysis of CaCIPK25 Promoter for Tissue-specific Expression

The 2.2 kb promoter sequence/5′UAS was analyzed for the presence of putative *cis*-acting elements. There were several dehydration responsive element/C-repeat (GTCGAC), ABA-responsive element (ACGTG), ARR1AT (NGATT), auxin responsive elements (TGTCTC), and W-box element (TTGAC/TGAC) are present within this region of the promoter explaining differential expression of CaCIPK25 upon treatments with salt, PEG, ABA, BAP, and IAA. In addition to those, multiple copies of ROOTMOTIFTAPOX1 (ATATT) (24 copies in the positive strand) and POLLEN1LELAT52 (AGAAA) (9 copies in the positive strand) elements, which drive root-specific and anther-specific expression, respectively, were found. Detail analysis of *cis*-acting elements in the 5′UAS of CaCIPK25 is presented in Supplementary Text. ROOTMOTIFTAPOX1 element was first identified in the promoter region of the rolD gene of *Agrobacterium rhizogenes*. The GUS gene driven by rolD promoter strongly expressed in the roots and expressed at a very low level in stem and leaves of tobacco plants. The distinctive expression pattern of the rolD promoter-GUS construct was that the strongest GUS activity was observed in the root elongation zone and vascular tissue and a very low expression in the root apex (Elmayan and Tepfer, 1994), highly

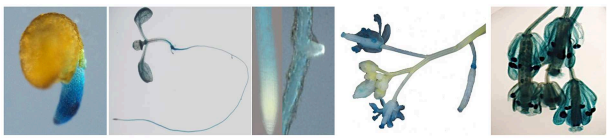


FIGURE 3 | Activity of *CaCIPK25* gene promoter in different tissues in transgenic *Arabidopsis*. CaCIPK25 gene promoter up to the translation start codon was fused to GUS gene and introduced in *Arabidopsis* to study promoter activity in different tissues. Representative samples were shown after histochemical GUS staining. Tissues shown from left were radicle, whole seedling, root apex, lateral root initial flower buds and flowers, flower at higher magnification.

consistent with the expression pattern of pCaCIPK25-GUS construct. A high GUS activity driven by CaCIPK25 promoter was observed in the root, cotyledon and reproductive organs and a very low GUS activity was observed in the true leaves. The pollen-specific *cis*-acting element POLLEN1LELAT52 was previously reported in the 5′UAS of tomato endo-β-mannase 5 gene (LeMAN5). Transgenic Arabidopsis plants expressing GUS driven by LeMAN5 promoter showed strong GUS activity in the anthers and pollens. In anthers, the highest LeMAN5 mRNA expression was observed in the later stages of flower development (Filichkin et al., 2004), similar to pCaCIPK25-GUS expression in flower. To delineate the *CaCIPK25* promoter regions driving the root and flower-specific expression, two promoter deletion constructs were used. The first deletion construct (pCaCIPK25D1) was made by retaining −1 to −1047 bases of the promoter. The first deletion of 1150 bases from the 5′-end removed 13 ROOTMOTIFTAPOX1 elements out of twenty-four and one POLLEN1LELAT52 elements out of nine (**Figure 4A**). Arabidopsis plants expressing GUS driven by pCaCIPK25D1 showed a substantial reduction of GUS-staining in cotyledons, petals, anthers, and stigma. However, decrease in GUS stain in roots was not so profound, suggesting the −1046 to −2196 region of the promoter was more relevant for the expression of the gene in cotyledon, petal and anther tissues. The second deletion construct (pCaCIPK25D2) removed next 700 bases and all the ROOTMOTIFTAPOX1 and POLLEN1LELAT52 elements. This deletion totally abolished GUS expression in petals and cotyledons. There was a substantial reduction in GUS expression in roots and anthers, but the removal of all the ROOTMOTIFTAPOX1 and POLLEN1LELAT52 elements did not totally abolish the GUS expression in these two tissues, suggesting that the immediate 378 bases from the transcription start site of the promoter was also responsible, although modestly, for root- and anther-specific expression (**Figure 4B**).

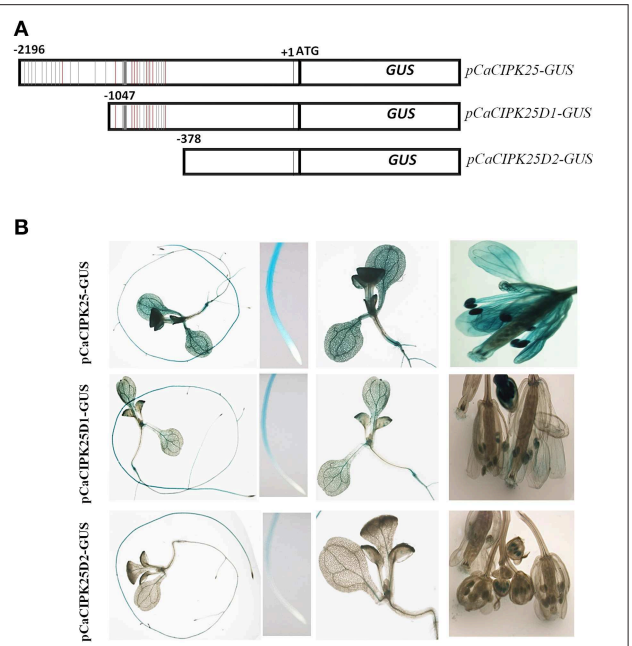


FIGURE 4 | Analysis of *CaCIPK25* promoter for tissue-specific expression. (A) Schematic diagram of CaCIPK25 promoter-GUS reporter construct (*pCaCIPK25-GUS*) and its two truncated versions (*pCaCIPK25D1-GUS* and *pCaCIPK25D2-GUS*). Positions (not to scale) of ROOTMOTIFTAPOX1 and POLLEN1LELAT52 elements are shown in gray and red lines. Transcription and translation start sites (+1 and ATG) are marked. **(B)** Expression of GUS driven by different deletion constructs of *CaCIPK25* promoters in whole seedlings, root apex, cotyledon, and leaves and flowers are shown.

Kinase Activity of CaCIPK25

In order to biochemically characterize the CaCIPK25 protein, the CDS was expressed in *E. coli* as a glutathione-S-transferase (GST)-fused protein. The conserved threonine (T171) residue located at the activation domain was substituted with aspartic acid (D) and mutated recombinant protein was expressed similarly. The purified recombinant proteins were tested for auto- and substrate phosphorylation. GST-CaCIPK25 showed a low level of auto-kinase activity. The T171D substitution increased the autokinase activity of the protein by about 2-fold. GST-CaCIPK25 was able to use myelin basic protein as a substrate and the aspartic acid substitution increased the kinase activity protein by about 2.5-fold using this substrate (**Figure 5**).

Increased Root Length of CaCIPK25-expressing Tobacco Plants

To investigate *in planta* function, CaCIPK25 and CaCIPK25T171D (henceforth referred to as CaCIPK25T/D) were expressed in tobacco plants under the control of 35S promoter. More than 10 transgenic lines were raised for each construct along with the lines harboring only pBI121 (vector-control) using the *Agrobacterium*-mediated transformation method. T_3 homozygous plants were selected for experiments on the basis of expression analysis by RT-PCR (Supplementary Figure 1). Seeds of two individual lines for each construct along with the vector-control seeds were sown on ½MS-agar plates for germination. No difference was observed among the lines with respect to the percentage of and time taken for germination, however, all the lines expressing CaCIPK25 or CaCIPK25T/D displayed longer root length within 2 days of germination. After 15 days, the primary roots of the CaCIPK25— and CaCIPK25T/D-overexpressing lines were 52 and 60% longer than the roots of the control line (**Figures 6A,B**). The difference

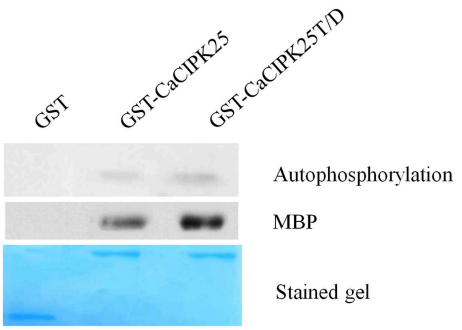


FIGURE 5 | Kinase assay of CaCIPK25 protein. Autophosphorylation and substrate phosphorylation assay of bacterially expressed GST-CaCIPK25 and CaCIPK25T/D proteins. GST protein was used as control. Myelin basic protein (MBP) was used as substrate. A Coomassie blue-stained gel shows the amount of CaCIPK25 protein loaded.

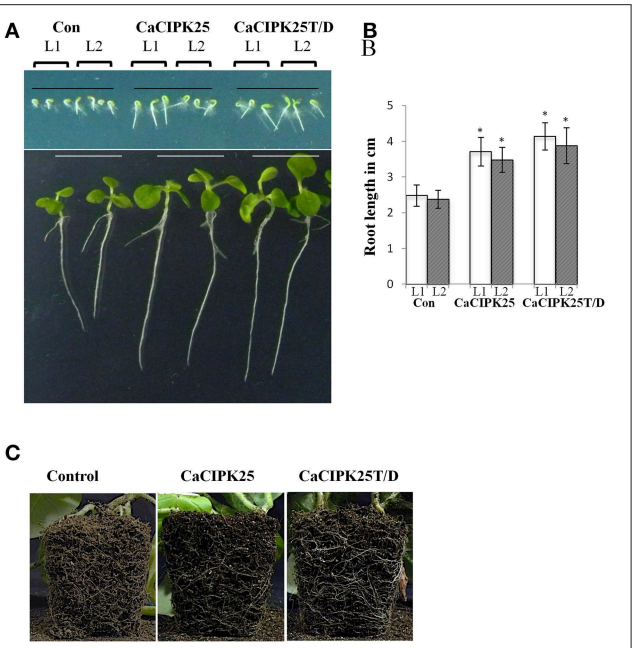


FIGURE 6 | Morphology of CaCIPK25 overexpressing transgenic tobacco lines. (A) Morphological comparison of vertically grown tobacco seedlings transformed with empty vector (Con), *CaCIPK25* and *CaCIPK25T*^{171}*D* genes. Transgenic seeds from two lines (L1 and L2) are represented for all the constructs, upper and lower panels are showing 2− and 15−days old seedlings after germination, respectively. **(B)** Comparison of root lengths of 15-days old seedlings. Averages of three measurements of 15 seedlings each are shown. * indicates statistically significant difference ($p < 0.05$) from the control sample. **(C)** Root system of soil-grown 50 days-old transgenic tobacco plants. Roots exposed to the soil surface are shown.

in root morphology was more evident in the matured plants. The CaCIPK25− and CaCIPK25T/D− overexpressing plants showed an enlarged root system as compared to the control plants when grown in soil for 50 days (**Figure 6C**). Although, the leaves of the seedlings expressing both the CaCIPK25 constructs were larger than those of the control seedlings at the early stages after germination probably due to longer root, there was no significant difference in leaf sizes observed between the control and CaCIPK25-expressing tobacco plants in the later stages of growth (Supplementary Figure 2).

Improved Salinity and Water Deficit Tolerance in CaCIPK25-overexpressing Plants

To investigate the effect of the CaCIPK25 expression on germination efficiency of the transgenic seeds under high salinity condition, seeds were sown on normal growth medium supplemented with 200 mM sodium chloride and allowed to germinate for 15 days. The tobacco seeds transformed with the empty vector and both the constructs of CaCIPK25 germinated simultaneously and showed similar growth in normal growth medium. Both the CaCIPK25-transformed seeds took 6 days more to germinate on the salt-supplemented medium as compared to their germination on the normal growth medium. Only 7% of vector-control seeds germinated in comparison to 60 and 64% seed germination of CaCIPK25− and CaCIPK25T/D-transformed lines, respectively, after 15-day exposure on high-salt medium (**Figure 7A**). To assess salt-tolerance of the seedlings, 8-day-old seedlings were exposed to medium supplemented with 250 mM sodium chloride. After 10 days of exposure, the seedlings were transferred to the normal growth medium for recovery for 8 days (**Figure 7B**). Leaves were scored for chlorosis and the relative fresh weights for each line were assessed by comparing the fresh weights of the corresponding lines continuously grown on the normal growth medium. Approximately 80% leaves of the control plants have undergone chlorosis in contrast to about 36 and 26% chlorosis in the lines expressing CaCIPK25 and CaCIPK25T/D, respectively (**Figure 7C**). The relative fresh weight of the control seedlings grown in high salinity was about 20% of those grown in normal medium, while the relative fresh weights of the CaCIPKL25− and CaCIPK25T/D− expressing plants were 53 and 57%, respectively (**Figure 7D**). Enhanced tolerance to salt and drought was also observed in plants grown in pots. Fifty days-old plants of one line for each of the constructs, three in a pot in duplicate, were irrigated with 300 mM sodium chloride twice a week for 2 weeks. All the vector-control plants were etiolated, displayed severe chlorosis and died. The plants expressing both the forms of CaCIPK25 showed a moderate level of chlorosis and etiolation. Similarly, 50-day-old plants in pots were not irrigated for 25 days. All the control plants died within this period while all the plants expressing the either form of CaCIPK25 showed a moderate level of etiolation (**Figure 8**) and nine out of 12 plants recovered upon further irrigation for 2 weeks.

Expression levels of the known genes related to abiotic stress signaling and tolerance were assessed in the transgenic plants under control and stress treatments. Five genes, namely NtERD10B (GenBank:AB049336), NtERD10C (AB049337), NtDREB1 (EU727155), NtDREB2 (EU727156), NtAPX1 (U15933.1), and were selected on the basis of their reported enhanced expression in transgenic plants showing tolerance to such stresses (Shukla et al., 2006; Tripathi et al., 2009; Bao et al., 2015; Zhou et al., 2015). NtERD10B and NtERD10C encode dehydrins, NtDREB1 and NtDREB2 encode dehydration responsive element (DRE)/C-repeat element (CRE) binding

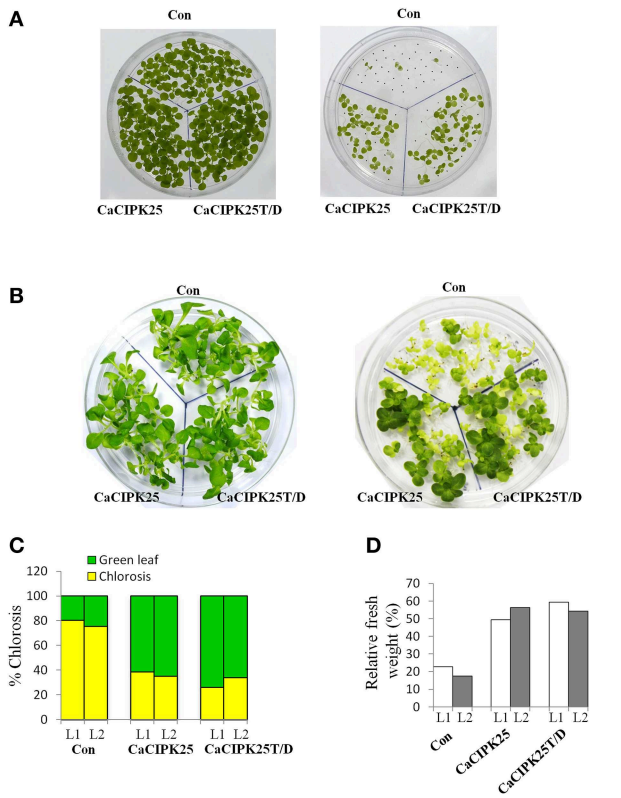


FIGURE 7 | Effect of salt stress on transgenic tobacco seedlings. **(A)** Control (vector only), *CaCIPK25*- and *CaCIPK25T/D*- expressing tobacco seeds were germinated on media without (left) and with (right) 200 mM sodium chloride for 15 days. **(B)** 8-day-old seedlings of control, CaCIPK25– and CaCIPK25T/D-expressing lines were transferred to media without (left) and with (right) 250 mM sodium chloride for 10 days and then transferred to normal medium for 8 days. **(C)** Leaves of the seedlings of two representative transgenic lines (L1 and L2) exposed to salt were scored for chlorosis and bleaching and presented as relative to total number of leaves. **(D)** Fresh weights of the seedlings from the same experiment were presented relative to the fresh weights of corresponding seedlings grown in normal medium for the same period.

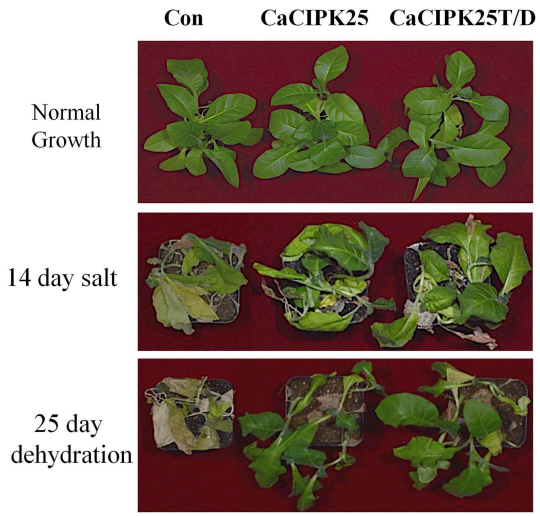


FIGURE 8 | Effect of salt on transgenic tobacco plants. Fifty-day-old soil-grown vector-control, *CaCIPK25*- and *CaCIPK25T/D*- overexpressing tobacco plants **(top)** were irrigated with 300 mM sodium chloride for 2 weeks **(middle)** or, not irrigated with water for 25 days **(bottom)**.

proteins and are transcription factors, and NtAPX1 encode ascorbate peroxidase. Only NtERD10B and NtERD10C showed more than 2-fold increase in expression level with respect to the vector-control plants only in the CaCIPK25T/D-overexpressing line in the control condition. Three other genes showed less than 2-fold increase in this line. Expression of all the five genes was not significantly increased in the CaCIPK25-overexpressing plants. Upon treatment with 20% PEG or 250 mM sodium chloride, expression of these genes increased several folds in the vector-transformed plants. In the CaCIPK25- or CaCIPK25T/D-expressing tobacco lines, increase in expression level of all the five genes was more than 2-fold as compared to the vector-transformed plants when exposed to PEG and sodium chloride. Further, expression of all the five genes were always higher in the CaCIPK25T/D lines than that in the CaCIPK25 lines, suggesting that the higher kinase activity of the protein resulted in higher expression of stress-related genes and, thereby, further enhanced tolerance level (**Figure 9**).

Discussions

In this report, we proposed CaCIPK25, a chickpea ortholog of Arabidopsis CIPK25, as a positive regulator of root development and tolerance to water deficit and high salinity. This is the first report on the function of CIPK25 from any plant. This gene was cloned from one of the two ESTs identified in a screening of dehydration-induced ESTs in chickpea. Under normal growth condition, CaCIPK25 expression is restricted to root and flower. Expression of a reporter gene driven by the 5′-UAS of CaCIPK25 in Arabidopsis also supported this expression pattern, suggesting that similar factors controlled this promoter activity in these two plants. The expression pattern surprisingly corresponded to that of the rolD gene of *Agrobacterium rizogenes*. CaCIPK25 promoter, like that of rolD, possesses multiple copies of similar root– and flower-specific *cis*-acting elements. Infection with *A. rhizogenes* with mutated *rolD* gene resulted in attenuated root growth and formation of callus (White et al., 1985). A fine balance of auxin and cytokinin concentrations in root regulates natural root growth. While auxin promotes cell division at the root apical meristem, cytokinin promotes cell differentiation at the elongation zone of the root (Chapman and Estelle, 2009). Therefore, it appears that rolD functions to maintain the balance between auxin and cytokinin and, thereby, promotes root growth. Antagonistic expression pattern of CaCIPK25 in response to auxin and cytokinin, absence of its expression at the root apex and lateral root initials having high auxin concentration and enhanced root growth in the overexpressing lines indicates its involvement in maintaining balance between auxin and cytokinin. This was further supported by the presence of auxin-responsive and cytokinin-responsive *cis*-acting elements in the 5′-UAS of the gene. The other organ that showed high CaCIPK25 expression was the flower. CaCIPK25 expression in

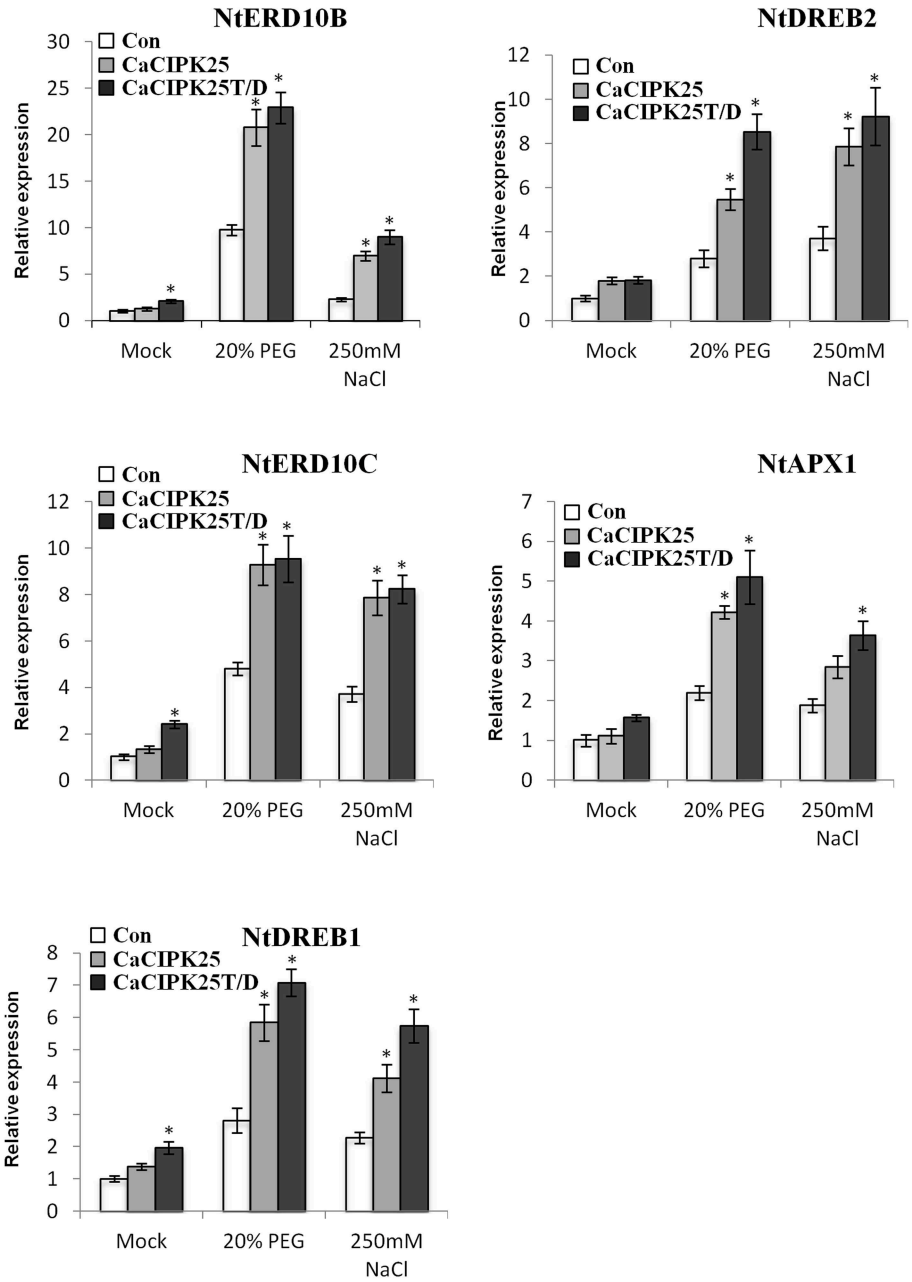


FIGURE 9 | Expression analysis of abiotic stress marker genes in CaCIPK25 overexpressing plants. Expression of known abiotic stress marker genes determined by qRT-PCR in 20-day-old control, *CaCIPK25*– and *CaCIPK25T/D*– overexpressing tobacco plants in response to treatments mentioned. Y-axis described the fold change of expression. Tobacco actin gene was used as internal control. * indicates statistically significant difference ($p < 0.05$) from the control sample.

flower was growth stage dependent, similar to that of rolD. Transgenic tobacco plants expressing rolD were early flowering and displayed earlier and enhanced organogenesis of flowers (Mauro et al., 1996). We did not observe any apparent differential morphology or period taken for flowering. Most probably, enhanced expression of CIPK25 alone was not enough to bring about altered morphology in the reproductive organs. On the other hand, CIPK25 activity in reproductive tissues was already saturated and, therefore, overexpression could not alter the morphology or period taken for flowering.

CaCIPK25 overexpressing plants showed enhanced root growth in normal growth condition. It suggested that there was a potential for the root to grow longer. CIPK25 activity for root growth was limiting and overexpression of the

protein must activated a signaling pathway that regulates root growth. Overexpression of high active CaCIPK25 mutant (CaCIPK25T/D) caused a further increase in the root length suggesting the kinase activity was required for this function. The larger leaf size of the CaCIPK25-overexpressing seedlings with respect to the control seedlings at the early stage of growth was most probably due to faster root growth and, thereby, absorption of more nutrient from the medium. The shoot size of the control plants was recovered at the later stage. This result and a very low expression of *CaCIPK25* in stem and leaf suggested that associated factors required for CaCIPK25 to promote shoot growth were absent. Fold increases in the marker gene expression in CaCIPK25T/D-overexpressing plants were higher than those in the CaCIPK25-overexpressing plants. Higher fold increase in the marker gene expression and in the limit of tolerance in the *CaCIPK25T/D*-overexpressing plants suggested that the full potential of CaCIPK25 activity was not achieved even after stress treatment. We assessed the expression level of a few marker genes related to abiotic stress tolerance. Expression of only NtERD10B and NtERD10C was increased by 2-fold in the CaCIPK25T/D-overexpressing lines as compared to the vector-control plant in normal growth condition. Expression of all the marker genes increased many folds only after stress treatment. This result suggested that just overexpression of CaCIPK25 alone or longer root length was not enough for introducing stress tolerance. It appeared that stress tolerance mechanism of CaCIPK25-overexpressing plants was activated only after stress treatment. Similar observation was previously reported in case of *Arabidopsis* DREB2A protein, where stress treatment was required to nullify the negative regulatory effect of a negative element in the DREB2A protein (Sakuma et al., 2006). There are several reports demonstrating the importance of DREB protein family in drought and salinity tolerance (Agarwal et al., 2006; Morran et al., 2011; Jiang et al., 2014). CaCIPK25 overexpression caused enhanced expression of tobacco DREB1 and DREB2. A previous report described that the overexpression of a calcium binding peptide CBP in *Arabidopsis* resulted in the increase in total Ca^{+2} store in the cell and provided salinity tolerance to the plant. CBP-overexpressing plants showed higher expression of DREB1A and CIPK6. However, when crossed with cipk6 mutant, the CBP-overexpressing plants did not show any enhancement in the salinity tolerance and expression of DREB1A, suggesting CIPK6 was involved in Ca^{+2}-mediated expression of DREB1A (Tsou et al., 2012). This observation, and the enhanced expression of tobacco DREB1 and DREB2 in the CaCIPK25-overexpressing plants after stress treatment indicated that there might be some connections between the CIPKs and DREBs in the stress-regulated Ca^{+2}-mediated signaling. Cytosolic Ascorbate peroxidase1 (APX1) was previously reported to play a key role in the stress acclimation of *Arabidopsis* (Koussevitzky et al., 2008).

In this report, we have presented an expression analysis of chickpea *CaCIPK25* gene and characterized the transgenic tobacco plants overexpressing this gene. *CaCIPK25* expressed preferentially in the root, except in root apex, and flower. Its overexpression in tobacco plants resulted in enlarged root system with a normal shoot morphology. Although, highly expressed in flower, the overexpressing plant did not show any apparent morphological disorder in the reproductive organ and the flowers were fertile. Overexpression of *CaCIPK25* in tobacco enhanced the tolerance of the transgenic plant to water deficit and high salinity. Replacement of a conserved threonine residue with aspartic acid in the activation domain of the protein increased its kinase activity. Overexpression of this active kinase caused further enhancement of root growth and tolerance to stress treatments as compared to that of the wild-type protein suggesting that the kinase activity was important for these functions. Altogether, we have reported for the first time a functional characterization of CIPK25 from a plant.

Author Contributions

MM, SG, VD, and AR conducted the experiments, interpreted the results, and prepared the first draft. DC conceptualized the study, designed the experiments, interpreted the results and made the final draft. All the authors approved the final version.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00683

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G-protein Signaling Components GCR1 and GPA1 Mediate Responses to Multiple Abiotic Stresses in *Arabidopsis*

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G-protein signaling components have been implicated in some individual stress responses in *Arabidopsis*, but have not been comprehensively evaluated at the genetic and biochemical level. Stress emerged as the largest functional category in our whole transcriptome analyses of knock-out mutants of GCR1 and/or GPA1 in *Arabidopsis* (Chakraborty et al., 2015a,b). This led us to ask whether G-protein signaling components offer converging points in the plant's response to multiple abiotic stresses. In order to test this hypothesis, we carried out detailed analysis of the abiotic stress category in the present study, which revealed 144 differentially expressed genes (DEGs), spanning a wide range of abiotic stresses, including heat, cold, salt, light stress etc. Only 10 of these DEGs are shared by all the three mutants, while the single mutants (GCR1/GPA1) shared more DEGs between themselves than with the double mutant (GCR1-GPA1). RT-qPCR validation of 28 of these genes spanning different stresses revealed identical regulation of the DEGs shared between the mutants. We also validated the effects of cold, heat and salt stresses in all the 3 mutants and WT on % germination, root and shoot length, relative water content, proline content, lipid peroxidation and activities of catalase, ascorbate peroxidase and superoxide dismutase. All the 3 mutants showed evidence of stress tolerance, especially to cold, followed by heat and salt, in terms of all the above parameters. This clearly shows the role of GCR1 and GPA1 in mediating the plant's response to multiple abiotic stresses for the first time, especially cold, heat and salt stresses. This also implies a role for classical G-protein signaling pathways in stress sensitivity in the normal plants of Arabidopsis. This is also the first genetic and biochemical evidence of abiotic stress tolerance rendered by knock-out mutation of GCR1 and/or GPA1. This suggests that G-protein signaling pathway could offer novel common targets for the development of tolerance/resistance to multiple abiotic stresses.

Keywords: ***Arabidopsis*, G-protein, GPA1, GCR1, abiotic stress, enzyme assays, qPCR**

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Abbreviations: GPA1, G-protein α subunit 1; GCR1, G-protein Coupled Receptor 1; DEGs, Differentially Expressed Genes; MDA, Malondialdehyde; RWC, Relative Water Content; APx, Ascorbate Peroxidase; SOD, Superoxide Dismutase.

INTRODUCTION

Plants encounter a variety of abiotic and biotic environmental stresses, which result in substantial loss in yield of crops worldwide. Greenhouse gas emissions and climate change could further exacerbate various stresses that plants have to encounter (Challinor et al., 2014). The abiotic stresses include temperature variations (both low and high), flood, drought, and salinity. The molecular mechanisms of stress response have been extensively researched and reviewed (Cabello et al., 2014; Suzuki et al., 2014; Tanveer et al., 2014; Parihar et al., 2015). However, the search for new genetic targets for crop improvement toward stress tolerance is far from complete. Signaling mechanisms in plant stress response are of particular interest in this regard, as their extensive cross talk in plants could reveal common genetic targets to deal with multiple stresses. The signaling mechanisms in plants during low and high temperature, drought, and salinity are different and yet related to each other (Nakashima et al., 2014; Smékalová et al., 2014). Each of these stresses provide different cues and elicit changes from the plant at different levels, including physiological and biochemical levels, as well as at the level of gene expression. The physiological changes are easily measurable in terms of germination, root length, shoot length, etc., whereas the biochemical changes are measured by markers such as lipid peroxidation, SOD assay, APx assay, etc. (Cabello et al., 2014).

G-proteins and GPCR have been associated with several stress-signaling pathways in plants (Pandey et al., 2015). G-proteins transmit the signal through downstream effectors like ion channels, phospholipases, kinases/phosphatases and other GTPases (Xu et al., 2015). G-proteins regulate the activity of many enzymes like phosphatidylinositol-phospholipase C (PLC) and phospholipase D (PLD) (Apone et al., 2003). They in turn modulate the expression of stress-responsive genes like LEA and LEA-like genes under different stress conditions in *Arabidopsis* (Zhao, 2015), clearly indicating the involvement of G-proteins in stress signaling. Similarly, the loss-of-function mutant of GCR1 in *Arabidopsis* was reported to be resistant to drought stress and also showed higher expression levels of few known drought- and ABA-regulated genes (Pandey and Assmann, 2004). This was also consistent with their finding that GCR1 acts as a negative regulator of GPA1-mediated ABA responses in *Arabidopsis* guard cells. In tobacco, transgenic lines overexpressing Gα and Gβ from pea revealed the role of Gα in salinity and high temperature stress response, while Gβ was linked to heat tolerance (Misra et al., 2007). Though, most of the stress-related studies on GPA1 have been done on ABA and biotic stresses (Alvarez et al., 2011; Wang et al., 2011; Urano et al., 2013), recent studies in *Arabidopsis* revealed that G-proteins are also involved in growth under salt stress (Colaneri et al., 2014), as well as cellular senescence and cell division in rice and maize (Urano et al., 2014).

Though important, these scattered findings were not enough to suggest widespread role of G-protein signaling components in transducing multiple stress signals. Recently, our whole transcriptome analyses of loss-of-function mutants of GCR1 and GPA1 in *Arabidopsis* revealed stress response pathways as the largest functional cluster of differentially expressed genes (Chakraborty et al., 2015a,b). Our analysis of the GCR1-GPA1 double mutant further confirmed the higher functional overlap of stress response as a category at the process level, despite the limited overlap between the mutants in terms of the DEGs themselves (Chakraborty et al., submitted). These results led us to ask whether G-protein signaling components offer converging points in the plant's response to multiple abiotic stresses (especially GCR1 and GPA1) in *Arabidopsis*. The present paper tested this hypothesis by a thoroughly detailed analysis of the abiotic stress-related category of DEGs revealed in our functional genomic analyses of the single and double mutants, as well as by validating them parallelly under three stresses *viz.*, cold, heat, and salt.

MATERIALS AND METHODS

In-silico Analysis of Stress Responsive Genes

For this study, we used our transcriptome data obtained from the single and double mutants of GPA1 and GCR1 (GSE 40217). The list of DEGs from the transcriptome of each of the mutants was used separately as input data to generate an abiotic stress responsive dataset for each of the mutant by comparing against the stress responsive transcription factor database (STIFDB2.0). These abiotic stress responsive genes were then subjected to Venn selection to check their overlap in the mutants. Further each of these gene list were put as an input to expression browser against abiotic stress series of AtGenexpress (Toufighi et al., 2005) as background data to check their expression profile in previous expression data.

Plant Material and Stress Treatments

Arabidopsis thaliana wild type, Ws2 and knock-out mutants devoid of either GPA1 (*gpa1-5*) or GCR1 (*gcr1-5*) or both (*gpa1-5gcr1-5*) were grown on 1X B5 medium hydroponically in a growth chamber at 22 ± 1°C with a light intensity of 150 μM s^{-1} m^{-2} and a photoperiod of 16:8 h of light:dark cycle. The seeds were vernalized prior to inoculation at 4°C for 2–3 days. The seedlings were allowed to grow for 10 days followed by stress treatments. For cold stress, the seedlings were placed at 4°C for overnight (Al-Quraan et al., 2012); heat stress was given at 37°C for 4 h (Barah et al., 2013); and salt stress was given using 100 mM NaCl for 12 h (Colaneri et al., 2014). RWC was performed immediately after stress treatments. Rest of the tissues of the control as well as stressed plants were harvested in liquid nitrogen and kept at −80°C till further use. For germination studies, vernalized seeds were inoculated onto 1X B5 plates solidified using 0.4% ClariGel (Hi-Media, India) and incubated at the appropriate temperatures. For salt stress, seeds were plated on 1X B5 plates containing 100 mM NaCl and incubated at 22 ± 1°C. We used 5 plates for each of the conditions. The plates were scored for germination after 3 days. For, measurement of root and shoot length under stress and control conditions, seeds were placed on similar kind of plates as given above and incubated in vertical position at appropriate conditions. The root and shoot lengths were measured after a week.

RT-qPCR Validation of Stress Responsive Genes

In order to validate the stress response of the wild type and the three mutants, 28 DEGs were picked from all the mutants in such a way that some of them were common to at least two of the three mutants and some were unique to any one of the three mutants. This resulted in 16, 13, and 20 DEGs picked from gcr1-5, gpa1-5, and gpa1-5gcr1-5, respectively, including some well-characterized stress-responsive genes like RD29A, RD26, ERF13, CML38, etc. Their sequences were obtained from TAIR and primers were designed using PrimerQuest tool of IDT. Total RNAs were isolated from the control and stressed tissues and the RNA samples were analyzed using spectrophotometer and electrophoresis to determine the quantity and quality. The total RNAs were used for qPCR with gene-specific primers. GPA1 and/or GCR1 responsive DEGs were verified by RT-qPCR using the instrument Stratagene Mx3000P (Agilent technologies) using standard conditions. Typically, total RNA was digested by RNase free DNase (Fermantas), repurified, quantified, and 5 μg of RNA was used for cDNA preparation for each biological replicate using Oligo(dT) primers and RevertAid reverse transcriptase (Fermentas). Sequences for designing the primers were obtained from TAIR. PCR amplifications were performed in 20 μl by using the BrilliantIII Ultrafast SYBR Green QPCR mastermix (Agilent Technologies) with 1.0 μl of sample cDNA and 100 n moles of each gene-specific primer. Primer efficiency was determined by serial dilution of the template and only primers that worked at 90–110% efficiency were used for all qPCR analyses (**Supplementary Table S1**). The specificity of primer pairs was obtained by melting curve analysis of the amplicons. Actin2 (ACT2) was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed by using the 2–11 CT method (Pfaffl, 2001).

Relative Water Content (RWC)

Relative water content of the mutants and the wild type were measured (Slavík, 1974) after the control and stress treatments. A seedling was removed and weighed (W). The seedling was then floated on de-ionized water in a Petridish/pre-weighed vial and kept at 10°C for 4 h. The seedling was then removed and wiped the surface water using a paper towel. This surface dried seedling was weighed again (TW). The seedling was then kept for drying in an 80 °C hot air oven overnight/for 24 h. The dried seedling was weighed again (DW) and RWC was calculated using the below mentioned formula:

$$RWC\,(\%) = \frac{W-DW}{TW-DW} \times 100$$

Proline Content

Proline was extracted by heating the tissue (250 mg) twice in 80% ethanol and once with 50% ethanol, to obtain the final extract in a 70:30 mixture of ethanol and water. Proline standards (0.04–1 mM) were prepared by dissolving standard proline in 70:30 ethanol:water mixture. 50 μl of extract/standard was added to 100 μl of reaction mixture containing 1.0% (w/v) ninhyrin in 60% acetic acid and 20% (v/v) ethanol (Reaction mixture must be protected from light). Then the tubes were sealed and heated at 95°C for 20 min and then allowed to cool to room temperature. The mixture was then centrifuged at 25,000 rpm for 1 min. One hundred microliter of this mix was then transferred to a microplate well and absorbance was taken at 520 nm (Bates et al., 1973). The proline content was estimated against the standard curve generated.

Malondialdehyde Assay (MDA)/Lipid Peroxidation Assay

Plant tissue (0.1 g) was crushed to fine powder in liquid nitrogen, added to 3 ml of 10% TCA and mixed well. The tube was then centrifuged at 12,000 rpm for 20 min. 2 ml of supernatant was taken and 2 ml solution of 10% TCA containing 0.6% TBA was added to it. The mixture was then heated at 85°C for 30 min and allowed to cool to room temperature. Absorbance was then taken at 450, 532, and 600 nm. MDA content was calculated using the formula (Hodges et al., 1999):

$$MDA\ content = (Z \times 6.45) - (A_{450} \times 0.56)\ \mu M/gFw$$

where, $Z = (A_{532} - A_{600})$, gFw = Fresh weight (in g)

Catalase Assay

Plant tissue (0.25 g) was crushed using liquid nitrogen to fine powder and added to 1 ml 0.1% (w/v) TCA in an ice-bath. The mixture was then centrifuged at 12,000 g for 15 min at 4°C and the supernatant (100 μl) was taken. To it, 50 μl of 10 mM potassium phosphate (pH 7.0) and 100 μl 1 M potassium iodide were added, vortexed and absorbance was measured at 390 nm (Velikova et al., 2000). Final calculation was done against the standard curve generated using commercial hydrogen peroxide.

Ascorbate Peroxidase (APx) Estimation

Tissue (0.25 g) was ground to a fine powder with liquid nitrogen and added to 1 ml extraction buffer containing 50 mM sodium phosphate (pH 7.5), 1 mM PEG, 1 mM PMSF, 8% (w/v) PVPP, and 0.01% (v/v) Triton X-100. The mix was centrifuged at 18,000 rpm for 20 min and the supernatant was transferred to a fresh tube. The extract (20 μl) was added to 1 ml of reaction mixture (0.2 M Tris-Cl, pH 7.8; 0.25 mM ascorbic acid, and 0.5 mM H_2O_2), mixed by inversion and absorbance recorded after 10 min at 290 nm till the absorbance stabilized (Nakano and Asada, 1981). The enzyme activity was calculated as follows:

$$APx\ activity = \frac{A_2-A_1}{T_2-T_1} \text{per mg protein}$$

Superoxide Dismutase (SOD) Assay

The tissue (0.25 g) was homogenized in 100 mM TEA buffer (pH 7.4), centrifuged at 16,000 rpm for 20 min and the supernatant was used as the crude extract. The assay mixture was prepared by adding 10 mM TEA buffer (pH 7.4), 7.5 mM NADH, 100 mM/50 mM EDTA/$MnCl_2$, 10 mM 2-mercaptaethanol and the crude extract. Decrease in absorbance was monitored at

340 mM for 15 min (Beauchamp and Fridovich, 1971). The enzyme activity was calculated as:

$$SOD\ activity = \frac{A_2 - A_1}{T_2 - T_1} \text{per mg protein}$$

Statistical Analyses

The data were analyzed statistically using ANOVA (analysis of variance) and the differences among the mean values were compared with Duncan's Multiple Range Test (DMRT) ($P < 0.05$) using Sigmaplot ver. 11 (Wass, 2009). All the results were expressed as mean ± SD of three independent experiments.

RESULTS

In-silico Analysis GPA1/GCR1-responsive Genes in Abiotic Stress

In this study, our comparison of DEGs from GPA1/GCR1-responsive transcriptomes to the known list of abiotic stress-responsive genes (STIFDB2.0) (Naika et al., 2013) revealed 57 DEGs (49 up/8 down) from the *gcr1-5 mutant*, 45 (30 up/15 down) from the *gpa1-5 mutant*, and 94 (68 up/26 down) from the *gpa1-5gcr1-5* double mutant (**Supplementary Table S1**), relative to the wild type (Ws2) in each case. When these stress responsive gene lists obtained from each of the mutant was compared to each other, we found that 10 DEGs are shared by all the three mutants, while 4 additional genes were only shared between the two single mutants. Interestingly, each of the single mutants share many more DEGs with the double mutant, with 15 of them from *gcr1-5* and 13 from the *gpa1-5* (**Figure 1**). All these DEGs span a wide range of abiotic stresses, including heat, cold, salt, light stress etc. (**Supplementary Table S2**). Further, when each of the abiotic stress responsive gene lists from the mutants were subjected to Expression Browser tool of Bio Array Resource, the expression value of each of the genes under different abiotic stresses like cold, oxidative, salt, heat, etc. was shown as a heatmap (**Supplementary Figures S1–S3**). We found that each of the genes in the input list not only showed different fold change value under different stress conditions but also varied based on the duration of the treatment given (0.5–24 h) under each stress.

qPCR Validation of GPA1/GCR1-responsive Genes in Abiotic Stress

Most of the genes were down-regulated under heat stress in Ws2 as well as all the mutants. Both wild- type and all 3 mutants behaved similarly in terms of up/down regulation of genes under stress conditions, though the extent of such regulation varied occasionally. The extent of regulation was much higher in the stress treatment conditions than in the control in all the plants (**Figure 2**). Out of all the 28 genes validated, only 4 were found to be down-regulated and only 5 were found to be up-regulated in all the conditions in both the wild-type and the mutants. Under cold stress, 7 genes were found to be highly up-regulated and 6 genes were highly down-regulated in all. The up-regulated genes include well-known stress responsive genes like AT-PP2A5, ERF6, CML38, RD29A, and RD26; while the down-regulated ones include YLS9, VSP2, RRTF1, and a peroxidase family protein. Only 9 genes were found to be up-regulated under heat stress in all the plants and the rest were down-regulated. The up-regulated genes include MLO12, ELIP1, RD29A, RD26, ASN1 etc. The highly down-regulated genes included ERF6 and 13, PDF1.2, ZAT11, LDOX, peroxidase family protein, etc. Most of the genes were found to be up-regulated under salt stress with only 6 genes being down-regulated. The up-regulated ones included ERF13, CML37, RRTF1, etc. while the down-regulated ones included NRT2.1, SPX1, PDF1.2, ASN1, and two members of peroxidase family. The final fold change values of each of the selected genes with standard error and statistical significance is given in **Supplementary Table S3**.

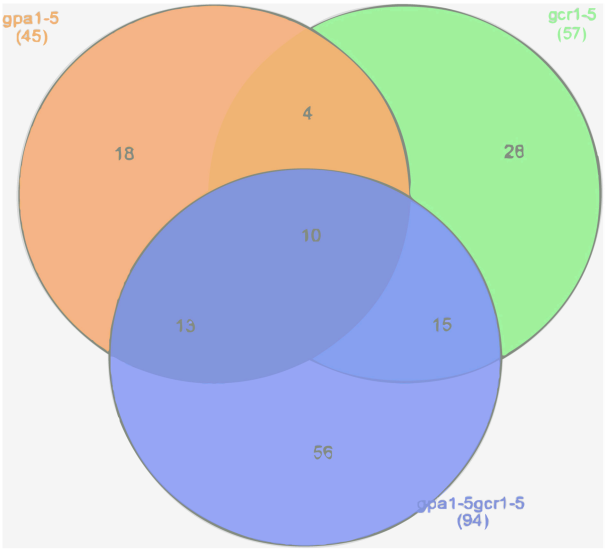


FIGURE 1 | Venn selection of abiotic stress responsive DEGs in GPA1/GCR1 mutants. DEGs from the transcriptome of each of the above mutant (GEO accession no. GSE 40217) was compared to abiotic stress responsive genes from STIFDB2.0 to obtain GPA1/GCR1 regulated stress responsive genes. Their distribution in the mutants was checked using Venn selection.

Phenotypic Validation of Tolerance to Different Abiotic Stresses in all Three Mutants

The phenotypes of the wild-type (Ws2) and the mutants (*gcr1-5*, *gpa1-5*, and *gpa1-5gcr1-5*) under control and stress conditions were measured in terms of % germination, root and shoot length. We found that under control conditions, *gcr1-5* showed better germination (∼50%) than Ws2 (∼45%) while the other two mutants had lower germination rate (∼33% and 27%) than Ws2 (**Figure 3A**). Germination percentage reduced drastically in the heat and salt stressed seeds of all, though the mutants had slightly higher germination percentages. *gcr1-5* showed better germination under cold stress than any other mutant and

	control	control	control	control	cold (4 °C)	cold (4 °C)	cold (4 °C)	cold (4 °C)	heat (37 °C)	heat (37 °C)	heat (37 °C)	heat (37 °C)	NaCl (100 mM)	NaCl (100 mM)	NaCl (100 mM)	NaCl (100 mM)
	Ws2	gcr1-5	gpa1-5	gpa1-5gcr1-5	Ws2	gcr1-5	gpa1-5	gpa1-5gcr1-5	Ws2	gcr1-5	gpa1-5	gpa1-5gcr1-5	Ws2	gcr1-5	gpa1-5	gpa1-5gcr1-5
CMPG1	0.00	1.39	0.82	0.70	3.84	5.09	5.10	4.13	-1.52	-2.27	-2.17	-2.73	2.93	5.79	3.07	3.07
SPX1	0.00	-1.19	-0.54	-0.42	3.23	2.03	2.77	2.90	-2.10	-2.77	-2.70	-2.83	-1.10	-2.37	-1.60	-1.70
YLS9	0.00	-0.57	-1.20	-0.89	-2.97	-3.87	-4.53	-4.57	-2.10	-3.07	-3.17	-4.10	5.07	4.83	5.30	3.85
VSP2	0.00	-0.02	-1.19	-1.17	-2.03	-2.10	-3.03	-3.03	-2.37	-2.40	-3.33	-3.03	2.17	2.10	1.73	1.97
KCS2	0.00	-0.31	-0.44	-1.10	-3.43	-4.13	-2.41	-4.27	1.57	2.53	1.47	2.20	5.80	6.07	5.90	4.40
ZAT11	0.00	2.22	1.33	2.31	2.40	5.20	3.93	5.07	-3.10	-1.60	-3.90	-2.07	6.43	8.23	3.60	6.87
WRKY46	0.00	1.06	1.31	1.09	3.87	4.97	4.10	4.50	-1.77	-1.73	-2.47	-3.03	5.67	6.07	6.90	5.03
MLO12	0.00	2.21	0.89	1.10	1.50	4.37	1.83	2.35	1.15	5.53	1.43	1.63	3.75	6.97	2.90	3.17
At5g19890	0.00	0.83	0.77	1.19	-1.50	-2.23	-2.00	-2.33	-1.53	-2.37	-1.50	-2.53	-4.50	-4.53	-4.97	-4.97
ASN1	0.00	-0.72	1.24	1.79	-3.67	-4.20	-2.09	-4.17	2.47	3.00	2.27	3.90	-2.43	-2.63	-3.37	-3.07
ELIP1	0.00	0.09	-0.44	-1.03	4.37	4.20	2.10	2.87	3.63	3.63	2.97	2.73	4.73	4.93	5.00	4.40
RD26	0.00	0.74	0.43	1.15	6.47	7.03	7.37	8.07	2.70	3.97	3.97	3.13	5.10	4.90	6.07	5.93
At1g55450	0.00	1.24	0.65	1.29	3.03	3.93	3.13	5.07	-2.50	-4.87	-2.93	-2.83	3.67	5.07	3.70	6.10
At4g30170	0.00	0.14	1.59	0.88	-2.73	-2.70	-2.80	-2.77	-3.20	-3.17	-4.33	-3.07	-2.83	-3.13	-3.87	-3.03
PDF1.2	0.00	1.53	0.37	-0.56	-1.73	-3.93	-1.87	-2.07	-3.87	-5.80	-4.13	-4.07	-2.13	-3.67	-2.09	-2.37
ERF6	0.00	0.68	0.70	1.12	8.80	9.07	8.10	11.47	-6.73	-6.95	-5.57	-5.90	5.40	5.87	5.97	6.00
LOX4	0.00	1.33	0.58	1.57	5.10	7.03	5.47	5.97	-2.20	-2.90	-2.63	-2.97	6.90	7.93	5.03	9.83
NRT2	0.00	1.07	2.00	0.16	-2.87	-3.89	-2.14	-2.97	-5.13	-5.06	-2.99	-4.74	-3.00	-4.02	0.15	-2.88
RRTF1	0.00	4.00	0.69	0.51	-3.60	0.09	-4.00	-2.90	-2.98	-1.97	-2.87	-3.27	8.25	12.33	9.07	6.93
LDOX	0.00	-0.43	-2.65	-1.95	-2.63	-3.53	-5.03	-4.19	-4.70	-5.43	-5.94	-4.09	3.07	1.89	4.03	4.11
ERF13	0.00	1.60	1.45	1.91	4.23	5.13	5.93	6.10	-4.87	-2.20	-3.24	-1.88	9.53	11.90	11.37	8.93
CML37	0.00	1.26	1.03	0.78	5.17	6.29	5.33	5.08	2.23	4.00	3.10	3.65	7.83	10.81	9.23	8.11
CNI1	0.00	1.26	0.71	1.25	1.90	2.45	2.92	4.15	2.06	3.87	1.77	5.97	3.30	4.09	3.79	5.29
LTP4	0.00	-0.28	-1.00	-1.49	-2.60	-2.67	-3.16	-4.29	4.73	4.23	2.94	2.10	10.64	10.71	8.61	8.26
AT-PP2A5	0.00	1.92	1.13	2.04	7.73	9.51	8.90	10.07	-3.80	-1.99	-1.31	-1.79	2.61	4.19	5.06	5.97
CML38	0.00	1.29	1.35	1.65	7.53	7.89	7.92	9.26	-4.36	-4.23	-2.68	-2.04	1.89	3.67	4.17	1.65
RD29A	0.00	0.44	0.48	1.11	7.67	7.49	7.28	8.61	2.23	2.78	2.84	5.54	5.89	5.14	5.15	6.87
CRK11	0.00	1.37	0.70	2.01	-1.41	0.15	-2.18	-2.16	-1.58	0.39	-2.73	-1.85	5.14	9.81	5.74	7.27

FIGURE 2 | qPCR of stress responsive genes validate the role GPA1 and GCR1 in regulating abiotic stresses. These genes have been implicated in various abiotic stress response previously and also found to be differentially regulated in our transcriptome data (GEO accession no. GSE 40217). The values are average log2 fold change values obtained from 3 independent experiments each having technical triplicates (The final values as log2 fold change ±SE and statistical significance is given as Supplementary Table). Red represents up-regulation; green represents down-regulation; yellow is non-differential. The intensity of color represents the level of differential regulation.

wild-type. Germination under stressed conditions was lowest in the double mutant (*gpa1-5gcr1-5*) while *gpa1-5* showed germination level similar to the wild type under salt and heat stress. Reduction in root length of both wild type and the mutants were observed when grown under stress conditions but the change was almost similar in them (**Figure 3B**). When shoot lengths of the wild-type and the mutants under control and stress treatments were compared, we found that the shoot length were almost comparable in Ws2 and the mutants, but the effect of heat and salt were more severe in all. The single mutants (*gpa1-5* and *gcr1-5*) showed better shoot length under cold conditions than the double mutant (**Figure 3C**).

Validation of Abiotic Stress Tolerance by Non-enzymatic Stress Markers

When treated with different stresses in parallel, the RWC in the wildtype (Ws2) decreased to 55, 36, and 46% in cold, heat and salt stress respectively, while the mutants showed higher RWC under the same conditions. The mutant RWC values under cold, heat and salt stress were found to be 87, 78, and 66% respectively in *gcr1-5*, 77, 57, and 56% in *gpa1-5* and 85, 53, and 45% in the double mutant *gpa1-5gcr1-5* (**Figure 4A**). Similarly, we found that proline content was much higher in all the three mutants relative to WT under all three stresses, with maximum proline accumulation under cold stress (**Figure 4B**).

Validation of Abiotic Stress Tolerance by Enzymatic Stress Markers

In this study, MDA was found to be much higher in the stressed WT plants than in any of the three mutants (**Figure 2B**), suggesting higher membrane injury and accumulation of free radicals in the WT plants. Even in the absence of any stress, *gcr1-5* mutant showed lower amount of MDA than the WT. However, the other mutants, *gpa1-5* and *gpa1-5gcr1-5* did not show significant difference in their levels of MDA relative to WT (**Figure 5A**). We also assayed other stress-related enzymes, catalase, ascorbate peroxidase, and superoxide dismutase, in both wild-type and the three mutants under control and stress treatments. In the absence of any stress, the activities of all these enzymes were similar in the WT and all the three mutants. When exposed to stress, these enzyme levels increased in the mutants under all the stresses tested, with maximum activities under cold stress followed by heat and salt, while there was no significant change in the wild type (**Figures 5B–D**).

DISCUSSION

The involvement G-protein α subunit in plants in individual abiotic stress responses is either known directly in relation to heat (Misra et al., 2007) and salt (Colaneri et al., 2014; Urano et al., 2014) or indirectly in relation to ABA signaling

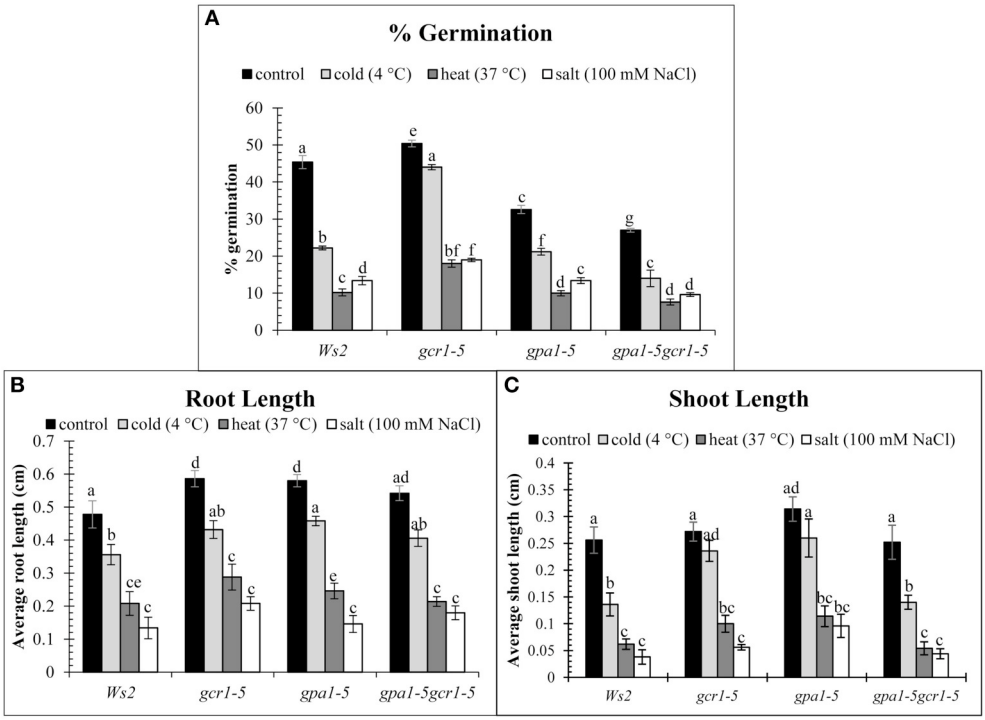


FIGURE 3 | Phenotypic characterization of mutants under control and stress conditions. Germination (%) **(A)**, Average root length **(B)** and Average shoot length **(C)**. The values are mean of 3 independent experiments. Each reading was average of atleast 10 independent plants/plates. Data is represented as mean ± SE. Values followed by different letters are significantly different at 5% level as determined by Duncan's test.

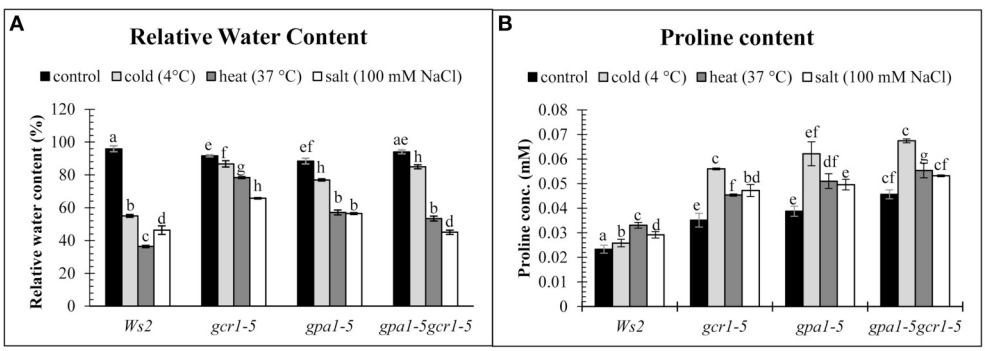


FIGURE 4 | Non-enzymatic stress markers, RWC (A) and Proline content (B) validate the roles of GCR1/GPA1 in abiotic stress. The values are mean of 3 independent experiments each having technical triplicates. Data is represented as mean ± SE. Values followed by different letters are significantly different at 5% level as determined by Duncan's test.

(Pandey et al., 2010; Alvarez et al., 2011) or oxidative stress (Booker et al., 2012). In addition, the β subunit has been implicated in heat response in pea (Misra et al., 2007), whereas γ subunit has only been implicated in biotic stress so far (Trusov et al., 2007; Trusov and Botella, 2012; Thung et al., 2013). However, comprehensive and/or comparative assessment of the involvement of any heterotrimeric G-protein subunit in all the major abiotic stresses has not been tested in any single plant so far. The best known candidate for a plant G-protein coupled receptor, the *Arabidopsis* GCR1 was implicated in drought stress (Pandey and Assmann, 2004), but the annotation of GCR1 as a GPCR, its interaction with GPA1 as well as its role in G-protein signaling was contested (Urano et al., 2013; Urano and Jones, 2013). This made it difficult to link any role of GCR1 in abiotic stress with that of G-protein signaling.

Functional Genomic Identification and qPCR Validation of the Role of G-protein Signaling Components in Abiotic Stress

Our parallel transcriptome analyses of *Arabidopsis* single and double mutants of GCR1 and GPA1 (Chakraborty et al., 2015a,b;

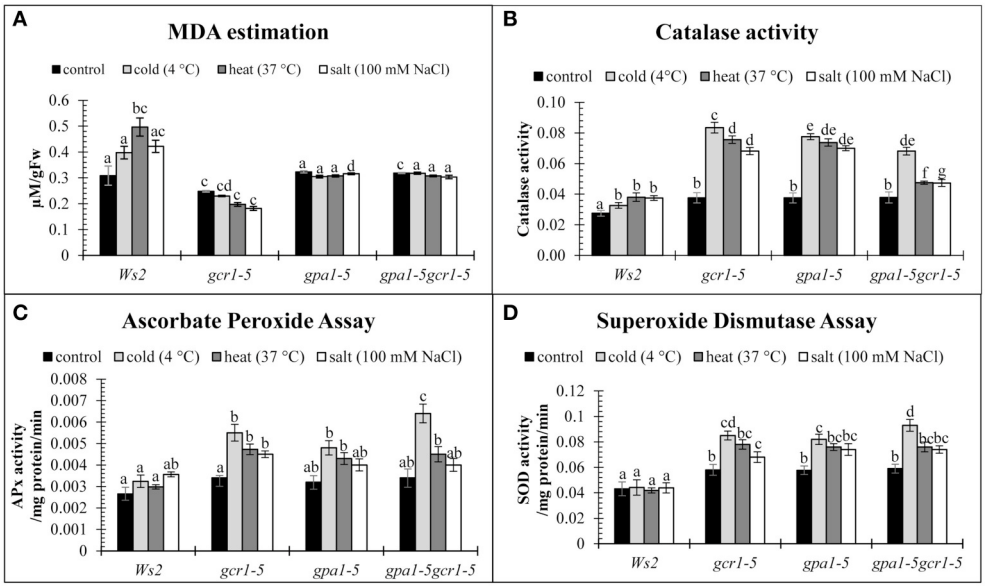


FIGURE 5 | Enzymatic stress markers, MDA (A), Catalase assay (B), Ascorbate peroxidase assay (C), and Superoxide dismutase assay (D) to validate the roles of GCR1/GPA1 in abiotic stress. The values are mean of 3 independent experiments each having technical triplicates. Data is represented as mean ± SE. Values followed by different letters are significantly different at 5% level as determined by Duncan's test.

Chakraborty et al., submitted) under identical conditions gave the strongest indication of their substantial partnership on a genomewide basis for the first time, including in stress. Response to stress and response to stimulus emerged as the largest affected process in the transcriptomes of the single mutants of *gpa1-5*, *gcr1-5* as well as the double mutant *gpa1-5gcr1-5*. This not only revived the role of GCR1-GPA1 partnership in regulating a number of genes and an even higher number of processes, but also indicated that GCR1 and GPA1 may also work independently, possibly with other GCR/GPA isoforms or entirely different partners, to regulate some of the genes. The indication that genes related to stress-response figured in both shared and independent categories led us to hypothesize that G-protein signaling components may be the common conduits for responding to multiple stresses and could therefore be attractive targets for developing stress tolerance. In order to test this hypothesis, we thoroughly examined the stress-related DEGs *in silico*, and also validated them experimentally in a comprehensive manner in the present study. The experimental validation was done by investigating the impact of different stresses on the wild type, single and double mutants of GCR1 and GPA1 simultaneously under identical conditions for the first time.

Our comparison of DEGs from GPA1 and/or GCR1-responsive transcriptomes revealed 144 DEGs spanning a wide range of abiotic stresses, including heat, cold, salt, light stress etc. (**Supplementary Table 1**). Out of them, only 10 DEGs are shared by all the three mutants. Interestingly, each of the single mutants shared many more DEGs with the double mutant than between themselves (**Figure 1**). RT-qPCR validation of 28 of these genes spanning different stresses revealed identical regulation of the DEGs shared between the mutants (**Figure 2**). This can be best explained by GCR1-GPA1 partnership in regulating abiotic stress response in a classical G-protein signaling pathway. The seemingly independent regulation of the remaining unshared DEGs between the 3 mutants could either be due to the GCR1/GPA1 partnership with other (known or unknown) GPA/GCR isoforms, or entirely different signaling pathways.

GCR1 and/or GPA1 Mutants are Tolerant to Multiple Abiotic Stresses

Plants have specialized regulatory networks which mediate sensing, response and adjustment of plant to change in environmental conditions such as change in temperature, amount of water, presence of salt and other minerals, etc. (Bailey-Serres et al., 2012). These networks are also linked to gene networks related to plant growth (Hirayama and Shinozaki, 2010). Therefore, we sought to validate the stress-related gene clusters predicted from our mutants by testing their physiological and biochemical response to stress. This was done by exposing the wild type, single and double mutants parallelly to three different stresses, *viz.* salt (100 mM NaCl), cold (4°C) and heat (37°C). Out of all the 3 stresses checked, heat and cold caused significant reduction in germination in all while the effect of cold was minimal (**Figure 3A**). The effect of all the stresses on root length in all wild type and the mutants was similar (**Figure 3B**). Effect on the shoot length under different stress was similar to that observed in % germination (**Figure 3C**), with heat and salt causing drastic reduction of shoot length. The only difference observed was that the single mutants had longer shoots than the double mutant under cold stress. All these results suggest that the mutants were able to withstand the stress conditions better than the wild type. This not only confirms our hypothesis

that G-proteins signaling components could mediate the plant's response to multiple stresses, but also prove that their knock-out mutation renders the plants more tolerant to multiple abiotic stresses. In other words, G-protein signaling may enhance the sensitivity of the plant to abiotic stresses.

This was further confirmed by studying the non-enzymatic and enzymatic stress markers. For example, relative water content (RWC), which influences water relations of a plant (Slavík, 1974), decreased to a much lesser extent under different stress conditions in the mutants than in the wildtype, with the *gcr1-5* mutant being more tolerant to any stress than others (**Figure 4A**), Moreover, all the 3 mutants are more tolerant to cold stress than heat or salt stress. Proline accumulation is widely accepted as an indicator of abiotic stress and higher levels of proline accumulation are associated with abiotic stress tolerance (Ashraf and Foolad, 2007). In our study, proline content was much higher in all the three mutants (relative to WT) in all the stresses, with all 3 mutants showing maximum proline accumulation under cold stress (**Figure 4B**).

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems of plant and animal origin and is measured in units of MDA (Hodges et al., 1999). MDA is used as an index to measure membrane injury in plants under any stress. In this study, MDA was found to be much higher in the stressed WT plants than in the mutants (**Figure 5A**), suggesting higher membrane injury and accumulation of free radicals in the WT plants. Even in the absence of any stress, *gcr1-5* mutant showed lower amount of MDA than the WT. However, the other mutants, *gpa1-5* and *gpa1-5gcr1-5* did not show significant difference in their levels of MDA relative to WT.

During stress, plant cells produce large quantities of reactive oxygen species (ROS), which cause damage to protein, lipids and DNA (Schützendübel and Polle, 2002). Under normal conditions, the level of ROS remains low due to the presence of active free radical scavenging enzymes like superoxide dismutase (SOD), catalase, and ascorbate peroxidase. We assayed these enzymes all the three mutants and found that relative to WT, all of them have higher activity of these enzymes under control conditions. When exposed to stress, these enzyme levels increased even further in the mutants as compared to the wild type, with maximum activities under cold stress followed by heat and salt (**Figures 5B–D**). These results indicate that functional GPA1 and GCR1 may subdue the ROS-scavenging ability and make the plant stress-sensitive and build up ROS. Their loss of function in the mutants makes them more tolerant to stress by enhanced activity of ROS-scavenging enzymes.

Significantly, our transcriptome data on the single and double mutants revealed none of the genes coding for ROS scavenging enzymes as differentially regulated. This indicates the role of GCR1-GPA1-regulation of these enzymes at the post-translational level.

CONCLUSIONS AND PROSPECTS

Our detailed analysis of the stress related category of DEGs identified from our *Arabidopsis* whole transcriptome microarray data on the GPA1 and GCR1 single and double mutants, as well as their comprehensive parallel validation in response to cold, heat and salt stresses clearly confirms the roles of GCR1 and GPA1 in abiotic stress response for the first time. This implies a role for classical g-protein signaling pathways involving GCR1 and GPA1 in stress sensitivity in the normal plants of Arabidopsis. The identical response of each of the 3 mutants to each of the 3 stresses is striking, despite the fact that they do not share majority of the genes belonging to stress response in their transcriptomes (Chakraborty et al., submitted). Indeed, this is an ample proof of our recent prediction that even if all the 3 mutants do not share majority of their DEGs, they may achieve the same regulatory outcomes, wherever their unshared DEGs belong to shared biological processes (Chakraborty et al., submitted). Another important contribution of this paper is to revive the role of GCR-GPA coupling in abiotic stress signaling in Arabidopsis. Most importantly, our findings also offer G-protein signaling pathway as a potential source of novel common targets for the development of tolerance/resistance to multiple abiotic stresses. At the same time, it would be of interest to examine the genomewide response of these mutants to individual or combined stresses, so as to estimate what proportion of a genomewide stress response can be attributed to G-protein signaling. Efforts are underway.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01000

Supplementary Figure S1 | *In-silico* analysis of all abiotic stress responsive genes in *gcr1-5* using Expression Browser. It shows the expression value for each genes in a color coded form obtained from all experiment categories, plant growth stages, tissues types, treatments, and identifiers, and thumbnail summary of expression levels and cluster results.

Supplementary Figure S2 | *In-silico* analysis of all abiotic stress responsive genes in *gpa1-5* using Expression Browser. It shows the expression value for each genes in a color coded form obtained from all experiment categories, plant growth stages, tissues types, treatments, and identifiers, and thumbnail summary of expression levels and cluster results.

Supplementary Figure S3 | *In-silico* analysis of all abiotic stress responsive genes in *gpa1-5gcr1-5* using Expression Browser. It shows the expression value for each genes in a color coded form obtained from all experiment categories, plant growth stages, tissues types, treatments, and identifiers, and thumbnail summary of expression levels and cluster results.

Supplementary Table S1 | List of genes used for validation of stress response data, with their primer sequences and efficiencies.

Supplementary Table S2 | Distribution of GPA1/GCR1 responsive genes in different abiotic stresses. It shows the distribution of DEGs in the transcriptome of the single and double mutants of GPA1 and GCR1 (*gpa1-5*, *gcr1-5,* and *gpa1-5gcr1-5*) in different abiotic stresses.

Supplementary Table S3 | qPCR of stress responsive genes validate the role GPA1 and GCR1 in regulating abiotic stresses. These genes have been implicated in various abiotic stress response previously and also found to be differentially regulated in our transcriptome data (GEO accession no. GSE 40217). The values are given as average of log2 fold change ±SE obtained from 3 independent experiments each having technical triplicates. Values followed by different letters are significantly different at 5% level as determined by Duncan's test.

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Microarray Analysis of Rice d1 (RGA1) Mutant Reveals the Potential Role of G-Protein Alpha Subunit in Regulating Multiple Abiotic Stresses Such as Drought, Salinity, Heat, and Cold

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The genome-wide role of heterotrimeric G-proteins in abiotic stress response in rice has not been examined from a functional genomics perspective, despite the availability of mutants and evidences involving individual genes/processes/stresses. Our rice whole transcriptome microarray analysis (GSE 20925 at NCBI GEO) using the G-alpha subunit (RGA1) null mutant (Daikoku 1 or d1) and its corresponding wild type (*Oryza sativa* Japonica Nipponbare) identified 2270 unique differentially expressed genes (DEGs). Out of them, we mined for all the potentially abiotic stress-responsive genes using Gene Ontology terms, STIFDB2.0 and Rice DB. The first two approaches produced smaller subsets of the 1886 genes found at Rice DB. The GO approach revealed similar regulation of several families of stress-responsive genes in RGA1 mutant. The Genevestigator analysis of the stress-responsive subset of the RGA1-regulated genes from STIFDB revealed cold and drought-responsive clusters. Meta data analysis at Rice DB revealed large stress-response categories such as cold (878 up/810 down), drought (882 up/837 down), heat (913 up/777 down), and salt stress (889 up/841 down). One thousand four hundred ninety-eight of them are common to all the four abiotic stresses, followed by fewer genes common to smaller groups of stresses. The RGA1-regulated genes that uniquely respond to individual stresses include 111 in heat stress, eight each in cold only and drought only stresses, and two genes in salt stress only. The common DEGs (1498) belong to pathways such as the synthesis of polyamine, glycine-betaine, proline, and trehalose. Some of the common DEGs belong to abiotic stress signaling pathways such as calcium-dependent pathway, ABA independent and dependent pathway, and MAP kinase pathway in the RGA1 mutant. Gene ontology of the common stress responsive DEGs revealed 62 unique molecular functions such as transporters, enzyme regulators, transferases, hydrolases, carbon and protein metabolism, binding to nucleotides, carbohydrates, receptors and lipids, morphogenesis, flower development, and cell homeostasis. We also mined 63 miRNAs that bind to the stress responsive transcripts identified in this study, indicating their post-transcriptional regulation. Overall, these results indicate the potentially extensive role of RGA1 in the regulation of multiple abiotic stresses in rice for further validation.

Keywords: G-protein, heat, cold, salt, drought, stress, rice, RGA1

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INTRODUCTION

Abiotic stress responses in plants are being increasingly addressed on a genome-wide scale to find newer gene targets for protecting crop yields in the era of climate change (Pandey et al., 2015). Rice has been a crop of particular interest in this regard, not only because of its popularity as a post-genomic model crop, but also its importance as a staple food for half of the world's population. In rice, transcriptome-wide analyses of abiotic stress response have been reported in terms of either specific stresses, or specific families of genes that respond to multiple stresses, or both. They include drought-responsive (Wang et al., 2011) and salinity-responsive (Jiang et al., 2013) rice transcriptomes spanning multiple gene families, pathways, and transcription factors. Studies that examined multiple stresses in parallel include transcriptome-wide response to water-deficit, cold, and salt stress in rice (Ray et al., 2011; Venu et al., 2013).

There have been many other whole transcriptome microarray studies in rice under different abiotic stress conditions, but they reported only specific gene families that responded to various stresses. They include the MADS-box transcription factor family (Arora et al., 2007), F-Box Proteins (Jain et al., 2007), calcium-dependent protein kinase (CDPK) gene family (Ray et al., 2007), auxin-responsive genes (Jain and Khurana, 2009), protein phosphatase gene family (Singh et al., 2010), Sulfotransferase (SOT) gene family (Chen et al., 2012), thioredoxin gene family (Nuruzzaman et al., 2012), half-size ABC protein subgroup G (Matsuda et al., 2012), class III aminotransferase gene family (Sun et al., 2013), Ca^{2+}ATPases gene family (Kamrul Huda et al., 2013), Rice RING E3 Ligase Family (Lim et al., 2013) etc.

Hetetrotrimeric G-protein signaling components have often been implicated in stress response in plants. For example, in pea, Gα subunit was shown to be up-regulated by heat, as well as to impart heat and salt tolerance when overexpressed in transgenic tobacco, whereas the Gβ subunit imparted only heat tolerance (Misra et al., 2007). The role of α subunit in salt stress has also been shown in Arabidopsis (Colaneri et al., 2014), rice, and maize (Urano et al., 2014). Recently, we demonstrated that stress-related genes/pathways constitute the largest functional cluster of GPCR/G-protein-regulated genes in *Arabidopsis* using whole transcriptome analyses of knock-out mutants of GCR1 and GPA1 (Chakraborty et al., 2015a,b).

The rice G protein subunits are well characterized as RGA1 for Gα subunit (Ishikawa et al., 1995), RGB1 for Gβ subunit (Ishikawa et al., 1996) and RGG1 and RGG2 for the Gγ subunits (Kato et al., 2004). The expression of rice Gα subunit (RGA1) gene was reported to be up-regulated by salt, cold, and drought stresses, and down regulated by heat stress (Yadav et al., 2013). However, the regulation of the two Gγ subunits was different—while both RGG1 and RGG2 were up-regulated in salt, cold, heat, and ABA treatments, only RGG1 was up-regulated in drought stress (Yadav et al., 2012). While these two studies demonstrated that abiotic stresses regulate the expression of Gα and Gγ genes in rice, the role of G-proteins in mediating various stress responses in rice remains uncharacterized on a genome-wide scale. The availability of a natural mutant of RGA1 (D1) in rice (Ashikari et al., 1999) makes a functional genomic approach particularly attractive in this regard. We carried out a microarray analysis of this RGA1 mutant in comparison with the wild type in rice (GSE 20925 at NCBI GEO), which provided a convenient starting point for the present study, to examine the stress-related genes in the genome-wide response to the RGA1 null mutation in rice. In specific terms, we asked what proportion of the RGA1-regulated transcriptome corresponds to abiotic stress response in rice and how are these genes distributed in terms of major individual abiotic-stresses or in terms of their differential regulation in the RGA1 mutant or normal rice plants. We report here an integrative analysis of our experimental RGA1 mutant microarray data with the *in silico* meta data analysis of the known response of normal rice plants to various abiotic stresses.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the rice d1 mutant (devoid of Gα subunit or RGA1) and its corresponding wild type (*Oryza sativa* japonica Nipponbare) were obtained from the Faculty of Agriculture, Kyushu University, Japan. They were surface-sterilized with 70% ethanol and 0.01% Triton-X 100 and grown on 0.5x B5 media containing 0.7% agar at 25 ± 1°C with fluorescent white light intensity of 1 kilo lux and a 12/12 photoperiod for 25 days till the emergence of the tertiary leaves and used for microarray analysis.

RNA Isolation and Analysis

Total RNA was isolated by hot phenol extraction and lithium chloride precipitation method as described (Pathak and Lochab, 2010). Total RNA was qualitatively and quantitatively analyzed by spectrophotometry and agarose gel electrophoresis. Prior to microarray experiments, RNA integrity values (RIN) of the total RNA samples were determined using the Agilent 2100 Bionalyzer equipment as per the manufacturer's instructions and only samples with RIN values higher than 5 were used for microarray experiments.

Whole Transcriptome Microarray

cRNA labeling of total RNAs from the RGA1 mutant and its corresponding wild type was carried out using Agilent Low RNA Input Fluorescent Linear Amplification Kit (USA) as per the manufacturer's instructions, using Cy3 and Cy5 dyes (Perkin-Elmer, USA). Amplified samples were purified using Qiagen's RNeasy mini spin columns. The quantity and specific activity of cRNA was determined by using NanoDrop ND-1000 Spectrophotometer. Samples with specific activity >8 were hybridized with Agilent rice whole genome 60-mer microarrays (4 × 44 K, Ver 2) at 65°C for 17 h using Agilent Microarray Hybridization materials and equipment, as per the manufacturer's instructions. Slides were washed for 1 min each with Agilent Gene expression Wash Buffer I and II at RT and 37°C, respectively, and rinsed with acetonitrile for cleaning up and drying. They were scanned on an Agilent scanner (G2565B) at 100% laser power. Data extraction was carried out with Agilent Feature Extraction software (version 9.1).

The raw data was normalized using the recommended "Per Chip and Per Gene Normalization" feature of the software GeneSpring GX Version 11.5. The correlation of replicates was checked using principal component analysis and correlation coefficients were obtained. The geometric mean (geomean) fold change values are represented as $\log_2$. The average data of biological replicates were used for final calculations. Log_2 fold change value of 1.0 with a *p*-value of 0.05 was taken as the cut-off to identify the differentially regulated genes (DEGs).

Data Mining and Meta-Analysis of the Stress Related Genes

The stress-related genes were segregated from the above RGA1-regulated DEGs using the GO term "stress." This was done using rice genome annotation version 7 and also validated with the "manually curated database for rice proteins" (Gour et al., 2013). Further data mining was done using the genes corresponding to individual stresses downloaded from the stress responsive transcription factor database (STIFDB2.0, Naika et al., 2013), to find RGA1-regulated DEGs corresponding to heat, drought, salt, and cold. In order to identify additional stress-related genes among RGA1-responsive genes, our entire RGA1-regulated transcriptome was used as an input at the online database RiceDB (Narsai et al., 2013) to identify all the rice genes that responded to at least one of the four abiotic stresses i.e., cold, heat, drought, and salt. These genes were sorted into up-regulated and down-regulated sets and subjected to various Venn selections (Oliveros, 2007–2015) to generate a core list of 1498 stress-responsive genes common to all four stresses in rice. The core gene list was further classified into various functional categories, pathways and processes using a GO enrichment analysis tool, AGRIGO (Du et al., 2010) with binomial statistical test and cut-off for FDR-adjusted *P*-value of 0.05. Hierarchical clustering was done using average linkage based on Euclidean distance subsets of individual stress conditions such as heat, cold, drought/dehydration, salt, submergence, and shift from aerobic to anaerobic germination, cold, and drought. Biclustering was done with a threshold value of 1 and the largest bicluster was used for the analysis. Expression data were obtained for both the clustering analyses using Genevestigator (Zimmermann et al., 2004).

RT-PCR Validation of the Stress-Related Genes

In order to validate the stress-responsive genes identified from the microarray results, quantitative RT-PCR experiments were carried out using total RNAs isolated from two biological replicates of the wild type and RGA1 mutant rice plants grown and harvested under similar conditions. Two technical replicates were used to set up RT-PCR from each of the biological replicates, using gene-specific primers designed in-house for the selected genes. The primer sequences are provided in Supplementary Table 2. PCR amplifications were performed in 20 μl by using the KAPA SYBR FAST universal QPCR kit (KAPA BIOSYSTEMS) with 1.0 μl of sample cDNA prepared by using iScript cDNA synthesis kit (Cat#170-8891) from BIORAD and 100 n moles of each gene-specific primer. Actin (ACT) was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed by using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

RESULTS

Whole transcriptome microarray analysis of the rice RGA1 (Gα) null mutant in comparison with its WT yielded a total of 2270 differentially expressed genes under MIAME compliant conditions, using stringent cut-off values (geomean 1.0 with *p*-value of 0.05) and removing redundancies. The raw data of this entire microarray experiment are reported at NCBI GEO (GSE 20925). Among these RGA1-regulated genes, a large number of abiotic stress-responsive genes have been identified using their annotation information or online databases for further bioinformatic analysis as detailed below.

Stress-Responsive Genes Identified by GO-Terms

Our search for stress-related genes among these RGA1-regulated DEGs using the GO terms related to stress yielded 94 abiotic stress-related DEGs that are nearly equally distributed in terms of up/down regulation (49 up/45 down). A vast majority of these genes could be clustered into <40 related families (20 up/20 down) showing identical mode of up/down regulation, despite wide variation in the extent of their regulation (**Table 1**). For example, all the RGA1-regulated members of gene families such as DREB seem to be uniformly up-regulated, albeit to varying extents, ranging between +3.99 and +1.18. In addition, there are 21 stress-related DEGs that are individually regulated in the RGA1 mutant with no other family member, including up-regulated genes such as CDPK, MAP kinase kinase 2, DnaJ like protein, and down-regulated genes such as Myb factor, phytochelatin synthetase, and water-stress inducible protein (RAB21).

Stress-Responsive Genes Identified at STIFDB2.0

Data mining for all abiotic stress-responsive genes of rice at STIFDB2.0 yielded 626 genes in all, corresponding to heat (522), drought (101), salt (37), and cold (15), as shown in the left panel of **Figure 1**. A Venn selection between these 626 stress responsive genes and the 2270 RGA1-regulated genes identified on our microarray yielded 106 genes (**Figure 1**, inset), indicating the role of RGA1 in mediating their stress regulation. A significant majority of them respond to heat (94), followed by drought (13), salt (6), and cold (4), with the 25 genes being common to salt and drought stresses (**Figure 1**, right panel). But this order becomes very different when seen in terms of what proportion of each of the stress responses was RGA1-regulated: With four out of all 15 cold-responsive genes listed at STIFDB2.0 being regulated by RGA1, cold-response has the highest proportion of genes under the regulation of RGA1 (27%), followed by heat (18%), salt (16%), and drought (13%).

TABLE 1 | RGA1-regulated stress-responsive gene families with their fold changes in mutant.

RGA1 up regulated stress responsive families	Fold change	RGA1 down regulated stress responsive families	Fold change
DRE-binding protein 1A,1B,1C	3.99–1.18	Peroxidase	−6.09 to −1.42
Metallothionein-like protein type 2	3.88–3.47	Catalase isozyme	−3.33 to −3.09
Catalase isozyme 2	2.89–2.6	Haem peroxidase family protein.	−2.88 to −1.47
Glutathione S-transferase GST 19,6	2.49–1.55	LRR-like protein	−2.71 to −2.54
Heat shock proteins (70,82,90)	2.31–1.47	Pathogen-inducible alpha dioxygenase	−2.60 to −2.58
MAP Kinase	2.19–2.06	Triosephosphate isomerase	−1.89 to −1.63
Endo-1, 3; 1, 4-beta-D-glucanase	2.06–1.55	Anth (Pollen-specific desiccation-associated LLA23 protein)	−1.86 to −1.59
Rossmann-like alpha/beta/alpha sandwich fold domain containing protein	2.05–1.28	Cytosolic 6-phosphogluconate dehydrogenase	−1.81 to −1.72
Zinc finger, domain containing proteins (AN1-type, TAZ-type)	2.04–1.50	Glycine-rich RNA-binding protein 1	−1.79 to −1.72
Heat stress transcription factor Spl7 (RHSF10)	1.99–1.11	Beta-glucanase precursor	−1.72 to −1.64
Sucrose synthase 2	1.84–1.33	Cytosolic Glyceraldehyde-3-phosphate dehydrogenase	−1.70 to −1.50
Anthranilate synthase beta chain	1.75–1.44	Superoxide dismutase	−1.69 to −1.49
Manganese-superoxide dismutase precursor	1.52–1.26	WW/Rsp5/WWP domain containing protein	−1.59 to −1.33
6-phosphogluconate dehydrogenase	1.46–1.35	Serine/threonine-protein kinase SAPK3 (Osmotic stress/abscisic acid-activated protein kinase 3)	−1.46 to −1.36
Serine/threonine-protein kinase SAPK2/9	1.34–1.05	Alcohol dehydrogenase 1.	−1.42 to −1.23
UspA domain containing protein	1.30–1.18	Class III peroxidase GvPx2b	−1.39 to −1.08
GTP-binding nuclear protein Ran1B	1.15–1.04	Nucleoside diphosphate kinase I	−1.36 to −1. 33
Multiple stress-responsive zinc-finger protein ISAP1 (Stress-associated protein 1)	1.14–1.09	Hypothetical conserved gene. Similar to peroxidase (Os03t0339300-02)	−1.25 to −1. 07
OSIGBa0145M07.4 protein	1.83–1.14	Aldehyde dehydrogenase	−1.21 to −1.16

From the differentially regulated genes (DEGs) identified in our microarray analysis of the RGA1 mutant, 94 genes with GO terms related to stress were segregated and categorized into families.

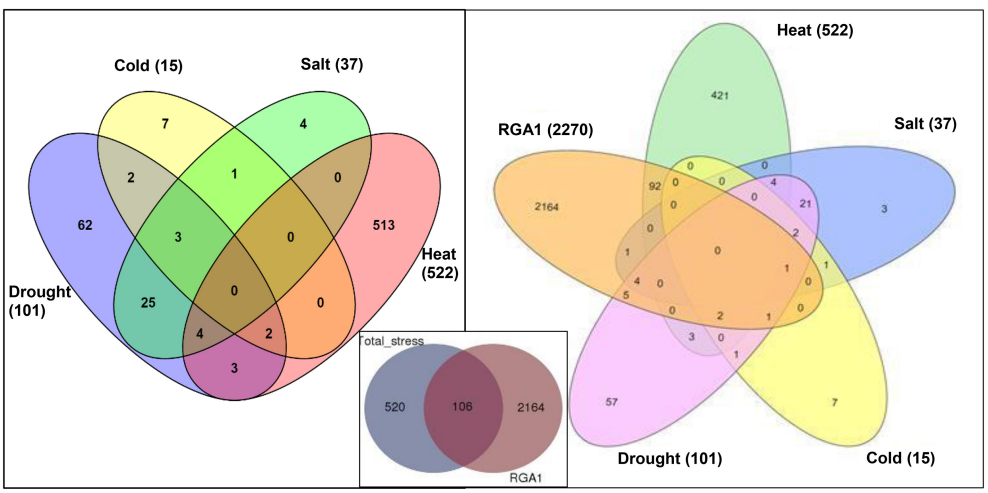


FIGURE 1 | Stress responsive genes among RGA1-regulated genes in rice. The left panel shows Venn selections between the subsets of all rice abiotic stress-responsive genes listed at STIFDB2.0. The inset shows Venn selection between all 626 abiotic stress-responsive genes listed at STIFDB2.0 and 2270 RGA-1-regulated DEGs identified on our microarray. The left panel shows the break-up of the 106 RGA-regulated stress-responsive genes identified in the inset in terms of individual stresses viz., heat (94), drought (13), salt (6), and cold (4).

Expression Profiles of RGA1-Regulated Stress-Responsive Genes

Hierarchical clustering of the transcripts of the 106 RGA1-regulated stress responsive genes using Genevestigator revealed their differential expression under 132 perturbations related to abiotic stress studies reported in literature. Out of them, the data in **Figure 2** include only 118 perturbations such as heat, cold, drought/dehydration, salt, submergence, and shift from aerobic to anaerobic germination, that have affected the expression of the vast majority of 106 genes queried based on our study. This

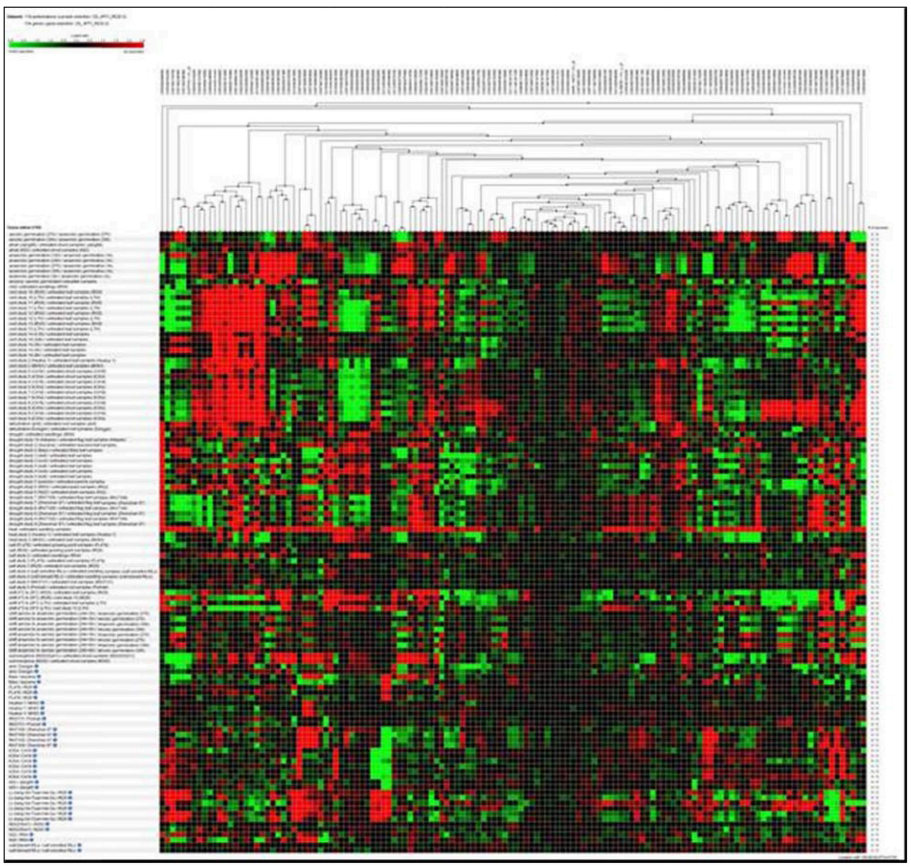
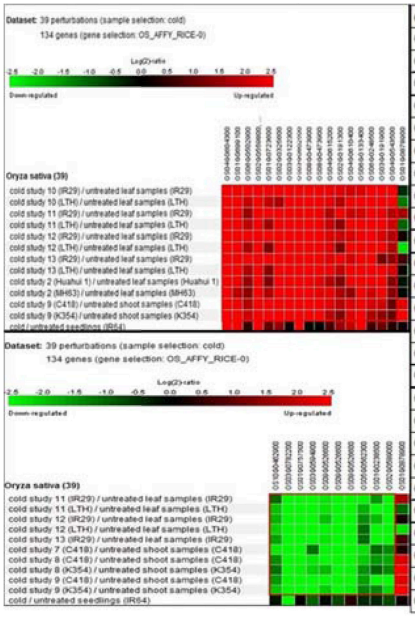


FIGURE 2 | Hierarchical clustering of the transcripts of 106 RGA1-regulated stress-responsive genes in various abiotic stress studies in rice (118 perturbations). The red and green colors indicate up-regulation (log2 [2.5]) and down-regulation (log2 [−2.5]), respectively as shown in the color bar. Hierarchical clustering was done using average linkage based on Euclidean distance subsets of individual stress conditions such as heat, cold, drought/dehydration, salt, submergence, and shift from aerobic to anaerobic germination cold and drought. The expression data were obtained using Genevestigator (Zimmermann et al., 2004).

revealed a prominent cluster of over 25 genes that are highly up-regulated (over 2.5-fold) and a similar number of highly down-regulated (over 2.5-fold) under 24 cold stress studies in literature on rice. There are an even larger number of genes that are differentially regulated under drought, of which the down-regulated genes are both predominant and better clustered, relative to the up-regulated genes. Though there are a smaller number of heat responsive genes, they are neither well clustered not consistent between different studies. With respect to salt, the results from nine studies show that very few of our 106 RGA-regulated genes respond to salt stress in rice. In view of these findings, further *in silico* analysis of transcript profiles was restricted to cold and drought stress conditions.

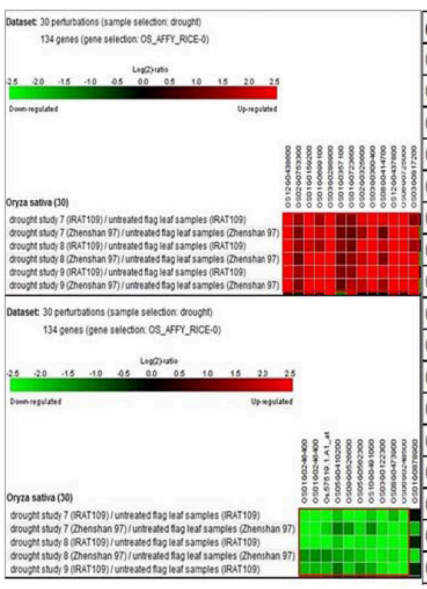
Biclustering analysis of the expression profiles of 106 RGA1-regulated, stress-responsive genes in various studies on cold stress revealed that 17 genes were up-regulated and 11 genes were down-regulated in 39 different perturbations/studies (**Figure 3**, left panel). Their comparison with the actual fold-change values of those genes on our microarray revealed that about half of them are similarly up-regulated in both RGA1 mutant (without stress) as well as in normal rice plants under cold stress. The remaining genes include seven up-regulated genes and six down-regulated genes in the RGA1 mutant with opposite pattern of regulation under cold stress in normal rice plants in literature (**Figure 3**, right panel). The genes up-regulated in the RGA1 mutant but down-regulated by cold stress in the normal plants include mitochondrial chaperonin-60, 4,5-DOPA dioxygenase extradiol-like protein, isoform 2 of heat stress transcription factor B-2c, cytochrome P450 family protein, calcyclin-binding protein, DnaJ-like protein. The genes down-regulated in the RGA1 mutant but up-regulated in normal plants include amino acid transporter-like protein and alpha-amylase isozyme 3D precursor. The opposite pattern of regulation of these genes could be due to the RGA1 mutation, which indicates that RGA1 may mediate the response of these genes to cold stress.

A similar biclustering analysis of the expression patterns of 106 RGA1-regulated stress-responsive genes in studies on drought stress revealed that 13 genes were up-regulated and 10 genes were down-regulated in 30 different perturbations/studies (**Figure 4**, left panel). When their up/down regulation was compared with the actual fold-change values obtained on our



GeneSymbol	RGA1 FOLD Change	Stress fold change	Gene_Description
Os04g0604300	2.13	Cold up	Xyloglucan endotransglucosylase/hydrolase protein 24 precursor
Os01g0878900	3.66	Cold up	Similar to 4,5-DOPA dioxygenase extradiol-like protein
Os02g0559800	2.77	Cold up	E3 ubiquitin ligase EL5
Os03g0122300	2.27	Cold up	Similar to Flavanone 3-hydroxylase-like protein
Os04g0610400	1.89	Cold up	Similar to AP2 domain containing protein RAP2.6
Os04g0610400	1.89	Cold up	Similar to AP2 domain containing protein RAP2.6
Os01g0699100	1.64	Cold up	Serine/threonine protein kinase domain containing protein
Os04g0543500	1.46	Cold up	Hypothetical conserved gene
Os01g0723600	1.43	Cold up	Ribose-phosphate pyrophosphokinase 3
Os02g0325600	1.43	Cold up	Similar to Phosphate starvation response regulator-like protein
Os06g0248500	1.40	Cold up	U box domain containing protein
Os03g0802500	1.22	Cold up	ATPase, AAA-type, core domain containing protein.
Os06g0133400	1.22	Cold up	Conserved hypothetical protein. (Os06t0133400-01)
Os02g0191300	-1.02	Cold up	Similar to Amino acid transporter-like protein
Os02g0191300	-1.02	Cold up	Similar to Amino acid transporter-like protein
Os04g0615200	-1.41	Cold up	Conserved hypothetical protein. (Os04t0615200-01)
Os08g0473900	-3.33	Cold up	Alpha-amylase isozyme 3D precursor
Os10g0462900	1.42	Cold down	Similar to mitochondrial chaperonin-60
Os01g0878900	3.66	Cold down	Similar to 4,5-DOPA dioxygenase extradiol-like protein
Os03g0782200	2.14	Cold down	Conserved hypothetical protein
Os09g0526600	1.81	Cold down	Similar to Isoform 2 of Heat stress transcription factor B-2c.
Os03g0594900	1.64	Cold down	Cytochrome P450 family protein
Os01g0757500	1.33	Cold down	Similar to Calcyclin-binding protein
Os05g0562300	1.02	Cold down	Similar to DnaJ-like protein
Os01g0238500	-1.00	Cold down	Similar to Branched-chain-amino-acid aminotransferase-like protein 3
Os02g0589000	-1.08	Cold down	Lecithin:cholesterol acyltransferase family protein

FIGURE 3 | Expression profiles of 106 RGA1-regulated stress-responsive genes in cold stress (39 perturbations from literature). The red and green colors indicate up-regulation (log2 [2.5]) and down-regulation (log2 [−2.5]), respectively, as shown in the color bar. The expression data in the left panel were obtained using Genevestigator. The table compares their regulation in normal plants under stress in literature with actual fold-change values in the RGA1 mutant.



GeneSymbol	RGA1 FOLD Change	Stress fold change	Gene_Description
Os01g0357100	4.41	Drought up	Ferredoxin-nitrite reductase
Os12g0438600	3.59	Drought up	Similar to Chloride channel protein CLC-a
Os03g0286900	2.29	Drought up	Similar to Low-temperature induced protein
Os02g0753300	1.82	Drought up	Lipoxygenase, LH2 domain containing protein
Os01g0723600	1.43	Drought up	Ribose-phosphate pyrophosphokinase 3
Os01g0159200	1.31	Drought up	Conserved hypothetical protein
Os06g0725000	-1.13	Drought up	Similar to Ntdh
Os08g0414700	-2.42	Drought up	Similar to Trehalose-6-phosphate synthase
Os12g0437800	-2.85	Drought up	Similar to MPI
Os01g0878900	3.66	Drought down	Similar to 4,5-DOPA dioxygenase extradiol-like protein
Os01g0246400	3.27	Drought down	Similar to Low molecular mass early light-inducible protein HV90
Os03g0122300	2.27	Drought down	Similar to Flavanone 3-hydroxylase-like protein.
Os09g0526600	1.81	Drought down	Similar to Isoform 2 of Heat stress transcription factor B-2c.
Os05g0410200	1.66	Drought down	Alpha/beta hydrolase fold-3 domain containing protein
Os06g0248500	1.40	Drought down	U box domain containing protein. (Os06t0248500-00)
Os10g0491000	1.32	Drought down	Plant Basic Secretory Protein family protein. (Os10t0491000-01)
Os08g0473900	-3.33	Drought down	Alpha-amylase isozyme 3D precursor

FIGURE 4 | Expression profiles of 106 RGA1-regulated stress- responsive genes in drought stress (30 perturbations from literature). The red and green colors indicate up-regulation (log2 [2.5]) and down-regulation (log2 [−2.5]), respectively, as shown in the color bar. The expression data in the left panel were obtained using Genevestigator. The table compares their regulation in normal plants under stress in literature with actual fold-change values in the RGA1 mutant.

microarray, six of the up-regulated genes and one of the down-regulated genes from literature are similarly up-regulated in both RGA1 mutant (without stress) as well as in normal rice plants under drought stress. Among the rest, seven up-regulated and three down-regulated genes in the RGA1 mutant showed opposite pattern of regulation under drought stress in normal rice plants in literature (**Figure 4**, right panel). The genes up-regulated in the RGA1 mutant but down-regulated by drought stress in the normal plants include flavanone 3-hydroxylase-like protein, Isoform 2 of heat stress transcription factor,

B-2cAlpha/beta hydrolase fold-3 domain containing protein, U box domain containing protein, and plant basic secretory protein family protein. The genes down-regulated in the RGA1 mutant by up-regulated in normal plants include Trehalose-6-phosphate synthase, MPI, and Ntdin. The opposite pattern of regulation of these genes could be due to the RGA1 mutation, which indicates that RGA1 may mediate the response of these genes to drought stress.

Interestingly, ribose phosphate pyrophosphokinase 3 is up-regulated in the RGA1 mutant as well as in response to cold and drought stress in literature, whereas isoform 2 of the heat stress transcription factor is up-regulated in the RGA1 mutant, but down-regulated in drought and cold stresses.

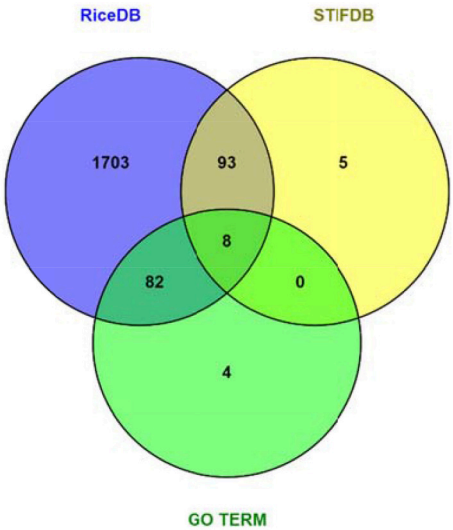


FIGURE 5 | Venn selection of RGA1 regulated stress responsive genes mined from RiceDB, STIFDB, and GO term. The overlap among the three sets revealed that the genes mined using GO term stress and stress responsive genes from STIFDB are largely subsets of the 1886 DEGs identified using Rice DB.

Meta-Data Analysis

Data mining using our entire non-redundant list of 2270 RGA1-regulated DEGs (1242 up and 1028 down) as input query at the Rice DB Oryza information portal revealed a much larger number of 1886 stress-related genes as differentially regulated in our RGA1 mutant. This prompted a comparison of various stress-responsive gene lists identified using different approaches in this study, such as gene ontology (94), STIFDB2.0 (106), and Rice DB (1886). A Venn selection of all three sets revealed that the former two are largely subsets of the 1886 DEGs identified using Rice DB (**Figure 5**). Therefore, the rest of the meta-data analysis was carried out using these 1886 genes.

The distribution of these 1886 RGA1-regulated, stress-responsive DEGs in terms of individual stresses was found to be 1730 DEGs in salt stress (889 up/841 down), 1719 DEGs in drought (882 up/837 down), 1690 DEGs in heat (913 up/777 down), and 1688 DEGs in cold (878 up/810) down with 1498 genes (773 up/725 down) common to all four stresses (**Figure 6**). In other words, as many as 1886 G-protein-regulated genes are responsive to one or more of these stresses, indicating their possible regulation through G-protein (RGA1) signaling.

Interestingly, the largest majority of 1886 with 1498 genes (or 80%) are common to all four abiotic stresses, followed by 137 genes common to cold, drought and salt stresses, followed by 38 genes common to drought, heat, and salt and so on, indicating their common regulation through G-proteins (**Table 2**). Even more interesting is the fact that as many as 111 heat-responsive genes are not common to any other stress and are uniquely regulated through G-proteins by heat only, followed by eight genes each in cold only and drought only, and two genes in salt stress only (**Table 2**). Some of the exclusively heat-responsive RGA1-regulated genes include superoxide dismutase, chitin-inducible gibberellin-responsive protein, brassinosteroid insensitive 1-associated receptor kinase 1, Hsp70 heat shock family protein, GTP-binding nuclear protein Ran1B (fragment), low affinity sulfate transporter 3, mitochondrial chaperonin-60, nucleoside diphosphate kinase I (EC 2.7.4.6) (NDPK I), wound responsive protein, and auxin response factor 2 (ARF1-binding protein).

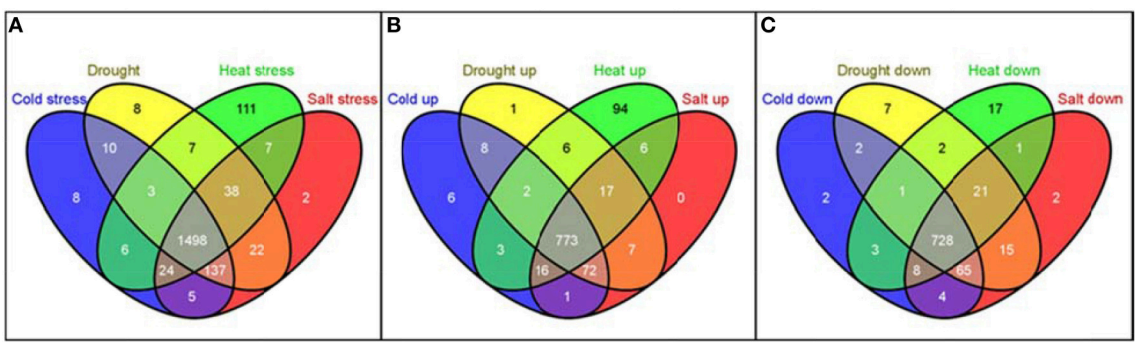


FIGURE 6 | Meta-data analysis of RGA1-regulated genes regulated under various abiotic stresses. The 2270 RGA1-regulated genes (1242 up and 1028 down) were used as input query at Rice DB to generate genes responsive to cold (878 up/810 down), drought (882 up/837 down), heat (913 up/777 down), and salt stress (889 up/841 down) with 1498 genes common to all four stresses and totaling 1886 unique genes. Their Venn selections are depicted as total **(A)**, up-regulated **(B)** and down-regulated **(C)** sets, using the online tool Venny (Oliveros, 2007–2015).

In order to validate the stress-responsive genes identified from the microarray results, quantitative RT-PCR experiments were carried out using total RNAs isolated from the wild type and RGA1 mutant rice plants grown and harvested under similar conditions. Out of the 1498 RGA1-regulated genes identified as common to multiple abiotic stresses on the microarray, 12 of the most up/downregulated genes were validated by qRT-PCR. Their fold change data are shown in **Figure 7** along with microarray results. The data clearly show the broad correspondence between the microarray data and RT-PCR results for both upregulated and downregulated sets of genes.

TABLE 2 | Distribution of RGA1-regulated genes among major abiotic stresses in Rice DB.

Stress categories	Up regulated in RGA1 mutant	Down regulated in RGA1 mutant	Total
Cold, drought, heat, and salt	773	728	1498*
Cold, drought, and salt	72	65	137
Drought, heat, and salt	17	21	38
Cold, heat, and salt	16	8	24
Drought and salt	7	15	22
Cold and drought	8	2	10
Drought and heat	6	2	8
Heat and salt	6	1	7
Cold and heat	3	3	6
Cold and salt	1	4	5
Cold, drought, and heat	2	1	3
Heat only	94	17	111
Cold only	6	2	8
Drought only	1	7	8
Salt only	0	2	2

*The 1886 RGA1-regulated genes identified as responsive to abiotic stresses at Rice DB have been categorized in terms of shared/unique stress categories and their up/down regulation in the RGA1 mutant. *Three genes out of 1501 were redundant or common to up/down categories, hence 1498.*

Gene ontology analysis of the core list of 1498 genes shared by all four stresses revealed 62 unique GO terms associated with molecular functions such as transporter activity, enzyme regulator activity, transferase activity, hydrolase activity, metabolic processes (carbon and protein), binding to nucleotides, carbohydrates, receptors and lipids, anatomical structure morphogenesis, flower development, and cell homeostasis (Supplementary Table 1). Further analysis using AGRIGO showed that many of these 1498 shared stress-responsive genes also share many GO terms of biological process, such as response to stimuli (GO: 0050896) with 49 genes out of the 95 genes (or 51%) accepted by AGRIGO for the query; 29 genes (30%) in response to chemical stimulus (GO: 0042221), 49 genes (51%) in response to stress (GO:0006950); 25 genes (26%) belong to oxidation reduction (GO:0055114); five genes (5%) belong to the category cellular response to chemical stimulus (GO:0070887), and 25 genes (26%) belong to response to oxidative stress (GO:0006979; **Figure 8**). This reveals the role of RGA1 in regulating a diverse range of processes related to stress response. GO terms of molecular function such as electron carrier activity had 80 genes (4%) and 61 genes (3%) in calcium ion binding out of a total of 1942 genes, indicating the role of RGA1 in their regulation. Its role also seems to be important in regulating the products of diverse cellular locations, such as etioplasts (130 genes), mitochondria (33 genes), plastid (16 genes), nucleus (15 genes), chloroplast (12 genes), and three genes each in endoplasmic reticulum, vacuole, and golgi apparatus (**Figure 8**).

Mining for miRNAs Targeting RGA1-Regulated, Stress Responsive Genes

Data mining for miRNAs at Rice DB using the GO terms of 1498 RGA1-regulated genes shared by all four stresses revealed that 63 of them could be targets of miRNAs. This indicates the role of RGA1 in post-transcriptional regulation of 63 target genes

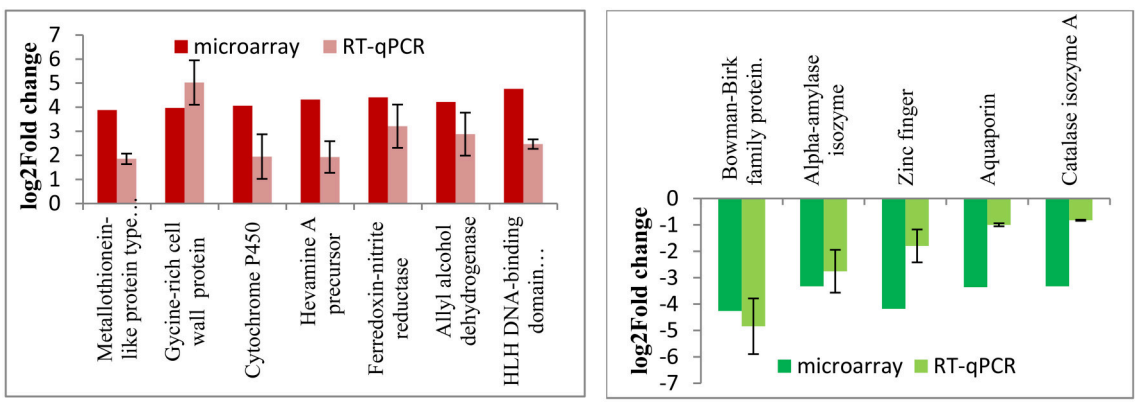


FIGURE 7 | qRT-PCR validation of RGA1-regulated genes identified as common to various abiotic stresses. Out of the 1498 RGA1-regulated genes identified as common to multiple abiotic stresses on the microarray, 12 of the most up/down-regulated genes were validated by qRT-PCR. Their fold change data are shown based on averages of two biological replicates and two technical replicates of total RNA, along with microarray results. The left panel in red shows the up-regulated genes and the right panel in green shows the down-regulated genes.

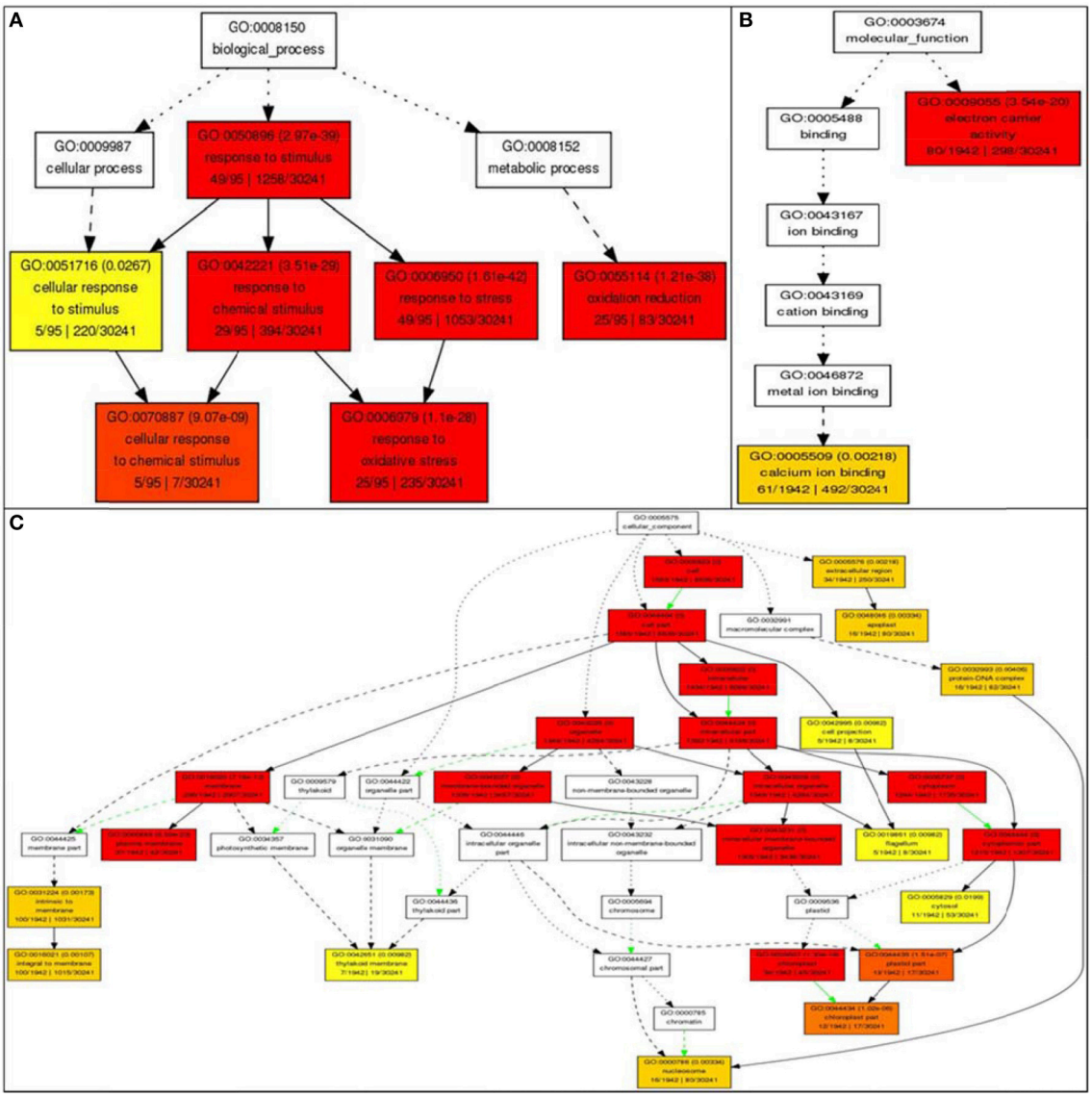


FIGURE 8 | Gene Ontology enrichment of RGA1-regulated, stress responsive genes from Rice DB. The 1498 genes common to all four major abiotic stresses were subjected to GO enrichment using AgriGO with default settings. **(A)** Biological process categorization of the RGA1-regulated genes shared by salt, heat, cold, and drought stresses. **(B)** Molecular function categorization and **(C)** Subcellular localization of the RGA1-regulated genes shared by all four abiotic-stresses.

TABLE 3 | miRNA targets among RGA1-regulated stress-responsive genes.

Gene identifier	miRNA_gene	Rap_description	GOID
Os11g0119100	OsSBS99	Catalytic/hydrolase	GO:0008152
Os11g0115400	OsSBS97	Lipid transfer protein LPT IV	NA
Os01g0679600	OsSBS87	THAP domain-containing protein 4	GO:0003674
Os03g0206400	OsSBS73	Conserved hypothetical protein	GO:0003674
Os10g0181200	OsSBS68	Protein prenyltransferase	GO:0006139
Os08g0562600	OsSBS67ab	C2 calcium-dependent membrane targeting	GO:0003674
Os10g0391400	OsSBS58	Tify domain containing protein	GO:0006950
Os03g0818400	OsSBS53	40S ribosomal protein S23 (S12)	GO:0005840

(Continued)

TABLE 3 | Continued

Os05g0128200	OsSBS45	Hypothetical conserved gene	GO:0009058
Os04g0396800	OsSBS4	Serine carboxypeptidase K10B2.2	GO:0005618
Os03g0126000	OsSBS27	Phosphorybosylanthranilatetransferase 1	GO:0008152
Os08g0561700	osa-MIR552	Superoxide dismutase	GO:0008152
Os03g0738400	osa-MIR530-5p′	Serine hydroxymethyltransferase,)	GO:0005739
Os02g0761400	osa-MIR530-3p	ATPREP2	GO:0005576
Os08g0137400	osa-MIR530	Cupredoxin domain containing protein	GO:0003674
Os09g0428000	osa-MIR529b	Glycosyltransferase, family 2 domain protein	GO:0016740
Os01g0823600	osa-MIR444f.17	Conserved hypothetical protein	GO:0003674
Os02g0324400	osa-MIR444c-5p	FON2 SPARE1	GO:0005576
Os02g0274900	osa-MIR444bc.9	Metabolite transport protein csbC	GO:0006810
Os07g0583600	osa-MIR444bc.25	Chitin-inducible gibberellin-responsive protein	GO:0007165
Os03g0802500	osa-MIR444ad.2,e	ATPase, AAA-type, core domain	GO:0005783
Os08g0482700	osa-MIR444	Conserved hypothetical protein	GO:0003674
Os04g0459600	osa-MIR442	Mog1/PsbP, alpha/beta/alpha sandwich domain	NA
Os05g0414700	osa-MIR403	Brassinosteroid insensitive 1 receptor kinase 1	GO:0005102
Os05g0557700	osa-MIR399j	Conserved hypothetical protein	GO:0005575
Os01g0850700	osa-MIR397b′	Cupredoxin domain containing protein	GO:0009056
Os01g0121600	osa-MIR396c-3p	Conserved hypothetical protein	GO:0006810
Os01g0180800	osa-MIR396c	Heat shock protein Hsp70 family protein	GO:0005634
Os03g0225500	osa-MIR395f′	Nucleoporin, Nup133/Nup155-	GO:0005515
Os05g0574500	osa-MIR395c′o′	GTP-binding nuclear protein Ran1B	GO:0005515
Os03g0195300	osa-MIR395	Low affinity sulfate transporter 3	GO:0016020
Os03g0559700	osa-MIR393ab′	Conserved hypothetical protein	NA
Os03g0388900	osa-MIR319a.2	Peptidase C14, caspase catalytic protein	GO:0019538
Os01g0323600	osa-MIR2055′	S-adenosylmethionine synthase 2	GO:0016020
Os03g0409100	osa-MIR1884b-3p	PUA-like domain domain protein	GO:0019538
Os08g0417000	osa-MIR1884b	2OG-Fe (II) oxygenase domain protein	GO:0008152
Os10g0462900	osa-MIR1879′	Mitochondrial chaperonin-60	GO:0005739
Os08g0512400	osa-MIR1879	Unknown function DUF296 domain protein	GO:0003677
Os07g0492000	osa-MIR1862	Nucleoside diphosphate kinase I (NDK I)	GO:0005576
Os01g0763200	osa-MIR1861d	Transcription factor PCF7 (Fragment)	GO:0005575
Os02g0830700	osa-MIR1860-5p	Leucine-rich repeat	GO:0005623
Os09g0530700	osa-MIR1860-3p	Hypothetical conserved gene	GO:0003674
Os02g0580900	osa-MIR1858b	TGF-beta receptor	NA
Os03g0667100	osa-MIR1851	BTB/POZ domain containing protein	GO:0006950
Os10g0510000	osa-MIR1850	Actin	GO:0009719
Os01g0720600	osa-MIR1847.7	Starch synthase IVa	GO:0016740
Os03g0101300	osa-MIR1847.10	Hexose transporter	GO:0005829
Os03g0821100	osa-MIR1846d-5p	Non-cell-autonomous HS cognate protein 70	GO:0016020
Os07g0684800	osa-MIR1846c-5p	NAM/CUC2-like protein	GO:0005634
Os08g0357000	osa-MIR172c	Wound responsive protein	GO:0006950
Os04g0442000	osa-MIR172b	Auxin response factor 2 (ARF1-BP)	GO:0009058
Os03g0784000	osa-MIR172ad	FAD dependent oxidoreductase family protein	GO:0008152
Os02g0662700	osa-MIR171i	Scl1 protein (Fragment)	GO:0008150
Os03g0696300	osa-MIR169no	Nuclear transcription factor Y subunit A-1	GO:0007275
Os03g0687000	osa-MIR168a	Predicted protein	GO:0016020
Os03g0640800	osa-MIR166m	(Homeodomain-leucine zipper protein 14)	GO:0003677
Os12g0147800	osa-MIR164d	Phytosulfokines 5 precursor	NA
Os05g0580000	osa-MIR162b	ADP-glucose pyrophosphorylase (EC 2.7.7.27)	GO:0009058
Os03g0140200	osa-MIR1441	Cytochrome P450 86A1	GO:0005623
Os02g0821200	osa-MIR1428	Ribosomal protein L28e domain protein	GO:0005840
Os01g0967800	osa-MIR1328	Conserved hypothetical protein	NA
Os10g0503800	osa-MIR1322	Remorin	GO:0005575
Os06g0195900	NA	NOG, C-terminal domain containing protein	GO:0016020

Data mining at Rice DB using them are targets for miRNA regulation.

for the first time. They include 38 up-regulated genes and 25 down-regulated genes identified in the RGA1 mutant (**Table 3**).

DISCUSSION

Heterotrimeric G-protein subunits or their interacting partners have either been implicated in stress signal transduction or have been shown to respond to stress themselves (Urano et al., 2013). Experimental approaches, including genome-wide studies, were generally focused on the response to individual stresses or individual components of G-protein signaling. The role of the G-protein α subunit in individual abiotic stress responses has been in particular focus, in relation to heat/salt stress in pea (Misra et al., 2007) and salt stress in Arabidopsis (Colaneri et al., 2014), rice and maize (Urano et al., 2014), or indirectly in ABA signaling (Pandey et al., 2010; Alvarez et al., 2011) or oxidative stress (Booker et al., 2012). The expression of rice Gα subunit (RGA1) gene itself was reported to be up-regulated by salt, cold, and drought stresses, and down regulated by heat stress (Yadav et al., 2013). However, there are no comprehensive studies on the genome-wide involvement of any heterotrimeric G-protein subunit in all the main abiotic stresses in any plant, except Arabidopsis (Chakraborty et al., 2015a,b). Comprehensive functional genomic analyses are particularly lacking on the genome-wide role of RGA1 or other G-protein subunits in multiple abiotic stress responses in rice.

In view of our own recent findings reported elsewhere in this issue on the growing importance of G-protein signaling components in abiotic stress response in *Arabidopsis* (Chakraborty et al., 2015c), as well as the importance of abiotic stress in rice crop improvement, we sought to examine the abiotic stress component of our RGA1 transcriptome microarray data in detail. This was done by combining our experimental functional genomic data with *in silico* meta data analysis to answer the following questions: Does abiotic stress figure prominently in the genome-wide response to RGA1 null mutation in rice and if yes, what are the various genes involved and how are they distributed in terms of major individual abiotic-stresses or in terms of their differential regulation in the RGA1 mutant? How do they compare with the known genome-wide response of normal rice plants to various abiotic stresses? Can *in silico* transcriptome meta-data analyses provide adequate insights for integrative understanding on abiotic stress signaling components in rice as possible converging points for interventions?

Our microarray experiments under MIAME compliant conditions using the Japonica rice RGA1 mutant and wild type (GSE 20925 at NCBI GEO) revealed 2270 differentially expressed genes, out of which the stress responsive data set was identified and analyzed using three approaches: Gene Ontology terms, data mining from STIFDB, and meta-data analysis from Rice DB. Firstly, segregation using Gene Ontology terms yielded 94 genes corresponding to various abiotic stress categories, most of which belonged to less than 40 families (**Table 1**), indicating their regulation by RGA1. The fact that majority of these families showed similar patterns of up/down regulation indicates that their regulation by RGA1 is also uniform, while there are a few families such as those related to oxidative stress response that show differential regulation of their members in the RGA1 mutant. The uniform mode of up/down regulation of multiple members of the same family of stress-responsive genes reveals the inherently coordinated pattern of gene regulation in response to a stress signal (e.g., DREB), whereas the varied extent of that regulation reveals the fine tuning of the signal/response flux through a regulatory cascade. Such patterns of regulation may be amenable to deeper network analysis.

Secondly, data mining for genes specifically categorized as stress-responsive genes from Japonica rice at STIFDB yielded 626 genes, out which 106 genes belonging to various abiotic stresses—heat drought, salt cold, were RGA1-regulated (**Figure 1**). Together, these 106 abiotic stress-responsive genes constitute less than 5% of all the G-protein (RGA1) regulated genes. But they constitute a far higher proportion (17%) of the 626 abiotic stress-responsive genes, indicating the larger role for G-proteins in regulating them, even though mediating abiotic stress seems to be a smaller part of the genome-wide role of G-proteins. However, this difference may also be an artifact arising out of the relatively lesser coverage of 626 rice stress-responsive genes on the STIFDB, as compared to 3150 genes in *Arabidopsis*, as similar analysis on its GPA1 mutant produced more consistent ranking with cold>salt>drought (Chakraborty et al., 2015c).

Hierarchical clustering of the 106 RGA1-regulated, stress-responsive genes mined from STIFDB2.0 using Genevestigator revealed prominent clusters of cold and drought responsive genes (**Figure 2**), which were subjected to further analysis by biclustering using the same software. While hierarchical clustering helps in grouping genes with similar profiles across all abiotic stress conditions, Biclustering identifies groups of genes that exhibit similarity only in a subset of conditions such as cold or drought, irrespective of their expression profiles in other conditions. The regulation of genes identified as highly differentially regulated by biclustering in both cold and drought conditions was compared with the fold-change values obtained on our microarray (**Figures 3**, **4**). This revealed that some of the genes follow similar pattern of regulation between the stress response in normal rice plants and the RGA1-response in mutants unexposed to stress. While these may indicate independent regulation, the remaining genes that follow opposite pattern of regulation could be due to the RGA1 mutation, suggesting that RGA1 may mediate the response of these genes to cold or drought stresses.

Thirdly, metadata analyses based on data mining at Rice DB using the 2270 genes we identified in the RGA1-transcriptome microarray revealed a much larger number of 1886 stress-related genes as differentially regulated in our RGA1 mutant. A Venn selection of the stress-responsive gene lists identified by all three approaches used in this study viz., gene ontology (94), STIFDB2.0 (106) and Rice DB (1886) revealed that the former two are largely subsets of the 1886 DEGs identified using Rice DB (**Figure 5**). Their Venn selections in terms of individual abiotic stress categories and by up/down regulation on our microarray (**Figure 6**) revealed 1498 genes as common to all four stresses, with fewer common genes in smaller combinations of stresses

(**Table 2**). Out of them, 12 of the most up/down-regulated genes have been validated by qRT-PCR (**Figure 7**), confirming the broad trends of up/down regulated genes identified on the microarray. These include the well-known stress-responsive genes such as catalase and aquaporin. Among the individual stresses, the sheer number of RGA1-regulated genes that only respond to heat (and no other abiotic stress) is striking, and needs further analysis. Comparative microarray or RT-PCR profiling of the RGA1 mutant and wild type rice plants exposed to various abiotic stresses would reveal more details in this regard.

Gene Ontology enrichment of the 1498 RGA1-regulated genes shared by all four abiotic stresses using AGRIGO revealed their molecular functions, cellular localizations, and biological processes (**Figure 8**). In terms of processes, genes from the various abiotic stress signaling pathways such as calcium-dependent pathways, ABA dependent or independent pathways, and MAP kinase pathways, as well as various pathways involved in the production of osmoprotectants, heat shock proteins, metallothioneins, antioxidants etc., were found to be differentially regulated in the RGA1 mutant as elaborated below. Together, they clearly indicate the crucial role of G-protein alpha subunit signaling in transducing/mediating the response of rice to multiple abiotic stresses.

Calcium-Dependent Pathways in G Protein-Mediated Abiotic Stress Signaling

Calcium is a well-known second messenger in abiotic signal transduction and various calcium binding proteins such as calmodulins, calcineurin, CDPKs, and calcineurin B-like interacting protein kinases (CIPK) play an important role in calcium-dependent abiotic stress (Batistič and Kudla, 2012). The CBL proteins form a complex network with their target kinases CIPKs and regulate target gene expression (Das and Pandey, 2010). Some genes related to calcium signaling were shown to be involved in stress signaling in rice (Batistič and Kudla, 2012), and transgenic manipulation of some such genes has been shown to improve stress-tolerance in rice (Campo et al., 2014). In our study, calcium dependent protein kinase (Os02g0685900) is up regulated while Calmodulin-like protein CaML3 (Os11g0141400) is down regulated in the RGA1 mutant. Their further validation could help determine their potential as candidate genes for development of rice plants tolerant to multiple abiotic stresses.

Map Kinase Pathways in G-Protein-Mediated Abiotic Stress Signaling

Many MAPKs have been reported in rice for various abiotic stresses (Danquah et al., 2014). The MAPK gene OsMSRMK2 is highly induced by a variety of stresses including ABA, JA, SA, drought, and salt but not by cold (Danquah et al., 2014). In this study, for example, we found MAP kinase (Os03g0285800) to be up regulated. A receptor-like kinase, or *O. sativa* stress-induced protein kinase gene 1, which is known to be involved in drought and salt stress tolerance is also induced in the RGA1 mutant.

ABA Signaling in G-Protein Mediated Abiotic Stress Response

ABA is involved in the regulation of many aspects of plant growth and development and also is the major hormone that controls plant responses to abiotic stresses (Danquah et al., 2014), especially drought stress. ABA is also one of the most studied hormones in relation to G-protein signaling (Zhao et al., 2010). We found a related gene encoding the SNF1-related protein kinase regulatory gamma subunit 1 (AKIN gamma1Os04g0382300) to be suppressed in the RGA1 mutant. Similarly, drought- responsive element binding protein (DREB) is a part of ABA-independent pathway, from which both DREB2 and CRT/DRE binding protein were up regulated in our data. Among the ABA signaling pathway genes, we also found that MYB expression was enhanced in the RGA1 mutants as compared with wild-type plants. Abscisic acid responsive element-binding factors belong to the ABA dependent pathway, of which AREB2 was up regulated in our data. Members of TF families that are involved in both ABA-independent (AP2/ERF and WRKY) and ABA dependent pathways are also involved in stress tolerance (Song et al., 2012).

Transcription Factors and miRNAs in G Protein-Mediated Abiotic Stress Response

The expression of many stress responsive genes is mediated by transcription factors that bind to specific cis-elements in the promoters of their target genes. We found various transcription factors such as ADH1, OsNAC5, OsWRKY45, bZIP23/72 to be differentially regulated in our RGA1 mutant. Further characterization and validation of the transcription factors identified in our study may reveal their potential as candidate genes to engineer tolerance to various abiotic stresses in rice. At the post-transcriptional level, miRNAs are also known to play important regulatory roles in plant development and stress. miRNAs, such as miR168, miR171, and miR396, are regulated by abiotic stresses such as salinity, drought, and cold in rice (Mal et al., 2015). So far, no study has reported RGA1-responsive miRNAs involved in stress. In this study, we have mined 63 RGA1-regulated target genes for miRNAs that are also stress responsive. Further validation of their role in stress-response could reveal if they have any potential in crop improvement.

Osmoprotectant Genes, Lea Genes, Heat Shock Proteins, and Others

Several genes found to be differentially regulated in the RGA1 mutant belong to biosynthetic pathways of osmoprotectants such as polyamine, glycine-betaine, proline, and trehalose. Three genes (Os08g0445700, Os02g0661100, and Os01g0749400) involved in the trehalose synthesis pathway were up regulated in the RGA1 mutant. A major pathway that is significantly down regulated is the betanidin degradation pathway with 21 genes being down regulated. Two genes (Os03g0738400, Os12g0409000) from the glycine betaine synthesis were also up regulated in the RGA1 mutant. These genes are known to be involved in various stress

responses such as increased submergence tolerance, drought, and cold resistance (Marco et al., 2015).

Heat shock proteins and molecular chaperone proteins like metallothionein proteins are involved in heat and drought tolerance. Their genes were highly up regulated in the RGA1 mutant with fold changes up to three and validated by qRT-PCR (**Figure 7**). Late embryogenesis abundant (LEA protein) genes are known to help in drought and salinity tolerance (Mondini and Pagnotta, 2015). In our study, Lea14-A was up-regulated in the RGA1 mutant, indicating its potential importance in rice stress. Similarly, among the hormone regulatory genes, we found RGA1-regulation of IPT and ABA hydroxylase, which delay senescence and yield under drought and reduce sterility under cold stress. Oxidative stress related genes such as Glutathione S-transferase and superoxide dismutase genes are involved in salt and cold stress (Marco et al., 2015). In our study, superoxide dismutase was down regulated in the RGA1 mutant, indicating the important role of G-protein alpha subunit in SOD-mediated regulation of oxidative stress. Genes encoding proton pumps, antiporters, and ion transporters like vacuolar Na+/H+ antiporter and aquaporins are also known to enhance salt and cold tolerance. Our data shows that aquaporins are down regulated 3.36 times in the RGA1 mutant and is validated by qRT-PCR (**Figure 7**).

CONCLUSION

Overall, our results clearly indicate the potentially crucial role of the G-protein α subunit (RGA1) in regulating the response of the rice plant to multiple abiotic stresses for further experimental validation. The 1886 RGA1-regulated and stress-responsive genes we mined in this study may represent only a subset of overall-stress responsive genes in rice, but they do constitute a G-protein (RGA1)-regulated subset that was never described in any plant so far, except in Arabidopsis elsewhere in this issue (Chakraborty et al., 2015c). The fact that as many as 1498 RGA1-regulated, stress-responsive genes are common to the four abiotic stresses (drought, salt, heat, cold), and that relatively fewer genes are uniquely regulated by RGA1 in response to individual stresses indicates that RGA1-signaling could be a converging point for the regulation of multiple abiotic stress responses. Its experimental validation, as well as that of the exceptionally large number of 111 unique genes regulated by RGA1 in heat stress (unshared with the other three stresses) could offer glimpses into the commonalities and differences in heat stress signaling vis-à-vis other stresses.

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Class-Specific Evolution and Transcriptional Differentiation of *14-3-3* Family Members in Mesohexaploid *Brassica rapa*

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14-3-3s are highly conserved, multigene family proteins that have been implicated in modulating various biological processes. The presence of inherent polyploidy and genome complexity has limited the identification and characterization of 14-3-3 proteins from globally important *Brassica* crops. Through data mining of *Brassica rapa*, the model *Brassica* genome, we identified 21 members encoding 14-3-3 proteins namely, BraA.GRF14.a to BraA.GRF14.u. Phylogenetic analysis indicated that *B. rapa* contains both ε (epsilon) and non-ε 14-3-3 isoforms, having distinct intron-exon structural organization patterns. The non-ε isoforms showed lower divergence rate ($Ks < 0.45$) compared to ε protein isoforms ($Ks > 0.48$), suggesting class-specific divergence pattern. Synteny analysis revealed that mesohexaploid *B. rapa* genome has retained 1–5 orthologs of each *Arabidopsis 14-3-3* gene, interspersed across its three fragmented sub-genomes. qRT-PCR analysis showed that 14 of the 21 *BraA.GRF14* were expressed, wherein a higher abundance of non-ε transcripts was observed compared to the ε genes, indicating class-specific transcriptional bias. The *BraA.GRF14* genes showed distinct expression pattern during plant developmental stages and in response to abiotic stress, phytohormone treatments, and nutrient deprivation conditions. Together, the distinct expression pattern and differential regulation of *BraA.GRF14* genes indicated the occurrence of functional divergence of *B. rapa* 14-3-3 proteins during plant development and stress responses.

Keywords: 14-3-3, *Brassica rapa*, expression differentiation, gene divergence, polyploidy

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INTRODUCTION

14-3-3 proteins derived their unique name from the studies of fractionation of bovine brain proteins on DEAE cellulose and their electrophoretic mobility on starch gel electrophoresis (Moore and Perez, 1967). These regulatory proteins are present in all eukaryotes and involved in protein interactions mediated signal transduction pathways. In plants, 14-3-3 proteins function by binding to numerous "client" proteins in a phosphorylation-dependent manner to modulate their activities, degradation, or sub-cellular localization (Rosenquist et al., 2001; Paul et al., 2012). So far, more than 300 putative 14-3-3 interacting client proteins have been reported in plants out of which nitrate reductase, sucrose-phosphate synthase, plasma membrane H^+-ATPase, EmBP1, and VP1 transcription factors, the RSG transcription activator, a lipoxygenase from barley, a membrane

bound ascorbate peroxidase and a outward-rectifying K^+ channel in tomato are well-characterized (reviewed in Oecking and Jaspert, 2009; Denison et al., 2011; de Boer et al., 2013). This high number reflects the potential role of the 14-3-3s in controlling various signaling and developmental processes in plants. Current literatures clearly suggest the involvement of 14-3-3 proteins in various physiological processes including primary carbon and nitrogen metabolism (Comparot et al., 2003), abiotic and biotic stress responses (Roberts et al., 2002; Umezawa et al., 2004; Yan et al., 2004; Chen et al., 2006; Yang et al., 2013; Catalá et al., 2014; Zhou et al., 2014; He et al., 2015; Li et al., 2015), signaling pathways of phytohormones like ABA, GA, and BR (Testerink et al., 1999; Igarashi et al., 2001; Ryu et al., 2007; Kim et al., 2009; Zhou et al., 2015), and also during plant growth and development (Radwan et al., 2012; de Boer et al., 2013; Sun et al., 2014; van Kleeff et al., 2014).

To carry such diverse roles, almost all eukaryotes harbor multiple isoforms of *14-3-3* genes, with two present in yeast, seven in humans, and more than a dozen in vascular plants. The model monocot (*Oryza sativa*) and dicot (*Arabidopsis thaliana*) plant genomes encode 8 and 13 expressed *14-3-3* genes, respectively (DeLille et al., 2001; Rosenquist et al., 2001; Chen et al., 2006; Yao et al., 2007). Data mining of sequenced plant genomes has led to the identification of much higher number of *14-3-3* genes, particularly from polyploid genomes. A total of thirty-one *14-3-3* cDNAs encoding 25 unique proteins were identified from allotetraploid cotton (Sun et al., 2011), whereas the diploidized tetraploid soybean genome has eighteen *14-3-3* gene homologs, of which 16 are expressed (Li and Dhaubhadel, 2011). The chromosomal/segmental duplications and the evolutionary diversification are largely known to shape the quantitative variability of functional 14-3-3 proteins across plant species. Even though high sequence similarity exists among multiple copies of 14-3-3s, the specificity of these protein isoforms harboring definite subcellular localization, tissue-specific expression, and dynamic regulation in response to environmental changes is well-reviewed (Kjarland et al., 2006). Also each isoform, expressing in different subcellular location inside the cell, interacts with different client partners and relay the downstream signaling. This partly explains the versatility of so many isoforms in a plant species regulating a wide range of biological processes and functions.

Brassica species, the closest crop relatives to *Arabidopsis*, play an important role in global agriculture and horticulture. *Brassica rapa* (field mustard) is one of the globally important *Brassica* crop because of its enormous genetic and morphological diversity, and being utilized as leafy vegetables, vegetable oils, turnips roots, turnip greens, turnip tops, and fodder turnip. Besides, it is one of the diploid progenitor species ($n = 10$) which contributed the "A" genome to the important oilseed crops, *Brassica juncea* ($n = 18$, AABB) and *Brassica napus* ($n = 19$, AACC). Because of its pivotal position among the *Brassica* species, the recent sequencing of *B. rapa* genome offers an excellent opportunity to study the structural and functional evolution of candidate genes (The *B. rapa* Genome Sequencing Project Consortium; Wang et al., 2011). Sequence level studies although reflect high similarity in functional genes present between *Arabidopsis* and *B. rapa*, quantitative variation and evolutionary divergence in members of gene families present in polyploid *Brassica* genome, however, may contribute to the remarkable phenotypic plasticity and environmental adaptability of economically important *Brassica* species.

To investigate the important roles played by 14-3-3 protein isoforms in *Brassica* crops, comprehensive analysis of *14-3-3* gene homologs was undertaken from *B. rapa*. Present study through data mining of the recently sequenced *B. rapa* genome identified 21 divergent *14-3-3* genes providing their chromosomal and sub-genomic localization, phylogenetic relationship, and divergence analysis. We further carried out comprehensive expression profiling of 14 expressed *14-3-3* gene family members in *B. rapa* across plant development stages, abiotic stress conditions, phytohormone treatments and under nutrient deprivation conditions. We observed highly coordinated tissue- and condition-specific expression of *B. rapa 14-3-3* transcripts, suggesting their multifarious roles across plant growth, development, and environmental cues. The study provides an excellent base for conducting further in-depth research on various signaling pathways regulated by *B. rapa* 14-3-3 proteins, which could be utilized for agricultural improvements of the mustard crop.

MATERIALS AND METHODS

Plant Materials and Growth Condition

B. rapa genotype YID1 was grown in a controlled growth conditions set as day (22°C, 10 h)/night (15°C, 14 h) cycle and 70% relative humidity. Different developmental stages namely, seedling, root, stem, leaf, and silique (20 days post anthesis), were harvested and immediately frozen in liquid nitrogen and stored at −80°C.

Identification of the 14-3-3 Isoforms and Phylogenetic Comparisons

Arabidopsis 14-3-3 gene sequences were used to search against the *B. rapa* genome database (http://brassicadb.org/brad/; Cheng et al., 2011). The retrieved sequences were then named according to the existing nomenclature as *BraA.GRF14.a* to *BraA.GRF14.u*. Multiple sequence alignments of the encoded proteins were done using Clustal W and the phylogenetic trees were constructed using the Neighborhood-joining method of MEGA5 (Tamura et al., 2011). The human 14-3-3 theta isoform (NP_006817) was used as an out-group protein.

RNA Isolation and cDNA Synthesis

RNA was extracted from plant tissues using the Spectrum Plant Total RNA Kit (Sigma Life Sciences, USA) according to manufacturer's instructions. The quantity and quality of RNA sample was checked using Nano spectrophotometer (ND-1000 Thermo scientific); and RNA samples with 260/280 ratio (1.9–2.1) and 260/230 ratio (2.0–2.5) were used for further analysis. First strand cDNA was synthesized by reverse transcribing 2 μg of total RNA with random primers of high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) in a 20 μl

reaction according to manufacturer's instructions. Diluted cDNA (1:50) was used for the real-time qRT-PCR reaction.

Expression Profiling of *B. rapa 14-3-3* Genes

Real-Time PCR was performed using standard cycling conditions (95°C for 10 min, 40 cycles of 15 s at 95 and 60°C for 60 s) in final volume of 20 µl in a 7900 HT real time PCR machine (Applied Biosystems). Reaction mixture contained SYBR Green Master Mix (Kapa Biosystems), 10 pmol of gene-specific forward and reverse primers, and 2 µl of the diluted cDNA (∼200 pg). To check for the specificity of PCR amplification dissociation curve was generated (Figure S3). The *Ct*-values were determined for each reaction using SDS version 2.3 and RQ manager version 1.2 (Applied Biosciences) software with default parameters. *GAPDH* and *ACT2* genes of *Brassica* origin were used as endogenous control (Chandna et al., 2012). Three independent sets of experiments were conducted with two technical replicates each to confirm results. Primers used for qRT-PCR analysis are tabulated in Table S4.

Elicitor and Stress Treatments

For elicitor and stress treatments, seedlings were grown initially for 5 days on agar plates containing one-half strength Murashige and Skoog (MS) medium. Before elicitor induction, seedlings were adapted to liquid MS culture containing 1% sucrose for 24 h. For hormones induction, methyl jasmonate (MeJA, 0.2 mM), salicylic acid (SA, 0.2 mM), indole-3-acetic acid (IAA, 0.1 mM), and abscisic acid (ABA, 0.1 mM) were independently added to the medium (Chandna et al., 2012). Seedlings were also subjected to different stress conditions such as heat shock (37°C), cold (4°C), salinity (300 mM NaCl), and dehydration (between folds of tissue paper). The plants were harvested at 15 min, 30 min, 3 h, and 6 h durations and the mock treated seedling for same time interval served as control. For elicitor treatment experiments, the expression cut-off of 1.5-fold change (w.r.t. corresponding control) was used to identify the up- and down-regulated transcripts.

Nutrient Deprivation Experiments

Plants were initially grown in MS agar medium for 3 days at 22°C with 10 h daylight at 200 µmol m^{-2} s^{-1}. Seedlings were then transferred and adapted for 2 days on hydroponic nutrient solution containing 5 mM KNO_3, 2.5 mM KH_2PO_4, 2.5 mM Fe-EDTA, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, and the following micronutrients: 1 mM NaCl, 1.4 mM $MgCl_2$, 0.001 mM $CaCl_2$, 7 mM H_3BO_3, 0.1 mM $ZnSO_4$, 0.05 mM $CuSO_4$, and 0.002 mM Na_2MoO_4. For nutrient deprivation experiments, the 2 days old hydroponically adapted seedlings were then transferred to nutrient solution lacking either Phosphorus (P), or Potassium (K), or Nitrogen (N). For the "P" deprivation, 2.5 mM KH_2PO_4 was replaced with 2.5 mM K_2SO_4. For the "K" deprivation, 5 mM KNO_3 was replaced with 3 mM $Ca(NO_3)_2$; whereas 2.5 mM KH_2PO_4 was replaced with 0.25 mM K_2SO_4. For the "N" deprivation, 5 mM KNO_3, and 2 mM $Ca(NO_3)_2$ were replaced with 5 mM KCl and 2 mM $CaCl_2$, respectively. The untreated control and nutrient deprived seedlings were harvested at 1, 6, 24, and 48 h after treatments, immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

RESULTS

Identification and Sequence Analysis of *14-3-3* Gene Family from *B. rapa*

The availability of initial draft of *B. rapa* genome project (http://brassicadb.org/brad/) led us to perform the comprehensive search of *14-3-3* gene sequences. Using *Arabidopsis* GRF (General Regulatory Factor) cDNA sequences as query, BLAST search in *B. rapa* genome database resulted in the identification of 21 putative gene sequences encoding 14-3-3 proteins (**Table 1**). The 21 *BraA.GRF14* genes were located on eight out of 10 linkage groups (LG) of *B. rapa* genome, with A07 LG containing up to six *BraA.GRF14* genes (Figure S1, **Table 1**). Adopting the standard gene nomenclature for *Brassica* species (Ostergaard and King, 2008), the genes were named as *BraA.GRF14.a* to *BraA.GRF14.u*, in order of their identification. The 21 *BraA.GRF14* genes were variable in their sizes ranging from 998 to 1857 bp. The coding DNA sequences (CDS) of *BraA.GRF14* genes ranged from 738 to 918 bp and shared 41.8–92.6% sequence identity among them (**Table 1**, Table S1).

The *BraA.GRF14* genes encoded proteins ranging from 245 to 305 amino acids, with their calculated molecular masses and pI that ranged from 27.79 to 35.28 kDa and 4.34 to 4.77, respectively. Amino-acid sequence alignment of the deduced BraA.GRF14 proteins indicated that these are highly conserved proteins (**Figure 1**) sharing a high level of sequence identity (45.4–96.8%) among them (Table S2). Further, when we queried the deduced BraA.GRF14 proteins at ExPASy proteomics tool (www.expasy.org) all proteins, except for BraA.GRF14.e (Bra035020), showed the presence of highly conserved 14-3-3 signature motifs RNL(L/V)SV(G/A)YKNV and YKDSTLIMQLLRDNLTLWTS, thereby confirming their identity as 14-3-3 proteins. The divergence observed in the deduced Bra035020 (BraA.GRF14.e) protein sequence might be an evolutionary consequence; although the possibility of its mis-annotation in the current version of *B. rapa* genome assembly cannot be ruled out completely. In addition, the conserved phosphorylation sites reported earlier for plant's 14-3-3 proteins were also present in BraA.GRF14 proteins. The pY137 of maize GF14-6 known to decreases binding of the H^+-ATPase (Giacometti et al., 2004); and pS216, pS220, pT221 reported to be phosphorylated during the seed development in oilseed rape (Agrawal and Thelen, 2006), were all found to be conserved across BraA.GRF14 proteins. The pS93/95 residue, identified as being phosphorylated by SnRK2.8 in roots of the three *Arabidopsis* GRFs (χ, κ, ψ; Shin et al., 2007), was however found to be present in 7 of the 21 BraA.GRF14 proteins, thereby suggesting isoform-specific phosphorylation pattern and functional specificity of 14-3-3 proteins in *B. rapa*.

Genomic Structure and Phylogenetic Relationships of *B. rapa 14-3-3* Genes

The presence of multiple *14-3-3* type sequences in *B. rapa* having significant sequence divergence led us to investigate the

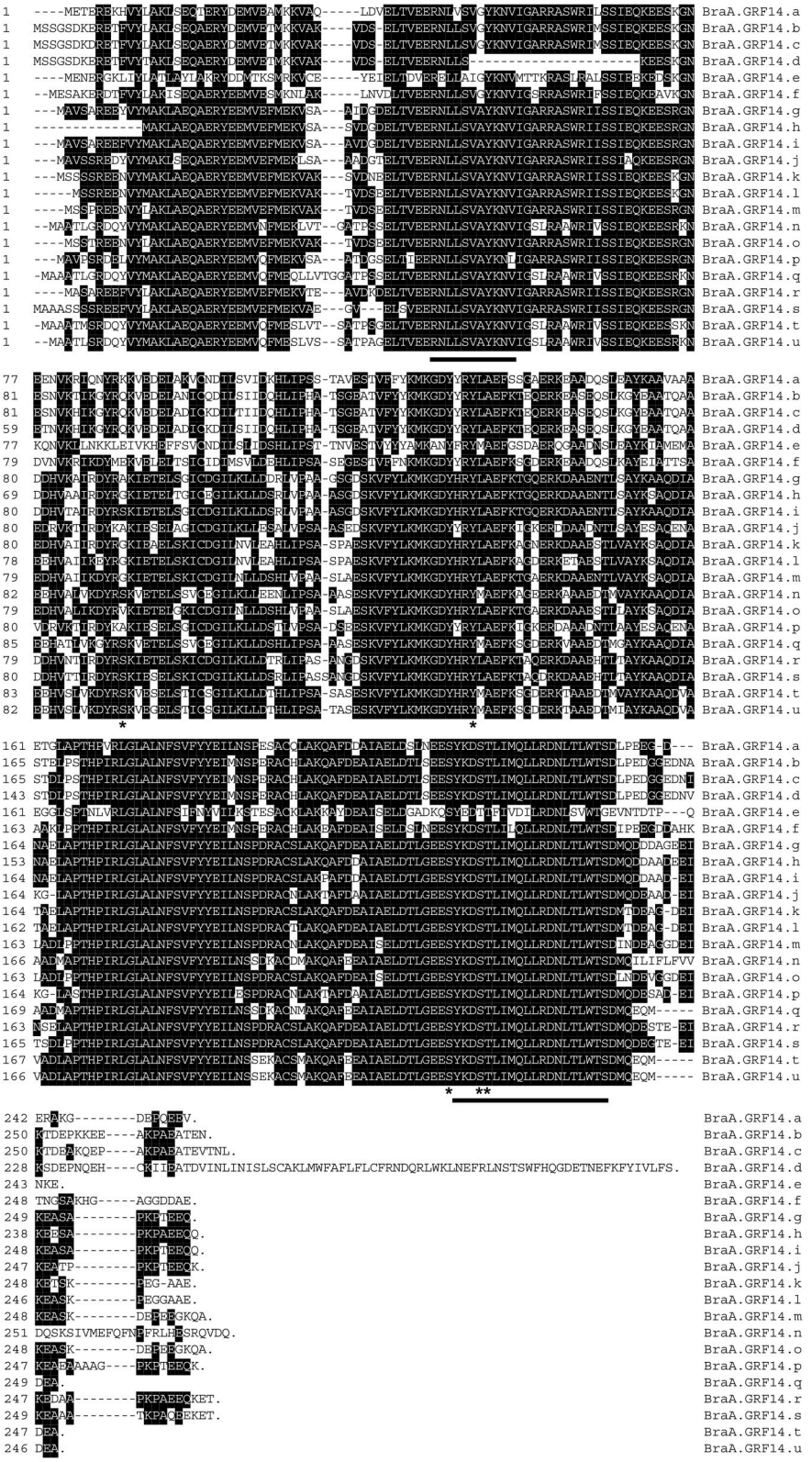


FIGURE 1 | Amino acid alignment of the deduced 14-3-3 proteins of *B. rapa* (BraA.GRF14). The sequence alignment of the 21 BraA.GRF14 proteins was performed using ClustalW. The 14-3-3 signature motifs RNL(L/V)SV(G/A)YKNV and YKDSTLIMQLLRDNLTLWTS, are underlined. The phosphorylation sites reported earlier for plant 14-3-3 proteins (Paul et al., 2012) are marked with asterisk.

TABLE 1 | Summary of gene structure attributes of the twenty-one 14-3-3 proteins identified in *B. rapa* genome (http://brassicadb.org/brad/).

S. No.	Name	Gene ID in *B. rapa* database	*B. rapa* linkage group	Gene size (bp)	CDS size (bp)	Protein size (aa)	No of exons	No of introns	Group
1	*BraA.GRF14.a*	Bra031383	A5	1647	762	253	6	5	ε
2	*BraA.GRF14.b*	Bra024713	A9	1278	804	267	7	6	ε
3	*BraA.GRF14.c*	Bra016273	A8	1334	813	270	6	5	ε
4	*BraA.GRF14.d*	Bra012455	A7	1351	918	305	7	6	ε
5	*BraA.GRF14.e*	Bra035020	A7	1328	738	245	6	5	ε
6	*BraA.GRF14.f*	Bra000268	A3	1595	789	262	6	5	ε
7	*BraA.GRF14.g*	Bra035027	A7	1857	783	260	3	2	non-ε
8	*BraA.GRF14.h*	Bra008355	A2	1386	753	250	4	3	non-ε
9	*BraA.GRF14.i*	Bra003649	A7	1386	783	260	4	3	non-ε
10	*BraA.GRF14.j*	Bra016380	A8	1372	780	259	4	3	non-ε
11	*BraA.GRF14.k*	Bra028409	A7	998	777	258	4	3	non-ε
12	*BraA.GRF14.l*	Bra028187	A4	1029	774	257	4	3	non-ε
13	*BraA.GRF14.m*	Bra040592	A5	1082	786	261	4	3	non-ε
14	*BraA.GRF14.n*	Bra028586	A2	1730	828	275	4	3	non-ε
15	*BraA.GRF14.o*	Bra001049	A3	1053	786	261	4	3	non-ε
16	*BraA.GRF14.p*	Bra012325	A7	1207	792	263	4	3	non-ε
17	*BraA.GRF14.q*	Bra006061	A3	1382	756	251	4	3	non-ε
18	*BraA.GRF14.r*	Bra028068	A9	1159	786	261	4	3	non-ε
19	*BraA.GRF14.s*	Bra034430	A5	1058	792	263	3	2	non-ε
20	*BraA.GRF14.t*	Bra037818	A9	1346	750	249	4	3	non-ε
21	*BraA.GRF14.u*	Bra024384	A6	1566	747	248	4	3	non-ε

evolution of *BraA.GRF14* gene family. We therefore, analyzed the genomic structure and phylogenetic relationship of *BraA.GRF14* gene family members. Analysis of genomic structure of 21 *BraA.GRF14* genes showed that the members of *14-3-3* gene in *B. rapa* contain 3–7 exons, interspersed by highly divergent introns (**Figure 2**). On the basis of organization of exons and introns, the *BraA.GRF14* genes were broadly categorized into two distinct sub-groups namely, the ε (epsilon) and non-ε (non-epsilon) groups. The six genes belonging to ε group contained 6–7 exons each, whereas 3–4 exons were present in the 15 non-ε group genes. Furthermore, the length of the exons was highly conserved among most of the 15 non-ε group genes; whereas genes belonging to ε group showed comparably higher divergence in their exon length. The extreme conservation of the exon organization in both ε and non-ε *BraA.GRF14* genes possibly suggest independent evolution and expansion of the ε and non-ε groups genes in *B. rapa*. In comparison, the introns in both ε and non-ε groups genes of *B. rapa* were highly divergent in their sizes and sequences.

To get a better insight into expansion of *B. rapa 14-3-3* gene family, a phylogenetic tree was constructed based on the multiple sequence alignment of full-length protein sequences from *B. rapa* (21), *Arabidopsis* (13) and *Oryza sativa* (7). Phylogenetic analysis showed that 14-3-3 protein isoforms from the three plant genomes were clustered into distinct ε and non-ε groups (**Figure 2**, Figure S2). Within each class, the 14-3-3 proteins from *Arabidopsis* and *B. rapa* genomes were grouped together, whereas the rice 14-3-3 proteins formed separate branches. This indicates that the 14-3-3 proteins of each class existed before the divergence of monocots and dicots, and have expanded independently in species-specific manner, as also observed for other gene families in plants (Zhang et al., 2005; Jain et al., 2007).

Sub-Genomic Location and Divergence Analysis of *B. rapa 14-3-3* Genes

It is well-known that the whole genome triplication (WGT) event in *Brassica* lineage had formed multiple homologs (paralogs) in each *Brassica* species. To analyze the degree of expansion of *14-3-3* genes between *B. rapa* and its nearest model plant *Arabidopsis*, we identify the sub-genomic location of the 21 *BraA.GRF14* genes on *B. rapa* genome (http://brassicadb.org/brad/). The *BraA.GRF14* genes were identified in all the three sub-genomes of *B. rapa*, with both least fractionated (LF) and most-fractionated (MF2) sub-genomes containing eight genes each, and the moderately fractionated (MF1) sub-genome having five genes only (**Table 2**).

Of the 13 *AtGRF14* genes, syntenic homologs for 10 could be identified in the *B. rapa*, with each *Arabidopsis* gene having variable number of *B. rapa* paralogs (**Table 2**; Figure S2). A total of five and three *B. rapa* paralogs was identified for *AtGRF2* (omega) and *AtGRF12* (iota) genes, respectively; whereas two paralogs were identified for five *Arabidopsis 14-3-3* genes in *B. rapa* genome. Only one *B. rapa* homologs was identified for three *AtGRF* genes. The gene-fractionation events in *Brassica* lineage in all possibility have led to the presence of variable number of *14-3-3* gene orthologs in *B. rapa*. Surprisingly, for three *14-3-3* genes of *Arabidopsis*, namely *AtGRF1*, *AtGRF5,* and *AtGRF11* no syntenic homolog could be identified in the *B. rapa* genome. Phylogenetic

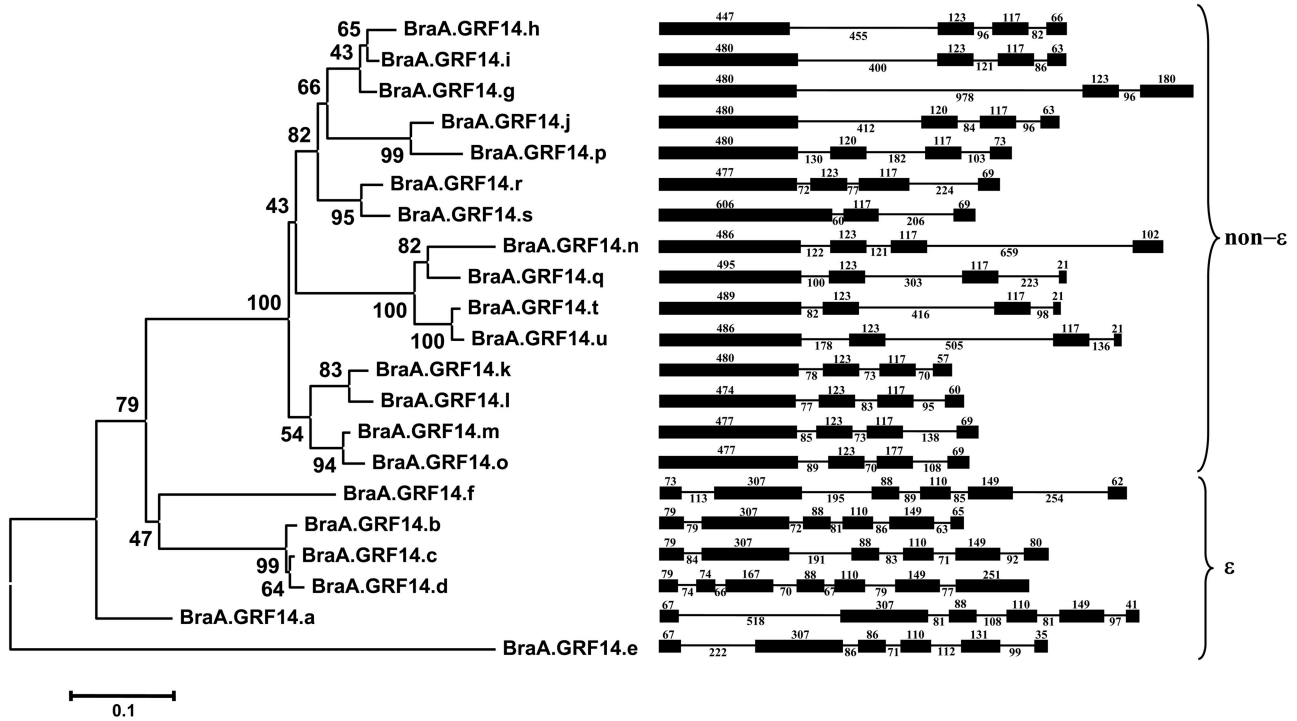


FIGURE 2 | Phylogenetic analysis and gene structures of the deduced 14-3-3 proteins of *B. rapa* (BraA.GRF14). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model conducted in MEGA5 (Tamura et al., 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sizes (in bp) and organization of exons (dark boxes) and introns (lines) of *BraA.GRF14* genes are marked along with.

TABLE 2 | Gene fractionation and divergence analysis of syntenic *14-3-3* genes identified in the three sub-genomes of *B. rapa* (http://brassicadb.org/brad/) with their corresponding *Arabidopsis* orthologs.

14-3-3 protein class	Gene	Protein name	Least fractionated sub-genome (LF)	Moderately fractionated sub-genome (MF1)	Most fractionated sub-genome (MF2)
non-ε	*AtGRF1*	Chi	–	–	–
	AtGRF2	Omega	*BraA.GRF14.g* (0.81; 26.84)	*BraA.GRF14.h* (0.87; 29.12)	*BraA.GRF14.i* (0.57; 19.12)
				BraA.GRF14.j (0.99; 32.84)*	*BraA.GRF14.p* (0.94; 31.40)*
	AtGRF3	Psi	*BraA.GRF14.l* (0.70; 23.33)	*BraA.GRF14.k* (0.73; 24.34)	–
	AtGRF4	Phi	*BraA.GRF14.r* (0.78; 25.84)	–	*BraA.GRF14.s* (0.78; 26.15)
	AtGRF5	Upsilon	–	–	–
	AtGRF6	Lambda	–	*BraA.GRF14.q* (0.82; 27.37)	*BraA.GRF14.n* (0.98; 32.63)
	AtGRF7	Nu	*BraA.GRF14.m* (0.66; 21.93)	–	*BraA.GRF14.o* (0.48; 15.92)
	AtGRF8	Kappa	*BraA.GRF14.u* (0.90; 29.94)	–	*BraA.GRF14.t* (0.85; 28.23)
ε	*AtGRF9*	Mu	–	–	*BraA.GRF14.f* (0.45; 15.04)
	AtGRF10	Epsilon	*BraA.GRF14.a* (0.41; 13.82)	–	–
	AtGRF11	Omicron	–	–	–
	AtGRF12	Iota	*BraA.GRF14.b* (0.35; 11.56)	*BraA.GRF14.c* (0.32; 10.62)	*BraA.GRF14.d* (0.38; 12.71)
	AtGRF13	*Pi*	*BraA.GRF14.e* (0.45; 14.98)	–	–

The Ks (synonymous substitution) and divergence time (mya, million years ago) of a B. rapa gene with its syntenic Arabidopsis ortholog, are given within parenthesis.
**BRAD database assign these as non-syntenic homologs of AtGRF10.*

analysis showed that *AtGRF1/AtGRF4*, *AtGRF5/AtGRF7*, and *AtGRF11/AtGRF12* gene pairs formed individual branches, and could have arisen as the result of *Arabidopsis* specific At-α WGD event, dating around 24–40 mya (Franzke et al., 2011). Divergence analysis of these proteins pairs also showed that at least *AtGRF1/AtGRF4*, and *AtGRF5/AtGRF7* gene pairs might

have duplicated recently in *Arabidopsis* lineage around 20.92 and 24.76 mya, respectively, after the *Arabidopsis–Brassica* split.

We further estimated the divergence of *BraA.GRF14* genes retained in the extant *B. rapa* genome by performing the pairwise comparisons to estimate the synonymous base substitution (*Ks*)-values between the duplicated *B. rapa* and *Arabidopsis* genes. Divergence times were calculated assuming a mutation rate of 1.5×10^{-8} synonymous substitutions per year (Koch et al., 2000). The *Ka/Ks* ratios of *Arabidopsis–B. rapa 14-3-3* orthologs were less than 1, suggesting purifying selection on these duplicated pairs (**Table 2**; Table S3). The *Ks*-values of *BraA.GRF14* genes ranged from 0.32 to 0.99, which indicated that the *BraA.GRF14* genes might have diverged somewhere between 10.62 and 32.84 million years ago (mya). Interestingly, the ε protein orthologs shared between the *Arabidopsis–B. rapa* genomes showed lower range of *Ks*-values (0.32–0.45) compared to the non-ε protein orthologs with higher range of *Ks*-values (0.48–0.99). This class-specific divergence pattern indicated that the non-ε proteins might have diverge recently (10.62–15.04 mya) compared to the ε proteins (>15.92 mya) during the evolution of extant *B. rapa* genome.

Tissue Specific Expression of *BraA.GRF14* Genes

The study on gene expression patterns of all the members of a gene family provides insight about their functional diversification. The multiplicity of *14-3-3* genes, therefore led us to investigate if all the 21 *BraA.GRF14* genes are expressed in *B. rapa*. Gene specific primers (Table S4) were designed for each *BraA.GRF14* genes and real time quantitative PCR (qRT-PCR) was performed using the cDNA samples prepared from different tissue types representing various stages of plant development.

It is interesting to note that the genes belonging to ε (epsilon) group, in general, showed lower levels of transcripts abundance in all the tissue type tested. For example, only two genes namely *BraA.GRF14.a* and *BraA.GRF14.f* showed moderate transcript abundance, when compared to the expression of *BraGAPDH*, whereas four genes namely *BraA.GRF14.b*, *BraA.GRF14.c*, *BraA.GRF14.d*, and *BraA.GRF14.r* showed almost negligible transcript abundance across different tissue types tested. In contrast, among the 15 non-ε group genes, only three genes (*BraA.GRF14.n*, *BraA.GRF14.s,* and *BraA.GRF14.u*) showed lower transcript accumulation across plant development. This observation was confirmed using multiple primer pairs from different regions of the representative genes (Table S4). The quantitatively higher transcript abundance of non-ε group genes compared to the ε group genes obtained for *B. rapa 14-3-3* gene family, is somewhat similar to the expression observed for *AtGRF* genes in *Arabidopsis* (Table S5).

The *BraA.GRF14* genes also exhibited a high degree of tissue specificity (**Figure 3**). Hierarchal clustering based on gene expression profile suggested that *BraA.GRF14* genes, in general, are abundantly expressed in root, stem and seedling stages, compared to leaf and silique stages where moderate level of transcript abundance was detected. In contrast, two of the genes namely, *BraA.GRF14.k* and *BraA.GRF14.l* showed a high level of transcript abundance in the developing leaf stages only, thereby

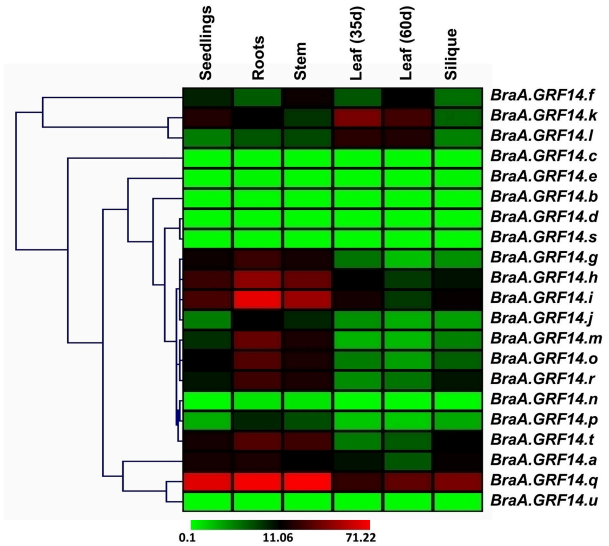


FIGURE 3 | Expression profile of *B. rapa 14-3-3* genes (*BraA.GRF14*) across plant developmental stages. A heat map representing hierarchical clustering of average fold values of *BraA.GRF14* genes expression (w.r.t. *GAPDH*, set at 100) in various developmental tissues (mentioned at the top of each lane) is shown. The color scale representing average signal is shown at the bottom of the heat map.

suggesting that these members may play a specialized role in these tissue types.

We also examined the expression pattern of multiple paralogs of each *GRF* gene, resulted from WGT event in *Brassica* lineage. Hierarchical clustering of three paralogs of *GRF2* namely *BraA.GRF14.g, BraA.GRF14.h,* and *BraA.GRF14.i* showed almost similar expression levels and tissue specificity when tested across different tissue types of *B. rapa* (**Figure 3**). The *B. rapa* paralogs of *GRF3* (*BraA.GRF14.k* and *BraA.GRF14.l*) and *GRF7* (*BraA.GRF14.m* and *BraA.GRF14.o*) also showed similar expression patterns, thereby suggesting that these *B. rapa* paralogs, resulted from polyploidization, had conserved their expression levels and tissue-specificity during the evolution of *Brassica* species. However, in other cases the paralogs of *GRF4* (*BraA.GRF14.r* and *BraA.GRF14.s*), *GRF6* (*BraA.GRF14.q* and *BraA.GRF14.n)*, and *GRF8* (*BraA.GRF14.u* and *BraA.GRF14.t*), showed contrasting variation in their transcript abundance in different tissue type tested, suggesting tissue-specific transcriptional sub-functionalization of *B. rapa 14-3-3* paralogs.

Expression Analysis of *14-3-3* Genes in Response to Abiotic Stresses and Hormone Treatments in *B. rapa*

To investigate the effects of various abiotic stress conditions and hormone treatments on the expression of *BraA.GRF14* genes, *B. rapa* seedlings were treated with different abiotic stress conditions (dehydration, cold, heat, salt) and exogenously supplied hormones (IAA, MeJA, SA, and ABA) for different time points (15 min, 30 min, 3 h, and 6 h). The qRT-PCR expression

analysis was performed to detect the transcriptional regulation of 14 *BraA.GRF14* genes, having detectable transcripts levels, compared to the untreated control seedlings.

The expression of *BraA.GRF14* genes were altered differentially in response to different abiotic stress treatments (**Figure 4A**). The expression of *BraA.GRF14* genes was found to be unaltered or down-regulated, particularly during the early stages (15 and 30 min) of dehydration and heat stress treatments compared to higher induction observed during later time points (3 and 6 h; **Figures 4B,C**). Most of the *BraA.GRF14* genes were found to be highly induced by salt treatment at all tested time points, thereby suggesting their crucial roles during salt stress conditions (**Figure 4A**). In response to cold, *BraA.GRF14.t* was up-regulated within 15 min, whereas transcript accumulation of eight *BraA.GRF14* genes was found to be induced after delayed cold treatment. In response to dehydration, none of the *BraA.GRF14* genes were up-regulated during early time points, whereas three *BraA.GRF14* genes namely, *BraA.GRF14.k*, *BraA.GRF14.l,* and *BraA.GRF14.m* showed up-regulation in their transcripts during later time points (**Figures 4B,C**). Under cold and salt treatments, five *BraA.GRF14* genes namely, *BraA.GRF14.h*, *BraA.GRF14.i,* *BraA.GRF14.k*, *BraA.GRF14.l,* and *BraA.GRF14.m*, were found to be commonly induced during late time points (**Figures 4A,C**). Similarly, the three common genes namely *BraA.GRF14.k*, *BraA.GRF14.l,* and *BraA.GRF14.m* were also found to be up-regulated during the later time points of cold, dehydration and salt stresses. However, using the stringent criterion, expression of only one *BraA.GRF14* gene i.e., *BraA.GRF14.m* was found to be up-regulated during late time points under all the four tested abiotic stress conditions (**Figure 4C**).

The transcriptional regulation of *BraA.GRF14* genes in response to exogenously supplied phytohormones was also analyzed. Our results showed that most of the *BraA.GRF14*

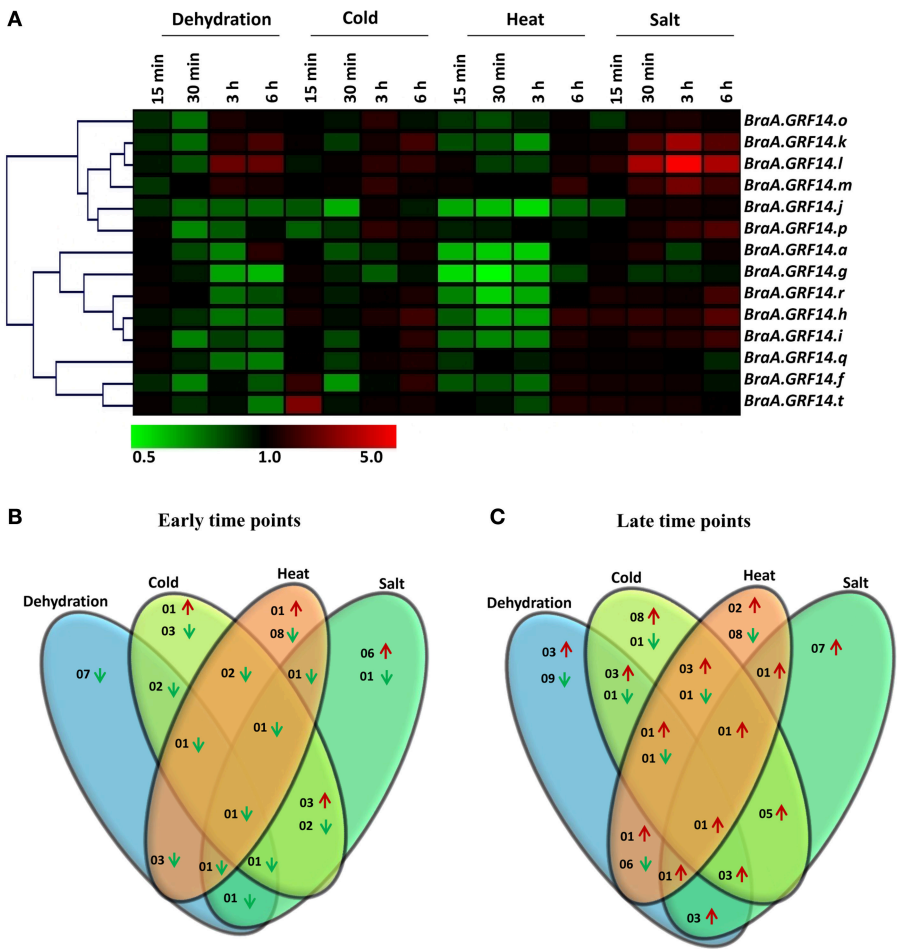


FIGURE 4 | Expression profile of *BraA.GRF14* genes in 6 days old *B. rapa* seedlings in response to abiotic stress conditions. (A) A heat map representing hierarchical clustering of average fold change of *BraA.GRF14* genes expression in response to dehydration, cold (4°C), heat (37°C), and salt (300 mM NaCl) treatments at different time points (15 min, 30 min, 3 h, and 6 h) is shown (mentioned at the top of each lane). The mock treated seedlings of same time interval served as control (set at 1) and expression was normalized with *ACT2* gene. The color scale representing average signal is shown at the bottom of the heat map. The Venn diagrams represent the total number of *BraA.GRF14* genes which were upregulated (red upward arrow) and down-regulated (green downward arrow) during **(B)** early (15 and 30 min), and **(C)** late (3 and 6 h) abiotic stress conditions.

genes were differentially expressed and showed up-regulation in their transcript particularly during later time points (3 and 6 h; **Figures 5A–C**). Of 14 *BraA.GRF14* genes, a total of 13, 5, 13, and 13 genes were found to be up-regulated during later time points on IAA, MeJA, SA, and ABA treatments, respectively, thereby suggesting that *B. rapa* 14-3-3 proteins could mediate various plant responses *via* phytohormone sensing and signaling. In response to IAA treatment, the *BraA.GRF14* genes showed up-regulation of their transcripts at a later time point (6 h); whereas the exogenous treatment of ABA showed a pronounced up-regulation of almost all the *BraA.GRF14* genes except *BraA.GRF14.f* within few minutes, suggesting differential transcriptional response of *B. rapa 14-3-3* genes during abiotic stress. In response to SA treatment, a hormone mimicking the biotic stress condition, the expression of most of the *BraA.GRF14* genes was found to be induced within 15 min and up to 6 h of treatment. On contrary to this, with the application of MeJA on *B. rapa* seedlings, the expression of only two *BraA.GRF14* genes namely, *BraA.GRF14.j* and *BraA.GRF14.k* showed up-regulation in their transcript during early time points (**Figures 5A,B**). In response to all the tested phytohormone treatments, although two genes (*BraA.GRF14.j* and *BraA.GRF14.k*) were found to be up-regulated during early time points; a total of five genes (*BraA.GRF14r; BraA.GRF14.i, BraA.GRF14.j, BraA.GRF14.k, and BraA.GRF14.l*) were up-regulated during later time points. None of the single *BraA.GRF14* gene was found to be commonly down-regulated in response to all the phytohormone treatments, during both early and late time points.

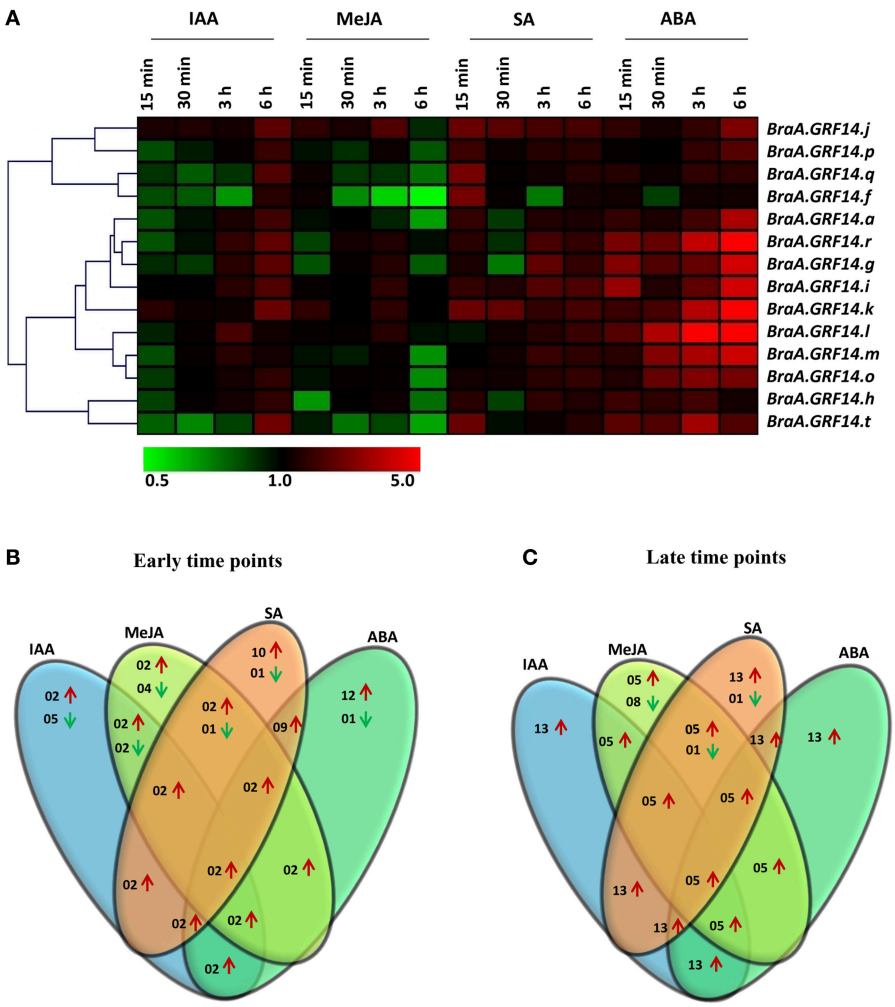


FIGURE 5 | Expression profile of *BraA.GRF14* genes in 6 days old *B. rapa* seedlings in response to phytohormone treatments. (A) A heat map representing hierarchical clustering of average fold change of *BraA.GRF14* genes expression in response to methyl jasmonate (MeJA, 0.2 mM), salicylic acid (SA, 0.2 mM), 1-aminocyclopropane carboxylate (ACC, 0.1 mM), indole-3-acetic acid (IAA, 0.1 mM), and abscisic acid (ABA, 0.1 mM) at different time points (15, 30 min, 3 and 6 h) is shown (mentioned at the top of each lane). The mock treated seedlings of same time interval served as control (set at 1) and expression was normalized with *ACT2* gene. The color scale representing average signal is shown at the bottom of the heat map. The Venn diagrams represent the total number of *BraA.GRF14* genes which were upregulated (red upward arrow) and down-regulated (green downward arrow) during **(B)** early (15 and 30 min), and **(C)** late (3 and 6 h) phytohormone treatments.

Expression Analysis of *14-3-3* Genes during Nutrient Deprivation Conditions in *B. rapa*

Previous findings suggest a connection of *14-3-3* isoforms with plant nutrient metabolism and signaling (Xu and Shi, 2006; Shin et al., 2011). To better understand the transcriptional regulation of *14-3-3* genes toward changes in plant nutrient status, *B. rapa* seedlings were deprived of nitrogen (N), phosphorus (P), and potassium (K) from early (1 and 6 h) to late (24 and 48 h) time intervals, and the expression of *BraA.GRF14* genes was analyzed using qRT-PCR.

The *BraA.GRF14* genes showed differential transcriptional variation in response to the tested nutrient deprivation conditions. It was observed in case of nitrogen deprivation condition (-N), the transcript abundance of most of the *BraA.GRF14* isoforms was found to be down-regulated by less than two-folds at early time points (**Figures 6A–C**). Interestingly, during delayed nitrogen deprivation (24 and 48 h), the expression of almost all *BraA.GRF14* genes showed pronounced up-regulation. In response to phosphorus deprivation condition (-P), the expression of 12 *BraA.GRF14* genes were found to be down-regulated during early time points, whereas after delayed phosphorus deficiency only *BraA.GRF14.g* was found to be up-regulated (**Figures 6B,C**). In contrast, the potassium deficient (-K) *B. rapa* seedlings showed a profound up-regulation of most of the *BraA.GRF14* genes within 6 h of treatment (**Figure 6A**). For example, expression of *BraA.GRF14.g*, *BraA.GRF14.j*, *BraA.GRF14.m*, *BraA.GRF14.o*, and *BraA.GRF14.r* genes were significantly up-regulated during early time points. During the prolonged K deprivation condition, the expression of seven *BraA.GRF14* was also found to be up-regulated, suggesting a profound yet differential transcriptional response of *B. rapa 14-3-3* genes in response to -K condition (**Figures 6B,C**). Overall, in response to all the tested nutrient deprivation conditions, two genes namely, *BraA.GRF14.f* and *BraA.GRF14.h* were commonly down regulated during early time points; whereas *BraA.GRF14.g* was only found to be up-regulated during later time points (**Figures 6B,C**).

Analysis of *cis*-Regulatory Divergence of 5′ Upstream Sequences of *B. rapa 14-3-3* Genes

The differential transcriptional regulation of *BraA.GRF14* genes during plant growth and developmental stages and in response to various elicitor treatments tested in this study could be attributed to sequence divergence and *cis*-regulatory elements present in their 5′ upstream regulatory sequences. Approximately 1.5 kb sequence upstream of transcription start site of *BraA.GRF14* genes were obtained from the BRAD database and analyzed using ClustalW. The 5′ upstream sequence of *BraA.GRF14* genes were highly divergent showing a low level of sequence identity ranging from 18.5 to 31.7% (Table S6), which is quite in agreement with the differential expression pattern obtained among *BraA.GRF14* genes.

To identify various *cis*-acting regulatory elements present in the 5′ upstream sequences, the *BraA.GRF14* genes were further analyzed using the PLACE database (http://www.dna.affrc.go.jp/PLACE/). *In-silico* analysis revealed the presence of various motifs involved in phytohormones, abiotic and biotic stress responses (Table S7). In general, the *BraA.GRF14* genes, which were found to be up-regulated under ABA treatment in current study, showed abundance of ABA responsive elements like ABRELATERD1, DRE1COREZMRAB17, and ABREZMRAB28, thereby indicating the involvement of *B. rapa 14-3-3* gene family members in various ABA mediated cellular responses. Similarly, upstream sequence of *B. rapa 14-3-3* genes like *BraA.GRF14.g*, *BraA.GRF14.h*, *BraA.GRF14.p,* and *BraA.GRF14.m*, which were significantly up-regulated under salt stress, were found to have GT1GMSCAM4 response element known to be involved in salt stress and plant defense. Various pathogen and elicitor response elements like ELRECOREPCRP1, GCCCORE, T/GBOXATPIN2, and TATCCACHVAL21 were also present, which confirm up-regulation of few *BraA.GRF14* genes under MeJA and SA treatments. A significant up-regulation of most of the *BraA.GRF14* genes during late time point of IAA treatment (6 h) can also be correlated with the presence of different types of auxin inducible *cis*-acting elements like ARFAT, CATATGGMSAUR, D3GMAUX28, and NTBBF1ARROLB in their 5′ upstream sequences. Expression of all the *B. rapa 14-3-3* genes were either unaltered or down-regulated during different time point of heat stress, which could be due to the presence of only few heat stress related *cis*-acting elements. Although the upstream sequence of *B. rapa 14-3-3* genes showed the presence of few *cis*-acting elements for nutrient deprivation conditions, but interestingly all the *B. rapa 14-3-3* genes were highly expressed during late time of nutrient deprivation, which could be due to the presence of still unknown regulatory motifs involved in the nutrient sensing in *B. rapa*. Nonetheless, the differential transcriptional alteration observed in the expression of *B. rapa 14-3-3* genes could be nicely attributed to the presence of various *cis*-regulatory elements and their quantitative variability, across multiple *BraA.GRF14* genes.

DISCUSSION

The 14-3-3 proteins are a family of highly conserved regulatory proteins present across phyla, which function by binding to the phosphorylated target proteins (effectors) to play vital roles in many biological processes in plants, including primary metabolism and hormone signaling as well as in response to the abiotic and biotic stresses. In this study, through data mining we identified 21 genes encoding 14-3-3 like proteins from the recently sequenced *B. rapa*, the model *Brassica* genome.

Evolutionary Expansion of *B. rapa 14-3-3* Multigene Family

It is quite expected that the inherent polyploidy in plants has shaped the expansion of *14-3-3* gene family. For example, Li and Dhaubhadel (2011) identified 18 genes encoding 14-3-3 proteins in soybean, an allotetraploid genome (ca. 1115 Mb), having undergone two whole genome duplication events (ca. 14 and 42 mya). Comparative genomics study in cotton, an allotetraploid crop species (ca. 2500 Mb), identified the highest thirty-one *14-3-3* cDNAs encoding 25 unique proteins, resulting from a recent duplication event after the divergence of cotton from its progenitor species (Sun et al., 2011).

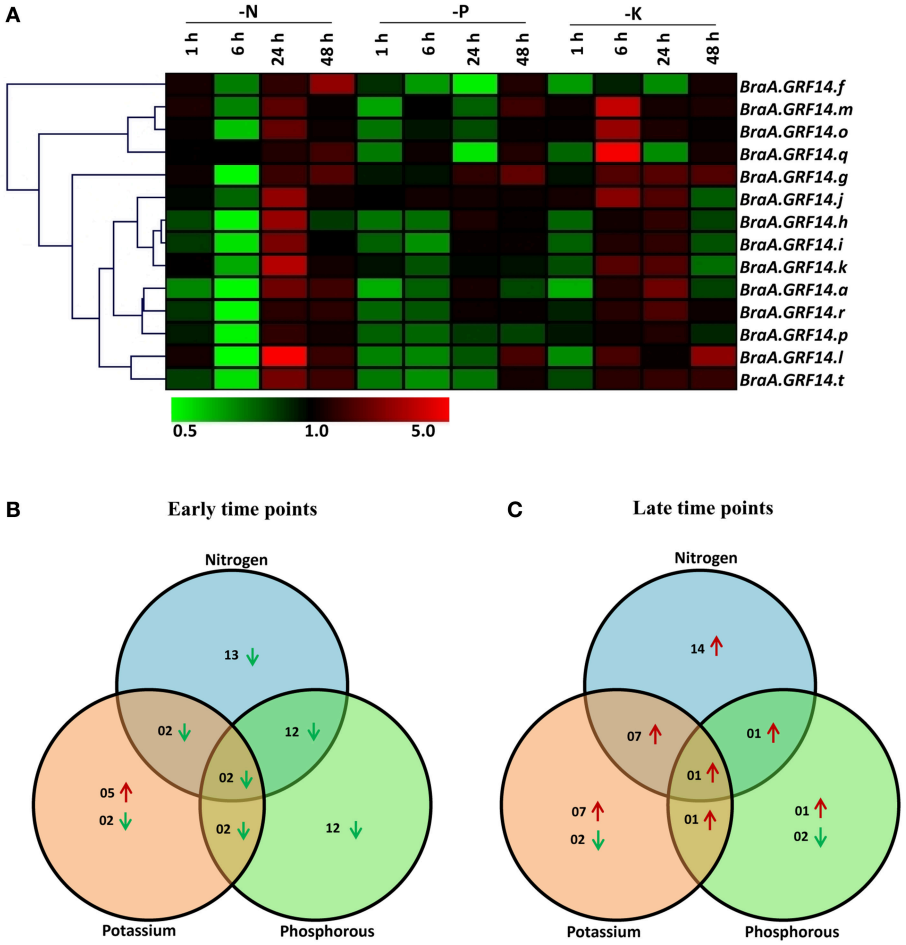


FIGURE 6 | Expression profile of *BraA.GRF14* genes during nutrient deprivation conditions in 5 days old *B. rapa* seedlings. (A) A heat map representing hierarchical clustering of average fold change of *BraA.GRF14* gene expression in response to nutrient deprived conditions at different time points (mentioned at the top of each lane). The 5 days old *B. rapa* seedlings were harvested at different time points (1, 6, 24, and 48 h) after they were exposed to nutrient solution lacking Nitrogen (N), Phosphorus (P), and Potassium (K). The mock treated seedlings of same time interval served as control (set at 1) and expression was normalized with *ACT2* gene. The color scale representing average signal is shown at the bottom of the heat map. The Venn diagrams represent the total number of *BraA.GRF14* genes which were upregulated (red upward arrow) and down-regulated (green downward arrow) during **(B)** early (1 and 6 h), and **(C)** late (24 and 48 h) nutrient deprived conditions.

It is interesting that given the higher size (haploid genome ca. 500 Mb) and ploidy (mesohexaploidy) level of the *B. rapa* genome compared to that of the diploid *A. thaliana* (ca. 120 Mb), the *B. rapa* has most likely only 21 isoforms of 14-3-3 proteins, in comparison with 13 expressed isoforms reported in the closest dicot model (**Figure 1**). Various comparative genomics studies have clearly suggested that the *Arabidopsis* and the cultivable *Brassica* species had split from a common ancestral Brassicaceae around 13–17 million years ago (mya; Lysak et al., 2007; Panjabi et al., 2008; Franzke et al., 2011; Wang et al., 2011; Cheng et al., 2013). The *Brassica* lineage has further undergone whole genome triplication (WGT) event after the *Arabidopsis–Brassica* split, as a result of which the so called diploid *Brassica* species, including *B. rapa*, are paleohexaploid containing three sub-genomes. Sequence level studies in recent year strongly suggested that among these three sub-genomes, biased gene-fractionation (gene-loss) phenomenon has occurred, which resulted in the formation of least fractionized (LF), moderate gene fractionized (MF1), and the most gene fractionized (MF2) sub-genomes in *B. rapa* (Cheng et al., 2013). Thus, all *Brassica* species analyzed to date are supposed to contain multiple copies of orthologous genomic regions of *A. thaliana* (Lysak et al., 2007; Panjabi et al., 2008). We presume that the variable copies (1–5) of each *14-3-3 Arabidopsis* genes identified in *B. rapa* could be the consequence of differential level of gene-fractionation (or gene-loss) phenomenon that occurred after the WGT event in the extant *B. rapa* genome (**Table 2**). As extreme cases, for few *Arabidopsis 14-3-3* genes no syntenic ortholog were observed in *B. rapa*. Nonetheless, polyploidy coupled with genomic shrinkage and rearrangements have caused noteworthy expansion of *14-3-3* gene family members (21 isoforms) in mesohexaploid *B. rapa*, compared to the *A. thaliana*, rice, soybean, and cotton having 13, 8, 18, and 25 members, respectively.

Sequence alignment revealed that the orthologous genes shared between *Arabidopsis–B. rapa* genomes have a high level of amino-acid similarity, suggesting possibility of functional conservation of the 14-3-3 proteins across the two genomes. The *B. rapa* 14-3-3 proteins could be classified into two groups namely epsilon (ε) and non-epsilon (non-ε), with each sub-group showing extreme conservation of the intron-exon organization, and a distinct divergence pattern (*Ks*-values) between the two sub-groups, which in all possibility suggest independent evolution and expansion of the ε and non-ε groups *14-3-3* genes in *B. rapa*. Such class specific divergence pattern is also reported recently for the three types of plant G-gamma (Gγ) subunits (Type-A, -B, and -C) of the heterotrimeric G-proteins (Trusov et al., 2012; Arya et al., 2014; Kumar et al., 2014), an important class of signaling proteins, wherein the divergent Gγ subunits are known to provide functional selectivity to G-proteins in plants. We presume that the quantitative variation as well as the divergent residues present across *14-3-3* isoforms could shape some degree of specificity with regard to their expression profiles and the target protein(s) with which they interact, thereby contributing to the remarkable phenotypic plasticity and environmental adaptability of *B. rapa*.

B. rapa 14-3-3 Genes Exhibit Redundant and Divergent Expression Patterns

Gene duplication although raises the functional redundancy of duplicated genes, it is known to serve as a mechanism to increase the functional diversity. The duplicated genes often evolved *cis*-regulatory divergence in their regulatory regions; as a consequence there exist both immediate and long-term alterations in the expression of genes arising from polyploidy, such as differential expression, transcriptional bias, or gene silencing (Adams, 2007; Chaudhary et al., 2009). As a result, the duplicated genes may undergo diversification of gene function(s) such as neo-, sub- or, non-functionalization.

The *BraA.GRF14* genes are ubiquitously expressed during *B. rapa* growth and developmental stages (**Figure 3**). Since 14-3-3 proteins represent one of the key components of the plant signaling cascade (reviewed in Sehnke et al., 2002; de Boer et al., 2013), the ubiquitous activity of BraA.GRF14 proteins *vis-à-vis* their interaction with their client proteins, are quite necessary for regulating a wide variety of biological processes in *B. rapa*. Interestingly, the class specific transcription abundance of the *B. rapa* non-ε group genes compared to the ε group genes observed in this study, is somewhat similar to that of the *Arabidopsis 14-3-3* genes (Paul et al., 2012). Three of the five ε group genes of *Arabidopsis* have significantly low expression intensities compared to the highly expressed non-ε group genes (Table S4). This evolutionary conservation of expression pattern of the *Arabidopsis–Brassica* orthologs in all possibility suggests that non-ε group *14-3-3* genes might have retained functional dominance in regulating various growth and development processes across Brassicaceae.

Recent studies in polyploid *Brassica* species have functionally demonstrated that members of a multigene family can be expressed at different levels and can respond differentially to polyploidy in various organs of the plant or in response to various environmental stimuli (Higgins et al., 2012; Augustine et al., 2013; Meenu et al., 2015). Our study also suggest that multiple paralogs of few *GRF* genes, resulted from WGT event in *Brassica* lineage, although show almost similar expression patterns and tissue specificity; whereas in other cases the paralogs have contrasting variation in their transcript abundance and pattern across plant developmental stages. The overlapping and divergent expression patterns of *BraA.GRF14* genes suggest that the multiple members have evolved to perform redundant yet tissue-specific functions during plant growth and development in *B. rapa*.

B. rapa 14-3-3 Genes are Differentially Regulated in Response to Various Stimuli

There are evidences that plant 14-3-3 proteins show changes in their gene expression in response to various environmental stresses (Roberts et al., 2002). These stresses often have variable effects on different isoforms in terms of change in expression level and the time period. Our study also demonstrates that the expressed *B. rapa 14-3-3* genes behave differentially in response to the tested abiotic stress conditions, exogenously supplied phytohormones, and nutrient deprivation conditions indicating that different members of this gene family might have undergone differential transcriptional regulation to play specific roles during altered environmental conditions.

In general, most of the *BraA.GRF14* genes were significantly induced during high salt treatment both during early and late time points (**Figure 4**). The rice *OsGF14f* was originally identified as the *14-3-3* transcript that accumulated in the callus and seedling of rice when exposed to high salt or cold temperature (Kidou et al., 1993). Similarly, the up-regulation of *14-3-3* genes has been reported earlier under NaCl treatment in tomato, cotton and rice (Chen et al., 2006; Xu and Shi, 2006; Yao et al., 2007; Sun et al., 2011). In *Arabidopsis*, two *14-3-3* genes namely, *RCI1A* and *RCI1B* were shown to be involved in cold and freezing stress tolerance (Jarillo et al., 1994; Catalá et al., 2014). Under cold stress condition, eight *BraA.GRF14* genes were up-regulated during later time points (**Figure 4C**). Likewise, the expression of few members of *Phaseolus vulgaris 14-3-3* gene family has been recently reported to be up-regulated by cold stress (Li et al., 2015). Over-expression of the *Arabidopsis 14-3-3* "*lambda*" gene into cotton has been shown to impart enhanced tolerance to drought in transgenic lines, as determined by less wilting and visible damage to the leaves (Yan et al., 2004). Interestingly, we found only few *BraA.GRF14* genes were up-regulated under dehydration and heat stress both during early and later time points (**Figure 4**), suggesting that *BraA.GRF14* gene members have evolved to perform condition-specific functions.

It has been widely accepted that *14-3-3* genes act as key components in regulating phytohormone mediated plants responses (reviewed in Denison et al., 2011). Our study shows a profound up-regulation of *BraA.GRF14* genes under MeJA and SA treatments during later time points (**Figure 5**), indicating that *B. rapa 14-3-3* multigene family plays regulatory roles in response to biotic stress. Earlier, it has been shown that *14-3-3* genes are involved in plant defense response in poplar (Lapointe et al.,

2001). Similarly, altered expression pattern of *14-3-3* genes under various biotic stress conditions has been reported in rice (Chen et al., 2006; Yao et al., 2007). The constitutive up-regulation of *BraA.GRF14* genes under ABA treatment, coupled with the presence of high number of ABA responsive *cis*-regulatory elements in their 5′ upstream region, suggests that the *B. rapa* 14-3-3 proteins play a vital role in ABA mediated cellular responses. The 14-3-3 proteins are also known to interact with the ABA signaling pathway in barley, cotton and rice, by interacting with the AREB-like transcription factors (ABF1, ABF2, ABF3, and ABI5), that binds to ABA-responsive elements (Schoonheim et al., 2007; Zhang et al., 2010; Hong et al., 2011). The differential transcriptional regulation of *BraA.GRF14* genes in response to the tested phytohormones suggests their complex cross-talk with phytohormone signaling components to regulate wide range of physiological processes.

In higher plants, 14-3-3 proteins play a significant role in response to nutrient sensing. The phosphorus deprivation (-P) condition, in general, causes a significant down-regulation of *B. rapa 14-3-3* gene expression (**Figure 6**). This is in agreement with that observed for *Arabidopsis GRF* orthologs (Cao et al., 2007), thereby suggesting that P deficiency potentially affects transcript levels of *14-3-3* isoforms and their dependent processes in different cellular compartments. In response to nitrogen deprivation (-N) condition, the expression of *B. rapa 14-3-3* genes was induced at later time points. In contrast, potassium deprivation (- K) treatment seems to up-regulate the expression of most of the *14-3-3* isoforms as also reported for tomato 14-3-3 proteins (Wang et al., 2002; Xu and Shi, 2006). The expression of *BraA.GRF14.t* gene was increased under potassium deprivation (-K) condition during the late time points. Similarly, the expression level of *Arabidopsis 14-3-3* κ increased after "K" deprivation in leaves (Shin et al., 2011). The plant's 14-3-3 proteins are known to interact with regulatory enzymes involved in nutrient sensing, metabolism and transport in plants. Glutamate synthase (GS) and nitrate reductase, key enzymes that regulate N metabolism, were identified as a 14-3-3 interacting proteins (Bachmann et al., 1996; Shin et al., 2011). Various signaling proteins including 14-3-3 interact with phosphorus deficiency response factors including protein kinases, phosphatases (Baldwin et al., 2008; Xu et al., 2012). Regulatory evidences of *14-3-3* genes were also observed of plant K^+ channels, which are known to play a role in potassium homeostasis in the plants (Bunney et al., 2002; Wijngaard et al., 2005). The differential transcriptional response of *14-3-3* genes in response to abiotic stress conditions, phytohormones treatments, and nutrient deprivation conditions indicated that each member of this family participate for condition specific function in multiple signaling pathways.

It is well-known that various post-translational modifications of proteins increase the functional diversity of the proteome by the covalent addition of functional groups or proteins, proteolytic cleavage of regulatory subunits or degradation of the entire protein. Recent studies in *Arabidopsis* and other plants suggested evidences for phosphorylation of several isoforms at conserved as well as divergent serine (S) and tyrosine (Y) residues, providing the potential for phosphorylation to affect 14-3-3 proteins in an isoform-specific manner (reviewed by Paul et al., 2012). Thus, in addition to the transcriptional regulation observed in the current study, the isoform-specific phosphorylation patterns could also provide a key post-translational regulation of the multiple BraGRF14 proteins toward controlling their binding with "client" proteins and the functional diversity in *B. rapa*.

In conclusion, our study provides a comprehensive classification together with a structural and evolutionary analysis of the *BraA.GRF14* gene family in *B. rapa*. A total of 21 isoforms of *BraA.GRF14*s are identified from *B. rapa,* of which 14 isoforms were found to be expressed throughout the plant developmental tissues. The *BraA.GRF14* genes, on the basis of introns and exons, were broadly categorized into two distinct sub-groups namely the ε (epsilon) and non-ε (non-epsilon) groups. It was also observed that multiple orthologs for most of the *Arabidopsis 14-3-3* genes existed in *B. rapa.* Expression analysis of the 14 *BraA.GRF14* genes in response to abiotic stress, hormone stress and nutrient deficiency in the *B. rapa* seedlings suggest that *B. rapa* 14-3-3s are either directly or indirectly involved in the regulation of majority of physiological and metabolic pathways. A systemic and comparative analysis of the target proteins of the *B. rapa* 14-3-3 isoforms, in future, would contribute to fundamental understanding of the conservation and divergence of biological processes controlled by these key signaling proteins.

AUTHOR CONTRIBUTIONS

RC, RA conducted the real time PCR experiments and data analysis. Kanchupati P, RK, Kumar P, GA carried out identification and *in-silico* analysis of candidate genes. RC, RK, and NB compiled the data and writing of manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00012

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Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role as a stress signal molecule

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Methylglyoxal (MG) is a toxic metabolite produced primarily as a byproduct of glycolysis. Being a potent glycating agent, it can readily bind macromolecules like DNA, RNA, or proteins, modulating their expression and activity. In plants, despite the known inhibitory effects of MG on growth and development, still limited information is available about the molecular mechanisms and response pathways elicited upon elevation in MG levels. To gain insight into the molecular basis of MG response, we have investigated changes in global gene expression profiles in rice upon exposure to exogenous MG using GeneChip microarrays. Initially, growth of rice seedlings was monitored in response to increasing MG concentrations which could retard plant growth in a dose-dependent manner. Upon exposure to 10 mM concentration of MG, a total of 1685 probe sets were up- or down-regulated by more than 1.5-fold in shoot tissues within 16 h. These were classified into 10 functional categories. The genes involved in signal transduction such as, protein kinases and transcription factors, were significantly over-represented in the perturbed transcriptome, of which several are known to be involved in abiotic and biotic stress response indicating a cross-talk between MG-responsive and stress-responsive signal transduction pathways. Through *in silico* studies, we could predict 7–8 bp long conserved motif as a possible MG-responsive element (MGRE) in the 1 kb upstream region of genes that were more than 10-fold up- or down-regulated in the analysis. Since several perturbations were found in signaling cascades in response to MG, we hereby suggest that it plays an important role in signal transduction probably acting as a stress signal molecule.

Keywords: methylglyoxal, rice, signal transduction, signaling, transcriptome

Introduction

Methylglyoxal (MG) is a highly reactive cytotoxic α-oxoaldehyde that is formed endogenously via both enzymatic and non enzymatic reactions, mainly as a byproduct of glycolysis from triose phosphates. In addition, MG is also generated as a side-product of amino acid and acetone metabolism (Kalapos, 1999). At higher than physiological levels, MG inhibits cell proliferation

(Ray et al., 1994) which is likely due to its ability to readily react with and modify macromolecules such as DNA, RNA, and proteins, forming advanced glycation end products (AGEs) (Thornalley, 2008). In animals, MG-mediated post-translational protein modifications are believed to be one of the causative factors of aging as well as of a number of diseases, including cancer and diabetes (Ramasamy et al., 2006). MG levels also rise to toxic concentrations in plants on exposure to abiotic and biotic stresses. Its concentration in rice at physiological conditions is ∼2 μmol/g fresh weight and rises two- to six-fold in response to salinity, drought, and cold conditions (Yadav et al., 2005a; Ghosh et al., 2014). To keep its levels under control, several detoxification mechanisms exist in living systems. The glutathione (GSH)-dependent glyoxalase system comprising glyoxalase I and glyoxalase II enzymes, is believed to be the major detoxification route for MG, converting it to D-lactate. Other enzymes involved in MG detoxification include GSH-independent glyoxalase III enzyme, aldo-keto reductase, and dehydrogenases (Kalapos, 1999).

Interestingly, despite its adverse effects on cell growth, *E. coli*, and other microorganisms possess enzymes which generate MG through catalytic reactions. MG synthase is one such enzyme which converts dihydroxyacetone phosphate, a glycolytic intermediate (Ferguson et al., 1998) to MG and inorganic phosphate. When MG is so toxic, its catalytic synthesis raises questions regarding its physiological role in the living systems. It is however proposed that in bacteria, MG production might allow cells to control carbon influx in case an imbalance in metabolism occurs (Booth et al., 2003). Also, MG is known to induce Ca^{2+} transients in *E. coli*; apparently by opening Ca^{2+} channels in a dose-dependent manner (Campbell et al., 2007). This suggests an important role for this metabolite in bacterial-host cell signaling. Likewise in yeast, MG has been shown to act as a signal initiator during oxidative stress (Maeta et al., 2005). On the other hand, the probable role of MG as a signal molecule in plants is yet to be examined. Nonetheless, some indications can be taken from the previous reports which demonstrate that MG is never completely depleted from the system, maintaining a threshold level under normal growth conditions (Singla-Pareek et al., 2003, 2006; Yadav et al., 2005a,b; Hossain et al., 2009), pointing toward its potential role in signal transduction.

In order to understand the physiological and molecular details underlying MG response, in the present study, we have investigated changes in rice transcriptome upon exogenous application of MG. We analyzed physiological changes in MG-exposed rice seedlings followed by a whole genome microarray analysis. Several genes were found to be up- and down-regulated upon MG application. Interestingly, we observed a significant alteration in genes involved in signal transduction. Further, analysis of upstream sequences of MG-responsive genes (up- and down-regulated genes) led to the identification of few conserved motifs which may serve as MG-response element (MGRE). Overall, the present study strongly indicates an important role of MG in signal transduction and provides first information on global expression profile of MG-responsive transcriptome in plants, suggesting diverse biological functions of MG in plants.

Material and Methods

Plant Material and Growth Conditions

Plants of rice cultivar IR64 were grown in controlled conditions in growth chamber at 28 ± 2°C and 16 h photoperiod. The seeds were surface sterilized with 1% Bavistin for 20 min and allowed to germinate in a hydroponics culture system. Germinated seeds were supplied with the modified Yoshida medium (Yoshida et al., 1972).

Morphological Analysis

For seedling growth experiments, 7 day old seedlings of IR64 rice were transferred to Yoshida medium supplemented with different concentrations of MG (5, 7.5, 10, 15, and 20 mM) for 16 h. Untreated seedlings were used as control. After 16 h, seedlings were rescued from MG medium and transferred to Yoshida medium for recovery. Growth of the seedlings was monitored for 4 days after recovery and root and shoot length were measured from control and MG treatment to assess plant growth.

RNA Extraction and GeneChip Microarray Experiment

IR64 rice cultivar was chosen for microarray analysis. Seven day old seedlings were treated with 10 mM MG for 16 h in hydroponics. Shoot tissue was harvested and total RNA was isolated from the control and MG-treated samples as described previously (Kaur et al., 2013). RNA samples were used for Affymetrix rice chip hybridization experiment and data analysis. A total of four hybridizations were carried out as two biological replicates for each condition (control and MG treatment). Single color Affymetrix rice chip was used, where only one dye (Cy3 dye) was used per sample/per hybridization and the arrays were processed further as per the GeneChip microarray (Affymetrix) manufacturer's protocol. Quality control analysis was carried out prior to cDNA synthesis followed by its labeling and hybridization and the expression data of 57,381 probe sets was generated. Overall quality of the prepared hybridized chip was assessed using sample correlation and principle component analysis (PCA) (Table S1 and Figure S1).

Microarray Data Analysis

The Robust Multiarray Average (RMA) algorithm was used for normalization and probe summarization. The resultant normalized expression values were $\log_2$ transformed and utilized for further analysis. In order to reduce false positives which can arise due to poor reproducibility among the replicates, we used statistical stringency of $p \leq 0.05$.

Linear modeling approach was used for the assessment of differential expression. The limma library from R package was used to construct linear models with arbitrary coefficients and contrasts of interest. To obtain differentially expressed genes, moderated t-statistic has been implemented. The multiplicity of testing was performed using Benjamin and Hochberg (BH) correction adjusting for the false discovery rate (FDR). A threshold adjusted *p*-value was set to 0.05, and the fold-change threshold was set to 1.5. Only the transcripts with a minimum

1.5-fold increase or decrease in signal over the control were identified as "MG-responsive" genes. The functional categories were obtained from the annotation of the probes using NetAffx software developed for Affymetrix microarray chips. Further, clustering analysis was performed within conditions using Euclidean distance metric and Centroid linkage rule (Figure S2).

Prediction of MG-responsive Motifs

For the identification of probable MG-responsive *cis*-regulatory elements in the promoter region of genes being 10-fold or more induced or repressed upon MG treatment, we downloaded 1 kb sequences upstream of ATG initiation codon of "MG-responsive" genes from the Rice genome annotation project database (http://rice.plantbiology.msu.edu/index.shtml). The conserved motifs were predicted using the Multiple Expectation for Motif Elicitation (MEME) software (Bailey et al., 2009) and validated *in silico* across the members of glyoxalase family reported in Mustafiz et al. (2011).

Results

Exogenously Supplied Methylglyoxal Inhibits Growth of Rice Seedlings

To study the effect of exogenous application of MG on growth of rice plants, 7 day old seedlings of IR64, a high yielding but salt-sensitive cultivar of rice were subjected to different concentrations (0, 5, 7.5, 10, 15, and 20 mM) of MG for 16 h. After 16 h, seedlings were allowed to recover for 4 days following which root and shoot length was measured to determine the effect of MG application on plant growth. MG exposure adversely affected growth of rice seedlings with profound effect on root elongation (**Figure 1**). Upto 10 mM MG concentration, MG exposure caused reduction in growth of rice seedlings in a dose-dependent manner. But at higher than 10 mM concentration, growth inhibition was observed to be very severe causing more than 50% decrease in both root and shoot length (**Figure 1**). Thus, MG could be clearly seen to affect the growth of rice seedlings with severe retardation in growth of plants exposed to concentrations greater than 10 mM MG. Hence, for subsequent analysis, 10 mM MG concentration was used.

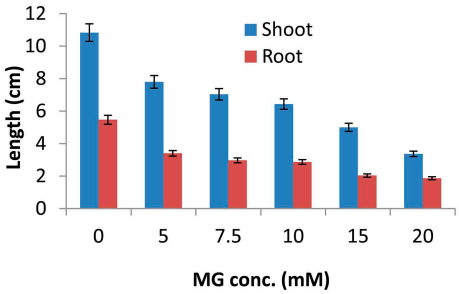


FIGURE 1 | Methylglyoxal inhibits growth of rice seedlings. One week-old IR64 rice seedlings were subjected to various concentrations of MG (0, 5, 7.5, 10, 15, and 20 mM) for 16 h and assessment of growth parameters (shoot and root length) was done after 4 days of recovery.

MG Causes a Global Change in Rice Gene Expression Profile

Gene expression profiles were determined using 1 week-old IR64 rice seedlings exposed to 10 mM MG for 16 h. Detailed analysis of the transcriptome profile obtained through microarray experiment revealed several intriguing facts. The rice transcriptome showed a global alteration upon MG exposure. Using an Affymetrix GeneChip microarray containing 57,381 probe sets, we identified 1685 probe sets which displayed greater than 1.5-fold change in expression upon application of exogenous MG compared to the untreated control. Of these, 719 probe sets were down-regulated and 966 were up-regulated. While main emphasis is usually given to the identification of the up-regulated genes, the down-regulation of gene expression also contributes to the adaptation of plants to a given stimuli. A reasonable assumption is that genes are down-regulated because their product may not be suited to the new physiological conditions caused by the external stimuli (Chandler and Robertson, 1994). A *ProDH* gene is down-regulated during water stress, leading to the accumulation of proline which helps in restoring osmotic balance during stress (Yoshiba et al., 1997). Also, down-regulation of some genes may be required for reprogramming of protein synthesis under stress such as, a 60S ribosomal protein L32 encoding gene, *rpL32,* is down-regulated at the transcriptional level under abiotic stress through the removal of transcription factors from the *cis*-elements in its promoter region (Mukhopadhyay et al., 2011) in turn contributing to reprogramming mechanisms.

The MG-responsive genes obtained from the microarray experiment could be classified into 10 categories: (1) Signal transduction, (2) Transcription and translation, (3) Transposons/retrotransposons, (4) Transport, (5) Stress and defense response, (6) Metabolism, (7) Cell structure and biogenesis, (8) Protein degradation/apoptosis, (9) Growth and development, and (10) Unknown function (**Figure 2**). The genes encoding unknown proteins formed the largest category. About 32% of the total up-regulated and 40% of the total down-regulated genes lied in this category (**Figure 2**). These expressed proteins can open up new avenues for the identification of novel proteins important for MG response. The next largest category belonged to genes involved in metabolism, with 17% of genes being up-regulated and equal percentage being down-regulated upon MG application. This category included genes involved in primary metabolism, i.e., carbohydrate (e.g., hexokinase, triose phosphate isomerase), lipid (e.g., fatty acid hydroxylase, omega-3 fatty acid desaturase), and amino acid (e.g., shikimate kinase, serine hydroxymethyltransferase) metabolism; and also secondary metabolism. An alteration in the metabolic pathways is expected since MG is an inevitable byproduct of glycolysis, a carbohydrate metabolic pathway. A rise in MG concentration possibly acts as a signal for the system to adjust its energy needs through alteration in metabolic pathways as a whole.

Further, genes involved in signal transduction formed another significant category, with 18% of the total genes being up-regulated and 14% being down-regulated indicating a

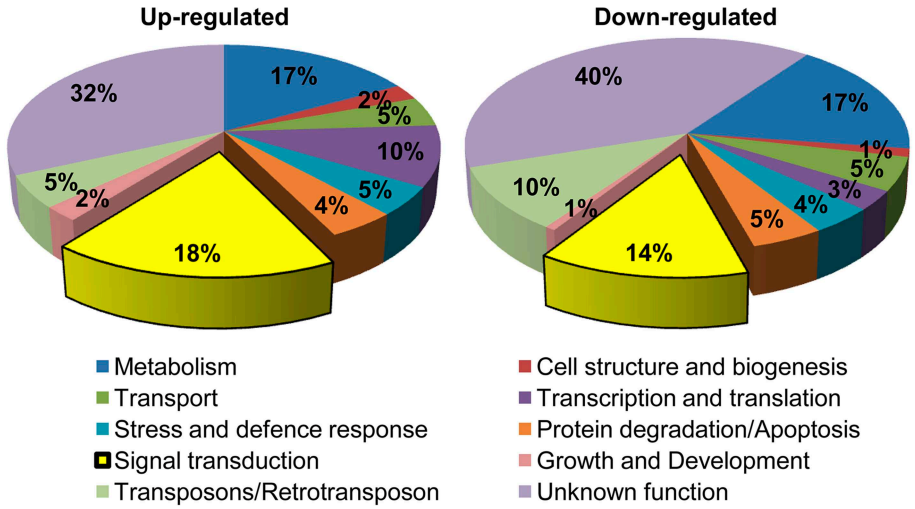


FIGURE 2 | Functional classification of MG-responsive genes. Genes showing more than 1.5-fold change in expression were grouped into 10 functional categories. In total, 966 probe sets were up-regulated and 719 were down-regulated in response to MG.

large-scale alteration in signaling pathways upon exposure to exogenous MG. We also observed a change in the expression of around 13% genes involved in transcription and translation processes, which is probably required for reprogramming cellular machinery during adaptation to MG. Notably, 9% of the MG-responsive transcriptome involved genes required for degradation and apoptosis. This is because MG can irreversibly modify proteins at arginine and lysine residues, with arginine having the highest probability among all amino acids for location at functional sites of proteins often leading to functional impairment, and in turn triggering cellular proteolysis (Rabbani and Thornalley, 2014). In this context, a dicarbonyl proteome has been defined which includes proteins susceptible to modifications by MG such as albumin, hemoglobin, transcription factors, mitochondrial proteins, and other proteins linked to mitochondrial dysfunction, oxidative stress, and apoptosis (Rabbani and Thornalley, 2012). Furthermore, several stress and defense responsive genes were also altered possibly as a part of general response to combat adverse conditions. Overall, we could observe a global alteration in rice transcriptome upon MG application.

Identification of MG-responsive Signal Transduction Pathways

Of all the genes affected by exogenous application of MG, the cluster comprising genes involved in signal transduction formed a significant part (32% of the total up/down regulated genes) (**Figure 2**). The signal transduction pathway is a complex cascade of genes working in a fine-tuned manner to regulate gene transcription patterns in response to extracellular stimuli. The gene regulatory signals are transmitted through the cytoplasm via protein kinases, which are essential for communication between the cytoplasm and nucleus, and act by carrying out signal-dependent phosphorylation/dephosphorylation of transcription factors.

Detailed analysis revealed perturbations in the expression pattern of various transcription factors as well as protein kinases. Also, other signal transduction related genes such as, those involved in hormone signaling, chromatin remodeling, and cell-cell signaling were affected. Importantly, transcription factors constituted a major fraction of MG-responsive signal transduction category, with 49% of the up-regulated and 41% of the down-regulated genes in the signal transduction category being transcription factors (**Figure 3**). Several transcription factors, such as, bZIP, AP2 domain-containing protein, NAM, WRKY, and zinc finger proteins were found to be induced in response to exogenous MG (**Table 1**). We could also identify several protein kinases being perturbed in response to MG, with 25% down-regulated and 12% up-regulated genes in the signal transduction category belonging to the kinase family (**Figure 3**). Various protein kinases such as, MAP kinase (Mitogen-activated protein kinase), calcium/calmodulin-dependent protein kinase (CDPKs), Ser/Thr protein kinase, histidine kinase, and receptor–like kinase showed perturbations in expression. A complete list of protein kinases affected by MG is given in **Table 2**. Notably, about seven genes of the MAPK pathway were found to be regulated in response to MG. The MAPK superfamily is known to play important roles in response to a variety of cellular stresses (Mizoguchi et al., 1997). In animals and yeast, some reports describe the activation of MAPK pathway even in response to MG (Miyata et al., 2002; Maeta et al., 2005), thereby indicating a role of MAPK pathway in MG-responsive signaling cascade.

Another important observation was the effect of elevated MG levels on the expression of stress-responsive components of the signal transduction machinery. Transcript levels of several stress-inducible transcription factors like DREB, MYB, NAC, WRKY, and AP2 domain containing proteins, and protein kinases such as OsRR2 type-A response regulator, were altered upon MG exposure (**Tables 1**, **2**). In case of MYB family transcription

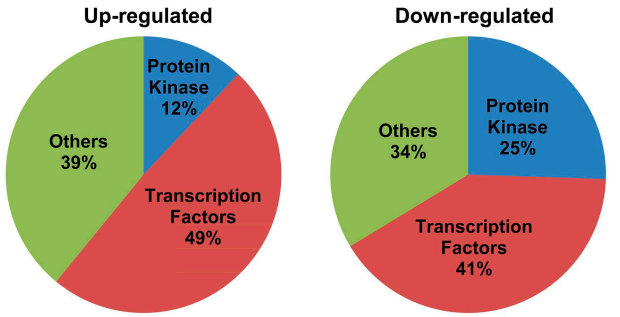


FIGURE 3 | Signal transduction genes majorly constitute transcription factors and protein kinases. The genes included in the signal transduction category have been grouped into three classes: transcription factors, protein kinases, and others. Percentage composition of each category has been indicated.

factors, 8 different genes were up-regulated and three were down-regulated following MG treatment. Likewise, nine NAM genes, eight zinc finger C_3HC_4 type domain-containing protein and seven C_2H_2 zinc finger protein encoding genes were found to be differentially regulated after MG application (**Table 1**). Since MG levels increase under various abiotic and biotic stress conditions, it is now believed to be a common consequence of stress and thus, it is not surprising to observe a change in expression of the above mentioned stress-inducible transcription factors and protein kinases upon MG treatment. Also, it is very likely that MG-responsive transcriptome significantly overlaps with the abiotic stress-responsive transcriptome as we could find various stress-inducible genes being affected by MG.

Identification of Conserved Motifs in the Upstream Region of MG-responsive Genes

Being a potent glycating agent, MG can readily modify DNA, protein, and phospholipid moieties. Importantly, DNA is susceptible to irreversible modifications by MG, with deoxyguanosine being the most reactive nucleotide under physiological conditions (Thornalley, 2008). The ability of MG to directly react with DNA prompted us to identify conserved motifs in the 1 kb upstream region of MG-responsive genes which can potentially act as MG-responsive elements (MGREs). For this purpose, genes whose expression was altered (either up- or down-regulated) more than 10-fold in response to MG were selected for analysis.

We retrieved the upstream sequences of around 160 genes being more than 10-fold altered in expression upon MG treatment including the promoter sequences of the members of the glyoxalase family as well. Screening of the upstream region using Multiple Expectation for Motif Elicitation (MEME) software predicted the presence of 7–8 bp long conserved motifs (**Figure 4**). Most of the predicted motifs were found to be C/G rich regions, like CTXXCTC and GGCGGCGX. Though, as an exception an all A/T rich motif was also observed during the motif search. The results of the *in silico* analysis however, need to be validated through biological experiments. Nevertheless, the data gives some indication toward the presence of MG-responsive elements which regulate gene expression upon perception of increasing MG levels.

Discussion

Despite the recognized toxic nature of MG in living systems, little is known about the molecular mechanisms determining MG response and subsequent growth arrest in plants. In the present study, we have demonstrated the toxic effects of MG on growth of rice seedlings followed by a microarray-based expression profiling study to investigate changes in the gene expression patterns upon exogenous MG application.

MG affected the growth of rice seedlings in a dose-dependent manner similar to that observed in Arabidopsis (Hoque et al., 2012), with severe retardation at concentrations higher than 10 mM MG. Analysis of gene expression patterns revealed large-scale perturbations in the rice transcriptome upon exposure to 10 mM MG concentration affecting diverse biological processes such as, metabolism, transport, signal transduction, transcription, and translation. A similar MG-mediated genome-wide alteration in expression profile has been earlier reported in human endothelial cells as well (Lee et al., 2011). MG-derived modifications can be either through direct interaction with DNA and/or RNA or indirectly by modifying the activity of proteins involved in diverse biological pathways (Thornalley, 2008).

What is more interesting is the strong representation of genes involved in signal transduction pathway. MG, like nitric oxide (NO) and hydrogen peroxide (H_2O_2) perturbs a significant proportion of signaling genes. NO and H_2O_2 are important signaling molecules and in fact, many similarities can be drawn between the two related to transcriptional responses induced by them, with both exhibiting significant cross-talk with general stress-responsive pathways (Neill et al., 2002). Like NO and H_2O_2, MG could induce the transcript levels of various protein kinases and transcription factors, with many known to be involved in abiotic stress response. MG is probably perceived as a stress signal by the system through some sensor proteins, as happens in yeast through Sln1 (Maeta et al., 2005) and subsequently signals the activation of several components of signaling pathways. For MG to really act as a specific signaling molecule, mechanisms must exist to sense elevation in its levels in cells, which is possible through MG-mediated reversible modification at cysteine residues within proteins (Thornalley, 2008). This redox modulation in turn can alter the protein conformation and thereby triggering a cellular response. For example, MG is known to modify Akt1/PKB, a protein kinase, at Cys residue in L6 muscle cells thereby increasing its phosphorylation and hence affecting its activity; subsequently initiating downstream cellular responses (Chang et al., 2011). In yeast, role of MG as a signal initiator is well-defined, activating the Hog1-MAP kinase cascade through Sln1 branch (Maeta et al., 2005). Sln1 is an osmosensor with histidine kinase activity that functions as a sensor of MG. Upon MG stimulation, Hog1 is phosphorylated by Pbs2 and translocated into the nucleus. The nuclear Hog1 recruits transcription factors (such as, Msn2 and Msn4) to the promoter region, thereby activating transcription of the genes under its control, including MG-detoxifying GLYI

TABLE 1 | List of transcription factor genes differentially regulated by methylglyoxal (MG) treatment along with their fold change.

Probe ID	Accession No.	Description	*p*-value	Fold change
UP-REGULATED				
Os.7329.1.S1_s_at	LOC_Os08g42550	AP2 domain containing protein, expressed	0.04	2.58
Os.6367.1.S1_at	LOC_Os01g48060	Auxin response factor, putative, expressed	0.02	2.75
Os.10817.3.S1_x_at	LOC_Os11g32110	Auxin response factor, putative, expressed	0.01	2.28
Os.6367.1.S1_a_at	LOC_Os01g48060	Auxin response factor, putative, expressed	0.05	1.82
OsAffx.26536.1.S1_at	LOC_Os04g49230	B3 DNA binding domain containing protein, expressed	0.05	8.80
OsAffx.15137.1.S1_at	LOC_Os05g47650	B3 DNA binding domain containing protein, expressed	0.03	2.11
OsAffx.25130.1.S1_x_at	LOC_Os03g18210	Basic helix-loop-helix, putative, expressed	0.02	2.54
Os.11132.1.S1_s_at	LOC_Os08g08120	B-box zinc finger family protein, putative, expressed	0.03	4.60
Os.27522.1.S1_x_at	LOC_Os03g58330	BHLH transcription factor, putative, expressed	0.03	1.89
Os.1503.1.S1_at	LOC_Os06g45140	bZIP transcription factor domain containing protein, expressed	0.03	2.35
OsAffx.13332.1.S1_x_at	LOC_Os03g43800	DIRP family protein, putative, expressed	0.02	1.53
Os.54721.1.A1_at	LOC_Os05g51550	DNA binding protein, putative, expressed	0.00	2.50
Os.14023.2.S1_at	LOC_Os06g01370	DNA-binding protein-related, putative, expressed	0.05	2.36
Os.14023.2.S1_x_at	LOC_Os06g01370	DNA-binding protein-related, putative, expressed	0.02	2.30
Os.7574.1.S1_s_at	LOC_Os06g48534	GATA transcription factor 25, putative, expressed	0.02	2.29
OsAffx.24974.1.S1_x_at	LOC_Os03g05160	GATA zinc finger domain containing protein, expressed	0.05	5.97
Os.49626.1.S1_at	LOC_Os03g57260	GRF zinc finger family protein, expressed	0.03	2.37
Os.50790.1.S1_at	LOC_Os03g52320	GRF-interacting factor 1, putative, expressed	0.02	4.14
OsAffx.29777.1.S1_at	LOC_Os09g09460	harpin-induced protein 1 domain containing protein, expressed	0.00	9.11
Os.38400.1.S1_at	LOC_Os09g29830	Helix-loop-helix DNA-binding domain containing protein, expressed	0.05	3.78
OsAffx.6120.1.S1_x_at	LOC_Os08g43070	Helix-loop-helix DNA-binding domain containing protein, expressed	0.05	2.66
Os.14287.1.S1_at	LOC_Os01g64560	Helix-loop-helix DNA-binding protein, putative, expressed	0.03	1.64
OsAffx.23994.1.S1_at	LOC_Os01g70890	Histone-like transcription factor and archaeal histone, putative, expressed	0.03	5.63
Os.49747.1.S1_at	LOC_Os04g55590	Homeobox domain containing protein, expressed	0.02	2.83
Os.35731.1.S1_at	LOC_Os07g38080	Homeodomain-like, putative, expressed	0.04	2.73
Os.13960.2.S1_x_at	LOC_Os01g73560	Leucine rich repeat domain containing protein, putative, expressed	0.04	2.04
Os.19190.3.A1_a_at	LOC_Os03g21230	Leucine-rich repeat family protein, putative, expressed	0.04	1.78
Os.14823.1.S1_at	LOC_Os03g20090	MYB family transcription factor, putative, expressed	0.00	16.99
Os.55708.1.A1_at	LOC_Os02g42870	MYB family transcription factor, putative, expressed	0.03	5.34
Os.55636.1.S1_at	LOC_Os05g48010	MYB family transcription factor, putative, expressed	0.03	3.22
Os.47453.1.A1_s_at	LOC_Os12g39640	MYB family transcription factor, putative, expressed	0.05	2.06
Os.14008.1.S1_at	LOC_Os02g53670	MYB family transcription factor, putative, expressed	0.05	2.03
OsAffx.6404.1.S1_at	LOC_Os09g26170	MYB family transcription factor, putative, expressed	0.03	1.79
Os.52524.1.S1_at	LOC_Os07g48596	Myb transcription factor, putative, expressed	0.04	2.69
OsAffx.2486.1.S1_at	LOC_Os02g02370	Myb-like DNA-binding domain containing protein, putative, expressed	0.04	8.53
OsAffx.24305.1.A1_at	LOC_Os02g18460	NAM, putative, expressed	0.02	2.85
OsAffx.13243.2.S1_at	LOC_Os03g39100	No apical meristem protein, expressed	0.04	2.57
OsAffx.30184.1.S1_at	LOC_Os09g38000	No apical meristem protein, putative, expressed	0.04	2.54
Os.6124.1.S1_a_at	LOC_Os10g21560	No apical meristem protein, putative, expressed	0.01	2.06
OsAffx.23563.1.S1_s_at	LOC_Os01g38780	Nucleic acid binding protein, putative, expressed	0.04	3.44
Os.28015.1.S1_at	LOC_Os01g52680	OsMADS32—MADS-box family gene with MIKCc type-box, expressed	0.02	1.82
Os.4175.1.S1_at	LOC_Os01g69850	OsMADS65—MADS-box family gene with MIKC* type-box, expressed	0.02	1.67
Os.53339.1.S1_a_at	LOC_Os07g49380	PWWP domain containing protein, expressed	0.01	2.62
OsAffx.15318.1.S1_at	LOC_Os06g08820	RING-H2 finger protein, putative, expressed	0.03	2.90
OsAffx.17313.1.S1_at	LOC_Os08g33530	TCP family transcription factor, putative, expressed	0.04	2.17
Os.57558.1.S1_at	LOC_Os04g51000	Transcription factor FL, putative, expressed	0.03	2.23
OsAffx.1957.1.S1_at	LOC_Os01g07880	Transcription factor HY5, putative, expressed	0.04	2.83
Os.6016.1.A1_s_at	LOC_Os08g37810	Transcription factor like protein, putative, expressed	0.04	2.38
OsAffx.11931.1.S1_at	LOC_Os02g07800	Transcription factor, putative, expressed	0.03	5.31
OsAffx.17359.1.S1_at	LOC_Os08g36450	Transcription regulator, putative, expressed	0.03	2.16
Os.50527.1.S1_a_at	LOC_Os03g50110	Transcription regulator, putative, expressed	0.00	2.02

(Continued)

TABLE 1 | Continued

Probe ID	Accession No.	Description	*p*-value	Fold change
Os.26946.1.S1_at	LOC_Os08g09900	WRKY118, expressed	0.03	3.93
Os.19013.1.S1_at	LOC_Os12g40570	WRKY94, expressed	0.02	2.13
Os.49773.1.S1_at	LOC_Os03g11600	YABBY domain containing protein, putative, expressed	0.01	2.29
Os.11823.1.S1_at	LOC_Os06g41590	YIF1B, putative, expressed	0.03	5.30
Os.15553.1.S1_at	LOC_Os02g06584	Zinc finger C-x8-C-x5-C-x3-H type family protein, expressed	0.04	2.81
OsAffx.13605.1.S1_at	LOC_Os04g01160	Zinc finger family protein, putative, expressed	0.02	4.61
Os.27108.1.S1_at	LOC_Os08g36774	Zinc finger family protein, putative, expressed	0.02	3.40
OsAffx.24591.1.S1_at	LOC_Os02g36000	Zinc finger protein, putative, expressed	0.03	1.90
Os.33152.1.S1_x_at	LOC_Os11g07450	Zinc finger, C3HC4 type domain containing protein, expressed	0.01	2.97
Os.37432.1.S1_at	LOC_Os03g08920	ZINC finger, C3HC4 type domain containing protein, expressed	0.04	2.88
Os.33683.1.S1_at	LOC_Os12g43930	Zinc finger, C3HC4 type domain containing protein, expressed	0.02	2.62
Os.22596.1.S1_s_at	LOC_Os05g28730	Zinc finger, C3HC4 type domain containing protein, expressed	0.02	2.06
OsAffx.26842.1.S1_at	LOC_Os05g11860	Zinc finger, C3HC4 type domain containing protein, expressed	0.02	2.02
Os.7498.1.S1_at	LOC_Os08g42640	Zinc finger, C3HC4 type domain containing protein, expressed	0.05	2.01
OsAffx.17384.1.S1_at	LOC_Os08g37760	Zinc finger, C3HC4 type domain containing protein, expressed	0.05	1.88
Os.212.1.S1_at	LOC_Os01g16950	Zinc finger, C3HC4 type domain containing protein, expressed	0.02	1.70
Os.9372.1.S1_at	LOC_Os07g05610	Zinc ion binding protein, putative, expressed	0.04	1.71
Os.24550.1.S1_at	LOC_Os03g57160	Zinc ion binding protein, putative, expressed	0.05	1.56
OsAffx.11292.1.S1_at	LOC_Os01g32920	ZOS1-08—C2H2 zinc finger protein, expressed	0.05	7.91
OsAffx.23943.1.S1_s_at	LOC_Os01g66570	ZOS1-19—C2H2 zinc finger protein, expressed	0.02	1.65
OsAffx.3743.1.S1_s_at	LOC_Os04g08060	ZOS4-03—C2H2 zinc finger protein, expressed	0.03	1.93
Os.50189.2.S1_at	LOC_Os06g46910	ZOS6-07—C2H2 zinc finger protein, expressed	0.02	7.30
DOWN-REGULATED				
OsAffx.11389.1.S1_at	LOC_Os01g39430	Anthocyanin regulatory protein, putative	0.04	3.38
Os.38983.1.S1_at	LOC_Os03g05590	AP2 domain containing protein, expressed	0.05	10.15
Os.30907.1.S1_at	LOC_Os08g45110	AP2 domain containing protein, expressed	0.03	4.25
OsAffx.5131.1.S1_at	LOC_Os06g46995	Armadillo/beta-catenin repeat family protein, putative, expressed	0.01	13.10
Os.5537.1.S1_s_at	LOC_Os09g34880	Basic region leucine zipper domain containing protein, expressed	0.05	2.83
OsAffx.17824.1.S1_at	LOC_Os09g21410	BED zinc finger family protein, expressed	0.03	5.26
OsAffx.27283.2.S1_s_at	LOC_Os01g57580	bHelix-loop-helix transcription factor, putative, expressed	0.03	1.70
OsAffx.11963.1.S1_at	LOC_Os02g09830	bZIP transcription factor domain containing protein, expressed	0.04	7.71
OsAffx.20062.1.S1_at	LOC_Os12g40920	bZIP transcription factor domain containing protein, expressed	0.01	3.36
Os.16422.1.S1_s_at	LOC_Os08g15050	CCT/B-box zinc finger protein, putative, expressed	0.00	3.01
OsAffx.6090.1.S1_at	LOC_Os08g40030	CUP-SHAPED COTYLEDON3, putative, expressed	0.02	5.77
OsAffx.27442.1.S1_at	LOC_Os06g03670	Dehydration-responsive element-binding protein, putative, expressed	0.02	4.16
OsAffx.23629.2.S1_at	LOC_Os01g42050	DNL zinc finger domain containing protein, putative, expressed	0.01	4.78
Os.16203.1.S1_at	LOC_Os01g74590	MYB family transcription factor, putative, expressed	0.00	6.69
OsAffx.14536.1.S1_at	LOC_Os05g04210	MYB family transcription factor, putative, expressed	0.03	1.72
Os.623.2.S1_x_at	LOC_Os01g09640	Myb transcription factor, putative, expressed	0.04	2.10
OsAffx.7264.1.S1_at	LOC_Os11g31360	No apical meristem protein, putative, expressed	0.00	6.10
Os.27469.1.S1_a_at	LOC_Os01g09550	No apical meristem protein, putative, expressed	0.00	5.31
Os.18595.1.A1_at	LOC_Os07g48450	No apical meristem protein, putative, expressed	0.04	2.78
Os.4385.1.S1_at	LOC_Os11g08210	No apical meristem protein, putative, expressed	0.04	2.30
Os.36651.1.S1_at	LOC_Os04g43560	No apical meristem protein, putative, expressed	0.01	1.55
Os.48040.1.A1_at	LOC_Os05g11414	OsMADS58—MADS-box family gene with MIKCc type-box, expressed	0.01	1.70
Os.4153.1.S1_a_at	LOC_Os08g41950	OsMADS7—MADS-box family gene with MIKCc type-box, expressed	0.02	1.88
Os.52097.1.S1_at	LOC_Os04g44440	TCP family transcription factor, putative, expressed	0.04	22.10
Os.15421.1.S1_at	LOC_Os02g55000	Zinc finger CCCH-type with G patch domain-containing protein, putative, expressed	0.01	7.44
Os.55651.1.S1_at	LOC_Os06g11450	Zinc finger, C3HC4 type domain containing protein, expressed	0.02	2.68
Os.19944.1.S1_at	LOC_Os12g43560	Zinc finger, putative, expressed	0.04	1.78
Os.12918.2.S1_x_at	LOC_Os04g58320	Zinc finger, RING-type, putative, expressed	0.03	1.79
Os.49588.1.S1_at	LOC_Os03g41390	ZOS3-15—C2H2 zinc finger protein, expressed	0.04	1.81
Os.15874.1.A1_at	LOC_Os03g60570	ZOS3-22—C2H2 zinc finger protein, expressed	0.05	18.64
Os.56344.1.S1_at	LOC_Os06g20020	ZOS6-03—C2H2 zinc finger protein, expressed	0.04	2.79

TABLE 2 | List of protein kinase genes differentially regulated by methylglyoxal (MG) treatment along with their fold change.

Probe ID	Accession No.	Description	*p*-value	Fold change
UP-REGULATED				
Os.5636.1.S1_at	LOC_Os05g14750	AGC_PVPK_like_kin82y.12—ACG kinases include homologs to PKA, PKG, and PKC, expressed	0.03	8.97
OsAffx.9832.1.S1_x_at	LOC_Os08g27780	OsWAK77—OsWAK receptor-like cytoplasmic kinase OsWAK-RLCK, expressed	0.04	5.70
Os.338.1.S2_a_at	LOC_Os03g17980	CAMK_KIN1/SNF1/Nim1_like_AMPKh.2—CAMK includes calcium/calmodulin depedent protein kinases, expressed	0.00	3.43
Os.338.3.S1_x_at	LOC_Os03g17980	CAMK_KIN1/SNF1/Nim1_like_AMPKh.2—CAMK includes calcium/calmodulin depedent protein kinases, expressed	0.01	2.86
Os.52734.1.S1_at	LOC_Os11g17080	OsMPK15—Putative MAPK based on amino acid sequence homology, expressed	0.05	2.72
OsAffx.30096.1.S1_x_at	LOC_Os09g30190	Receptor-like protein kinase 2 precursor, putative, expressed	0.03	2.43
Os.338.2.S1_x_at	LOC_Os08g37800	CAMK_KIN1/SNF1/Nim1_like_AMPKh.4—CAMK includes calcium/calmodulin depedent protein kinases, expressed	0.01	2.29
Os.436.1.S1_at	LOC_Os01g17250	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative, expressed	0.04	2.25
Os.17915.1.S1_at	LOC_Os06g17290	Phosphatidylinositol 3- and 4-kinase family protein, putative, expressed	0.01	2.24
Os.17824.1.S1_at	LOC_Os02g42620	Protein kinase, putative, expressed	0.05	2.22
Os.26917.1.A1_at	LOC_Os02g47220	CPuORF20—conserved peptide uORF-containing transcript, expressed	0.01	2.18
Os.3356.1.S1_at	LOC_Os10g25090	STRUBBELIG-RECEPTOR FAMILY 6 precursor, putative, expressed	0.05	2.18
Os.8740.1.S1_at	LOC_Os02g14530	Protein kinase domain containing protein, expressed	0.05	2.05
Os.56163.1.S1_at	LOC_Os04g04000	ECT protein, putative, expressed	0.01	2.03
Os.49462.1.S1_at	LOC_Os02g44642	STE_MEKK_ste11_MAP3K.10—STE kinases include homologs to sterile 7, sterile 11, and sterile 20 from yeast, expressed	0.00	1.89
Os.52830.1.S1_at	LOC_Os02g34430	Serine/threonine-protein kinase, putative, expressed	0.04	1.86
Os.11626.1.S1_at	LOC_Os04g56530	STE_MEKK_ste11_MAP3K.1—STE kinases include homologs to sterile 7, sterile 11, and sterile 20 from yeast, expressed	0.01	1.80
Os.19371.1.S1_at	LOC_Os12g29580	AGC_PVPK_like_kin82y.19—ACG kinases include homologs to PKA, PKG, and PKC, expressed	0.01	1.72
Os.23942.1.A1_at	LOC_Os02g35180	OsRR2 type-A response regulator, expressed	0.02	1.65
Os.20399.1.S1_s_at	LOC_Os01g62080	Serine/threonine-protein kinase AFC1, putative, expressed	0.03	1.63
Os.12648.1.S1_at	LOC_Os09g08420	AGC_PVPK_like_kin82y.15—ACG kinases include homologs to PKA, PKG, and PKC, expressed	0.01	1.63
DOWN-REGULATED				
OsAffx.15056.1.S1_s_at	LOC_Os05g41270	CAMK_CAMK_like.4—CAMK includes calcium/calmodulin depedent protein kinases, expressed	0.05	5.97
Os.10274.4.S1_at	LOC_Os12g03810	CAMK_KIN1/SNF1/Nim1_like.37—CAMK includes calcium/calmodulin depedent protein kinases, expressed	0.02	3.87
Os.55776.1.S1_x_at	LOC_Os01g06876	Cf-2, putative, expressed	0.02	2.55
Os.14999.3.S1_x_at	LOC_Os01g47530	CGMC_MAPKCMGC_2.6—CGMC includes CDA, MAPK, GSK3, and CLKC kinases, expressed	0.02	2.12
OsAffx.10273.1.S1_at	LOC_Os07g03970	Lectin-like receptor kinase 7, putative, expressed	0.01	23.36
OsAffx.29778.1.S1_at	LOC_Os09g09500	Lectin-like receptor kinase, putative, expressed	0.01	6.57
Os.12660.1.S1_at	LOC_Os07g38800	Lectin-like receptor kinase, putative, expressed	0.02	4.74
Os.50228.1.S1_at	LOC_Os07g15940	Legume lectins beta domain containing protein, expressed	0.05	11.24
Os.29772.1.S1_at	LOC_Os03g40250	Leucine Rich Repeat family protein, expressed	0.03	2.72
OsAffx.31317.1.S1_at	LOC_Os11g35960	Leucine rich repeat N-terminal domain containing protein, putative, expressed	0.05	3.69
OsAffx.27458.1.S1_at	LOC_Os06g04810	Leucine rich repeat protein, putative, expressed	0.03	1.51
Os.50342.1.S1_at	LOC_Os04g52780	Leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed	0.02	1.82
OsAffx.20258.1.S1_at	LOC_Os10g33080	Leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed	0.03	2.55
OsAffx.18277.1.S1_at	LOC_Os10g17910	OsWAK114—OsWAK receptor-like cytoplasmic kinase OsWAK-RLCK, expressed	0.00	7.20
Os.19575.1.A1_at	LOC_Os04g30010	OsWAK45—OsWAK receptor-like protein kinase, expressed	0.02	4.60
Os.14874.2.S1_at	LOC_Os01g47430	Protein of unknown function DUF1296 domain containing protein, expressed	0.01	5.02
OsAffx.7310.1.S1_at	LOC_Os11g36090	Receptor kinase, putative, expressed	0.01	1.75

(Continued)

TABLE 2 | Continued

Probe ID	Accession No.	Description	*p*-value	Fold change
Os.46331.1.S1_s_at	LOC_Os01g66040	Receptor kinase, putative, expressed	0.02	3.15
Os.49804.1.S1_at	LOC_Os11g47310	Receptor kinase-like protein, identical, putative, expressed	0.03	1.88
Os.27053.2.S1_s_at	LOC_Os11g07200	Receptor protein kinase CLAVATA1 precursor, putative, expressed	0.04	1.95
OsAffx.12352.1.S1_at	LOC_Os02g34770	Receptor-like protein kinase 2 precursor, putative	0.01	2.49
Os.11775.1.S1_a_at	LOC_Os01g53920	Receptor-like protein kinase 5 precursor, putative, expressed	0.01	7.95
Os.27084.1.A2_at	LOC_Os04g52640	SHR5-receptor-like kinase, putative, expressed	0.01	2.10
Os.49145.1.S1_at	LOC_Os03g27990	STRUBBELIG-RECEPTOR FAMILY 7 precursor, putative, expressed	0.00	2.91
Os.26816.1.A1_s_at	LOC_Os07g35410	TKL_IRAK_DUF26-lc.18—DUF26 kinases have homology to DUF26 containing loci, expressed	0.01	2.20

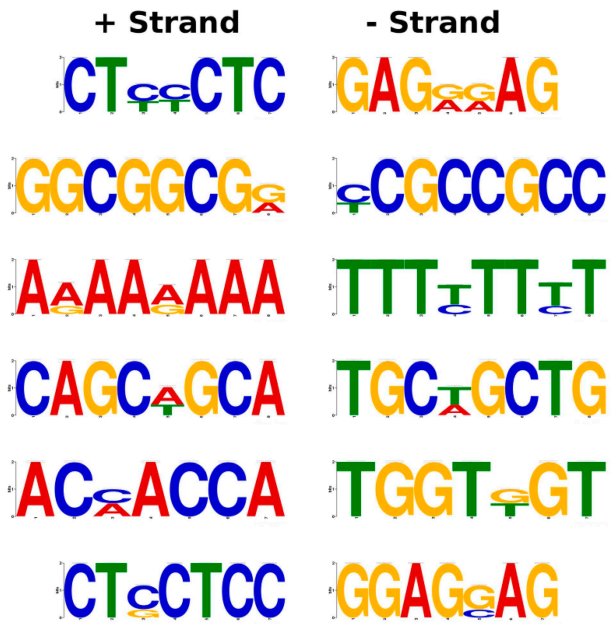


FIGURE 4 | Prediction of putative MG-responsive elements (MGREs) as conserved motifs in the promoter region of MG-responsive genes. The genes with more than 10-fold alteration in expression upon MG treatment were selected for analysis. The 1 kb upstream region of MG-responsive genes was used for the identification of motifs using MEME software.

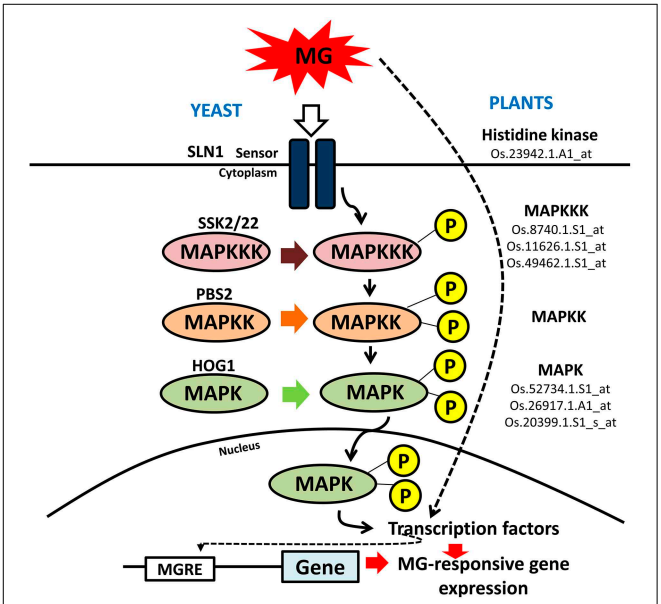


FIGURE 5 | Schematic representation of MAPK signaling cascade in yeast and plants. Mitogen-activated protein kinase (MAPK) signaling cascade is triggered in yeast in response to MG. A similar pathway is proposed to operate in rice as well in response to MG. The rice counterparts found to be induced by MG application have been shown along with their probe IDs. MG can modify expression of genes either by inducing the signal cascade through sensor proteins or it can directly affect the activity of transcription factor that goes and binds the promoter region at MG-responsive elements (MGRE) and regulate gene expression.

(Maeta et al., 2005). In addition, MG also stimulates Yap1, a bZIP transcription factor that is predominantly distributed in the cytoplasm under normal conditions in yeast but upon MG stimulation translocates to the nucleus and functions in regulating gene expression (Maeta et al., 2004). In plants, little to nothing is known regarding the role of MG in signaling. Based on the existing reports in animals and yeast (reviewed by Kaur et al., 2014a), we suggest that even in plants similar MAPK pathway might be involved in MG signaling (**Figure 5**). In agreement, we observed an induction in the transcript levels of a putative histidine kinase gene and six MAPK genes (mentioned in **Figure 5**). In plants, MAPKs are usually known to be activated in response to various environmental signals such as drought, cold, osmotic stress, and pathogen challenge (Mizoguchi et al., 1997). In fact, MG has also been shown to induce the p38/MAPK pathway in animals, regulating various cellular processes (reviewed by Kaur et al., 2014a). The MAPK cascade is now considered as a central pathway mediating tolerance to various stresses (Smékalová et al., 2014), and may be to MG stress also. Further, even in response to NO and H_2O_2, MAPK cascade is believed to be the focal point of signal transmission and also convergence with various stress-inducible signaling pathways (Neill et al., 2002).

Since MG and abiotic stresses are inextricably linked (Yadav et al., 2005a; Kaur et al., 2014b), we could detect various stress-inducible transcription factors to be induced upon MG application. For instance, several NAC genes were found to

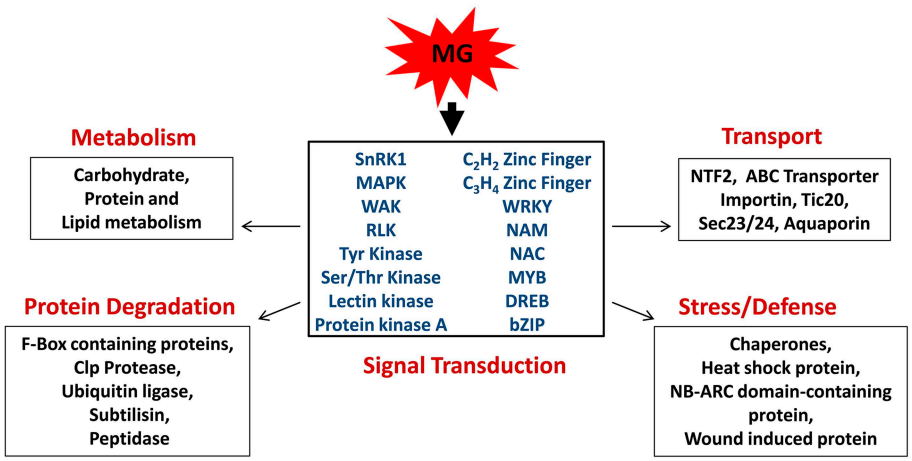


FIGURE 6 | Model depicting the role of methylglyoxal as a signaling molecule in rice. MG induces the expression of signal transduction genes, mainly protein kinases, and transcription factors, which subsequently regulate various cellular processes, such as metabolism, transport, stress, and defense response, and protein degradation.

be up-regulated which can bind to the promoter region of stress-responsive genes including glyoxalase and promote their transcription in order to lower MG levels and associated stress (Fujita et al., 2004). The WRKY transcription factors also have well-established roles in abiotic and biotic stress response and even participate in several developmental and physiological processes, leaf senescence, regulation of biosynthetic pathways and hormone signaling (Chen et al., 2012). Similarly, C_2H_2-type zinc finger protein genes are known to activate stress-related genes and enhance tolerance to salt, dehydration, and/or cold stresses (Sun et al., 2010; Kiełbowicz-Matuk, 2012). A cell wall-associated receptor-like kinase (OsWAK77) was also up-regulated. WAK genes are involved in various functions in plants, including pathogen resistance (He et al., 1998), abiotic stress response (Gao and Xue, 2012), heavy-metal tolerance (Sivaguru et al., 2003), and plant development (Lally et al., 2001). Additionally, MG was also found to induce the expression of genes involved in metabolic signaling, such as, a SnRK1-type of kinase, which encodes an energy sensor protein that regulates gene expression in response to energy depletion in plants (Cho et al., 2012). Furthermore, our motif search predicted 7–8 bp long conserved elements in the promoter region of genes showing more than 10-fold change in expression in the microarray analysis that may serve as possible MG-responsive element (MGRE).

Taken together, through this study we provide first report on the effect of MG on global gene expression profile of rice. We propose that plants perceive MG as a stress signal and trigger a response via induction of several protein kinases and transcription factors which then affect the expression of downstream targets involved in various biological pathways, thereby causing a global change in plant transcriptome (**Figure 6**). In addition, other mode of action may also exist, involving direct modification of effector proteins by MG, thereby regulating their expression and activity.

Author Contributions

SS conceived the idea and designed the experiments. CK, HK, and AM performed the analysis and wrote the manuscript. SLS, AP, and SKS edited the manuscript. All the authors approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00682

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Tissue specific and abiotic stress regulated transcription of histidine kinases in plants is also influenced by diurnal rhythm

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Two-component system (TCS) is one of the key signal sensing machinery which enables species to sense environmental stimuli. It essentially comprises of three major components, sensory histidine kinase proteins (HKs), histidine phosphotransfer proteins (Hpts), and response regulator proteins (RRs). The members of the TCS family have already been identified in *Arabidopsis* and rice but the knowledge about their functional indulgence during various abiotic stress conditions remains meager. Current study is an attempt to carry out comprehensive analysis of the expression of TCS members in response to various abiotic stress conditions and in various plant tissues in *Arabidopsis* and rice using MPSS and publicly available microarray data. The analysis suggests that despite having almost similar number of genes, rice expresses higher number of TCS members during various abiotic stress conditions than *Arabidopsis*. We found that the TCS machinery is regulated by not only various abiotic stresses, but also by the tissue specificity. Analysis of expression of some representative members of TCS gene family showed their regulation by the diurnal cycle in rice seedlings, thus bringing-in another level of their transcriptional control. Thus, we report a highly complex and tight regulatory network of TCS members, as influenced by the tissue, abiotic stress signal, and diurnal rhythm. The insights on the comparative expression analysis presented in this study may provide crucial leads toward dissection of diverse role(s) of the various TCS family members in *Arabidopsis* and rice.

Keywords: abiotic stress, *Arabidopsis*, histidine kinase, histidine phosphotransfer protein, response regulator, rice, two-component system

Introduction

Growth potential of the plants are severely affected under various abiotic stress conditions especially salinity and drought. Since plants are rooted to a place, they have to make adjustments in their genetic and metabolic machinery in order to survive under abiotic stress conditions. Under stress conditions, plants use specific signaling machineries to relay the stress signals in order to "switch on" the adaptive responses which assist plants in developing tolerance toward abiotic stress. Some

of the signaling machineries are conserved across various genera. One such signaling machinery is the two-component system (TCS) or His-to-Asp phosphorelay which is well-known and conserved machinery for signal transduction in the cells (Mochida et al., 2010; Nongpiur et al., 2012). Apart from stress signaling, TCS has been one of the key regulators for many biological processes such as cell division, cell growth and proliferation, and responses to growth regulators in both prokaryotic and eukaryotic cells (Stock et al., 2000; Hwang et al., 2002; Mizuno, 2005; Pareek et al., 2006; Schaller et al., 2008; Pils and Heyl, 2009).

The TCS signaling system essentially comprise of sensory histidine kinases (HKs) and their cognate response regulators (RRs) substrates, which have been reported in almost all the sequenced bacterial genomes except for mycoplasma (Mascher et al., 2006; Laub and Goulian, 2007). In a simple prototypical TCS regulatory system, HK protein senses the environmental signals, autophosphorylates a histidine residue (H), and signals to its corresponding cytosolic RR protein by transferring the phosphate to an aspartate residue (D) (**Figure 1**). Phosphorylated RR further mediates the downstream signaling (Urao et al., 2000). Some of the bacteria, yeast, slime molds and plants possess a more complex form of TCS or His-to-Asp phosphorelay system. This is due to the presence of "hybrid" type of kinases which possess both His-kinase (HK) domain and a receiver domain (RD) in one protein. Another protein namely, His-containing phosphotransfer (Hpt) protein is involved which acts as a signaling module connecting to the final RRs (Schaller et al., 2008). Hpts allows species to have multistep phosphorelays which has a major advantage of having multiple regulatory checkpoints for signal crosstalk or negative regulation by specific phosphatases (Urao et al., 2000).

Structurally, Histidine kinase (HK) protein is a dimeric protein and is regulated by receptor-ligand interactions (Grebe and Stock, 1999; Koretke et al., 2000). The HK proteins consist of highly conserved domains, the dimerization and histidine phosphotransfer domain (DHp), which contains the conserved histidine, and the catalytic and ATP binding (CA) domain (Cheung and Hendrickson, 2010). Apart from other functional domains, the HK protein has a sensory domains like HAMP (Histidine Kinases, adenyl cyclases, methyl accepting proteins, and phosphatases), GAF (cGMP-specific phosphodiesterases, adenyl cyclases and FhlA), PAS (Per Arnt Sim), and phytochrome domains for sensing wide range of environmental cues. Response regulators share a common, well conserved receiver domain RD that catalyzes phosphotransfer from its cognate HK (Capra and Laub, 2012). The differential gene expression is the consequence of protein-protein interaction or protein-DNA interaction which is mediated by the C-terminal effector (or output) domain of the RR thus giving rise to the appropriate cellular response (Mascher et al., 2006). In hybrid type kinases, phosphate is first transferred from the histidine residue in the transmitter to the aspartate residue of the attached RD, then to a histidine residue on a histidine phosphotransfer domain (**Figure 1**). Finally, the phosphate is relayed from the Hpt domain to the RD of a down-stream response regulator protein (RRs), which results in the output response. Based on

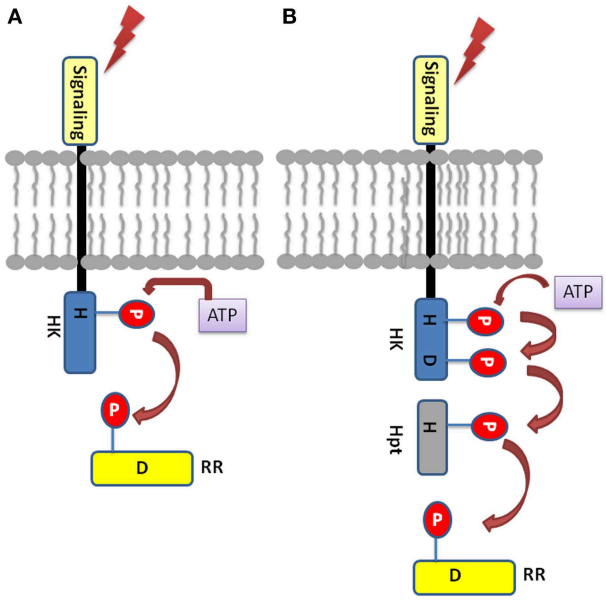


FIGURE 1 | Basic Two-component system architecture (A) A simple TCS member: The sensory domain of histidine kinase sense the extracellular signal which leads to the phosphorylation of the conserved His in its transmitter domain. Further, the conserved Asp in the receiver domain of the RR is autophosphorylated resulting in signal output. **(B)** Hybrid-type TCS member: The conserved His and Asp are present in the same protein which is membrane bound. The Hpt protein acts as mediator for the transfer of the phosphoryl group between the HK and the RR.

highly conserved residues which HK proteins possess conserved sequence fingerprints, namely H, N, D, F, and G-boxes can be identified. The H-box bears the histidine that get phosphorylated while the N, D, F, and G-boxes are located at the ATP binding site (Kofoid and Parkinson, 1988; Stock et al., 1988, 1995).

Several plant species, including model plant *Arabidopsis*, are known to possess TCS signaling machinery (Hwang and Sheen, 2001; Grefen and Harter, 2004). Crucial processes such as cytokinin signaling, ethylene signaling, and light perception involves members of the TCS (Hwang et al., 2002). The presence of TCS system in eukaryotes was anticipated in *Arabidopsis* with the characterization of ethylene receptor ETR1 (Chang et al., 1993), photoreceptors (Schneider-Poetsch, 1992; Li et al., 2011) and yeast osmosensor SLN1 (Ota and Varshavsky, 1993) which was earlier considered to be restricted only to prokaryotes. The characterization of multi-step TCS machinery in *Arabidopsis* as the key element of plant cytokinin signaling revealed TCS machinery in plants (To and Kieber, 2008). In *Arabidopsis*, AtHK1 of the TCS family is indicated to be involved in the osmosensing mechanism (Urao et al., 1999). Earlier, we have performed whole genome analysis of the TCS members in rice in comparison to *Arabidopsis* (Pareek et al., 2006). Further, current advances have shown role of TCS machinery in various environmental stresses such as drought, cold, osmotic stress and abscisic acid (ABA) (Tran et al., 2010; Ha et al., 2011; Hwang et al., 2012).

The current investigation presents the comprehensive expression analysis of various members of TCS in *Arabidopsis thaliana* and *Oryza sativa* using massively parallel signature sequencing (MPSS) and publicly available microarray data under various abiotic stress conditions. The analysis would be able to enhance our understanding about the role of TCS members in the two plant species.

Materials and Methods

Search of TCS Members in *Oryza sativa and Arabidopsis*

Earlier, all the members of TCS signaling machinery were identified and characterized in rice (TIGR rice database version 4.0) and were compared to the TCS members present in *Arabidopsis* (Hwang and Sheen, 2001; Grefen and Harter, 2004). The TCS signaling members were retrieved for *Arabidopsis* and rice as done earlier (Pareek et al., 2006) using TIGR rice database version 7.0 and TAIR version 10 for *Arabidopsis*, in order to rule out any new member or deleted member protein from the updated genome database versions.

Analysis of MPSS Database for Expression Profiles

With the representation of more number of signature libraries in the MPSS database (Brenner et al., 2000), we have extracted expression evidence from the most recent MPSS tags for both *Arabidopsis* and *Oryza* gene models (Database release 2008). With high specificity, the signature sequence uniquely represents a gene and shows perfect match (100% identity over 100% length of the tag). The expression of the gene is estimated by the normalized abundance (tags per million, tpm) of specific signatures in a given library. Class 1, 2, 5, and 7 were used for sense coding sets while Class 3 and 6 were used for antisense coding sets. For both the genomes, 20-nt tags were used for determining the tissue specific expression of TCS members.

In *Arabidopsis*, the tissue specific signature libraries considered for analysis are as follows: for Callus—CAF, CAS; for inflorescence—INF, AP1, AP3, AGM, INS, SAP; for leaves—LEF, LES, S04, S52; for roots—ROF, ROS; for Silique—SIF, SIS; for seeds—GSE. These libraries were earlier considered for analysis of CBS family protein in *Arabidopsis* (Kushwaha et al., 2009).

In rice, the tissue specific signature libraries considered for analysis are as follows: for leaves—I9LA, I9LB, I9LC, I9LD, FLA, FLB, FLC, FLD, NDL, NCL, NLA, NLB, NLC, NLD, NYL, NSL, PLA, PLW, PLC; for meristem—NME, I9ME, FME; for roots—I9RO, I9RR, FRO, FRR, NYR, NRA, NRB, NDR, NCR, NSR; for callus—NCA; for panicle—NIP; for stigma—NOS; for pollen—NPO; for stem—NST; for seeds—NGD, NGS, PSC, PSI, PSL, PSN, PSY. These libraries were earlier considered for analysis of CBS family protein in rice (Kushwaha et al., 2009). The quantitative values obtained for respective TCS genes were used for making the heatmap using open source R software.

Expression Analysis using Microarrays

In order to analyze the gene expression for various abiotic stress conditions the latest microarray data for cold, UV, wound, heat, genotoxic, drought, osmotic, salt, and oxidative stress were retrieved from the *Arabidopsis* Information Resource (Lamesch et al., 2012). The tissue (root and shoot) specific datasets were obtained for different time sets namely 30 min, 1 h, 3 h, 6 h, 12 h and 24 h of various abiotic stresses and analyzed, as performed earlier (Kushwaha et al., 2009). The pre-normalized data thus obtained, was used for analysis of fold change expression in *Arabidopsis*. The expression datasets for the rice were obtained from NCBI-GEO database (Supplementary Table 1). The microarray expression data was downloaded using Bioconductor package. The array quality of the experiment was assessed by MA and RNA degradation plot for individual arrays. The individual GEO raw data sets were normalized using RMA method. The normalized datasets were integrated and differentially expressed genes were identified using RankProd package in Bioconductor (Hong et al., 2006). The expression matrix thus obtained, was used to extract expression values for TCS members. Fold increase in transcript abundance under stress conditions were calculated with respect to their respective controls. The expression with respect to the control was calculated using in-house PERL programs. The hierarchical clustering analysis and the heatmaps were made using R software.

Plant Material and Growth Conditions

Seeds of *Oryza sativa* L. cv "IR-64" were washed with deionized water and allowed to germinate in half Yoshida medium (Yoshida et al., 1972) under hydroponic system with continuous air bubbling for 48 h in dark and then transferred to light for further growth for 14 days under control conditions ($28 \pm 2^{\circ}C$, 12 h light and 12 h dark cycle) in plant growth chamber, having 70% relative humidity. To find out the rhythmic expression of TCS genes in rice, shoot samples were harvested for 2 days at an interval of 3 h starting from the dawn of 15th day from rice seedlings grown under 12 h light/12 h dark cycle. After harvesting, each sample was immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$ till further use.

Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated from the harvested plant samples using RaFlex™ solution I and solution II (GeNei, India) as per the manufacturer's protocol. Two hundred milligram of each sample was used for RNA extraction. The quantity and quality of RNA was estimated by determining absorbance at a wavelength of 260 nm. Concentration of RNA was calculated using OD_{260} nm formula ($OD_{260} = 1$, corresponds to 40 μg/ml of RNA). Quality of RNA was checked by A_{260}/A_{280} ratio. Five microgram of total RNA of each sample was checked by electrophoresis. EtBr stained formaldehyde agarose gel showed the presence of two distinct bands of 28S rRNA and 18S rRNA in each sample of total RNA. It confirmed the integrity of RNA of each sample which was then used for subsequent cDNA synthesis. First strand cDNA was synthesized using first strand cDNA synthesis kit (Fermentas). Total RNA was treated with DNase to remove genomic DNA contamination. For DNase treatment, 5 μg RNA was incubated with 1 unit of DNase in 1X buffer for 30 min at $37^{\circ}C$. The DNase was then denatured by heating at $75^{\circ}C$ for 5 min. Before heating 1 μl of 25 mM EDTA, which works as a chelating agent,

was added to the reaction mixture to prevent RNA break down. After this treatment, RNA samples were used for first strand cDNA synthesis. The primers of TCS members were designed using Primer 3 express (Applied biosystem, USA) and NCBI primer BLAST software. The sequences for these primers are listed in Supplementary Table 2. All Primers were specific to the unique regions in the 3′-UTR of their respective genes. These primers were rechecked for their uniqueness via primer BLAST at NCBI database. For primer designing the transcript nucleotide sequences were downloaded from TIGR rice database. Designed primers were ordered to Sigma-Aldrich, India for synthesis.

Quantitative RT-PCR

The rice translation elongation factor 1α (eEF-1α) gene was taken as the reference gene for the analysis. The quantitative RT-PCR reaction mixture contained 5 μl of 10 fold diluted cDNA, 10 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems, USA), and 100 nM of each gene-specific primers in a final volume of 20 μl. No template controls (NTCs) were also taken for each primer pair. The real-time PCR reactions were performed employing ABI Prism 7500 Sequence Detection System and software (PE Applied Biosystems, USA). All the reactions of quantitative real-time PCR were performed under following conditions: 10 min at 95°C, and 40 cycles of denaturation at 95°C for 25 s, annealing and extension at 59°C for 1 min in 48-well optical reaction plates (Applied Biosystems, USA). The specificity of amplification was tested by dissociation curve analysis. The experiment was repeated with two biological replicates, each of them having three technical replicates. Data analysis was performed using ddCT method (Livak and Schmittgen, 2001).

Results

The analysis of TCS members has been carried out using latest version of genomes of *Arabidopsis thaliana* (TAIR ver. 10) and *Oryza sativa* (TIGR ver. 7) using pfam profiles (Pareek et al., 2006). In comparison to the earlier report (Pareek et al., 2006), some new members have been found in both *Arabidopsis* and rice, in the current analysis (Supplementary Tables 3A–F). Earlier analysis suggested 54 genes coding for 63 proteins in *Arabidopsis* while current analysis found 54 genes coding for 73 proteins. Similarly in *O. sativa*, 51 genes encoding 73 putative proteins was reported earlier but the current analysis showed 52 genes coding for 81 proteins (**Table 1**). The new members added to the list of histidine kinase have been named as histidine kinase like (*OsHKL1*) gene because of the presence of only histidine kinase domain in the protein sequence. The increase in the number of protein products has been attributed to the presence of more alternative spliced products. In order to avoid any ambiguity in nomenclature, the previous nomenclature has been retained as reported in Pareek et al. (2006).

TABLE 1 | Comparison of TCS architecture in *Arabidopsis* and *O. sativa*.

Gene Family	*Arabidopsis thaliana*		*Oryza sativa*	
	No. of genes	No. of proteins	No. of genes	No. of proteins
TCS	54	73	52	81
HK	15	19	13	22
Hpt	6	9	5	7
RR	33	45	34	52

Analysis of Expression Profiles for TCS Members in *Arabidopsis* and Rice using MPSS Database

Sensitive measures of expression of all genes in the genome can be assessed using MPSS (Brenner et al., 2000). MPSS has been used for the analysis of genome-level expression analysis in various plant systems including rice, *Arabidopsis* and grapes (Meyers et al., 2004). To extract information about the relative abundance of transcripts of TCS members in various tissues/organs of *Arabidopsis* and rice, analysis was carried out using MPSS database (http://mpss.udel.edu). Analysis of TCS-specific mRNA tags (measured as transcript per million; TPM) in various libraries showed considerable variability in their abundance in various tissues.

Among the HKs, *ERS1* showed considerably large accumulation of transcripts in untreated 21-days roots while it showed moderate rise in transcripts in 28–48 h post fertilized silique, untreated 21-day leaf and actively growing callus in *Arabidopsis* (**Figures 2A,B**). Another HK, *CRE1* showed moderate accumulation of transcripts in untreated 21-days roots. Low transcripts accumulation was observed in *AHK2*, *AHK3*, *ETR1*, *PHYA,* and *PHYB* in *Arabidopsis*. On the other hand, *OsHK5* showed considerably high accumulation of transcripts in leaves. The transcripts accumulation was also observed in leaves, 60 days mature leaf for *OsPHYc* in rice. The accumulation of transcripts was also observed in 60 days mature roots for *OsPHYA* gene. Among the Hpts in *Arabidopsis*, *AHP2* showed accumulation of transcripts in 24–48 h post fertilized silique while it shows moderate accumulation in callus. On the contrary, *OsHpt3* in rice showed high accumulation in developing seeds (**Figures 2C,D**). For the RRs, in *Arabidopsis*, *APRR1* showed high accumulation of transcripts in agamous inflorescence and leaves while *ARR4* showed transcript abundance in callus. In rice, *OsPRR3* and *OsPRR1* showed accumulation of transcripts in beet armyworm damaged; water weevil damaged and mechanically damaged leaves. Another RR, *OsRRA2* and *OsRRA3* showed transcripts accumulation in 60 days mature leaves (**Figures 2E,F**). These observations suggest that the TCS-related transcriptome of *Arabidopsis* and rice is complex, showing a tissue-specific differential regulation.

Analysis of Expression Profiles for TCS Members in *Arabidopsis* and Rice using Microarray

For analysis of expression of the TCS members in *Arabidopsis*, we used data available on the TAIR database consisting time series analysis performed in root and shoot tissues under various abiotic stress conditions while for rice, the expression was analyzed using various abiotic stress experiment data available at NCBI-GEO database.

Histidine Kinase Proteins (HKs)

The expression profile of the histidine kinase genes in root tissues of *Arabidopsis* under cold conditions showed downregulation

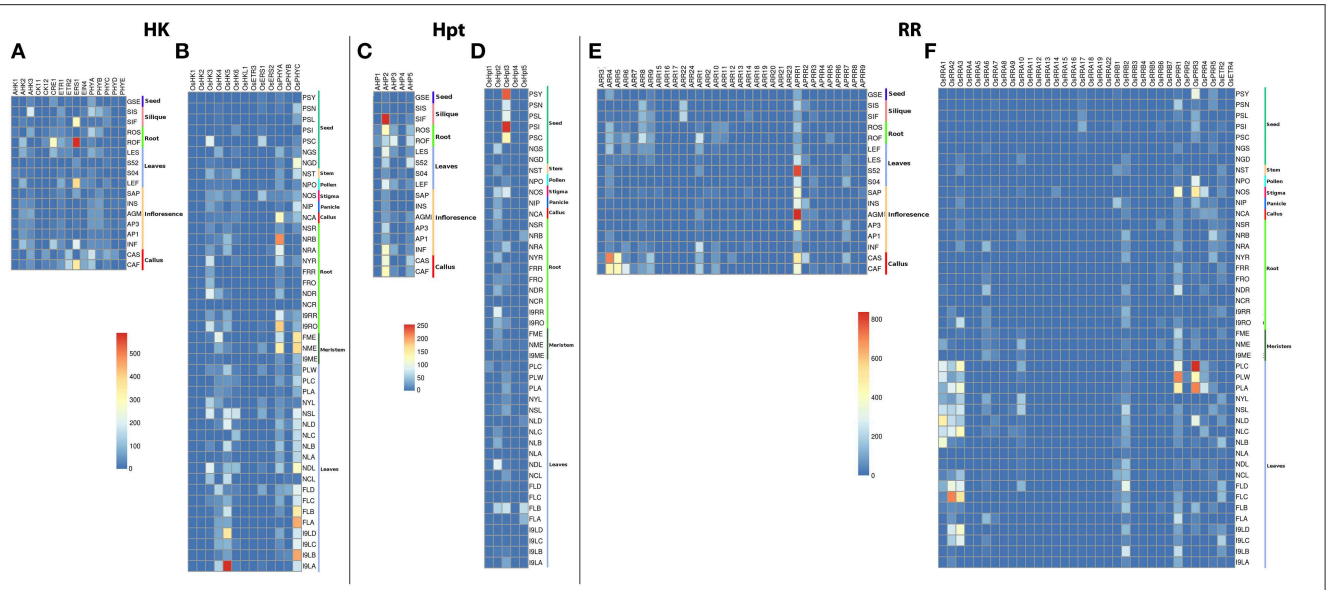


FIGURE 2 | Heatmap depicting the tissue-specific transcript accumulation for Histidine Kinases (HKs) for (A) *Arabidopsis* and (B) rice, Histidine phosphotransfer proteins (Hpts) for (C) *Arabidopsis* and (D) rice, Response regulators (RRs) for (E) *Arabidopsis* and (F) rice. These heatmaps are based on the MPSS database. The heatmap was made using open source R software.

of *AHK1* and *AHK2* genes at 24 h of stress, while at the other time spans, its expression remained unchanged. On the other hand, in shoots, *AHK1* maintained a basal level of expression in all the cold stress time points but the transcripts for *AHK2* showed two fold downregulation at 12 and 24 h of cold stress conditions (**Figure 3A**). Another cytokinin signaling gene, *CK11* showed changing expression during all time-series. The gene was observed to be downregulated at 30 min of cold stress and two fold upregulated at 1 h of stress. The expression of this gene gets normalized only to get further upregulated by over two folds at 24 h of cold stress. Further, in shoots, *CK11* gene showed similar behavior where the basal level of expression is maintained at 30 min of cold stress, which gets upregulated upto two folds in 1 h of stress. The expression further goes down two folds at 3 h of stress and again gets two fold upregulated at 6 h and 12 h of stress. Finally, it again shows downregulation at 24 h of cold stress (**Figure 3A**). *CK11* and *AHK1* have been found to play major role in the cytokinin signaling and osmosensing process respectively in *Arabidopsis* (Urao et al., 2000). In roots, ethylene receptor, *ETR2* showed downregulated response as the time span of the cold stress increases from 30 min to 24 h while, in shoots, it shows an upregulated response till 12 h of cold stress and finally maintains a basal expression at 24 h of cold stress. In shoots, another member of TCS, namely *AHK3*, showed downregulated response at 24 h of cold stress and *CK12* showed upregulation in 30 min, 1 h and 6 h of cold stress while it gets downregulated in 3 h of cold stress. *ETR2* also showed upregulation in response to 12 h of cold stress. Among the photoreceptors, *PHYC* showed downregulation in 12 and 24 h of cold stress in both root and shoot tissues. In roots, photoreceptor *PHYE* showed upregulation in 6 h of cold stress, while it maintained basal level expression in the shoot tissues.

Under drought conditions, in the root tissues, all the histidine kinase genes in *Arabidopsis* were found to be upregulated. *AHK1* was found to be upregulated in 30 min of drought stress followed by its downregulation in 1, 3, 6, and 12 h of drought stress, after which it again got upregulated by two folds in 24 h. Ethylene receptor *ETR2*, showed two fold upregulation at 30 min, 12 h and 24 h of drought stress, photoreceptor *PHYB* showed upregulated response at 30 min of drought stress. On the other hand, in shoots of *Arabidopsis*, most of histidine kinase genes showed downregulation in response to drought stress. Only *CK11* showed three fold upregulation in 12 h of drought stress and then got downregulated in continuation of drought stress for 24 h.

Under the genotoxic stress conditions, in root tissue, genes namely, *AHK1*, *AHK2*, *CK12*, *CRE1*, and *ETR2* showed downregulated response. Rest all the members of the HK family showed minimal level of expression at all the time points in both root and shoot tissues. Only *CK11* showed three fold upregulation in 1 h of genotoxic stress and got downregulated only to get upregulated again at 24 h of stress. In shoot tissues, *CK11* gene showed upregulation at 6 and 12 h of genotoxic stress. Another gene, *CK12* showed 1.5-fold upregulation at 6 h of stress.

Similar response of the members of the HK family was found in response to heat stress. In root tissues, all genes of HK family maintained minimal to high expression at all time points of the stress. Specifically, gene *ETR2* showed three fold upregulation at 6 h of stress while *AHK1* showed downregulation at 3 h and 6 h of heat stress condition. On the other hand, in shoot tissues, the entire HK family members showed exactly the reverse of the expression as it showed in root tissues, that is, mainly down

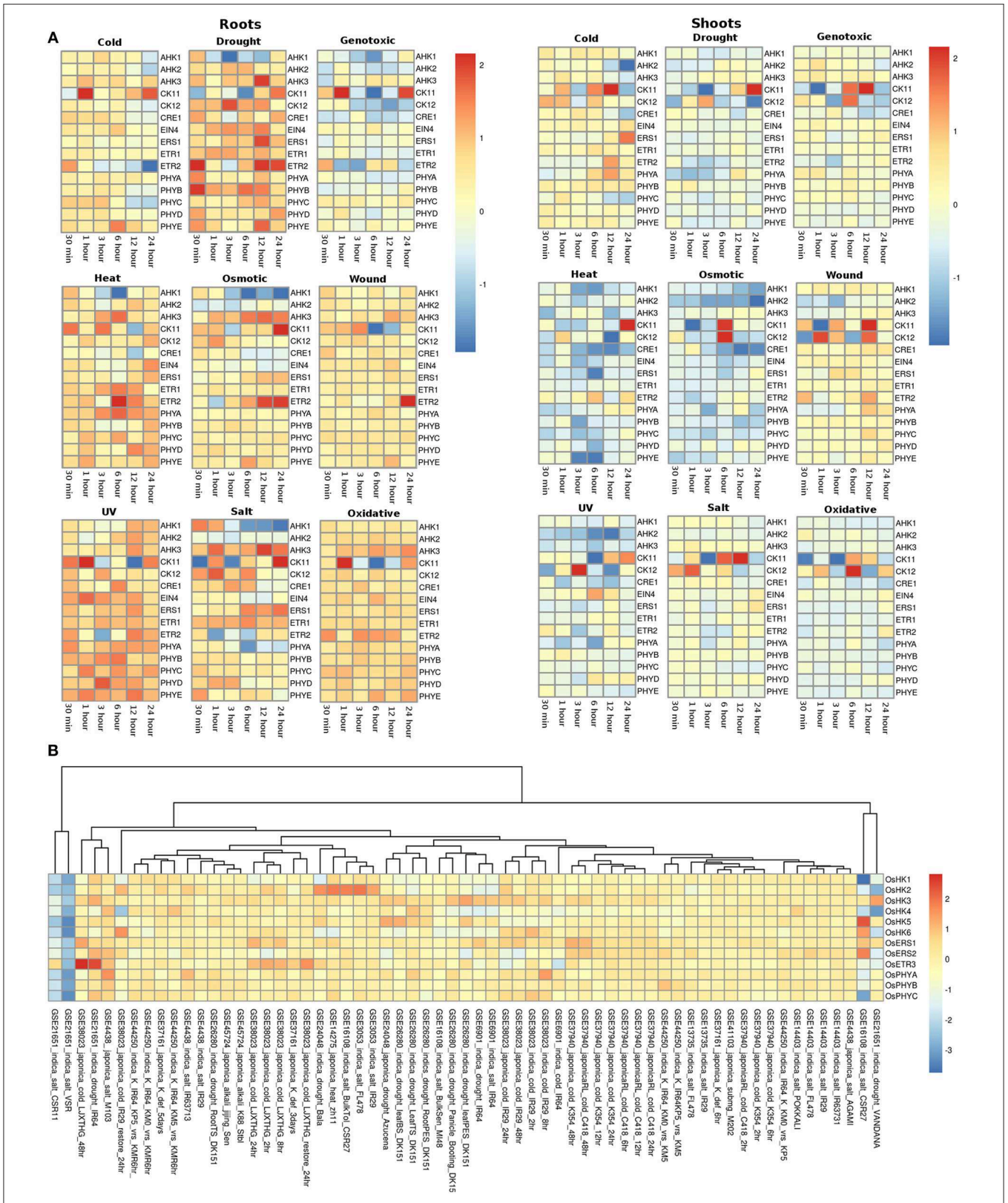


FIGURE 3 | Heatmap depicting the stress-induced expression of Histidine Kinase (HKs) genes from root and shoot tissues obtained using microarray data for *Arabidopsis* (A) and *O. sativa* (B). The heatmap was made using open source R software.

expression for most of the members. Only, *CK11* showed two fold upregulated expression at 24 h of heat stress.

Under osmotic stress, in root tissues, *CK11* and *ETR2* showed three fold upregulation during 24 h of osmotic stress while *AHK1* showed downregulated expression during the 3, 6, 12, and 24 h of osmotic stress. *CK11* and *CK12* showed upregulation during 6 h of osmotic stress in the shoot tissues. All the other members showed similar level of expression as in the heat stress, that is, downregulation for most of the members. Under the wounding stress, *CK11* and *CK12* showed upregulated response in 1 h and 12 h of stress in the shoot tissues while in root tissues, only *ETR2* showed two fold upregulation in the expression. Rest of the members of the HK family maintained unchanged expression levels at all time points in both root and shoot tissues.

Similar to heat and osmotic stress conditions, HK members showed minimal to high expression under UV stress in the root tissues while in shoot tissues, *CK12* showed three fold upregulation in 3 h of stress. In roots, *AHK1* showed downregulation after 6 h of UV stress condition. Further, under salt stress conditions, all the HK members were found to be upregulated except *AHK1* and photoreceptor, *PHYA* in the root tissue while in shoot tissues, the expression of HK members remained unchanged with respect to the control conditions. *CK11* and *CK12* showed high expression in the salt stress condition in shoot tissues. Similar expression profile was observed in oxidative stress conditions in both root and shoot tissues. Overall, the HK family members in *Arabidopsis* appear to be more active in the root tissues than in the shoot tissues in all the stress conditions.

In rice (in all the experiments and genotypes of rice) histidine kinases (HKs) showed similar behavior of basal expression under cold conditions. Only in GEO dataset, GSE38023, ethylene receptors *OsERS1*, *OsERS2,* and *OsETR3* showed one to two fold upregulation at various time points (**Figure 3B**). Under drought conditions, in indica genotype of rice Vandana (GSE21651), *OsHK3* showed 1.5-fold upregulation while *OsHK2* and *OsHK3* showed similar fold downregulation in the expression. In IR64 genotype (GSE21651), *OsETR3* showed two fold upregulation under drought stress. All the other members of histidine kinase gene family showed basal expression levels in all other experiments. In salt stress conditions, all the members of the HK gene family maintained basal expression in all the experimental conditions. In two of the rice genotypes, VSR and CSR11 all the HK members were found to be downregulated under salt stress conditions.

Histidine Phosphotransfer Proteins (Hpts)

The expression profiles of the Histidine phosphotransfer (Hpt) genes in *Arabidopsis* in root and shoot tissues appear to show reverse behavior as compared to the HK family members under all the stress conditions. Overall, Hpt family members appear to show higher expression in shoots than in roots under all the considered abiotic stress conditions (**Figure 4A**). Under osmotic stress conditions, in the root tissues, all the Hpt members appear to be downregulated except for *AHP4* which showed three fold upregulation. Under osmotic stress, Hpt members namely *AHP1*, *AHP2,* and *AHP3* showed downregulation at 30 min and 1 h of stress but they maintained a basic level of expression at other time points in the root tissues. While in shoot tissues, all the genes were observed to be expressed at all the time points especially, *AHP4* and *AHP6* showed high (two or three fold) upregulation under osmotic stress. Under drought stress conditions, all the Hpt members showed similar expression in root as well as shoot tissues. Again, *AHP4* showed high upregulation at 1, 6, and 12 h of drought stress in roots and at 3 h in shoots. Under genotoxic conditions, all the members, with an exception of *AHP4* showed downregulation in the root tissues. In shoot tissues, members of the Hpt family showed unchanged minimal expression with respect to the control conditions. Similar to cold stress, all the members showed downregulation all time points except for *AHP4* which showed three fold upregulation during 1 h of stress in the root tissues. However, in the shoot tissues, all the Hpt members were observed to be upregulated except for *AHP6* which showed two fold downregulation in 12 h cold stress (**Figure 4A**). On the other hand, all the members of Hpt family maintained minimal expression except for *AHP4* which showed three fold upregulation at 30 min of heat stress. Under wounding stress, in roots, all the members were downregulated while in shoot tissues all the Hpt genes were upregulated. *AHP4* was found to be downregulated under the wounding stress at all the time points in the shoot tissues. *AHP1* showed higher expression under the wounding stress in the shoot tissues. Under the UV stress, Hpt members showed exactly reverse expression behavior in the root and shoot tissues with the expression of *AHP4* showing periodic pattern in the expression. Under salt and oxidative stress conditions, the expression levels remained same in both root and shoot tissues showing that the salt stress nearly leads to the oxidative stress as well. Overall, it appears that the *AHP4* gene of the Hpt family is most active members among all, showing expression in all the considered abiotic stress conditions.

In rice, the members of the Hpt family showed expression in all the considered experimental conditions in all the considered genotype suggesting their potential importance in the rice plant (**Figure 4B**). *OsHpt4* showed three fold expression in drought and salt stress conditions in Bala (GSE24048) and IR29 (GSE13735) genotypes of rice respectively. *OsHpt4* showed three folds expression under salt stress in the CSR27 genotype of rice (GSE16108) while *OsHpt5* showed three folds expression under similar conditions in IR64 genotype of rice (GSE6901). In other genotypes namely, VSR and Vandana, *OsHpt4* showed two fold downregulation under salt and drought stress (GSE21651).

Response Regulator Proteins (RRs)

The RR gene family members in root and shoot tissues of *Arabidopsis* showed similar pattern of expression under various abiotic stresses considered in this investigation. Under the cold stress, *APRR9* showed three fold upregulation in 12 h of stress in both root and shoot tissues. Most of the members of the RR family were found to be downregulated under cold stress conditions in both root and shoot tissues (**Figure 5A**). Under drought conditions, most genes of the RR family maintained minimal expression in both root and shoot conditions. Genes namely *APRR4* (*Arabidopsis* pseudo response regulator), *APRR9* and *ARR15* were upregulated in the root tissues while *APRR4*,

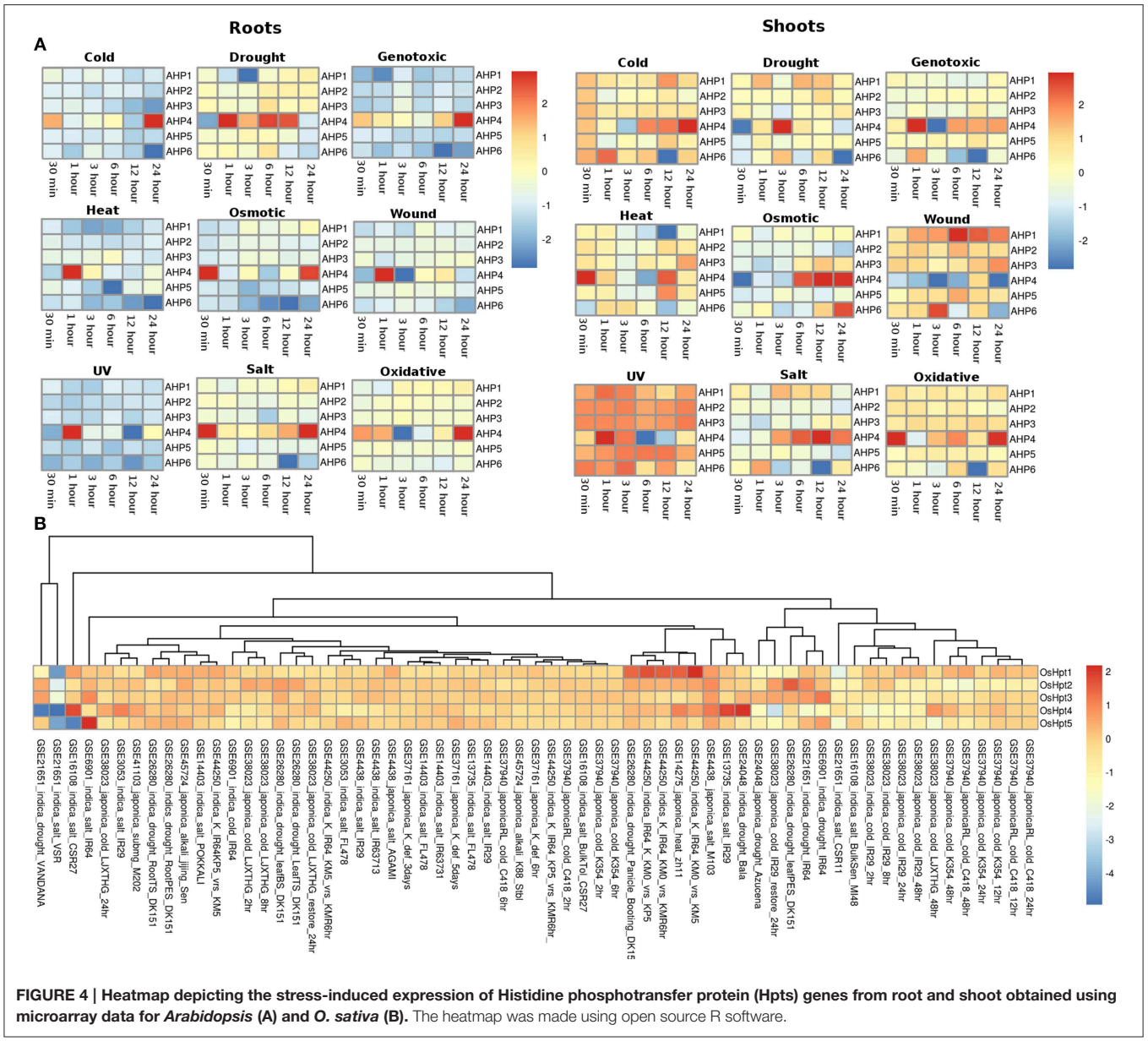


FIGURE 4 | Heatmap depicting the stress-induced expression of Histidine phosphotransfer protein (Hpts) genes from root and shoot obtained using microarray data for *Arabidopsis* (A) and *O. sativa* (B). The heatmap was made using open source R software.

APRR9, *ARR11,* and *ARR21* were upregulated in the shoot tissues under cold stress conditions at various time points. In genotoxic stress conditions, most gene members of the family were downregulated in the root tissues, while in shoots, most of the genes maintained unaltered expression at various time points. Similar expression pattern was observed in response to heat and osmotic stress. Only *ARR23* in the root tissues was found to be upregulated while in shoots, it maintained unchanged expression with respect to control conditions. Under osmotic stress, *APRR9* in the shoot tissues got upregulated in 12 h of stress. Under wounding stress, most of the members maintained unaltered expression at various time points in the root tissues while in shoot tissues, RR gene family members showed mixed response ranging from downregulation to unaltered expression. In response to UV stress, RR family members maintained unchanged expression with respect to the control conditions with only *APRR4*, *APRR9,* and *ARR3* showing upregulated expression in the root tissues while in shoot, only *APRR9* showed three fold upregulation. In salt stress conditions, RR gene family members in shoot tissues were found to have marginally higher expression levels than in the root tissues. Pseudo response regulator, *APRR9* showed two fold and three fold high expression in the shoot and root tissues respectively. Similar pattern of expression was found in the oxidative stress of both root and shoot tissues of *Arabidopsis*.

In rice, similar to *Arabidopsis*, all the members of RR gene family showed unaltered expression under all the abiotic stress conditions in all the genotypes. *OsRRA1* showed downregulation in the indica genotype under salt and drought conditions (**Figure 5B**). Some genes of the RR gene family showed downregulation in the indica genotype in VSR

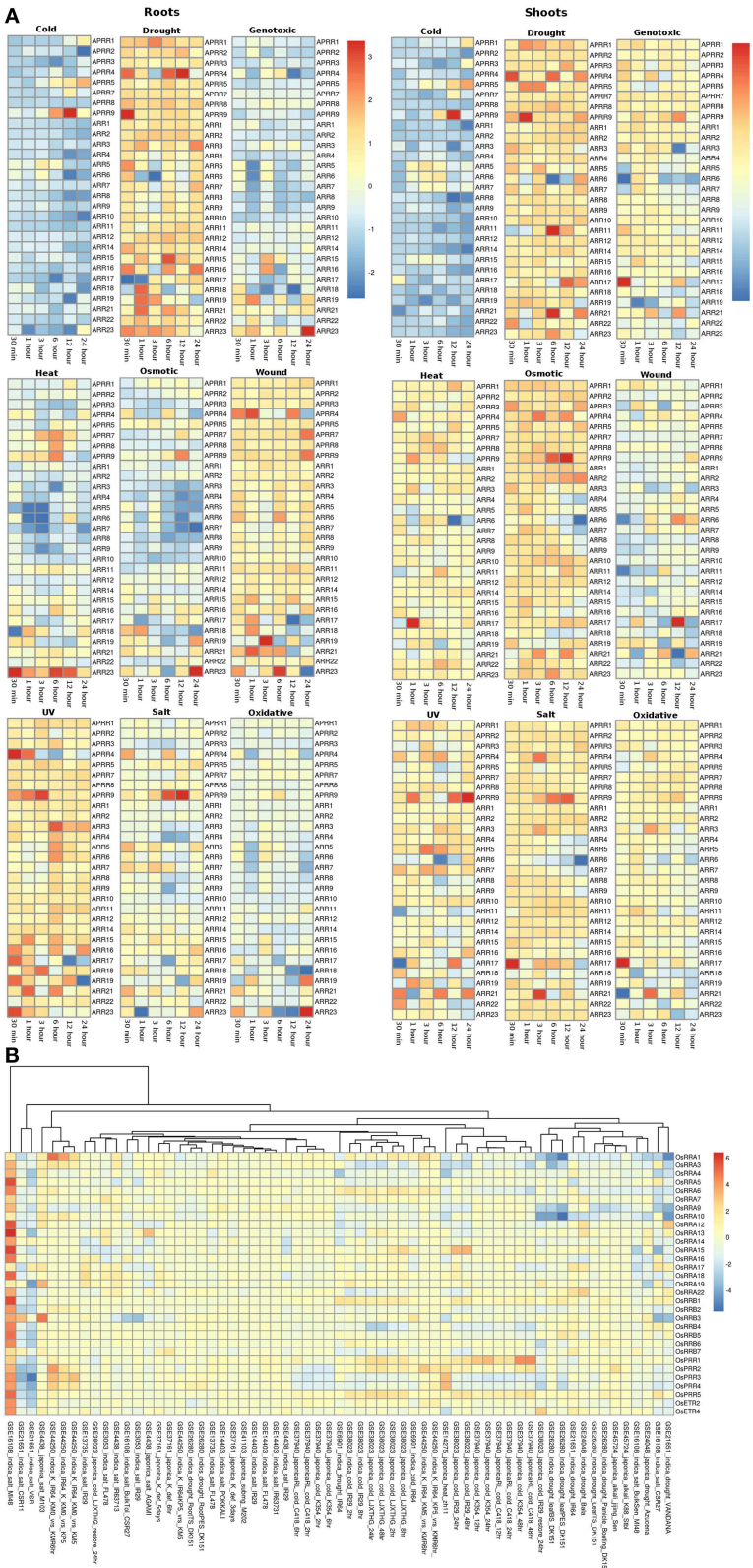


FIGURE 5 | Heatmap depicting the stress-induced expression of Response regulator (RRs) genes from root and shoot obtained using microarray data for *Arabidopsis* (A) and *O. sativa* (B). The heatmap was made using open source R software.

and CSR11 (GSE21651) variety of rice. Another variety of the indica genotype, *MI48* showed two to three fold upregulation of various gene members of the RR gene family (GSE16108).

qRT-PCR-based Analysis of TCS Members Toward Diurnal Rhythm in Rice

The circadian clock controls many aspects of plant physiology such as flowering, photosynthesis and growth. The diurnal expression profiles of representative genes of TCS family were analyzed under conditions of 12 h light (L)/12 h dark (D) with a constant temperature 28 ± 2°C using IR64 genotype of rice at the seedling stage. Histidine kinases showed rhythmic expression in diurnal manner in response to light and dark cycle (**Figure 6A**). *OsHK1*, *OsHK2,* and *OsHK5* genes showed comparatively low expression while *OsHK4* and *OsHK6* showed comparatively high expression. *OsHK4* and *OsHK6* showed same pattern of expression, with a phase of 24 h and peak at transition period of night to light during morning. It was evident from the expression analysis that the HKs followed diurnal cycle. Similar to the rhythmic cycle of HKs, Hpts, and RRs also showed expression in diurnal cycle (**Figures 6B,C**). Among the Hpts, *OsHpt5* showed comparatively low expression than other members of the family. Changes in expression levels of *Hpts* also coincide with dark to light transition in diurnally regulated manner. Level of mRNAs of all *OsHpts* genes except *OsHpt4* oscillated during the 24 h cycle of light-dark, peaking in the morning. *OsHpt3* expression oscillated with higher amplitude in comparison to that of other *Hpts.*

Among the RRs, A type RR- *OsRRA10* and B type RR- *OsRRB1* showed comparatively high expression (**Figure 6C**). *OsPRR1*, *OsPRR2*, *OsPRR3* and *OsPRR4* belong to pseudo-response regulator family. Expression of each member of this family rhythmically oscillated in the given 24 h period (**Figure 6D**). Interestingly, the level of each mRNA reached its maximum at a distinctive time. *OsPRR1* showed evening specific peak. mRNA of members of PRR family started accumulating after dawn sequentially at 3 h intervals in the order of *OsPRR3*→*OsPRR4*→*OsPRR2*→*OsPRR1*. *OsPRR2*>*OsPRR1*>*OsPRR3*>*OsPRR4* is the order of their expression amplitude.

In summary, most of the considered TCS members except pseudo-response regulators, showed similar pattern of expression, with a phase of 24 h and peak at transition period of night to light during morning.

Discussion

TCS is considered as one of the most crucial signal transduction system in plants. Evidence suggest that TCS pathways are involved in sensing the environmental stimuli, ethylene signaling, light perception, circadian rhythm and cytokinin-dependent processes which include shoot and root development, vascular differentiation and leaf senescence (Hwang et al., 2002; Kakimoto, 2003; Tran et al., 2010). Cytokinin signaling has been associated with the variety of stress response (Hare et al., 1997). Histidine kinase of the TCS is known to function as an oxidative stress sensor (Singh, 2000). *ERS1* gene provides ethylene

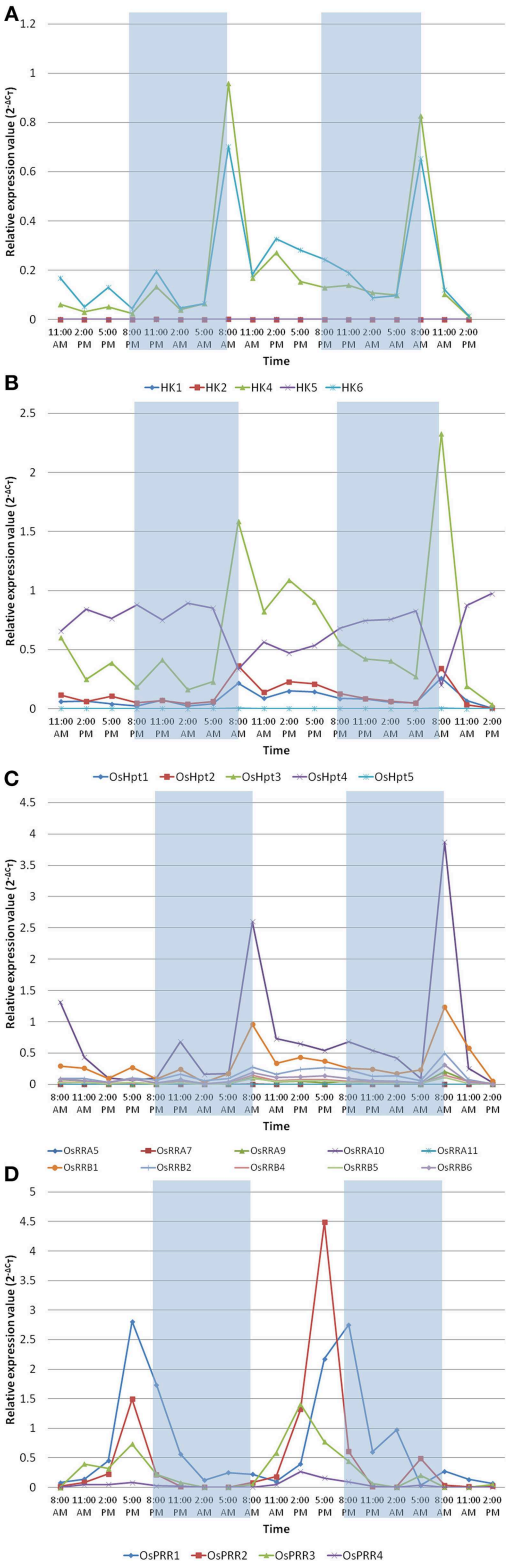


FIGURE 6 | Expression analysis of the representative family members of two-component systems, (A) OsHKs, (B) OsHpts, (C) OsRRAs and OsRRBs, (D) OsPRRs in seedlings of *Oryza sativa* L. (cv IR-64) during day and night cycles. The rice seedlings were subjected to 12 h of dark followed by the 12 h of light period. The shaded area shows dark and non shaded area shows the light period.

sensitivity to the plants. Analysis has shown the accumulation of ERS1 in the leaves of *Nicotiana tabacum* L. on exogenous ethylene treatment, while transcripts were observed in root, shoot, and leaf of the plant (Terajima et al., 2001). MPSS analysis also showed the accumulation of transcripts in roots and leaves in *Arabidopsis*. Cytokinin receptor, CRE1 transcripts were observed to accumulate in root tissues. Recent analysis has shown that CRE1 cytokinin pathway is differentially recruited depending on the root environmental conditions in *Medicago truncatula* (Laffont et al., 2015). Further, the expression of *AHK2*, *AHK*3, and *AHK*4 was observed in several organs of the plant species (Ueguchi et al., 2001). In *Arabidopsis*, various histidine kinases namely AHK2, AHK3, and CRE1 (cytokinin response1/AHK4) are considered as principle cytokinin receptors. Mutant analysis of these cytokinin receptors confirmed their role in response toward low water potential and salt stress (Kumar and Verslues, 2015). Expression analysis showed little or no change in the expression of *AHK2* and *CRE1* genes in various abiotic stresses in both root and shoot tissues. In the present analysis, *AHK3* showed two fold expression at 12 h of salt stress. *AHK2* and *AHK3* were found to be negatively controlling osmotic stress responses in *Arabidopsis*. *CRE1* also negatively regulates osmotic stress in the presence of cytokinin (Tran et al., 2007). It was found that cold stress did not significantly induce *AHK2* and *AHK3* expression which indicates that these proteins may mediate cold temperatures for A-type RR expression (Jeon et al., 2010). Our expression analysis of the *AHK2* and *AHK3* genes in cold stress in both root and shoot tissues corroborated with the earlier result. Previously, *AHP1* was shown to be expressed in roots; *AHP2* and *AHP3* were found to express more in roots, stems, leaves, flowers, and siliques (Suzuki et al., 1998; Hradilova and Brzobohaty, 2007). Our MPSS data analysis showed the accumulation of *AHP2* and *AHP3* transcripts in the root, silique and inflorescent tissues. Analysis shows that Hpt proteins in *Arabidopsis* namely *AHP2*, *AHP3*, and *AHP5* control the response toward drought stress in negative and redundant manner. Also, the downregulated expression of these genes was observed under dehydrating conditions which is assumed to be due to the stress induced reduction of the endogenous cytokinin levels (Nishiyama et al., 2013). Analysis using microarray also showed downregulated expression of these genes under various abiotic stress pertaining to dehydrating conditions such as osmotic and salt stress. Recently, a knock-down analysis of two histidine phosphotransfer (*OsHpt2* and *OsHpt3*) via RNA interference (RNAi) showed that OsHpts function as positive regulators of the cytokinin signaling pathway and play different roles in salt and drought tolerance in rice (Sun et al., 2014). A 1.5-fold expression of these genes in the various rice genotypes in microarray, as reported in the present analysis, also supports the earlier results. Earlier, type-A response regulator genes in rice were shown to have an overlapping/differential expression patterns in various organs and in response to light (Jain et al., 2006). Previously, under short day conditions, B-type RR, *Ehd1* (Early heading date 1) from rice has been shown to be a floral inducer (Doi et al., 2004). In *Arabidopsis*, B-type RRs are involved in cytokinin and ethylene signaling (Hwang et al., 2002) while in rice they are involved in developmental and environmental signals mediated by light, cytokinin, and ethylene (Doi et al., 2004). Multiple A type ARRs were found to be upregulated by cold stress (Argueso et al., 2009). The upregulation of A type RR was also observed in the expression analysis in both *Arabidopsis* and rice. In *Arabidopsis*, the expression of *ARR4* and *ARR5* is found to be induced by the low temperature, dehydration and high salinity (Urao et al., 1998). Triple mutant analysis among the pseudo-RRs (APRRs) showed *APRR5*, *APRR7* and *APRR9* as the negative

TABLE 2 | Table showing genes of the TCS family which were found to be altered significantly (≥1.5 fold; upregulation/downregulation) under various abiotic stress conditions.

	Oryza sativa		*Arabidopsis thaliana*	
	Up-regulated	**Down-regulated**	**Up-regulated**	**Down-regulated**
HK	OsHK5 (1), OsHK6 (1), OsERS2 (1), OsPHYA (1), OsHK2 (4), OsETR3 (3)	OsHK4 (4), OsPHYB (2), OsPHYC (3), OsERS1 (1), OsHK5 (2), OsHK6 (3), OsERS2 (2), OsPHYA (2), OsHK2 (4), OsETR3 (2)	CK12 (6), CK11 (12), ETR2(1)	AHK1 (4), CRE1 (1), AHK2 (1), CK12 (2), CK11 (13), ETR2 (1)
Hpt	OsHpt1 (3), OsHpt2 (1), OsHpt4 (3), OsHpt5 (1)	OsHpt1 (2), OsHpt3 (1), OsHpt2 (2), OsHpt4 (5), OsHpt5 (4)	AHP6 (1), AHP4 (23)	AHP5 (1), AHP1 (1), AHP3 (1), AHP6 (8), AHP4 (8)
RR	OsRRA1 (5), OsRRA3 (3), OsRRA9 (1), OsRRA10 (2), OsPRR3 (5), OsRRB4 (1), OsRRA4 (1), OsRRA14 (1), OsRRA16 (1), OsRRB2 (1), OsRRA19 (2), OsRRA5 (2), OsRRA15 (5), OsRRB5 (2), OsRRA18 (3), OsPRR4 (5), OsRRB6 (1), OsETR2 (1), OsRRB3 (5), OsRRA22 (3), OsRRA13 (3), OsRRA6 (4), OsRRA12 (5), OsETR4 (2), OsRRA7 (2), OsRRA17 (5), OsRRB1 (7), OsPRR5 (11), OsPRR1 (12), OsPRR2 (11), OsRRB7 (3)	OsRRA1(14), OsRRA3(12), OsRRA9(10), OsRRA10(11), OsPRR3(12), OsRRB4(6), OsRRA4(6), OsRRA14(5), OsRRA16(3), OsRRB2(3), OsRRA19(1), OsRRA5(1), OsRRA15(1), OsRRB5(1), OsRRA18(1), OsPRR4(1), OsRRB6(1), OsETR2(1), OsRRB3(1), OsRRA22, OsRRA13(1), OsRRA6(1), OsRRA12(1), OsETR4(1), OsRRA7(1), OsRRA17(1), OsRRB1(1), OsPRR5(1), OsPRR1(1), OsPRR2(1)	APRR9 (22), APRR4 (12), ARR23 (17), ARR7 (5), ARR17 (9), ARR6 (6), ARR18 (3), ARR19 (6), ARR11 (1), ARR3 (2), ARR22 (1), APRR3 (1), ARR16 (3), ARR5 (7), APRR5 (7), ARR15 (4), APRR8 (2), ARR2 (1), APRR1 (3), APRR7 (5), ARR21 (10)	APRR9 (2), APRR4 (4), ARR23 (9), ARR7(11), ARR17(17), ARR6 (29), ARR18 (15), ARR19 (20), ARR11 (9), ARR3 (6), ARR22 (5), APRR3 (3), ARR16 (4), ARR5 (9), APRR5 (2), ARR15 (2), ARR9 (4), ARR1 (1), APRR2 (2), ARR10 (2), ARR8 (5), ARR4 (8), ARR14 (2), ARR21 (11)

The numbers in parenthesis with the gene name shows the number of conditions in which their alteration was observed.

regulators in the abiotic stress conditions (Nakamichi et al., 2009).

All the organisms have a natural time keeping mechanism popularly known as circadian clocks that is used for the coordination of the physiology of organism with its surrounding environment. In plants, circadian clocks have been shown to play a major role in regulating numerous stress and growth response mechanism (Dodd et al., 2005). Regulation of signaling of phytohormones like auxin and ABA by circadian clock has been reported (Covington and Harmer, 2007; Seung et al., 2012). Earlier, in *Arabidopsis*, two Myb-related transcription factors, *circadian clock associated* (*CCA1*) and *late elongated hypocotyls* (*LHY*) have been shown to induce the expression of *PRR7* and *PPR9* in circadian rhythm (in morning cycles) and *PRR1* (in evening cycles) which also, in turn bind and repress the expression of the formers (Alabadí et al., 2001; Nakamichi et al., 2010). Earlier, *prr9/prr7/prr5* triple mutant analysis revealed the molecular link between metabolism and the circadian clock (Fukushima et al., 2009). In *Arabidopsis*, pseudoresponse regulators have been shown to be involved in circadian rhythms, control of flowering time and also photo-sensory signal transduction (Devlin and Kay, 2001; Mouradov et al., 2002). Our data show that the expression of *OsPrr* genes is under diurnal control in indica rice IR64. Murakami et al. (2003) did similar analysis in japonica *O. sativa* (var Nipponbare) and observed similar results. It again indicates both the dicotyledonous (e.g., *Arabidopsis thaliana*) and monocotyledonous (e.g., *Oryza sativa*) plants might share the evolutionarily conserved molecular mechanism underlying the circadian rhythm. Our expression analysis of representative members of the TCS family showed that not only PRRs, but also other members like HKs, Hpts and RRs are also regulated by the diurnal clock. The TCS members have been shown to play a major role in the abiotic stress response mechanism. The result provides a crucial input related to molecular link between abiotic stress response and diurnal clock.

Conclusions

The progress made over a decade has enhanced our understanding about the two-component signaling system and the crucial role played by its members in perceiving environmental stimuli. Even though the members of the TCS system have been characterized in many plant species but their functional involvement in various environmental stress conditions is still a conundrum. The current analysis has assembled all the expression data for all the TCS members, in order to understand their functional complexity. Further, MPSS data analysis presented an overview of the transcript abundance of the TCS members in various plant tissues under various stress conditions. Expression analysis suggest that rice involves more number of TCS members (HKs, Hpts, and RRs) in these responses, despite having comparable number of genes with respect to *Arabidopsis* (**Table 2**). Also, the diurnal and rhythmic expression of the TCS gene family members in response to the day and night cycle provides a crucial information about the complexities of the process that are regulated by various TCS members in response to various abiotic stress conditions. The analysis presented in this study provides interesting insights about the functional involvement of the TCS members in growth and stress response in plants.

Author Contributions

AP and SLS-P conceived the idea and designed the experiments. PS and HG did the real time PCR work and its analysis. AS and HK performed the MPSS and microarray database analysis and wrote the manuscript. AP and SLS-P edited the manuscript. All the authors approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00711

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Expression of *Arabidopsis FCS-Like Zinc finger* genes is differentially regulated by sugars, cellular energy level, and abiotic stress

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Cellular energy status is an important regulator of plant growth, development, and stress mitigation. Environmental stresses ultimately lead to energy deficit in the cell which activates the SNF1-RELATED KINASE 1 (SnRK1) signaling cascade which eventually triggering a massive reprogramming of transcription to enable the plant to survive under low-energy conditions. The role of *Arabidopsis thaliana FCS-Like Zinc finger* (*FLZ*) gene family in energy and stress signaling is recently come to highlight after their interaction with kinase subunits of SnRK1 were identified. In a detailed expression analysis in different sugars, energy starvation, and replenishment series, we identified that the expression of most of the *FLZ* genes is differentially modulated by cellular energy level. It was found that *FLZ* gene family contains genes which are both positively and negatively regulated by energy deficit as well as energy-rich conditions. Genetic and pharmacological studies identified the role of *HEXOKINASE 1*- dependent and energy signaling pathways in the sugar-induced expression of *FLZ* genes. Further, these genes were also found to be highly responsive to different stresses as well as abscisic acid. In over-expression of kinase subunit of *SnRK1*, *FLZ* genes were found to be differentially regulated in accordance with their response toward energy fluctuation suggesting that these genes may work downstream to the established *SnRK1* signaling under low-energy stress. Taken together, the present study provides a conceptual framework for further studies related to *SnRK1-FLZ* interaction in relation to sugar and energy signaling and stress response.

Keywords: *Arabidopsis*, *FLZ* gene family, sugar signaling, energy signaling, SnRK1, low-energy stress, abiotic stress, *HXK1*

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Introduction

Life is modulated by an array of internal and external factors which act as signals to control the fate of the organisms. In response to these factors, organisms tone their growth and reproduction so that they will effectively survive and reproduce. Cellular energy status is directly dependent on the supply of sugars. Internal sugar status is an important factor which controls the lifespan and aging in metazoans, yeast and plants (Heilbronn and Ravussin, 2005; Minina et al., 2013). Adequate sugar supply is required for the better stress tolerance. Glucose regulates a large number of genes involved in both biotic and abiotic stress response (Price et al., 2004). Sucrose promotes the survival

of plants under salt stress through G-protein signaling (Colaneri et al., 2014). Many of the mutants defective in sugar responses were found to be allelic to mutants of abscisic acid (ABA) and ethylene signaling suggesting a large interplay between sugar and stress signaling (Leon and Sheen, 2003).

In plants, stressful environment ultimately leads to energy deprivation in the cell which activates the evolutionarily conserved multi-subunit serine-threonine kinase SNF1-RELATED KINASE 1 (SnRK1). SnRK1 acts a central integrator of energy metabolism, stress, and developmental cues (Baena-González et al., 2007). Similar to its mammalian homolog AMP-activated protein kinase (AMPK), SnRK1 was also found to be phosphorylated on its T-loop and its dephosphorylation is inhibited by 5′-AMP (Sugden et al., 1999; Hey et al., 2007). In response to low-energy stress, SnRK1 promotes catabolism and minimize anabolism through reprogramming the transcriptional machinery which enables the plants to survive under low-energy conditions (Baena-González et al., 2007). It was also found that SnRK1 imparts tolerance to various biotic as well as abiotic stresses via controlling the transcription of genes involved in stress tolerance (Baena-González et al., 2007; Baena-González and Sheen, 2008; Cho et al., 2012; Confraria et al., 2013). SnRK1 activity is essential for establishing flooding stress tolerance in rice (Cho et al., 2012). A PP2C hub is involved in the regulation of both SnRK1 and ABA signaling suggesting an integrated molecular pathway in the mitigation of low-energy condition and abiotic stresses (Rodrigues et al., 2013).

The plant-specific FCS-like zinc finger (FLZ) domain proteins are implicated in the regulation of various biotic and abiotic stresses. *FCS-LIKE ZINC FINGER 9 /MEDIATOR OF ABA-REGULATED DORMANCY 1* (*FLZ9/MARD1*) is found to be involved in the ABA-mediated seed dormancy and up-regulated during leaf senescence (He et al., 2001; He and Gan, 2004). Over-expression of *FCS-LIKE ZINC FINGER 4 /INCREASED RESISTANCE TO MYZUS PERSICAE 1* (*FLZ4/IRM1*) rendered plants shorter which creates mechanical resistance to aphid attack (Chen et al., 2013). Over-expression of a wheat *FLZ* gene, salt-related hypothetical protein (*TaSRHP*) in *Arabidopsis thaliana* resulted in enhanced resistance to salt and drought stress (Hou et al., 2013). The expression analysis from the publically available microarray data suggests that members of *A. thaliana FLZ* gene family is responsive to ABA, JA, various abiotic stresses, glucose, and nitrogen and phosphorous deficiency (Nietzsche et al., 2014). Protein–protein interaction studies identified that all 18 *A. thaliana* FLZ proteins interact with kinase subunits of SnRK1 (Arabidopsis Interactome Mapping Consortium, 2011; Nietzsche et al., 2014).

The available evidence indicates the possible role of *FLZ* gene family in stress tolerance and adaptive growth. Similarly, their physical interaction with kinase subunits of SnRK1 suggests their relation with SnRK1 signaling. However, their relation with SnRK1 signaling particularly during energy fluctuations in the cell and abiotic stresses is not explored yet. In this study, we analyzed the transcriptional regulation of *FLZ* gene family during low-energy stress and energy rich conditions. We also identified that different sugar signaling pathways regulate the sugar-dependent transcription of *FLZ* genes. We also analyzed the expression of these genes under ABA treatment and different stresses with particular emphasize on salt stress. Over-expression of kinase subunit of SnRK1 resulted in differential regulation of some of the *FLZ* genes suggesting that SnRK1 signaling transcriptionally regulates these genes in the plants.

Materials and Methods

Plant Material and Growth Conditions

The *A. thaliana* Columbia (Col-0) and Landsberg erecta (Ler) ecotypes were used as controls in the experiments. All the experiments were done in Col-0 unless stated. Seeds of *gin2-1* (CS6383) were obtained from ABRC (https://abrc.osu.edu; Moore et al., 2003). *KIN10 OE2* seeds were provided by Prof. Filip Rolland (Metabolic signaling group, KU Leuven, Baena-González et al., 2007). For all experiments, seeds were surface sterilized and stored at 4°C for 48 h in dark for stratification. The imbibed seeds were grown on square petri plates containing 0.5X MS medium with 1% sucrose and 0.8% agar. The plates were kept vertically for germination and growth in climate-controlled growth room under 16:8 h photoperiod with 22 ± 2°C temperatures and 60 μmol m^{-2} s^{-1} light intensity unless stated. Five-days old seedlings grown in standard growth conditions were used for all experiments and at least 40 seedlings were harvested for each sample. *KIN1OE2* and Ler seedlings grown for 5 days in the standard growth conditions were used for gene expression analysis.

Sugar Starvation and Replenishment Assay

Five-days old uniformly grown Col-0 seedlings under standard growth condition were used for sugar starvation and replenishment assay. Plants were starved in 0.5X MS liquid medium without sucrose in 22°C at 140 rpm in darkness. Samples were collected after 3, 6, 12, and 24 h time points of starvation. After 24 h time point, the plants were transferred to 0.5X liquid medium with sucrose and grown under 22°C at 140 rpm in the light. Samples were collected after 3, 6, 12, and 24 h time points of replenishment.

Sugar Sensitivity Assays and Treatment with Chemical Inhibitors

The sugar sensitivity assay is done as described previously using 3% glucose/sucrose/3-*O*-methylglucose/mannose/mannitol individually (Mishra et al., 2009). For sugar sensitivity assay in glucose signaling mutant *gin2-1*, the Ler and *gin2-1* seedlings were subjected to the same treatment. For sugar sensitivity assay along with metabolic inhibitors, Col-0 seedling were transferred to 0.5X MS medium with 3% glucose or sucrose with 3% 2-Deoxy-D-glucose (2DG)/5 μM Antimycin-A (AmA)/50 μM 2,4-Dinitrophenol (DNP)/10 μM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma–Aldrich) individually in the same treatment condition mentioned above. For 2DG treatment, 5-days old Col-0 seedlings grown in standard growth conditions were treated with 25, 50, and

100 mM 2DG in 0.5X liquid MS medium in the dark for 3 h at 140 rpm.

Abscisic Acid and Salt Treatments

Five-days old uniformly grown Col-0 seedlings grown under standard growth condition were used for ABA as well as NaCl treatment. The seedlings were treated with 10 μM ABA in 0.5X liquid MS medium for 30 min, 3 and 6 h time points at 22°C at 140 rpm in the light. For salt treatment, the seedlings were subjected to 150 mM NaCl treatment in 0.5X liquid MS medium for 3 h at 22°C at 140 rpm in the light.

Gene Expression Analysis

RNA isolation and cDNA preparation were performed as described previously (Mishra et al., 2009). qRT-PCR were done with 1:50 diluted cDNA samples with SYBR-Green PCR master mix in 384-well optical reaction plates employing Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The primers were prepared from the transcript sequence of genes using PRIMER EXPRESS version v3.0 (Applied Biosystems, USA) with default parameters. *18S rRNA* and *UBQ10* were as used endogenous controls and relative quantification of the mRNA level of candidate genes were calculated by ΔΔCT method (Livak and Schmittgen, 2001). Primers used for qRT-PCR experiments are given in Supplementary Table S1. The heat maps were generated from the gene expression data using MultiExperiment Viewer (MeV, v4.8; Saeed et al., 2006). The hierarchical clustering of genes was performed by Pearson correlation algorithm with average linkage clustering.

The digital gene expression analysis under ABA and abiotic stress treatments was done using the AtGenExpress data available through *Arabidopsis* eFP Browser (Kilian et al., 2007; Winter et al., 2007; Goda et al., 2008). The expressions in the treatment time points were calculated using control expression values and heat map and hierarchical clustering were done as described above.

Promoter: *GUS* Line Construction and Starvation Assay

For promoter: *GUS* transcriptional fusion constructs (p*FLZ1*::*GUSA*, p*FLZ6*::*GUSA*, and p*FLZ8*::*GUSA*), primers designed from 2 kb 5′ UTR upstream region of *FLZ1* and *FLZ6* and 1.5 kb 5′ UTR upstream region of *FLZ8*. Primers used for cloning are listed in Supplementary Table S1. The amplified products were cloned in pMDC164 vector and transformed into Col-0 plants through floral dip transformation method (Clough and Bent, 1998; Curtis and Grossniklaus, 2003). The transformants were selected on 0.5X MS plates supplemented with 15 μg/ml hygromycin A and homozygous lines were identified in T3 generation. For starvation assay of promoter:: GUS lines, leaves of the same developmental stage from the rosette plants grown in standard growth conditions were detached and kept in petri plates with soaked filter paper in the dark for 1 and 2 days. The leaves were GUS stained for 1 h as described previously (Jefferson et al., 1987).

Results

Response of *Arabidopsis thaliana FLZ* Gene Family Genes Toward Cellular Energy Fluctuation

The microarray-based expression analysis and protein–protein interaction analysis with KIN10/11 suggests the involvement of *FLZ* genes in low-energy stress (Nietzsche et al., 2014). In order to study the response of *FLZ* gene family genes in low-energy stress at the transcriptional level, we did an extensive energy depletion and replenishment assay using 5-days old Col-0 seedlings (Supplementary Figure S1A). The expression of all 18 *A. thaliana FLZ* genes was analyzed at different time points of low-energy stress and energy replenishment conditions (**Figure 1A**). It was found that the transcript levels of a group of genes were rapidly decreased on the onset of low-energy stress and these genes formed a distinct cluster in the heat map. The expression of these genes was significantly down-regulated in response to mild as well as prolonged energy depletion and their expressions were rapidly up-regulated in response to sugar replenishment (Supplementary Figure S1B). *FLZ1, FLZ2, FLZ3, FLZ5, FLZ8,* and *FLZ14* belong to this cluster of genes whose levels are rapidly perturbed in response to energy depletion and replenishment. Among other members of this gene family, *FLZ12*, *FLZ7,* and *FLZ15* do not show any significant difference in the expression in all treatments studied. *FLZ4*, *FLZ10*, *FLZ11,* and *FLZ16* showed significant up-regulation of transcript level in the mild energy depletion. However, prolonged energy depletion significantly reduced their expression and sugar replenishment restored their expression to control level in most of these genes. *FLZ9* showed a mild repression of transcript level at the onset of energy depletion; however, it showed significant repression of transcript levels after sugar replenishment. Interestingly, prolonged starvation significantly induced the transcript levels of *FLZ6* and *FLZ17*/18 suggesting that these genes may be involved in the regulation of growth under prolonged low-energy stress. Consistent with this, it was found that the level of these two genes gradually reached the control level after energy is replenished to the plant. The levels were further reduced during prolonged incubation in energy-rich conditions (**Figure 1A**).

In order to validate the results obtained from energy depletion and replenishment assay, the expression of two selected *FLZ* genes was analyzed in samples treated with increasing concentrations of glycolysis inhibitor 2DG (**Figure 1B**). 2DG is a non-metabolizable glucose analog which is also a substrate of hexokinase and blocks the glycolytic pathway through competitive inhibition which ultimately leads to energy depletion in the cell (Wick et al., 1957). Increasing concentrations of 2DG significantly repressed the level of *FLZ2* which was found to be negatively regulated during low-energy stress in the previous experiment. *FLZ6*, which was found to be positively regulated by prolonged low-energy stress, was found to be significantly up-regulated in response to 2DG treatment. These

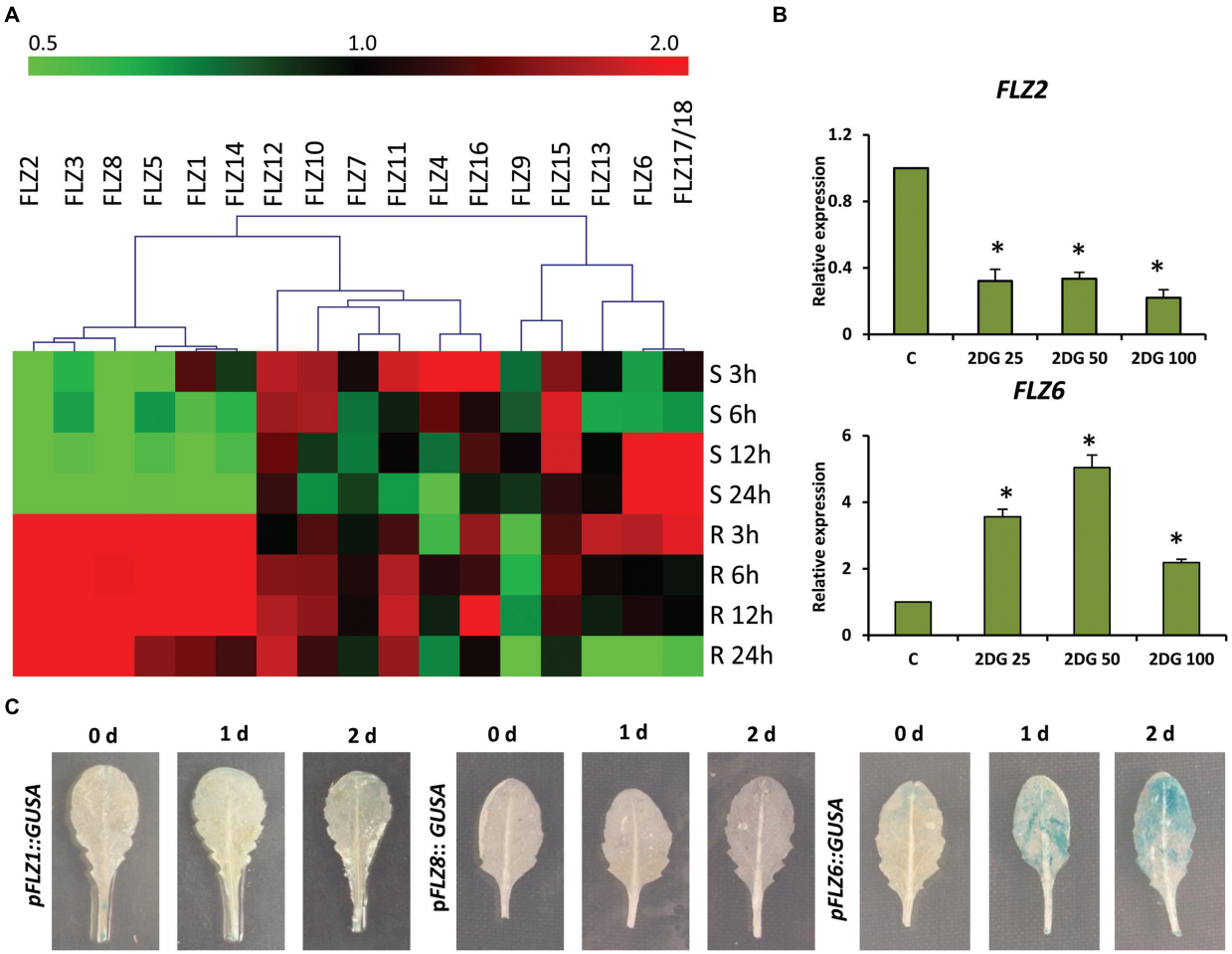


FIGURE 1 | Expression of *Arabidopsis thaliana FLZ* gene family during low-energy stress and replenishment. (A) Expression of *A. thaliana FLZ* gene family under energy starvation and replenishment studied with qRT-PCR. S denotes 'starvation' and R denotes 'recovery.' The expression in 0.5X MS grown plants was taken as the control to compare the expression in low-energy levels and replenishment series. The genes were subjected to hierarchical clustering based on their response toward these treatments. *UBQ10* was used as endogenous control. **(B)** Expression of *FLZ2* and *FLZ6* in response to increasing concentration of 2DG (25, 50, and 100 mM). The bars represent the average of two independent biological experiments with three technical replicates each and error bars represent SE. *UBQ10* was used as endogenous control. Asterisk indicates a significant difference of expression in the treatment compared to control ($P < 0.005$, Student's *t*-test). **(C)** The response of *pFLZ1*::*GUSA*, *pFLZ8*::*GUSA*, and *pFLZ6*::*GUSA* transcriptional-reporter lines toward energy starvation for 0, 1, and 2 days.

results validate the observations of the previous experiment. In order to further confirm these results, we used reporter lines of starvation-repressible and sugar-inducible genes *FLZ1* and *FLZ8* (*pFLZ1*::*GUSA* and *pFLZ8*::*GUSA*) and prolonged starvation-inducible gene *FLZ6* (p*FLZ6*::*GUSA*). Uniformly grown leaves of the same developmental stage were subjected to low-energy stress for 1 and 2 days and the GUS activity was compared with the leaves grown in normal conditions (**Figure 1C**). The GUS activity was found to be too low in the untreated leaves which is consistent with the earlier observation that the expression of these genes are too low in the mature leaves compared to other tissues (Jamsheer et al., 2015). The starvation could not induce the GUS activity in the leaves of *pFLZ1*::*GUSA* and p*FLZ8*::*GUSA* lines. However; low-energy stress induced GUS expression in p*FLZ6*::*GUSA* line. All these results conclusively suggest that *FLZ* gene family contain genes which are positively and negatively regulated during low and high energy levels in the plant.

Response of *FLZ* Genes Toward Sugars

In the energy depletion and replenishment assay, it was found that the expression of many genes was positively or negatively regulated by sucrose replenishment. In order to dissect how the *FLZ* genes respond to sugar and energy rich condition in the cell, 24 h energy-starved Col-0 plants were treated with glucose, sucrose, mannose, and sugar alcohol mannitol. In order to rule out the effects of sugars other than the nutrient effect, seedlings were also treated with a non-metabolizable and non-toxic glucose analog, 3-*O*-methyl glucose (3-OMG; Cortès et al., 2003). Further, mannitol was used as an additional osmotic control to rule out the involvement of osmotic regulation of *FLZ* genes in the results. Among sugars, glucose, and sucrose profusely

altered the transcript level of many genes (Supplementary Figure S2).

Based on their response toward glucose and/or sucrose treatment, the *FLZ* gene family was divided into three distinct classes. The transcript level of class I genes was induced by ≥10 fold by glucose and/or sucrose (**Figure 2A**). *FLZ1, FLZ2, FLZ5, FLZ8,* and *FLZ14* genes belong to this class of high sugar-inducible genes. Class II genes are medium sugar-inducible genes which are induced by ≥2 fold by glucose and/or sucrose (**Figure 2B**). Class III includes sugar non-responsive genes as well as sugar-repressible genes (**Figure 2C**). *FLZ6* could not display any significant response toward sugar treatments (Supplementary Figure S2). Interestingly, the levels *FLZ7*, *FLZ9*, *FLZ12*, and *FLZ17*/18 were found to be significantly decreased in glucose, sucrose or in both sugars suggesting that these genes are sugar-repressed genes.

Mannose could also affect the transcription of many genes albeit to a lesser extent (Supplementary Figure S2). Among the sugar-induced genes, *FLZ1*, *FLZ2*, *FLZ3*, *FLZ4* were found to be up-regulated in the presence of mannose. Similarly, sugar down-regulated genes such as *FLZ7*, *FLLZ9*, and *FLZ17*/18 were found to be also downregulated by mannose. *FLZ12*, which is specifically repressed by sucrose showed highest positive response toward mannose treatment.

Role of Different Sugar Signaling Pathways in the Sugar Responsiveness of *FLZ* Genes

It is already identified that different sugar signaling pathways act individually or along with other sugar signaling pathways to regulate the expression of sugar-responsive genes. The role of HXK1-dependent glucose signaling pathway is attributed to many glucose-mediated responses in plants (Mishra et al., 2009). Consistent with this, the glucose-induced up-regulation of key transcription factors involved in the aliphatic glucosinolate biosynthesis is completely abolished in the *gin2-1* mutant (Miao et al., 2013). Similarly, the glucose-dependent repression of *TANDEM ZINC FINGER 1* transcription is dependent on HXK1-signaling (Lin et al., 2011). These reports suggest that downstream effects of glucose at the transcriptional level are partly mediated by HXK1-dependent glucose signaling pathway. Similarly, the role of TOR and SnRK1 dependent energy signaling pathway is also implicated in the regulation of many sugar responsive genes (Baena-González et al., 2007; Ramon et al., 2008; Xiong et al., 2013).

Genetic and pharmacological approaches were used to identify the role of different sugar signaling pathways on the sugar responsiveness of *FLZ* genes. To study whether HXK1-dependent glucose signaling has any role in the transcriptional up-regulation of *FLZ* genes, we checked the glucose responsiveness of sugar-inducible *FLZ* genes in the *gin2-1* mutant. It was found that the transcript induction of *FLZ* genes after glucose treatment was significantly reduced in the *gin2-1* mutant (**Figure 3**). Abolition of the HXK1-dependent glucose signaling pathway could only partially affect the sugar-dependent activation of *FLZ* genes suggesting the involvement of other sugar signaling pathways in this response. The treatment of 2DG significantly reduced the transcript level of *FLZ2* which suggest that ultimately cellular energy level regulates the expression of sugar-induced *FLZ* genes. To confirm this observation, sugar depleted seedlings were treated with sugars alone or in combination with chemicals which inhibit glycolysis and various steps of oxidative phosphorylation. The glycolysis inhibitor 2DG, the mitochondrial electron transport blocker AMA and mitochondrial uncoupling agents CCCP and DNP were used for studying the effect of metabolic signaling on the induction of *FLZ* genes (Xiong et al., 2013). It was found that the induction of transcript level by sugars was severely abolished when sugars were given in combination with metabolic inhibitors (**Figure 4**).

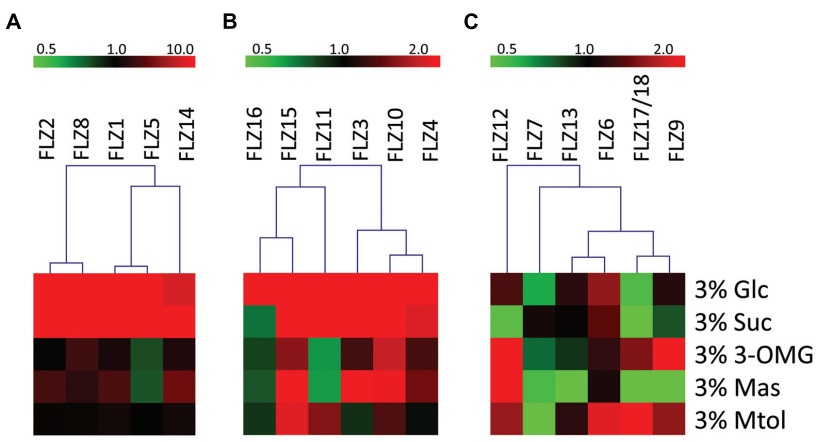


FIGURE 2 | Sugar sensitivity of *Arabidopsis thaliana FLZ* gene family. The *A. thaliana FLZ* gene family was classified into three groups based on their response toward sugar treatments in qRT-PCR experiments. The expression in 0% sugar grown plants was taken as the control for comparing the expression in other treatments and *18S rRNA* was used as endogenous control. **(A)** High sugar-inducible genes (Class I, induced by ≥10 fold by glucose and/or sucrose). **(B)** Medium sugar-inducible genes (Class II, induced by ≥2 fold by glucose and/or sucrose). **(C)** Sugar non-responsive and sugar-repressible genes (Class III, significantly repressed by glucose and/or sucrose).

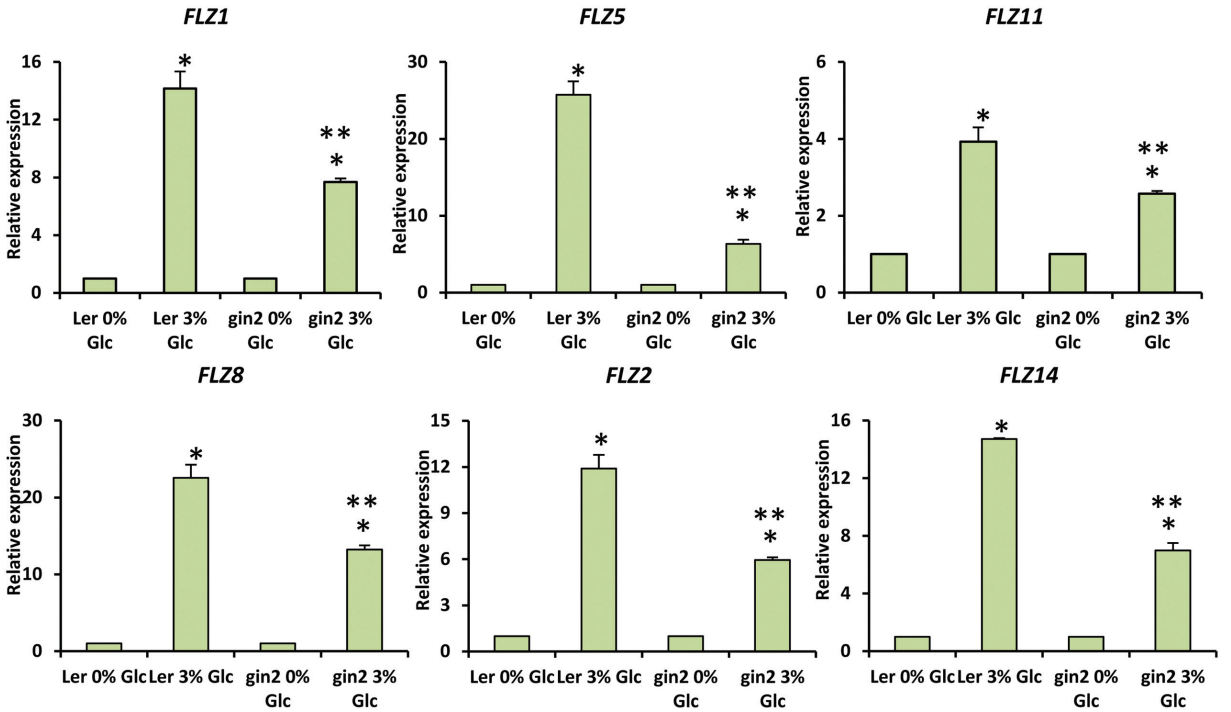


FIGURE 3 | Involvement of HXK1-dependent glucose signaling in the regulation of glucose-dependent up-regulation of *FLZ* genes. The effect of *gin2-1* mutation on the expression of glucose-responsive *FLZ* genes was studied with qRT-PCR. The expression in 0% sugar treatment in Ler and *gin2-1* were taken as the control individually for comparing the expression in 3% glucose treatment in WT and mutant respectively. *18S rRNA* was used as endogenous control. The bars represent the average of two independent biological experiments with three technical replicates each and error bars represent SE. Single asterisk indicates a significant difference of expression in the treatment compared to control 0% sugar. Double asterisk indicates a significant difference of expression in the *gin2-1* mutant after the treatment compared to the treatment in Ler ($P < 0.005$, Student's *t*-test).

Response of *FLZ* Genes towards Different Abiotic Stresses and ABA Treatment

Over-expression of a wheat *FLZ* gene *TaSRHP* in *A. thaliana* resulted in enhanced salt stress tolerance. *TaSRHP* found to be a positive regulator of many stress-related genes (Hou et al., 2013). This result suggests an involvement of *FLZ* genes in salt stress response. Most of the *FLZ* genes were found to be responsive to the cellular energy deficit which is also caused by stresses. We made use of available microarray data to investigate whether salt stress directly regulates the expression of *FLZ* genes and identified that salt stress differentially regulate the expression of all *FLZ* genes (**Figure 5A**). Genes like *FLZ2*, *FLZ10*, *FLZ11*, and *FLZ15* were found to be constantly up-regulated during salt stress. However, most of the genes showed temporal response toward the salt treatment. In order to validate the microarray data, 5-days old seedlings were subjected to salt stress for 3 h and the expression level of 4 *FLZ* genes which were shown differential regulation in the microarray data was analyzed (**Figure 5B**). As observed in the microarray data, *FLZ10* and *FLZ11* were found to be moderately up-regulated in response to 3 h salt stress. Similarly, moderate down-regulation of *FLZ13* was observed in the qRT-PCR data also. *FLZ17/18*, which were found to be profusely up-regulated in almost all stages in the microarray data was found to be profusely up-regulated in the salt treated 5-days old seedlings too.

We also analyzed the expression pattern of *FLZ* genes under other abiotic stress using available microarray data and it was found that the expression of these genes were considerably fluctuated in response to different abiotic stresses (Supplementary Figure S3). In general, it can be seen that the response of these genes toward different stresses are more or less spatiotemporal. The varied response of *FLZ* genes toward different abiotic stress prompted us to investigate the response of these genes toward ABA. Digital expression analysis identified that many members are of this gene family are differentially regulated in response to ABA treatment (**Figure 5C**). Most of the genes were found to be positively regulated by ABA while few genes like *FLZ13* were found to be down-regulated. In order to validate the microarray data, we analyzed the response of two *FLZ* genes in 5-days old seedlings treated with ABA at three different time points (**Figure 5D**). Among these two genes, the ABA-responsiveness of *FLZ9* is already reported earlier which can be used as excellent marker for validating microarray data (He and Gan, 2004). As observed in the microarray data, the expression of *FLZ9* was found to be gradually increased in response to ABA treatment. Similarly, the expression of *FLZ17/18* was also found to be significantly increased in response to 3 h ABA treatment. Taken together, all these results suggest the possible involvement on *FLZ* genes in plant growth under stress.

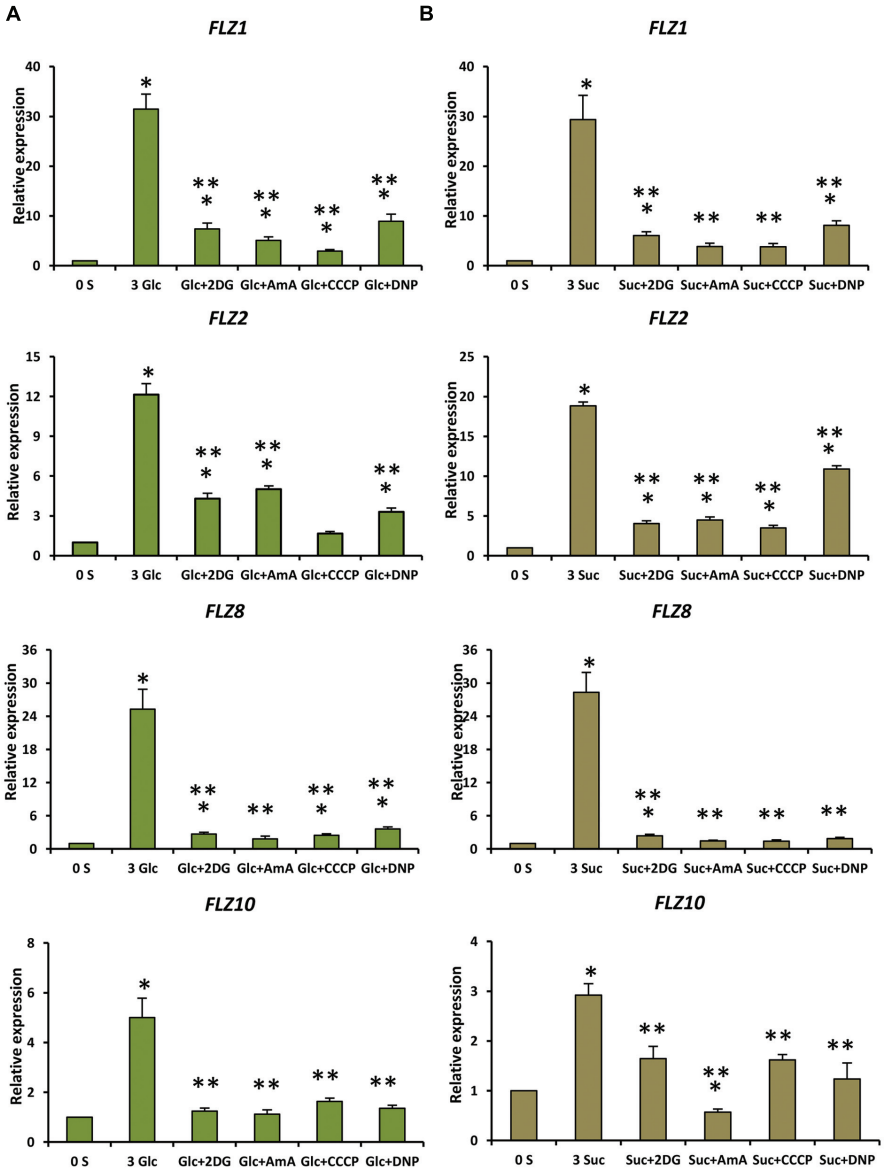


FIGURE 4 | Involvement of energy signaling in the regulation sugar-dependent up-regulation of *FLZ* genes. The effect of metabolic inhibitors on the sugar-mediated transcriptional up-regulation of *FLZ* genes was studied by qRT-PCR. **(A)** The effect of metabolic inhibitors on the glucose-sensitivity of *FLZ* genes. **(B)** The effect of metabolic inhibitors on the sucrose-sensitivity of *FLZ* genes. The expression in 0% sugar grown plants was taken as the control for comparing the expression in 3% sugar treatment alone or with metabolic inhibitors. *UBQ10* was used as endogenous control. The bars represent the average of two independent biological experiments with three technical replicates each and error bars represent SE. Single asterisk indicates a significant difference of expression in the treatment compared to control 0% sugar. Double asterisk indicates a significant difference of expression in the treatment compared to 3% Glc or 3% Suc ($P < 0.005$, Student's *t*-test).

Involvement of SnRK1 in the Regulation of *FLZ* Genes

It is already established that SnRK1 regulates the expression of genes involved in the mitigation of multiple stresses including low-energy stress (Baena-González et al., 2007). The over-expression of SnRK1.1 in *A. thaliana* resulted in enhanced starvation tolerance, late senescence, and perturbation in the normal developmental processes (Baena-González et al., 2007). In order to investigate whether the *FLZ* genes have any connection with the conserved SnRK1 signaling cascade, we checked the expression of selected *FLZ* genes from all three classes in *KIN10* over-expression line *KIN10 OE2* (Supplementary Figure S4). It was found that SnRK1 repress the transcription of sugar-inducible *FLZ* genes (**Figure 6A**). The degree of repression in high sugar-inducible genes (Class I) like *FLZ2*, *FLZ3*, and *FLZ8* were found to be more severe compared to the repression in medium sugar-inducible gene (Class II) *FLZ11*. The expression of sugar-repressible genes (Class III) *FLZ9* and

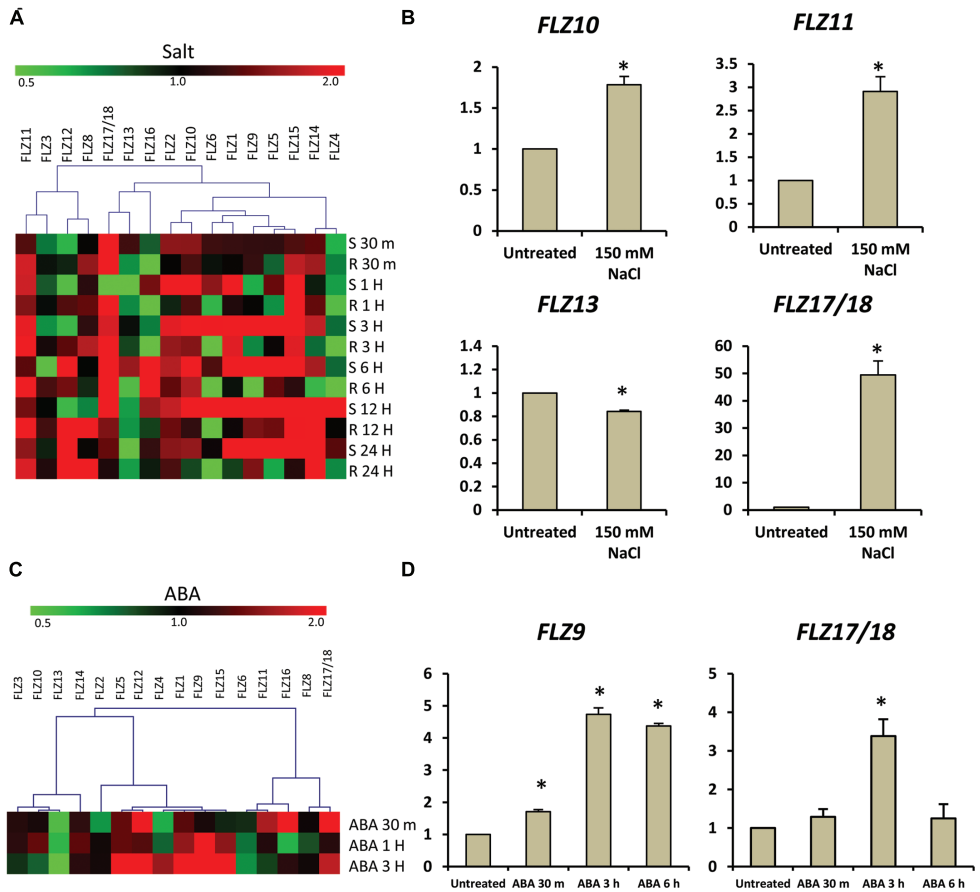


FIGURE 5 | Expression of *FLZ* genes during salt stress and abscisic acid. The impact of salt stress and ABA on the expression of *FLZ* genes was studied using available microarray data and by qRT-PCR. **(A)** The expression of *FLZ* genes during salt stress from available microarray data. The expression data was downloaded from the AtGenExpress global stress expression data set (Kilian et al., 2007) available through *Arabidopsis* eFP Browser (Winter et al., 2007). The genes were subjected to hierarchical clustering based on their response toward salt stress. S denotes 'shoot' and R denotes 'root.' **(B)** The expression of selected *FLZ* genes in 5-days old seedlings under salt stress. **(C)** The response of *FLZ* genes toward ABA from available microarray data available through *Arabidopsis* eFP Browser (Winter et al., 2007; Goda et al., 2008). **(D)** The expression of two selected *FLZ* genes in 5-days old seedlings treated with ABA at three different time points. In both salt stress and ABA treatments, the expression in 5-days old Col seedlings grown on 0.5X MS was used as the control to compare the transcript level of *FLZ* genes under salt stress and ABA. *18S rRNA* was used as endogenous control. The bars represent the average of two independent biological experiments with three technical replicates each and error bars represent SE. Asterisk indicates a significant difference of expression in the treatment compared to control ($P < 0.005$, Student's *t*-test).

FLZ17/18 were found to be significantly increased in *KIN10* over-expression line (**Figure 6B**). However, the expression of sucrose-repressible and mannose-inducible gene *FLZ12* was found to be unperturbed. Mannose-repressible *FLZ13* level was also found to be unchanged in the *KIN10* over-expression lines suggesting that the regulation of SnRK1 on *FLZ* gene is directly dependent on their response toward metabolizable sugars such as glucose and sucrose.

Discussion

FLZ Genes are Transcriptionally Regulated by Sugars, ABA, and Abiotic Stresses

It is already reported that *FLZ9* expression is induced during leaf senescence and nutrient deficiency differentially regulates *FLZ* genes (He et al., 2001; Nietzsche et al., 2014). In our analysis, it was found that the expression of most of the *FLZ* gene family members is differentially regulated in response to sugar treatment and under energy depleted and replenished conditions. The expressions of these genes were found to be highly regulated by glucose, sucrose and to a very less extent by mannose which confirms that this response is dependent on sugar signaling and metabolism but not dependent on the osmotic effects of sugars. We could classify the *FLZ* genes into three classes based on their response toward sugars. Each class contains genes in which some of them is phylogenetically closer and some of them are phylogenetically very distant suggesting that the specific response toward sugars is not confined to any specific clad defined in an earlier study (Jamsheer and Laxmi, 2014). The expression of all high sugar-inducible genes (Class I) was repressed in response to low-energy stress and their levels were rapidly increased during

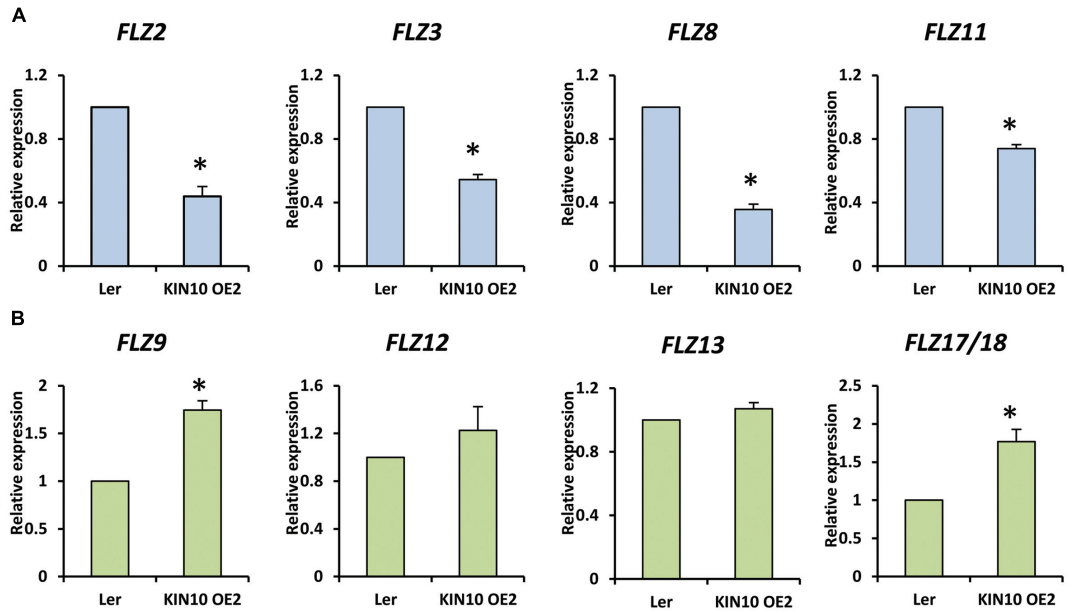


FIGURE 6 | Expression of *FLZ* genes in SnRK1.1 over-expression line. The transcript level of selected *FLZ* genes in *KIN10 OE2* seedlings were studied with qRT-PCR. **(A)** The expression of sugar-inducible (Class I and II) *FLZ* genes in *KIN10 OE2*. **(B)** The expression of Class III *FLZ* genes in *KIN10 OE2*. The expression in 5-days old Ler was used as the control to compare the transcript level of *FLZ* genes in *KIN10 OE2*. *UBQ10* was used as endogenous control. The bars represent the average of two independent biological experiments with three technical replicates each and error bars represent SE. Single asterisk indicates a significant difference of expression in *KIN10 OE2* compared to Ler ($P < 0.005$, Student's *t*-test).

sugar replenishment. Similarly, *FLZ3* which is a paralog of high sugar-inducible genes *FLZ1* and *FLZ2* showed a similar response. In medium sugar-inducible group (Class II), many genes found to be were up-regulated in response to mild low-energy stress while prolonged stress repressed their levels. Some of the sugar-repressible genes (Class III) identified from the sugar sensitivity assay showed induction of transcript levels in response to low-energy stress. *FLZ6* and *FLZ17/18* were found to be responsive to prolonged starvation. These results suggest that majority of *FLZ* gene family members are responsive to sugar levels and cellular starvation. These results were further supported by GUS assay and 2DG treatment.

It is already well known that sugars transcriptionally regulate a large number of genes which belongs to diverse cellular processes. These sugar-regulated genes include genes involved in primary and secondary metabolism, hormone signaling and developmental processes such as phase transition, flowering, senescence etc. (Price et al., 2004; Li et al., 2006; Mishra et al., 2009; Xiong et al., 2013). Functional analysis of these genes deciphered molecular mechanisms by which sugars regulate different aspects of plant development. For example, the sugar-induced expression of nucelolin-1 in *A. thaliana* is important for ribosome synthesis (Kojima et al., 2007). Similarly, the glucose-TARGET OF RAPAMYCIN (TOR) signaling cascade regulates the transcription of many genes including cell cycle genes which is important in the establishment of early seedling growth (Xiong et al., 2013). From our analysis, it has been found that the cellular sugar and energy level is an important regulator of transcription of many *FLZ* genes. Similar functional analysis of individual *FLZ* genes will give more information on the molecular aspects of the sugar-mediated regulation of plant growth. Interestingly, in an earlier study, we have identified that most of the *FLZ* genes are differentially regulated during vegetative-to-reproductive phase transition (Jamsheer et al., 2015). The role of sugars on the developmental phase transition and flowering is already known. Sugars known to regulate these processes by controlling the activity of microRNAs and other well know regulators involved in the developmental phase transition and flowering (Seo et al., 2010; Wahl et al., 2013; Yang et al., 2013; Yu et al., 2013). The differential regulation of *FLZ* genes during vegetative-to-reproductive phase transition further supports the possible role of *FLZ* gene family in the sugar-mediated regulation of plant growth. The interaction of these proteins with kinase subunits of SnRK1 and regulatory subunit of TOR kinase also suggest their involvement of *FLZ* genes in the sugar-mediated regulation of plant growth (Arabidopsis Interactome Mapping Consortium, 2011; Nietzsche et al., 2014). Our transcriptional studies and earlier protein: protein interaction studies could suggest a link between sugars and *FLZ* gene family; however, more molecular studies are needed to establish this connection.

Transcriptional studies also identified that different abiotic stresses and ABA can regulate the expression of many *FLZ* genes. These results suggest the possible involvement of *FLZ* genes in mediating adaptive growth under abiotic stress conditions. There are already few reports which link *FLZ* genes in providing stress tolerance and involvement in ABA-directed processes. Over-expression of a wheat *FLZ* gene, *TaSRHP* in *A. thaliana* positively regulated the expression of many stress-related genes

and enhanced resistance toward different abiotic stresses (Hou et al., 2013). The *FLZ9/MARD1* gene of *A. thaliana* was found to involved in ABA-mediated seed dormancy (He and Gan, 2004). Similarly, the interplay between sugars and stress signaling is well established. A large overlap between sugar- and stress-responsive genes is already reported by different groups suggesting the role of sugars and cellular energy in providing stress-tolerance (Price et al., 2004; Li et al., 2006). Disruption of many genes resulted in perturbation of both ABA- and sugar-mediated responses further suggest an overlap between glucose and stress signaling (Arenas-Huertero et al., 2000; Brocard et al., 2002). Our transcriptional studies firmly established that the *FLZ* genes are regulated by sugars, ABA, and abiotic stresses. Since sugar and cellular energy status is important in dealing stresses, this class of genes which are co-regulated by both sugars and stresses might be important in the regulation of adaptive responses toward stress and stress-derived energy depletion (**Figure 7**). The studies in this direction may open a novel molecular pathway by which sugars regulate adaptive growth during stress.

The Sugar-Induced Expression of *FLZ* Genes is Dependent on HXK1-Dependent Signaling and Metabolism-Dependent Energy Signaling

Different glucose signaling pathways are implicated in the control of sugar-regulated gene expression in both distinct and overlapping manner (Miao et al., 2013; Xiong et al., 2013). In our study, we found that the sugar-induced expression of *FLZ* genes is regulated by two different sugar signaling pathways. Abolition of HXK1-dependent pathway reduced the expression of *FLZ* genes considerably after sugar treatment suggesting that HXK1-dependent signaling acts as a positive regulator of glucose-induced expression of *FLZ* genes. This pathway is independent of the kinase activity of HXK1 and decoupled from the sugar metabolism-dependent signaling (Moore et al., 2003). HXK1 form complex with H^+-ATPase B1 and the 19S regulatory particle of proteasome subunit in the nucleus and sugar-regulated expression of many genes are reported to be dependent on this pathway (Cho et al., 2006; Lin et al., 2011; Miao et al., 2013). However, in case of sugar-induced *FLZ* genes, the abolition of the HXK1-dependent pathway could only partially hamper the transcript induction suggesting the involvement of other sugar signaling pathways.

The expression of *FLZ* genes was severely abolished when the cellular respiratory pathway was blocked independently at glycolysis and oxidative phosphorylation stages implying the role of metabolism-dependent energy signaling as the pivotal regulator of the sugar-induced *FLZ* gene expression. This observation is further supported by the contrasting response of *FLZ2* and *FLZ6* toward 2DG treatment. The metabolism dependent energy signaling pathway is implicated in controlling TOR and SnRK1 signaling (Nunes et al., 2013; Xiong et al., 2013). It is earlier reported that glucose-control of cell division, meristem activation, and the developmental transition is dependent on metabolism-dependent energy signaling pathway and independent of HXK1-dependent glucose signaling pathway. Understandably, all major hormone signaling pathways and the HXK1-dependent glucose signaling found to be downstream to the metabolism-dependent control of plant growth (Xiong et al., 2013). Our results suggest that induction of *FLZ* genes under sugar and energy rich conditions is controlled by metabolism-dependent energy signaling. The HXK1-dependent glucose signaling acts as a positive regulator of glucose-induced transcriptional up-regulation of *FLZ* genes. This regulation may be important for the fine tuning of *FLZ* gene function in response to sugar and energy signaling. Besides, the dual regulation of sugar-dependent transcriptional induction of *FLZ* genes by both HXK1-dependent glucose signaling and metabolism-dependent energy signaling suggest the possibility that *FLZ* genes might be acting as a cross-talking hub for different sugar signaling pathways established in plants.

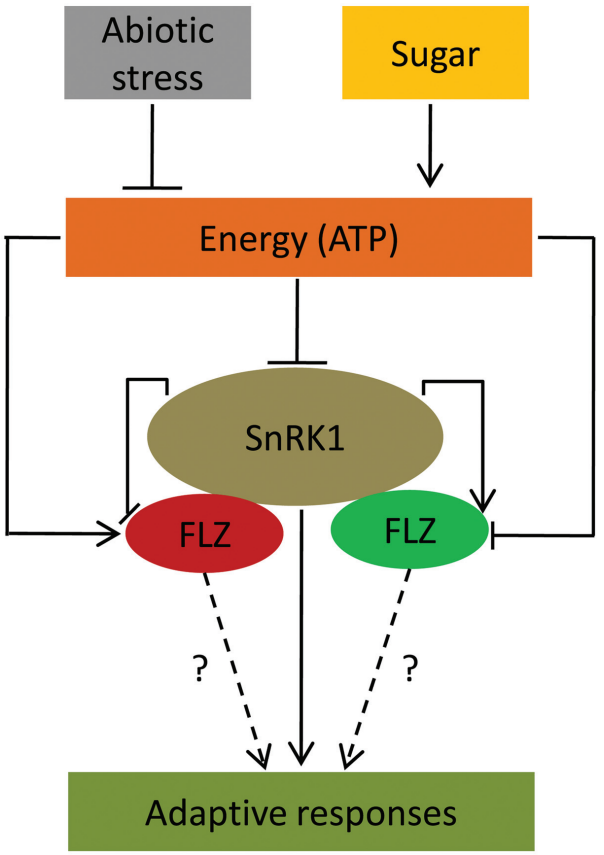


FIGURE 7 | A conceptual model suggesting *FLZ* genes as a factor involved in the regulation of adaptive response under energy fluctuations and abiotic stress. Sugars, cellular energy level and abiotic stress regulate the expression of *FLZ* genes. Sugar-induced *FLZ* genes are shown in red color and sugar down-regulated genes are shown in green color. Stress-driven energy deficit activates SnRK1 which in turn activates the genes involved in the adaptive responses toward stresses (Baena-González et al., 2007; Cho et al., 2012; Confraria et al., 2013). *FLZ* genes are also involved in the stress mitigation (Hou et al., 2013). SnRK1 transcriptionally regulates *FLZ* genes. FLZ proteins physically interact with kinase subunits of SnRK1 (Arabidopsis Interactome Mapping Consortium, 2011; Nietzsche et al., 2014); however, the biological significance of these interactions is still unknown.

SnRK1 Regulation of *FLZ* Gene Transcription

The gene expression analysis in *KIN10* over-expression identified that SnRK1 regulates the transcription of *FLZ* genes. Interestingly, this regulation of SnRK1 on the transcription of *FLZ* genes found to have a connection with the sugar-response of these genes. The sugar-inducible *FLZ* genes were found to be repressed while the sugar-repressible genes were found to up-regulated in the *KIN10* over-expression line. The repression was found to be more severe in high sugar-inducible genes (Class I) compared to medium sugar-inducible gene *FLZ11* (Class II). Consistent with this hypothesis, it was found that *KIN10* positively regulates the expression of class III sugar-repressible genes *FLZ9* and *FLZ17*/*18*. These results provide a framework for the further studies regarding the role of *FLZ* gene in the regulation of stress responses, particularly during low-energy stress. This result suggests that SnRK1 is possibly working upstream to regulate the transcription of *FLZ* genes. It is already known that SnRK1 undertake a massive reprogramming of transcription during low-energy stress (Baena-González et al., 2007). Interestingly, the contrasting regulation of Class I and II genes and Class III genes in the *KIN10* over-expression line add more complexity to the situation. Molecular study of this sugar-response dependent regulation of *FLZ* genes by SnRK1 would reveal the further intricacies of adaptive growth under energy stress. The genetic studies using *FLZ* gene mutants and over-expression studies can tell whether these genes work downstream to SnRK1 in this pathway. Besides, it is known that both kinase subunits of SnRK1 physically interact with all FLZ proteins in *A. thaliana* (Arabidopsis Interactome Mapping Consortium, 2011; Nietzsche et al., 2014). Identification of biological significance of these interactions will be crucial in the elucidation of the molecular regulation of energy signaling-SnRK1 interaction. The FLZ proteins can be a downstream factor by acting as kinase substrate of SnRK1 or it is possible that this interaction regulate the kinase activity of SnRK1. Considering the regulation of sugar and energy level on SnRK1 activity and *FLZ* gene expression, both hypotheses deserve the merit for further studies. Low light and various abiotic stresses ultimately lead to energy deficit in the cell and the response of *FLZ* gene toward energy fluctuation and stress suggest that they are possibly involved in the regulation of adaptive responses which enable the plant to survive in these non-favorable conditions. More genetic, molecular and physiological studies are needed to decipher this pathway.

Author Contributions

Conceived and designed the experiments: MJK and AL. Performed the experiments: MJK. Analyzed the data: MJK and AL. Contributed reagents/materials/analysis tools: MJK and AL. Wrote the paper: MJK and AL.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00746

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NAC transcription factors in plant multiple abiotic stress responses: progress and prospects

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Abiotic stresses adversely affect plant growth and agricultural productivity. According to the current climate prediction models, crop plants will face a greater number of environmental stresses, which are likely to occur simultaneously in the future. So it is very urgent to breed broad-spectrum tolerant crops in order to meet an increasing demand for food productivity due to global population increase. As one of the largest families of transcription factors (TFs) in plants, NAC TFs play vital roles in regulating plant growth and development processes including abiotic stress responses. Lots of studies indicated that many stress-responsive NAC TFs had been used to improve stress tolerance in crop plants by genetic engineering. In this review, the recent progress in NAC TFs was summarized, and the potential utilization of NAC TFs in breeding abiotic stress tolerant transgenic crops was also be discussed. In view of the complexity of field conditions and the specificity in multiple stress responses, we suggest that the NAC TFs commonly induced by multiple stresses should be promising candidates to produce plants with enhanced multiple stress tolerance. Furthermore, the field evaluation of transgenic crops harboring *NAC* genes, as well as the suitable promoters for minimizing the negative effects caused by over-expressing some *NAC* genes, should be considered.

Keywords: abiotic stress, multiple stresses, NAC, transcription factors, transgenic plant

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INTRODUCTION

As sessile organisms, plants continuously suffer from a broad range of environmental stresses including abiotic and biotic stresses. Abiotic stresses such as drought, salinity, heat and cold, adversely affect plant growth and agriculture productivity, and cause more than 50% of worldwide yield loss for major crops every year (Boyer, 1982; Bray et al., 2000; Shao et al., 2009; Ahuja et al., 2010; Lobell et al., 2011). Further to this, plants are also attacked by a vast range of pests and pathogens, including fungi, bacteria, viruses, nematodes, and herbivorous insects (Hammond-Kosack and Jones, 2000). In addition, current climate prediction models indicate the deterioration of climate including an increasing average temperature, a changing distribution of annual precipitation, a rise of sea level, and so on. This will be concurrent with an increased frequency of drought, flood, heat wave, and salinization (Easterling et al., 2000; IPCC, 2007, 2008; Mittler and Blumwald, 2010). Climate change will also affect the spread of pests and pathogens. For example, the increasing temperature can facilitate pathogen spread (Bale et al., 2002; Luck et al., 2011; Nicol et al., 2011), and many abiotic stress can weaken the defense mechanism of plants and

increase their susceptibility to pathogen infection (Amtmann et al., 2008; Goel et al., 2008; Mittler and Blumwald, 2010; Atkinson and Urwin, 2012). Taken together, crop plants will face a greater range and number of environmental stresses, which are likely to occur simultaneously. So it is very urgent to breed stress-tolerant crop varieties to satisfy an increasing demand for food productivity due to global population increase (Takeda and Matsuoka, 2008; Newton et al., 2011).

To cope with these recurrent environmental stresses, plants can activate a number of defense mechanisms which include signal perception, signal transduction through either ABA-dependent or ABA-independent pathways, stress-responsive gene expression, in turn the activation of physiological and metabolic responses (Xiong et al., 2002; Chaves et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006; Perez-Clemente et al., 2013). To date, a large array of stress responsive genes have been identified in many plants, including *Arabidopsis* and rice. These genes are generally classified into two types (Shinozaki et al., 2003). One is functional genes encoding important enzymes and metabolic proteins (functional proteins), such as detoxification enzyme, water channel, late embryogenesis abundant (LEA) protein, which directly function to protect cells from stresses. The other is regulatory genes encoding various regulatory proteins including transcription factors (TFs) and protein kinases, which regulate signal transduction and gene expression in the stress response. In the signal transduction processes, TFs play pivotal roles in the conversion of stress signal perception to stress-responsive gene expression. TFs and their interacting cis-elements function in the promoter region of different stress-related genes acting as molecular switches for gene expression. In plants ∼7% of the genome encodes for putative TFs, which often belong to large gene families, such as *WRKY*, *bZIP*, *MYB*, *AP2/EREBP*, and *NAC* families (Udvardi et al., 2007; Golldack et al., 2011). In light of the key importance of TFs in controlling a wide range of downstream events, lots of studies have aimed to identify and characterize various TFs involved in stress responses. However, these studies have mostly focused on understanding the responses of model plants and crops to a single stress such as drought, salinity, heat or cold, pathogen infection, and so on (Hirayama and Shinozaki, 2010; Chew and Halliday, 2011). Unlike the controlled conditions in the laboratory, crops and other plants are often simultaneously subjected to multiple stresses in the field conditions (Ahuja et al., 2010). Recent studies have showed that plant response to a combination of drought and heat is not a simple additive effect of the individual stress, and the combination of multiple stresses produces a unique pattern of gene expression, which is distinct from the study of either stress individually (Rizhsky et al., 2002, 2004; Prasch and Sonnewald, 2013; Rasmussen et al., 2013). Therefore, the results of studies performed under individual stress factors are not suitable for the complex field conditions, and it is crucial to characterize the response of plants to multiple stresses and identify multiple stress responsive genes by imposing multiple stresses simultaneously as an entirely new stress (Mittler, 2006). Maybe, manipulation of these multiple stress responsive genes, especially multifunctional TFs, will provide the opportunity to breed the broad-spectrum tolerant crops with high yields. Based on these considerations above, this paper reviews the progress of NAC TFs involved in plant abiotic stress responses, and also prospects the future study direction for the challenge of multiple environmental stresses in agriculture, particularly concerning their potential utilization for plant multiple stress tolerance in the field conditions.

NAC TRANSCRIPTION FACTORS IN PLANTS

As one of the largest family of TFs in plants, the NAC TFs comprise a complex plant-specific superfamily and are present in a wide range of species. The NAC acronym is derived from three earliest characterized proteins with a particular domain (NAC domain) from petunia NAM (no apical meristem), *Arabidopsis* ATAF1/2 and CUC2 (cup-shaped cotyledon; Souer et al., 1996; Aida et al., 1997). By the availability of an ever-increasing number of complete plant genomes and EST sequences, large numbers of putative *NAC* genes have been identified in many sequenced species at genome-wide scale (As shown in **Table 1**), such as 117 in *Arabidopsis*, 151 in rice, 74 in grape, 152 in soybean, 204 in Chinese cabbage, 152 in maize, and so on. The large size of *NAC* family inevitably complicates the unraveling of their regulatory process.

The *NAC* family has been found to function in various processes including shoot apical meristem (Takada et al., 2001), flower development (Sablowski and Meyerowitz, 1998), cell division (Kim et al., 2006), leaf senescence (Breeze et al., 2011), formation of secondary walls (Zhong et al., 2010), and biotic and abiotic stress responses (Olsen et al., 2005; Christianson et al., 2010; Tran et al., 2010; Nakashima et al., 2012). Nonetheless, only

TABLE 1 | *NAC* family in various plant species.

Species	Number of *NAC* family	References
Arabidopsis thaliana	117	Nuruzzaman et al., 2010
Rice (*Oryza sativa*)	151	Nuruzzaman et al., 2010
Grape (*Vitis vinifera*)	74	Wang et al., 2013
Soybean (*Glycine max*)	152	Le et al., 2011
Pigeonpea (*Cajanus cajan*)	88	Satheesh et al., 2014
Foxtail millet (*Setaria italica L.*)	147	Puranik et al., 2013
Chinese cabbage (*Brassica rapa*)	204	Liu et al., 2014
Brachypodium distachyon	101	You et al., 2015
Physic Nut (*Jatropha curcas L.*)	100	Wu et al., 2015
Maize (*Zea mays*)	152	Shiriga et al., 2014
Apple (*Malus domestica)*	180	Su et al., 2013
Chickpea (*Cicer arietinum L.*)	71	Ha et al., 2014
Potato (*Solanum tuberosum*)	110	Singh et al., 2013
Poplar (*Populus trichocarpa*)	163	Hu et al., 2010
Banana (*Musa acuminata*)	167	Cenci et al., 2014
Tobacco (*Nicotiana tabacum*)	152	Rushton et al., 2008
Tomato (*Solanum lycopersicum*)	104	Su et al., 2015
Cassava (*Manihot esculenta Crantz*)	96	Hu et al., 2015
Gossypium raimondii	145	Shang et al., 2013

a few of these genes have been characterized to date and most of the *NAC* family members have not yet been studied, even though these genes are likely to play important roles in plants, and a great deal of work will be required to determine the specific biological function of each *NAC* gene. The intensive study on model plants including *Arabidopsis* and rice reveals that a typical NAC protein contains a highly conserved N-terminal DNA-binding NAC domain and a variable transcriptional regulatory region in the C-terminal region. The NAC domain with ∼150–160 amino acids is divided into five sub-domains (A to E; Ooka et al., 2003). The function of the NAC domain has been associated with nuclear localization, DNA binding, and the formation of homodimers or heterodimers with other NAC domain-containing proteins (Olsen et al., 2005). In contrast, the highly diverged C-terminal region functions as a transcription regulatory region, acting as a transcriptional activator or repressor, but it has frequent occurrence of simple amino acid repeats and regions rich in serine and threonine, proline and glutamine, or acidic residues (Olsen et al., 2005; Puranik et al., 2012). Some NAC TFs also contain transmembrane motifs in the C-terminal region which are responsible for anchoring to plasma membrane or endoplasmic reticulum, and these NAC TFs are membrane-associated and designated as NTLs (Seo et al., 2008; Seo and Park, 2010).

The expression of *NAC* genes can firstly be regulated at the level of transcription because there are some stress-responsive cis-acting elements contained in the promoter region such as *ABREs* (ABA-responsive elements), *DREs* (Dehydration-responsive elements), jasmonic acid responsive element and salicylic acid responsive element. Then the complex post-transcriptional regulation involves microRNA-mediated cleavage of genes or alternative splicing. *NAC* TFs also undergo intensive post-translational regulation including ubiquitinization, dimerization, phosphorylation or proteolysis (Nakashima et al., 2012; Puranik et al., 2012). These regulatory steps help NAC TFs playing multiple roles in the majority of plant processes as mentioned above. The NAC TFs regulate the transcription of downstream target genes by binding to a consensus sequence in their promoters. The *NAC* recognition sequence (NACRS) containing the CACG core-DNA binding motif has been identified in the promoter of the drought inducible EARLY RESPONSE TO DEHYDRATION1 (*ERD1*) gene in *Arabidopsis* (Simpson et al., 2003; Tran et al., 2004). The rice drought-inducible ONAC TFs also can bind to a similar NACRS, demonstrating that the *NACRS* might be conserved across plants at least for stress-inducible NAC TFs (Hu et al., 2006; Fang et al., 2008). In addition, other sequences have also been reported as NAC binding sites (*NACBS*), such as an *Arabidopsis* calmodulin-binding NAC with GCTT as core-binding motif (Kim et al., 2007), the iron deficiency-responsive *IDE2* motif containing the core sequence CA(A/C)G(T/C) (T/C/A) (T/C/A) (Ogo et al., 2008) and the secondary wall NAC binding element (SNBE) with (T/A)NN(C/T) (T/C/G)TNNNNNNNA(A/C)GN(A/C/T) (A/T) as consensus sequence (Zhong et al., 2010). The sequences flanking the core site in promoter of target genes may define the binding specificity of different NAC TFs. Thus, the NAC TF family can recognize a vast array of DNA-Binding sequences and regulate multiple downstream target genes. These target genes regulated by NAC TFs comprise regulatory genes encoding regulatory proteins which function in signal transduction and regulation of gene expression and functional genes encoding proteins which are involved in osmolyte production, reactive oxygen species scavenging and detoxification, macromolecule protection and ubiquitination (Puranik et al., 2012). Taken together, the existence of NACRS in promoter of some of these genes makes them to be the potential direct targets, whereas those that do not have this motif may not be direct targets. In future more other novel NACRS remain to be elucidated by microarrays combined with chromatin immunoprecipitation (Taverner et al., 2004).

NAC TRANSCRIPTION FACTORS FUNCTION IN ABIOTIC STRESS

The NAC TFs play a vital role in the complex signaling networks during plant stress responses. Because of the large number of NAC TFs from different plants and their unknown roles, it is still a great challenge to uncover their roles in abiotic stress. Recently, whole-genome expression profiling and transcriptome studies have enabled researchers to identify a number of putative NAC TFs involved in abiotic stress responses. For example, 33 *NAC* genes changed significantly in *Arabdopsis* under salt treatment (Jiang and Deyholos, 2006), 38 *NAC* genes were involved in response to drought in soybean (Le et al., 2011), 40 *NAC* genes responded to drought or salt stress in rice (Fang et al., 2008), 32 *NAC* genes responded to at least two kinds of treatments in *Chrysanthemum lavandulifolium* (Huang et al., 2012). It appears that a significant proportion of *NAC* genes function in stress response according to the expression data from genome-wide transcriptome analyses in many plants. Phylogenetic analyses of NAC TFs showed that most of the stress responsive NAC TFs appeared to contain a closely homologous NAC domain (Ernst et al., 2004; Fang et al., 2008). Moreover, the stress-responsive *NAC* genes exhibit a large diversity in expression patterns, indicating their involvement in the regulation of a wide spectrum of responses to different abiotic stresses. The precise regulations of *NAC* genes during plant abiotic stress responses contribute to the establishment of complex signaling networks, and the important roles of *NAC* genes in plant abiotic stress responses make them promising candidates for the generation of stress tolerant transgenic plants. The functional studies of NAC TFs by over-expression techniques will directly improve our understanding of the regulatory functions of NAC members to abiotic stresses. Transgenic constructs over-expressing the selected *NAC* genes have been made in *Arabidopsis*, rice and other plants. Some successful examples are summarized in **Table 2**.

CONCLUSIONS AND PERSPECTIVES

Considerable information has been gained about NAC TFs since the discovery of NAC TFs, but the research in this area is still in its infancy. Genome-wide identification and expression profiling will undoubtedly open new avenues for describing the

TABLE 2 | Abiotic stress tolerance of transgenic plant over-expressing *NAC* genes.

Transgenic plant	Genotype	Enhanced tolerance	References
A. thaliana	*ANAC019* overexpression	Drought, high-salinity, ABA signaling	Tran et al., 2004
	ANAC055 overexpression	Drought, high-salinity, ABA signaling	Tran et al., 2004
	ANAC72 overexpression	Drought, high-salinity, ABA signaling	Tran et al., 2004
	RD26 overexpression	Drought, salt, ABA signaling	Fujita et al., 2004
	ANAC019 overexpression	Cold, ABA signaling	Jensen et al., 2010
	ATAF1 overexpression	Positive regulator of drought tolerance	Wu et al., 2009
	ONAC063 overexpression	Higher seed germination under high salinity and osmotic stress	Yokotani et al., 2009
	GmNAC20 overexpression	Salt and freezing tolerance	Hao et al., 2011
	ZmSNAC1 overexpression	Low temperature, high-salinity, drought, and ABA signaling	Lu et al., 2012
	TaNAC2 overexpression	Drought, salt, and freezing stresses	Mao et al., 2012
	ANAC042 overexpression	Heat stress	Shahnejat-Bushehri et al., 2012
O. sativa	*SNAC1* overexpression	Increased stomatal closure and drought resistance in dry field conditions, salt toleranc	Hu et al., 2006
	SNAC2 overexpression	Salt, drought, disease resistance drought, salinity, cold, wounding, and ABA treatment	Sindhu et al., 2008
	OsNAC4 overexpression	Drought, salt, cold tolerance	Zheng et al., 2009
	OsNAC5 overexpression	ABA, salt, cold tolerance, grain filling	Sperotto et al., 2009
	OsNAC6 overexpression	Drought and salt tolerance	Nakashima et al., 2007
	ONAC10 overexpression	Drought, high salinity, low temperature toleranc	Jeong et al., 2010
	ONAC045 overexpression	Drought and salt tolerance	Song et al., 2011
N. tabacum	*TaNAC2a* overexpression	Drought tolerance	Tang et al., 2012
	DgNAC1 overexpression	ABA, NaCl, drought, and cold	Liu et al., 2011a
	EcNAC1 overexpression	Water-deficit and salt stress	Ramegowda et al., 2012
T. aestivum	*TaNAC69* overexpression	PEG-induced dehydration and mild salt tolerance	Xue et al., 2011
G. max	*GmNAC11* overexpression	Salt tolerance in soybean transgenic hairy roots	Hao et al., 2011

key features of NAC TFs. As a result, our current understandings of the regulatory functions of the NAC TFs in various plant species will be definitely accelerated. In particular, the stress-responsive *NAC* TFs can be used as promising candidates for generation of stress tolerant transgenic plants possessing high productivity under adverse conditions. As a matter of fact, many transgenic studies have been proved successful by gene manipulation of *NAC* TFs for conferring different stresses tolerance to plants (As shown in **Table 2**), but there are still some problems to be solved. Firstly, the constitutive overexpression of *NAC* genes occasionally may lead to negative effects in transgenic plants such as dwarfing, late flowering and lower yields (Fujita et al., 2004; Nakashima et al., 2007; Hao et al., 2011; Liu et al., 2011b). Secondly, the transgenic plants overexpressing *NAC* genes may occasionally have antagonistic responses to different stresses. For example, drought tolerant *Arabidopsis* plants overexpressing *ATAF1* were highly sensitive to ABA, high-salt, oxidative stress and necrotrophic fungus (*B. cinerea*; Wu et al., 2009). Overexpressing *ANAC019* and *ANAC055* not only increased drought tolerance but also decreased resistance to *B. cinerea* (Fujita et al., 2004; Bu et al., 2008). Thirdly, only a few of transgenic plants overexpressing *NAC* genes were evaluated in the field trials so far, and most of them were tested in greenhouse conditions and focused on plant vegetative stages rather than reproductive stages (Valliyodan and Nguyen, 2006). Lastly, most of the studies on *NAC* TFs only investigated the molecular mechanisms of individual occurring stress situations. Although recent studies have conducted multi-parallel stress experiments and identified different *NAC* TFs responding to single stress situations (Huang et al., 2012), the knowledge concerning responses to combinations of several stress factors is scarce, especially interactions among stress factors.

As everyone knows, one of the most important aims for plant stress research is to provide targets for the improvement of stress tolerance in crop plants. With the forecast changes in climatic conditions leading to a more complex stress environment in the fields, we will face new challenges in creating the multiple stress-tolerant crops. Breeding such plants will depend on understanding the crucial stress-regulatory networks and the potential effects of different combinations of adverse conditions. Studies of multiple stress responses in *Arabidopsis* have provided us with several possible avenues. Master regulatory genes such as members of the *MYC*, *MYB*, and *NAC* TF families that act in multiple abiotic stress responses are excellent candidates for manipulating multiple stress tolerance. So in the future,

it is crucial to impose multiple stresses simultaneously that simulate natural field conditions and regard each set of stress combinations as an entirely new stress in order to identify the corresponding *NAC* TFs commonly induced by multiple stresses. Manipulation of these genes should be the major target of attempts to produce plants with enhanced multiple stress tolerance. Furthermore, the potential *NAC* genes which confer multiple abiotic stress tolerance in model plant species must be tested in crop plants and greater emphasis should be placed on the field evaluation of the transgenic crops harboring *NAC* genes, especially focusing on their reproductive success.

Another lesson is the selection and/or improvement of suitable promoters (such as a stress-inducible promoter) which can maximize the positive effects and minimize the negative effects caused by over-expressing some *NAC* genes. In summary, NAC TFs are the key components of the signaling pathway in stress response which carry out their function by interacting with both downstream and upstream partners (**Figure 1**). Understanding the molecular mechanisms of NAC TFs networks integrating multiple stress responses will be essential for the development of broad-spectrum stress tolerant crop plants that can better cope with environmental challenges in future climates.

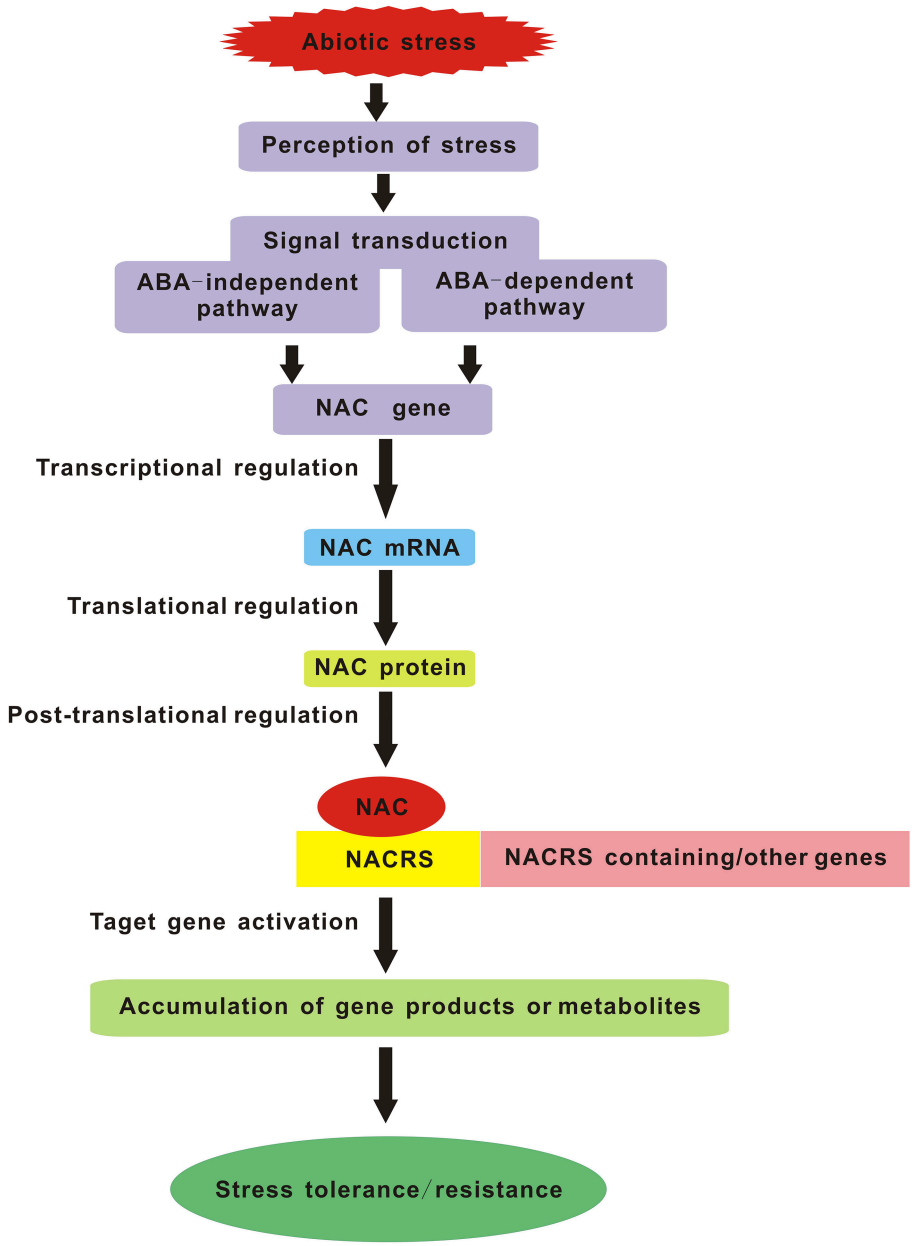


FIGURE 1 | Schematic diagram of NAC TFs as key components in transcriptional regulatory networks during abiotic stress. NACRS is NAC recognition sequence.

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REVIEW
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Transcriptional regulation of drought response: a tortuous network of transcriptional factors

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Drought is one of the leading factors responsible for the reduction in crop yield worldwide. Due to climate change, in future, more areas are going to be affected by drought and for prolonged periods. Therefore, understanding the mechanisms underlying the drought response is one of the major scientific concerns for improving crop yield. Plants deploy diverse strategies and mechanisms to respond and tolerate drought stress. Expression of numerous genes is modulated in different plants under drought stress that help them to optimize their growth and development. Plant hormone abscisic acid (ABA) plays a major role in plant response and tolerance by regulating the expression of many genes under drought stress. Transcription factors being the major regulator of gene expression play a crucial role in stress response. ABA regulates the expression of most of the target genes through ABA-responsive element (ABRE) binding protein/ABRE binding factor (AREB/ABF) transcription factors. Genes regulated by AREB/ABFs constitute a regulon termed as AREB/ABF regulon. In addition to this, drought responsive genes are also regulated by ABA-independent mechanisms. In ABA-independent regulation, dehydration-responsive element binding protein (DREB), NAM, ATAF, and CUC regulons play an important role by regulating many drought-responsive genes. Apart from these major regulons, MYB/MYC, WRKY, and nuclear factor-Y (NF-Y) transcription factors are also involved in drought response and tolerance. Our understanding about transcriptional regulation of drought is still evolving. Recent reports have suggested the existence of crosstalk between different transcription factors operating under drought stress. In this article, we have reviewed various regulons working under drought stress and their crosstalk with each other.

Keywords: ABA, drought, regulons, cross-talk, transcription factors

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INTRODUCTION

Plants being sessile organisms frequently encounter a wide range of unfavorable conditions during their life cycle. These conditions have deleterious effects on their physiology leading to reduced growth and development. Such adverse conditions along with some other factors play a crucial role in determining the yield and geographical distribution of plants. These different unfavorable conditions are generally termed as stress.

Plants face both abiotic as well as biotic stresses during their life cycle. Various climatic factors such as extreme temperature, drought, salinity, and chemical contamination of soil fall in the category of abiotic stresses. However, stresses caused by various pathogens and other biological

agents are grouped into biotic stress. Both kinds of stresses have detrimental effects on plants. Especially, abiotic stresses alter various cellular processes such as photosynthesis, growth, carbon partitioning, carbohydrate and lipid metabolism, protein synthesis, gene expression, and osmotic homeostasis. Thus, to survive under stress conditions, plants have evolved a wide range of mechanisms to avoid or tolerate these stresses.

Drought is an imperative factor limiting the crop productivity across the globe (Bray et al., 2000). It can be characterized by below normal precipitation for a certain period of months to year and drying winds leading to reduced soil water available to plants. In addition to this, it is generally accompanied with high temperature. In recent years, frequency of drought stress has increased due to irregular rain fall. Almost every year drought occurs in some part of the earth reducing the crop yield. The condition is going to be worse in coming years due to global warming responsible for increasing desertification. On the other hand, world population is anticipated to reach 9 billion by 2050 (http://www.fao.org/wsfs/world-summit/en). Taking into consideration the increase in population, it is important to increase crop yield. Therefore, it is important to understand the mechanism of drought stress tolerance in plants in order to improve crop productivity under stress conditions.

Understanding the mechanism underlying drought stress tolerance has been an active area of research. Till date, drought stress responses have been studied in various plants; including crops, vegetables, trees as well as horticulture plants. Plants respond to environmental stresses at various levels such as cellular responses, metabolic changes, molecular adaptations as well as epigenetic regulation (Krasensky and Jonak, 2012). Although, plant response to drought has been analyzed at all these levels but in past few years focus has shifted toward the molecular mechanism. Drought stress affects the expression of many genes. Most of the molecular studies have been done using *Arabidopsis thaliana* as a model plant (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000). Genome sequence of *Arabidopsis* has provided valuable information pertaining genes, gene families, *cis* elements and other related factors; resulting in rapid progress regarding molecular responses of plants to drought (Hirayama and Shinozaki, 2010). Later on, in addition to genomics, incorporation of advanced omics approaches such as transcriptomics, proteomics and metabolomics have increased our knowledge in this area (Hirayama and Shinozaki, 2010).

During drought and other osmotic stresses, the phytohormone ABA (abscisic acid) plays a pivotal role in plant adaptation. Effect of ABA on plant response to stress has been extensively researched. ABA is accumulated under drought stress condition due to induction of ABA biosynthetic genes (Iuchi et al., 2001). ABA regulates the expression of many genes leading to some important physiological as well as biochemical changes that help plant to survive under stress (Umezawa et al., 2010). Molecular and genomic analyses have revealed the existence of ABA-independent signal transduction pathway in conjunction to the ABA-dependent signal transduction pathway during drought stress (Yamaguchi-Shinozaki and Shinozaki, 2006).

TRANSCRIPTIONAL REGULATORY NETWORK

Plants respond to various environmental stresses including drought through changes ranging from physiological to molecular level. These changes help plants to optimize their growth and stress resistance. Drought stress changes the expression of many genes that are thought to play an important role in stress response and tolerance. Many of these genes have been identified and characterized (Yamaguchi-Shinozaki and Shinozaki, 2006; Todaka et al., 2015). Microarray analyses by various groups have revealed thousands of genes that are upregulated and downregulated in response to drought stress. A significant number of drought-inducible genes are also induced by high salinity, suggesting a cross-talk between drought and salt stress. Comparatively lesser number of drought-inducible genes are induced by cold stress (Yamaguchi-Shinozaki and Shinozaki, 2006). There is a very small overlap of only 27 genes that were found to be commonly induced in microarray studies (Bray, 2004). This lack of commonality may be attributed to the fact that different sets of probes were used during these microarray experiments and variations in conditions of plant growth and stress. Recently, 17 microarray experiments of *Arabidopsis,* rice, wheat, and barley were compared using a novel **C**ross-**S**pecies meta-**A**nalysis of progressive **Drought** stress at the reproductive stage (**CSA:Drought**); and 225 differentially expressed genes were identified that were shared across studies and taxa (Shaar-Moshe et al., 2015).

Stress inducible genes in *Arabidopsis* can be classified into two categories: functional and regulatory genes (Yamaguchi-Shinozaki and Shinozaki, 2006). Genes encoding proteins required for cellular stress tolerance fall into the former category, for example, LEA (late embryogenesis abundant) proteins, molecular chaperones, reactive oxygen species detoxifying enzymes, and sugars or proline biosynthetic enzymes. Whereas, genes encoding proteins that are involved in signal transduction and gene expression come under the latter category, such as protein kinases, components of ABA signaling, enzymes for lipid signaling, and various transcription factors (Yamaguchi-Shinozaki and Shinozaki, 2006).

As stated above, plant hormone ABA plays an important role in response to water deficit including regulation of transcriptional network (Yamaguchi-Shinozaki and Shinozaki, 2006). A large number of genes that are induced by water deficit are also highly induced by exogenous application of ABA. Conversely, there are several genes that are induced by water deficit but are not affected by exogenous ABA. These findings suggested that transcriptional response to water deficit is regulated by both ABA-dependent and ABA-independent signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki, 2006).

Transcription factors are regulatory proteins that can modulate expression of a specific set of genes through binding to their promoter. They play an important role in converting the stress-induced signals to cellular responses. A single transcription factor can modulate the expression of a number of genes. A collection of genes under regulation of the same regulatory protein is called regulons. Plants activate many regulons under

drought and other stresses to optimize plant growth, some of them have been well determined in *Arabidopsis* (Nakashima et al., 2009). Both ABA-dependent and ABA-independent pathways regulate the transcriptional response by affecting one or more regulons active under drought stress (Nakashima et al., 2009). In the following section, we will discuss in brief about water deficit induced regulons and pathways affecting them.

AREB/ABF Regulon

Abscisic acid-responsive element binding protein (AREB) /ABF (ABRE binding factor) regulon function in ABA-dependent regulation of gene expression under drought stress (Nakashima et al., 2009; Yoshida et al., 2015), (**Figure 1**). Many genes that are affected by water deficit also respond to the exogenous application of ABA (Nakashima et al., 2009). Promoter analysis revealed that most of these ABA-responsive genes are regulated by ABRE (ABA-responsive element) in their promoter region. ABRE is a conserved, 8 bp long *cis* element (PyACGTGG/TC) with a core ACGT sequence (Nakashima et al., 2009; Fujita et al., 2011). A single copy of an ABRE is not sufficient to induce ABA-responsive gene expression. To function as an active *cis*-acting element, ABRE requires in proximity other copies of ABRE or another specific *cis*-acting element, which is termed as the coupling element. Certain sequences have been shown to function as coupling elements such as, CE1 (coupling element1) and CE3 (coupling element3); and DRE (dehydration-responsive element)/CRT (C-repeat) *cis* element (major *cis* element in ABA-independent pathway; Shen et al., 1996; Narusaka et al., 2003). CEs are usually GC-rich sequence and they are similar to ABRE (Yamaguchi-Shinozaki and Shinozaki, 2006).

Abscisic acid-responsive element binding protein or ABF transcription factors were found to bind to ABRE element in yeast one-hybrid screening. These are major transcription factors that bind to ABRE and regulate ABA-responsive gene expression (Choi et al., 2000; Uno et al., 2000), (**Figure 1**). AREB/ABF is a subfamily of the basic leucine zipper (bZIP) family that consists of 9 members in *Arabidopsis*. All AREB/ABF transcription factors contain four conserved domains in addition to bZIP domain (Fujita et al., 2011, 2013). In *Arabidopsis, AREB1/ABF2, AREB2/ABF4*, *ABF1,* and *ABF3* are expressed mainly in the vegetative tissues in response to ABA and osmotic stress (Fujita et al., 2011). In contrast, some other members are expressed during seed maturation such as *Arabidopsis ABI5*, *AREB3*, *DPBF2,* and *EEL* (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Bensmihen et al., 2002). Transgenic plants overexpressing *AREB1/ABF2, AREB2/ABF4,* or *ABF3* exhibit enhanced drought tolerance and increased ABA sensitivity (Kang et al., 2002; Fujita et al., 2005). Triple AREB/ABF mutant *areb1 areb2 abf3* exhibits reduced tolerance to drought and decreased sensitivity to exogenous ABA compared to that of wild-type, single mutants, or double mutants (Yoshida et al., 2010). Transcriptome analysis of the triple mutant under osmotic stress conditions showed reduced levels of many osmotic stress-inducible genes (Yoshida et al., 2010). Recently, ABF1 has also been reported to play an important role in ABA-mediated gene expression under drought stress. Although, it is expressed in lesser quantity as compared to those three AREB/ABFs, the *areb1 areb2 abf3 abf1* quadruple mutant plants show an increase in drought sensitivity and decreased ABA sensitivity in comparison to *areb1 areb2 abf3* mutant. In quadruple mutant many dehydration-inducible genes including LEA protein genes and transcription factors show reduced expression (Yoshida et al., 2015). Both triple mutants, as well as quadruple mutants, exhibited decreased inflorescence heights as compared to wild-type; as well as delayed bolting. Except for this, all of them showed phenotypes similar to wild-type (Yoshida et al., 2010, 2015). Thus, these four AREB/ABFs have been shown to be central transcription factors that cooperatively function in ABA-dependent transcriptional activation through their ABREs under these abiotic stress conditions (**Figure 1**).

Abscisic acid-responsive element binding protein/ABF transcription factors are fully activated only after phosphorylation of their conserved regions (Fujita et al., 2011). The phosphorylation is catalyzed by serine/threonine kinase SnRK2s (SNF1-related protein kinase) that are induced by ABA. The importance of phosphorylation was suggested by the observation that overexpression of *AREB1/ABF2* activated downstream gene expression only when phosphorylated active form of this gene was used (Furihata et al., 2006). Thus, under water deficit, cellular ABA concentration is increased that is recognized by the ABA receptors PYR/PYL/RCARs (pyrabactin resistance/pyrabactin resistance1- like/regulatory component of ABA receptors) leading to the inhibition of phosphatase activity of PP2C (protein phosphatase 2C). PP2C is a negative regulator of ABA signaling which dephosphorylates and thereby inactivates subclass III SnRK2s. Subsequently, released subclass III SnRK2s get accumulated in the cell and phosphorylate AREB/ABF thereby inducing expression of AREB/ABF regulon genes (reviewed in Cutler et al., 2010; Umezawa et al., 2010), (**Figure 1**). There are 10 members of SnRK2 family in *Arabidopsis* that are divided into three groups including three members in subclass III SnRK2s, SRK2D/SnRK2.2, SRK2E/OST1/SnRK2.6, and SRK2I/SnRK2.3 (Umezawa et al., 2010). Out of these 10 SnRK2s, nine are activated by osmotic stress. However, subclass III SnRK2s are also strongly induced by ABA and mediate most of the ABA responses (Yoshida et al., 2014). Subclass III SnRK2s have been shown to phosphorylate AREB/ABFs *in vitro* as well as co-localize and interact with them in plant cell nuclei (Furihata et al., 2006; Fujii et al., 2007; Fujita et al., 2009; Yoshida et al., 2010). Studies involving triple mutant *srk2d/e/i* showed that expression of most of the AREB1/ABF2, AREB2/ABF4, and ABF3 regulated genes are highly reduced and ABA-dependent phosphorylation of AREB/ABFs is completely abolished (Fujii and Zhu, 2009; Fujita et al., 2009). All these results indicate that subclass III SnRK2s regulate ABA-responsive gene expression under drought stress by phosphorylating AREB/ABFs.

DREB1/CBF and DREB2 Regulons

Dehydration-responsive element binding protein 1 (DREB1)/CBF (C-repeat binding factor) and DREB2 regulons function in ABA-independent regulation of gene expression under drought stress (Nakashima et al., 2009), (**Figure 1**). In *Arabidopsis*, the *RD29A/COR78/LTI78* gene is induced by drought and cold. This gene has been found to be ABA-inducible

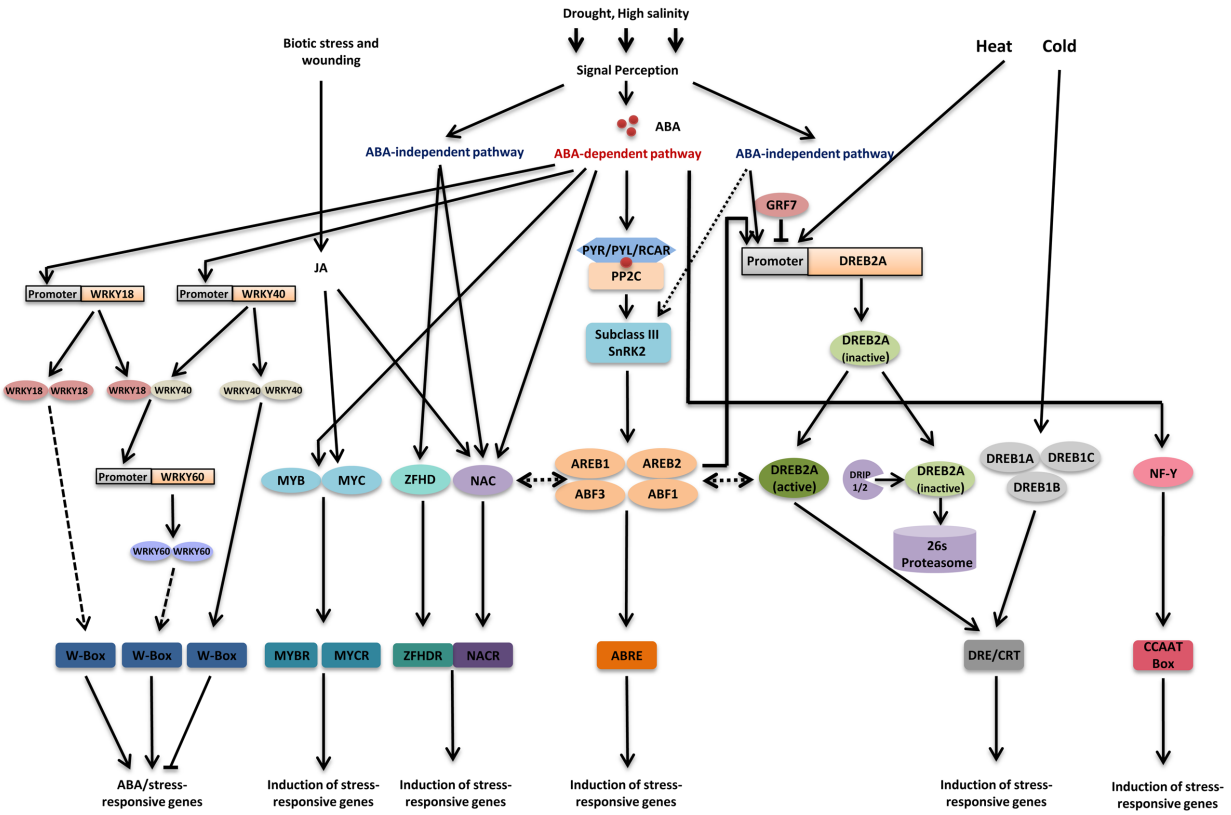


FIGURE 1 | Major transcriptional regulatory networks of transcription factors involved in drought stress. Drought signal perception leads to activation of both abscisic acid (ABA)-dependent and ABA-independent pathways. In ABA-dependent pathway, accumulation of ABA leads to activation of sub class III SnRK2s through PYR/PYL/RCAR-PP2C receptor complex. Four transcription factors ABA-responsive element (ABRE) binding protein 1 (AREB1), AREB2, ABRE binding factor 3 (ABF3), and ABF1 are mainly phosphorylated through sub class III SnRK2s under drought stress and regulate most of the downstream genes by binding to the ABRE *cis*-element present in their promoter region. In addition to this, ABA also modulates the activity of MYB/MYCs, NACs, WRKYs, and NF-Y transcription factors. MYC2 and NAC proteins are also involved in JA signaling. ABA signal perception leads to induction of WRKY18 and WRKY40 and their product could bind to W-box present in WRKY60 and thereby induce it. DREB2A plays a pivotal role in ABA-independent gene expression regulation under drought stress. *DREB2A* expression is regulated by GRF7 under unstressed condition. Additionally, DRIPs regulate levels of DREB2A protein under unstressed condition. DREB2A also participate in gene expression regulation under heat stress. Three DREB1 proteins, DREB1A, DREB1B, and DREB1C are the key factors regulating gene expression under cold stress. Some NAC transcription factors also regulate gene expression in ABA-independent manner. Recently, it has been reported that AREB/ABFs can induce DREB2A and AREB/ABFs interact with DREB2A, based on these observations, ABA-dependent and ABA-independent pathways are thought to crosstalk under drought stress. Apart from this, some recent reports suggest a possible interaction between NACs and AREB/ABFs. Transcription factors and DNA-binding proteins are shown in colored ellipses. Dashed lines indicate possible although unconfirmed routes.

but at the same time it can be induced by drought and cold stress in mutants defective in ABA biosynthesis and signaling (Yamaguchi-Shinozaki and Shinozaki, 2006). The analysis of its promoter, together with expression studies suggested that the dehydration inducibility of this gene is regulated by both ABA-independent and ABA-dependent pathways through different *cis*-acting elements (Yamaguchi-Shinozaki and Shinozaki, 2006).

The promoter of *RD29A* gene contains DRE/CRT *cis* element in addition to ABRE (Yamaguchi-Shinozaki and Shinozaki, 1994, 2005). DRE element is responsible for ABA-independent induction of many genes in response to osmotic and cold stress in many plants including *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 2005). DRE is a conserved 9 bp long (TACCGACAT) *cis* element. Unlike ABRE, a single copy of DRE is sufficient to induce genes under osmotic and cold stress (Yamaguchi-Shinozaki and Shinozaki, 1994). Similar *cis*-acting elements, named C-repeat (CRT) and low-temperature responsive element (LTRE), were identified in low-temperature-inducible genes. These sequences share a common core sequence, A/GCCGAC, which is referred to as the DRE/CRT core sequence (Baker et al., 1994; Jiang et al., 1996; Thomashow, 1999). DRE/CRTs are found in promoters of many stress-inducible genes.

Dehydration-responsive element binding protein 1/CBF and DREB2 transcription factors recognize DRE/CRT and activate downstream genes (**Figure 1**). Both DREB1/CBF and DREB2 belong to the plant-specific AP2 (APETALA2)/ ERF (ethylene-responsive element-binding factor) family having AP2/ERF DNA-binding motif. There are 145 members in AP2/ERF transcription factor family in *Arabidopsis* (Sakuma et al., 2002). DREB transcription factors constitute a subfamily of AP2/ERF family. *Arabidopsis* has six and eight *DREB1/CBF*-type and *DREB2*-type genes, respectively. Among them, three

DREB1/CBF-type transcription factors, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 are rapidly induced by low temperature and act as major transcription factors that activate gene transcription through DRE/CRT in response to cold stress (Nakashima et al., 2009), (**Figure 1**). Transgenic plants overexpressing *DREB1* show enhanced tolerance to cold and accumulate osmoprotectants such as proline and various sugars (Gilmour et al., 2000). These transgenic plants also exhibit "dwarf" phenotype and pronounced prostrate growth habits. They have shorter petiole in comparison to wild-type plants with leaves having bluish-green tint. Additionally, plants overexpressing *DREB1* show delayed bolting and flowering as well as lower yield in comparison to wild-type plants (Gilmour et al., 2000).

In contrast to DREB1, two DREB2-type transcription factors, DREB2A and DREB2B, are highly induced in response to osmotic stress conditions and are considered to be involved in DRE-mediated gene transcription in response to water deficit (Liu et al., 1998), (**Figure 1**). Later, it was found that DREB2A plays a pivotal role in gene expression regulation under salt stress whereas DREB2B regulates gene expression in response to drought stress (Nakashima et al., 2009). However, weak induction of several *DREB1* genes such as *DREB1D/CBF4*, *DREB1E/DDF2,* and *DREB1F/DDF1* under dehydration stress suggests that DREB1/CBF and DREB2 regulons interact with each other (Haake et al., 2002; Sakuma et al., 2002; Magome et al., 2004).

Although DREB2A and DREB1A were isolated together (Liu et al., 1998), later, it was discovered that both of them have slight difference in their downstream genes (Maruyama et al., 2009). Microarray analysis has suggested that products of most of the genes downstream to DREAB1A and DREB2A have similar putative functions, but carbohydrate metabolism genes have different expression pattern in DREB1A and DREB2A transgenic plants (Maruyama et al., 2009). Plants overexpressing *DREB1A* exhibit changes in expression of genes responsible for starch degradation, sucrose metabolism and sugar alcohol synthesis similar to that observed in dehydration and cold stress. These changes lead to accumulation of many kinds of sugar and sugar alcohols in plants that might be responsible for enhanced dehydration and cold stress tolerance. In contrast plants overexpressing *DREB2A-CA* (constitutively active form of *DREB2A*) do not exhibit the increase in these metabolites level (Maruyama et al., 2009). The reason for this might be the difference in their DNA-binding specificity (Nakashima et al., 2009). DREB1A has a high affinity to A/GCCGACNT sequences, whereas DREB2A preferentially binds ACCGAC motifs (Sakuma et al., 2006a) resulting in slight variation in target genes.

Although DREB2A regulates the expression of many genes involved in stress response and tolerance, it causes growth retardation and reduced reproduction rate in plants, therefore, its expression is tightly regulated (Yoshida et al., 2014). *DREB2A* expression is negatively regulated by GRF7 (growth-regulating factor7). GRF proteins are a family of putative transcription factors that consist of nine members in *Arabidopsis* (Kim et al., 2003). Among these nine members, GRF7 inhibits expression of *DREB2A* under normal conditions by binding to its short promoter region (**Figure 1**). Both knockdown and knockout mutants of *GRF7* exhibit enhanced DREB2A expression under non-stressed condition (Kim et al., 2012a). These plants also exhibit enhanced salinity tolerance and retarded growth. Microarray analysis revealed that a large number of osmotic stress-responsive genes were upregulated in *grf7* knockout mutants under non-stressed condition. These shreds of evidence suggest that GRF7 regulates a large number of osmotic stress responsive genes by regulating *DREB2A* expression (Kim et al., 2012a).

In addition to transcriptional regulation, DREB2A is also regulated at post-transcriptional level. Transgenic plant overexpressing DREB1/CBF under *Arabidopsis* stress-responsive RD29A promoter showed strong tolerance to stresses mainly against cold (Kasuga et al., 1999). However, transgenic plant overexpressing *DREB2A* did not exhibit any significant phenotypic change (Liu et al., 1998). Domain analysis revealed that central region of DREB2A protein has a negative regulatory domain (NRD), and removal of the NRD from DREB2A converts the protein into its constitutively active form (DREB2A-CA). The DREB2A-CA proteins are more stable in the nucleus than the wild-type protein. In contrast to wild-type *DREB2A*, overexpression of *DREB2A-CA* shows enhanced drought tolerance as well as a slight improvement in cold stress tolerance and upregulation of many stress-inducible genes (Sakuma et al., 2006a). Transgenic plants overexpressing *DREB2A-CA* exhibited growth retardation in comparison to wild-type as well as rounded, slightly darker leaves with short petiole. Additionally, extent of retardation and these mentioned phenotypes was in correlation to the expression of transgene (Sakuma et al., 2006a). All these reports suggest that stability control is a posttranslational regulatory mechanism of DREB2A.

Furthermore, DRIP1 (DREB2A-interacting protein 1), a ubiquitin E3 ligase is thought to degrade the leaky expression under normal conditions (Qin et al., 2008). DRIP1 harbors a C3HC4-type RING domain and has been found to interact with DREB2A in yeast two-hybrid screening. Transgenic *Arabidopsis* plants overexpressing *DRIP1* exhibit delayed expression of DREB2A-regulated drought responsive genes, however, double knockout mutants of *DRIP1* and its homolog *DRIP2* exhibit increased expression of these genes. DRIP1 and DRIP2 function as E3 ubiquitin ligase and target DREB2A to 26S proteasome proteolysis and thereby negatively regulate the expression of drought-responsive genes (Qin et al., 2008), (**Figure 1**). Recently, it has been shown that stress signal play important role in stabilization of DREB2A (Morimoto et al., 2013). In *drip1drip2* mutant, DREB2A protein levels were induced rapidly under heat stress suggesting their involvement in DREB2A degradation but the protein levels reduced in the mutant by prolonged heat stress (Morimoto et al., 2013). It has also been shown that stabilization of DREB2A is important but not sufficient for induction of downstream genes (Morimoto et al., 2013). All these results suggest that apart from DRIPs some other factors might be involved in degradation and activation of DREB2A.

Recently, transcription factor ERF53 (ethylene response factor53) and two homologous C3HC4-type RING E3 ligases,

RGLG2 (RING domain ligase2) and RGLG1 has been identified that also function similar to DREB2A and DRIPs and regulate drought stress-responsive genes (Cheng et al., 2012). ERF53 is an AP2/ERF transcription factor that belongs to the non-DREB2 subfamily. *AtERF53* expression increase significantly under drought and high salinity but it exhibit mild induction to exogenous ABA (Cheng et al., 2012; Hsieh et al., 2013). Overexpression of *AtERF53* induces unstable drought tolerance. AtERF53 has been found to interact with RGLG2 and RGLG1, both of which act as E3 ubiquitin ligase and target it to proteasomal degradation. AtERF53-GFP fusion protein accumulates more stably in double mutant *rglg1 rglg2* leading to enhanced drought stress tolerance. All these reports suggest that AtERF53 and RGLGs function in combination to regulate osmotic stress-responsive genes (Cheng et al., 2012; Hsieh et al., 2013).

Dehydration-responsive element binding protein 2A regulon also operates under heat shock stress (**Figure 1**). Overexpression studies of *DREB2A-CA* showed improved thermotolerance by inducing expression of heat shock related genes (Sakuma et al., 2006b; Mizoi et al., 2012). Similarly, transgenic plants overexpressing *DREB2C* showed increased expression of heat shock stress-inducible genes, leading to thermotolerance (Lim et al., 2007). Therefore, it is clear that DRE/CRT is involved in gene expression not only during dehydration but also under conditions of low and high temperature.

NAC Regulon

NAM, ATAF, and CUC (NAC) are plant-specific transcription factors that constitute one of the largest families of plant transcription factors. There are more than a hundred members in *Arabidopsis* and rice that have been classified in 10 groups based on their phylogenetic relationship (Jensen et al., 2010). NAC transcription factors have a highly conserved N-terminal DNA-binding domain and variable C-terminal region this C-terminal region is thought to play a crucial role in determination of their target genes (Nuruzzaman et al., 2013). NAC transcription factors are involved in various developmental processes from shoot meristem development to auxin signaling (Olsen et al., 2005). There are reports demonstrating their involvement in abiotic and biotic stress signaling (reviewed in Olsen et al., 2005; Nakashima et al., 2012; Nuruzzaman et al., 2013). NAC transcription factors involved in stress response and tolerance have been classified in stress-responsive NAC (SNAC) group.

The *ERD1* (*early response to dehydration1*) gene encodes a ClpA homolog of *Arabidopsis* (Nakashima et al., 1997). ERD1 was found to be induced by osmotic stress such as dehydration and salt stress as well as during senescence; however, exogenous ABA application could not strongly induce it (Nakashima et al., 1997). Two different *cis*-acting elements, a MYC-like sequence (CATGTG) and a 14-bp *rps1* site 1-like sequence are necessary for dehydration induced expression of *ERD1* (Simpson et al., 2003). Three NAC transcription factors; ANAC019, ANAC055, and ANAC072/RD26 were reported to bind MYC-like sequence present in the promoter of ERD1 (Tran et al., 2004). These three proteins are included in the SNAC group of NAC transcription factors (Nakashima et al., 2012). Detailed DNA binding assay of these NAC transcription factors determined NACRS (NAC recognition sequence) ANNNNNTCNNNNNNNACACGCATGT, containing CATGT and harboring CACG as the core DNA-binding site (Tran et al., 2004), (**Figure 1**). These NAC genes were found to be expressed within 1–2 h of ABA treatment suggesting that they are induced through ABA-independent pathway under drought stress (Tran et al., 2004). Transgenic plants overexpressing *ANAC019* and *ANAC072* showed phenotype and the time course of growth similar to that of vector control (Tran et al., 2004). In contrast, plants overexpressing *ANAC055* exhibited growth rate similar to that of vector control until they reached rosette stage; after this point, plants in which the expression of transgene was at the medium level, showed a little delay in bolting as compared to vector control whereas; plants in which the transgene was overexpressed at high level remained at rosette stage for an additional few days before first bolting (Tran et al., 2004). Transgenic plant overexpressing *ANAC019*, *ANAC055*, or *ANAC072/RD26* induced expression of many stress-inducible genes but failed to up-regulate *ERD1* (Fujita et al., 2004; Tran et al., 2004).

Subsequently, a zinc-finger homeodomain (ZFHD) transcription factor, ZFHD1, was identified as a transcriptional activator that recognizes the 14-bp rps1 site1-like sequence (CACTAAATTGTCAC) and this sequence was termed as ZFHDR (zinc finger homeodomain recognition sequence; Tran et al., 2007), (**Figure 1**). Expression of the *ERD1* gene was induced only when both the NAC and ZFHD proteins were overproduced simultaneously in a transgenic plant. Thus, these transcription factors cooperatively activate the transcription of the *ERD1* gene (Tran et al., 2007). Studies suggest that in addition to the role of ZFHD1 in cooperation of NAC transcription factor it can also function as transcriptional activator alone (Tran et al., 2007).

Overexpression of several stress-responsive NAC factors in *Arabidopsis* and rice has imparted drought tolerance in transgenic plants. For example, transgenic plants overexpressing *ANAC072/RD26* exhibited enhanced drought tolerance as well as increased sensitivity to ABA (Fujita et al., 2004; Tran et al., 2004). Microarray analysis of *ANAC072/RD26* overexpressing plants showed upregulation of stress-inducible genes and ABA-responsive genes suggesting that RD26 is involved in regulation of drought-responsive genes in ABA-dependent manner (Fujita et al., 2004). Similarly, overexpression of another NAC factor *ATAF1* resulted in improved drought tolerance (Wu et al., 2009). Recently, NAC genes such as *ANAC096* and *ANAC016* are associated with drought response and tolerance (Xu et al., 2013; Sakuraba et al., 2015). In case of rice, overexpression of *NAC* genes such as *SNAC1, OsNAC6/SNAC2*, *OsNAC5,* and *OsNAC10* improved drought tolerance (reviewed in Nakashima et al., 2014). NTLs (NAC with transmembrane motif 1-like) such as NTL4 and NTL6 are also involved in drought stress. Transgenic plants overexpressing *NTL6* and *ntl4* null mutants exhibit drought

tolerance, suggesting that these two work antagonistic to each other during drought stress (Lee and Park, 2012; Kim et al., 2012b).

Several stress-inducible NAC genes are also induced by jasmonates and/or during senescence in *Arabidopsis* and rice (**Figure 1**). Thus, these stress-responsive NAC transcription factors not only function in the transcriptional response to abiotic stress conditions, including water stress, but are likely involved in the cross talk between abiotic and biotic stress responses (Nakashima et al., 2012).

Other Transcriptional Pathways Involved in Water Stress Responses

In addition to the above-mentioned pathways of *cis*-acting elements and transcription factors, many other transcriptional pathways function in water stress responses. The *Arabidopsis RD22* gene is inducible by drought stress in ABA-dependent manner (Yamaguchi-Shinozaki and Shinozaki, 1993). Although it is induced by ABA, it does not have ABRE *cis*-element in its promoter region; in spite of that its expression is regulated by two *cis*-acting elements, MYC and MYB recognition elements (Abe et al., 1997). A MYC like transcription factor, MYC2, and a MYB transcription factor, MYB2 bind to these *cis*-acting elements and cooperatively activate the transcription of this gene (Abe et al., 1997, 2003), (**Figure 1**). Transgenic plants overexpressing *AtMYC2* and *AtMYB2* exhibit higher ABA sensitivity as well as osmotic tolerance (Abe et al., 2003). Transgenic plants overexpressing *AtMYC2* show morphology similar to wild-type. However, overexpression of *AtMYB2* causes growth retardation in transgenic plants growing on soil. Similarly, overexpression of both transcription factors causes severe growth retardation in plants growing on soil (Abe et al., 2003). Although, *AtMYC2* overexpressing plants have the morphology similar to wild-type, they have characteristically irregular shaped leaf epidermal cells. In contrast, plants overexpressing *AtMYB2* and both transcription factors have leaf epidermal as well as parenchymal cells similar to wild-type but smaller in shape (Abe et al., 2003). Microarray analysis has suggested that their target genes include many ABA-inducible genes. Conversely, a mutation in MYC2 decreased the expression of target genes, including *RD22* (Abe et al., 2003). All these results suggest that in addition to ABRE mediated gene regulation, MYB and MYC transcription factors regulate gene expression in response to ABA under drought stress (**Figure 1**).

MYB transcription factors are one of the largest transcription factor families in plants that have characteristic MYB domain in their DNA binding region (Lindemose et al., 2013). Analysis of transcriptome data present in GENEVESTIGATOR database showed that 51% of *Arabidopsis MYB* genes are upregulated and 41% are downregulated under drought stress (Baldoni et al., 2015). Furthermore, many MYB genes have been found to be involved in drought stress responses (reviewed in Baldoni et al., 2015).

MYC transcription factors belong to bHLH (basic-helix-loop-helix) transcription factor family of plants that have a characteristic bHLH domain (Kazan and Manners, 2013). Guard cell transcriptome analysis showed that ABA-responsive genes having MYC-binding motifs in the promoter region are present in large number in these cells (Wang et al., 2011). MYC2 protein has emerged as a master player in jasmonic acid signaling as well as cross-talk between jasmonic acid and ABA signaling (Kazan and Manners, 2013).

The involvement of WRKY transcription factors in drought stress has been reported recently. WRKY transcription factors constitute a family that has one or two WRKY domains which is involved in DNA binding. WRKY transcription factors bind to a conserved sequence named as W box and regulate gene expression (Rushton et al., 2010), (**Figure 1**). WRKY transcription factors are involved in various plant processes including biotic stress responses (Ulker and Somssich, 2004; Rushton et al., 2010). Recently, they have been reported to be involved in abiotic stress responses (Rushton et al., 2010; Banerjee and Roychoudhury, 2015). Various WRKY transcription factors have been found to be involved in ABA signaling. WRKY18 and WRKY60 act as positive regulators of ABA signaling during seed germination, and stress response while WRKY40 has the opposite effect on ABA signaling. WRKY18 and WRKY60 act as weak transcriptional activator whereas WRKY40 binds to the promoters of multiple stress-inducible transcription factor genes, including *DREB1A/CBF3*, *DREB2A*, and *MYB2*, and represses their expression (Chen et al., 2010; Shang et al., 2010). ABA signal perception leads to induction of WRKY18 and WRKY40 and their product could bind to W-box present in WRKY60 promoter and thereby induce it (Chen et al., 2010), (**Figure 1**). In another report, WRKY gene, *WRKY63/ABO3* (*ABA Overly Sensitive3*) has been demonstrated to be involved in drought responses. *abo3* mutant exhibits hypersensitive response for ABA in the seedling stage as well as reduced drought tolerance. WRKY63 has been shown to bind to the promoter of *AREB1/ABF2* and thereby positively regulating its expression (Ren et al., 2010). Apart from these WRKY genes, many other have been reported to be involved in drought and salt stress responses (Bakshi and Oelmüller, 2014; Banerjee and Roychoudhury, 2015)

In addition to these, NF-Y (nuclear factor-Y) transcription factors also take part in drought stress response and tolerance mechanisms. NF-Y transcription factors are heterotrimeric proteins with three distinct subunits NF-YA, NF-YB, and NF-YC and bind to CCAAT box in the promoter region of target genes (**Figure 1**). NF-Y transcription factors are crucial factors in nodulation in nitrogen-fixing plants and nitrogen assimilation but there are several reports suggesting their role in stress responses especially in drought stress response and tolerance (Li et al., 2008; Petroni et al., 2012; Laloum et al., 2013; Xu et al., 2014; Quach et al., 2015). Under drought stress, *AtNF-YA5* has been shown to be upregulated in ABA-dependent manner in leaf and roots of *Arabidopsis* plants (Li et al., 2008), (**Figure 1**). Transgenic plants overexpressing *AtNF-YA5* exhibited improved drought resistance and reduced water loss; whereas, *Atnf-ya5* mutant plants were found to be hypersensitive to drought (Li et al., 2008).

INTERACTION BETWEEN DIFFERENT TRANSCRIPTION FACTORS INVOLVED IN DROUGHT STRESS RESPONSE

Evidence suggests that drought-responsive transcription factors work cooperatively to regulate gene expression. The subclass III SnRK2s play a very important role in this context. These subclass III SnRK2s are induced by both ABA and drought stress. In-gel kinase assay using ABA-insensitive and –deficient mutants has suggested that drought stress activate SnRK2s independent of ABA (Yoshida et al., 2006; Boudsocq et al., 2007), (**Figure 1**). Transcriptome analysis of *srk2d/e/i* triple mutants suggests that they modulate the expression of genes involved in ABA-dependent as well as ABA-independent pathways (Fujita et al., 2009). Recently, phosphoproteome study has shown that 5 min of ABA treatment results in phosphorylation of phosphopeptide corresponding subclass III SnRK2s, but osmotic stress fails to do the same. However, short osmotic stress led to phosphorylation of a phosphopeptide corresponding to subclass I SnRK2s, which are not activated by ABA, and phosphopeptides corresponding to MAP3K and MAP4K (E Stecker et al., 2014). Novel proteins are thought to be involved in osmotic stress-dependent activation of SnRK2s.

As mentioned earlier, the target site of DREB/CBF, i.e., DRE/CRT motif also function as a coupling element for ABRE and is present in many ABA-inducible drought responsive genes (Narusaka et al., 2003). AREB/ABF proteins have been shown to physically interact with DREB/CBFs including DREB1A, DREB2A, and DREB2C (Lee et al., 2010), (**Figure 1**). Recent reports have shown that ABRE sequence in the promoter region is required for induction of *DREB2A* under osmotic stress. Furthermore, transient-expression analyses coupled with ChIP (Chromatin Immunoprecipitation) assays has shown AREB/ABFs such as AREB1, AREB2, and ABF3 can bind to the promoter of DREB2A and thereby induce them in an ABRE-dependent manner (Kim et al., 2011), (**Figure 1**). In *grf7* mutant expression of ABA-inducible genes and osmotic stress-responsive genes is upregulated (Kim et al., 2012a). All these reports suggest a complex interaction between the AREB and DREB regulons that need to be further studied in order to create a more comprehensive picture of transcriptional regulation under drought stress.

There are several reports indicating the interaction between AREB/ABFs and NACs. SNAC transcription factor *ATAF1* has been reported to bind to the promoter of NCED3 and thereby regulating ABA hormone levels, giving rise to a probability those SNACs may be involved in regulation of ABA-dependent gene expression of AREB/ABF regulons (Jensen et al., 2013). Conversely, ABRE sequences have been reported in the promoter region of SNAC genes (Nakashima et al., 2012). ANAC096 directly interacts with ABF2 and ABF4 but not with ABF3. Evidence suggests that ANAC096 acts cooperatively with ABFs in activation of ABA-inducible drought responsive genes (Xu et al., 2013), (**Figure 1**). Sakuraba et al. (2015) found that ANAC016 negatively regulates drought stress tolerance. They also found that ANAC016 directly binds to the promoter of *AREB1* and represses its expression. In addition to these two, five other drought-responsive NAC transcription factors (NAC019, NAP, NAC053/NTL4, NAC055, and NAC072) have been found to be associated with ABA signaling (Tran et al., 2004; Lee and Park, 2012; Zhang and Gan, 2012); suggesting they might act as additional tier in regulation of ABA-dependent drought responsive genes.

CONCLUSION

Plant response to drought is a complex process comprising many changes from morphological to molecular level. Under drought stress, many transcription factors operate both exclusively and cooperatively forming a web of interactions. In this review, we have summarized major transcription factors that play a pivotal role in drought stress response and tolerance.

Drought activates many pathways in plants that have been broadly classified in two categories, i.e., ABA-dependent pathways and ABA-independent pathways. AREB/ABFs, DREBs and NACs are the vital transcription factors regulating a large fraction of drought inducible genes (**Figure 1**). Along with these, some other transcription factors such as MYB/MYC factors, WRKY and NF-Y have also been demonstrated to be involved in one or more drought responsive mechanisms (Abe et al., 1997, 2003; Yamaguchi-Shinozaki and Shinozaki, 2006; Baldoni et al., 2015). Four AREB/ABFs, AREB1/ABF2, AREB2/ABF4, ABF3, and ABF1 are the central players of ABA-mediated regulation of gene expression. These AREB/ABFs carry out ABA-regulated responses through binding to the ABRE *cis*-elements present in the promoter region of target genes (Yoshida et al., 2015). On the other hand, DREB1/CBF and DREB2 transcription factors regulate gene expression in ABA-independent manner. Three DREB1/CBFs, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 regulate cold-responsive gene expression whereas, DREB2A and DREB2B are mainly involved in regulation of osmotic stress-responsive gene expression (Nakashima et al., 2009). Additionally, heat stress-inducible genes are also regulated by DREB2A (Mizoi et al., 2012). However, under unstressed conditions, DREB2A levels are tightly regulated at the transcriptional and post-transcriptional levels through GRF7 and DRIPs, respectively (Yoshida et al., 2014). These DREBs bind to DRE/CRT element present in the promoters of genes acting downstream to them (Nakashima et al., 2009). Different NAC factors regulate drought-inducible gene expression by binding to NACRS *cis* elements (Nakashima et al., 2012, 2014).

Drought-responsive transcription factors interact with each other as well as components of other stress pathways resulting in overlap of target genes of these pathways. In this context, subclass III SnRK2s might act as a nodal point as these kinases can regulate the expression of AREB/ABFs as well as DREB2A (Yoshida et al., 2014). Furthermore, there are several pieces of evidence suggesting that AREB/ABF transcription factors interact with DREB and NAC transcription factors (Nakashima et al., 2014).

Transcription factors are potent candidates for engineering stress tolerant plants as a single transcription factor can modulate

a large set of genes. Many of drought-responsive transcription factors have been used to improve drought tolerance in different crops such as rice, wheat, soybean, and maize (Nakashima et al., 2014; Krannich et al., 2015).

All these transcription factors along with other interacting partners constitute a complex network that has been extensively studied but not yet completely understood. In future, further studies about transcriptional regulatory network should provide a more comprehensive picture of the pathways as well as cross-talks. Identification of crucial factors of these pathways along with evolution of new technologies to produce genetically engineered plants should lead to development of plants with improved drought tolerance under field condition with minimal negative effects on crop yield and development.

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REVIEW
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Importance of Mediator complex in the regulation and integration of diverse signaling pathways in plants

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Basic transcriptional machinery in eukaryotes is assisted by a number of cofactors, which either increase or decrease the rate of transcription. Mediator complex is one such cofactor, and recently has drawn a lot of interest because of its integrative power to converge different signaling pathways before channeling the transcription instructions to the RNA polymerase II machinery. Like yeast and metazoans, plants do possess the Mediator complex across the kingdom, and its isolation and subunit analyses have been reported from the model plant, Arabidopsis. Genetic, and molecular analyses have unraveled important regulatory roles of Mediator subunits at every stage of plant life cycle starting from flowering to embryo and organ development, to even size determination. It also contributes immensely to the survival of plants against different environmental vagaries by the timely activation of its resistance mechanisms. Here, we have provided an overview of plant Mediator complex starting from its discovery to regulation of stoichiometry of its subunits. We have also reviewed involvement of different Mediator subunits in different processes and pathways including defense response pathways evoked by diverse biotic cues. Wherever possible, attempts have been made to provide mechanistic insight of Mediator's involvement in these processes.

Keywords: transcription, RNA polymerase II, mediator complex, development, defense signaling, abiotic stress, Arabidopsis, rice

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Introduction

The process of transcription in eukaryotic organism is an immensely complex and highly orchestrated phenomenon, and is mediated by a plethora of proteins wherein primary role is played by RNA polymerase II (RNAP II) (Lee and Young, 2000). The process is regulated both at the transcription initiation and elongation stages by a seemingly endless collections of regulatory proteins involved in different mechanisms (Woychik and Hampsey, 2002). Over the past 30 years, elegant biochemical, genetic, and structural biology works have established a core set of six general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) along with RNAP II as the core elements, which are obligatory to initiate and sustain any successful gene transcription event. On the other hand, among the numerous co-activators characterized till date to facilitate the initial recruitment of RNAP II to the core promoter and the subsequent transcript elongation, the

Abbreviations: MED, Mediator; RNAP II, RNA Polymerase II; BR, Brassinosteroid; SA, Salicylic acid; JA, Jasmonic acid; ET, Ethylene; MudPIT, Multi-dimensional Protein Identification Technology; TAP, Tandem Affinity Purification; LC-MS/MS, Liquid Chromatography-Mass Spectrometry; HT-ChIP, High-Throughput Chromatin Immunoprecipitation.

Mediator complex has emerged as potentially the most crucial by virtue of its essentiality in RNAP II-mediated transcription (Myers and Kornberg, 2000; Conaway et al., 2005; Kornberg, 2005; Malik and Roeder, 2010). The Mediator complex is a highly conserved and integral part of RNAP II-mediated transcriptional machinery of the eukaryotes. In the past, the composition of the Mediator complex and the functions of different Mediator subunits have been reviewed several times focusing on yeasts and metazoans. In plant biology, the central role of Mediator complex in RNAP II-mediated transcriptional event has already been recognized by its discovery in Arabidopsis and other crop plants. The recent time has experienced a flush of interesting reports on the plant Mediator subunits detailing its quintessential role not only in growth and developmental processes, but also in biotic and abiotic stress responses (**Figure 1**). Realizing the need for an updated and critical analysis of roles of Mediator subunits in plants' life, this review summarizes the functions of Mediator subunits and provides insight about the depth and complexity of involvement of Mediator complex in transcriptional regulations of plant genes.

Discovery of Mediator Subunits in Plants

Mediator complex was first discovered in yeast in 1990, and within few years also reported in human (Kelleher et al., 1990; Flanagan et al., 1991; Thompson et al., 1993; Kim et al., 1994; Fondell et al., 1996; Ito et al., 1999). It took more than a decade to purify and characterize first Mediator complex from Arabidopsis cell suspension culture (Bäckström et al., 2007). Bioinformatics predictions including 16 plant species

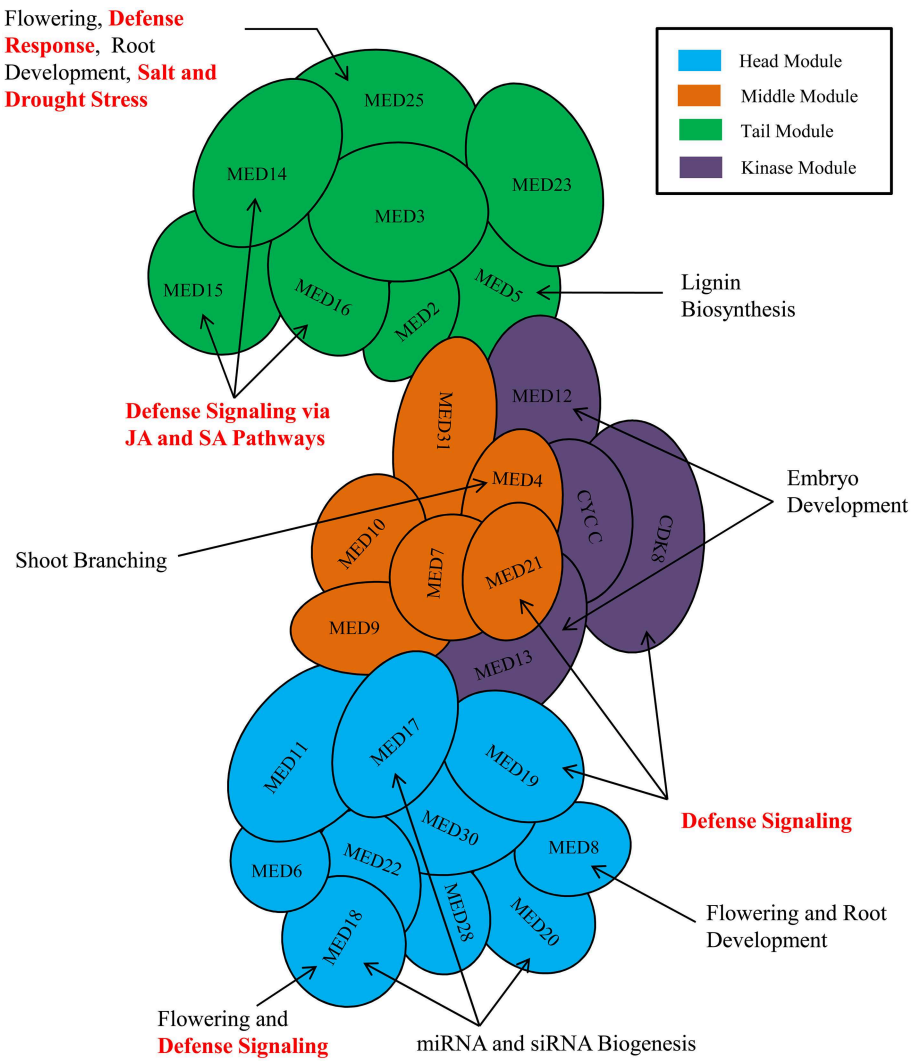


FIGURE 1 | Involvement of plant Mediator subunits in development, and various biotic and abiotic stress responses. Arrangement of Mediator subunits is modular in nature. Mediator complex subunits are arranged into four modules-Head module (Cyan), Middle module (Orange), Tail module (Green), and a separable Kinase module (Purple). Only the known and important functions of plant Mediator complex are shown in the figure. MED26 and plant-specific Mediator subunits (MED34, MED35, MED36, and MED37) are not shown in the figure because of insufficient information about their module positions.

representing the entire plant kingdom ascertained its existence in major crop species including rice (Mathur et al., 2011). Despite low sequence similarity among the orthologs of the Mediator subunits in different organisms because of its rapid evolution, orthologs of all the yeast Mediator subunits reported to be present in plants too (Levine and Tjian, 2003; Bourbon, 2008; Mathur et al., 2011). MED1 is not found in higher plants, but is encoded by the genome of red algae. Also, many of the subunits which were earlier reported to be plant-specific, are actually present in organisms of other kingdom (Bäckström et al., 2007; Bourbon, 2008; Mathur et al., 2011). Thus, most of the Mediator subunits are conserved across the eukaryotic organisms. Structure of Mediator complexes from different organisms have been analyzed with the help of electron microscopy and seemed to be astonishingly similar (Cai et al., 2009; Tsai et al., 2014; Wang et al., 2014). Alignment of secondary structures of the individual plant Mediator subunits with orthologs in other organisms also suggests quite high structural resemblance (Mathur et al., 2011). However, as in the case of many other proteins, plant genomes code for more number of paralogs of several Mediator subunits. Four paralogs of MED15 are encoded by the genes present in *MED15* cluster on chromosome 1 of Arabidopsis (Pasrija and Thakur, 2012). Though functional significance of the presence of multiple paralogs of a particular Mediator subunit is not demonstrated yet, they might help in broadening the regulatory capability of the complex. The spatio-temporal regulation of the expression level of different paralogs of a particular Mediator subunit can make the Mediator structure more dynamic depending upon the external milieu and the growth and developmental phase of the plant.

Modular Organization of Mediator Complex and Its Functions

Mediator is a multi-protein conglomerate, which is enormous in size and complex in composition. The individual protein identity is termed as MED subunit, and the numbers can vary from 25 to 30 depending upon the species. Another salient feature of the Mediator complex is its modular structure. The entire array of subunits of the Mediator complex is arranged into three modular structures; head module, middle module, and tail module (Asturias et al., 1999; Dotson et al., 2000; Chadick and Asturias, 2005; Bourbon, 2008). These three modules together form the Mediator core. The RNAP II-bound Mediator complex is called "Holoenzyme." In addition, there is also a separable kinase or CDK8 module in the Mediator complex, which consists of CDK8, cyclin C, MED12, and MED13 (Wang et al., 2001; Spahr et al., 2003; Elmlund et al., 2006). The Mediator core associates with RNAP II favoring transcription whereas the kinase module-bound Mediator complex dissociates from RNAP II to repress transcription. The Mediator can shuttle between these two different forms (Mediator core and Mediator core-kinase complex) depending upon the cellular contexts. It is worth mentioning here that the kinase or the CDK module subunits were characteristically absent from the first-ever Mediator complex purified from Arabidopsis (Bäckström et al., 2007). The mechanism of Mediator functioning is manifested in different ways. Mediator acts as a bridge between the *cis*-element bound transcription factors and the promoter bound RNAP II, hence recruits the RNAP II machinery to the promoter of the transcriptionally active genes. However, recent progress suggests that Mediator is not just an adaptor molecule between the transcription factor and the basic transcriptional machinery, but provides a platform for recruitment of other cofactors, GTFs, and TFs for the formation of Pre-Initiation Complex (PIC). These interactions can bring changes in the structure of the resultant complex which may affect transcription. Thus, Mediator acts as a docking site for many other transcriptional regulators and plays critical role in relaying regulatory signals from them to RNAP II machinery (Takahashi et al., 2011, 2015).

In the beginning, Mediator complex was thought to be involved only in the initiation step of transcription as evident by its interaction with the components of the transcription initiation complex (Mittler et al., 2001; Baek et al., 2002; Cantin et al., 2003; Johnson and Carey, 2003; Wang et al., 2005). However, in last few years, Mediator has been reported to be involved in the regulation of many other steps of transcription like promoter escape (Malik et al., 2007; Cheng et al., 2012; Jishage et al., 2012), elongation (Takahashi et al., 2011; Conaway and Conaway, 2013; Galbraith et al., 2013), termination (Mukundan and Ansari, 2011, 2013), as well as in other co-transcriptional RNA processing events (Kim et al., 2011; Huang et al., 2012; Oya et al., 2013). Mediator has also been implicated in epigenetic and architectural modification of chromatin leading to changes in gene expression (Kagey et al., 2010; Zhu et al., 2011; Fukasawa et al., 2012; Liu and Myers, 2012; Lai et al., 2013; Tsutsui et al., 2013; Zhang et al., 2013a). Thus, it seems that Mediator is critical for almost every aspect of transcription of eukaryotic genes.

Mediator complex was first discovered as an entity required for enhanced transcription of an *in vitro* transcription system which contained RNAP II and essential general transcription factors (Flanagan et al., 1991; Kim et al., 1994). Now, it is well-established that a number of transcription factors need Mediator to enhance the process of transcription. At this point of time, it is not clear if this positive effect in the activation of transcription is direct or indirect. However, the role of Mediator complex in the repression of gene expression has also been reported in many cases. The repressor activity of Mediator is primarily attributed to the kinase module. Association of this module with the core Mediator complex occludes the RNAP II from the PIC exerting a repressive role in the RNAP II-mediated transcriptional events (Holstege et al., 1998; Samuelsen et al., 2003; Elmlund et al., 2006; Knuesel et al., 2009). However, contrary to this, researchers from different laboratories have also reported the positive effect of kinase module on gene expression (Donner et al., 2007, 2010; Belakavadi and Fondell, 2010). Mechanistically, the activation property of CDK8 module may partly be attributed to its ability to recruit transcription elongation factor, P-TEFb, and to release the promoter-proximally paused RNAP II into productive elongation phase (Takahashi et al., 2011, 2015). The added complexity of the Mediator functions is brought about by the presence of multiple isoforms of the kinase module, which might help the Mediator

complex to fine-tune the gene expression in a tissue, cell, or even pathway specific manner (Sato et al., 2004; Bourbon, 2008; Conaway and Conaway, 2011; Mathur et al., 2011).

Mediator as a Global Regulator of Gene Expression vs. Its Gene Selective Functions

Even after two decades of its discovery, it is still debatable whether Mediator complex is a general transcription factor or just a cofactor of gene expression. Although, initially identified as an entity that supports activator-dependent transcription, now, according to several evidences, Mediator complex can also be categorized as general transcription factor. The human Mediator complex can support basal level transcription of many genes by playing important roles in assembly of PIC and transcription initiation (Mittler et al., 2001; Baek et al., 2002). Mediator complex enhances the RNAP II recruitment to the protein coding genes and provides stability to the transcription machinery assembled at the promoter region (Cantin et al., 2003; Baek et al., 2006). In yeast, deletion of *MED17* makes Mediator structurally unstable, and in the conditional yeast *med17* knockout expression of protein-coding genes is severely compromised on a genome-wide scale (Thompson and Young, 1995; Ansari et al., 2009). In plants, comparison of transcriptomes between *nrpb2-3* (mutant in second largest subunit of RNAP II) and *med20a* plants revealed 84% overlap in the down-regulated genes, implying that Mediator complex is as important as the RNAP II for gene expression (Kim et al., 2011). Thus, the Mediator complex should be regarded as an integral component of the basal transcriptional machinery in the eukaryotes, and the roles are manifested in the forms of RNAP II recruitment and activation, co-ordination of PIC assembly, control of TFIIH-dependent RNAP II CTD phosphorylation within the PIC, and sustained or transient repression of transcription initiation via Mediator-CDK8 module interactions (Taatjes, 2010). Nevertheless, increasing number of reports of deletions of certain Mediator subunits affecting particular phenotype suggest that several individual Mediator subunits possess specific functions as well (**Tables 1**–**3**). This dilemma could be explained by taking into account the modular nature of Mediator complex and by assigning the "division of labor" principles to each module. The head module subunits might be involved in the more basic functions of the Mediator complex, whereas the tail module subunits residing on the periphery might be controlling gene-specific functions by contacting the specific transcription factors.

Functional Analyses of Mediator Subunit Genes

Expression Analyses of the Mediator Subunit Genes

Tight transcriptional regulation of gene expression is very important for proper growth and development of plant and its protection from adverse environmental conditions. After the basic transcriptional machinery, Mediator complex probably can be considered as the second most important regulatory hub for different signaling networks in response to different developmental as well as environmental changes both in animals and plants as suggested by the work of many laboratories including ours. Before we describe the functions of individual Mediator subunit genes, the following is an account of changes in the transcript level of Mediator subunit genes in different tissues, and also how they are affected by different stages of growth and development. We have also discussed the changes in the transcript level of Mediator subunit genes in response to different hormones and abiotic stress treatments.

Tissue-specific and Developmental Regulation of Mediator Subunit Genes

In an attempt to answer the question of what affect the levels of expression of individual Mediator subunits, analyses of differential expression of *MED* genes in different tissues and during different stages of plant development were performed. Several *MED* genes were significantly regulated during panicle and seed development stages as compared to root and leaf of the rice plants (Mathur et al., 2011). The enrichment of seed storage-specific promoter elements in certain *MED* genes raises possibilities of important function of MED subunits during embryo development and seed maturation. The increased abundance of *OsMED8* and *OsMED11_1* at early panicle and seed stages implicates their probable roles in reproductive development of rice. Middle module subunit, *AtMED21_1* showed approximately two-fold upsurge in the advanced stages of seed development which supports the reported role of AtMED21 in embryo development and cotyledon expansion. OsMED21 might be involved prominently in the early stages of panicle development (Dhawan et al., 2009; Mathur et al., 2011). OsMED31_1 is expressed more in leaf as compared to root. The tail module subunit, *AtMED14* is significantly expressed in leaf as compared to other parts of the plants, and has been implicated in the control of cell cycle duration and root elongation (Autran et al., 2002; Krichevsky et al., 2009). In rice, *OsMED26* is expressed more in root as compared to leaves. Significant up-regulation of *OsMED15_1* during different stages of seed development in different rice cultivars supports its probable role in seed development (Thakur et al., 2013). In this process, interaction of OsMED15_1 with seed-specific transcription factors could be predicted. SNP analysis of this gene sequence among several rice cultivars significantly segregated long and short grain varieties. Thus, an important gene regulatory role of this Mediator subunit in seed development and size determination is highly anticipated (Thakur et al., 2013). Several plant-specific Mediator subunits like *MED34*, *MED35*, *MED36*, and *MED37*, which have not been assigned to any module yet, are expressed more in reproductive stages as compared to vegetative parts implying its tissue-specific functions. The Mediator subunit, *MED36* is expressed more in the root of Arabidopsis and is anticipated to be involved in root-specific gene regulatory functions (Pasrija and Thakur, 2013). Thus, Mediator complex as a whole is a dynamic entity, and its

TABLE 1 | Plant Mediator subunits involved in growth and development.

Growth and development	Gene name	Functions in brief	Interacting proteins	References
Embryonic development	*MED12/GCT*	Mediates embryo pattern formation repressing the transcriptional program mediated by KANADI 1 and KANADI 2	Unknown	Gillmor et al., 2010, 2014
	MED13/CCT/Macchi Bou2 (MAB2)	Mediates embryo pattern formation. Additionally, involved in auxin signaling	Unknown	Gillmor et al., 2010, 2014; Ito et al., 2011
Flower development	*AtMED25/PFT1*	Positively controls Constant (*CO*) and *FT* expression, promotes self-destruction by proteasome-mediated degradation	MBR1 and MBR2	Inigo et al., 2012a,b
	AtMED8	Involved in flowering. Transcript of *FLC* and *FT* were high and low, respectively in *Atmed8*	Unknown	Kidd et al., 2009
	AtMED12	Positive regulator of flowering	Unknown	Imura et al., 2012
	AtMED18	Has role in flowering. Binds with the promoter of *FLC* along with AtSUF4, keeps the expression level of *FLC* under control	AtSUF4	Zheng et al., 2013; Lai et al., 2014
Root development	*AtMED25*	The mutant is root hairless. The expression level of class III peroxidases got affected	Unknown	Sundaravelpandian et al., 2013
	AtMED8	Mechanistically similar to MED25, but may be in a different pathway	Unknown	Sundaravelpandian et al., 2013
Other growth and developmental events	*AtMED25/PFT1*	Mutants form large organs, partly because of increasing expression levels of expansions genes, *AtEXP1, AtEXP3, AtEXP5, AtEXP9, AtEXP11,* and *AtEXPB3*	Unknown	Xu and Li, 2011
	AtMED8	Positively controls the organ size	Unknown	Xu and Li, 2012
	AtMED14/SWP	The mutant has reduced leaf number and size, and disorganized SAM	LEUNIG; SMP1 and SMP2 (Probable)	Autran et al., 2002; Clay and Nelson, 2005; Gonzalez et al., 2007
	AtMED18	The mutant has floral deformities. It controls expression of floral homeotic genes like *AP1*, *PI*, and *AG*	Unknown	Kim et al., 2011; Zheng et al., 2013
	AtMED16	Regulates iron homeostasis in plants. Controls marker genes of iron homeostasis like *IRT1, FRO2*	AtMED25, FIT	Yang et al., 2014; Zhang et al., 2014
	OsMED4	Mutants are embryonic lethal. Speculated to be involved in rice tiller growth	OsSAD1 (RPA 34.5)	Li et al., 2014
	AtCDK8/HEN3	Controls the specification of stamen and carpel. Has an inhibitory role on *AG*, *AP1,* and *AP2* expression	CTD domain of RNAP II	Wang and Chen, 2004
	AtMED5a and *AtMED5b*	Involved in active transcriptional processes which inhibit growth and lignin biosynthesis in plants	Unknown	Bonawitz et al., 2012, 2014

composition may fluctuate in different tissues at different stages of growth and development.

Stress and Hormone-induced Regulation of Mediator Subunit Genes

In the process of delineating how hormones affect the expression of different *MED* subunits in Arabidopsis, it was found that brassinosteroid (BR) and abscissic acid (ABA) have more significant impact on the transcription of *MED* genes as compared to other hormones including auxin and jasmonic acid (JA) (Pasrija and Thakur, 2012). *AtMED37*, which was discovered as a plant-specific Mediator subunit, is the most highly up-regulated *MED* in response to BR treatment. The reported 2.5-fold increase of *AtMED12* in response to BR treatment might also shed some light on its role in embryo development (Gillmor et al., 2010; Pasrija and Thakur, 2012). Among the other significant expression changes of Mediator subunits in response to phytohormone treatments, more than two-fold build-up in the transcript level of *AtMED18* in response to JA deserves special mention. AtMED18 has been reported to be involved not only in flower development but also in disease signaling (Zheng et al., 2013; Lai et al., 2014). On the other hand, we noted significant down-regulation (>40%) of tail module subunit genes, like *AtMED15*, *AtMED14*, and *AtMED5* in response to auxin treatment (Pasrija and Thakur, 2012). Although auxin and BR are known for their synergistic effects on plant growth and development, transcription of a set of Mediator genes was different in response to these hormones. For instance, *AtMED15*

TABLE 2 | Plant Mediator subunits involved in stress signaling.

Biotic and abiotic stresses	Gene name	Functions in brief	Interacting proteins	References
Biotic stress	*AtMED25/PFT1*	Regulates the jasmonate pathway	MYC2, AP2/ERF, bHLH, MYB, WRKY, bZIP	Kidd et al., 2009; Çevik et al., 2012; Chen et al., 2012
	AtMED8	Same as AtMED25. The mutant shows more disease susceptibility as compared to AtMED25	Unknown	Kidd et al., 2009
	AtMED16/SFR16	Involved in SA and JA pathway of disease signaling	Unknown	Wathugala et al., 2012; Zhang et al., 2012
	AtMED21	Provides resistance against the necrotrophic fungal pathogens	HUB1	Dhawan et al., 2009
	AtMED15/NRB4	May be involved in SA response pathway of disease signaling. No specific transcriptomic changes observed in mutant plants	Unknown	Canet et al., 2012
	AtMED14/SWP	Effectors of SAR were down-regulated in mutant plant. Both positive and negative regulators of SAR pathway were affected significantly in *Atmed14* mutant	Unknown	Zhang et al., 2013b
	AtMED19a	Provides resistance against powdery mildew pathogen, *Hyaloperonospora arabidopsidis*	HaRxL44	Caillaud et al., 2013
	AtCDK8	Binds with the promoter of the *AGMATINE COUMAROYLTRANSFERASE* to increase expression of defense active bio-compounds	AtMED25	Zhu et al., 2014
	AtMED18	Plays positive regulatory role in defense signaling inhibiting the expression of disease susceptibility genes, glutaredoxins and thioredoxin	YIN YANG1 (YY1)	Lai et al., 2014
Abiotic stress	*AtMED25*	AtMED25 controls salinity stress and drought stress antagonistically	DREB2A, ZFHD1, and MYB	Elfving et al., 2011
	AtMED16/SFR16	The mutant plants are defective in cold acclimation	Unknown	Knight et al., 1999, 2008, 2009; Hemsley et al., 2014

was up-regulated by BR but severely down-regulated by auxin. It seems that these two hormones show their transcriptional effects by a combination of different set of Mediator subunits (Pasrija and Thakur, 2012). In rice, there was not much effect on the transcript abundance of *MED* genes in response to different stresses like drought, salt, and salinity, but one, *OsMed37_6*, exhibits around two-fold change in response to different stresses (Mathur et al., 2011). However, in Arabidopsis, significant transcriptomic reprogramming of the Mediator subunit genes in response to high light, dark, and high salinity conditions was documented (Pasrija and Thakur, 2012). Interestingly, MED16 has been reported to be involved in cold signaling pathways, but the expression level of both *AtMED16* and *OsMED16* remains unchanged in response to cold treatment (Warren et al., 1996; Knight et al., 1999; Mathur et al., 2011). Like its role in cold signaling, more than two-fold increase of *MED16* transcript in response to salinity stress may imply its role as a converging point of both salt and cold signaling pathways. The important functions of AtMED12 in light and salt signaling pathways can not be ruled out because of its two-fold up-regulation in response to high light and salt conditions. Induction of *AtMED37* in response to BR and low light suggests a probable link between shade and BR signaling, and the process may be mediated by Endoplasmic Reticulum-Associated Degradation (ERAD) (Hong et al., 2008; Pasrija and Thakur, 2012). The up-regulation of *AtMED37* in response to cold and salinity stresses provokes an intriguing hypothesis that AtMED37 may act as an integrative hub of many different signaling pathways, which is supported by the near ubiquitous, high expression level of *AtMED37* in all the tissues tested so far (Pasrija and Thakur, 2012, 2013).

Compositional Dynamics of Mediator Complex

Accumulating evidences suggest that Mediator complex is a dynamic and highly flexible entity, and its structural composition alters depending upon the context. Based on the spatio-temporal regulations of transcription of individual Mediator subunit genes in response to different stimuli, we predicted enrichment of specific structural arrangement composed of specific Mediator subunits during certain developmental stages (Pasrija and Thakur, 2013). However, as the Mediator stimulates basal transcription by participating in the recruitment of RNAP II at specific sites all over the genome, a basic, core structure should always be maintained irrespective of tissue, cell, development stage, or any environmental condition. That is the reason that transcription of a set of *MED* genes is not affected by hormones, stresses, or developmental cues. In animal cells, this has been

TABLE 3 | Plant Mediator subunits involved in associated nuclear functions.

Associated nuclear functions	Gene name	Functions in brief	Interacting proteins	References
	AtMED17, AtMED18, and *AtMED19*	Regulation of miRNA and siRNA biogenesis by preventing the binding of RNAP II on the promoters of these genes	Unknown	Kim et al., 2011
	AtMED34/AtRecQ2	Involved in replication related phenomena like D-loop and Holiday structure disruption, maintenance of genomic stability	Unknown	Kobbe et al., 2008
	MED36/FIB2	Processing of rRNA by regulating its methylation	AtPRMT1a and AtPRMT1b (Probable)	Barneche et al., 2000; Yan et al., 2007; Huang et al., 2009
	MED37a/BIP	Helps in female gametophyte development mediating polar nuclei proliferation. Promotes degradation of BRI1-5	BRI 1-5 (Brassinosteroid Receptor)	Hong et al., 2008; Maruyama et al., 2010
	AtMED 35/AtPRPa	Probably takes part in RNA processing	CTD domain of RNAP II	Kang et al., 2009

well-illustrated by the presence of a simpler Mediator complex made of just 6–8 members in differentiated cells as compared to a 26-member Mediator complex in the cancerous and stem cells (Deato et al., 2008).

Genetic and Mutational Analyses of Mediator Subunit Genes

A large portion of total protein coding genes in eukaryotes requires the presence of Mediator complex even to sustain basal level of transcription. This proves unequivocally that Mediator constitutes important part of the basal transcriptional machinery. However, drastic morphological changes in mutants of individual Mediator subunits suggest that Mediator could also act as selective gene regulator both in metazoans and plants (Malik and Roeder, 2010; Taatjes, 2010; Kidd et al., 2011; Mathur et al., 2011; An and Mou, 2013; Poss et al., 2013; Allen and Taatjes, 2015). As the present review is plant specific, the following is an account and critical analyses of important functions of Mediator subunits reported from different plant species through mutational and genome-wide transcriptom analyses (**Tables 1**–**3**).

Embryonic Development

In Arabidopsis and other plants, different phases of embryo development and maturation are marked by specific patterns and shapes. The Mediator subunits, MED12 and MED13, also known as GRAND CENTRAL (GCT) and CENTER CITY (CCT), respectively, mediate the embryo pattern formation, albeit in a transient manner (Gillmor et al., 2010). Mutations in these two genes disrupt the central and peripheral identity of the embryo along with the inhibition of globular to heart transition (Gillmor et al., 2010). Further investigations led to the prediction that the aberrant pattern during early embryo development might be due to a transient transcriptional repression of important genes like those encoding KANADI 1 and KANADI 2 transcription factors. AtMED13, also known as Macchi Bou2 (MAB2), has also been reported to be involved in embryo patterning and cotyledon development (Ito et al., 2011). In this case, the mutant shows aberrations in auxin response. The inability of *Atmed13* embryo to perceive and respond to auxin signals might account for its defective cotyledon formation. Recently, these two kinase module subunits have been shown to be involved in three more developmental transitions, i.e., germination, vegetative phase change, and flowering (Gillmor et al., 2014). Interestingly, the delay in vegetative phase change occurs largely due to over-expression of miR156 and the delay in flowering is caused by the increased production of *FLC*. On the whole, AtMED12 and 13 act as a global regulator of temporal genes making the developmental transitions a tightly controlled phenomenon.

Flower Development

One of the most well-characterized Mediator subunits in plants is AtMED25, which has been described earlier as PFT1. MED25/PFT1 was discovered as a positive regulator of shade avoidance in Arabidopsis (Cerdán and Chory, 2003). It was postulated to be involved in control of flowering by phytochrome B pathway, which is dependent on light quality. The *Atmed25* plants flower late as compared to the wild type (Kidd et al., 2009). It has been demonstrated that AtMED25 positively regulates CONSTANT (CO) and FLOWERING LOCUS T (FT), two important flowering regulators in Arabidopsis (Inigo et al., 2012a). AtMED25 seems to be subjected to the phenomena of "activation by destruction." AtMED25 is an unstable protein that is targeted by two RING H2 proteins, MBR1, and MBR2 for degradation by proteosomal pathway (Inigo et al., 2012b). The high turnover of AtMED25 is required for the activation of FT which promotes flowering. The phenomena elegantly demonstrate how a Mediator subunit follows "activation by destruction" principle to control a plant-specific event, and also adds a new dimension to Mediator function.

Few other Mediator subunits have also been implicated to be involved in flowering process (**Table 1**). Along with AtMED25, the delayed flowering phenotype was also observed in *Atmed8* mutants both under short and long day conditions (Kidd et al., 2009). In *Atmed8* mutants, the level of *FT*, a positive regulator of flowering, is low whereas *FLC*, a negative regulator of flowering, is expressed more. Enhanced phenotype in the double mutant of *Atmed25*/*Atmed8* suggests that AtMED25 and AtMED8 work

independently and they might be controlling flowering process by responding to two different signaling pathways in synergy.

Mediator subunit MED12 (also known as CRYPTIC PRECOCIOUS, CRP) is a positive regulator of flowering and affects multiple genes working upstream and downstream of FT (Imura et al., 2012). As AtMED12 is a part of the kinase module and could interact with histone H3K9 methyltransferase, there is a possibility that it is involved in the epigenetic regulation of *FLC* and *FT* genes (Ding et al., 2008).

Very recently, another Mediator subunit, AtMED18, has been reported to be involved in flowering (Zheng et al., 2013; Lai et al., 2014). The loss-of-function mutant showed delayed flowering, and has altered level of *FLC* and *FT*. AtMED18 has been reported to interact with SUPPRESSOR OF FRIGIDA 4 (SUF4), and together binds to the promoter of *FLC* gene (Lai et al., 2014). Normally, AtSUF4 is a positive regulator of *FLC* gene. AtMED18 probably acts as suppressor of AtSUF4 activity.

The process of flowering requires the transition of vegetative primordia to reproductive primordia, and the region is marked with constant cell division. As the Mediator complex is often connected with dynamic cellular activities, it is quite obvious that Mediator plays significant role in the process of flowering and that is why several subunits affect this process (**Table 1**). Mostly, the loss-of-function mutations of Mediator subunits led to late and abnormal floral development, which is attributed to the perturbation in the transcript level of important flowering regulators like *FLC*, *FT*, and floral identity regulators like *AG*. But the missing link is how Mediator subunits control the expression of these genes. The non-coding RNAs play important role in epigenetic regulation of *FLC* gene (Crevillén and Dean, 2011; De Lucia and Dean, 2011). Given the reported association of non-coding RNA with the Mediator complex, a similar kind of mechanism in flowering time control could be envisaged (Lai et al., 2013).

Root Development

A search for the role of Mediator subunits in root morphogenesis revealed the pivotal role of MED25 and MED8 in the production of root hairs in Arabidopsis (Sundaravelpandian et al., 2013; Raya-González et al., 2014). The absence of root hairs in *Atmed25* and *Atmed8* is due the inappropriate distribution of hydrogen peroxides (H_2O_2) and superoxides (O_2^-) over the surface of tap root system. In fact, the comparison of the transcriptome of wild type and the *Atmed25* plants revealed that class III peroxidases are the worst affected ones in the mutant, perturbing the ROS homeostasis across the root length. The more severe phenotype of *med25/med8* double mutant eliminates the possibility of these two genes interacting in the same pathway. It will be interesting to find out if other MED subunits assist MED25 and MED8 in root hair development. Also, knowledge of transcription factors targeting these subunits will be helpful in understanding the mechanisms of transcriptional regulation of this process.

Other Growth and Developmental Events

Mediator subunit CDK8 (or HEN3) of the kinase module plays important role in specification of stamen and carpel in Arabidopsis (Wang and Chen, 2004). Mechanistically, like in yeast and mammals, AtCDK8 phosphorylates the CTD domain of largest subunit of RNAP II and represses transcription. This leads to an enhanced expression of *AG*, *AP1*, and *AP2* in cdk8 mutant. CDK8 is abundantly expressed in the proliferating tissues suggesting its involvement in mediating cell division and cell fate specification. Alternatively, as the RNA transcription and RNA processing are coupled and CDK8 interacts with CTD domain of RNAP II, the perturbed alternative transcript of *AG1* in the mutant plant indicates its probable role in alternative splicing. What it warrants at this moment is to identify the transcription factors that interact with these Mediator subunits and the immediate target genes for a better understanding of the regulatory circuitry that controls cell number and size.

Another Mediator subunit AtMED18 contributes to the organ identity and number. Other than being short in stature and late flowering, *Atmed18* plants have altered number of floral parts. In mutant plants, sepals and petals are more and anthers are less. There are two carpels, and the pollen maturation is delayed (Kim et al., 2011; Zheng et al., 2013). The down-regulation of floral homeotic genes like *AP1*, *PI*, and *AG* in *Atmed18* mutant plants indicates crucial regulatory role of AtMED18 in homeotic gene expression (Zheng et al., 2013). Additionally, AtMED18 may control the organ identity genes through its association with HEN3/CDK8, which also controls organ identity and shows similar loss-of-function phenotypes (Wang and Chen, 2004).

Cell number over the entire arial parts of Arabidopsis is decreased if there is a mutation in another Mediator subunit *MED14*, more popularly known as *STRUWWELPETER (SWP)* (Autran et al., 2002). Both the leaf number and size in the heterozygous mutant lines are reduced whereas homozygous mutant lines are sterile. The importance of *AtMED14* in leaf development is also evident by its strong expression in leaves. The mutant plant also carries a disorganized Shoot Apical Meristem (SAM). The arrest of cell division in *Atmed14* plants may result from the endoreduplication of the chromosomal DNA. Mechanistically, AtMED14 may interact with SMP1, SMP2 which encode step II splicing factors as both the mutants show similar phenotypes (Clay and Nelson, 2005). LEUNIG, a GroTLE transcription corepressor, has been reported to interact with AtMED14 and controls multiple physiological processes (Gonzalez et al., 2007).

MED4 is a subunit in the middle module, and has recently been speculated to be involved in growth of the tillers in rice (Li et al., 2014). Its homozygous mutants are embryonic lethal. Surprisingly enough, it interacts with SAD1, an ortholog of RNA polymerase I subunit RPA 34.5 in rice, and is involved in rRNA biosynthesis. It is worth mentioning here that SAD1 was isolated as a component of Mediator complex during the complex purification study in Arabidopsis (Bäckström et al., 2007). It also interacts with the counter parts of the other RNA polymerases like pol II and pol III. Thus, this is the first example which shows the interaction between the Mediator complex and the RNA pol I and III, and thus extends the function of Mediator beyond RNAP II-mediated transcription.

Cell proliferation and cell expansion are two important basic processes in any organism, which ultimately determine the organ size, hence the entire body size. DA1 is an ubiquitin receptor and restricts cell proliferation to control final size of organs in Arabidopsis (Li et al., 2008). In a genetic screen to find the enhancer of *DA1* mutation, *AtMed25* mutant was characterized (Xu and Li, 2011). AtMED25 too negatively controls the cell proliferation and cell enlargement. Loss-of-function mutant of *MED25* has large organs, with larger and slightly increased numbers of cells as a result of an increased period of cell proliferation and cell expansion. The observed phenotype in *Atmed25* mutant plants may be partly because of the up-regulation of expansin genes like *AtEXP1, AtEXP3, AtEXP5, AtEXP9, AtEXP11,* and *AtEXPB3*. Consistent to this, plants over-expressing *MED25* have small organs owing to decrease in both cell number and size. Further analysis eliminated the possibility of higher ploidy level in the mutant plants as the cause of larger organ size. The genetic and physiological data suggest that MED25 acts to limit cell and organ growth independently of its involvement in phytochrome and JA signaling pathways (Cerdán and Chory, 2003; Kidd et al., 2009; Xu and Li, 2011; Chen et al., 2012; Inigo et al., 2012a,b). Rather, MED25 functions synergistically with DA1 to control organ growth by restricting cell proliferation. In contrast to MED25, MED8 positively controls the organ size (Xu and Li, 2012). The mutant *Atmed8* plants have shorter flowers because of reduced cell expansion. Analysis of *med25med8* double mutants revealed the antagonistic behavior of MED25 and MED8, at least in the case of cell expansion and cell proliferation, hence in organ size determination.

Getting rid of lignins from the crops for its usage as forage, pulp, and paper production poses a significant challenge because most of the lignin related mutants are stunted and growth defective. Two such mutants, *ref8-1* and *ref8-2*, which are deficient in lignin content, are short and display little vegetative growth. Two Mediator subunits, AtMED5a (REF4), and AtMED5b (RFR1), have been shown to negatively regulate plant height and lignin content (Bonawitz et al., 2012). Interestingly, the mutants of either of these subunits rescue the phenotype of *ref8-1* or *ref8-2* without any yield penalty on biomass production (Bonawitz et al., 2014). Importantly, the mutants are free from biomass recalcitrance. Thus, the domain of Mediator function also encompasses the regulation of cell wall biosynthesis, which is of great practical value.

Iron is one of the essential elements in plants, and its uptake and assimilation are tightly controlled. Two Mediator subunits, AtMED16 (YID), and AtMED25, have been reported to control iron homeostasis is plants (Yang et al., 2014; Zhang et al., 2014). The mutants of these Mediator subunits display hypersensitivity toward iron deficiency resulting in leaf chlorosis. AtMED16 directly interacts with FIT, the master regulator of iron homeostasis in plants. In chromatin immunoprecipitation analysis, AtMED16 was found to be present on the promoter of the iron acquisitions genes like *FRO2* and *IRT1*, probably by interacting with FIT (Zhang et al., 2014). FIT also interacts with other bHLH proteins forming heterodimers and these heterodimers bind to *FRO2* and *IRT1* promoters. The binding of AtMED16 probably confers stability to the FIT/bHLH complex (Zhang et al., 2014). On the other hand, MED25 interacts with two transcription factors, EIN3 and EIL1, which are involved in ethylene signaling. EIN3 and EIL1 directly interact with FIT. FIT is a highly unstable protein and the interaction of MED25 with EIN3 and EIL1 provides stability to FIT enabling it to regulate downstream iron regulatory genes like *FRO2* and *IRT1* (Yang et al., 2014). Interaction between AtMED16 and AtMED25 has also been reported (Zhang et al., 2014). However, the effects of double mutations of these two genes are yet to be investigated. Probably, AtMED16, AtMED25, EIN3, EIL1, FIT, and other bHLH proteins form a stable activator complex on the promoter of *FRO2* and *IRT1* leading to their activation during iron deficient conditions.

Defense Signaling

Plants in its natural environments are being constantly challenged by myriad of insect pests and pathogens, which together constitute the biotic stresses. A survivor plant activates its defense arsenal quickly and efficiently in order to counter the invading and inflicting biotic agents. Such an orchestrated and rapid response is only achievable by the timely activation of key defense genes. Emerging reports have established Mediator complex as an essential component for regulation of genes involved in defense pathways (An and Mou, 2013). In comparison to other pathways, higher number of Mediator subunits has been shown to be involved in defense signaling (**Table 2**).

The first Mediator subunit reported to be involved in defense response was AtMED25 (Kidd et al., 2009). AtMED25 bears similarity with the mammalian MED25, which also plays important role in defense response (Leal et al., 2009). In Arabidopsis, MED25 directly affects JA-dependent gene expression (*PDF1.2, HEL, CHIB,* and *ESP*), and provides resistance against the leaf-infecting necrotrophic fungi, *Alternaria brassicicola*, and *Botrytis cinerea* (Kidd et al., 2009). The complementation of *Atmed25* by its homologs from wheat strengthened the view that functions of some of the Mediator subunits may be conserved in higher plants (Kidd et al., 2009). A group of 12 transcription factors (TFs) have been shown to interact with AtMED25, which includes AP2/ERF, bHLH, MYB, WRKY, and bZIP. Among these transcription factors, many have previously been demonstrated to be involved in JA signaling pathway (Çevik et al., 2012). Furthermore, AtMED25 takes part in ERF1- and ORA59-dependent activation of *PDF1.2* gene as well as MYC2-dependent activation of *VSP1* gene, which are some important genes in the JA signaling pathway (Çevik et al., 2012). In fact, MED25 physically associates with the bHLH transcription factor, MYC2 in promoter regions of its target genes to elicit a positive effect on their transcription (Chen et al., 2012). The head module subunit mutant, *Atmed8*, behaves like *Atmed25* but shows pronounced susceptibility toward *A. brassicicola* (Kidd et al., 2009). These two mutants, however, do not interact genetically, suggesting that AtMED25 and AtMED8 might be acting in two independent pathways controlling the same response and phenotype (Kidd et al., 2009).

The middle module subunit MED21 is an essential requirement for survival of Arabidopsis plants as its T-DNA insertional homozygous lines are embryonic lethal (Dhawan et al., 2009). The RNAi lines of *MED21* are highly susceptible to *A. brassicicola* and *B. cinerea.* The detailed study revealed that MED21 interacts with RING E3 ligase, Histone Monoubiquitination1 (HUB1), which mediates the H2B ubiquitination, thus establishing a link between Mediator and the chromatin remodeling. The induced expression of both *MED21* and *HUB1* in response to chitin treatment, an important constituent of fungal cell wall, suggests their probable role in defense signaling (Dhawan et al., 2009).

The head module subunit, AtMed19a interacts with nuclear localized fungal effector (HaRxL44) of powdery mildew pathogen, *Hyaloperonospora arabidopsidis* (Hpa). This leads to proteasome-dependent degradation of AtMed19a and shift the balance from SA-mediated disease resistance to ET/JA-mediated transcriptomic changes making the plants more vulnerable to bitrophs (Caillaud et al., 2013). This highlights how pathogens can break plant immune barrier by hijacking the important resistance mechanisms offered by Mediator complex. Another head module subunit, AtMED18, plays a positive regulatory role toward necrotropic fungal infection by interacting with YYI keeping the expression of glutaredoxin and thioredoxin genes suppressed (Lai et al., 2014).

Three tail module subunits, AtMED14, AtMED15, and AtMED16 have been reported to be involved in defense signaling as well (Canet et al., 2012; Wathugala et al., 2012; Zhang et al., 2012, 2013b) (**Table 2**). The Arabidopsis plants carrying mutation in *MED16* are compromised for SA- and JA-dependent defense responses (Wathugala et al., 2012). The *Atmed16* mutant plants are more susceptible to *Pseudomomas syringae* attack, and exhibit lower expression of defense-related genes like those coding for PR (Pathogenesis Related) proteins and defensins. Moreover, the expression levels of the important SAR (systemic acquired resistance) markers like *PR1, PR2, PR5, GST11, EDR11, SAG21* are severely reduced in *Atmed16* mutant (Zhang et al., 2012). Hence, MED16 acts as a positive regulator of SA-induced gene expression. Similarly, the *Atmed16* mutation also blocks the induction of the JA/ET-dependent gene expression making the plants vulnerable to necrotrophic fungi like *A. brassicicola* and *B. cinerea* (Zhang et al., 2012). Thus, MED16 seems to function as an integrative hub for both SA and JA signaling pathways. The tail module subunit, AtMED15, also dubbed as NRB4 (Non-recognition of BTH4, a salicylic acid analog), has recently been shown to be involved in defense signaling via its involvement in SA pathway (Canet et al., 2012). The mutant plants with defective MED15 do not show any noticeable phenotypic change except its attenuated response to SA, reminiscent of the effects of *npr1* mutation in plants' defense signaling. NPR1 (non-expresser of *PR* genes) plays a pivotal role and takes the center stage in the SA-mediated defense pathways (Dong, 2004). However, neither a genetic nor a biochemical interaction has been reported between MED15 and NPR1. The additive phenotypes of *Atmed15/npr1-70* plants indicate that they might work at different point of SA signaling pathway. Moreover, *Atmed15* affects neither the localization of NPR1 nor its stability. Thus, mechanistically, MED15/NRB4 might be functioning downstream of NPR1 in the regulation of SA response pathway. The exact position of MED15 in SA signaling pathway is not known, and it warrants detailed molecular and genetic investigations. A mutation in *AtMED14* subunit gene suppresses the SA-dependent expression of defense genes (Zhang et al., 2013b). AtMED14 prevents *PR1* expression without interfering the binding of NPR1, the master regulator of defense gene expression, to its promoter. This leads to the speculation that AtMED14 might be responsible for the recruitment of RNAP II to the promoter of *PR1* gene. Further investigation is needed to delineate the exact mechanism involved in the process. Thus, it seems that most of the subunits in the tail module play significant role in the regulation of defense gene expression during pathogen attack. However, the mechanisms employed by the three different Mediator subunits (MED14, MED15, and MED16) differ considerably toward controlling the expression of defense genes. The *Atmed16* mutation differentially affects the expression of different positive and negative regulators of SAR, whereas *Atmed14* mutation inhibits expression of similar genes. Moreover, defense-related transcriptomic change in the case of *Atmed14* is much smaller as compared to that in the case of *Atmed16*.

The kinase module component, AtCDK8, has recently been reported to be a positive regulator of disease response (Zhu et al., 2014). The mutant plants are highly susceptible to *A. brassicicola*. Mechanistically, it interacts with another Mediator subunit, AtMED25, and regulates JA-mediated gene expression during pathogen signaling. Additionally, it binds with the promoter of *AGMATINE COUMAROYLTRANSFERASE (AACT1)* gene whose products are involved in the biosynthesis of defense active bio-compounds like hydroxycinnamic acid amides in plants.

Abiotic Stress Signaling

Plants are sessile organisms. They cannot run away to safer places during inclement weather. On the other hand, growth and development of the plant is profoundly influenced by the environment. A robust, surviving plant must translate the vagaries of the surrounding environments into proper signals relaying them to the transcriptional machinery ensuring the adaptability of the plants to the changed milieu. Of late, Mediator has emerged as an integrative hub for the different signaling pathways leading to the transcription regulation by RNAP II. So it is highly anticipated that the Mediator will also play a crucial role in the integration of signals originated in response to stresses like drought, cold, salinity etc. So far two Mediator subunits (**Table 2**), which also play important roles in biotic stresses, have been reported to be involved in abiotic stress signaling. The *Atmed25* mutant seeds display increased sensitivity toward salt stress during germination. The importance of MED25 in high salinity is conserved across the plant species (Elfving et al., 2011). In a yeast two hybrid screen, three stress-specific transcription factors, DREB2A, ZFHD1, and MYB like proteins were found to be interacting with the ACID (Activator Interacting Domain) domain of AtMED25. The plants carrying mutations in any of these genes also display severe salt sensitivity. Mechanistically, ACID domain of MED25 might be targeted by these transcription factors for communication with the RNAP II transcriptional

machinery for effective salt-responsive transcriptomic changes in plants. Surprisingly, MED25 negatively regulates drought tolerance in plants (Elfving et al., 2011). The mutant plants display huge increase in the expression level of drought responsive marker genes like *RD29A*, *RD29B*, and *DREB2A*. AtMED25 has been projected as a co-repressor interacting with the repressor domain of DREB2A making the plants vulnerable to drought stress (Elfving et al., 2011). Thus, it is one of those examples, where the same Mediator subunit, AtMED25, controls salt and dehydration stresses in an antagonistic manner.

MED16, originally discovered as SFR6 in Arabidopsis before being identified as a part of Mediator complex, has been reported as an important component involved in acclimation to cold (Knight et al., 1999, 2008; Wathugala et al., 2011). The mutant plants fail to embrace freezing temperature following its exposure to subzero temperature. At the molecular level, the plants are incapable of switching on the COR (cold on regulation) regulon including the expression of *LTI78*, *COR15A*, and *KIN1/2*. Microarray analysis revealed that a subset of cold-responsive genes bearing CRT/DRE motifs in their promoter regions gets miss-regulated in *Atmed16* mutant plants (Knight et al., 1999). These genes are involved in freezing tolerance and controlled by CBF transcription factors (Boyce et al., 2003). However, neither the expression of CBF nor its localization is affected in *Atmed16* mutant plants (Knight et al., 2009). Thus, it provokes the intriguing speculation that MED16 might modulate the activity of CBFs through post-transcriptional modulation.

Associated Nuclear Functions

One of the most significant discoveries of Mediator function in plants is related to miRNA and siRNA biogenesis (Kim et al., 2011). The loss-of-function mutants of three Mediator subunits, *Atmed17*, *Atmed18*, and *Atmed20a*, are short in stature, late flowering, and bear small fruits as compared to the wild types. The in-depth, detailed analyses revealed that these mutants are defective in the regulation of miRNA and siRNA at the transcriptional level. The occupancy of RNAP II at the promoters of miRNA and siRNA genes was also highly reduced in these mutants. The role of these Mediator subunits has also been implicated in the silencing of transposons and repeat sequences. These elements normally undergo siRNA-mediated transcriptional gene silencing, and were de-repressed in *med17*, *med18*, and *med20a*. On the other hand, co-purification of MED36 with the largest subunit of RNA pol V led to the intriguing hypothesis that Mediator complex may act in cooperation with other RNA polymerases in the production of non-coding RNA (Huang et al., 2009). Although it is a matter of debate, the same study also advocated the role of the Mediator complex as a general transcription factor. The discovery brought a paradigm shift in the understanding of Mediator functions beyond the regulation of subunit specific functions (**Table 3**).

The newest entrants into the expanding list of plant Mediator subunits are MED34 to MED37 (Bäckström et al., 2007). The phenomenon that provokes curiosity is that a DNA helicase, AtRecQ2, which takes part in replication related phenomena like genome stability, D-loop and Holliday structure disruption, turned out to be MED34 (Kobbe et al., 2008). The Arabidopsis Mediator subunit, MED36/FIB2 has been shown to encode a Fibrillarin (FIB2), which is involved in rRNA processing (Barneche et al., 2000). It interacts with and is methylated by histone methyltransferases, AtPRMT1a and AtPRMT1b, and co-purified with RNA pol V (Yan et al., 2007; Huang et al., 2009). MED37a (also known as BiP) was first characterized as one of the HSP70 family members, and is homologous to yeast Ig-binding protein (Rose et al., 1989). It is involved in polar nuclei fusion during female gametophyte development, and is essential for the regulation of endosperm nuclei proliferation (Maruyama et al., 2010). In Arabidopsis, it also interacts with BR hormone receptor, BRI1, facilitating its proteasome-independent endoplasmic reticulum–associated degradation (ERAD) (Hong et al., 2008). Among the three AtPRP40s (*Arabidopsis thaliana* pre-mRNA processing protein 40), AtPRP40a has been recently named as AtMED35 of the Mediator complex. It interacts both with the phosphorylated and the unphosphorylated forms of the largest subunit of RNAP II. In Arabidopsis, it has its characteristic high expression level in roots and cauline leaves as compared to the other parts. The mutant does not show any phenotype, probably because of its redundancy with AtPRP40b and AtPRP40c (Kang et al., 2009).

The key importance of the Mediator complex lies in its ability to act as an adaptor molecule between transcription factors and the RNAP II, and hence the on-going research has so far been directed toward its role in the initial processes of transcription. The recent findings regarding its probable role in elongation and termination have not only expanded its arena of functionality, but have given fresh impetus toward the possibility of involvement of Mediator complex in other co-transcriptional processes like RNA processing (splicing, capping, polyadenylation), alternative splicing and epigenetic regulation (**Table 3**). Hence, the functional association of some Mediator subunits in these processes seems quite natural, and these issues need to be addressed more critically in future. As expected Mediator complex has critical control over miRNA and siRNA biogenesis as these are also transcribed by RNAP II. However, the association of other RNA polymerases with the Mediator complex, and its role in other RNA polymerase-mediated transcriptional events need to be examined further. Currently, we lack explanations for the Mediator subunits, which take part in phenomena like replication, protein degradation etc.

Complex System of Mediator as Target of Diverse Transcription Factors to Regulate Different Processes and Pathways

Mediator acts as an intermediary between the *cis*-element bound transcription factor and the RNAP II-mediated transcriptional machinery relaying the information from the transcription factor to the transcription apparatus. Recently, a couple of reports in plants have made the picture more complicated as the interaction between the transcription factor and the Mediator complex is not a simple binary one-one interaction. The Arabidopsis Mediator subunit, MED25 can interact with several transcription factors (DREB2A, ZFHD1, and MYB like proteins), that function in

the same pathway. The mutants of all these three genes show increased sensitivity to salinity stress (Elfving et al., 2011). On the other hand, it has been also shown that AtMED25 can differentially control two seemingly different pathways, JA and ABA signaling, by interacting with two different transcription factors like MYC2 and ABI5, respectively (Chen et al., 2012). Similarly, AtMED18 has been shown controlling multiple plant responses by interacting with different transcription factors (Lai et al., 2014). An analogous situation also happens in yeast where different nuclear receptor-like transcription factors like Oaf1, Pdr1, and Pdr3 target the same Mediator subunit, MED15, to control different processes like fatty acid metabolism and multi-drug resistance (Thakur et al., 2008, 2009). On the contrary, a single characteristic/phenotype in plants can also be controlled by the concerted actions of more than one Mediator subunits (**Tables 1**, **2**). Detailed investigation is needed to figure out whether these Mediator subunits do take part in the same developmental pathway while controlling a specific character or they control different developmental programs converging to a single phenotype. There are copious examples in animals where distinct Mediator subunits can control specific developmental and signaling pathways (Ito et al., 2000; Stevens et al., 2002; Ge et al., 2008). We suggest that the permutations and combinations of transcription factors with the Mediator subunits probably generate a Mediator code which dictates the downstream gene expression phenomena in co-ordination to the developmental stage and the prevailing environmental conditions. It might also involve Mediator complex undergoing a great deal of structural adjustment and alignment after binding with the transcription factors, which need to be studied in detail in the future.

Conclusion

The universality of the Mediator complex in the transcription of protein coding genes has ushered a new era in the understanding of transcriptional regulations in yeast and human. The plant science community is not lagging far behind in Mediator research. The achievement includes not only the first Mediator complex isolation from Arabidopsis but also the discovery of ubiquitous presence of Mediator complex in almost all the phyla of plant kingdom.

A general revelation from different studies is that the repertoire of Mediator subunits has been expanded in plant species to cope up with the increased number of plant transcription factors. This provides better resilience power to the sessile plants against the vagaries of the biotic and abiotic stresses. However, a note of caution should be shown regarding the discovery of new Mediator subunits. Until now there are no defined parameters to designate a protein as Mediator subunit. The Mediator acts as a scaffold for the interaction of a number of transcriptional regulatory proteins. Does mere co-purification with the Mediator complex qualify a protein to be regarded as Mediator subunit? Recently, six new plant-specific Mediator subunits (AtMED32–AtMED37) were discovered in Arabidopsis, but later AtMED32 and AtMED33 were found to be AtMED2 and AtMED5, respectively. As some of their functions are not directly related to Mediator functions or transcription (as for example, AtMED34 or AtRecQ2), concern has been expressed regarding how truly these proteins represent Mediator subunits.

Many of the Arabidopsis Mediator subunits were characterized earlier, but not in consideration of its Mediator membership. Over the time, several Mediator subunits have been characterized in Arabidopsis and many more may follow. In most of the cases, phenotypes of a particular Mediator subunit mutant has been described, but its association with transcription factors and the set of genes under its control are yet to be discovered in majority of the cases. What is lacking more is the understanding of how the Mediator subunits interact with components of the basic transcriptional machinery resulting in the regulated transcription.

Recently, many of the hitherto unknown but interesting functional aspects of Mediator has been unveiled in other organisms further broadening the horizon of its roles. Mediator not only takes part in the recruitment of RNAP II on the promoters of the active genes but also in transcription elongation and termination, chromatin remodeling, alternative splicing, small, and long non-coding RNA biogenesis, heterochromatin formation. All these developments are taking place in the arena of yeast and metazoan biology. Except characterization of few Mediator subunits, studies involving the Mediator complex as a whole or the mechanistic dexterity of Mediator complex in general or gene-specific regulation has not been addressed with proper emphasis and interest in plants. So, besides characterization of the every Mediator subunits in model species, attention should also be focused to address how the Mediator controls different steps of transcription in terms of mechanical intricacies.

Presence of more than one paralog has been reported for some Mediator subunits. Another level of complicacy may arise regarding which paralog remains with the complex, which most probably is controlled in a temporal and spatial manner. The presence of more than one paralog at a time in the Mediator complex has not been reported by any group. The more interesting question which has just been started to be answered is how stable is the Mediator structure in terms of its subunit composition. We postulate that the structure of Mediator complex changes depending on the composition of Mediator subunits, which again is controlled by different biotic and abiotic stimuli. Mediator complex isolation and its structural comparison from different stages of growth and development hold the key to the questions of how the structural shifts due to changes in Mediator composition are translated into transcriptomic changes of a species in response to intrinsic and extrinsic factors. Armed with the tools of modern molecular biology like TAP, MudPIT, LC-MS/MS, and HT-ChIP; the aforementioned questions are anticipated to be answered at an accelerated speed in near future.

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Correlation between differential drought tolerability of two contrasting drought-responsive chickpea cultivars and differential expression of a subset of *CaNAC* genes under normal and dehydration conditions

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Drought causes detrimental effect to growth and productivity of many plants, including crops. NAC transcription factors have been reported to play important role in drought tolerance. In this study, we assessed the expression profiles of 19 dehydration-responsive *CaNAC* genes in roots and leaves of two contrasting drought-responsive chickpea varieties treated with water (control) and dehydration to examine the correlation between the differential expression levels of the *CaNAC* genes and the differential drought tolerability of these two cultivars. Results of real-time quantitative PCR indicated a positive relationship between the number of dehydration-inducible and -repressible *CaNAC* genes and drought tolerability. The higher drought-tolerant capacity of ILC482 cultivar vs. Hashem cultivar might be, at least partly, attributed to the higher number of dehydration-inducible and lower number of dehydration-repressible *CaNAC* genes identified in both root and leaf tissues of ILC482 than in those of Hashem. In addition, our comparative expression analysis of the selected *CaNAC* genes in roots and leaves of ILC482 and Hashem cultivars revealed different dehydration-responsive expression patterns, indicating that *CaNAC* gene expression is tissue- and genotype-specific. Furthermore, the analysis suggested that the enhanced drought tolerance of ILC482 vs. Hashem might be associated with five genes, namely *CaNAC02*, *04*, *05*, *16,* and *24*. *CaNAC16* could be a potential candidate gene, contributing to the better drought tolerance of ILC482 vs. Hashem as a positive regulator. Conversely, *CaNAC02* could be a potential negative regulator, contributing to the differential drought tolerability of these two cultivars. Thus, our results have also provided a solid foundation for selection of promising tissue-specific and/or dehydration-responsive *CaNAC* candidates for detailed *in planta* functional analyses, leading to development of transgenic chickpea varieties with improved productivity under drought.

Keywords: chickpea, NAC transcription factors, differential expression, differential drought tolerability, RT-qPCR

Introduction

Drought has been considered as a major environmental constraint commonly encountered by plants, which cause significant losses to crop yield (Shao et al., 2009; Stolf-Moreira et al., 2011; Osakabe et al., 2013). Intensive research conducted in the past two decades has provided an insight into molecular mechanisms that control plant responses to drought (Shao et al., 2008; Ni et al., 2009; Hadiarto and Tran, 2011; Jogaiah et al., 2013; Albacete et al., 2014; Shanker et al., 2014). Various transcription factors (TFs) and their DNA binding sites, the so-called *cis*-acting elements, have been identified as molecular switches of stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006; Tran et al., 2007). Among the TF families, the plant-specific NAC [no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF), and cup-shaped cotyledon (CUC)] TF family members have been intensively studied owing to their functions in a wide range of biological processes in plants, including regulation of plant responses to environmental stimuli (Olsen et al., 2005; Tran et al., 2010; Nakashima et al., 2012; Puranik et al., 2012). Increasing number of reports have shown convincing evidence correlating drought tolerance of various plant species and expression of *NAC* genes (Tran et al., 2004; Hu et al., 2006; Nakashima et al., 2007; Thao et al., 2013; Thu et al., 2014a), suggesting their potential for genetic engineering of improved drought-tolerant crop varieties.

Chickpea (*Cicer arietinum* L.) is a nutritionally important legume crop cultivated in many countries in the Asian–African region, supplying a great source of mineral-, vitamin-, protein-, and carbohydrate-rich food for animal feeding and human consumption (Rubio, 2005; Bampidis and Christodoulou, 2011; Jukantil et al., 2012; Ngwe et al., 2012). However, drought imposes a detrimental impact on chickpea productivity worldwide, leading to a significant yield loss which has necessitated the load of chickpea research programs with the aim to develop drought-tolerant chickpea cultivars (Molina et al., 2008; Jain and Chattopadhyay, 2010; Nasr Esfahani et al., 2014). Seeing the great potential of the NAC TFs in conferring plant tolerance to drought, we recently took the advantage of the availability of the chickpea whole genomic sequence (Jain et al., 2013; Varshney et al., 2013) to identify all the *CaNAC* genes annotated in the chickpea genome (Ha et al., 2014). A total of 71 and 62 potential *CaNAC* genes was identified in the genome of the sequenced chickpea "kabuli" and "desi" cultivars, respectively (Jain et al., 2013; Varshney et al., 2013), many of which showed dehydration-responsive patterns, suggesting their involvement in regulation of drought responses in chickpea, and thus potentially playing important roles in chickpea adaptation to drought stress (Ha et al., 2014).

In this study, we further examined the functions of *CaNAC* genes in chickpea by comparing the expression levels of a subset of *CaNAC* genes in two chickpea cultivars with contrasting drought tolerance using real-time quantitative PCR (RT-qPCR) under normal and dehydration conditions. Such correlation analysis of expression levels, dehydration-responsive expression patterns and drought-tolerant degrees will enable us to identify *CaNAC* genes that are potentially associated with drought tolerance for in-depth *in planta* functional characterization prior to using them in genetic engineering for development of transgenic chickpea, as well as other crop, cultivars with superior yield under water-limited conditions.

Materials and Methods

Plant Growth, Treatments, and Collection of Tissues

Seeds of chickpea (*Cicer arietinum* L.) drought-sensitive Hashem and drought-tolerant ILC482 "kabuli" cultivars were received from International Center for Agricultural Research in the Dry Area (ICARDA), Syria. Hashem was developed by the Seed and Plant Improvement Institute, Karaj, Iran (Sabaghpour et al., 2005), whereas ILC482 was released by ICARDA, Syria (Singh et al., 1992). The drought-tolerant ILC482 and drought-sensitive Hashem cultivars used in this study are well-known for their contrasting drought tolerance. Their differential drought tolerability was demonstrated by the comparison of the stress tolerance index (STI), geometric mean productivity (GMP), mean productivity (MP), and harmonic mean (HM) that were determined based on their yields obtained from a field study under irrigated (well-watered) and rainfed (drought stress) conditions (Rozrokh et al., 2012, 2013). For treatments, 9-days-old chickpea seedlings grown in pots containing vermiculite under greenhouse conditions (continuous 30°C temperature, photoperiod of 12 h/12 h, 150 μmol m^{-2} s^{-1} photon flux density and 60% relative humidity) as described by Ha et al. (2014) were used. The plants were carefully removed from pots, gently washed to remove soil from roots, then subjected to either dehydration or water (control) treatments for a period of 2 and 5 h according to the methods published earlier (Tran et al., 2009). For dehydration treatment, washed plants were dried on Kim Towels (Nippon Paper Crecia Ltd.) papers, while for water treatment plants were kept in water for indicated time points. Subsequently, leaf and root samples of three biological replicates were carefully collected and frozen in liquid nitrogen for expression analysis.

RNA Isolation, DNaseI Treatment, cDNA Synthesis

Total RNA was purified from collected leaf and root samples using RNeasy Plant Mini Kit and QIAcube system (Qiagen) according to the manufacture's instruction. Determination of RNA concentration, DNaseI digestion, and cDNA preparation for real-time quantitative PCR (RT-qPCR) were performed as previously described (Le et al., 2011a).

RT-qPCR and Statistical Analyses

Gene-specific primers, which were designed by Ha et al. (2014; **Table 1**), were used in the RT-qPCR analysis of 3 biological replicates to assess the expression of 19 selected dehydration-responsive *CaNAC* genes under various treatment conditions. Detailed information about the RT-qPCR reactions was described in (Le et al., 2011a). The RT-qPCR reactions were run using Stratagene MX3000P system (Agilent Technologies, Santa Clara, CA, USA) with the following thermal profile: 95°C for 1 min,

TABLE 1 | Primer pairs of 19 *CaNAC* genes used in RT-qPCR analysis.

#	Gene name	Forward primers*	Reverse primers*
1	*CaNAC02*	CCATGGGAGCTACCAAAGAA	TTTCGATCTCTCGGGCTAAA
2	*CaNAC04*	AACAAGACCACCTGACCCTG	AATGCGTCGATTTCTCAACC
3	*CaNAC05*	CTAAGGCAACGTTCGGAGAG	TTTGGCCTAGCACCATTAGG
4	*CaNAC06*	GTCCCTTCTGTGTCCACGAT	GCTCCACCACTCTGAACCTC
5	*CaNAC16*	CACCAAAGGGCCTCAAGACAG	GCCTCATGGATCCAATTTGCCTAT
6	*CaNAC19*	AGAGGTTTGGTTTGTTGGTG	CCAAACACATGGTGAGGAAA
7	*CaNAC21*	CTTACCCTTTACCCGCTTCC	TCTTCTCCCAAATCACCTGG
8	*CaNAC24*	TGCCACCAGGTTTTAGGTTC	AATGATGGAAACAGGCAAGG
9	*CaNAC27*	GCTTTGTTTGGGGATGAAGA	ACCTGCACCAGCTGCTCTAT
10	*CaNAC40*	ACGATCCTTGGGATCTTCCT	ATATTTCCTGTCTCGTGGCG
11	*CaNAC41*	CCTGAAGAGGCAATTGACAGA	TCACCACTGCAGTCAAAGGT
12	*CaNAC43*	CACTGGTGTTCTACGCTGGA	GCCGGCTGATCTATCAACAT
13	*CaNAC44*	CCCACATGGTACTCGTACTGG	TTGCAAGCCAGAAGAAGGAT
14	*CaNAC46*	TATTGGAAGGCAACAGGGTC	TTTCTTAGGCCAACAATGCC
15	*CaNAC47*	TTTCACACGGATTCAAGCTG	ACAAATTCGTTCCACTTGGG
16	*CaNAC50*	CCCACCGATGAAGAACTTGT	TACTGGAAGGGGTGCAGAAG
17	*CaNAC52*	GCTACATCAAAGCCATGCCC	GGCCTCACTCCATTTGGGTA
18	*CaNAC57*	GTGGTATGCAGGACCAAGCA	GGTGGTGGACGATGGTGATT
19	*CaNAC67*	ACAGGAGGAGAAGCTCGGAT	TCCTCATCCCGCTTTGAACC

**The primer sequences were obtained from Ha et al. (2014).*

40 cycles at 95°C for 15 s and at 60°C for 1 min. After the last PCR cycle, the melting curves were obtained using the thermal profile of 95°C for 1 min followed by a constant increase in the temperature between 55 and 95°C. The *IF4a* gene, with specific RT-qPCR primers F: 5′-TGGACCAGAACACTAGGGACATT-3′ and R: 5′-AAACACGGGAAGACCCAGAA-3′, was selected as reference gene according to a report published earlier (Garg et al., 2010), and $2^{-\Delta\Delta Ct}$ method was used in analysis of RT-qPCR data (Le et al., 2012). Statistical significance of the differential expression within a cultivar or between 2 cultivars under well-watered or dehydration treatment was assessed using the Student's *t*-test (one tail, unpaired, equal variance). A gene was considered as dehydration-responsive if it had at least two-fold expression change (P-value < 0.05) at least at one time point under dehydration. For comparison of expression levels of *CaNAC* genes between drought-tolerant ILC482 and drought-sensitive Hashem, differential expression ratio with at least two-fold (P-value < 0.05) was considered as significant.

Criteria for Selection of Potential Dehydration-Responsive *CaNAC* Genes for In-Depth *In Planta* Functional Analyses and Genetic Engineering

The method was adopted from a previously published research (Thu et al., 2014b). Briefly, the selected candidate genes could be classified into two groups based on the following selection criteria. Group 1 of candidate genes are those being considered to be potential for development of improved drought-tolerant transgenic plants using overexpression approach, if they meet one of the following criteria: (i) being dehydration-inducible in tolerant cultivar vs. unchanged in sensitive cultivar and possessing higher expression levels in the tolerant cultivar under well-watered and/or dehydration conditions, (ii) showing up-regulation tendency by dehydration in both tolerant and sensitive cultivars with higher up-regulated expression change in the drought-tolerant cultivar under well-watered and/or dehydration conditions, (iii) being up-regulated in tolerant cultivar vs. unchanged in sensitive cultivar, or up-regulated/unchanged in tolerant cultivar vs. down-regulated in sensitive cultivar. Group 2 of candidate genes are those being unchanged or down-regulated by dehydration in both cultivars and showing lower expression levels in tolerant cultivar under well-watered and/or dehydration conditions. These genes could be considered for creation of improved drought-tolerant transgenic plants using gene suppression approach, such as RNA interference (RNAi).

Results

Expression Patterns of Selected *CaNAC* Genes in Leaves and Roots of Drought-Tolerant ILC482 Cultivar under Dehydration

The availability of natural germplasm and genetic diversity of crop varieties provides an essential key for biotechnological programs toward abiotic stress tolerance. As a means to gain a further understanding of relevant contributions of *CaNAC* genes to drought tolerance of chickpea and to identify candidate *CaNAC* genes for transgenic study, we obtained the drought-tolerant ILC482 and drought-sensitive Hashem chickpea varieties from ICARDA for comparative expression analysis of a subset of *CaNAC* genes. In a previous study, we found that expression of 19 of 23 *CaNAC* genes examined was significantly altered in leaves and roots of the drought-sensitive Hashem chickpea plants by dehydration (Ha et al., 2014), suggesting that these genes

may play an important role in drought responses of chickpea. These 19 *CaNAC* genes, representing 26.76% (19/71 *CaNAC* genes identified in chickpea genome) of the *CaNAC* members in chickpea (Ha et al., 2014), were then selected to examine whether there is a correlation between their dehydration-responsive expression patterns in the drought-tolerant ILC482 and drought-sensitive Hashem and the differential drought tolerability of these two cultivars.

As a first step toward this objective, we determined the expression of the 19 selected *CaNAC* genes in the leaf and root tissues of the drought-tolerant ILC482 cultivar that was grown and subjected to dehydration treatment in parallel with the drought-sensitive Hashem cultivar. All the 19 selected *CaNAC* genes also displayed dehydration-responsive in ILC482 as observed in Hashem, out of which 13 and 19 genes showed altered expression in roots and leaves of ILC482, respectively, by dehydration treatment according to the pre-defined criterion (fold-change in expression ≥ 2 and $P < 0.05$; **Figures 1** and **2**). A significant overlap was observed among the dehydration-responsive *CaNAC* genes identified in ILC482 roots and leaves, with 10 and 1 genes being induced and repressed, respectively, in both root and leaf tissues (**Figure 3**).

Specifically, we found 11 (*CaNAC06*, *16, 19*, *24*, *27*, *40*, *43*, *47*, *50*, *52,* and *67*) and 17 (*CaNAC05*, *06*, *16, 19*, *21*, *24*, *27*, *40*, *41*, *43*, *44*, *46*, *47*, *50*, *52*, *57,* and *67*) up-regulated *CaNAC* genes in dehydrated roots and leaves of ILC482, respectively, whereas 2 (*CaNAC02* and *46*) and 2 (*CaNAC02* and *04*) down-regulated *CaNAC* genes in the corresponding dehydrated root (**Figure 1**; **Table 2**) and leaf tissues (**Figure 2**; **Table 3**). Noticeably, *CaNAC27* and *CaNAC67* were the two most significantly induced genes in ILC482 roots and leaves by over 300- and 400-fold, respectively, whereas *CaNAC02* was the most highly repressed gene in both roots (17.5-fold) and leaves (9.2-fold) of ILC482 after 5 h of dehydration. It is also interesting to note that *CaNAC24* displayed opposite expression patterns in dehydrated ILC482 leaf tissues at 2 and 5 h, with down-regulation of 3.8-fold at 2 h but then up-regulation of 2.1-fold at 5 h of dehydration (**Figure 2**; **Table 3**). This gene was then not included in the Venn analysis to study the overlap in expression responsiveness of dehydration-responsive genes in ILC482 roots and leaves (**Figure 3**). In addition, *CaNAC46* was noteworthy to be mentioned as its expression was repressed by 3.9-fold (at 5 h) in dehydrated ILC482 roots (**Figure 1**; **Table 2**) but induced by 3.3-fold (at 2 h) in dehydrated ILC482 leaves (**Figure 2**; **Table 3**). Such opposite dehydration-responsive expression profiles in roots and leaves indicate the diverse and tissue-specific functions of *CaNAC46* in regulation of ILC482 chickpea cultivar to drought in a way that would provide the best survival of chickpea plants under water deficit conditions.

Differential Expression of the *CaNAC* Genes in Roots of ILC482 and Hashem

As reported earlier by Ha et al. (2014), among the 19 tested *CaNAC* genes, seven (*CaNAC06*, *16*, *19*, *24*, *40*, *50,* and *67*) and two (*CaNAC02* and *04*) genes were up-regulated and down-regulated, respectively, in roots of Hashem cultivar by 2 h dehydration, whereas 11 (*CaNAC06*, *16*, *19*, *24*, *27*, *40*, *43*, *44*, *50*, *52,* and *67*) and 3 genes (*CaNAC02*, *04,* and *46*) were induced and repressed, respectively, in the same tissues by 5 h dehydration (**Figure 1**; **Table 2**). In comparison with drought-tolerant ILC482, our data demonstrated that more *CaNAC* genes were up-regulated, whereas less *CaNAC* genes were down-regulated by dehydration in the drought-tolerant ILC482 roots than in the drought-sensitive Hashem roots. Specifically, we detected 9 and 7 dehydration-induced, as well as 1 and 2 dehydration-repressed *CaNAC* genes in roots of ILC482 and Hashem, respectively, after 2 h of dehydration (**Table 2**). As for 5 h dehydration, we recorded the same number (11) of up-regulated *CaNAC* genes in roots of ILC482 and Hashem, whereas less down-regulated *CaNAC* genes in roots of ILC482 than in roots of Hashem (2 vs. 3; **Table 2**).

A comparative analysis of expression levels of the *CaNAC* genes in the roots of drought-tolerant ILC482 vs. those in the roots of drought-sensitive Hashem revealed that under normal conditions, 2 (*CaNAC16* and *24*) and 7 (*CaNAC02*, *06*, *27*, *40*, *43*, *47,* and *50*) *CaNAC* genes had higher and lower expression levels, respectively, in ILC482 roots than Hashem roots after 2 h water control treatment. The same 7 *CaNAC* genes showed lower expression levels by 5 h water treatment, while 2 *CaNAC* genes, namely *CaNAC04* and *16*, displayed higher expression levels in ILC482 roots vs. Hashem roots (**Table 2**). On the other hand, under dehydration conditions, 3 and 4 *CaNAC* genes showed higher expression levels, whereas 5 and 3 genes exhibited lower expression levels in ILC482 roots than Hashem roots after 2 and 5 h treatments, respectively (**Table 2**). Specifically, *CaNAC04*, *16*, and *24* and *CaNAC02*, *06*, *27*, *43,* and *50* were found to possess higher and lower expression levels, respectively, in ILC482 roots than Hashem roots after 2 h water control treatment. With regard to 5 h treatment, we recorded the same three genes *CaNAC04*, *16*, and *24* in addition to the *CaNAC27* showing higher expression levels, whereas *CaNAC02*, *06,* and *50* displaying lower expression levels in ILC482 roots vs. Hashem roots, as in the case of 2 h dehydration treatment. With the exception of *CaNAC04*, which was down-regulated in Hashem roots by both 2 and 5 h dehydration treatments, *CaNAC16*, *24,* and *27* were up-regulated by dehydration in ILC482 roots, as well as Hashem roots.

Differential Expression of the *CaNAC* Genes in Leaves of ILC482 and Hashem

With regard to the expression of the tested *CaNAC* genes in leaves, Ha et al. (2014) reported that among 19 selected *CaNAC* genes, 6 (*CaNAC06*, *19*, *47*, *50*, *57,* and *67*) and 3 (*CaNAC02*, *04,* and *24*) genes showed up-regulated and down-regulated expression, respectively, in the leaves of Hashem cultivar by 2 h dehydration (**Figure 2**; **Table 3**). On the other hand, they detected more dehydration-responsive genes in 5-h-dehydrated Hashem leaves. Namely, they found 13 (*CaNAC05*, *06*, *16*, *19*, *21*, *27*, *40*, *41*, *43*, *50*, *52*, *57,* and *67*) and 3 genes (*CaNAC02*, *04,* and *46*) displaying up-regulated and down-regulated expression patterns, respectively, in 5-h-dehydrated Hashem leaves (**Figure 2**; **Table 3**). Similar to our observation in roots, when comparing the dehydration-regulated expression patterns of the 19 tested *CaNAC* genes in the leaves of ILC482 and Hashem, we found that a higher number of *CaNAC* genes were up-regulated, whereas a lower number of *CaNAC* genes

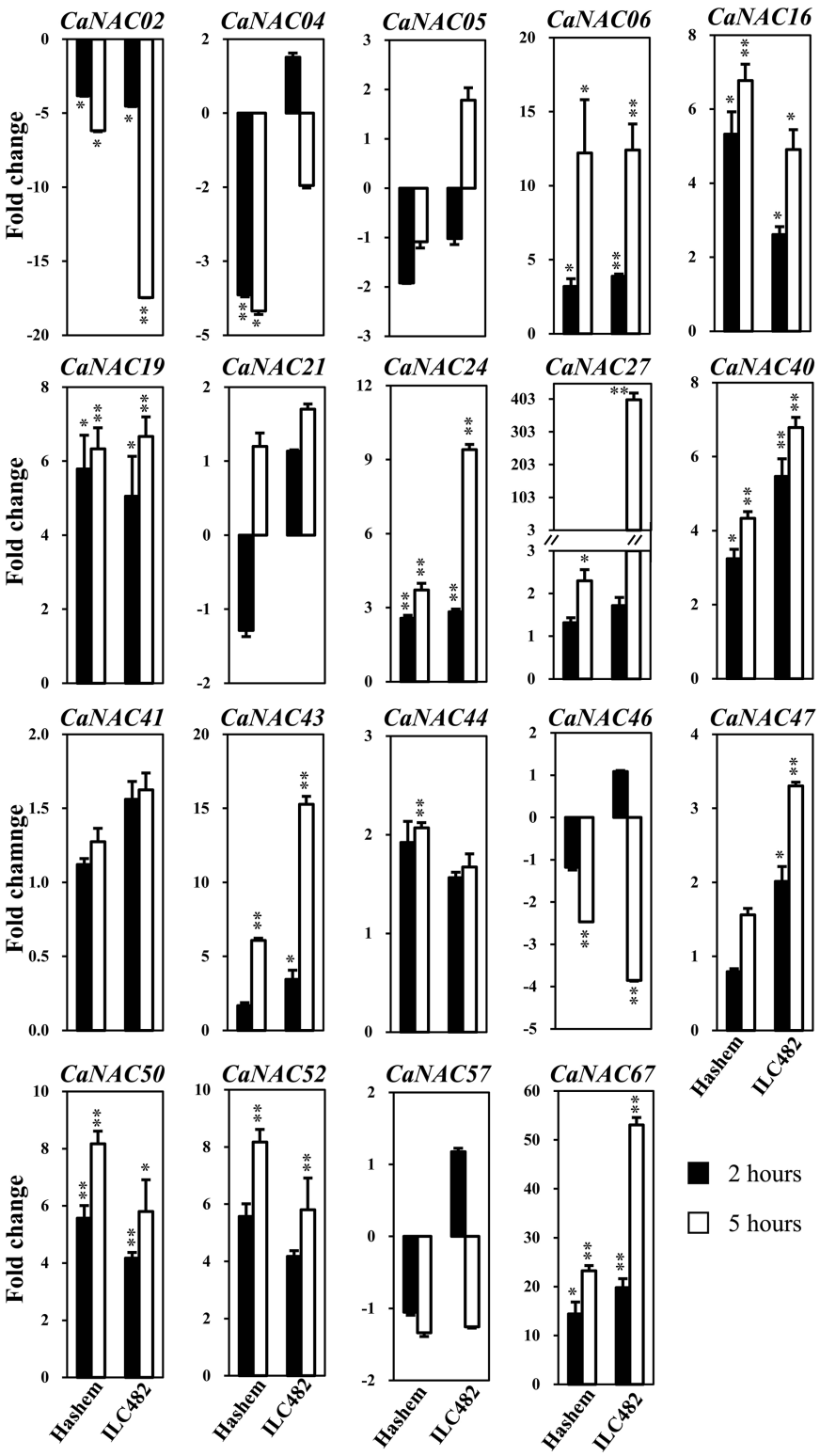


FIGURE 1 | Expression of 19 selected *CaNAC* genes in roots of drought-tolerant ILC482 and drought-sensitive Hashem cultivars under dehydration. Expression data of the *CaNAC* genes in ILC482 roots were obtained by RT-qPCR of root samples treated with well-water control or dehydration for 2 or 5 h. For convenient comparison, expression data of the *CaNAC* genes in Hashem roots were extracted from Ha et al. (2014) and displayed. Mean relative expression levels normalized to a value of 1 in water-treated control root samples. Error bars = SE values of 3 biological replicates. Asterisks indicate significant differences as determined by a Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

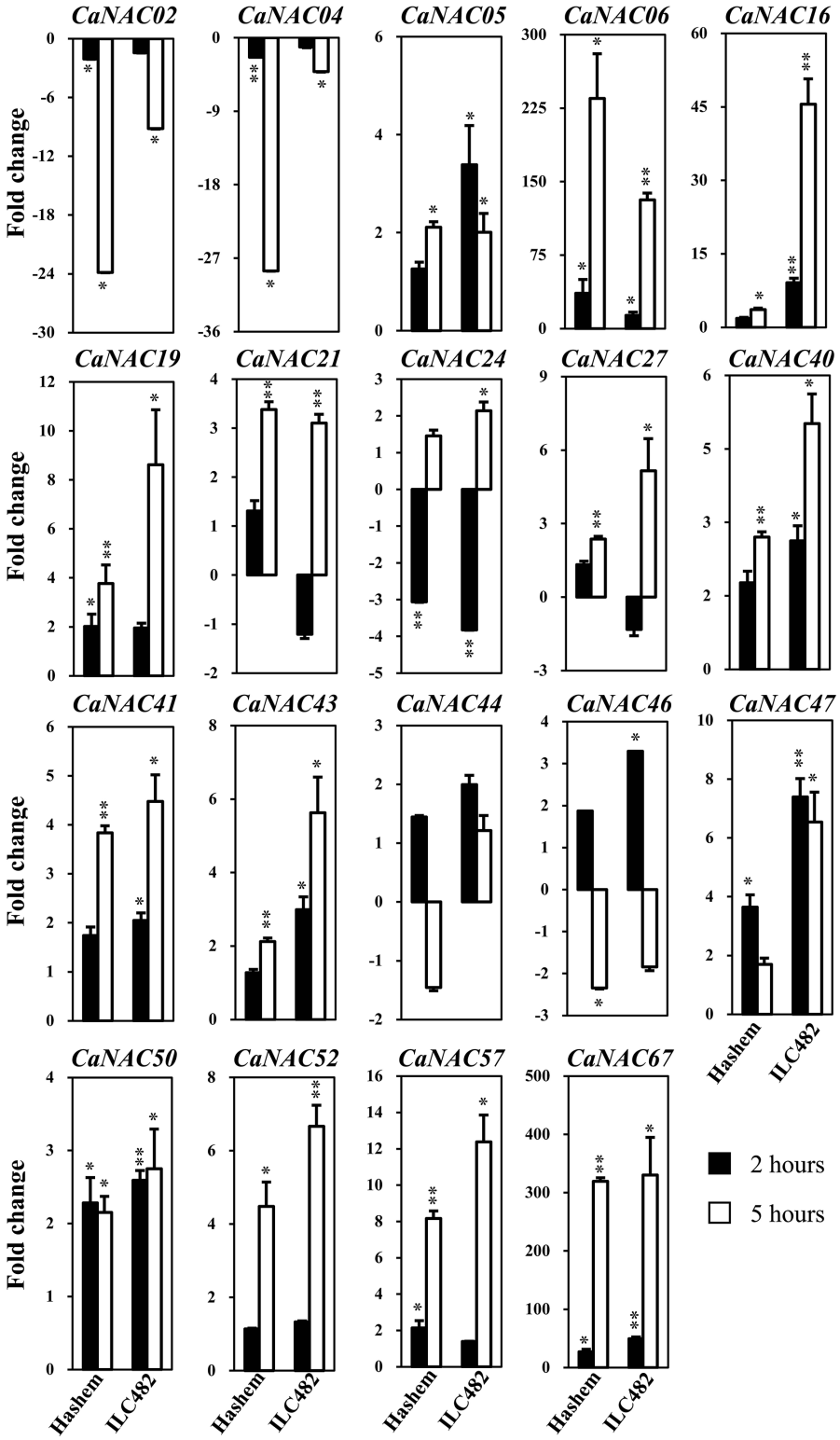


FIGURE 2 | Expression of 19 selected *CaNAC* genes in leaves of drought-tolerant ILC482 and drought-sensitive Hashem cultivars under dehydration. Expression data of the *CaNAC* genes in ILC482 leaves were obtained by RT-qPCR of leaf samples treated with well-water control or dehydration for 2 or 5 h. For convenient comparison, expression data of the *CaNAC* genes in Hashem leaves were extracted from Ha et al. (2014) and displayed. Mean relative levels were normalized to a value of 1 in water-treated control leaf samples. Error bars = SE values of 3 biological replicates. Asterisks indicate significant differences as determined by a Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

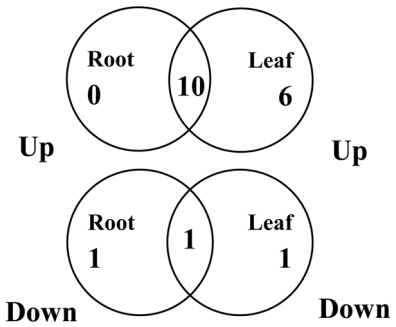


FIGURE 3 | Venn diagram analysis of expression of 19 selected *CaNAC* genes in roots and leaves of ILC482 under dehydration. *CaNAC24* was not included in the analysis because it displayed opposite expression patterns in dehydrated ILC482 leaf tissues at 2 and 5 h.

were down-regulated in ILC482 leaves than in Hashem leaves by either 2 or 5 h dehydration treatment. Specifically, we recorded 11 and 15 up-regulated *CaNAC* genes in leaves of ILC482, while only 6 and 13 up-regulated *CaNAC* genes in leaves of Hashem after 2 and 5 h dehydration treatments, respectively (**Table 3**). As for the down-regulated *CaNAC* genes, we detected 1 and 2 down-regulated genes in ILC482 leaves, whereas 3 and 3 down-regulated genes in Hashem leaves after 2 and 5 h dehydration treatments, respectively (**Table 3**).

A comparison of the expression levels of the tested *CaNAC* genes in the leaves of ILC482 and Hashem revealed similar tendency as observed in the roots. Under well-watered conditions, 9 (*CaNAC02*, *06*, *27*, *40*, *43*, *46*, *47*, *50,* and *67*) genes showed lower expression levels, while 1 (*CaNAC16*) gene possessed higher transcript abundance in ILC482 leaves than Hashem leaves after 2 h water control treatment. The same number of genes (*CaNAC02*, *06*, *19*, *27*, *40*, *41*, *43*, *44,* and *50*) showing lower expression levels in ILC482 leaves than in Hashem leaves by 5 h water control treatment was found, whereas 2 (*CaNAC04* and *16*) genes were recorded with higher expression levels in the same comparison. Under dehydration conditions, 9 and 4 genes were noted to have lower expression levels in ILC482 leaves than Hashem leaves after 2 and 5 h treatments, respectively. On the other hands, 3 (*CaNAC04*, *05,* and *16*) and 2 (*CaNAC04* and *16*) genes showed higher transcript abundance in ILC482 leaves than Hashem leaves after 2 and 5 h treatments, respectively.

Selection of Potential *CaNAC* Candidate Genes for In-Depth *In Planta* Characterization

As a means to propose promising *CaNAC* candidate genes for further in-depth *in planta* functional analyses, which would lead to their application in generating improved drought-tolerant transgenic chickpea plants using genetic engineering, we applied the section criteria adopted from a study published previously (Thu et al., 2014b). Among the 19 *CaNAC* genes examined in this study, 5 genes could be suggested as top priorities for functional characterizations according to the selection criteria set in the Materials and Methods. Specifically, 3 (*CaNAC04*, *16,* and *24*) genes of Group 1 and 1 (*CaNAC02*) gene of Group 2 were found to be satisfied for overexpression and knock-down studies, respectively, based on the differential analysis of the root expression data. On the other hand, according to the differential analysis of the leaf expression data, 3 (*CaNAC04*, *05,* and *16*) genes and 1 (*CaNAC02*) gene were noted to meet the selection criteria to be classified to Groups 1 and 2, respectively.

Discussion

The plant-specific NAC TF family is one of the important TF families in plant kingdom, whose members play diverse functions during plant growth and development (Olsen et al., 2005; Tran et al., 2010; Nakashima et al., 2012; Puranik et al., 2012). The drought-related function of NAC genes was first discovered through the study of *ANAC019*, *ANAC055,* and *ANAC072* in *Arabidopsis* (Tran et al., 2004), which then has led to many other studies in different plant species, including crops. One of the best studies that reported the potential application of *NAC* genes in agriculture is the work of Hu et al. (2006), who reported that transgenic rice plants overexpressing *SNAC1* exhibited enhanced drought tolerance without yield penalty. Since then, an increasing number of studies, including transgenic or correlation analyses, have provided strong evidence for the correlation between *NAC* gene expression and drought-tolerant capacity of various crops (Nakashima et al., 2007; Zheng et al., 2009; Xue et al., 2011; Thao et al., 2013; Thu et al., 2014a; Zhu et al., 2014; Yang et al., 2015).

The root plasticity is an important root trait responding to various environmental stressors, including drought, to help plants adapt to adverse conditions. Primary root length, root biomass, and number of lateral roots are all important parameters for evaluation of drought tolerance in crops (Sharp et al., 2004; Manavalan et al., 2009; Nishiyama et al., 2011; Ha et al., 2013; Zhu et al., 2014). A recent study on *SlNAC4* gene of tomato (*Solanum lycopersicum*) has provided convincing evidence for the regulatory function of NAC TFs in modulation of root growth under abiotic stresses. Suppression of *SlNAC4* expression has resulted in hypersensitivity to drought and salt stress to *SlNAC4-RNAi* transgenic tomato plants, which was attributed to inhibition of root growth, as well as a decrease in water and chlorophyll contents (Zhu et al., 2014). Thus, studying expression of the *CaNAC* genes in roots of chickpea cultivars with contrasting drought-tolerant phenotype will enable us to determine the correlation between *CaNAC* gene expression and drought tolerability, which will subsequently aid us in identifying root trait-related *CaNAC* genes for further functional analysis. The comparative expression analysis of the 19 selected *CaNAC* genes has allowed us to detect a higher number of dehydration-inducible *CaNAC* genes (9 genes vs. 7 genes and 11 vs. 11 after 2 and 5 h dehydration treatments, respectively) and a lower number of dehydration-repressible *CaNAC* genes (1 gene vs. 2 genes and 2 genes vs. 3 genes after 2 and 5 h dehydration treatments, respectively) in the roots of drought-tolerant ILC482 than in the roots of drought-sensitive Hashem (**Figure 1**; **Table 2**). These findings suggested a correlation between drought tolerability of ILC482 and Hashem cultivars and the number of the dehydration-responsive *CaNAC* genes in their roots.

TABLE 2 | Comparison of the expression levels of 19 *CaNAC* genes in the roots of ILC482 and Hashem cultivars under normal and dehydration conditions.

Nomenclature	Dehydration-responsive expression* of *CaNAC* genes in each cultivar								Expression ratio** in ILC482 roots vs. Hashem roots							
	2 h dehydration				5 h dehydration				2 h dehydration				5 h dehydration			
	ILC482	*P*-value	Hashem***	*P*-value	ILC482	*P*-value	Hashem***	*P*-value	Normal	*P*-value	Dehydration	*P*-value	Normal	*P*-value	Dehydration	*P*-value
CaNAC02	−4.52	0.00453	−3.82	0.00295	−17.47	0.00025	−6.19	0.01054	−10.36	0.00121	−12.27	0.002168	−6.48	0.00758	−18.29	0.042676
CaNAC04	1.14	0.32335	−3.68	0.00058	−1.47	0.00398	−4.01	0.02542	1.81	0.03323	7.57	0.000346	3.47	0.00057	9.47	0.000312
CaNAC05	0.98	0.47366	−1.92	0.00205	1.79	0.06095	−1.09	0.37037	−1.29	0.0739	1.46	0.043666	−1.40	0.18456	1.39	0.121459
CaNAC06	3.87	0.00021	3.19	0.01257	12.41	0.00327	12.21	0.02305	−24.48	0.00098	−20.16	0.004008	−15.56	0.0233	−15.31	0.02125
CaNAC16	2.61	0.00215	5.33	0.00197	4.91	0.00221	6.77	0.00022	20.73	0.00012	10.15	0.000467	18.68	0.0045	13.55	0.001022
CaNAC19	5.05	0.01464	5.79	0.00152	6.67	0.00048	6.33	0.00020	−1.47	0.02349	−1.68	0.068642	−1.44	0.02158	−1.37	0.031867
CaNAC21	1.14	0.09328	−1.29	0.07359	1.70	0.00095	1.20	0.17106	−1.64	0.00345	−1.12	0.205391	−1.24	0.00358	1.14	0.289941
CaNAC24	2.83	0.00015	2.57	0.00053	9.40	2.8E-06	3.71	0.00085	2.11	0.00281	2.33	0.000204	−1.07	0.30848	2.37	0.000122
CaNAC27	1.72	0.03846	1.32	0.07125	404.50	2.0E-05	2.30	0.01121	−15.82	0.00048	−12.13	0.000477	−8.98	0.00317	19.61	2.53E-05
CaNAC40	5.46	0.00077	3.24	0.00105	6.79	3.1E-05	4.34	0.00008	−2.45	0.00042	−1.45	0.031305	−2.45	0.00224	−1.56	0.001794
CaNAC41	1.56	0.00865	1.12	0.08986	1.62	0.00704	1.27	0.05678	−1.77	0.00065	−1.27	0.035727	−1.44	0.01436	−1.13	0.188598
CaNAC43	3.44	0.01313	1.67	0.02792	15.28	1.4E-05	6.08	0.00004	−4.38	0.00088	−2.13	0.020537	−3.39	0.01826	−1.35	0.002143
CaNAC44	1.57	0.0017	1.92	0.01106	1.67	0.01384	2.07	0.00006	1.01	0.44808	−1.22	0.117385	1.21	0.09696	−1.02	0.445996
CaNAC46	1.08	0.19341	−1.18	0.16929	−3.85	7.4E-05	−2.47	0.00005	−1.67	0.01804	−1.30	0.033316	−1.14	0.05312	−1.77	0.000483
CaNAC47	2.01	0.00685	−1.26	0.02431	3.30	2.5E-06	1.56	0.05223	−3.36	0.0001	−1.32	0.053557	−2.63	0.02002	−1.25	0.021975
CaNAC50	4.17	0.00048	5.57	0.00070	5.80	0.0101	8.17	0.00012	−4.57	0.00813	−6.11	0.000519	−4.32	0.00848	−6.08	0.000206
CaNAC52	1.50	0.01086	1.51	0.03565	2.24	0.00059	2.02	0.00040	1.17	0.08358	1.16	0.2073	1.16	0.19327	1.29	0.001956
CaNAC57	1.18	0.07571	−1.05	0.31537	−1.26	0.06129	−1.34	0.03546	1.11	0.22924	1.37	0.007333	1.30	0.06767	1.38	0.007781
CaNAC67	19.79	0.00053	14.4	0.00468	53.1	4.8E-06	23.21	0.00004	−1.08	0.30451	1.28	0.195258	−1.25	0.24553	1.84	0.000312

*Data in blue and red colors indicate down- and up-regulated expression, respectively. Data in "ILC482" and "Hashem" columns indicate fold-changes (≥|2|, P-value < 0.05).

**Data in green and yellow colors indicate statistically significant difference in gene expression ratios (≥|2|-fold and P-value < 0.05). Higher and lower expression levels in ILC482 roots vs. Hashem roots were indicated by green and yellow colors, respectively.

***Expression data of CaNAC genes in the leaves of Hashem cultivar were obtained from Ha et al. (2014).

TABLE 3 | Comparison of the expression levels of 19 *CaNAC* genes in the leaves of ILC482 and Hashem cultivars under normal and dehydration conditions.

Nomenclature	Dehydration-responsive expression* of *CaNAC* genes in each cultivar								Expression ratio** in ILC482 roots vs. Hashem leaves							
	2 h dehydration				5 h dehydration				2 h dehydration				5 h dehydration			
	ILC482	*P*-value	Hashem***	*P*-value	ILC482	*P*-value	Hashem***	*P*-value	Normal	*P*-value	Dehydration	*P*-value	Normal	*P*-value	Dehydration	*P*-value
CaNAC02	−1.42	0.05106	−2.07	0.00752	−9.17	0.00469	−23.86	0.00157	−13.64	0.00071	−9.38	0.00042	−16.87	0.00163	−6.48	0.04476
CaNAC04	−1.14	0.26645	−2.38	0.00037	−4.16	0.00137	−28.57	0.00121	1.57	0.04406	3.30	0.00208	2.93	0.00232	20.11	0.00382
CaNAC05	3.39	0.02610	1.26	0.10376	2.01	0.07635	2.11	0.00344	−1.04	0.44168	2.60	0.03691	1.02	0.40680	−1.03	0.45923
CaNAC06	13.61	0.01292	36.17	0.03415	131.29	0.00006	235.02	0.00580	−19.93	0.01230	−52.95	0.03344	−16.64	0.00349	−29.79	0.00639
CaNAC16	9.08	0.00097	1.82	0.01889	45.57	0.00099	3.61	0.00135	17.31	0.00065	86.42	0.00064	9.51	0.00076	120.26	0.00094
CaNAC19	1.95	0.03314	2.02	0.01411	8.61	0.01979	3.77	0.00045	−1.98	0.01682	−2.05	0.01707	−3.06	0.00139	−1.34	0.23945
CaNAC21	−1.21	0.13293	1.31	0.16347	3.10	0.00052	3.38	0.00018	−1.88	0.02044	−2.97	0.01315	−1.45	0.02130	−1.58	0.00391
CaNAC24	−3.83	0.00001	−3.06	0.00001	2.14	0.01024	1.45	0.14400	1.77	0.00013	1.41	0.00135	1.14	0.39683	1.68	0.03029
CaNAC27	−1.32	0.29991	1.32	0.12226	5.16	0.02103	2.37	0.00099	−6.76	0.00535	−11.79	0.00148	−13.49	0.00085	−6.19	0.00023
CaNAC40	2.62	0.00624	1.77	0.02495	5.02	0.00263	2.70	0.00014	−3.54	0.00001	−2.38	0.01354	−3.33	0.00021	−1.79	0.00531
CaNAC41	2.05	0.00269	1.74	0.01117	4.48	0.00317	3.84	0.00006	−1.98	0.00004	−1.69	0.01748	−2.15	0.00299	−1.84	0.00430
CaNAC43	2.99	0.00494	1.28	0.19070	5.63	0.00750	2.12	0.00058	−3.65	0.01347	−1.56	0.02384	−5.10	0.00018	−1.92	0.01039
CaNAC44	2.00	0.02238	1.45	0.03113	1.22	0.24806	−1.45	0.05871	−1.38	0.18224	−1.00	0.46299	−2.51	0.00999	−1.42	0.13490
CaNAC46	3.29	0.00171	1.87	0.06866	−1.84	0.00992	−2.35	0.04115	−2.34	0.01068	−1.33	0.16198	−2.03	0.05211	−1.59	0.03775
CaNAC47	7.40	0.00055	3.65	0.00759	6.54	0.00493	1.69	0.29182	−4.06	0.04174	−2.00	0.01332	−3.75	0.10526	1.03	0.43462
CaNAC50	2.59	0.00043	2.28	0.01893	2.75	0.02719	2.15	0.00633	−16.34	0.00060	−14.39	0.00334	−17.84	0.00005	−13.96	0.00086
CaNAC52	1.33	0.00309	1.14	0.16492	6.67	0.00067	4.48	0.00564	−1.51	0.02076	−1.29	0.00072	−1.30	0.05864	1.14	0.30061
CaNAC57	1.39	0.00088	2.14	0.03178	12.38	0.00153	8.17	0.00010	−1.44	0.01039	−2.22	0.02830	−1.54	0.07144	−1.01	0.48783
CaNAC67	49.29	0.00011	26.97	0.00365	330.08	0.00589	319.57	0.00000	−2.06	0.01212	−1.13	0.26941	1.12	0.23757	1.15	0.23808

*Data in blue and red colors indicate down- and up-regulated expression, respectively. Data in "ILC482" and "Hashem" columns indicate fold-changes ($\geq|2|$, P-value < 0.05).

**Data in green and yellow colors indicate statistically significant difference in gene expression ratios ($\geq|2|$ -fold and P-value < 0.05). Higher and lower expression levels in ILC482 leaves vs. Hashem leaves were indicated by green and yellow colors, respectively.

***Expression data of CaNAC genes in the leaves of Hashem cultivar were obtained from Ha et al. (2014).

In addition, leaf-related traits, such as stomata aperture and leaf cell membrane stability, have been also well-known traits that influence drought tolerance (Kaiser, 2009; Manavalan et al., 2009; Guttikonda et al., 2014; Ha et al., 2014). Overexpression of *SNAC1* gene in rice was shown to enhance stomatal closure, thereby contributing to improved drought tolerance of transgenic plants (Hu et al., 2006). This finding suggested a close association of *NAC* gene expression and leaf-related traits. Thus, it was also our interest to examine the correlation between drought-tolerant levels of the two contrasting chickpea cultivars and expression levels of *CaNAC* genes in leaf tissues under dehydration. As shown in **Figure 2** and summarized in **Table 3**, more up-regulated *CaNAC* genes, whereas less down-regulated *CaNAC* genes were found in ILC482 leaves than in Hashem leaves. These data suggested a positive correlation between drought-tolerant degree of ILC482 and Hashem cultivars and the number of the dehydration-responsive *CaNAC* genes in leaves as well, which together with the results obtained in the roots (**Figure 1**; **Table 2**) firmly demonstrated this positive correlation. Taken together, the higher drought-tolerant capacity of ILC482 vs. Hashem might partly be attributed to their differential expression of the *CaNAC* genes in both root and leaf tissues. The more *CaNAC* genes are up-regulated and the less *CaNAC* genes down-regulated by dehydration, the higher drought-tolerant the cultivar is. In support of our results, previous studies in soybean (*Glycine max*) also identified positive correlation between the number of drought-inducible *GmNAC* genes and drought-tolerant capacity of 2 contrasting cultivars (Thao et al., 2013; Thu et al., 2014a).

From our comparative analyses of the expression of these selected 19 *CaNAC* genes, we also observed differential expression patterns between roots and leaves in the same cultivar, either ILC482 or Hashem, or between the same organs of the two contrasting chickpea cultivars (**Tables 2** and **3**). This finding suggested that the expression of *CaNAC* genes, at least of those examined in this study, is tissue- and genotype-dependent, which might then result in different phenotypes of different cultivars. Differential expression analyses of *GmNAC* genes in 3 soybean cultivars with different phenotypes also showed their tissue- and genotype-dependent expression patterns (Le et al., 2011b; Thao et al., 2013; Thu et al., 2014a,c), further supporting our observation.

One of the major aims of this study is to identify the best *CaNAC* candidate genes that have high potential for development of drought-tolerant chickpea cultivars by genetic engineering. On the basis of our analysis (**Tables 2** and **3**) and the selection criteria adopted from Thu et al. (2014b), 4 (*CaNAC04*, *05*, *16,* and *24*) genes belonging to Group 1, and 1 gene (*CaNAC02*) classified to Group 2 could be selected for detailed *in planta* functional analyses in model plant systems, such as *Arabidopsis*, prior to using them in genetic engineering of chickpea plants or other legume crops. *CaNAC04*, *16,* and *CaNAC02* are associated with both root and leave tissues, whereas *CaNAC05* and *CaNAC24* are specifically associated with leaves and roots, respectively (**Tables 2** and **3**). All these 5 genes might potentially play important roles in conferring higher drought tolerability to ILC482 than Hashem.

Out of these 5 genes, *CaNAC16* would be the best positive regulatory candidate gene as this gene was found (i) to be induced by dehydration in both roots and leaves of both ILC482 and Hashem cultivars, and (ii) to display higher expression levels in drought-tolerant ILC482 than drought-sensitive Hashem under both normal (20.73- and 18.68-fold in roots, and 17.31 and 9.51-fold in leaves at 2 and 5 h, respectively) and dehydration (10.15- and 13.55-fold in roots, and 86.42- and 120.26-fold in leaves at 2 and 5 h, respectively) conditions (**Tables 2** and **3**). On the other hand, *CaNAC02* is a promising negative regulatory gene, as this gene was strongly down-regulated by dehydration in both roots and leaves of both 2 chickpea cultivars, and showed lower expression levels in drought-tolerant ILC482 than drought-sensitive Hashem under both normal (10.36- and 6.48-fold in roots, and 13.64- and 16.87-fold in leaves at 2 and 5 h, respectively) and dehydration (12.27- and 18.29-fold in roots, and 9.38- and 6.48-fold in leaves at 2 and 5 h, respectively) conditions (**Tables 2** and **3**). Taken together, *CaNAC16* and *CaNAC02* are highly recommended for detailed functional characterization using overexpression and knock-down approaches, respectively, with the goal to lead to their application in development of chickpea varieties with improved drought tolerance.

Author Contributions

L-SPT conceived research and wrote the manuscript. KHN, CVH, YW, UTT, and MNE performed the experiments. DVN contributed research materials.

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Overexpression of *GhWRKY27a* reduces tolerance to drought stress and resistance to *Rhizoctonia solani* infection in transgenic *Nicotiana benthamiana*

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WRKY proteins constitute transcriptional regulators involved in various biological processes, especially in coping with diverse biotic and abiotic stresses. However, in contrast to other well-characterized WRKY groups, the functions of group III WRKY transcription factors are poorly understood in the economically important crop cotton (*Gossypium hirsutum*). In this study, a group III WRKY gene from cotton, *GhWRKY27a*, was isolated and characterized. Our data indicated that GhWRKY27a localized to the nucleus and that *GhWRKY27a* expression could be strongly induced by abiotic stresses, pathogen infection, and multiple defense-related signaling molecules. Virus-induced gene silencing (VIGS) of *GhWRKY27a* enhanced tolerance to drought stress in cotton. In contrast, *GhWRKY27a* overexpression in *Nicotiana benthamiana* markedly reduced plant tolerance to drought stress, as determined through physiological analyses of leaf water loss, survival rates, and the stomatal aperture. This susceptibility was coupled with reduced stomatal closure in response to abscisic acid and decreased expression of stress-related genes. In addition, *GhWRKY27a*-overexpressing plants exhibited reduced resistance to *Rhizoctonia solani* infection, mainly demonstrated by the transgenic lines exhibiting more severe disease symptoms, accompanied by attenuated expression of defense-related genes in *N. benthamiana*. Taken together, these findings indicated that *GhWRKY27a* functions in negative responses to drought tolerance and in resistance to *R. solani* infection.

Keywords: abscisic acid, cotton (*Gossypium hirsutum*), drought stress, *Rhizoctonia solani* infection, VIGS, WRKY transcription factor

Introduction

Due to their sessile growth habit, plants are constantly exposed to various biotic and abiotic stresses, such as pathogen infection and drought stress. To respond appropriately to these stresses, plants have evolved a highly sophisticated signaling network to perceive external signals and manifest adaptive responses with proper physiological and molecular changes (Asai et al., 2002; Smékalová et al., 2014). The transcriptional regulation of a multitude of defense-related genes is a key step during these processes. The regulation of these genes at the transcriptional level is largely mediated by the specific recognition of *cis*-acting promoter elements by *trans*-acting sequence-specific DNA

binding transcription factors (TFs) (Kim and Zhang, 2004). Among the several classes of TFs, the DNA-binding proteins containing WRKY domains have been shown to be associated with plant defense responses (Pandey and Somssich, 2009; Tripathi et al., 2014).

WRKY TFs are one of the largest families of transcriptional regulators in plants and are characterized by the presence of one or two 60-amino-acid WRKY domains (Rushton et al., 2010). A common feature of the WRKY domain is the highly conserved WRKYGQK sequence at its N-terminus along with a zinc-finger binding motif at its C-terminus. It is generally assumed that the WRKY domain can activate or repress the transcription of target genes by specific binding to various W-box elements with an invariant GAC core sequence present in the promoters (Brand et al., 2013a,b). In addition, based on the number of WRKY domains and the features of the zinc-finger motifs, WRKY proteins can be divided into three groups: group I contains two WRKY domains with a C2H2 zinc-finger motif; group II has one WRKY domain and a C2H2 zinc-finger motif; and group III contains one WRKY domain and a different C2HC zinc-finger motif (Eulgem et al., 2000).

To date, many group III WRKY TFs have been identified in *Arabidopsis*, rice, Pak-choi, *Thlaspi caerulescens*, and *Vitis pseudoreticulata* (Kalde et al., 2003; Xie et al., 2005; Wei et al., 2008; Li et al., 2010; Wang et al., 2012). Additionally, more group III WRKY TFs have been reported to be involved in plant responses to abiotic stress. For instance, *BcWRKY46* overexpression in tobacco enhanced the tolerance of transgenic tobacco to drought, cold, and salt stress (Wang et al., 2012). FcWRKY70, a WRKY protein of *Fortunella crassifolia*, functions in drought tolerance and modulates putrescine synthesis by regulating the arginine decarboxylase gene (Gong et al., 2015). WRKY70 and WRKY54 co-operate as negative regulators of stomatal closure and, consequently, osmotic stress tolerance in *Arabidopsis* (Li et al., 2013). Another group III WRKY TF, *AtWRKY46*, has been shown to play dual roles in regulating plant responses to drought and salt stress and light-dependent stomatal opening in guard cells (Ding et al., 2014). Furthermore, the phytohormone abscisic acid (ABA) plays central role in the stress responses of plants exposed to environmental challenges. A recent study has demonstrated that WRKY TFs constitute key nodes in ABA-responsive signaling networks (Rushton et al., 2012). Several rice WRKY proteins have been found to act as repressors or activators of an ABA-inducible promoter in aleuronic cells (Xie et al., 2005). Ren et al. (2010) also showed that *AtWRKY63* can bind the W-box in the promoter of *AREB1*/*ABF2 in vitro*, and the *wrky63* mutant is more sensitive to drought stress than wild-type plants.

In addition, emerging evidence has indicated that group III WRKY TFs are central components of many aspects of the plant innate immune system, including basal defense and systemic-acquired resistance (Rushton et al., 2010; Jiang et al., 2014). The WRKY TFs bind to and regulate the expression of several well-characterized plant defense-related genes, all of which contain W-box elements in their promoter regions (Yu et al., 2001). For example, Mao et al. (2007) reported that WRKY62 acts downstream of cytosolic NPR1 and negatively regulates JA-responsive gene expression in *Arabidopsis*. *Arabidopsis* WRKY70 has been shown to modulate the crosstalk between SA- and JA-mediated signaling by promoting SA-dependent and suppressing JA-dependent responses (Li et al., 2006). In rice plants, overexpression of the elicitor-induced *OsWRKY53* gene leads to enhanced resistance to the blast fungus *Magnaporthe grisea* (Chujo et al., 2007). OsWRKY31 acts as a transcriptional activator, and overexpression of the *OsWRKY31* gene enhances resistance against infection with *M. grisea* (Zhang et al., 2008). A pepper (*Capsicum annuum* L.) WRKY gene, *CaWRKY30*, is involved in pathogen stress responses (Zheng et al., 2011). Another group III member, AtWRKY52/RRS1, forms a receptor complex by combining with several NB-LRR proteins, and this receptor complex integrates a "decoy" domain that enables the detection of effectors that target WRKY proteins (Sarris et al., 2015). These findings further emphasize the significance of group III WRKY proteins for plant immunity.

Cotton (*Gossypium hirsutum*) is an important fiber and oil crop around the world, and its growth and yield are affected by various biotic and abiotic stress conditions. Previous studies have primarily focused on group I and II WRKY TFs in cotton. For example, a group I WRKY TF from cotton, *GhWRKY3*, was shown to be responsive to biotic stresses and various phytohormones (Guo et al., 2010). The group II WRKY TF, *GhWRKY17* responds to drought and salt stress through ABA signaling and the regulation of cellular reactive oxygen species (ROS) production (Yan et al., 2014), and *GhWRKY40* overexpression in tobacco results in enhanced susceptibility to biotrophic pathogen infections (Wang et al., 2014). However, few group III WRKY TFs have been functionally characterized in cotton. Here, we report the identification and functional characterization of a group III WRKY transcription factor, *GhWRKY27a*. The expression of *GhWRKY27a* was induced by various abiotic and biotic stresses. The silencing of *GhWRKY27a* enhanced the tolerance of cotton plantlets to drought stress. In addition, ectopic expression of *GhWRKY27a* in *Nicotiana benthamiana* led to enhanced susceptibility to drought stress and infection by the fungal pathogen *Rhizoctonia solani*. This study provides key clues toward understanding the roles of *GhWRKY27a* in plant defense responses to biotic and abiotic stresses.

Materials and Methods

Plant Growth and Various Treatments

Cotton (*G. hirsutum* L. cv. lumian 22) seedlings were grown in an environmentally controlled growth chamber at 26 ± 1°C with a 16 h light/8 h dark cycle (relative humidity of 60–75%). Seven-day-old cotton seedlings were collected for various treatments. For the temperature treatment, uniformly developed cotton seedlings were transferred to cold conditions (4°C) for indicated time periods. For other treatments, uniformly developed seedlings were cultured or sprayed with NaCl (200 mM), 15% poly(ethylene glycol) 6000 (w/v), H_2O_2 (10 mM), ABA (100 μM), MeJA (100 μM), SA (2 mM), or ET released from the ethephon (5 mM), or were wounded. For the fungal pathogen

treatment, the roots of cotton seedlings were dipped into *R. solani* conidial suspensions (10^5 conidia mL^{-1}). The treated cotyledons were collected for RNA extraction. Additionally, *N. benthamiana* seeds were surface sterilized and planted on Murashige and Skoog (MS) medium for germination under greenhouse conditions. *N. benthamiana* seedlings at the two- or three-leaf stage were transplanted into soil and maintained under a 16 h light/8 h dark photoperiod at 25°C. The resulting uniform seedlings were used for further study. Each treatment was performed at least three times.

Cloning of *GhWRKY27a*

The full-length cDNA and genomic sequence of *GhWRKY27a* were obtained as previously described (Yu et al., 2012). The general PCR procedures and primers are shown in **Tables S1**, **S2**. Multiple protein sequence alignments amongst homologs were conducted using DNAman 6.0.3 software and the NCBI bioinformatics tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA version 5.1) software using the neighbor-joining method.

Subcellular Localization of *GhWRKY27a*

The coding region of the *GhWRKY27a* gene without the stop codon was inserted at the 5′-terminal end of the GFP gene to generate pBI121-GhWRKY27a-GFP, which is driven by the *Cauliflower mosaic virus* 35S (CaMV35S) promoter. The *Agrobacterium tumefaciens* strain GV3101 carrying the pBI121-GhWRKY27a-GFP fusion construct or the positive control pBI121-GFP construct was inoculated into fully expanded leaves of 6-week-old *N. benthamiana*. The lower epidermis cells were analyzed using an LSM 510 confocal laser-scanning microscope (Carl Zeiss, Germany) operated with LSM Image Browser software.

Virus-induced Gene Silencing (VIGS) of *GhWRKY27a* in Cotton

For VIGS silencing of *GhWRKY27a*, the tobacco rattle virus (TRV)-based VIGS system was employed. A 481-bp fragment was inserted into the multiple cloning site in plasmid pTRV-RNA2 to produce pTRV-RNA2-GhWRKY27a. *A. tumefaciens* strain GV3101 carrying pTRV-RNA2-GhWRKY27a, the pTRV-RNA2-GhCLA fusion construct or the pTRV-RNA2 construct was combined with the pTRV-RNA1 strain (1:1 ratio; OD_{600} = 1.0) and co-infiltrated into two fully expanded cotyledons of cotton as described by Dang et al. (2013).

Vector Construction and Plant Transformation

Under the control of the CaMV35S promoter, the *GhWRKY27a* ORF was cloned into the *Xba* I/*Sal* I sites of the binary vector pBI121. The recombinant plasmid was then introduced into *A. tumefaciens* (strain LBA4404) for *N. benthamiana* transformation using the leaf disc method, and transformants were screened for kanamycin (100 mg L^{-1}) resistance and further confirmed by PCR. The transgenic T_3 lines were used in experiments. All of the primers used in this study are listed in **Table S1**.

Quantification of Endogenous ABA Content

Samples were homogenized in liquid nitrogen and extracted in ice-cold phosphate-buffered saline (PBS, pH 7.4). After centrifugation at 4000 g for 20 min, the supernatant was dried in N_2 and subsequently dissolved for ELISA assay using a kit (Fangcheng, Beijing, China) according to the manufacturer's instructions.

3,3′-Diaminobenzidine (DAB) and Nitro Blue Tetrazolium (NBT) Staining Assays

For DAB staining, *N. benthamiana* and cotton leaves were incubated in DAB solution (1 mg mL^{-1}, pH 3.8) for 15 h at 25°C in the dark. After staining, the leaves were soaked in 95% ethanol overnight to remove chlorophyll. For the NBT assays, leaves were incubated in NBT solution (0.1 mg mL^{-1}) for 15 h at 25°C in the dark. After staining, leaves were soaked in 95% ethanol overnight to remove chlorophyll.

Oxidative Stress Experiments

For oxidative stress analysis, uniform leaf discs were detached from healthy and fully expanded wild type and transgenic plants and floated in 12 mL of a solution containing one of three concentrations of methyl viologen (MV) (0, 400, or 600 μM) for 72 h. Subsequently, the chlorophyll contents were extracted using 95% ethanol and analyzed using spectrophotometry.

Pathogen Inoculation and Disease Resistance Test

Leaves of 7-week-old transgenic and wild-type plants were inoculated with *R. solani* spore suspensions (10^5 conidia mL^{-1}) prepared in 1% glucose. Inoculated leaves were kept in a transparent box under greenhouse conditions. Lesions were measured at 7 days after inoculation. Furthermore, infection was confirmed by inoculation with the above suspensions of *R. solani* spores using the trickle irrigation method (Li et al., 2014a).

RNA Extraction and Quantitative PCR

Total RNA was isolated from samples using the modified cetyltrimethylammonium bromide (CTAB) method (Lu et al., 2013) or TRIzol reagent (TaKaRa, Dalian, China). Next, the RNA was used to obtain first-strand cDNA using the EasyScript First-strand cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Real-time quantitative PCR (qRT-PCR) was carried out using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) and a CFX96TM Real-time System (Bio-Rad, Hercules, CA, USA) following the procedures described by Shi et al. (2014). The primers used in the qRT-PCR analyses are shown in **Table S3**. The *G. hirsutum* ubiquitin (*UBI*) and *N. benthamiana* *β-actin* genes were used as internal controls. Data were analyzed using the CFX Manager software, version 1.1, and significant differences were identified using Duncan's multiple range tests with Statistical Analysis System (SAS) software, version 9.1. All reactions were performed with three technical replicates.

Results

Sequence Analysis of GhWRKY27a

The full-length cDNA of the *GhWRKY27a* (GenBank accession number: KM453243) sequence consisted of 1513 nucleotides, including a 1068-bp open reading frame (ORF), a 319-bp 5′-untranslated region (5′-UTR) and a 126-bp 3′-UTR. The ORF encoded a protein composed of 356 amino acid residues with a predicted molecular mass and isoelectric point of 40.062 kDa and 5.46, respectively.

The deduced amino acid sequence of GhWRKY27a was closely related to those of *C. annuum* CaWRKY30 (GenBank accession number: ACJ04728.1, 48.49% protein sequence identity), *Populus trichocarpa* PtWRKY41 (GenBank accession number: XP_002297983.1, 66.11% protein sequence identity), *P. trichocarpa* PtWRKY53 (GenBank accession number: XP_002304549.1, 64.46% protein sequence identity), and *Jatropha curcas* JcWRKY54 (GenBank accession number: AGQ04248.1, 61.71% protein sequence identity). The WRKY domain and the C and H residues in the zinc-finger motif (C-X_7-C-X_{23}-H-X_1-C) were identified, indicating that GhWRKY27a belongs to group III of the WRKY family. Additionally, a putative nuclear localization signal (NLS), KKRK, was found at position 105–108 (**Figure 1A**).

To investigate the evolutionary relationship of the cloned WRKY protein to other known cotton WRKYs, a neighbor-joining analysis was performed with the obtained amino acid sequences. As shown in **Figure 1B**, GhWRKY27a was highly similar to group III WRKY family members, which is consistent with the results of the amino acid alignment analysis. These results strongly imply that GhWRKY27a is a member of WRKY group III.

To further elucidate the properties of *GhWRKY27a*, the genomic DNA sequence of *GhWRKY27a* (GenBank accession number: KM453244), which consisted of 1696 bp (containing three exons and two introns), was obtained. Comparative analysis of *GhWRKY27a* and the other group III WRKY TF genomic sequences revealed that the numbers and positions of the introns in these genes were highly conserved (**Figure S1**).

GhWRKY27a is Localized to the Nucleus

Bioinformatics analysis using the PSORT program predicted that GhWRKY27a would localize to the nucleus. To confirm this prediction, the *GhWRKY27a* ORF was fused in-frame to the green fluorescent protein (GFP) gene under the control of the cauliflower mosaic virus CaMV35S promoter (**Figure 2A**). As shown in **Figure 2B**, typical results indicated exclusive localization of GhWRKY27a-GFP to the nucleus in *N. benthamiana* epidermal cells, whereas GFP alone localized to multiple subcellular compartments, including the cytoplasm and nucleus. These results indicated that the GhWRKY27a protein localized to the nucleus.

Expression Profiles of *GhWRKY27a* under Stress Conditions

To examine the expression patterns of *GhWRKY27a* following various environmental stresses, 7-day-old cotton seedlings were exposed to various stresses. As shown in **Figure 3A**, NaCl treatment induced a slight increase in *GhWRKY27a* expression. Following poly(ethylene glycol) 6000 and cold treatments, *GhWRKY27a* transcription was dramatically elevated, peaking after 2 days and 4 h, respectively (**Figures 3B,C**). Conversely, the expression of *GhWRKY27a* was downregulated after wounding treatment (**Figure 3D**). In addition, to elucidate *GhWRKY27a*-related signal transduction mechanisms, we also examined the responsiveness of *GhWRKY27a* to diverse signaling molecules. As shown in **Figures 3E–I**, the expression of *GhWRKY27a* was notably increased at different time points by H_2O_2, ABA, methyl jasmonate (MeJA), salicylic acid (SA) and ethylene (ET) treatments, and it then decreased markedly. Moreover, the fungal pathogen *R. solani* increased *GhWRKY27a* transcript levels (**Figure 3J**). These results indicate that *GhWRKY27a* may be involved in responses to multiple abiotic and biotic stresses by mediating multiple plant defense signal transduction pathways.

Silencing *GhWRKY27a* Enhanced Drought Tolerance in Cotton

To evaluate the role of *GhWRKY27a* in the drought stress response, we employed a VIGS technique to knock down the expression of *GhWRKY27a* in cotton. The cotton *CLA* gene was used as an additional control to determine the efficiency of gene silencing (**Figure 4A**). The transcript levels of *GhWRKY27a* in *GhWRKY27a*-silenced (VIGS) and empty vector-treated cotton (CK) plants were analyzed via qRT-PCR. The downregulation of *GhWRKY27a* indicated that *GhWRKY27a* was successfully knocked down in the VIGS plants (**Figure S2**). As shown in **Figure 4B**, we did not observe any difference in morphology and growth between VIGS and CK plants. However, after mannitol treatment, the CK plants exhibited severe wilting compared with the VIGS plants (**Figure 4B**). Likewise, after 7 days of water-withholding treatment, the CK plants began wilting, while the VIGS plants were less affected (**Figure 4C**). In addition, the VIGS plants exhibited less water loss and a higher survival rate than CK plants (**Figures 4D,E**). Moreover, less H_2O_2 accumulation was observed via DAB staining in the VIGS plants after drought treatment (**Figure 4F**). These data above indicated that silencing of *GhWRKY27a* enhanced drought tolerance in cotton.

GhWRKY27a Overexpression Decreased Tolerance to Mannitol Treatments During Seed Germination and Root Elongation

To further confirm the function of *GhWRKY27a*, transgenic *N. benthamiana* plants overexpressing *GhWRKY27a* were generated. Eight independent transgenic lines were selected on kanamycin and the chromosomal integration of the transgene was confirmed through PCR detection using genomic DNA as a template (**Figure S3**). Three bona fide GhWRKY27a expressing transgenic lines (OE1, OE2, and OE3) were chosen randomly, and the T_3 transgenic plants were used for further experiments. The wild-type (WT) plants were germinated at the same time with the transgenic plants.

The potential effects of *GhWRKY27a* on osmotic stress were investigated by comparing *GhWRKY27a*-overexpressing (OE) plants with WT plants grown on 1/2 MS medium with

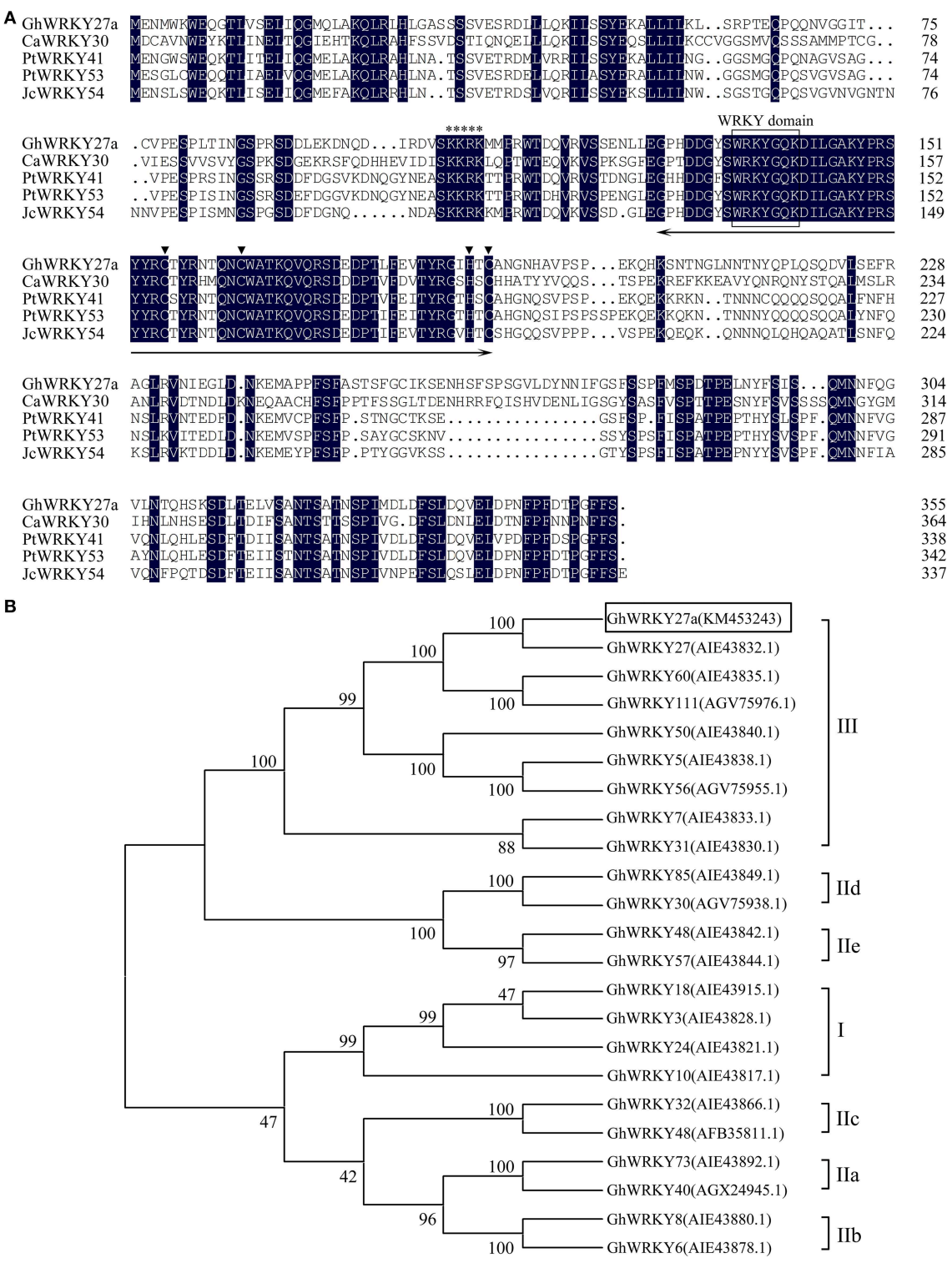


FIGURE 1 | Sequence analysis of GhWRKY27a. (A) Alignment of the amino acid sequence of GhWRKY27a with the sequences of CaWRKY30, PtWRKY41, PtWRKY53, and JcWRKY54. Identical amino acids are shaded in black. The approximately 60-amino acid WRKY domain and the C and H residues in the zinc-finger motif (C-X_7-C-X_{23}-H-X_1-C) are indicated with a two-headed arrow and inverted triangle, respectively. The highly conserved amino acid sequence WRKYGQK in the WRKY domain is boxed. The putative nuclear localization signal, KKRK, is indicated with an asterisk. **(B)** Phylogenetic analysis of GhWRKY27a in relation to other cotton WRKY TFs. A neighbor-joining phylogenetic tree was created using MEGA 5.1 software. GhWRKY27a is highlighted in the box, and each gene name is followed by its protein ID.

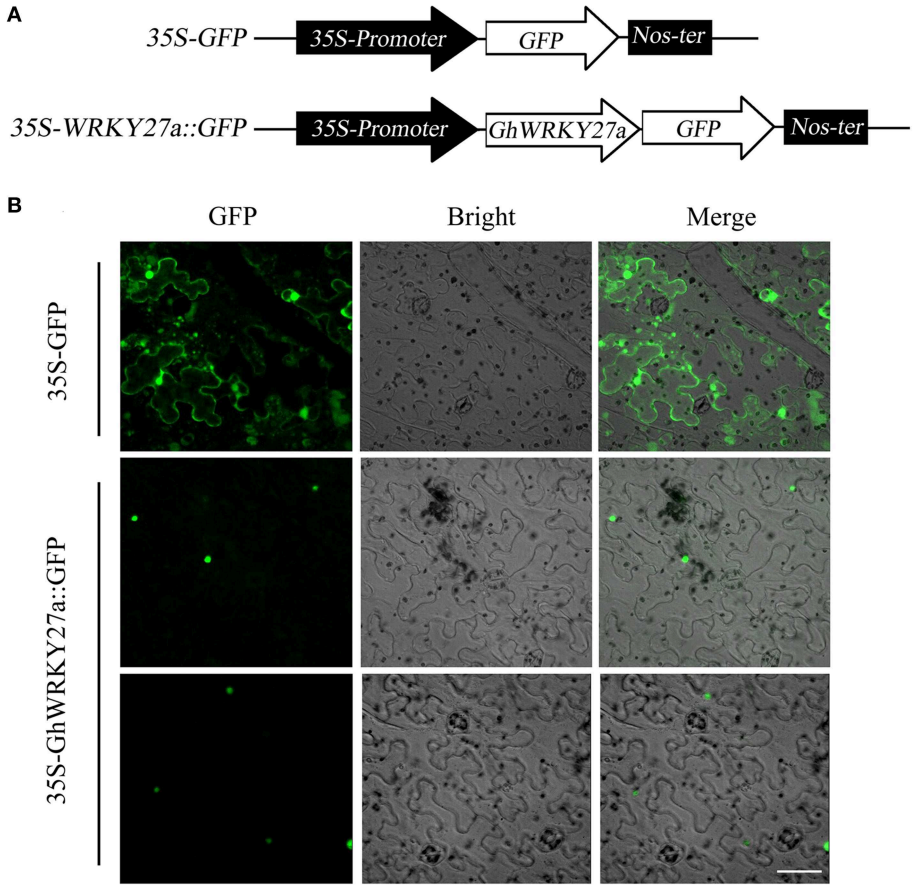


FIGURE 2 | Subcellular localization of the GhWRKY27a protein transiently expressed in *N. benthamiana* cells. (A) Schematic representation of the 35S-GhWRKY27a::GFP fusion construct and the control 35S-GFP construct. **(B)** The transient expression of 35S-GhWRKY27a::GFP in *N. benthamiana* cells was examined at 48 h after transformation under a Zeiss LSM 510 confocal laser-scanning microscope. The scale bar represents 50 μm.

or without mannitol. In the absence of mannitol, as shown in **Figures 5A,B**, no significant difference in seed germination rate was observed between WT and OE plants. However, in the presence of mannitol, OE plants showed enhanced sensitivity to mannitol-induced osmotic stress. The germination of OE seeds was more severely inhibited than that of WT lines. We next tested whether *GhWRKY27a* influences the growth of post-germinated tobacco seedlings under mannitol stress. At 3 days after sowing on 1/2 MS medium, seeds from the WT and OE lines showing radicle emergence were transferred to medium containing various mannitol concentrations ranging from 0 to 200 mM. The root length was observed to be shorter in all of the OE plants compared with WT plants in the presence of mannitol (**Figure 5C**). These data show that *GhWRKY27a* overexpression enhances osmotic sensitivity during seed germination.

GhWRKY27a Overexpression Reduced Tolerance to Drought Stress in Transgenic Plants

We next tested the phenotypes of the transgenic plants under drought stress. WT and OE plants at the vegetative growth stage were stopped to induce dehydration. After 3 days, the non-irrigated OE plants started to wilt, but the non-irrigated WT plants were still turgid (**Figure 6A**). The WT plants started to show wilting symptoms 2 days later. After 1 week of drought treatment, the plants were irrigated again. The WT plants recovered, while several of the OE leaves did not recover completely. In a further study, leaf water loss, the survival rate, and the stomatal aperture were compared in OE plants and WT plants after drought treatment. As shown in **Figure 6B**, the rate of water loss was higher in the OE plants than in WT plants. Moreover, the survival rate of the OE plants was lower than that of the WT plants (**Figure 6C**). The stomatal aperture of OE plants did not differ significantly from that of WT plants under normal conditions. However, the degree of stomatal closure observed in WT plants was greater than in OE plants under drought conditions (**Figure 6D**).

To further investigate the *GhWRKY27a*-associated mechanisms resulting in drought sensitivity, we measured the stomatal aperture in OE plants and WT plants in response to ABA because the ABA responsiveness of stomatal movement can modify drought sensitivity (Blatt, 2000). In the absence of ABA, there were no obvious differences in stomatal aperture

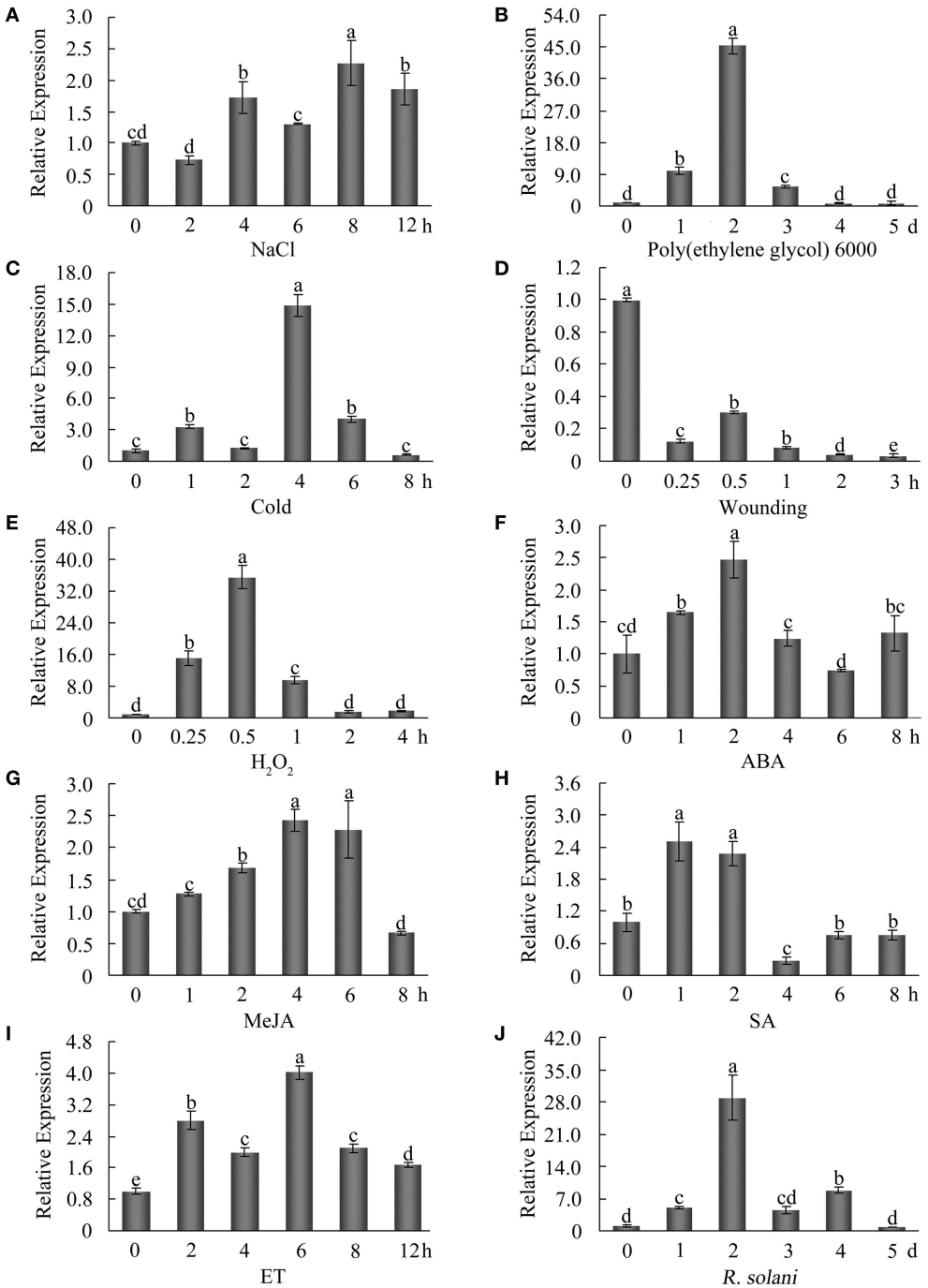


FIGURE 3 | Expression profiles of *GhWRKY27a* under diverse stress conditions. Seven-day-old cotton seedlings in hydroponic culture were subjected to the following treatments: 200 mM NaCl **(A)**, 15% poly(ethylene glycol) 6000 **(B)**, cold **(C)**, wounding **(D)**, 10 mM H_2O_2 **(E)**, 100 μM ABA **(F)**, 100 μM MeJA **(G)**, 2 mM SA **(H)**, 5 mM ET released from the ethephon **(I)**, and *Rhizoctonia solani* **(J)**. Total RNA was isolated at the indicated times after treatment and subjected to qRT-PCR analysis. The *ubiquitin* gene (GenBank accession number: EU304080) was employed as an internal control. The data are the mean ± SE of three independent experiments. The letters above the columns represent significant differences ($P < 0.01$) based on Duncan's multiple range test.

between WT and OE plants. Following treatment with 10 μM ABA for 3 h, the degree of stomatal closure was greater in WT plants than in OE plants (**Figures 7A,B**). In addition, we found that the endogenous ABA levels increased to a greater extent in WT plants than in OE plants under drought treatment, which is consistent with the higher degree of stomatal closure observed in the WT plants (**Figure 7C**). However, in the absence of the stress treatment, the ABA contents of WT

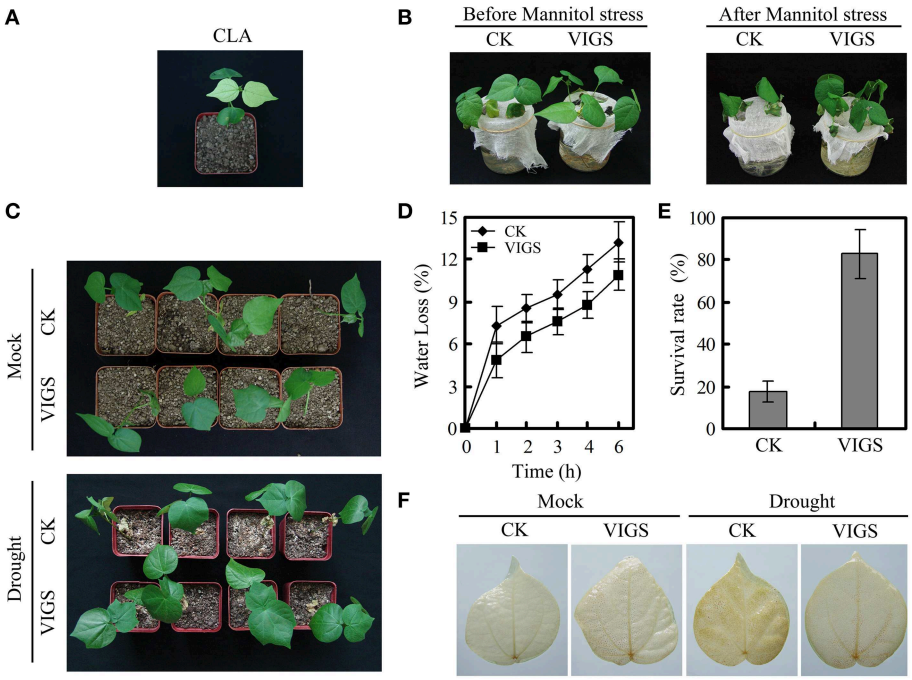


FIGURE 4 | Silencing *GhWRKY27a* enhanced drought tolerance in cotton. (A) Silencing of cotton CLA was phenotypically visible at 20 days after agroinfiltration. **(B)** Phenotype of mannitol-stressed plants. The plants were soaked with 400 mM mannitol solution for 12 h approximately 3 weeks post-inoculation. **(C)** Phenotype of drought-stressed plants. Water was withheld from CK and VIGS plants for 7 days. **(D)** Water loss from detached leaves of CK and VIGS plants. The rate of water loss was calculated based on the decrease in the fresh weight of the samples. The data are presented as the mean ± standard error of three independent experiments. **(E)** Survival rates of CK and VIGS plants under drought stress. The data are presented as the mean ± standard error of three independent experiments. **(F)** Drought-induced H_2O_2 as detected via DAB staining.

and OE plants were not significantly different (**Figure 7C**). Furthermore, many studies have shown correlations between drought sensitivity and the expression of ABA- or drought-related genes (Yan et al., 2014; Jia et al., 2015). In this study, *NbSnRK2.3* (sucrose non-fermenting 1-related protein kinase), *NbAREB1* (ABA-responsive element binding), *NbLEA* (late embryogenesis abundant), and *NbP5CS* (delta1-pyrroline-5-carboxylate synthetase) were used to monitor ABA and drought stress responses in OE plants. Under drought conditions, the levels of these genes in OE plants were lower than in WT plants (**Figure 7D**). Taken together, these data indicate that the drought-sensitive phenotype of OE plants is associated with enhanced stomatal opening and lower levels of ABA- or drought-related gene expression.

GhWRKY27a Overexpression in Transgenic Plants Reduced ROS Scavenging Ability under Drought Stress

Abiotic stress results in the accumulation of ROS in plants. Therefore, we evaluated the accumulation of H_2O_2 and superoxide radical anions (O_2^-) in the leaves of WT and OE plants under drought stress. Leaves detached from untreated WT and OE plants were used as controls. As shown in **Figures 8A,B**, under drought stress, OE plants showed greater accumulation of H_2O_2 and O_2^- than WT plants, as indicated by the accumulation of brown (DAB staining) and blue (NBT staining) pigments. Under normal growth conditions, no obvious differences in H_2O_2 or O_2^- were detected in WT vs. OE plants.

To confirm the ability of *GhWRKY27a*-overexpressing plants to scavenge ROS, the oxidative agent MV was used. As shown in **Figure 8C**, cotyledon bleaching or chlorosis was more severe in the OE plants than in the WT plants. This result was confirmed by measuring chlorophyll content after MV treatment. The WT plants demonstrated higher chlorophyll content than the OE plants (**Figure 8D**), suggesting that *GhWRKY27a* overexpression conferred decreased tolerance to oxidative stress.

To ascertain the possible mechanisms underlying the reduced antioxidant defense abilities observed in the transgenic plants, the levels of defense-related genes were assessed in WT and OE plants via qRT-PCR under drought stress. As shown in **Figure 8E**, the levels of superoxide dismutase gene (*SOD*), glutathione S-transferase gene (*GST*), ascorbate peroxidase gene (*APX*), and catalase gene (*CAT*), which encode ROS-scavenging enzymes, were increased to a greater extent in WT plants than in OE plants under drought conditions. However, the levels of the ROS producers (the respiratory burst oxidase homolog genes *RbohA* and *RbohB*) were increased to a greater extent in OE plants than in WT plants (**Figure 8E**). Together, these data demonstrate that *GhWRKY27a* may play a critical role in the regulation of the ROS network pathway.

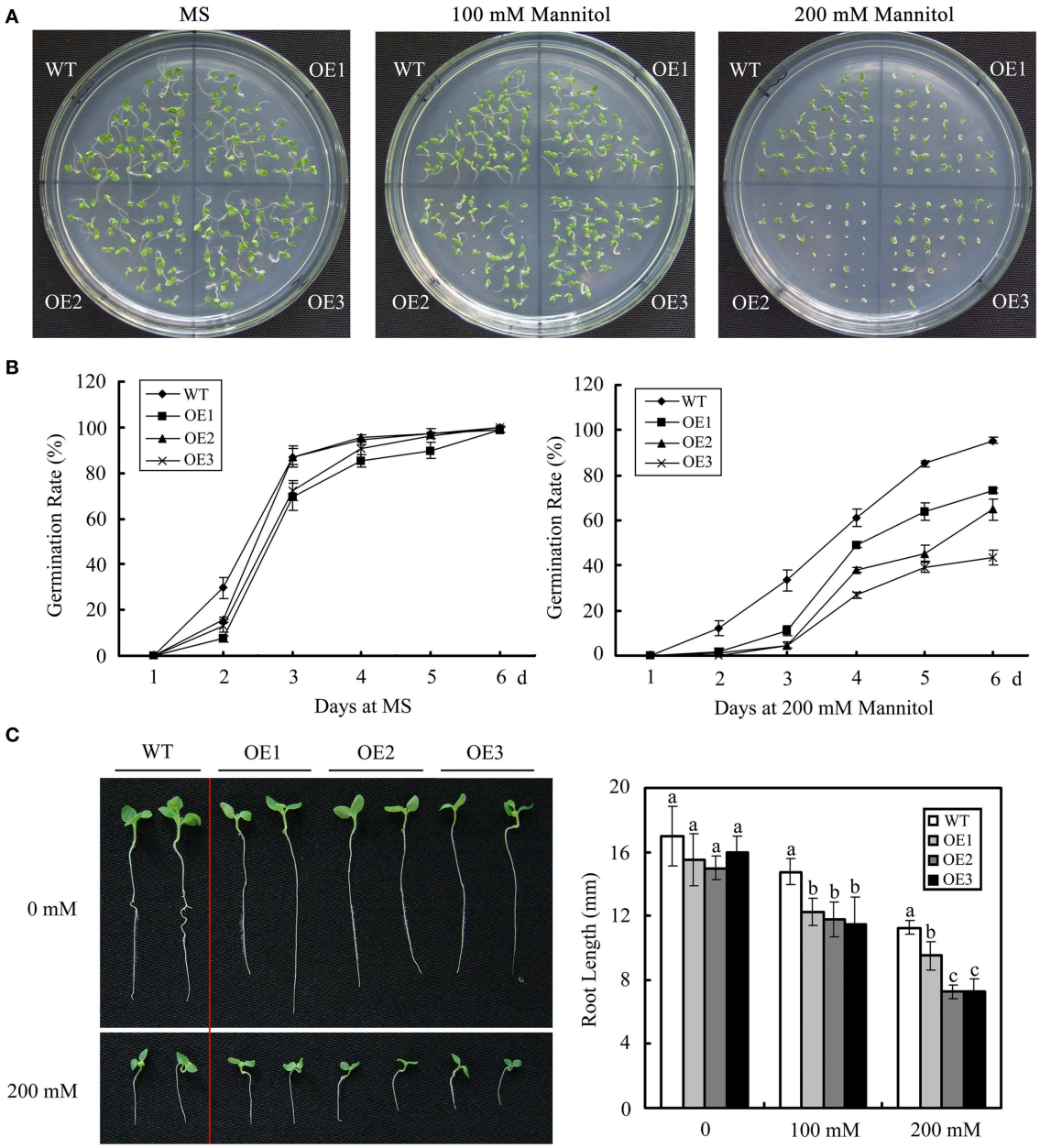


FIGURE 5 | *GhWRKY27a* overexpression enhances osmotic sensitivity during seed germination. (A) Seed germination on MS medium with 0, 100, and 200 mM mannitol. **(B)** Germination rates of the WT and OE lines under normal and 200 mM mannitol treatment conditions. Germination was scored daily. The data are the means ± SE of three independent experiments. **(C)** Photographs and measurements of the root lengths of WT and OE seedlings supplemented with mannitol. The data are the means ± SE of three independent experiments. The letters above the columns represent significant differences ($P < 0.05$) based on Duncan's multiple range test.

GhWRKY27a Overexpression Enhanced Susceptibility to *R. solani*

The upregulation of *GhWRKY27a* expression observed in response to *R. solani* inoculation and exogenous SA, ET, and MeJA application suggested a role for this gene in plant immunity. To investigate the role of *GhWRKY27a* in disease resistance in plants, detached leaves from 2-month-old T_3 generation transgenic plants were incubated with *R. solani*, which is a necrotrophic pathogen. As shown in **Figures 9A,B**, spreading necrosis and more severe disease symptoms were observed in OE plants. In contrast, WT plants were essentially resistant and exhibited only non-spreading local necrosis lesions at the inoculation sites. When the plants were infected via the trickle irrigation method for approximately 10 days, caudex rot was

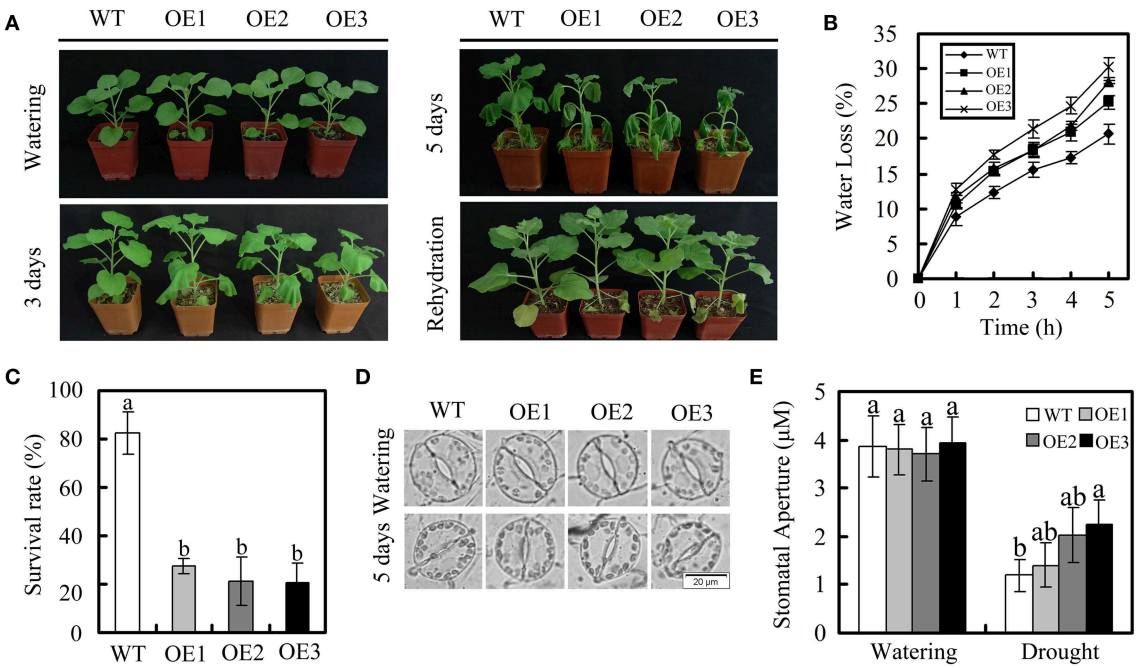


FIGURE 6 | Dehydration response of *GhWRKY27a*-overexpressing plants. (A) The WT and OE plants were grown until they produced eight expanded leaves after which irrigation was stopped to induce dehydration. Representative plants exposed to 3 or 5 days of drought are shown. After 1 week of drought stress, plants were rehydrated, and their recovery was recorded. **(B)** Water loss from detached leaves of WT and OE plants. The rate of water loss was calculated based on the decreases in the fresh weights of the samples. **(C)** Survival rates of WT and OE plants under drought stress. **(D,E)** Stomatal changes were observed with a microscope before and after drought treatment. The data are presented as the mean ± standard error of three independent experiments. The values indicated by the different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range test.

observed to be more serious in OE plants than in WT plants (**Figure 9C**).

To further elucidate possible mechanisms of *GhWRKY27a*-mediated disease sensitivity, we examined the effects of overexpressing *GhWRKY27a* on the accumulation of H_2O_2 and the transcript levels of defense genes following *R. solani* infection. As shown in **Figure 10A**, histochemical staining with DAB revealed that the *in situ* accumulation of H_2O_2 in OE leaves was higher than in the leaves of WT plants after *R. solani* infection. In addition, compared with WT plants, the expression levels of *Rboh A* and *Rboh B*, which encode ROS-generating enzymes, was increased to a greater extent in OE plants (**Figure 10B**). The levels of *APX*, *SOD*, *GST,* and *CAT*, which are involved in the scavenging of ROS, were repressed in the OE plants, as shown in **Figure 10B**. Likewise, compared with WT plants, the levels of the pathogenesis-related (PR) genes *PR1a* and *PR1c*, which are thought to be regulated by the SA-mediated signaling pathway (Dang et al., 2013), as well as the hypersensitivity-related (HSR) gene *HSR515*, which is considered to be associated with the hypersensitive response (Dang et al., 2013), were decreased significantly in OE plants following *R. solani* infection (**Figures 10B,C**). However, the levels of *JAZ1* and *JAZ3*, which are known to be associated with the JA signaling pathway (Pauwels and Goossens, 2011), were increased in OE plants compared with WT plants (**Figure 10C**). Similarly, the transcript levels of the ET-responsive gene *ACS6* were increased (**Figure 10C**). Furthermore, no significant differences in the levels of *nonexpresser of PR genes 1* (*NPR1*) was observed between WT and OE plants (**Figure 10C**). These results suggest that the enhanced susceptibility of *GhWRKY27a*-overexpressing plants to *R. solani* infection is associated with the altered expression of defense-related genes.

Discussion

WRKY TFs form one of the largest plant-specific TF families, exerting crucial roles in regulating the responses to biotic and abiotic stimuli. In cotton, thus far only several group I and II WRKY TFs have been characterized, while the functional roles of group III WRKY TFs remain elusive. In this study, we isolated a group III WRKY TF gene, *GhWRKY27a*, from cotton (*G. hirsutum*). The presence of conserved motifs and phylogenetic tree analysis further confirmed that *GhWRKY27a* was a member of group III (**Figure 1**). In addition, the subcellular localization of GhWRKY27a-GFP indicated that the fusion protein was located in the nucleus (**Figure 2**), which is consistent with previous studies on WRKY TFs from other species (Wang et al., 2013). These results indicate that *GhWRKY27a* may function in the nucleus.

Previous studies have shown that the expression of certain stress-induced proteins is associated with stress tolerance (Huang et al., 2011; Ma et al., 2013; Wang et al., 2014). The expression

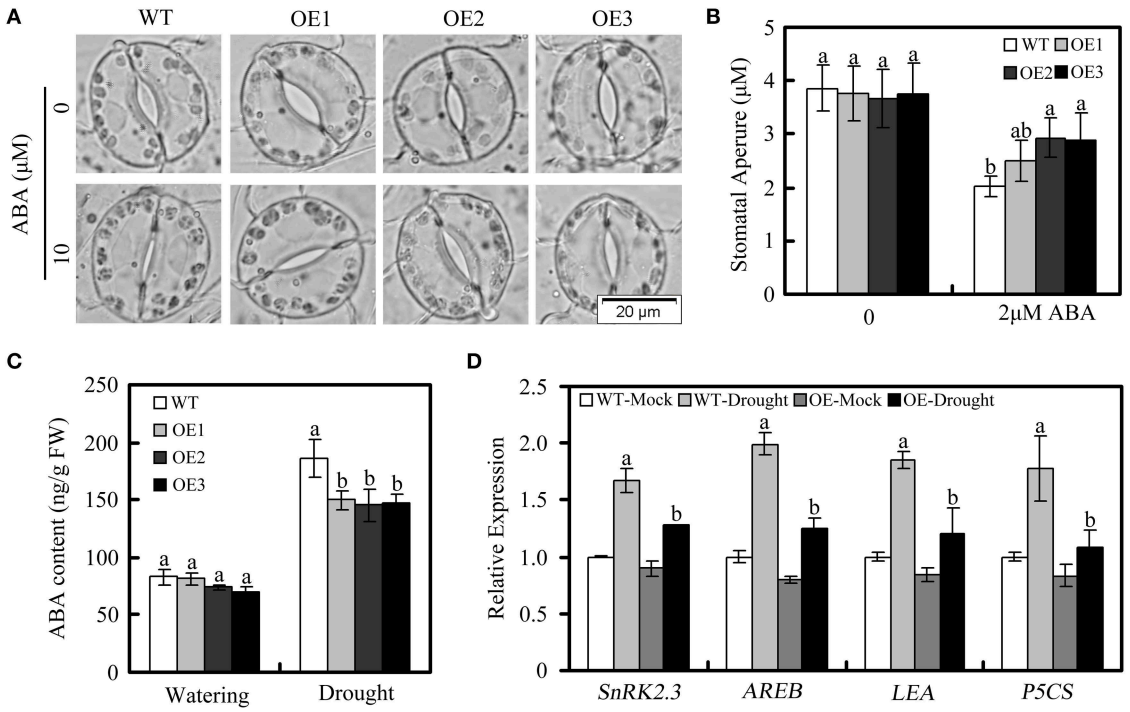


FIGURE 7 | Stomatal behavior in response to ABA and relative expression of ABA- and drought-responsive genes in WT and OE plants under drought treatment. (A,B) Stomatal closure in response to ABA treatment. **(C)** Endogenous ABA levels in WT and OE plants under normal and drought conditions. **(D)** Expression of ABA- and drought-responsive genes, determined via qRT-PCR. The *actin* gene was used to normalize the amount of template in each reaction. The transcript levels of the respective genes in mock-treated wild-type plants were used as a reference and set to a value of "1." The data are presented as the mean ± standard error of three independent experiments. The values indicated by the different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range test.

pattern of a gene is usually an indicator of its function (Li et al., 2015). In this study, the results from qRT-PCR analyses indicated that the transcription of *GhWRKY27a* in cotton was induced not only by abiotic stresses, pathogen infection, but also by multiple signaling molecules such as H_2O_2, ABA, SA, MeJA, and ET (**Figure 3**). These findings suggest that *GhWRKY27a* may function as a regulator that links multiple signaling networks in abiotic and biotic stress adaptation.

To expand our previous analysis of the biological roles of *GhWRKY27a*, we explored the possible contribution of *GhWRKY27a* to abiotic stress responses using drought stress as a model. Our results showed that silencing *GhWRKY27a* in cotton enhanced the tolerance to drought stress (**Figure 4**) and *GhWRKY27a* overexpression in *N. benthamiana* reduced its tolerance to drought stress (**Figure 6**), indicating that GhWRKY27a is a negative regulator of tolerance to drought stress. The function of GhWRKY27a in drought stress tolerance is similar to *Arabidopsis* WRKY53, which is phylogenetically related to GhWRKY27a (Dou et al., 2014). It was shown that activated expression of AtWRKY53 negatively regulated drought tolerance (Sun and Yu, 2015). The phytohormone ABA is essential in plant responses to drought stress, and an increased ABA content is beneficial for plants under drought stress as a result of ABA-induced changes at the cellular and whole-plant levels (Xiong and Zhu, 2003). The OE plants accumulated less ABA under drought stress compared with the WT plants (**Figure 7C**), which indicated that GhWRKY27a functioned in the ABA response in *N. benthamiana*. In addition, the transpirational water loss through the stomata is a key determinant of drought tolerance (Xiong et al., 2002). We found that the ectopic expression of *GhWRKY27a* in OE plants driven by the CaMV35S promoter did not result in the impairment of stomatal closure under normal conditions (**Figure 6D**). However, the more rapid water loss and impaired stomatal closure were observed in OE plants under drought stress (**Figure 6D**). We speculated that *GhWRKY27a* may not be expressed in guard cells of stomata, and impair stomatal closure indirectly. The regulation of stomatal movement may result from the interference of GhWRKY27a with the endogenous WRKY regulatory networks. Besides that, compared with normal condition, *GhWRKY27a* may interfere with more endogenous regulators under drought condition. Of course, further research is required to examine whether GhWRKY27a localizes to the guard cells of stomata and affects ABA level. Moreover, the results of this study showed that OE plants antagonistically regulated the expression of ABA- and drought-responsive genes, including *SnRK2.3*, *AREB*, *LEA,* and *P5CS* (**Figure 7D**). Under drought stress conditions, these stress-related genes are induced and are considered to

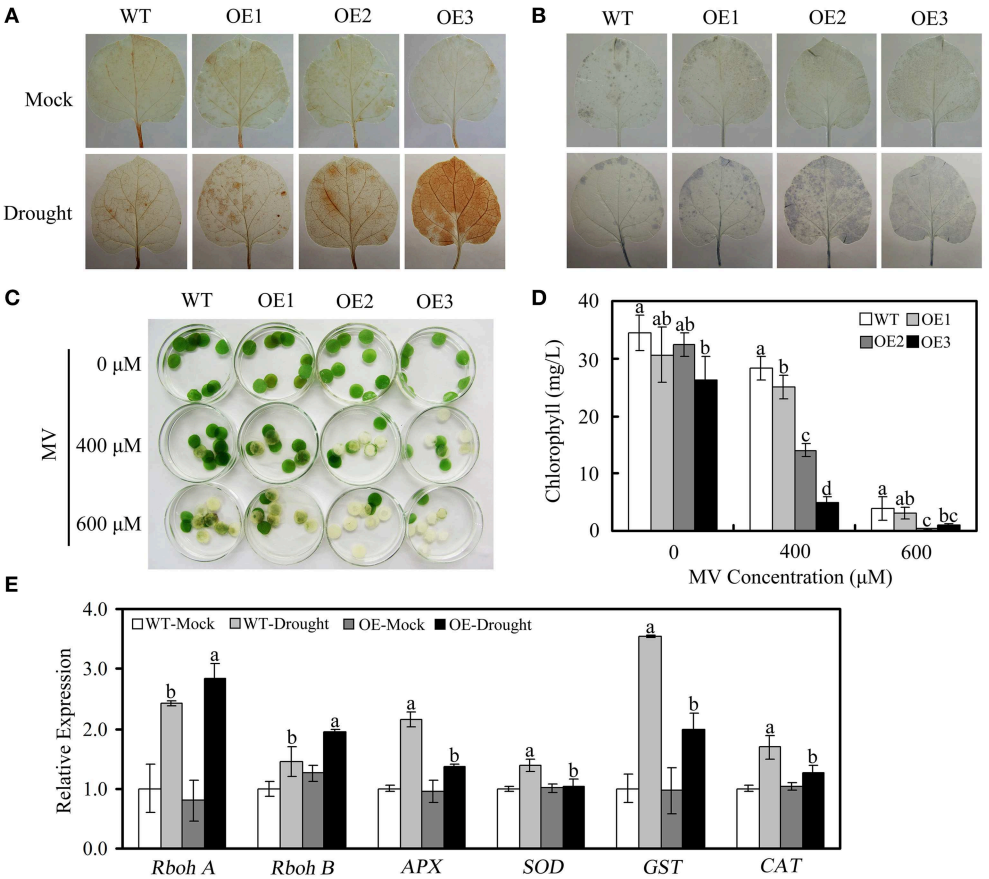


FIGURE 8 | ROS accumulation and expression of ROS-related genes in WT and OE plants under drought conditions. (A,B) Drought-induced H_2O_2 and O_2^- accumulation, as detected via DAB staining and NBT staining, respectively. **(C)** Phenotypes of leaf disks from WT and OE plants that were incubated in different concentrations of MV (0, 400, or 600 μM). **(D)** Relative chlorophyll content in the leaf disks from **(C)**. **(E)** Expression of ROS-related genes, determined via qRT-PCR. The *actin* gene was used to normalize the amount of template in each reaction. The transcript levels of the respective genes in mock-treated wild-type plants were used as references and set to a value of "1." The data are presented as the mean ± standard error of three independent experiments. The values indicated by the different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range test.

play a role in defense response (Shinozaki and Yamaguchi-Shinozaki, 2007). Based on these data, we infer that *GhWRKY27a* might confer reduced drought tolerance, which was coupled with impaired stomatal closure and downregulated expression of drought-responsive genes. On the other hand, we found that OE plants showed greater accumulation of ROS than WT plants after drought treatment, and *GhWRKY27a* overexpression resulted in reduced tolerance to oxidative stress (**Figure 8**), indicating that GhWRKY27a is also a negative regulator of tolerance to oxidative stress. It has been hypothesized that ROS production may be the primary symptom of phytotoxicity under abiotic stress (Choudhury et al., 2013). Thus, we infer that GhWRKY27a may function in drought stress by regulating production of ROS. This is similar to the observation that overexpression of *GhWRKY17* in *N. benthamiana* reduced drought stress tolerance by enhancing ROS accumulation (Yan et al., 2014).

In addition to the vital roles of WRKY TFs in abiotic tolerance, their role in disease resistance has been well documented (Cheng et al., 2015; Li et al., 2015). Intriguingly, certain pathogen-responsive group III WRKY genes function to repress plant basal disease resistance. For example, the WRKY domain in the group III WRKY TF AtWRKY52/RRS1 plays a negative role in defense signaling (Le Roux et al., 2015). AtWRKY38 and AtWRKY62 act as transcriptional activators and suppress disease resistance and defense gene expression in *Arabidopsis* (Kim et al., 2008). In this study, our results showed that *GhWRKY27a*-overexpressing plants exhibited enhanced susceptibility to the pathogen *R. solani* as measured by enhanced disease symptoms (**Figure 9**), implying that GhWRKY27a may play a negative role in the response to pathogen infection. However, there was no obvious difference in morphology between OE and WT plants under normal condition (**Figure 6A**). It is possible that certain *N. benthamiana* WRKY TFs positively regulate basal disease resistance (Yamamoto et al., 2004; Zhang et al., 2012), and this may interfere with the function of negative regulator of GhWRKY27a under normal condition. After pathogen infection, certain pathogen effectors can selectively target host WRKY

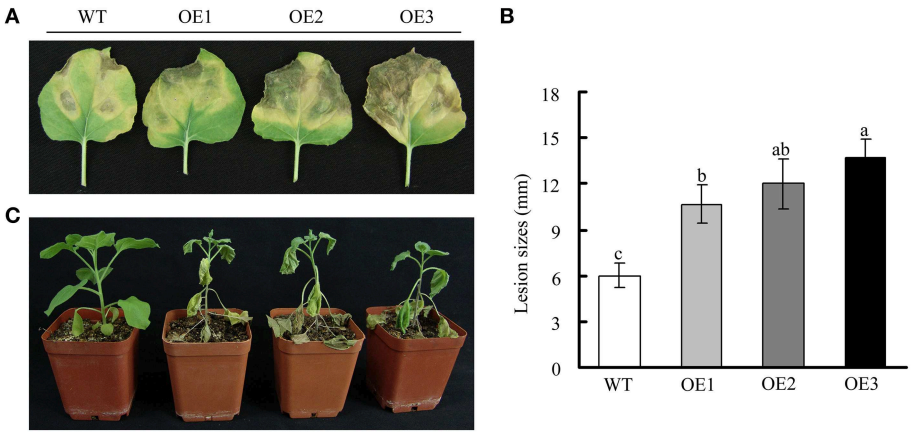


FIGURE 9 | Effects of constitutive *GhWRKY27a* overexpression on susceptibility to *R. solani*. (A) Disease symptoms in WT and OE plants at 7 days after inoculation with *R. solani*. **(B)** The lesion sizes induced by *R. solani* infection were assessed. Different letters above the columns indicate significant differences ($P < 0.001$) according to Duncan's multiple range test. **(C)** Symptoms of transgenic plants inoculated with *R. solani* using the trickle irrigation method.

TFs to disable defenses, and potentially avoid negative defense components whose inactivation would be disadvantageous for pathogen infection (Le Roux et al., 2015). This is also consistent with the observation that the OE plants were more susceptible to pathogen infection (**Figure 9**). The interactions between plants and pathogenic microbes are complex, thus further studies regarding WRKY TFs are necessary to parse the interactions between plants and pathogenic microbes.

The accumulation of ROS during infection of plants by necrotrophic pathogens has been implicated in susceptible response against these pathogens (Govrin and Levine, 2000). The results of this study indicated that OE plants demonstrated greater accumulation of ROS than WT plants after inoculation with *R. solani* (**Figure 10A**), consistent with the up-regulated expression of the ROS-producing genes *RbohA* and *RbohB* and the down-regulated expression of the antioxidant genes encoding SOD, GST, APX, and CAT (**Figure 10B**). Thus, it is likely that *GhWRKY27a* overexpression altered the expression of oxidation-related genes in response to *R. solani* infection and resulted in the accumulation of ROS, leading to susceptibility to this pathogen. On the other hand, the reduced expression of *HSR515* and *PR* gene (**Figures 10B,C**), reliable molecular markers of SA-dependent plant defense (Zheng et al., 2006), indicates that *GhWRKY27a* overexpression suppressed SA-mediated defense signaling pathways. Moreover, the JASMONAT-ZIM domain (JAZ) proteins are key repressors of JA signaling and represent a crucial interface in the JA signaling cascade (Pauwels and Goossens, 2011; Kazan and Manners, 2012). Notably, the levels of *NbJAZ1*/*NbJAZ3* were higher in OE plants than in WT plants (**Figure 10C**), suggesting that GhWRKY27a overexpression attenuated the JA-dependent signaling pathway, which is important for resistance to necrotrophic pathogens (Glazebrook, 2005). This is in agreement with the observations that cotton GbWRKY1 functions as a negative regulator of the JA-mediated defense response and plant resistance to the pathogens *Botrytis cinerea* and *Verticillium dahliae* by activating *JAZ1* expression (Li et al., 2014b). Li et al. (2015) also showed that the enhanced pathogen resistance observed in *SpWRKY1*-overexpressing tobacco plants is correlated with the SA-dependent and JA-dependent defense pathways. Therefore, we hypothesized that the disease susceptibility of OE plants may be related to the repression of SA-dependent and JA-dependent defense pathways.

The increased transcript levels of *GhWRKY27a* under drought stress and pathogen attack is paradoxical, because GhWRKY27a functions in negative responses to drought tolerance and in resistance to *R. solani* infection. Similar results have been reported in other plant species. In soybean, a stress-induced gene, *GmWRKY13*, negatively regulates drought stress responses (Zhou et al., 2008). *C. annuum CaWRKY1*, which is strongly induced by pathogen infections and the signal molecular SA, acts as a negative regulator to prevent spurious activation of defense responses at suboptimal concentrations of SA (Oh et al., 2008). In *Arabidopsis*, Wang et al. (2006) showed that *WRKY58* acts as a negative regulator in defense, and the *wrky58* mutant was more resistant to a pathogen than WT plants after treatment with a suboptimal level of benzothiadizole. We infer that the role of *GhWRKY27a* may be similar to that of these negative regulators, which is to prevent unnecessary activation of defense responses at suboptimal levels of signal molecules. In addition, another possibility is that *GhWRKY27a*, which is induced by multiple stresses, adjusts the intensity of defense responses in cooperation with stress-responsive positive regulators, and prevents overactivation of defense responses whenever stress diminishes. Further studies are needed to elucidate the role of GhWRKY27a in various biological processes.

Based on these data, we conclude that GhWRKY27a exerts important physiological functions in the negative regulation of tolerance to drought stress and resistance against *R. solani* infection, likely through modulating multiple signaling pathways. Although the complex regulatory mechanisms

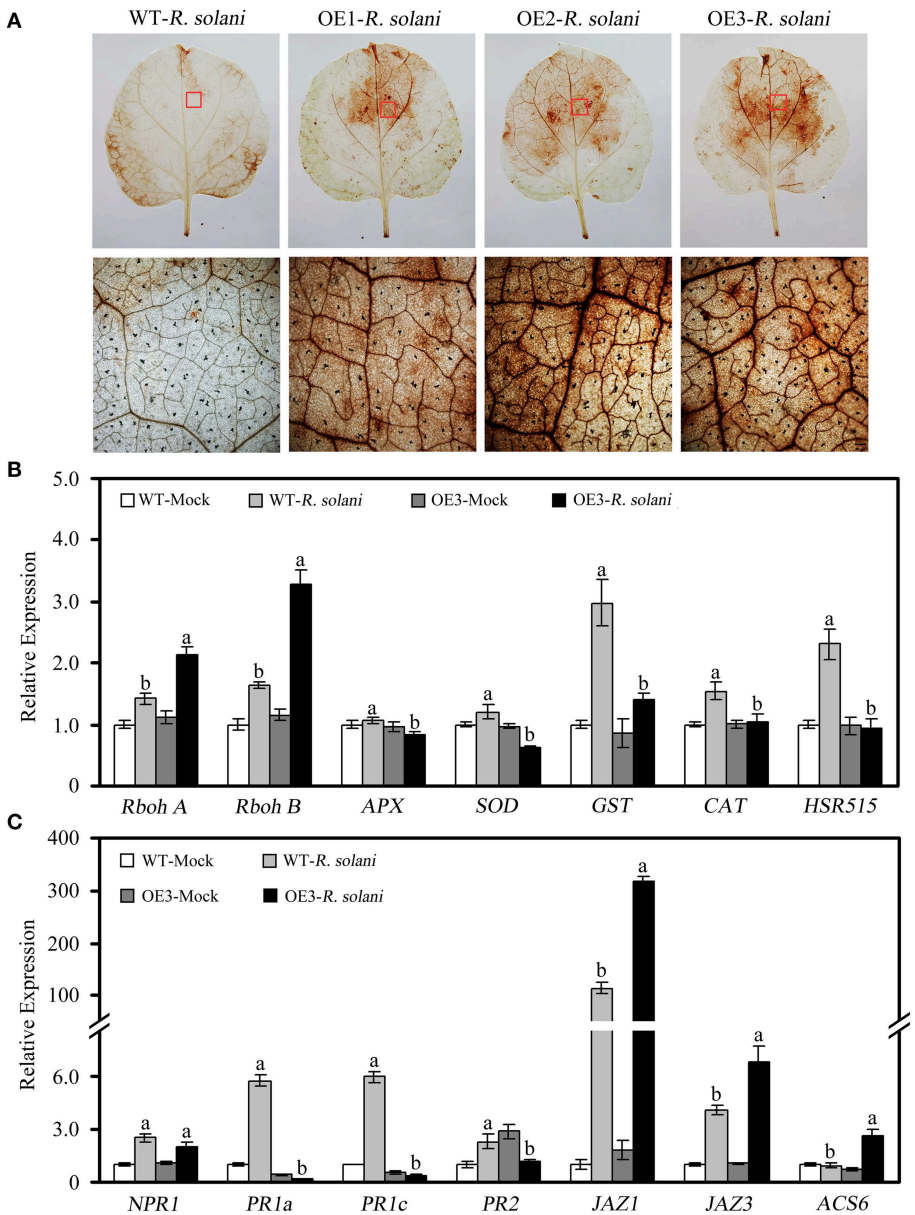


FIGURE 10 | The accumulation of H_2O_2 and relative expression of defense-related genes following *R. solani* infections. Leaf samples were collected at 7 days after inoculation with *R. solani*. **(A)** Accumulation of H_2O_2. **(B)** Relative expression of ROS production-related genes and HR-associated genes. **(C)** Relative expression of genes involved in different defense signaling pathways. The *actin* gene was used to normalize the amount of template in each reaction. The transcript levels of the respective genes in mock-treated wild-type plants were used as a reference and set to a value of "1." The data are presented as the mean ± standard error of three independent experiments. The values indicated by different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range test.

involving group III WRKY proteins in cotton remain unclear, this work provides further insight into the regulatory mechanisms of a group III WRKY protein.

Author Contributions

YY carried out most of the experiments, and drafted the manuscript. HJ participated in qRT-PCR analysis. HJ, FW, CW, and SL helped to revise the manuscript. XG designed the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00265

Figure S1 | Schematic representation of the DNA structures. The lengths of the extrons and introns of *GhWRKY27a* (GenBank accession number: KM453244), *AtWRKY41* (GenBank accession number: NW_003302550.1), *AtWRKY30* (GenBank accession number: NC_003076.8), and *AtWRKY54* (GenBank accession number: NC_003071.7) are shown according to the scale below.

Figure S2 | Silencing efficiency of the *GhWRKY27a* gene silenced plants. Seven-day-old seedlings were infiltrated with agrobacteria and leaf samples were collected 20 days after VIGS treatment. The silencing efficiencies of *GhWRKY27a* in wild-type (WT), vector control (CK), and *GhWRKY27a* gene silenced (VIGS) plants were analyzed via qRT-PCR. The *ubiquitin* gene (GenBank accession number: EU304080) was employed as an internal control. The data are presented as the mean ± standard error of three independent experiments.

Figure S3 | Characterization of transgenic tobacco plants. (A) The evaluation of transgenic plants in the T_0 progeny of transgenic plants by PCR. **(B)** Analysis of *GhWRKY27a* expression in wild-type (WT), T_1 *GhWRKY27a*-overexpressing (OE) plants, and T_3 *GhWRKY27a*-overexpressing (OE) plants. The *N. benthamiana* β-*actin* gene (GenBank accession number: JQ256516) was used as a loading control.

Table S1 | The primers used for general PCR in this study.

Table S2 | PCR amplification conditions.

Table S3 | The primers used for qRT-PCR in this study.

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Global analysis of WRKY transcription factor superfamily in *Setaria* identifies potential candidates involved in abiotic stress signaling

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Transcription factors (TFs) are major players in stress signaling and constitute an integral part of signaling networks. Among the major TFs, WRKY proteins play pivotal roles in regulation of transcriptional reprogramming associated with stress responses. In view of this, genome- and transcriptome-wide identification of WRKY TF family was performed in the C_4model plants, *Setaria italica* (SiWRKY) and *S. viridis* (SvWRKY), respectively. The study identified 105 SiWRKY and 44 SvWRKY proteins that were computationally analyzed for their physicochemical properties. Sequence alignment and phylogenetic analysis classified these proteins into three major groups, namely I, II, and III with majority of WRKY proteins belonging to group II (53 SiWRKY and 23 SvWRKY), followed by group III (39 SiWRKY and 11 SvWRKY) and group I (10 SiWRKY and 6 SvWRKY). Group II proteins were further classified into 5 subgroups (IIa to IIe) based on their phylogeny. Domain analysis showed the presence of WRKY motif and zinc finger-like structures in these proteins along with additional domains in a few proteins. All *SiWRKY* genes were physically mapped on the *S. italica* genome and their duplication analysis revealed that 10 and 8 gene pairs underwent tandem and segmental duplications, respectively. Comparative mapping of *SiWRKY* and *SvWRKY* genes in related C_4 panicoid genomes demonstrated the orthologous relationships between these genomes. *In silico* expression analysis of *SiWRKY* and *SvWRKY* genes showed their differential expression patterns in different tissues and stress conditions. Expression profiling of candidate *SiWRKY* genes in response to stress (dehydration and salinity) and hormone treatments (abscisic acid, salicylic acid, and methyl jasmonate) suggested the putative involvement of *SiWRKY066* and *SiWRKY082* in stress and hormone signaling. These genes could be potential candidates for further characterization to delineate their functional roles in abiotic stress signaling.

Keywords: WRKY transcription factors, *Setaria italica*, *Setaria viridis*, abiotic stress, stress signaling, expression profiling, comparative mapping

INTRODUCTION

Plants are exposed to diverse environmental stresses, which significantly affect their growth and development leading to drastic decrease in productivity. Among the different environmental stimuli, abiotic stresses are predominant, which includes drought, heat, salinity, and submergence. Climate change due to global warming is another aggravating challenge that influences the sustainability and productivity of crop plants (Kole et al., 2015). Plants have developed broad-spectrum defense responses to circumvent these stresses and exhibit stress tolerance or stress avoidance through acclimation and adaptation mechanisms (Mickelbart et al., 2015). On perception of stress, a complex signal transduction pathway (either abscisic acid-dependent or -independent) is induced, which initiates molecular, physiological and metabolic responses that ultimately enhance stress tolerance (Lata et al., 2015). Transcription factors (TFs) are a class of genes that predominate as tolerance determinants in plants (Mickelbart et al., 2015) by regulating the expression of stress-inducible genes. The TFs may constitute gene networks or signaling cascades, by which they regulate other TFs and/or other regulatory and/or functional genes (Tran and Mochida, 2010). Approximately 7% of the plant genome encodes for TFs (Udvardi et al., 2007), which are classified into 58 TF families (Jin et al., 2014). Among these TFs, WRKY is the seventh largest TF family (http://planttfdb.cbi.pku.edu.cn/). WRKY TFs are characterized by their unique WRKYGQK motif followed by a metal chelating zinc finger motif ($CX_{4-5}CX_{22-23}HXH$ or $CX_{5-8}CX_{25-28}HX_{1-2}C$) (Eulgem et al., 2000). These WRKY proteins bind to a specific domain called W-box in the promoter region with consensus sequence (C/T)TGAC[T/C], resulting in the expression of downstream target genes (Eulgem et al., 2000). In addition to W-box, WRKY TFs can also interact with a sugar responsive cis-element called SURE and activate transcription of downstream genes (Sun et al., 2003).

Several reports have shown the regulatory role of WRKY TFs in signaling pathways and modulation of diverse molecular and physiological processes including pollen development and function (Guan et al., 2014), seed dormancy (Rushton et al., 2010; Ding et al., 2014), seed development (Johnson et al., 2002; Sun et al., 2003; Luo et al., 2005), flowering time and plant height (Cai et al., 2014b), somatic embryogenesis (Alexandrova and Conger, 2002), biomass (Wang et al., 2010; Yu et al., 2013), secondary metabolite biosynthesis (Sun et al., 2003; Xu et al., 2004; Ma et al., 2009; Suttipanta et al., 2011), hormone signaling (Zhang et al., 2004) and leaf senescence (Miao et al., 2004). More importantly, WRKY TFs have been shown to get activated in response to different biotic (Dong et al., 2003; Muthamilarasan and Prasad, 2013) and abiotic stresses (Tang et al., 2013), including heat and drought (Rizhsky et al., 2002; Wu et al., 2009; Ren et al., 2010), cold (Huang and Duman, 2002; Pnueli et al., 2002), salinity (Jiang and Deyholos, 2006), wounding (Hara et al., 2000; Yoo et al., 2014), bacterial infection (Dellagi et al., 2000; Du and Chen, 2000; Chen et al., 2002; Chen and Chen, 2002; Deslandes et al., 2002; Kim et al., 2008), fungal invasion (Chen et al., 2002; Zheng et al., 2006; Marchive et al., 2007), virus attack (Wang et al., 1998; Yang et al., 1999; Chen et al., 2002, 2013; Huh et al., 2012) and defense against oomycetes (Beyer et al., 2001; Kalde et al., 2003).

Thus, considering the vital role of WRKY TFs in various molecular, biological and physiological processes, the *WRKY* gene family has been extensively characterized in various crop plants (Zhang and Wang, 2005), such as rice (Ross et al., 2007), cucumber (Ling et al., 2011), maize (Wei et al., 2012), tomato (Huang et al., 2012), Castor bean (Li et al., 2012), physic nut (Xiong et al., 2013), barley (Liu et al., 2014), *Brachypodium* (Wen et al., 2014), *Gossypium raimondii, G. hirsutum* (Cai et al., 2014a; Dou et al., 2014), grapevine (Wang et al., 2014), *G. arboretum* (Ding et al., 2015), cabbage (Yao et al., 2015), and in trees including rubber (Li et al., 2014), poplar (He et al., 2012; Jiang et al., 2014) and willow (Rao et al., 2015), and in *Arabidopsis* (de Pater et al., 1996; Deslandes et al., 2002; Song and Gao, 2014). However, no such studies have been reported in C_4 models, *Setaria italica* (foxtail millet) and *S. viridis* (green foxtail). Both *S. italica* and its wild progenitor *S. viridis* have collectively been accentuated as model crops for expediting functional genomics studies in Panicoideae, particularly C_4 photosynthesis, biofuel traits and abiotic stress tolerance (Brutnell et al., 2010, 2015; Li and Brutnell, 2011; Wang et al., 2011; Lata et al., 2013; Diao et al., 2014; Muthamilarasan and Prasad, 2015).

In view of their importance, the U.S. Department of Energy Joint Genome Institute and Beijing Genomics Institute, China have independently sequenced the genomes of *S. italica* and *S. viridis* (Bennetzen et al., 2012; Zhang et al., 2012). The availability of genome sequence information of *S. italica* in public domain has facilitated the identification of 2297 putative TFs belonging to 55 families (Bonthala et al., 2014). Of these 55 families, NAC (Puranik et al., 2012), AP2/ERF (Lata et al., 2014), MYB (Muthamilarasan et al., 2014a) and C_2H_2 zinc fingers (Muthamilarasan et al., 2014b) have been extensively characterized and their expression patterns in response to different abiotic stresses and hormone treatments have been investigated. However, no such global analysis of TFs has been performed in *S. viridis* due to non-availability of genome sequence in public domain (Muthamilarasan and Prasad, 2015). Recently, Xu et al. (2013) pooled the RNA isolated from *S. viridis* at three developmental stages, namely seed germination, vegetative growth, and reproduction in different tissues including leaf, stem, node, crown, root, spikelet, floret, and seed tissues. Subsequently, cDNA library was constructed from the pooled RNA and sequenced using Illumina HiSeq 2000 platform (Xu et al., 2013). Transcriptome-wide analysis of TFs has been demonstrated in important crop plants, namely barley (Tombuloglu et al., 2013), bread wheat (Okay et al., 2014), *Medicago sativa* (Postnikova et al., 2014), and *G. aridum* (Fan et al., 2015). In the present study, similar computational approach has been used to identify WRKY encoding transcripts from *S. viridis* transcriptome and the identified transcripts were analyzed with WRKY encoding genes of *S. italica*. Being the first comprehensive study on WRKY TFs in *S. italica* and *S. viridis*, the present study provides insights into the functional aspects of these TFs in response to abiotic stress, and highlights potential candidates for further characterization toward delineating their functional role in abiotic stress signaling.

MATERIALS AND METHODS

In Silico Mining of WRKY Proteins From *Setaria Italica and S. viridis*

The WRKY domain-containing protein sequences of *Setaria italica* and *S. viridis* were identified using the method of Plant Transcription Factor Database (Jin et al., 2014). *S. italica* protein sequences (v2.1) were retrieved from Phytozome v10.2 (Goodstein et al., 2012) and HMMER search was executed using the PFAM domain (PF03106) (Finn et al., 2011). The HMM profile generated with WRKY TFs of maize (Wei et al., 2012) and rice (Ross et al., 2007) were used to generate HMM profile and searched against the protein sequences of *S. italica* using HMMER (Finn et al., 2011). Both *de novo* and reference-based transcriptome sequences of *S. viridis* (kindly provided by Prof. Xin-Guang Zhu; Xu et al., 2013) were used to generate unique clusters using CD-Hit (Fu et al., 2012) with default parameters and the resultant sequences were subjected to ORF prediction using OrfPredictor (Min et al., 2005). The obtained peptide sequences were used for identification of WRKY domain-containing proteins using the methodology described for *S. italica*. The identified WRKY sequences were confirmed for the presence of PFAM domain PF03106 (WRKY DNA-binding domain) using HMMSCAN (http://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) and ScanProsite (http://prosite.expasy.org/scanprosite/; de Castro et al., 2006). The identified SiWRKY protein sequences were searched using BLASTP against *S. italica* database (v2.1) of Phytozome v10.2 to retrieve corresponding genomic, transcripts and coding sequences along with their chromosomal positions.

Protein Features, Multiple Sequence Alignment, and Phylogenetic Analysis

Protein features including molecular weight, isoelectric point (pI) and instability index were predicted using ProtParam tool of ExPASy (Gasteiger et al., 2005). Amino acid sequences of WRKY TFs belonging to *S. italica* (SiWRKY) and *S. viridis* (SvWRKY) were imported into BioEdit v7.2.5 (Hall, 1999) and multiple sequence alignment was performed using ClustalW at default parameters. The SiWRKY and SvWRKY sequences along with maize sequences (ZmWRKY; Wei et al., 2012) were imported into MEGA v6.06 (Tamura et al., 2013) to construct a phylogenetic tree by Neighbor-Joining method and the bootstrap test was performed with 1000 iterations.

Prediction of Gene Structure and Chromosomal Locations

The coding sequences and genomic sequences of SiWRKY proteins were analyzed using GSDS web server v2.0 (Hu et al., 2015) to identify the positions of introns and exons. Gene structure analysis for *SvWRKY* genes was not performed due to non-availability of genomic sequence of *S. viridis* in public databases. The information about chromosomal position of each *SiWRKY* gene was imported into MapChart v2.2 (Voorrips, 2002) and a physical map was constructed by mapping the genes in ascending order from short-arm telomere to long-arm telomere. MCScanX was used to identify tandem and segmental duplications of *SiWRKY* genes (Wang et al., 2012).

Gene Ontology Annotation and Promoter Analysis

SiWRKY and SvWRKY amino acid sequences were analyzed using Blast2GO v3.0.10 (Conesa et al., 2005) to obtain gene ontology (GO) annotation. The sequences were screened using BLASTN against *Oryza sativa* protein sequences following which, mapping, InterProScan, and annotation were performed. GO enrichment was conducted using BiNGO plugin of Cytoscape v2.6 based on Benjamini and Hochberg false discovery correction value (*Q*-value) at 0.05 for the genes (Shannon et al., 2003; Maere et al., 2005). The *SiWRKY* gene sequences were searched using BLASTN against *S. italica* database in Phytozome to retrieve 2 kb upstream sequences. These sequences were screened for cis-regulatory elements using PLACE web server (Higo et al., 1999).

Identification of Orthologs in C_4 Grass Genomes and Ks Dating

Orthologous genes of *SiWRKY* and *SvWRKY* in sequenced C_4 grasses including switchgrass (*Panicum virgatum*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*) were identified by BLAST analysis of the gene and protein sequences, respectively against these genomes. Sequences with >90% similarity were used for performing reciprocal BLAST and potential orthologs were identified. A comparative map was constructed using Circos (Krzywinski et al., 2009). Synonymous (Ks) and non-synonymous (Ka) substitution rates were calculated for paralogous and orthologous genes by PAL2NAL server (http://www.bork.embl.de/pal2nal/) and period of divergence was calculated using the equation $T = \mathrm{Ks}/2\lambda$, where λ was taken as 6.5×10^{-9} (Mishra et al., 2013; Puranik et al., 2013).

In silico Expression Profiling of *SiWRKY* and *SvWRKY* Genes

The transcriptome data of root (SRX128223), stem (SRX128225), leaf (SRX128224), spica (SRX128226), dehydration stress library (SRR629694), and control library (SRR629695) of *S. italica* were retrieved from European Nucleotide Archive (http://www.ebi.ac.uk/ena) (Zhang et al., 2012; Qi et al., 2013). *S. viridis* transcriptome data of pooled RNA isolated from samples across three developmental stages, namely seed germination, vegetative growth, and reproduction in different tissues including leaf, stem, node, crown, root, spikelet, floret, and seed tissues available under the accession number SRP019744 (Xu et al., 2013) was retrieved from DNA Data Bank of Japan (Tateno et al., 2002). The reads were filtered using NGS Toolkit (Patel and Jain, 2012), mapped on *S. italica* genome using CLC Genomics Workbench v4.7.1 and normalized by RPKM method. A heatmap was generated using MultiExperiment Viewer (MeV) v4.9 (Saeed et al., 2003).

Expression Profiling of Candidate Genes under Abiotic Stress and Hormone Treatments

Candidate *SiWRKY* genes were chosen for qRT-PCR expression analysis based on their *in silico* expression patterns. Primers were

designed for the 3′ UTR of each transcript using GenScript Real-time PCR Primer Design tool (https://www.genscript.com/ssl-bin/app/primer) (Supplementary Table S1). *S. italica* cv. "Prasad" was chosen for the study as the cultivar was reported to be tolerant to salinity and dehydration stress (Lata et al., 2010; Puranik et al., 2011). The seeds were grown in green house following conditions described by Lata et al. (2014). Twenty-one day old seedlings were treated with 250 mM NaCl (salinity) and 20% PEG 6000 (dehydration) for abiotic stress, and 100 μM methyl jasmonate (MJ), 100 μM salicylic acid (SA), and 100 μM abscisic acid (ABA) for hormone treatments (Lata et al., 2014). Samples were collected at 0 h (control), 1 h (early), and 24 h (late) intervals, immediately frozen in liquid nitrogen and stored at −80°C. Total RNA from each sample was isolated following the method described by Logemann et al. (1987) and treated with RNase-free DNase I (50 U/ml). The quality and purity of RNA was tested using NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) [OD_{260}:OD_{280} nm absorption ratio (1.8–2.0)] and integrity was checked by resolving on 1.2% agarose gel containing 18% formaldehyde. First strand complementary DNA was synthesized with random primers from 1 μg total RNA using Thermo Scientific Verso cDNA Synthesis kit (Thermo Fisher Scientific, USA) following manufacturer's instructions. qRT-PCR was performed in StepOne Real-Time PCR Systems (Applied Biosystems, USA). A constitutive *Act2* gene-based primer was used as the endogenous control (Kumar et al., 2013). The PCR mixtures and reactions followed by melting curve analysis and agarose gel electrophoresis were performed following Kumar et al. (2013). Three technical replicates for each biological replicate were maintained for qRT-PCR analysis.

RESULTS

WRKY Transcription Factors of *Setaria*

HMM search for WRKY proteins in *Setaria italica* showed the presence of 113 WRKY proteins (SiWRKY), which was in agreement with the numbers reported in Plant Transcription Factor Database v3.0 (Jin et al., 2014) and Foxtail millet Transcription Factor Database (Bonthala et al., 2014). Among these, four SiWRKY proteins (Si031469 m, Si030012 m, Si029764 m, and Si036581 m) were found to be the products of alternate transcripts. In case of *S. viridis*, 50 WRKY TF sequences were identified (SvWRKY). Domain analysis of both SiWRKY and SvWRKY proteins using HMMSCAN and ScanProsite web tools revealed that four SiWRKY and six SvWRKY proteins did not possess the consensus WRKY DNA-binding domain (PF03106). The resultant 105 SiWRKY and 44 SvWRKY sequences (Supplementary Table S2) were used in further studies. Among the 105 SiWRKY proteins, SiWRKY099 was identified to be the smallest protein with 93 amino acids (aa), whereas the largest one was SiWRKY011 (1290 aa). The molecular weights of the proteins also varied according to protein size ranging from 10.3 kDa (SiWRKY099) to 145.8 kDa (SiWRKY011). In case of SvWRKY, the smallest proteins were SvWRKY008 (204 aa) and SvWRKY025 (207 aa), while the largest protein was SvWRKY031 (1290 aa). The molecular weight of SvWRKY proteins ranged from 21.6 kDa (SvWRKY008) to 145.8238 kDa (SvWRKY031). Isoelectric point (pI) of SiWRKY and SvWRKY proteins ranged from 4.8 (SiWRKY056) to 10.1 (SiWRKY037) and 5 (SvWRKY026) to 11.8 (SvWRKY006), respectively. The large variation in protein features might denote the presence of putative novel variants. Instability index of these proteins showed that most proteins (99 SiWRKY and 41 SvWRKY) were unstable (Supplementary Table S2).

Classification of SiWRKY and SvWRKY Proteins

WRKY proteins are classified into three major groups (I, II, and III) based on the conserved WRKY domain and zinc finger-like structure (Rushton et al., 1995). Group I has two WRKY domains as well as $CX_{4-5}CX_{22-23}HXH$ structure, group II has one WRKY domain with conserved zinc-finger motif sequence, whereas group III has one WRKY domain and $CX_{4-5}CX_{22-23}HXC$ structure (Eulgem et al., 2000). Group II proteins are further classified into five sub-groups (IIa–IIe) based on the conservation of amino acid motifs outside the WRKY domain (Park et al., 2005). Sequence alignment of SiWRKY and SvWRKY showed that all proteins, except SiWRKY044, SiWRKY063, SvWRKY005, SvWRKY007, SvWRKY008, and SvWRKY011, possess conserved WRKY domain and zinc finger-like structure. These exceptional WRKY proteins were classified as group IV. However, these proteins could represent pseudogenes or sequencing and assembly errors (Xie et al., 2005; Ross et al., 2007).

Among the remaining 103 SiWRKY proteins, 10 belong to group I, 54 to group II and 39 to group III, whereas in case of SvWRKY proteins, 6 belong to group I, 23 to group II and 11 to group III (**Figure 1**). The first WRKY domain of group I proteins possesses a conserved WRKYGQK amino acid motif, whereas the second domain lacked the GQK signature. Both the WRKY domains were followed by conserved $CX_4CX_{22-23}HXH$ structure. Interestingly, SvWRKY004 was observed to possess three WRKY domains followed by zinc finger-like structures. In case of group IV proteins, the conserved WRKYGQK domain was present in the N-terminal region (**Figure 1**). Phylogenetic analysis of group I, II, and III proteins of SiWRKY, SvWRKY, and ZmWKRY (Wei et al., 2012) confirmed the group-wise classification and also enabled the sub-classification of group II proteins (**Figure 2**). Group IV proteins deduced through sequence alignment were not included in phylogenetic analysis as they represent the products of pseudogenes or sequencing and assembly errors (Xie et al., 2005; Ross et al., 2007; Wei et al., 2012). Among the 54 group II SiWRKY proteins, 5 belong to IIa, 8 to IIb, 20 to IIc, 9 to IId, and 12 to IIe. Similarly, two SvWRKY proteins belong to group IIa, 3 to IIb, 9 to IIc, 4 to IId, and 5 to IIe. Interestingly, group IIc was interrupted by the members of IIb and IIa (**Figure 2**). A similar observation was reported by Wei et al. (2012) in maize, wherein the phylogenetic tree of WRKY proteins from *Arabidopsis*, maize, rice, barley, and *Physcomitrella patens* showed the interruption in group IIc. Domain analysis using HMMSCAN and PROSITE tools revealed the presence of additional NB-ARC domain (PF00931) in SiWRKY011 and SvWRKY031, and domain of

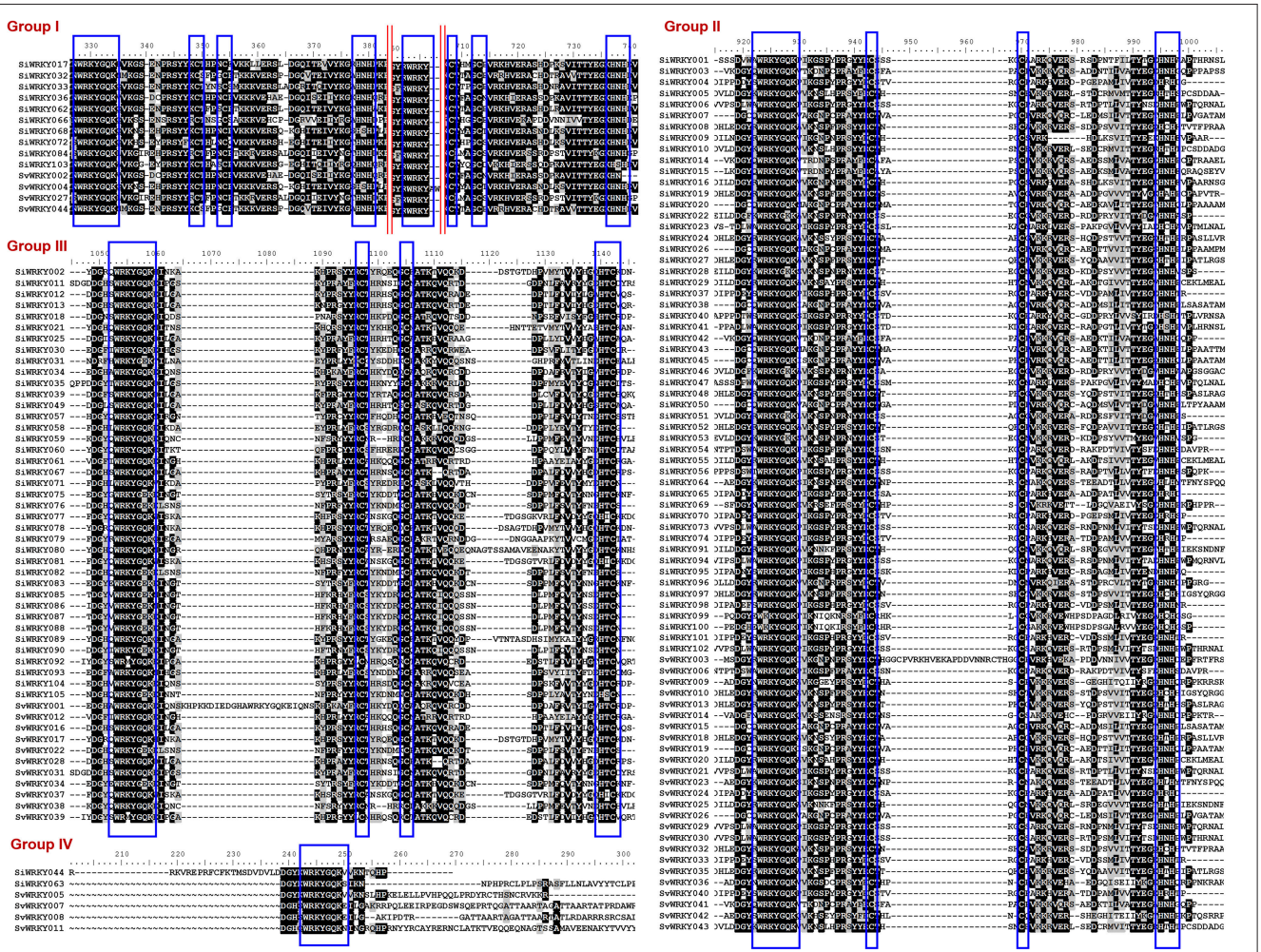


FIGURE 1 | Multiple sequence alignment of three major groups of SiWRKY and SvWRKY proteins. Group I, II, III, and IV proteins have been aligned separately and the consensus motifs are highlighted in blue boxes. Highly conserved amino acids have been shown in black boxes, less conserved in gray boxes, while amino acids with no similarly were indicated in black texts. The red lines in group I proteins indicate that the intermittent sequences, which were less conserved were not shown.

unknown function (PF12204) in SiWRKY011 and SiWRKY096 (Supplementary Table S3).

Structure, Location, and Duplication of *SiWRKY* Genes

Positions of introns and exons within the *SiWRKY* genes and their chromosomal locations were determined. However, this could not be performed for *SvWRKY* genes since the genome sequence data of *S. viridis* is not released in public database, till date. Gene structure prediction showed the numbers and arrangement of introns and exons within the *SiWRKY* genes (Supplementary Figure S1). The majority of *SiWRKY* genes (59; ~56%) were found to contain two introns, whereas 22 genes (~21%) have a single intron. Thirteen *SiWRKY* genes (~12%) have three introns, while 5 (~5%) and 4 (~4%) genes have four and five introns, respectively. A maximum of 10 introns were found to be present in *SiWRKY096* and the *SiWRKY065* gene was intronless (Supplementary Figure S1). The length of *SiWRKY* genes was also observed to be variable ranging from 0.6 kb (*SiWRKY019*) to 7.5 kb (*SiWRKY103*). Physical mapping of all the 105 *SiWRKY* genes onto nine chromosomes of *S. italica* revealed an uneven distribution of these genes in the genome (**Figure 3**). Among the four groups, members of group II and III were present in all the nine chromosomes, whereas group I *SiWRKY* genes were not present in chromosomes 1 and 4. Two members of group IV, namely *SiWRKY044* and *SiWRKY063*, were present in chromosome 5. Subsequently, the expansion of WRKY gene family in *S. italica* genome was examined using MCScanX tool, which showed that 10 and 8 *SiWRKY* gene pairs underwent tandem and segmental duplications, respectively (**Figure 3**). The tandemly duplicated genes include one pair of group I (in chromosome 3), two pairs of group II (in chromosomes 4 and 9), and seven pairs of group III genes (in chromosomes 1, 5, 7, and 8). Segmental duplication was found to occur between the *SiWRKY* genes of chromosome 3 and 5, and not in other chromosomes (**Figure 3**).

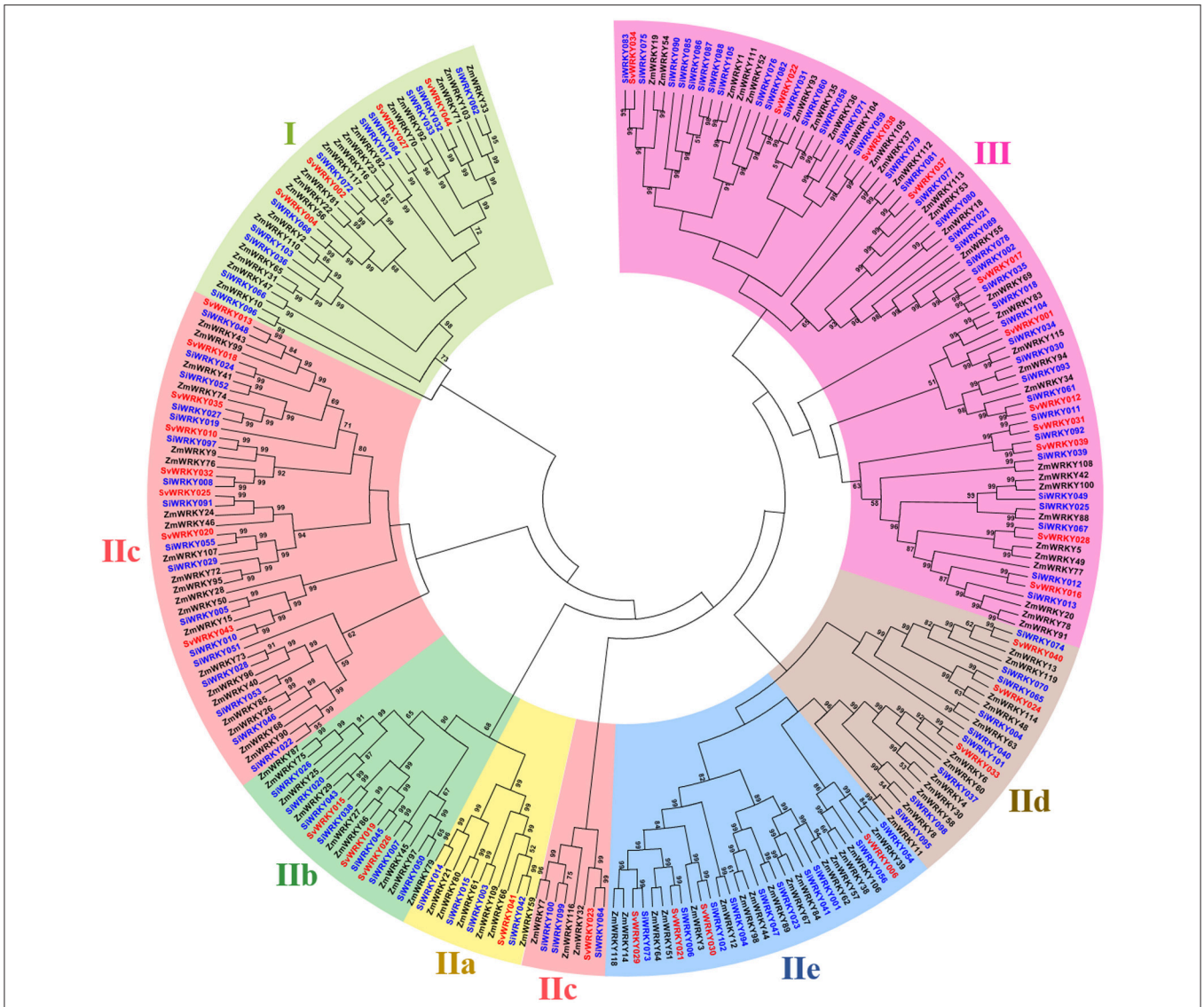


FIGURE 2 | Unrooted Neighbor-Joining tree constructed with WRKY proteins of *Setaria italica* (SiWRKY), *S. viridis* (SvWRKY), and *Zea mays* (ZmWRKY). SiWRKY and SvWRKY protein IDs are highlighted in blue color and groups are differentiated with different colors.

Gene Ontology Annotation and Analysis of cis-acting Elements

Gene ontology (GO) annotation of SiWRKY and SvWRKY proteins was performed using Blast2GO and Cytoscape tools and showed the involvement of these proteins in different biological processes and molecular functions (Supplementary Table S4). A majority of these proteins were predicted to be involved in response to stress as well as cellular, metabolic and biosynthetic processes (biological process; $P \leq 2.2 \times 10^{-6}$) (**Figure 4**). The molecular functions of these proteins corresponded to transcription regulator activity ($P \leq 4.2 \times 10^{-13}$). Further, cellular component analysis revealed the localization of these gene products in nucleus (**Figure 4**). Promoter analysis of *SiWRKY* genes showed the presence of 284 cis-regulatory elements (CREs), of which some elements were present in all the 105 genes, whereas a few were unique to one or two genes of the entire family (Supplementary Table S5). ARR1AT (element involved in cytokinin responsiveness), CAATBOX1 (element in enhancer regions of the promoter), CACTFTPPCA1 (element involved in mesophyll-specific gene expression of C_4 phosphoenolpyruvate carboxylase gene in C_4 plants), DOFCOREZM (target binding site of Dof proteins), EBOXBNNAPA (target binding site of bHLH and MYB-transcription factor), GATABOX (light responsive element), MYCCONSENSUSAT (MYC recognition site), and WRKY71OS (binding site of WRKY TFs) were present in the upstream region of all *SiWRKY* genes. In contrast, few CREs were found to be present in only one *SiWRKY* gene (Supplementary Table

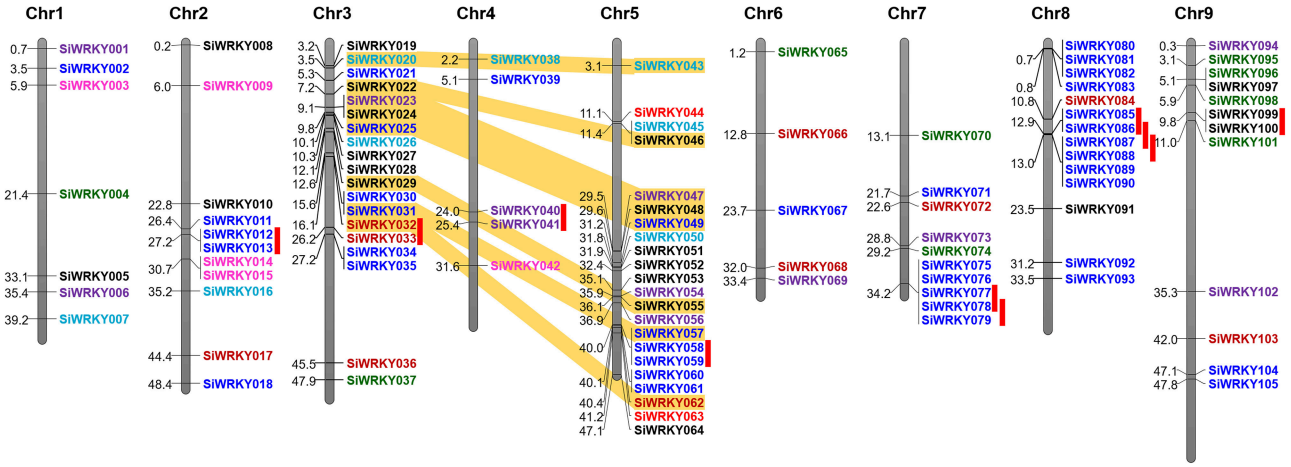


FIGURE 3 | Physical map of *SiWRKY* genes showing their chromosomal locations. Vertical bars represent the chromosomes and numbers at the left indicate the position of genes (in Mb). Tandemly duplicated gene pairs are indicated with red vertical bars and segmentally duplicated gene pairs are connected with yellow bands. Colors of *SiWRKY* IDs correspond to the different groups.

S5). This includes ABADESI2 (Synthetic element related to response to abscisic acid and to desiccation; in *SiWRKY009*), ABRE2HVA1 (ABA responsive element; in *SiWRKY053*), ACGTSEED3 (bZIP transcription activator binding site; in *SiWRKY015*), AGL2ATCONSENSUS (MADS binding site; in *SiWRKY034*), AUXRETGA2GMGH3 (auxin responsive element; in *SiWRKY017*), EREGCC (ethylene responsive element; in *SiWRKY051*), HSE (heat shock responsive element; in *SiWRKY047*), MREATCHS (MYB Recognition Element; in *SiWRKY094*), POLLEN2LELAT52 (required for pollen specific expression; *SiWRKY002*), and S2FSORPL21 (leaf-specific, light-independent regulatory element; in *SiWRKY094*; Supplementary Table S5).

Comparative Mapping in Related Grass Genomes and Ks Dating of Paralogs and Orthologs

All the 105 *SiWRKY* genes and 44 SvWRKY proteins were subjected to BLAST search against the database of switchgrass (*Panicum virgatum*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) to identify corresponding orthologs (>90% similarity). Potential orthologs were confirmed by reciprocal BLAST (**Figure 5**; Supplementary Tables S6–S11). A total of 60 *SiWRKY* genes (∼57%) showed syntenic relationship with maize (Supplementary Table S8), followed by switchgrass (∼54%; Supplementary Table S6) and sorghum (∼40%) (Supplementary Table S7). In case of SvWRKY proteins, maximum synteny was observed with switchgrass (31, ∼70%; Supplementary Table S9), followed by maize (24, ∼55%; Supplementary Table S11) and sorghum (20, ∼45%; Supplementary Table S10). Further, the effect of Darwinian positive selection in duplication and divergence of *WRKY* genes was examined by estimating the ratios of non-synonymous (Ka) vs. synonymous (Ks) substitution for paralogous as well as orthologous gene pairs. The Ka/Ks ratio for tandemly duplicated gene pairs ranged from 0.09 to 0.18 with an average of 0.13 (Supplementary Table S12), while for segmentally duplicated gene pairs, the ratio ranged from 0.06 to 0.14 with an average of 0.1 (Supplementary Table S13). Both the tandem and segmental duplications have been estimated to occur around 29 million years ago (mya) and 23 mya, respectively (Supplementary Tables S12, S13). Similarly, the average Ka/Ks ratios of orthologous gene pairs of *S. italica* - *P. virgatum*, *S. italica* - *S. bicolor,* and *S. italica* - *Z. mays* were estimated as 0.94, 0.19, and 0.19, respectively (Supplementary Tables S6–S8). In case of *S. viridis* - *P. virgatum*, *S. viridis* - *S. bicolor,* and *S. viridis* - *Z. mays* orthologs, the Ka/Ks ratios were 0.79, 0.21, and 0.18, respectively (Supplementary Tables S9–S11). This revealed that the orthologous gene pairs underwent natural selection (Ka/Ks $<$ 1). The estimated time of divergence of *S. italica* and *P. virgatum* was 4.7 mya, whereas *S. italica* and *S. bicolor* as well as *Z. mays* diverged around 27 mya. Similar estimates were observed in case of *S. viridis* - *P. virgatum* (4.7 mya), *S. viridis* - *S. bicolor* (26.8 mya), and *S. viridis* - *Z. mays* (27.8 mya) orthologs.

In silico Expression Profiling of *SiWRKY* and *SvWRKY* Genes

Expression pattern of *SiWRKY* genes in four tissues, namely root, leaf, spica, and stem revealed a differential expression pattern (**Figure 6**). A few genes including *SiWRKY003, SiWRKY017, SiWRKY033, SiWRKY034, SiWRKY056,* and *SiWRKY101* were found to be highly expressed in all the tissues. Tissue-specific higher expression of *SiWRKY028, SiWRKY032, SiWRKY042, SiWRKY045, SiWRKY060, SiWRKY062, SiWRKY078,* and *SiWRKY091* in root and *SiWRKY044* in stem was also observed. Some genes such as *SiWRKY006, SiWRKY019, SiWRKY026, SiWRKY039, SiWRKY057,* etc. did not show any expression in all the four tissues (**Figure 6**). In case of expression profiles

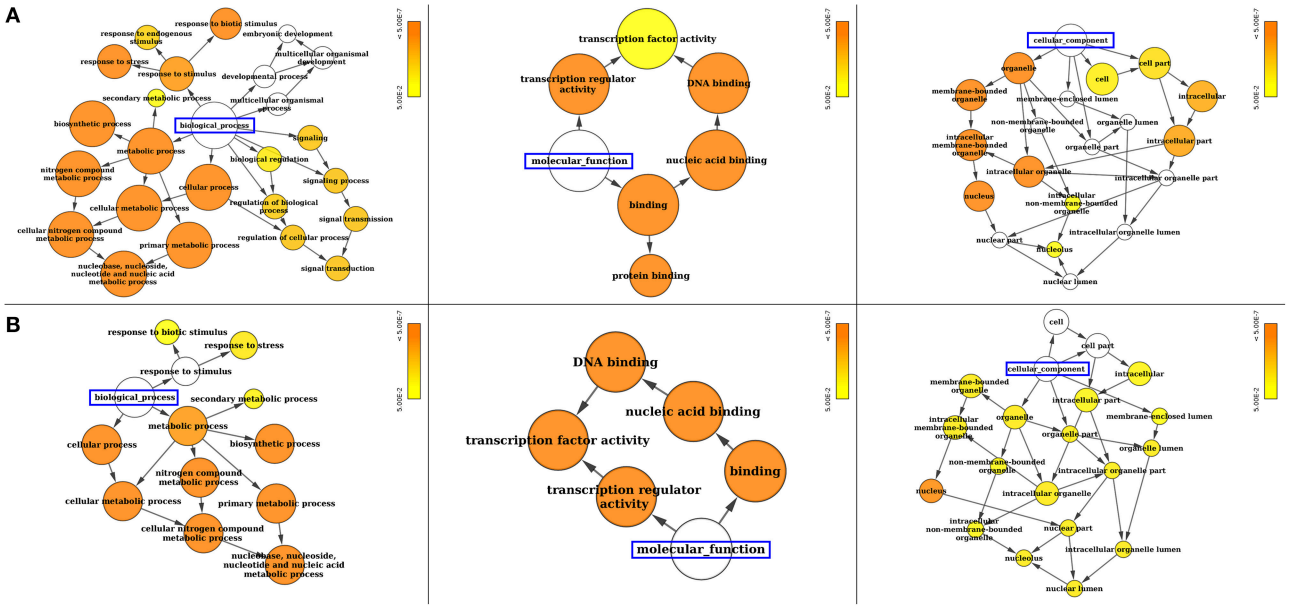


FIGURE 4 | Gene ontology (GO) enrichment analysis of (A) *SiWRKY* and (B) *SvWRKY* genes. The number of genes falling in each GO category is directly proportional to the node size. The nodes are color shaded according to the significance level (corrected *P*-value).

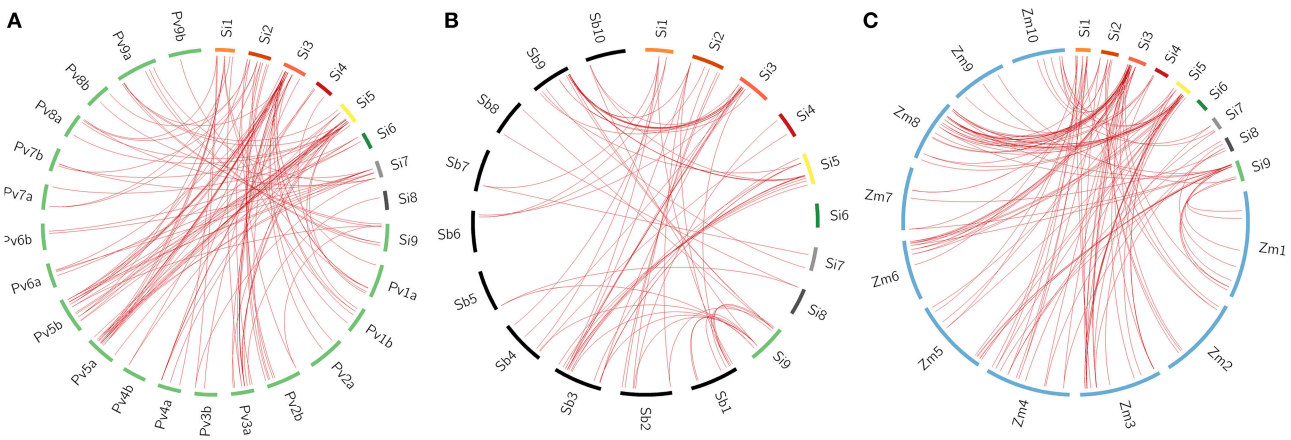


FIGURE 5 | Synteny map showing the orthologous gene positions of *WRKY* genes in *Setaria italica* (Si) and (A) *Panicum virgatum* (Pv), (B) *Sorghum bicolor* (Sb), (C) *Zea mays* (Zm). Each block represents individual chromosome and the orthologous genomic regions are marked with red lines.

in dehydration stress library, relatively higher expression of *SiWRKY004, SiWRKY024, SiWRKY046,* and *SiWRKY068* was observed in stressed sample as compared to control. Downregulation of a few genes viz., *SiWRKY60, SiWRKY61,* etc. was also seen (**Figure 6**). Expression patterns of *SvWRKY* genes in pooled RNA isolated from samples across three developmental stages, namely seed germination, vegetative growth, and reproduction in different tissues including leaf, stem, node, crown, root, spikelet, floret, and seed tissues showed higher transcript abundance of *SvWRKY001, SvWRKY027, SvWRKY033, SvWRKY037,* and *SvWRKY039*. However, the majority of *SvWRKY* genes showed no or negligible expression (**Figure 6**).

Expression Pattern of *SiWRKY* Genes in Response to Stress and Hormone Treatments

To investigate the expression of *SiWRKY* genes in response to abiotic stress and hormone treatments, 12 genes were selected based on their differential expression pattern in RNA-seq libraries of four tissues and under drought stress (**Figure 6**). Additionally, the genes were resourced from the nine chromosomes of *S. italica* in order to provide a genome-wide coverage (**Figure 2**). The expression profiles of the 12 candidate genes were examined during early (1 h) and late (24 h) stages of dehydration, salinity, ABA, SA, and MeJA

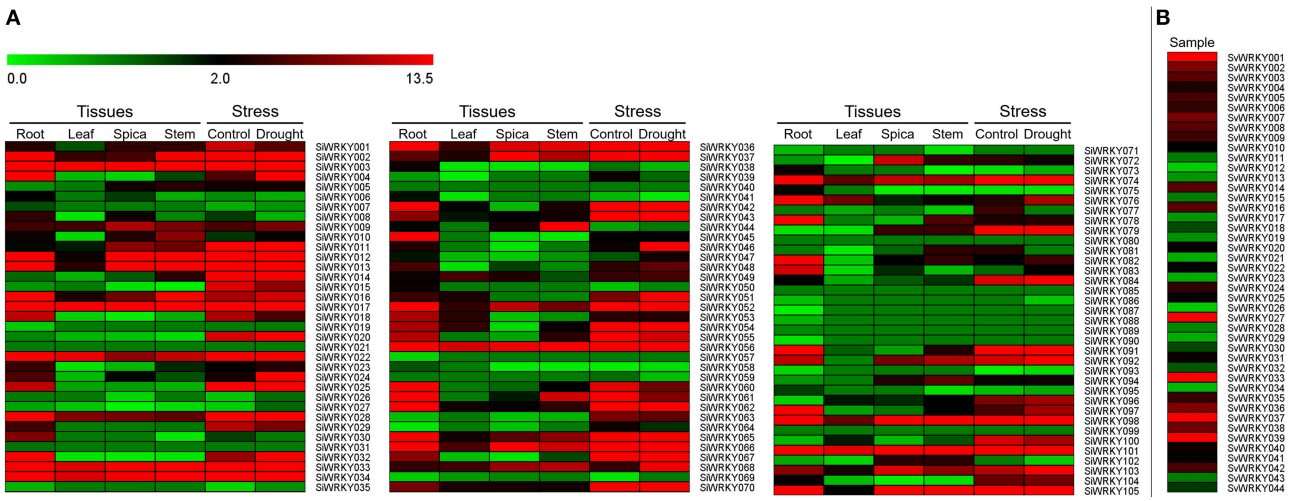


FIGURE 6 | Heatmap showing the expression pattern of (A) *SiWRKY* genes in four tissues, namely root, leaf, spica, stem, and dehydration stress library of *Setaria italica*, and (B) *SvWRKY* genes in pooled transcriptome of *S. viridis* samples across three developmental stages, namely seed germination, vegetative growth, and reproduction in different tissues including leaf, stem, node, crown, root, spikelet, floret, and seed tissues. The colored bar at top left represents relative expression value, where 0.0, 2.0, and 13.5 denotes low, medium, and high expression, respectively.

treatments. The relative transcript abundance assessed through qRT-PCR showed a differential expression pattern of all the *SiWRKY* genes (**Figure 7**). Few genes including *SiWRKY003, SiWRKY017, SiWRKY033, SiWRKY042,* and *SiWRKY056* did not show any significant expression throughout the experiments, whereas *SiWRKY034* was highly expressed only during the late phase of SA treatment. During dehydration and salinity stress, *SiWRKY064, SiWRKY066, SiWRKY074,* and *SiWRKY082* were found to be upregulated at both the time points, in which, significant upregulation of *SiWRKY064* and *SiWRKY082* at late phase of salinity stress, and *SiWRKY066* and *SiWRKY074* at both the phases of dehydration were observed (Supplementary Figure S2). In case of hormone treatments, all these four genes were found to be highly expressed during late phase. In addition to these, *SiWRKY101* was observed to be upregulated during late phase of dehydration and MeJA treatment. The fold expression of *SiWRKY064* and *SiWRKY082* were significantly higher during both the phases of stress and at late phase of hormone treatments, suggesting their potential as candidates for functional characterization.

DISCUSSION

WRKY transcription factors have been reported to play multiple roles in regulating normal growth and development, and in response to environmental stimuli in plants (Rushton et al., 2010). This class of TFs are one of the well-studied proteins whose mechanism of action, autoregulation and cross-regulation in signaling and evolution have been reported (Bakshi and Oelmüller, 2014). Though initially considered as vital players of biotic stress tolerance, WRKY TFs were later discovered to play significant roles in conferring tolerance to diverse abiotic stresses including salinity (Jiang and Yu, 2009; Chen et al., 2010), drought, heat (Li et al., 2009, 2011), cold (Zou et al., 2010), H_2O_2(Song et al., 2009), ozone oxidative stress (Jiang and Deyholos, 2009), UV radiation (Jiang and Deyholos, 2009), sugar starvation (Song et al., 2010), phosphate depreviation (Chen et al., 2009) and wounding (Shang et al., 2010). Further, numerous reports have indicated the response of a single WRKY gene to several stress factors, thus highlighting the diverse regulatory role of WRKY proteins in stress response (Wei et al., 2008; Jiang and Deyholos, 2009; Li et al., 2009, 2011; Chen et al., 2012). The expression of WRKY TFs in response to broad-spectrum abiotic stresses suggests their participation in regulation of signaling mechanisms associated with transcriptional reprogramming during environmental stress. Genome-wide identification of WRKY TFs has been performed in many crop plants and their expression profiling in response to various abiotic stresses have been studied.

Recently, C_4 crops are gaining momentum in stress biology research owing to their improved water-use efficiency and nitrogen-use efficiency (Sadras et al., 2011). C_4 photosynthesis also confers tolerance to crops against abiotic stress, particularly to drought and heat (Sadras et al., 2011). *Setaria italica* and its wild progenitor *S. viridis*, have recently been identified as model crops for studying C_4 photosynthesis and abiotic stress tolerance due to their small genome, short life span, inbreeding nature and ability to withstand adverse environmental conditions (Brutnell et al., 2010; Wang et al., 2011; Diao et al., 2014; Muthamilarasan and Prasad, 2015). Furthermore, both the crops share maximum genetic synteny with various biofuel grasses such as switchgrass, napiergrass and pearl millet and therefore, *S. italica* and *S. viridis* have also been regarded as model systems for bioenergy research (Li and Brutnell, 2011; Lata et al., 2013; Brutnell et al., 2015; Muthamilarasan and Prasad, 2015) and

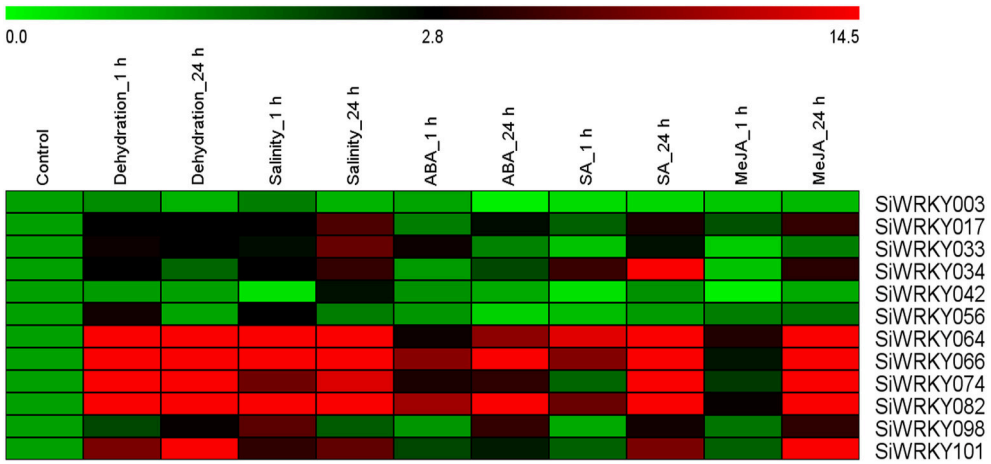


FIGURE 7 | Relative transcript levels of candidate *SiWRKY* genes in response to treatment with stresses and hormones. Expression profiles of *SiWRKY* genes in *Setaria italica* seedlings exposed to dehydration, salinity, abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA) for two time points (1 h and 24 h) were analyzed by qRT-PCR and presented as heatmap. The scale bar at the top represents relative expression value. Relative fold expression values are presented as bar diagram in Supplementary Figure S2.

nutrition studies (Muthamilarasan et al., in press). Therefore, in view of the importance of *S. italica* and *S. viridis* in abiotic stress biology, the present investigation was performed to identify and characterize WRKY TFs using computational tools and examine their expression patterns in response to abiotic stress and hormone treatments.

In this study, 105 *WRKY* genes from *S. italica* genome (*SiWRKY*) and 44 from *S. viridis* transcriptome (*SvWRKY*) were identified. Comparison of the number of *WRKY* genes in *S. italica* with other sequenced grass genomes namely maize (163 genes), sorghum (110 genes) and rice (*O. sativa* subsp. *indica*; 109 genes) has shown that *S. italica* has comparatively lesser number of genes. However, *Brachypodium* has a minimum of 87 genes, owing to its smaller genome size. Similar comparisons of the number of *WRKY* genes among all the sequenced plants showed that soybean has the maximum number of *WRKY* genes (233), followed by cotton (219), whereas the primitive plants of Chlorophyta have one to two genes. Interestingly, the genome of *Physcomitrella patens* has 41 *WRKY* genes. Only 44 WRKY proteins were identified from the transcriptome of *S. viridis* due to the non-availability of genome sequence information and this number is expected to increase when the whole genome sequence is released in public domain. Examining the protein properties of SiWRKY and SvWRKY TFs revealed large differences in amino acid length, molecular weight and isoelectric point of these proteins, and these variations could be attributed to the presence of putative novel variants, which needs to be validated.

Sequence alignment and phylogenetic analysis of SiWRKY and SvWRKY proteins classified them into three major groups (I, II, and III) based on the WRKY domain and conserved zinc finger-like motif. In addition, a distinct class of WRKY proteins classified as group IV has been identified with two members of SiWRKY and four members of SvWRKY. These proteins possess only the WRKY domain and not the zinc finger-like motif. Sequence alignment and phylogenetic analysis showed that a majority of SiWRKY proteins belong to group II (54) followed by group III (39) and group I (10). Similar case was observed in SvWRKY proteins, where a maximum of 23 proteins belong to group II, 11 to group III, and 6 to group I. This is in agreement with the distribution reported in maize (Wei et al., 2012). The position of WRKY domain and associated zinc finger-like structures in SiWRKY and SvWRKY was investigated through multiple sequence alignment and domain analyses tools, namely HMMSCAN and ScanProsite. The analyses revealed that the distribution of phylogenetic groups corresponds well with the domain structures and sequence conservation. It showed three interesting observations: (i) two proteins of SiWRKY (SiWRKY044 and SiWRKY063) and four SvWRKY proteins (SvWRKY005, SvWRKY007, SvWRKY008, and SvWRKY011) possess only WRKY domain and lack zinc finger-like structure. (ii) two additional domains, namely NB-ARC and DUF were present in SiWRKY011 and SvWRKY031, and SiWRKY011 and SiWRKY096, respectively. and (iii) SvWRKY004 has three WRKY domains followed by zinc finger-like structures.

Physical mapping of *SiWRKY* genes on the nine chromosomes of *S. italica* showed that maximum number of genes were present on chromosomes 5 (22 genes; ∼21%) and 3 (19 genes; ∼18%), and a minimum of 5 genes each (∼5%) were present on chromosomes 4 and 6. The maximum number of genes on chromosomes 5 and 3 could be attributed to the occurrence of segmental duplication, as revealed by MCScanX analysis. Eight genes in these chromosomes were segmentally duplicated, and in addition, 10 gene pairs were identified to be tandem duplicates. The Ks dating and estimation of Ka/Ks ratios of duplicated gene pairs showed that these genes underwent intense purifying selection. The time of duplication of tandemly and segmentally duplicated gene pairs were estimated as ∼26 and ∼23 million years ago (mya), which were in congruence with the whole

genome tandem and segmental duplication reported to have occurred around 25–27 and 18–22 mya (Zhang et al., 2012). This also demonstrates the effect of chromosomal duplication events in shaping the distribution and organization of *WRKY* genes in *S. italica* genome.

Comparative mapping of *SiWRKY* genes and SvWRKY proteins on the switchgrass, sorghum and maize databases was performed to understand the orthologous relationships between the grass genomes. *SiWRKY* genes showed maximum synteny with maize (∼57%), followed by switchgrass (∼54%) and sorghum (∼40%), whereas SvWRKY proteins showed maximum orthology with switchgrass (∼70%), followed by maize (∼55%) and sorghum (∼45%). Though higher percentage of orthology was expected between *Setaria* and switchgrass owing to their extensive gene-level synteny, *SiWRKY* genes were found to be more homologous to maize. However, SvWRKY revealed the syntenic pattern with respect to decrease in synteny with increase in phylogenetic distance, between these crops. Estimation of time of divergence of orthologous gene pairs revealed that *S. italica* and switchgrass *WRKY* genes diverged around 4.7 mya, whereas divergence between *S. italica WRKY* genes and those of maize and sorghum occurred around 27 and 27.5 mya, respectively. Similarly, *S. viridis* and switchgrass *WRKY* genes were predicted to have diverged around 4.7 mya, while *S. viridis* and maize, and sorghum *WRKY* genes diverged around 26.8 and 27.8 mya, respectively. These findings are in accordance with the period of divergence of Poaceae members as reported by Zhang et al. (2012). The comparative map constructed using orthologous *WRKY* genes demonstrated the frequent occurrence of nested chromosomal fusions in the grass genomes. Further, this comparative map would be useful in choosing candidate *WRKY* genes from these genomes for functional characterization.

The publicly available transcriptome data of four different tissues and dehydration stress library of *S. italica*, and pooled tissue library of *S. viridis* were processed using in-house perl scripts and computational tools to derive the RPKM expression values for *SiWRKY* and *SvWRKY* genes. The heatmap generated using these expression values showed tissue-specific and condition-specific expression patterns of *WRKY* genes. Relatively higher expression of few genes in all the tissues, or in any one tissue or only during dehydration stress suggested the multifaceted roles of *WRKY* genes in diverse molecular and physiological activities. This data could be exploited for selecting candidate genes showing distinct expression pattern for delineating their functional roles. Based on this heatmap and physical map data, twelve candidate *SiWRKY* genes were chosen for expression profiling under different abiotic stress (dehydration and salinity) and hormone (ABA, SA, and MeJA) treatments (at two time points). These genes showed differential expression pattern in the four tissues (root, stem, leaf, and spica) and drought stress library as deduced using RNA-seq expression data. Further, the genes were also chosen to represent all the nine chromosomes of foxtail millet, to provide a representative genome-wide coverage. The qRT-PCR analysis of these genes showed their differential expression patterns during exposure to stresses and hormones, and this suggested the putative involvement of *WRKY* genes in stress response mechanism and their regulation in response to phytohormones. Overall, the qRT-PCR analysis revealed that *SiWRKY066* and *SiWRKY082* could be potential candidates for further functional characterization and for delineating their roles in abiotic stress signaling.

CONCLUSIONS

With the advancement of high-throughput technologies and strategies, including physiology, chemical genetics, and computational approaches, the role of WRKY TFs in signal transduction and gene regulation has been well studied in all the major crops and tree species. However, no such study on WRKY TFs has been conducted in *S. italica* and *S. viridis*, which are now considered as model systems for investigating C_4 photosynthesis, biofuel traits and abiotic stress tolerance mechanisms. Considering the importance of these crops and WRKY TFs, the present study used comprehensive computational approaches to identify and characterize WRKY gene family members. The identified members were used for construction of a physical map, duplication studies, phylogenetic analysis, gene ontology annotation, promoter analysis, comparative mapping, and evolutionary studies. In addition, *in silico* expression profiling of *SiWRKY* and *SvWRKY* genes were performed to understand the expression pattern of these genes in different tissues and dehydration stress conditions. Expression profiling of candidate *SiWRKY* genes under abiotic stress and hormone treatments showed differential expression pattern of these genes, thus providing an indication of their regulatory functions under stress conditions.

AUTHOR CONTRIBUTIONS

MP conceived and designed the experiments. MM, VB, RK, JJ, SS, KN performed the experiments. MM analyzed the results and wrote the manuscript. MP approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00910

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OPINION
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isomiRs: Increasing Evidences of isomiRs Complexity in Plant Stress Functional Biology

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miRNAs AND isomiRs: RISE OF COMPLEX miRNA ISOFORMS

How and by which mechanism plants control post-transcriptional regulation? Reinhart et al. (2002) pinpointed the role of non-coding, small endogenous regulatory microRNAs (miRNAs) as the regulatory switch that controls the post-transcriptional regulation. Since then, several families of regulatory miRNAs including artificial miRNAs (Sablok et al., 2011) have been discovered and have shown to play a key role as system wide regulators, which are activated in response to several biotic and abiotic stress adaptations (Zhang, 2015), as well as regulators of plant development (Reinhart et al., 2002; Sun, 2012). Several reports on the identification and characterization of miRNAs have been published so far (for a review, see Budak et al., 2015), which established miRNAs as major post-transcriptional regulatory factors in plants system biology. Endogenous miRNAs perform regulatory role by binding specifically to the 3′-UTR of mRNAs, which in turns triggers the degradation of the targeted transcript, thereby leading to the shutdown of protein translation. Endogenous miRNAs originate from the stem of a single-stranded stem-loop precursor as a miRNA/miRNA* duplex with an approximately 2-nt 3′-end overhang. However, not all small RNAs expressed from a stem-loop are necessarily either the precise miRNA or precise miRNA*.

The advent of the high throughput small RNA sequencing approaches and concurrent development in the identification of miRNAs using advanced algorithmic-based computational approaches has led to the discovery of a new class of regulatory RNAs called isomiRs, which are canonical variants of miRNAs (Morin et al., 2008b). The biogenesis of these canonical miRNA sequence variants in plants might be due to the imprecise cleavage activity by the Rnase III enzyme or due to the post-transcriptional RNA editing events (Hackenberg et al., 2013) or by nucleotidyl transferases (Wyman et al., 2011). Morin et al. (2008a) hypothesized the origin of isomiRs as cleavage site variability in the pre-miRNA hairpin, which is cleaved by either DICER1 or DROSHA (Morin et al., 2008b).

Taking into account the length and nucleotide variation along with the non-templated additions (NTAs) such as adenylation and uridylation at the 3′-end with non-random functionality (Wyman et al., 2011), isomiRs can be classified into 5′ isomiR, 3′ isomiR, and polymorphic isomiRs (Neilsen et al., 2012; Jeong et al., 2013). Global analysis of the isomiRs and canonical miRNAs has shown uridine to be the preferential nucleotide at 5′- and 3′-ends. This has led to the conclusion that isomiRs commonly feature "U–C" at the 3′-end of the isomiRs as opposed to the addition of C at the 3′-end of the plant miRNAs (Zhang et al., 2013). However, isomiRs displayed a frequent truncation of the cytodine from both the ends, presenting a new complex cytodine balance in isomiRs (Xie et al., 2015), which suggests that uridylation plays a role in avoiding degradation. The primary sequences of identified isomiRs so far differ from the indexed high content miRBase miRNAs

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in their 5′-end (changing the "seed" region and suggesting a different target molecule) or in their 3′-end or both. The question that why this variant occurs is still intriguing and challenging, and needs to be addressed. However, recent reports have started suggesting the target cleavage efficiency of isomiRs, thus establishing them as another class of regulatory functional RNAs.

isomiRs IDENTIFICATION AND INTER-PLAY IN PLANT STRESS FUNCTIONAL GENOMICS

On the basis of accumulated knowledge about isomiRs, as well as their abundance and involvement in target cleavage (Ahmed et al., 2014), several algorithms either as web-based or standalone tool have been developed, using read mapping approaches to delineate the repertoire of isomiRs abundance and expression (**Table 1**). Previously, we developed the first web-based tool, isomiRex (bioinfo1.uni-plovdiv.bg/isomiRex/), which provides the high-throughput classification and differential expression of isomiRs and supports a broad range of organisms including plants (Sablok et al., 2013). Following isomiRex, several tools have been developed such as IsomiRage (Muller et al., 2014) and isomiRID (de Oliveira et al., 2013), which can distinguish isomiRs using template-based and non-template-based predictions. Since isomiRs can be a result of the adenylation or uridylation events, IsomiRage (Muller et al., 2014) implements algorithmic identification and classification of functionally relevant isomiRs based on their adenylation, uridylation, and other respective biological events (Muller et al., 2014).

Whether isomiRs are functional or just variants has been widely discussed and debated since the first reported evidence of isomiRs in *Oryza sativa* (Morin et al., 2008b). Although their functional role and capabilities are still unknown, isomiRs have the potential to extend the canonical miRNA regulatory network. This adds credence to the hypothesis that "mature miRNAs" cannot be related to only a single individual sequence and that a single precursor may cleave more than one functional product. Profiling of tissue-specific small RNAs in Peach (*Prunus Persica L.*) revealed an abundance of tissue-specific isomiRs (392 isomiRs—miRNA and miRNA*-related—corresponding to 26 putative miRNA coding loci), which supports the hypothesis that the origin of isomiRs is not just a random functional event in plant post-transcriptional machinery (Colaiacovo et al., 2012). Realizing the potential of isomiRs and their ability to cleave targets (Ahmed et al., 2014), several studies started exploring their functional role in plants. In *Phaseolus vulgaris,* as many as 57 functional isomiRs spanning across 25 families have been identified in nodule development and phasiRNAs generation (Formey et al., 2015).

TABLE 1 | Recently developed classification tools for identifying isomiRs.

Tool	Address	Type	References
SeqCluster	https://github.com/lpantano/seqbuster	Standalone	Pantano et al., 2011
miRSeqNovel	http://sourceforge.net/projects/mirseq/files	Standalone	Qian et al., 2012
isomiRID	http://www.ufrgs.br/RNAi/isomiRID/	Standalone	de Oliveira et al., 2013
isomiRex	bioinfo1.uni-plovdiv.bg/isomiRex/	Web-based	Sablok et al., 2013
miRspring	http://mirspring.victorchang.edu.au	Standalone	Humphreys and Suter, 2013
IsomiRage	http://cru.genomics.iit.it/Isomirage/	Standalone	Muller et al., 2014
sRNAtoolbox	http://bioinfo5.ugr.es/srnatoolbox	Standalone	Rueda et al., 2015

Alongside the discovery of isomiRs and their ability to cleave targets, studies pertaining to the relative differential expression of isomiRs have also been conducted. Jeong et al. (2013) reported strong expression of isomiRs with 5′ variations in *A. thaliana* using Parallel Analysis of RNA Ends (PARE-Seq), co-immunoprecipitation, and *ARGONAUTE* (AGO) loading data (Jeong et al., 2013). Using a reversed framework approach and degradome analysis, Shao et al. (2015) revealed the higher expression of the isomiRs as compared to their canonical miRNAs in *Oryza sativa* (Shao et al., 2015). Interestingly, they showed high abundance of iso-osa-miR528-5p in AGO1 complexes as compared to the osa-miR528-5p (1315 rpm vs. 165.72 rpm) (Shao et al., 2015). Ehya et al. (2013) observed differential expression of several isomiRs of canonical miRNAs involved in auxin signaling, such as miR160 (for miR160*), miR166, and miR167 suggesting miRNA-mediated auxin signaling, which might regulate the response of the Mexican Lime tree to Phytoplasma (Ehya et al., 2013). Taking these studies into account, it can be concluded that isomiRs are not random events and might play a key role in increasing the miRNAome complexity in plants.

In addition to the developmental and tissue-specific functions (Colaiacovo et al., 2012), isomiRs play an important role in modulating and regulating the miRNAome in biotic and abiotic stress conditions. However, as compared with the widely demonstrated regulatory roles of the differentially expressed, conserved and novel miRNAs in biotic and abiotic stress conditions in plants (Zhang, 2015), limited reports have shown the differential expression of the isomiRs (Baev et al., 2014). Nonetheless, it is worthwhile to mention that recent reports in stress conditions have presented the evidences of isomiRs expression along with the expression of the canonical miRNAs in model plants. The information gleaned from the recent phosphorus (P) deficiency stress studies in *Hordeum vulgare* (Barley) have highlighted the up-regulated isomiRs of miR399 and miR827 family under P deficiency significantly (Hackenberg et al., 2013). Understanding the post-transcriptional regulation of the signaling pathways during temperature stress response is critical in dealing global climate change. Baev et al. (2014) recently demonstrated the differential regulation of the miR160c isomiRs in high- and low-temperature conditions in *Arabidopsis thaliana* with the identified isomiRs showing substantially higher expression as compared to the canonical miRNAs. Such regulated isomiRs under P deficiency and high- and low-temperature stress suggest that isomiRs play a role in regulating the miRNAome in stress-induced biological gene regulation.

In addition to the relative recent increase in the knowledge gain about isomiRs, their biogenesis and expression, significant efforts have been made to understand the role of the functional target cleavage capacity of the isomiRs. Since reported variation in isomiRs sequences occurs at the 3′- or 5′-ends, they could potentially bind to a different repertoire of targets relative to their mature reference counterparts. The higher expression of the isomiRs as compared to the canonical mature miRNA may affect the target cleavage efficiency of that particular miRNA. Using PARE-Seq, Jeong et al. (2013) demonstrated the differential target cleavage capacity of miR161.1 isomiRs. In model plant *A. thaliana*, differential binding capacities of the isomiRs as compared to the canonical miRNAs, as well as high efficiency of the isomiRs in target cleavage as compared to the canonical miRNAs, have been recently demonstrated (Ahmed et al., 2014). In addition, higher target prediction efficiency using coupled combinations of miRNAs and isomiRs has been observed in *A. thaliana* (Ahmed et al., 2014). Although less evidence have been shown toward the functional gain or loss of the isomiRs in plants, they have been confirmed experimentally in other models systems including humans, thus representing the role of isomiRs as evolutionary and functionally important variants (Tan et al., 2014; Cammaerts et al., 2015). In conclusion, isomiRs act as canonical partners to miRNAs in regulating developmental and stress-associated post-transcriptional responses in stress. Realizing the emerging occurrences and the read-based support for the detection of the isomiRs, it can be presumed that post-transcriptionally isomiRs and canonical miRNAs act synergistically to regulate the developmental and signaling pathways in plants.

AUTHORS CONTRIBUTIONS

GS and AS conceived the idea, GS drafted the opinion article, PS, VB and PR provided revisions to the article.

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MINI REVIEW
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Small RNA mediated regulation of seed germination

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Mature seeds of most of the higher plants harbor dormant embryos and go through the complex process of germination under favorable environmental conditions. The germination process involves dynamic physiological, cellular and metabolic events that are controlled by the interplay of several gene products and different phytohormones. The small non-coding RNAs comprise key regulatory modules in the process of seed dormancy and germination. Recent studies have implicated the small RNAs in plant growth in correlation with various plant physiological processes including hormone signaling and stress response. In this review we provide a brief overview of the regulation of seed germination or dormancy while emphasizing on the current understanding of the role of small RNAs in this regard. We have also highlighted specific examples of stress responsive small RNAs in seed germination and discussed their future potential.

Keywords: seed germination, seed, small RNA, miRNA, stress response, seed dormancy

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Introduction

The seeds of higher plants contain the dormant embryos, as miniature new plants, along with adequate food reserves to sustain the growing seedlings until they establish themselves as self-sufficient, autotrophic organisms (**Figure 1A**). Germination is one of the most important physiological process of a seed which begins with the uptake of water by the quiescent dry seed and is completed when a part of the embryo, usually a radical oozes out of the seed coat (Bewley, 1997). Seed dormancy is regarded as the temporary failure or block of a viable seed to complete germination under seemingly unfavorable conditions and is an adaptive feature for optimizing the timing of germination (Bewley, 1997). The dynamic process of seed germination is triphasic (**Figure 1B**) and involves a complex coordination of many physiological, cellular and metabolic events (Bewley, 1997; Weitbrecht et al., 2011). Phase-I includes rapid leakage of solutes which paves the way for respiration and protein synthesis. Phase-II represents a plateau stage where new mRNAs and proteins are synthesized. There is also an accumulation of the mitochondrion to support the energy requirements at this stage. During phase-III, radicle cells elongate and divide. This is also the stage of rapid DNA synthesis and replication together with the mobilization of stored reserves (Bewley, 1997).

Various environmental factors such as light, temperature, moisture, oxygen, soil, humidity, stress etc and some physiological factors such as viability of seeds, thickness of seed coat, dormancy period etc also play vital role in seed germination stages (Martin et al., 2010; Weitbrecht et al., 2011). Several studies have implicated that the interactions between different phytohormones such as abscisic acid (ABA), gibberellins (GAs), ethylene, brassinosteroids (BRs), auxin, and cytokinins (CKs) play a key role in regulating the interconnected molecular processes that control dormancy release and activation of the stages of seed germination (Liu et al., 2007; Finkelstein et al., 2008). The activity of plant hormones needs to be precisely regulated, since some phytohormones exert crucial but

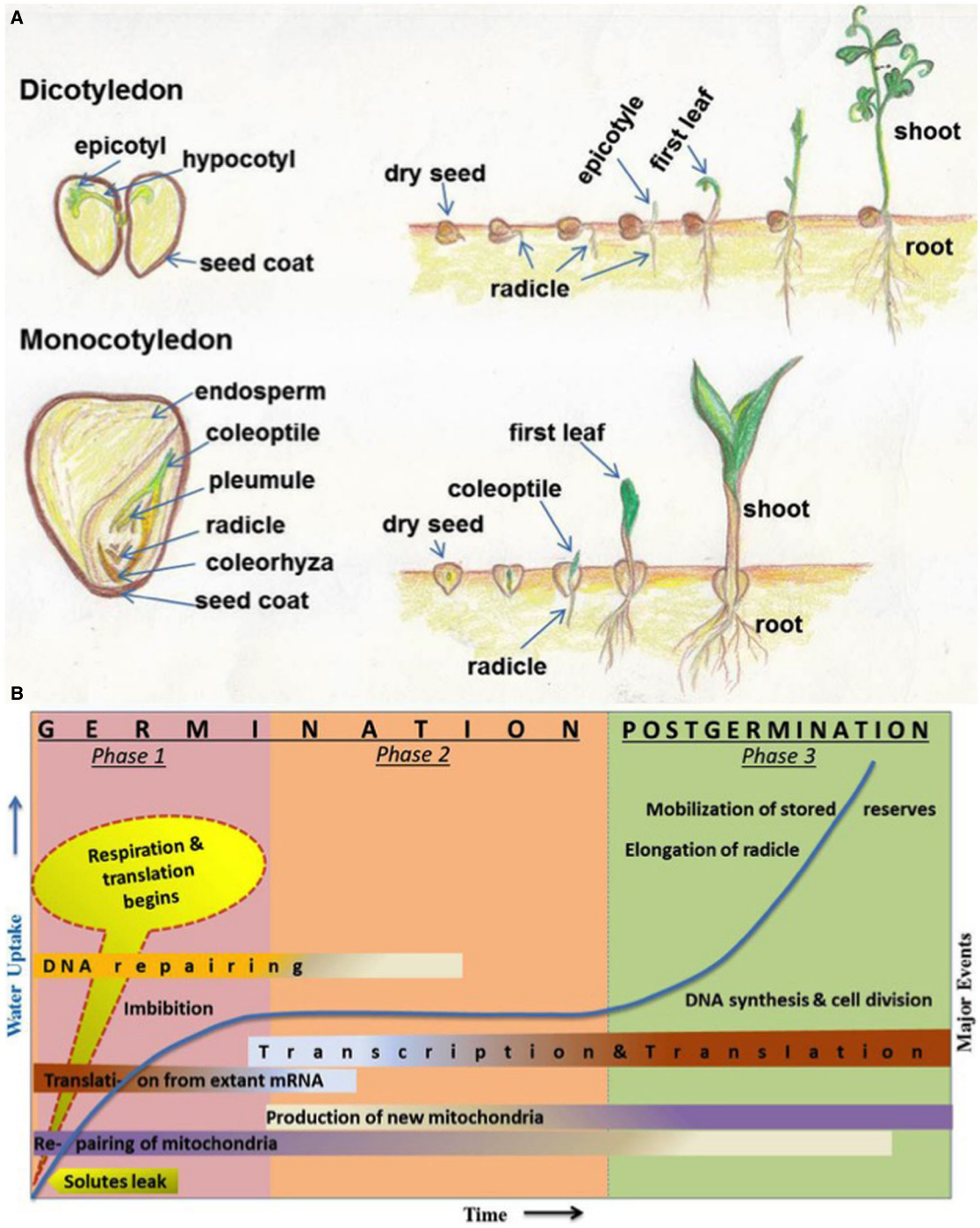


FIGURE 1 | (A) Schematic representation (hand drawn by SS) of different parts of seeds and seed germination stages. Seeds and germination stages of dicotyledonous (chickpea) and monocotyledonous (maize) plants have been shown in upper and lower panels, respectively. **(B)** Major events associated with seed germination and post-germinative growth phases. Germination stages are represented by phase 1 and phase 2; postgermination events includes phase 3. The time (x-axis) for events varies from several hours to many weeks, depending on different plant species and germination conditions. Uptake of water and related increase in biomass is indicated in y-axis and shown in line graph during three phases. Some events (such as DNA repairing, transcription, translation, and mitochondria production etc.) are spread over more than one phases and indicated with shaded color; dark colors indicate more activity and light colors indicate less activity. This figure has been reproduced with modification, after written permission of the corresponding author (Prof. J. D. Bewley) and the original publisher, American Society of Plant Biologists (ASPB).

contrasting influence on the process. ABA is a positive regulator of dormancy and its maintenance, while it is a negative regulator of germination (Finkelstein et al., 2008). The absence of, or insensitivity to ABA during seed development results in the production of viviparous or precociously germinating seeds as exemplified by maize *viviparous* (*vp*), tomato *sitiens* (*sit*), and *Arabidopsis ABA-deficient* (*aba*), and *ABA-insensitive (abi*) mutants (Finkelstein et al., 2008). GA releases dormancy and its role is analogous to that of Ethylene and BR in promoting germination by counteracting ABA effects. Recently, the crosstalk between ABA and auxin has also been highlighted (Finkelstein et al., 2008).

The discovery of small non-coding RNAs (of 19–24 nucleotides length) has added a new dimension to the understanding of the regulation of cellular environment (Bartel, 2004; Axtell et al., 2007). They have been shown to play diverse roles in growth, development, morphogenesis and stress responses of both plants and animals (Chen, 2012; Kamthan et al., 2015). The functional small RNAs are produced from double stranded RNA precursors through the activity of RNA-dependent RNA Polymerase (RDR), DICER-like (DCL), and ARGONAUTE (AGO) proteins (Allen et al., 2005; Mallory et al., 2008; Axtell, 2013). There are two major classes of small non-coding RNAs—short interfering RNAs (siRNAs) and microRNAs (miRNAs) that negatively regulate their target genes by binding to the complementary sequences. At the transcriptional level, the small RNAs may be involved in chromatin remodeling (Huettel et al., 2007; Pontier et al., 2012; Xie and Yu, 2015) while at the post-transcriptional level, depending upon the nature of homology they can bring about the cleavage of the target mRNA (Rajagopalan et al., 2006; Vaucheret, 2006) or block their translation (Poethig et al., 2006; Vaucheret, 2006; Bartel, 2009). The biosynthesis and function of many of these small RNA genes are also regulated by different plant hormones and environmental stress (Mallory et al., 2005; Reyes and Chua, 2007; Sunkar et al., 2007; Shukla et al., 2008; Martin et al., 2010; Khraiwesh et al., 2011; Sanan-Mishra et al., 2013).

Biogenesis of miRNA and ta-siRNA

miRNA biogenesis is a multistep process that is mainly resistricted to the nucleus in plants. Briefly, the miRNA gene is transcribed into a capped and poly-adenylated primary miRNA (pri-miRNA) by enzyme RNA polymerase II (Chen, 2012; Axtell, 2013). The pri-miRNA is processed to precursor miRNA (pre-miRNA), of around 70–100 nt long, by DCL protein (Axtell, 2013). The pre-miRNA is further processed to form miRNA and miRNA* duplex by the activity of DCL protein. The duplex is then methylated at the 2′OH of the 3′nucleotide end by HEN1 (Allen et al., 2005; Chen, 2009) and transported to the cytoplasm.

One strand of the duplex is loaded into RISC (RNA-induced silencing complex) containing AGO1. The strand selection widely depends on the relative stability of the two ends of the duplex (Axtell, 2013). It is observed that generally the strand whose 5′ end is comparatively loose, gets incorporated into the RISC (Allen et al., 2005; Axtell, 2013). The RISC complex containing the miRNA identifies its target transcripts based on perfect or nearly perfect sequence complementarity. In plants the stringency of target recognition is very high and the target transcripts are normally cleaved, however, the central mismatches in the miRNA:mRNA pair direct the inhibition of translation (Allen et al., 2005; Axtell, 2013).

Recently, the ta-siRNA (trans-acting small interferring RNAs) have also been implicated in plant development thereby attracting major research interest for many plant biologists (Nogueira et al., 2006; Axtell, 2013). ta-siRNAs are generated from TAS (Trans-Acting SiRNA locus) gene derived non-coding transcripts through specific miRNA guided cleavage. The cleaved precursors of ta-siRNAs are bounded and stabilized by SUPPRESSOR of GENE SILENCING3 (SGS3) and further synthesized into double stranded RNAs by RDR6 (Chen, 2009; Allen and Howell, 2010; Axtell, 2013). The double stranded RNAs are cleaved several times by DCL4 from the miRNA mediated cleavage sites, so that 21-nt long phased ta-siRNAs are produced. Similar to miRNAs, the ta-siRNAs are incorporated into RISCs, where they cleave the target mRNAs or repress translation (Allen et al., 2005; Allen and Howell, 2010). There are four families of TAS gene in *Arabidopsis*, namely *TAS1*, *TAS2*, *TAS3*, *TAS4* (Rajagopalan et al., 2006; Allen and Howell, 2010). For the initial processing *TAS1* and *2* require miR173 whereas *TAS3* and *TAS4* require miR390 and miR828, respectively for initial processing (Chen, 2009; Allen and Howell, 2010; Axtell, 2013).

The Role of miRNAs and ta-siRNAs in Plant Growth and Development

The miRNAs constitute a major class that play important and diverse roles in regulation of various aspects of plant development (Sanan-Mishra and Mukherjee, 2007; Chen, 2012; Sharma et al., 2015). The classical examples include regulation of *CUC1/CUC2* and *NAC1* transcripts by miR164 to affect reproductive and root development (Guo et al., 2005); determination of abaxial/adaxial leaf polarity and root development by miR166/165 mediated control of *Class III HD-ZIP* transcription factor mRNAs (Chen, 2012; Barik et al., 2014; Singh et al., 2014) and the regulation of flower development in *Arabidopsis thaliana* by miR172 targeted AP2 and other mRNAs (Wollmann et al., 2010). The function of miRs has been shown to be affected by hormones and stress responses (Mallory et al., 2005; Liu et al., 2007; Reyes and Chua, 2007)

The miRNA mediated, ta-siRNA production is also significantly altered in drought, salinity and hypoxia stresses, besides their regulation by auxin and other hormones (Moldovan et al., 2009; Matsui et al., 2014). This is evident by *TAS3* derived ta-siR-ARF that target different *AUXIN RESPONSE FACTOR2, 3 and 4 (ARF2,3,4)* and regulate various aspects of plant development such as vegetative to reproductive phase changes, leaf polarity and lateral root development in *Arabidopsis* (Peragine et al., 2004; Chitwood et al., 2007; Allen and Howell, 2010; Marin et al., 2010). Mutations in ta-siRNA biogenesis pathway lead to the upregulation of target mRNAs and affect the aforesaid aspects of plant development. The rice and maize ta-siRNA biogenesis mutants have been shown to have severely affected shoot and leaf development (Itoh et al., 2006; Nogueira et al., 2006; Nagasaki et al., 2007; Douglas et al., 2010). DCL4 is

TABLE 1 | List of miRNAs that are involved in seed germination and dormancy.

miRNAs	Targets of miRNA	Target regulation		Seed germination related function	References
		Up-regulation	Down-regulation		
miR395	ATP Sulfurylases (*APS1*, *APS3*, *APS4*); Sulfate transporter (*SULTR2:1*)	*APS1*, *APS4*, *SULTR2:1* by miR395c	*APS1*, *APS4*, *SULTR2:1* by miR395c and *APS3 by miR395e*	Regulatory effects on seed germination under salt and dehydration stress	Kim et al. (2010b)
miR402	DEMETER-LIKE protein 3 *(DML3*)		↓	Regulatory effect on seed germination and seedling growth under salt, dehydration and cold stress	Kim et al. (2010a)
miR417	Unknown	Unknown	Unknown	Plays a role as a negative regulator of seed germination in Arabidopsis thaliana under salt stress condition.	Jung and Kang (2007)
miR160	*ARF10*, *ARF16*, *ARF17*		↓	*ARF10* mutant show up regulation of ABA responsive genes during germination	Liu et al. (2007)
miR159	*MYB33*, *MYB65*, *MYB101*	*MYB33*, *MYB101*		*MYB33* and *MYB101* are the positive regulators of ABA signaling during seed dormancy and germination	Reyes and Chua (2007)
miR165/166	*PHB*, *PHV*, *REV* etc.	Unknown	Unknown	Maintain the auxin signal during seed development and maturation.	Huang et al. (2013)
miR164	*NAC1*, *CUC1/CUC2*	Unknown	Unknown	Maintain the auxin signal during seed development and maturation.	Huang et al. (2013)
miR167	*ARF6*, *ARF8*	Unknown	Unknown	Maintain the auxin signal during seed development and maturation.	
miR158	Unknown	Unknown	Unknown	Seed development and maturation.	
miR156	*SPL* 3, 4, 5		↓	Seed development and maturation.	Huang et al. (2013); Li et al. (2013)
miR172	*AP2*	↑		Seed development and maturation.	

The first five miRNAs in the gray shaded region of the table are also involved in mediating the stress response signals during germination.

suggested to redundantly regulate processing of some miRNAs, besides role in ta-siRNA biogenesis (Rajagopalan et al., 2006). ABA signaling is shown to be, at least partially, affect RDR6 accumulation (Zhang et al., 2013). Although ta-siRNA has not directly been implicated in seed germination, their cross talk with miRNA and hormone signaling in feed-back loops (Marin et al., 2010; Chen, 2012) as well as role in seed development (Zhang et al., 2013) indicate their potential function in seed maturation and germination. This remains to be an interesting area to be explored in the complex process of seed germination.

Molecular Network of Small RNAs in Seed Germination and Dormancy

Throughout the life cycle of an angiosperm plant, there are two major developmental phase transitional periods. One is germination (from seed to seedling stage; Huang et al., 2013) and another is flowering (from vegetative to reproductive stage; Wu et al., 2009). Recent studies indicate that genes, regulating phase transition to flowering are also involved in transition from dormancy to germination (Huang et al., 2013). The genes that regulate cellular phase transitions from embryo to seedling growth also play important role in the process. In addition, phytohormones and environmental factors affect expression of seed germination (Liu et al., 2007; Finkelstein et al., 2008). Recently, a role for small RNAs has been indicated in this process by characterizing the mutants of small RNA biogenesis pathway genes, such as *DCL1*, *HYL1*, *HEN1*, and *AGO1* that display severe defects in embryogenesis and seed development (Willmann et al., 2011). This can be illustrated by the *dcl1* mutant, which shows early seed maturation phenotype than the normal wild type seeds. The positive regulators of *DCL1* gene are leafy cotyledon (*LEC*) genes like *LEC2* and *FUS3*. Whereas the negative regulators or repressors of early embryo maturation are *ASIL1*, *ASIL2*, and *HDA6/SIL1* (Willmann et al., 2011).

Different miRNAs like are miR160, miR159, miR417, miR395, miR402, mir165/166, miR164, miR167, miR156, miR172, and miR158 (**Table 1**) are known to control both the activators and repressors of seed germination and dormancy (Jung and Kang, 2007; Liu et al., 2007; Reyes and Chua, 2007; Kim et al., 2010a,b; Martin et al., 2010; Huang et al., 2013). Increased level of miR156 and reduced level of SPLs and miR172 (**Table 1**) in the mature embryo could down regulate the developmental transition and keep seeds in dormant stages (Martin et al., 2010; Huang et al., 2013). The imbibition step itself has been shown to differentially down-regulate twelve miRNA families, miR156, miR159, miR164, miR166, miR167, miR168, miR169, miR172, miR319, miR393, miR394, and miR397; while four families, miR398, miR408, miR528, and miR529 were up-regulated during the seed germination (Li et al., 2013). Interestingly, miR156 and miR157 have also been implicated in vegetative to reproductive phase change (Wu et al., 2009), indicating their functional diversification.

The complex regulatory cross-talk between the hormones and the small RNAs, was evident by the identification of two ABA supersensitive mutants for germination viz. *absg1* and *absg2* as the alleles of *dcl1* and *hen1*. The *absg1* and *absg2* mutants show up regulation of the expression of ABA responsive

genes (Zhang et al., 2008). An important role for miR159 has been demonstrated in regulating the dynamic seed germination procedure by modulating GA and ABA hormone signaling (**Table 1**). The expression of miR159 is controlled by both GA and ABA (Martin et al., 2010). The GAMYB proteins act as the positive regulators, whereas DELLA proteins act as the negative regulators of the GA signaling cascade (Peng and Harberd, 2002; Finkelstein et al., 2008; Weitbrecht et al., 2011). The GAMYB mRNAs are regulated by miR159 during floral development, fertility and seed germination (Reyes and Chua, 2007). Recently, it was shown that alurone vacuolation, a GA-mediated (GAMYB protein) programmed cell death (PCD) process in alurone is required for seed germination (Peng and Harberd, 2002; Finkelstein et al., 2008; Alonso-Peral et al., 2010). The miR159 also regulates transcription factors MYB33 and MYB101, which are the positive regulators of ABA signaling during seed dormancy and germination (Reyes and Chua, 2007; Martin et al., 2010). miR159 expression is upregulated in *rdr2* and *dcl2 dcl3 dcl4* triple mutants. Interestingly, RDR2, DCL2, DCL3, DCL4 are the essential factors in case of siRNA biogenesis, especially heterochromatic siRNA biogenesis pathway (Allen and Howell, 2010; Axtell, 2013). This suggests that different kinds of small RNAs, besides miRNAs, could essentially play significant role in seed germination and dormancy.

The role of phytohormone Auxin in seed germination, became evident when Liu et al. (2007) showed that miR160 mediated down regulation of ARF10 plays crucial roles in seed germination (**Table 1**; Liu et al., 2007). ARFs are transcription factors involved in auxin signaling pathway during many plant growth and developmental stages. The miR160 also appears to be the converging point of Auxin and ABA mediated cross-talk during seed germination, since mutation in ARF10 results in developmental defects and overexpression of ABA responsive genes (Liu et al., 2007). Similarly, it was shown that over expression of miR160 caused hyposensitivity to ABA during germination (Liu et al., 2007). Auxin homeostasis is vital for embryo development and is mediated by the action of miR165/166, miR167, miR164, miR158, and miR160 (Martin et al., 2010). This suggests an important role for the miRNAs in mediating suitable auxin signaling during embryo and seed development (**Table 1**). Thus, it could be concluded that these miRNAs play important roles in maintaining dormancy and breaking of dormancy to promote embryo into seedling stage through seed germination (Martin et al., 2010; Huang et al., 2013; Zhang et al., 2013).

Gaseous hormone ethylene promotes seed germination through interaction with ABA signaling (Finkelstein et al., 2008) The two mutants namely *ethylene resistant1 (etr1)* and *ethylene insensitive2 (ein2)* or, *enhanced response to aba3 (era3)* show upregulation of ABA responsive genes and delay in seed germination (Finkelstein et al., 2008).Whereas wild type seeds treated with ethylene precursor ACC (1-aminocyclopropane -1-carboxylic acid) show downregulation of ABA response factors (Finkelstein et al., 2008). Again, *etr1-2* mutant show the over accumulation of GA content, which could be a compensation to over accumulation of ABA (Finkelstein et al., 2008). Since miR160 and miR159 both have regulatory effects on ABA and GA, and ethylene has a cross talk with ABA and GA, therefore, it is hypothesized that these miRNAs may have direct or indirect control over ethylene mediated regulation during seed germination and dormancy.

Plant steroid hormone BRs that mainly effect stem elongation and leaf unfurling also effect seed germination. The mutants for BR biosynthetic and signaling pathway are sensitive to ABA leading to decrease in the germination potential (Finkelstein et al., 2008). The possibility of a cross talk between BR and ABA signaling cannot be ruled out in the activation of the miR160 regulatory pathway in seed germination (Liu et al., 2007). Also, BRs induce the expression of distinct *EXPANSIN (EXP)* family members, which are cell wall loosening proteins that can indirectly influence seed germination (Bewley, 1997).

Parallel studies have shown that the small RNA biogenesis pathway mutants, that show high expression of ABA, are highly sensitive to salt and osmotic stresses (Zhang et al., 2008), thereby indicating the overlap with the environmental cues. Under abiotic stress conditions, miR395 (**Table 1**) acts both as a positive and negative regulator of seed germination (Kim et al., 2010b). miR395 has six family members in *Arabidopsis* genome, that target the proteins APS1, APS3, APS4, and SULTR, involved in sulfate assimilation and transport. It was shown that miR395e that differs from miR395c in a single nucleotide cannot target APS1 and APS4. These miRNAs have different effects on the seed germination of *Arabidopsis* under high salt or dehydration stress conditions (Kim et al., 2010b). Over expression of miR395c reduces the germination potential under high salt or dehydration stress condition; whereas over expression of miR395e enhances the germination potential under the same stress condition in *Arabidopsis thaliana* (Kim et al., 2010b). Similarly over expression of miR402 (**Table 1**) enhances the seed germination potential in *Arabidopsis* under salt, dehydration and cold stress conditions (Kim et al., 2010a). miR402 downregulates its target gene *DML3 (DEMETER-LIKE protein3)*, which is involved in DNA demethylation, an epigenetic regulatory process of plants in various stress conditions (Kim et al., 2010a). miR417 (**Table 1**) also exhibits a negative regulation over seed germination under salt stress condition (Jung and Kang, 2007). However, its mechanism of molecular action is not yet clear.

Conclusion and Future Perspective

Agriculture exclusively depends on growing crops; so the success of cultivation as well as productivity largely depends on seed viability, seed germination and efficiency of seed development. Small RNAs play critical roles in regulation of gene expression in developing and germinating seeds (Kamthan et al., 2015). In this review we describe that specific small RNAs, mainly miRNAs regulated nodes, play crucial roles in regulating seed germination in response to different phyto-hormones and abiotic stresses. But the mechanism of action and the interconnection of the various signaling cascades with their regulatory networks remain largely unknown till date. Thus, functional analysis of small RNAs expressed in seeds or during germination process will provide useful information for seed biology. Future studies are required to unravel the molecular details of small RNAs regulated pathways

in seed germination and viability maintenance, and their association with the stress responses and hormonal signals, especially in crop plants. Expression and functional analysis using transgenic approach, proteomic analysis and the use of different bioinformatics tools could also help to throw light on this issue.

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Role of bioinformatics in establishing microRNAs as modulators of abiotic stress responses: the new revolution

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microRNAs (miRs) are a class of 21–24 nucleotide long non-coding RNAs responsible for regulating the expression of associated genes mainly by cleavage or translational inhibition of the target transcripts. With this characteristic of silencing, miRs act as an important component in regulation of plant responses in various stress conditions. In recent years, with drastic change in environmental and soil conditions different type of stresses have emerged as a major challenge for plants growth and productivity. The identification and profiling of miRs has itself been a challenge for research workers given their small size and large number of many probable sequences in the genome. Application of computational approaches has expedited the process of identification of miRs and their expression profiling in different conditions. The development of High-Throughput Sequencing (HTS) techniques has facilitated to gain access to the global profiles of the miRs for understanding their mode of action in plants. Introduction of various bioinformatics databases and tools have revolutionized the study of miRs and other small RNAs. This review focuses the role of bioinformatics approaches in the identification and study of the regulatory roles of plant miRs in the adaptive response to stresses.

Keywords: microRNA, abiotic stress, high-throughput sequencing, microarray, bioinformatic approached, degradome, NGS

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ABIOTIC STRESSES AND THEIR IMPACT ON YIELD

Plants are exposed to a wide array of environmental fluctuations that lead to various physiological and metabolic changes, which in turn adversely affect the growth and productivity. Abiotic stresses are the principal cause of decrement in crop production globally and are responsible for lowering the average yield of major crops by more than 50% (Mahajan and Tuteja, 2005; Rodríguez et al., 2005). The World Meteorological Organization has reported that the years from 2001 to 2010 were considered to be the warmest period after 1850 (Oosterhuis, 2013). The climate change models have predicted that in coming time the occurrence and severity of such stresses will increase, leading to a decrease in agricultural production by about 70% (Cramer et al., 2011; Hasanuzzaman et al., 2013c; Ghosh and Xu, 2014).

The different abiotic stress conditions may be segregated into 35 different types that can be sorted as 11 groups, viz. cold, heat, drought, flooding, radiations (UV and light), wind, salinity, heavy metal toxicity, nutrient deprivation in soil, and oxidative stress (Mahajan and Tuteja, 2005). These stresses act by affecting plant growth at the molecular, biological, and physiological levels (**Figure 1**). The most studied abiotic stress conditions are cold, high temperature, salt, and drought stress. Plants cannot escape from these stresses because of their sessile nature

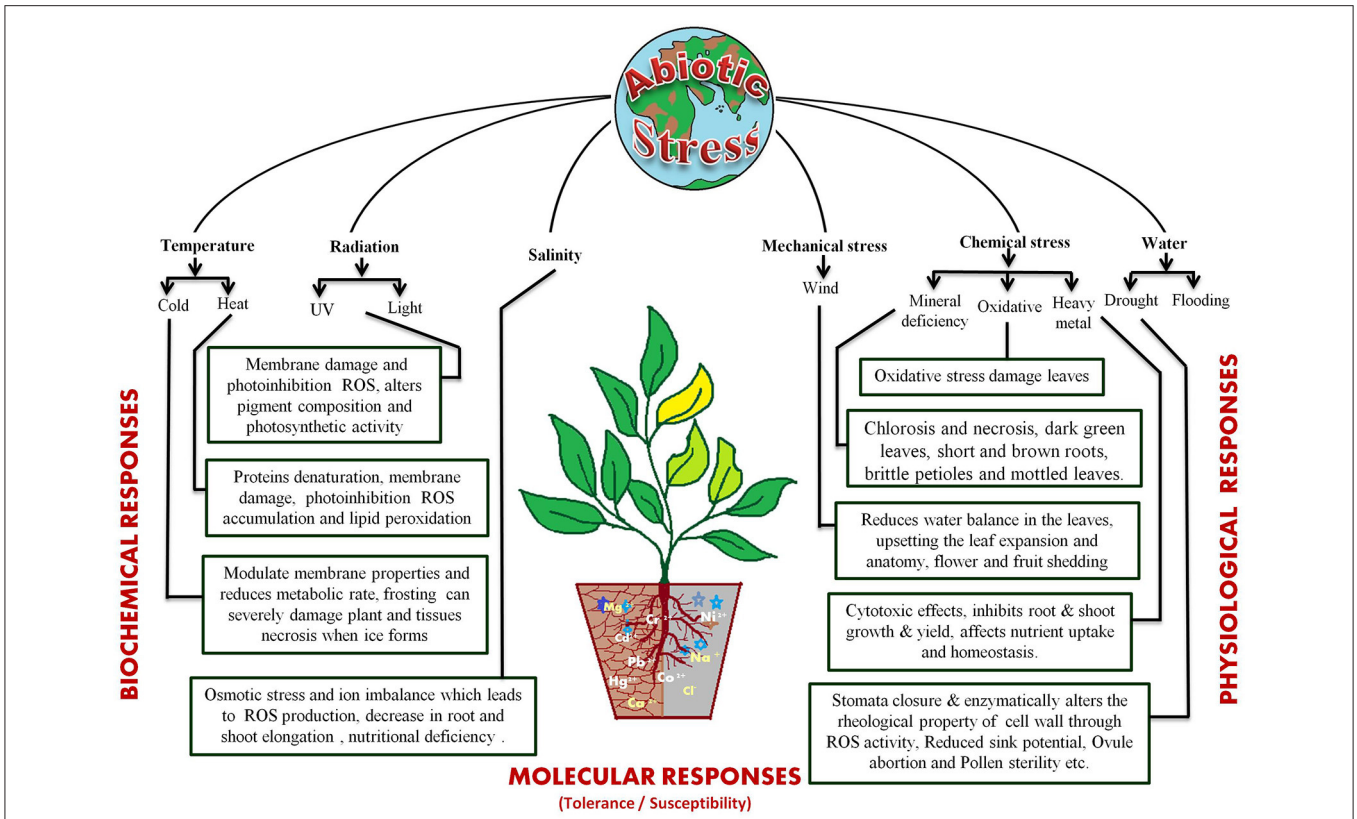


FIGURE 1 | Various abiotic stresses and their physiological effects on plants.

but, they have developed sophisticated systems to cope up with them (Nakashima et al., 2009; Pfalz et al., 2012; Upadhyaya and Panda, 2013). The response to abiotic stresses is usually multigenic and involves altering the expression of nucleic acids, proteins and other macromolecules (**Figure 1**). Several excellent reviews are available that discuss the impact of these stresses on plants in details (Cramer et al., 2011; Shanker and Venkateswarlu, 2011; Duque et al., 2013; Hasanuzzaman et al., 2013a; Rejeb et al., 2014; Petrov et al., 2015; Sodha and Karan, 2015).

Primarily fluctuations in available water, temperature and soil salt content are recognized as the basic environmental stress factors. The scarcity of water because of less rainfall, paucity of soil water and excessive evaporation, is probably the most common factor, limiting the crop's growth (de Oliveira et al., 2013). Water deficit negatively affects plant growth and development by modulating nutrient uptake, photosynthesis, hormonal levels, water potential etc. This often results in tissue dehydration leading to senescence (Kaiser, 1987; Aroca et al., 2001, 2012; Kacperska, 2004; Wahid and Close, 2007). Under low water conditions plants activate their protective machinery to enhance water uptake and reduce water loss. However, deficiency of sufficient water supply or drought limits the root hydraulic conductivity (Nobel and Cui, 1992; North and Nobel, 1997; Aroca et al., 2012) thereby affecting water uptake and resulting in physiological drought condition for the plant (Bréda et al., 1995; Duursma et al., 2008; Aroca et al., 2012). Similarly, when the water level goes above the optimal levels it results in flooding which causes hypoxic conditions, stimulate the reactive oxygen species (ROS) and induces ethylene production that restricts aerobic respiration (Bailey-Serres and Voesenek, 2008; Perata et al., 2011).

Fluctuations in atmospheric temperature due to climate change are also exerting an adverse affect at physical and cellular levels. High temperatures change the cellular state, lipid composition, membrane fluidity, and organelle properties. They induce oxidative stress and reduce the water content of the soil, causing physiological drought in plants (Wahid and Close, 2007; Giri, 2011; Hasanuzzaman et al., 2013b; Goswami et al., 2014). They also affect flowering by decreasing the number of flowers, reducing pollen viability and flower fertility (Matsui et al., 2000; Prasad et al., 2000, 2006; Suzuki et al., 2001) and cause embryo damage during the early stages of seed germination (Grass, 1994; Hasanuzzaman et al., 2013b). Low temperatures also confer osmotic and oxidative stress on plants (Chinnusamy et al., 2007; Aroca et al., 2012). They reduce metabolic rate, increase rigidification of the cellular membrane, cause flower abortion, fertilization breakdown and negatively impact seed filling (Thakur et al., 2010; Zinn et al., 2010; Hedhly, 2011).

The temperature increases along with poor irrigation practices increase soil salinity. This has emerged as an important stress which inhibits plant's growth at every stage by inducing osmotic stress and ion toxicity (Diédhiou and Golldack, 2006; Joseph

and Mohanan, 2013; Roychoudhury and Chakraborty, 2013). Salinity majorly affects roots by decreasing water use efficiency and ion exclusion, which adversely affects the root elongation, spike development and plant height (Choi et al., 2003; Alam et al., 2004; Diédhiou and Golldack, 2006; Mahmood et al., 2009; Aroca et al., 2012; Hakim, 2013; Pierik and Testerink, 2014).

The various environmental stresses result in osmotic and oxidative stresses, which inhibit metabolic reactions (Chinnusamy et al., 2007). Oxidative damage is one of the main reasons for loss of productivity and is triggered by increase in reactive oxygen species (ROS) that includes superoxide radicals ($2O^-$), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) (Mittler, 2002; Apel and Hirt, 2004; Bartels and Sunkar, 2005; Foyer and Noctor, 2005; Addo-Quaye et al., 2009). The ROS are responsible for nucleic acid damage, protein oxidation, and lipid peroxidation (Foyer et al., 1994). Plants have developed intrinsic mechanisms to avoid the oxidative stresses that includes recruitment of enzymatic scavengers, like superoxide dismutase (SOD), ascorbate peroxidase, glutathione peroxidase, glutathione S-transferase, catalase, and non-enzymatic low molecular mass molecules, such as ascorbate, tocopherol, carotenoids, and glutathione (Mittler, 2002; Mittler et al., 2004).

BASICS OF MICRORNA

The discovery of regulatory small RNAs (sRNAs) that block specific messenger RNAs (mRNAs) at the post-transcriptional levels (PTGS or post-transcriptional gene silencing) by cleavage or translational repression (Sunkar et al., 2006; Shi et al., 2012) or interfere with transcription (TGS or transcriptional gene silencing) by directing DNA methylation of genes (Wu and Zhang, 2010) have unlocked a new avenue in gene expression regulation. The sRNAs constitute a large family represented by many species of RNA molecules distinguished from each other by their size, biogenesis, mode of action, regulatory role etc. (Axtell and Bowman, 2008; Sanan-Mishra et al., 2009; Lima et al., 2011; Meng et al., 2011a; Zheng et al., 2012).

The microRNA (miR) represents a major sub-family of endogenously transcribed sequences, ranging in length from 21 to 24 nt (Carrington and Ambros, 2003; Eldem et al., 2013). They have been established as a major regulatory class that inhibits gene expression in a sequence-dependent manner. The *lin-4* and *let-7* regulatory RNAs are accepted as the naissance member of the miR family (Lee et al., 1993; Reinhart et al., 2002), which is conserved across animal and plant species. Though there is no conservation between the animal and plant sequences, but high conservation is observed among plant miRs (Reinhart et al., 2002). An exception is provided by Ath-miR854 and Ath-miR855, which regulate levels of transcript encoding the oligouridylate binding protein 1b (UBP1b) (Arteaga-Vázquez et al., 2006). The target transcript of miR854 performs similar functions in plants as well as in animals (Arteaga-Vázquez et al., 2006).

MicroRNA Biogenesis

Each miR arises in the nucleus from an independent transcription unit, comprising of its own promoter, transcribing region and terminator, by utilizing the basic machinery for DNA-dependent RNA polymerase II mediated transcription (Kurihara and Watanabe, 2004; Lee et al., 2004; Xie et al., 2005a; Kim et al., 2011). Plant miR genes are present throughout the genome, although majority of the loci in plants are generally found in genomic (intergenic) regions that are not protein coding (Jones-Rhoades et al., 2006; Wahid et al., 2010). Comparatively lesser number of plant miRs are present in the introns (Lagos-Quintana et al., 2001; Lau et al., 2001; Chen, 2008; Nozawa et al., 2010; Wahid et al., 2010) and are rarely found in the exons (Olena and Patton, 2010; Li et al., 2011). Two miRs, miR436, and miR444, were mapped to the exonic regions of the protein-coding genes J023035E19 (AK120922) and J033125N22 (AK103332), respectively (Sunkar et al., 2005). It is hypothesized that the miRs control the host gene expression via a negative feedback loop mechanism that affects alternative splicing and cytoplasmic movement of transcripts (Slezak-Prochazka et al., 2013). Recently, CDC5 was identified as a MYB-related DNA binding protein that positively regulates miR production (Zhang et al., 2013a) by binding to their promoters and through interaction with the RNase III enzyme DCL1 (Dicer-Like 1). The large pri-miRs (primary transcripts) contain a 5′-cap and 3′-polyA tail and are stabilized in the nucleus by DDL (Dawdle) which is a RNA binding protein (Yu et al., 2008).

The pri-miRs are further processed into hairpin loop structured pre-miRs (precursor miRs) in the D bodies (Dicing bodies) or SmD3-bodies (small nuclear RNA binding protein D3 bodies) (Kurihara et al., 2006; Fang and Spector, 2007; Fujioka et al., 2007) by a protein complex containing the DCL1 (Schauer et al., 2002) and the CBC (Cap-Binding protein Complex) (Kim et al., 2008). The accuracy of DCL1 mediated pri-miR processing is promoted by both HYL1 (Hyponastic Leaves 1), and the C_2H_2-zinc finger protein, SE (Serrate) (Kurihara et al., 2006; Dong et al., 2008; Manavella et al., 2012a). This activity is also aided by DRB (Double strand RNA-Binding) protein (Han et al., 2004; Kurihara et al., 2006; Vazquez, 2006). Recently the G-patch domain protein TGH (Tough) was identified as another active player which is responsible for enhancing the DCL1 activity (Ren et al., 2012). It has been shown that HYL1 binds double stranded (ds) region on the pri-miR (Hiraguri et al., 2005; Rasia et al., 2010; Yang et al., 2010), TGH binds the single-stranded (ss) RNA region (Ren et al., 2012) and SE possibly binds at ssRNA/dsRNA junctions (Machida et al., 2011). It was also observed that HYL1 is a phospho-protein that directly interacts with CPL1 (C-terminal domain Phosphatase-Like 1) protein, to maintain its hypo-phosphorylated state (Manavella et al., 2012a). Thus, CPL1 also plays a critical role in accurate miR processing though it is not directly required for DCL1 activity (Manavella et al., 2012a). It was observed that CPL1 directly interacts with SE and a mutation in SE can affect phosphorylation status of HYL1 by preventing recruitment of CPL1 (Manavella et al., 2012a). Thus, the proposed model for the pri-miR processing indicates association of multiple RNA binding proteins with definite regions to maintain the structural determinants for recruiting and directing DCL1 activity. The DCL1, HYL1, SE, and TGH seem to interact directly (Kurihara et al., 2006; Lobbes et al., 2006; Yang et al., 2006; Qin et al., 2010;

Machida et al., 2011; Ren et al., 2012) and are colocalized in the D bodies as shown by bimolecular fluorescence complementation. However, it has not been demonstrated whether they represent a stable plant microprocessor complex (Fang and Spector, 2007; Fujioka et al., 2007; Song et al., 2007; Manavella et al., 2012b; Ren et al., 2012).

The hairpin looped pre-miRs thus formed are further processed by DCL1 to produce miR/miR* duplex (Xie et al., 2005b; Sanan-Mishra et al., 2009; Naqvi et al., 2012). Recently a proline-rich protein, SIC (Sickle), was identified to co-localize with HYL1 foci (Zhan et al., 2012) and it was found to play an important role in the accumulation of mature miR duplex (Zhan et al., 2012). The strands of the duplex are protected from uridylation and degradation by the activity of a methyltransferase protein known as HEN1 (Hua Enhancer 1) which covalently attaches a methyl residue at the 3′ ribose of last nucleotide from each strand (Li et al., 2005a; Yu et al., 2005). The miR duplexes are transported to the cytoplasm by HST (Hasty), the ortholog of Exportin-5 (Park et al., 2005), where the miR strand guides the AGO1 (Argonaute 1) containing RNA-induced silencing complex (RISC) complex to the target transcript (Baumberger and Baulcombe, 2005; Qi et al., 2005).

microRNA Function

Plant miRs generally control the expression of their targets transcripts by cleavage and translational repression (Chen, 2009). Brodersen et al. concluded that central matches in miR:target-mRNA duplex tend to cleave target mRNA, regardless of a few mismatches in other regions, while central mismatches in miR:target mRNA duplex lead to translational repression (Brodersen et al., 2008). It was hypothesized that the rapid fine-tuning of the target transcripts by translation repression is required for the reversible modulation of the negative regulators of stress responses whereas the on-off switching of target gene expression by cleavage was important in regulating developmental processes, which require permanent determination of cell fates (Baumberger and Baulcombe, 2005).

In plants, miRs regulate various biological processes such as, growth and development, pattern formation, organ polarity, signal transduction, and hormone homeostasis etc. (Palatnik et al., 2003; Dugas and Bartel, 2004; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006; Mishra and Mukherjee, 2007; Cai et al., 2009; Voinnet, 2009; Sanan-Mishra et al., 2013). In past few years the role of miRs in response to diseases and environmental stresses has been highlighted (Fujii et al., 2005; Sunkar et al., 2007; Zhou et al., 2010; Lima et al., 2011; Meng et al., 2011a; Zheng et al., 2012; Mittal et al., 2013; Sharma et al., 2015). These are supported by reports on mutants of the miR biogenesis or pathways exhibiting defective phenotypes (Laufs et al., 2004; Zhong and Ye, 2004; Millar and Gubler, 2005; Ori et al., 2007; Chen et al., 2010; Rubio-Somoza and Weigel, 2011). The stress regulated miRs may be engaged in many biological pathways that re-program intricate procedures of physiology and metabolism (Khraiwesh et al., 2012) as suggested by their differential expression patterns in tissues in presence or absence of stress (Covarrubias and Reyes, 2010).

IDENTIFICATION OF STRESS-ASSOCIATED microRNAs

The identification of plant miR families began in the year 2000, with direct cloning and sequencing (Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002). However, this was an uphill task owing to their small size, methylation status and multiple occurrences in genome. The numbers however increased rapidly with the advancement in cloning techniques and computational algorithms. In the past few years high throughput sequencing and screening protocols has caused an exponential increase in number of miRs, identified and functionally annotated from various plant species (Rajagopalan et al., 2006; Fahlgren et al., 2007; Jagadeeswaran et al., 2010; Rosewick et al., 2013). This is best exemplified by the establishment of miRBase, a biological database that acts as an archive of miR sequences and annotations (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2014). The first release of miRBase in the year 2002 included total 5 miRs from only 1 plant species, *Arabidopsis thaliana.* This was followed by the inclusion of *Oryza sativa,* in miRBase in the year 2003. Thereafter miRs reported from *Medicago truncatula, Glycine Max,* and *Populus trichocarpa* were included in the year 2005. The current version (release 21) includes 48,496 mature plant miRs derived from 6992 hairpin precursors reported in 73 plant species (**Figure 2**).

The association of plant miRs with stress was first reported in 2004 (Sunkar and Zhu, 2004). Now there are a number of reports supporting the hypothesis for the function for miRs in the adaptive response to abiotic stress including drought (Liu et al., 2008b; Zhou et al., 2010), cold (Zhou et al., 2008), salinity (Liu et al., 2008a; Sunkar et al., 2008) and nutrient deficiency (Fujii et al., 2005). 1062 miRs have been reported to be differentially expressed in 35 different abiotic stress types in 41 plant species (Zhang et al., 2013b). The detailed list of these miRs is available as **Supplementary Table 1**. The comparative picture of stress-induced dis-regulations of Arabidopsis and rice miRs is compiled as **Figure 3**.

The survey of literature reveals that three major approaches have been employed for the identification and expression profiling of stress induced miRs. The first approach involves the classical experimental route that included direct cloning, genetic screening, or expression profiling. The second method involved computational predictions from genomic or EST loci and the third one employed a combination of both as it was based on the prediction of miRs from High Throughput Sequencing (HTS) data. Each of these was followed by experimental validations by northern analysis, PCRs or microarrays.

Experimental Approaches

Direct cloning was the principal and conventional method for the identification of miRs (Park et al., 2002; Reinhart et al., 2002). This method was of significant consideration as it was a sequence-independent approach where *a priori* knowledge of miR sequence was not required. Moreover, it provided more accuracy and efficiency by giving few false positives. Several

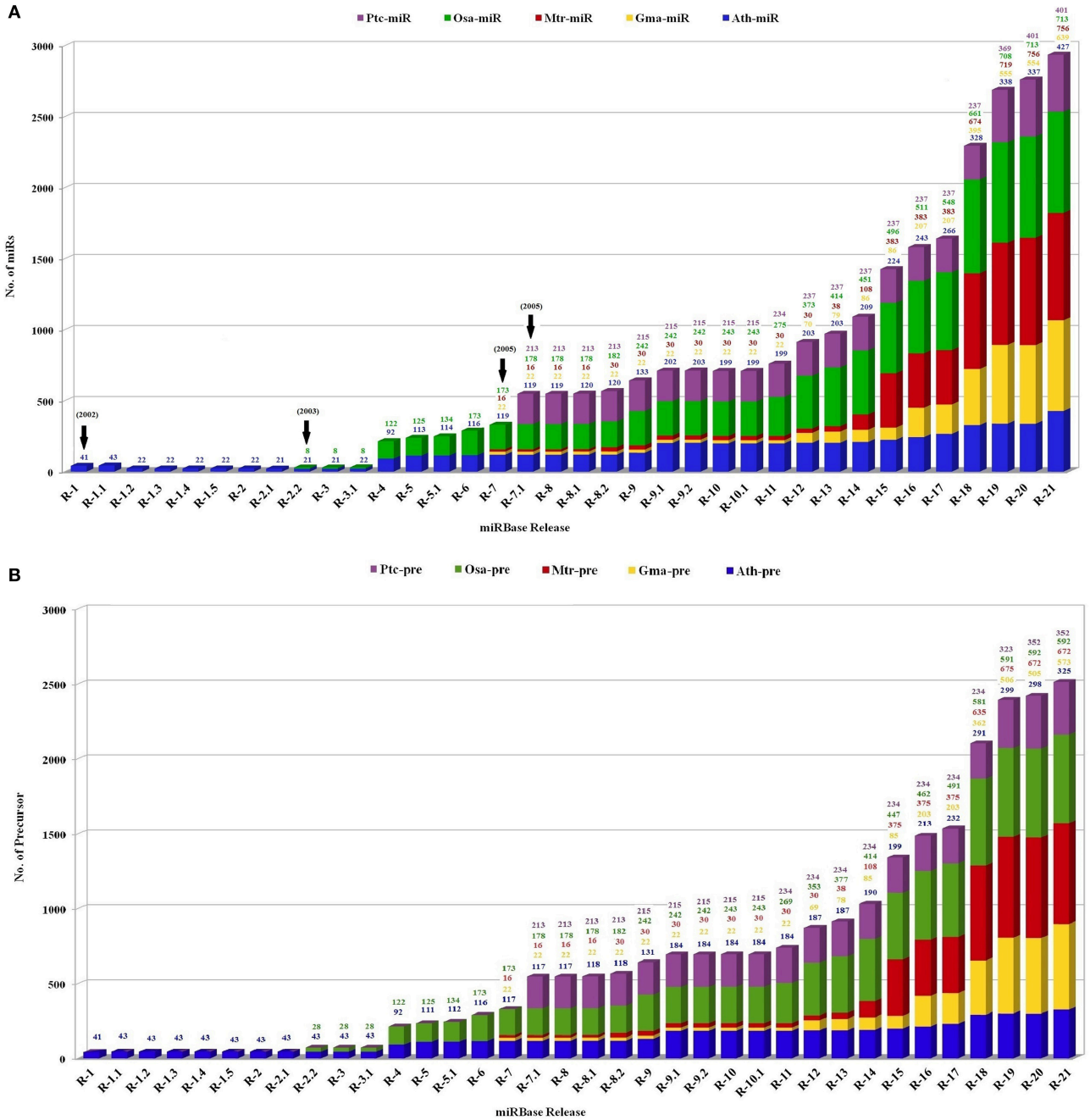


FIGURE 2 | The status of the number of (A) mature miRs and (B) precursor miR sequences in the miRBase registry in five plant species. The plot contains the number of miRs in *Medicago truncatula* (red), *Oryza sativa* (green), *Glycine max* (yellow), *Populus trichocarpa* (violet), and *Arabidopsis thaliana* (blue) calculated with respect to total number reported miRs. X axis represents number of respective sequences and Y axis denotes the released versions of miRBase.

related studies led to the establishment of different protocols for sRNA isolation and adaptor mediated synthesis of a cDNA library followed by their amplification and then cloning. The clones were screened and sequenced to identify the potential miRs (Llave et al., 2002; Reinhart et al., 2002; Sunkar and Zhu, 2004). Thus, it was portrayed as a time-consuming, low throughput, laborious, and expensive approach.

However, the first report indicating the role of miRs in plant responses to environmental stresses came from the sequencing and analysis of a library of sRNAs from Arabidopsis seedlings treated with cold, dehydration, salinity, and the plant stress hormone abscisic acid (ABA). It was observed that several miRs were up-regulated or down-regulated by the abiotic stresses (Sunkar and Zhu, 2004). This strategy

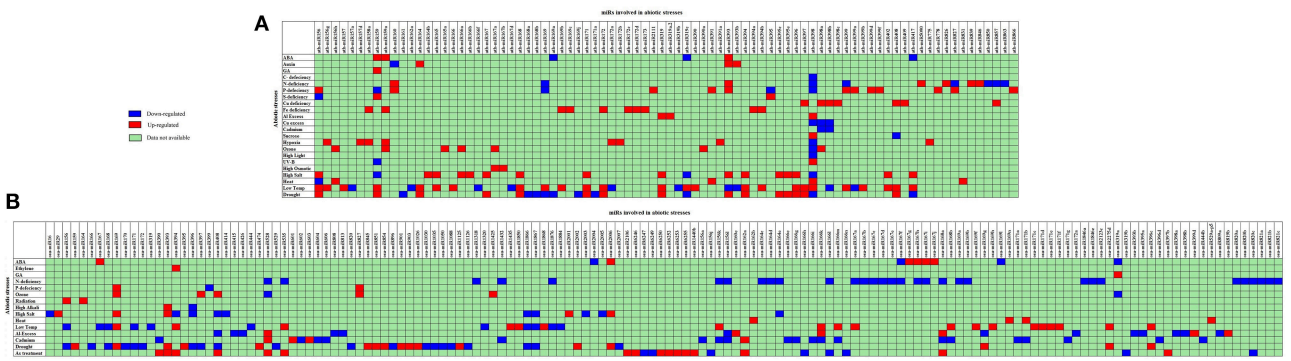


FIGURE 3 | Status of abiotic stress regulation for conserved miRs as reported from (A) Arabidopsis and (B) rice. The X axis contains the list of abiotic stress regulated miRs, and Y axis lists the different abiotic stress studied. ABA, Abscisic Acid; GA, Gibberellic Acid; C, carbon; N, nitrogen; P, Phosphorus; S, Sulfur; UV-B, Ultra violet; Al, aluminum; Cu, cupper; Fe, Iron. The miRs down-regulated in stress are shown in Blue and miRs up-regulated in stress are shown in Red. The miRs for which the stress induced status is not available are represented by green.

was used to clone miRs from the mechanical stress-treated Populus plants (Lu et al., 2005). A majority of these miRs were predicted to target developmental- and stress/defense-related genes. In our lab, 39 new miR sequences were cloned from salt-stressed basmati rice variety. This study also provided evidence for a converging functional role of miRs in managing both abiotic and biotic stresses (Sanan-Mishra et al., 2009).

The importance of miRs in abiotic stress responses was also implicated by the fact that several mutants such as *hyl1*, *hen1*, and *dcl1* which are defective in miR metabolism, exhibited hypersensitivity to ABA, salt, and osmotic stresses (Lu and Fedoroff, 2000). Nonetheless, the direct evidence was provided by studies monitoring the down-regulation of miR398 expression in response to oxidative stresses, in Arabidopsis. It was later shown that miR398 targeted two Cu/Zn superoxide dismutase (CSD) transcripts, cytosolic CSD1, and chloroplastic CSD2, so stress induced reduction of miR398 was expected to improve plant tolerance. This theory was proved subsequently by analysis of transgenic lines under oxidative stress conditions (Sunkar et al., 2006).

Expression analysis by northern blot analysis revealed that miR395 and miR399 were involved in sulfate and inorganic phosphate starvation responses, respectively (Jones-Rhoades and Bartel, 2004; Fujii et al., 2005). Similarly, RNA gel blot analysis identified miRs induced by cold, ABA, dehydration, and high salinity in 2-week-old Arabidopsis seedlings (Sunkar and Zhu, 2004). The results indicated that Ath-miR393 was highly up-regulated whereas Ath-miR397b and Ath-miR402 were slightly up-regulated and Ath-miR389a.1 was down-regulated under all the stress treatments. Similarly low temperature stress condition induced the expression of Ath-miR319c but no increase in response to dehydration, NaCl or ABA (Sunkar and Zhu, 2004). These and related findings not only helped in interpreting the role of miRs during stress but unraveled the role of specific members of the miR family. A comprehensive study of Ath-miR398, revealed that the expression of miR398 precursors (with identical mature sequences) is increased under high temperature stress and that heat stress induces expression of Ath-miR398b to a much higher level than that of the Ath-miR398a,c (Guan et al., 2013). Similarly in rice, Osa-miR169g, was proven as the only drought stress induced member among the ABA responsive miR169 family (Zhao et al., 2007).

The variable expression patterns of the miRs in response to different stresses were captured by reverse transcription quantitative PCR (RT-PCR) in several plants including Arabidopsis (Jung and Kang, 2007; Reyes and Chua, 2007; Li et al., 2008; Liu et al., 2008a; Jia et al., 2009), rice (Liu et al., 2009), *Phaseolus vulgaris*, (Arenas-Huertero et al., 2009), sugarcane (Thiebaut et al., 2012), and poplar (Rossi et al., 2015). These methods captured the similarities and differences in expression profiles of conserved miRs across different plants (Zhou et al., 2010). This is exemplified by identified molecules like miR393 that is consistently up-regulated during drought stress in many plants such as Arabidopsis, Medicago, common bean, and rice (Sunkar and Zhu, 2004; Zhao et al., 2007; Arenas-Huertero et al., 2009). Whereas miR169 was found to be induced by drought and high salinity in rice (Zhao et al., 2009), but was down-regulated by drought stress treatment in Arabidopsis (Li et al., 2008). High-throughput expression profiling analysis through one-tube stem-loop RT-PCR quantified the relative expression levels of 41 rice miRs under drought, salt, cold, or ABA treatments (Ding et al., 2011).

The need for genome wide characterization of miR expression profiles established the microarray analysis as a useful tool (Garzon et al., 2006; Zhao et al., 2007). The microarray technology is a hybridization based and a relatively cost-effective assay that allows analysis of large numbers of molecules in parallel. The tiling path microarray analysis was used to identify 14 stress-inducible Arabidopsis miRs after screening 117 miRs under high-salinity, drought, and low-temperature stress conditions (Liu et al., 2008a; Zhang et al., 2008b). The results were further validated to provide evidence for cross-talk among the high-salinity, drought and low temperature stress

associated signaling pathways (Liu et al., 2008a). Similar studies were performed to capture the expression patterns of miRs in response to Ultraviolet-B rays in Arabidopsis (Zhou et al., 2007), drought stress in rice (Zhao et al., 2007), cold stress in rice (Kang et al., 2010), cadmium stress in rice (Ding et al., 2011), and ABA and NaCl in *Populus tremula* (Jia et al., 2009).

The expression patterns also identified that tissue-specific regulation of miRs may be important for adaptation to stress. Under water deficit conditions, miR398a/b and miR408 were up-regulated in both roots and shoots of *Medicago truncatula* plant, but the increase was more pronounced in the shoots than in the roots. This was accompanied by the down-regulation of their corresponding targets, COX5b and plantacyanin, thereby suggesting that these miRs have a crucial role in regulation of plants responses against water deficiency (Trindade et al., 2010). In barley, miR166 was up-regulated in leaves, where as it was shown to be down-regulated in roots; and miR156a, miR171, and miR408 were induced in leaves, but unaltered in roots (Kantar et al., 2010).

The miR expression profiles were also used to compare the genotypic differences between varieties exhibiting contrasting stress sensitivities. Microarray profiles of salt-resistant and susceptible *Zea mays* identified 98 miRs belonging to 27 families (Ding et al., 2009). Zma-miR168 family members were induced in the salt-tolerant maize but suppressed in the salt-sensitive line. Interestingly this salt-responsive behavior of miR168 was found to be conserved in Maize and Arabidopsis (Liu et al., 2008a). miR microarray was also used to study drought-tolerant wild emmer wheat (*Triticum dicoccoides*) (Kantar et al., 2011), two cotton cultivars with high tolerance (SN-011) and high sensitivity (LM-6) to salinity (Yin et al., 2012) and for comparative analysis between drought-resistant and susceptible soybean (Kulcheski et al., 2011). A comparison of 12 salt-tolerant and 12 salt-susceptible genotypes in *Oryza sativa*, identified 12 polymorphic miR based simple sequence repeats (Mondal and Ganie, 2014). Only miR172b-SSR was different between the salinity stress tolerant and susceptible genotypes. The genotype-dependent miR profiles suggested that response of miRs to abiotic stresses varies among closely related genotypes with contrasting stress sensitivities. The result of this analysis showed that there was less diversity of miR genes in the tolerant as compared with susceptible cultivars (Mondal and Ganie, 2014).

Computational Predictions

The detection and validation of miRs by molecular cloning was supported by systematic approaches using computational techniques (Bonnet et al., 2004b). These approaches also complemented the experimental methods by identifying difficult to clone miR families such as miR395 and miR399 (Jones-Rhoades and Bartel, 2004; Adai et al., 2005) which were difficult to detect by experimental approaches due to their low expression levels. Computational predictions strategies have been quite useful in miR identification in various plant species such as Arabidopsis (Wang et al., 2004; Adai et al., 2005; Li et al., 2005b), rice (Li et al., 2005b; Zhang et al., 2005), maize (Zhang et al., 2006a, 2009b), tomato (Yin et al., 2008; Zhang et al., 2008b), foxtail millet (Khan et al., 2014), soybean (Zhang et al., 2008a), *Brassica napus* (Xie et al., 2007), apple (Gleave et al., 2008), grape (Carra et al., 2009), and some other plants (Zhang et al., 2005; Sunkar and Jagadeeswaran, 2008).

It had been verified that a majority of known miRs are evolutionarily conserved and are expected to have homologs or orthologs in other species. So search criteria allowed up-to three sequence mismatches while looking for conserved miRs in heterologous species. Using this approach 85 conserved sequences which were showing perfect match to miRs reported in miRBase (Release 19) were predicted from *Morus notabilis* tissues (Jia et al., 2014). Whereas in another study 35 miR families were identified in heat stressed *Brassica napus* by allowing two mismatches with *A. thaliana* miRs (Yu et al., 2012). Thus, the conserved sequence of plant miRs and other structural features were used for developing suitable strategies and rules for identifying and annotating (Discussed in Section The Influence of Bioinformatics Approaches on microRNA Nomenclature and Annotation) new miR genes (Lagos-Quintana et al., 2001; Reinhart et al., 2002; Floyd and Bowman, 2004; Wang et al., 2004; Adai et al., 2005; Zhang et al., 2006a; Lukasik et al., 2013). One of the early comprehensive computational analysis by Jones-Rhoades and Bartel (2004) systematically identified plant miRs and their regulatory targets that are conserved between Arabidopsis and rice. Using MIRcheck algorithm they predicted that the miRs could target mRNAs like superoxide dismutases (SOD), laccases, and ATP sulfurylases that are involved in plant stress responses. Such studies lead to identification of involvement of Ath-miR398 in the ROS pathway by targeting sites on Cu/Zn-SOD (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Lu et al., 2005; Sunkar et al., 2005) A similar approach was used in miRFinder computational pipeline, to identify 91 conserved plant miRs in rice and Arabidopsis (Bonnet et al., 2004a).

Another strategy was based on the property of miRs to bind with perfect complementarity to their target transcripts (Laufs et al., 2004). In plant species where the target sequence was available the conserved miRs could be easily predicted by using 20 mer genomic segments with not more than two mismatches as *in silico* probes. This target-guided strategy was adopted to identify 16 families of drought stress-associated miRs from *Physcomitrella patens* (Wan et al., 2011).

The computational predictions also utilized the criteria for conservation of miR sequence and key secondary structure features of pre-miRs like their characteristic fold-back structure, thermodynamic stability etc. to predict new miRs (Berezikov et al., 2006). Seventy-nine putative miRs were identified in wheat using traditional computational strategy, out of which 9 were validated by northern blot experiments (Jin et al., 2008). Subsequently bioinformatics tools like miRAlign were developed based on the requirement of structural similarity and sequence conservation between new candidates and experimentally identified miRs (Wang et al., 2005). Though numerous miR profiles were generated by the computational algorithms, this was not found to be appropriate for species with less annotated genomes (Chen and Xiong, 2012).

The non-availability of complete genome annotation was overcome by employing the Expressed Sequence Tags (EST)

database. These represented the true gene expression entities so they emerged as better indicators of dynamic expressions of the miR. A detailed study by identified 123 miRs from stress-induced ESTs of 60 plant species (Zhang et al., 2005). This study confirmed that irrespective of evolutionary divergence miRs are highly conserved in plant kingdom and miR genes may exist as orthologs or homologs in different species within the same kingdom (Weber, 2005; Zhang et al., 2006b). The EST database was also used to confirm some novel miRs identified earlier by computational strategies in citrus (Song et al., 2010) and peach (Zhang et al., 2012). In a recent study ESTs of abiotic stress treated libraries of *Triticum aestivum* were used to identify novel miRs in drought, cold, and salt stressed cDNA libraries by searching all mature sequences deposited in the miRBase (Release 19) (Pandey et al., 2013).

High Throughput Sequencing

The recent development of HTS approaches has invoked a new era by allowing the sequencing of millions of sRNA molecules. The HTS techniques employ sequencing-by-synthesis (SBS) technology, which enable accessing the full complexity of sRNAs in plants. In addition, it provides quantitative information of the expression profiles, since the cloning frequency of each sRNA generally reflects its relative presence in the sample. The signature-based expression profiling method such as massively parallel signature sequencing (MPSS) has identified miRs that have thus far proven difficult to find by using traditional cloning or *in silico* predictions. Sequencing technologies are rapidly emerging as the favored alternatives to the microarray-based approaches, since direct measures of gene expression can be obtained through sequencing of random ESTs, SAGE, and MPSS. The expression patterns of the identified miR targets can then be followed in the transcriptome sequencing data to gain novel insights into plant growth and development and stress responses (Wang et al., 2010; Li et al., 2013). Though currently an expensive technique, it is expected that as the technology grows, it will become more affordable.

Complex computational algorithms are used to rapidly and rigorously sift through the HTS data for identification of putative miRs (**Figure 5**). These datasets have been very successful in identification of conserved miRs where the sequence is well maintained across plant species. The targets for these miRs can also be easily predicted using Parallel Analysis of RNA End (PARE) sequencing, where miR and its target mRNA have often nearly perfect complementarily (Rhoades et al., 2002; Bonnet et al., 2004b; Jones-Rhoades and Bartel, 2004). The HTS data also provided a useful source to hunt for the non-conserved or species-specific miRs based on the criteria of miR annotation (Discussed in Section The Influence of Bioinformatics Approaches on microRNA Nomenclature and Annotation).

This HTS approach was initially used to visualize the repertoire of sRNAs in Arabidopsis (Rajagopalan et al., 2006; Fahlgren et al., 2007), followed by investigation on the rice miR expression profiles in drought and salt stress responses (Sunkar et al., 2008). Later, Liu and Zhang identified 67 arsenite-responsive miRs belonging to 26 miR families from *Oryza sativa* (Liu and Zhang, 2012). Solexa sequencing was also used to identify conserved and novel miRs in *Glycine max* libraries from water deficit and rust infections (Kulcheski et al., 2011), cold responsive miRs in trifoliate orange, *Poncirus trifoliate,* (Zhang et al., 2014a), drought and salinity responsive miRs in *Gossypium hirsutum* (Xie et al., 2015), heat stress induced miRs in *Brassica napus* (Yu et al., 2012), and salt stressed miRs in *Raphanus sativus* (Sun et al., 2015). Regulation of miRs in response to various abiotic stresses was studied in Arabidopsis, under drought, heat, salt, and metal ions such as copper (Cu), cadmium (Cd), sulfur (S) excess or deficiency, using sRNA NGS libraries. The search for most profound changes in miR expression patterns identified that miR319a/b, miR319b.2, and miR400 were responsive to most of the stresses under study (Barciszewska-Pacak et al., 2015).

Comparative profiles of miR expression during cold stress among *Arabidopsis*, *Brachypodium,* and *Populus trichocarpa* revealed that miR397 and miR169 are up-regulated. This indicated the presence of conserved cold responsive pathways in all the species. Whereas the differences in the pathways was highlighted by miR172 which was up-regulated in Arabidopsis and *Brachypodium* but not in poplar (Zhang et al., 2009a). Opposing patterns of miR regulation in different plant species during cold stress were observed for miR168 and miR171. The miRs are up-regulated in poplar (Lu et al., 2008) and Arabidopsis (Liu et al., 2008a) but down-regulated in rice (Lv et al., 2010). Likewise the HTS analysis of salt stressed sRNAome identified 211 conserved miRs and 162 novel miRs, belonging to 93 families between *Populus trichocarpa* and *P. euphratica* (Li et al., 2013). Using the approach of comparative miR profiling followed by experimental validation, our group identified 59 Osa-miRs that show tissue-preferential expression patterns and significantly supplemented 51 potential interactive nodes in these tissues (Mittal et al., 2013).

HTS technology has also played a crucial role in identification and characterization of the miR targets with PARE or Degradome sequencing. This involves sequencing of the entire pool of cleaved targets followed by mapping of the miR-guided cleavage sites (Ding et al., 2012). In Populus, 112 transcripts targeted by 51 identified miRs families were validated by using degradome sequencing (Li et al., 2013). These are several reports which used HTS of sRNA pools and degradome analysis to identify targets of stress induced miRs such as, in maize (Liu et al., 2014), tomato (Cao et al., 2014), *Raphanus sativus* (Wang et al., 2014), Populus (Chen et al., 2015), rice (Qin et al., 2015), *Phaseolus vulgaris* (Formey, 2015), and barley (Hackenberg et al., 2015).

It has been shown that plant miRs also act by inhibiting mRNA translation (Brodersen et al., 2008; Lanet et al., 2009), therefore such targets tend to get overlooked during degradome sequencing. The HTS techniques are also being employed for sequencing the whole transcriptome pools to identify the miR targets in *Medicago* (Cheung et al., 2006), *Zea mays* (Emrich et al., 2007), and Arabidopsis (Weber et al., 2007). The combined strategy of sRNAs and mRNAs (transcriptome) sequencing enabled the identification of new genes, involved in nitrate regulation and management of carbon and nitrogen metabolism in Arabidopsis. This study identified miR5640 and its target, *AtPPC3,* leading to the preposition that the NO_3^- responsive

miR/target might be involved in modulating the carbon flux to assimilate nitrate into amino acids (Vidal et al., 2013).

THE INFLUENCE OF BIOINFORMATICS APPROACHES ON microRNA NOMENCLATURE AND ANNOTATION

The *in silico* approaches have also played a dominant role in the identification of plant miRs and their targets. The advancement in molecular and computational approaches has not only resulted in the exponential growth in the discovery and study of sRNA biology but has also provided a deeper insight into the miR regulatory circuits. At the same time, they have been instrumental in defining and redefining the rules for annotating the miRs and their nomenclature.

A miR registry system was adopted in 2004 to facilitate a complete and searchable place for the published miRs and to provide a systematic rule so that the new miRs can be assigned with a distinctive name prior to publication of their discovery (Ambros et al., 2003; Griffiths-Jones, 2004). In miRBase the nomenclature of miRs starts with initial 3 letters signifying the organism, followed by a number which is simply a sequential numerical identifier based on sequence similarity, suffixed by "miR," trailed by alphabet letters which denotes the family member (**Figure 4**). It was later enforced that sequences showing homology within organisms and mature identical sequences coming from two or more different organism should be assigned the same family names (Meyers et al., 2008). Sequences with no similarity to previously reported sequence were considered novel and assigned next number in the series (Griffiths-Jones, 2004). It is observed that in miRBase *Medicago truncatula,* mtr-miR2592 is the largest miR family with 66 members, while in rice; the largest family is seen for Osa-miR395 with 25 members. The occurrence of more than 1 mature sequence from same precursor is designated by an integer followed by a dot at the end (Griffiths-Jones, 2004; Meyers et al., 2008). With the accumulation of HTS data and the experimental validation that both miR and miR* of same precursor can be functional, it was decided to add a suffix of 3p and 5p at the end of the sequence to represent the presence of miR on 3′ or 5′ arm of stem loop precursor (Meyers et al., 2008).

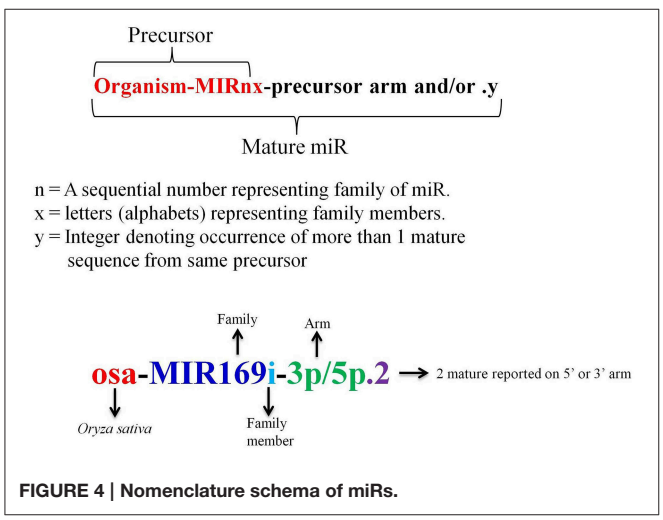


FIGURE 4 | Nomenclature schema of miRs.

The processing of biological information through bioinformatics tools and computational biology methods has now become crucial for elucidating complicated biological problems in genomics, proteomics, as well as in metabolomics. With the accumulation of huge sRNA sequencing datasets, it is almost impossible to analyze each and every sequence through direct experimental approaches. This has necessitated the role of bioinformatics tools and databases in analyzing and screening the huge data sets in a short time period, with minimum costs and without compromising on the specificity of analysis.

The primary criteria for annotation of plant miRs is the precise excision of a miR/miR* duplex from the stem of a single-stranded, stem-loop precursor. Computational algorithms use these criteria to predict the RNA secondary structure for the sequences identified from the genomic DNA, transcript or ESTs. Subsequently the annotation rules are followed to distinguish a miR from the sRNA pool. The first set of guidelines for miR annotation was based on specific expression and biogenesis criteria (Ambros et al., 2003). The expression criteria included the identification by cloning and/or detection by hybridization and phylogenetic conservation of the miR sequence. While the biogenesis criteria included the presence of a characteristic hairpin structured precursor transcript, conservation of the precursor secondary structure and increased accumulation of a precursor in absence or reduction in Dicer activity (Ambros et al., 2003).

The advancement in sequencing technologies provided with highly sensitive techniques for obtaining the complete small RNA profiles that could distinguish between fragments differing by a single base. This also provided an excellent medium to search for known and novel miR family members, their precursors, and modified versions. The bioinformatics based analysis of HTS datasets, made it feasible to predict the entire set of miRs present in a RNA sample. This was also utilized to retrieve the information on expression profiles, putative target transcripts, the miR isoforms, and sequence variants of miRs through differential expression profiling under various conditions (Moxon et al., 2008; Addo-Quaye et al., 2009; Yang and Li, 2011b; Neilsen et al., 2012). Dedicated web servers like isomiRex (Sablok et al., 2013) are available online for identification of the sequence variants using HTS data.

With the development in computational tools and the availability of genomic sequences the rules were further refined to include characteristics that are both necessary and sufficient for miR annotation. It was proposed that the prediction criteria should include that the miR and miR* are derived from opposite arm of same precursor such that they form a duplex with two nucleotide overhang at the 3′ end, base pairing of miR and miR* should have less than four mismatched bases, the asymmetric bulges are minimum in size and frequency specifically in miR/miR* duplex. sRNA-producing stem-loops that violate one of these criteria could still be annotated as miRs, provided that there is conclusive experimental evidence of precise miR/miR* excision (Meyers et al., 2008). In continuation

to the guidelines set by Ambros et al. (2003) it was recognized that conservation of miRs, assessed using either bioinformatics or direct experimentation, was still a powerful indicator of their functional relevance though it need not be necessary for annotation as many plant miRs lack homologs in other species. It was proposed that identification of a target is not necessary for miR annotation as targets could not be predicted for many of the less-conserved miRs or the predicted targets lacked experimental confirmation.

It is being observed that increased coverage of deep-sequencing results have resulted in capturing sequences of ever-lower abundance. This has made the identification of miRs even more challenging. A number of recent publications have attempted use additional criteria based on patterns of mapped reads (Hendrix et al., 2010). The consensus set of guidelines that have started to emerge lay importance to the presence of multiple reads with consistent processing of the 5′-end of the mature sequence preferably from several independent experiments. The mapped reads should not overlap other annotated transcripts as they may represent fragments of mRNAs or other known RNA types.

Various tools were developed based on the annotation guidelines to analyze the HTS data sets. The major steps adopted by various available tools for prediction of novel miRs and their target identification are discussed in **Figure 5**. Basically the sequenced reads are selected, based on the average quality score appended with each base, and subjected to 3′ adapter trimming. This can be achieved by designing specific scripts (using languages such as PERL) or by using various available tools such as NGSQC Toolkit (Patel and Jain, 2012), FASTX-Toolkit (Gordon and Hannon, 2010), CLC Genomics Workbench (Matvienko)[1] etc. Next the reads with length of 18–24 nucleotides are selected and aligned to the corresponding genome of the plant species under consideration using tools such as bowtie, soap, and bwa. The aligned reads are then used to filter out sequences mapping with other sRNAs such as, tRNA, rRNA, sRNA, snRNA, snoRNA, and known miRs. The remaining reads are used to retrieve the potential precursors from the reference genome and their secondary structure is predicted. Excellent softwares like Mfold (Zuker, 2003), RNAfold (Denman, 1993) etc. are freely available and have been useful in identifying the appropriately folded structures. Then these candidate precursors are evaluated on the basis of the annotation criteria (Meyers et al., 2008). The expression profiles of identified known and novel miRs from sequence pools are achieved by calculating the number of times a unique read occurred in the entire sRNA pool and normalized against total reads. Reads Per Million (RPM) for each sequence occurring in each sample is most common way to achieve the normalized expression of each sequence. RPM = (Actual read count/total

[1]Matvienko, M. CLC Genomics Workbench.

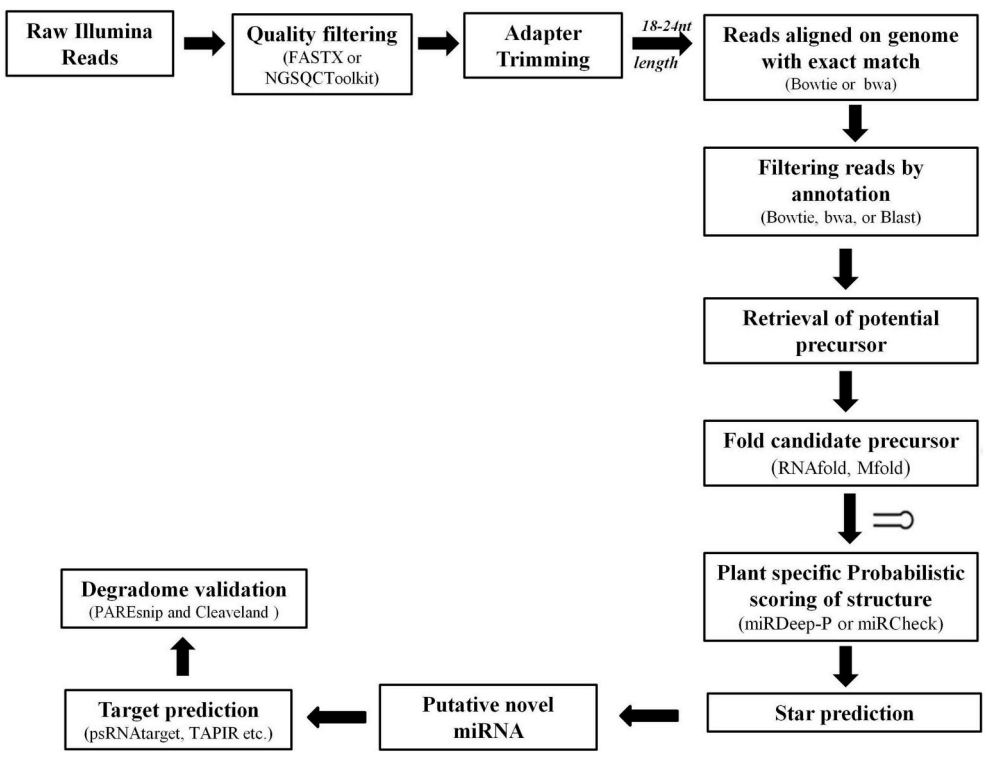


FIGURE 5 | Pipeline showing major steps for miR identification from high-throughput sequencing data.

number of reads in sample) × 1,000,000) (Motameny et al., 2010).

MICRORNA REPOSITORIES

The study of miR and their targets by analyzing the sRNA and transcriptome sequences is greatly facilitated by the availability of numerous freely accessible tools and databases, which can be used by experimental researchers without any specialization in bioinformatics. The various web-based tools and databases available for the prediction and analysis of plant miRs and their targets are listed in **Tables 1**, **2**, respectively. Each of these is based on different algorithms and methodologies and has their respective strengths and shortcomings. However, the major limitation in most of these techniques is the requirement for a known sequence and the search for a conserved hairpin loop structure (Unver et al., 2009). To overcome these limitations, Kadri et al. (2009) developed the Hierarchical Hidden Markov Model (HHMM) that employs region-based structural information of pre-miRs without relying on phylogenetic conservation. It obtains the secondary structures on the basis of minimum free energy and then classifies the sequence with HHMM (Kadri et al., 2009). Some of the popularly used tools are discussed below.

miRCheck

This is an algorithm written in the form of a PERL script for identifying 20 mers having potential to encode plant miRs. The tool requires input of a putative hairpin sequences and their secondary structures. The presence of candidate 20 mer sequences is then searched within the hairpin to predict potential plant miR. This algorithm was first used for identifying conserved miRs in Arabidopsis and rice (Jones-Rhoades and Bartel, 2004).

UEA sRNA Workbench

It is a comprehensive tool for the complete analysis of sRNA sequencing data and provides the convenience of using the facilities provided by different tools in one place. Its Graphical User Interface (GUI) makes it easy to use for researchers, do not needs any prior knowledge of computer programming (Moxon et al., 2008). It can be downloaded and installed locally, and it also has a web-based facility of doing the same analysis in form of UEA sRNA toolkit which is freely accessible. **Table 3** lists all the available tools at UEA sRNA Workbench.

TAPIR

This is an online web server for prediction of targets of plant miRs. It can characterize miR-targets duplexes with large loops which are usually not detectible by traditional target prediction tools. The prediction results are driven by a combination of two different algorithms. The first one is the fast and canonical FASTA local alignment program which cannot detect duplexes with large number of bulges and/or mismatches (Pearson, 2004) and second one is RNAhybrid (Krüger and Rehmsmeier, 2006) for detection of miR-mRNA duplexes (Bonnet et al., 2010). Though it is a good option for miR target prediction but is not preferred as the users face problem in analyzing large datasets on the online server.

CLC Genomics Workbench

It is a commercial software developed by QIAGEN that offers Quality Check (QC) and pre-processing of NGS data. Although it is a good tool for preprocessing of NGS data but it focuses more on other genomic areas such as *de novo* assembly and it doesn't provides the facility to process the sRNA data for miR and target identification. In relation to the sRNAs it has been majorly used in initial steps of quality filtering, adapter trimming and calculating abundances of sRNA libraries. It can also generate genome alignments by using standalone blast search. The workbench

TABLE 1 | Major Plant databases providing information on the miR and their targets.

Name	Description	Link	References
miRBase	Searchable database of published miR sequences and annotation	http://www.mirbase.org	Griffiths-Jones, 2004; Kozomara and Griffiths-Jones, 2010; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2014
deepBase	Database for annotating and discovering small and long ncRNAs (miRs, siRNAs, piRNAs) from high-throughput deep sequencing data.	http://deepbase.sysu.edu.cn/	Yang and Qu, 2012
PMRD	Database involving miRs and their target genes, especially model plants and major crops	http://bioinformatics.cau.edu.cn/PMRD/	Zhang et al., 2010
PNRD	It is an updated version of PMRD	http://structuralbiology.cau.edu.cn/PNRD/index.php	Yi et al., 2015
PMTED	Plant miR Expression Database	http://pmted.agrinome.org/	Sun et al., 2013
Plant MPSS	Measure's the expression level of most genes (including sRNA and their targets) under defined conditions and provide information about potentially novel transcripts. with the help of public HTS data	http://mpss.udel.edu/	Nakano et al., 2006
miRTarBase	The experimentally validated miR-target interactions database	http://mirtarbase.mbc.nctu.edu.tw/	Hsu et al., 2010
Rfam	A resource for predicted miR targets and expression	http://rfam.xfam.org/	Burge et al., 2013
ARMOUR	A Rice miRNA: mRNA Interaction Resource	http://armour.icgeb.trieste.it/	Unpublished

TABLE 2 | Major tools for analyzing plant miRs and their targets.

Name	Description	Link	References
PASmiR	A literature-curated database for miR molecular regulation in plant response to abiotic stress	http://pcsb.ahau.edu.cn:8080/PASmiR/	Zhang et al., 2013b
isomiRex	Web portal to identify miRs and their isoforms as well as differential expression of NGS datasets	http://bioinfo1.uni-plovdiv.bg/isomiRex/	Sablok et al., 2013
CLC genomics Workbench	Analyze, compares and visualizes NGS data	http://www.clcbio.com/products/clc-genomics-workbench/	
miRTarBase	The experimentally validated miR-target interactions database	http://mirtarbase.mbc.nctu.edu.tw/index.php	Hsu et al., 2010
MIRFINDER	Computational pre-miR prediction tool	http://www.bioinformatics.org/mirfinder/	Bonnet et al., 2004b
Targetfinder	Predicts small RNA targets in a sequence database using a plant-based scoring metric	http://carringtonlab.org/resources/targetfinder	
mirCheck	A PERL script designed to identify RNA sequences with secondary structures similar to plant miRs	http://bartellab.wi.mit.edu/software.html	Jones-Rhoades and Bartel, 2004
findmiRNA	Predicts potential miRs within candidate precursor sequences that have corresponding target sites within transcripts	http://sundarlab.ucdavis.edu/mirna/	Adai et al., 2005
MicroInspector	A web tool for detection of miR binding sites in a RNA sequence	http://bioinfo.uni-plovdiv.bg/microinspector/	Rusinov et al., 2005
RNAhybrid	Calculates a minimal free energy hybridization of RNA sequence(s) and miR(s)	http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid/	Krüger and Rehmsmeier, 2006
CleaveLand	A pipeline for using degradome data to find cleaved small RNA targets	http://axtell-lab-psu.weebly.com/cleaveland.html	Addo-Quaye et al., 2009
TAPIR	Target prediction for Plant miRs	http://bioinformatics.psb.ugent.be/webtools/tapir/	Bonnet et al., 2010
psRNATarget	A plant sRNA target analysis server	http://plantgrn.noble.org/psRNATarget/	Dai and Zhao, 2011
miRanalyzer	miR detection and analysis tool for next-generation sequencing experiments	http://bioinfo5.ugr.es/miRanalyzer/miRanalyzer.php	Hackenberg et al., 2011
PmiRKB	Plant miR knowledge base includes the miRs of two model plants, Arabidopsis and rice. Four major functional modules, SNPs, Pri-miRs, MiR-Tar and Self-reg, are provided	http://bis.zju.edu.cn/pmirkb/	Meng et al., 2011b
miRDeep-P	A computational tool for analyzing the miR transcriptome in plants	http://faculty.virginia.edu/lilab/miRDP/	Yang and Li, 2011b
C-mii	A tool for plant miR and target identification	http://www.biotec.or.th/isl/c-mii	Numnark et al., 2012b
Semirna	Searching for plant miRNAs using target sequences	http://www.bioinfocabd.upo.es/semirna/	Muñoz-Mérida et al., 2012
UEA sRNA Workbench	A suite of tools for analysing and visualizing NGS datasets	http://srna-workbench.cmp.uea.ac.uk/	Stocks et al., 2012
mirTool	A comprehensive web server providing detailed annotation information for known miRs and predicting novel miRs that have not been characterized before	http://centre.bioinformatics.zj.cn/mirtools/	Wu et al., 2013
miRPlant	An Integrated Tool for Identification of Plant MiR from RNA Sequencing Data	http://www.australianprostatecentre.org/research/software/mirplant	An et al., 2014
MTide	An integrated tool for the identification of miR-target interaction in plants	http://bis.zju.edu.cn/MTide/	Zhang et al., 2014b

provides an interactive visualization to the differential expression and statistical analysis of RNA-Seq and sRNA data.

C-mii

It uses a homology-based approach for plant miR and target identification. The tool aligns known miRs from different plant species to the EST sequences of the query plant species using blast homology search. The aligned sequences are allowed to fold in to the characteristic hairpin loop structures to identify the putative miRs. The predicted miR sequences are further used for identifying perfect or nearly perfect complimentary sites on the input transcript sequences to identify the putative targets. The tool has a unique feature of predicting the secondary structures of the miR-target duplexes. The identified targets can be annotated further by searching their functions and Gene Ontologies (GO) (Numnark et al., 2012a). It provides user friendly GUI, and is easily downloadable hence it can be easily used for analyzing large datasets. However, the major limitation lies in the search and availability of homologous sequences, so it cannot be used to analyze the NGS datasets.

TABLE 3 | Tools available on UEA sRNA Workbench and their functions in analyzing the sRNA sequencing data.

Tool	Function	References
Adapter removal	Removes the adapter sequence	Moxon et al., 2008
Filter	It filters already annotated sRNA (rRNA, tRNA. snRNA, snoRNA, miRNA etc) data	Moxon et al., 2008
Sequence alignment	Allows alignment of short reads to the genome	Moxon et al., 2008
CoLIde	It defines a locus as a combination of regions sharing same expression profiles, present in close proximity on genome	Mohorianu et al., 2013
miRCat	Predicts miRs from HTS data without requiring the precursor sequence	Moxon et al., 2008
miRProf	Determines normalized expression levels of sRNAs matching to known miR in miRBase	Moxon et al., 2008
PAREsnip	Finds target of sRNA using degradome data.	Folkes et al., 2012
SiLoCo	Compares expression patterns of sRNA loci among different samples	Moxon et al., 2008
ta-si Prediction	Trans-acting RNA prediction, by identifying 21nt characterstic of ta-siRNA loci by using sRNA dataset and respective genome	Moxon et al., 2008
RNA/Folding annotation	Predicts the secondary structure of RNA sequences and annotates it by highlighting up to 14 comma seperated short sequences	Moxon et al., 2008
VisSR	Used for sequence visualization	Moxon et al., 2008

miRdeep-P

It is a collection of PERL scripts that are used for prediction of novel miRs from deep sequencing data. It was developed by incorporating the plant-miR specific criteria to miRDeep (Friedländer et al., 2008). Its pipeline utilizes bowtie for sequence alignments and RNAfold for secondary structure prediction of putative precursors. The remaining steps such as extracting potential precursor sequences and identification of putative novel miR is regulated by specific scripts (Yang and Li, 2011a). Although it is a specialized tool for identification of plant miRs, but does not has a GUI interface. So the user needs to work through command line for its execution, which warrants knowledge on PERL scripting.

CleaveLand

It is a general pipeline, available as a combination of PERL scripts, for detecting miR-cleaved target transcripts from degradome datasets (Addo-Quaye et al., 2009). It can be executed by a single command and requires input of degradome sequences, sRNAs, and an mRNA database to yield an output of cleaved targets. The pipeline runs in command mode and requires the co-installation of several dependencies such as PERL, R, samtools, bowtie, RNAplex etc.

ARMOUR

The accumulation of sequencing data has generated the need for a comprehensive and integrated database of miR:mRNA, expression profile information and target information. Our group has developed ARMOUR database (A Rice miRNA: mRNA Interaction Resource) that consolidates extensive datasets of rice miRs from various deep sequencing datasets for examining the expression changes with respect to their targets. Development of such interactomes for different plant species shall provide a valuable tool to biologists for selecting miRs for further functional studies.

PERSPECTIVES

miRs are an extensive class of endogenous, small regulators of gene expression in the numerous developmental and signaling pathways. There is ample evidence for the role of miRs in abiotic stress mediated genomic changes that result in attenuation of plant growth and development. The different experimental approaches have identified the intriguing expression profiles of miRs in distinctive tissues and/or stages of development. The regulation of miR expression also varies between the domesticated plant species and their wild relatives. Sequence-based profiling along with computational analysis has played a pivotal role in the identification of stress-responsive miRs, although these results require independent experimental validations. sRNA blot and RT-PCR analysis have played an equally important part in systematically confirming the profiling data. The identification of putative targets for these miRs has provided robust confirmation of their stress responsiveness. This has also enabled quantification of their effect on the genetic networks, such that many of the stress regulated miRs have emerged as potential candidates for improving plant performance under stress. However, so many efforts are still required for in-depth analysis of the miR modulation of each gene product induced by abiotic stress(es) and its interacting partners. This requires development of reliable and rigorous assays for firm characterization of the spatio-temporal regulation of these miRs under stress conditions. The potential of computational biology needs to be tapped for performing an extensive comparison of miR expression profiles among agriculturally important crops during environmental stress conditions to tap key target nodes that need to be modulated for improving crop tolerance to environmental stress. The development and integration of plant synthetic biology tools and approaches will add new functionalities and perspectives in the miR biology to make them relevant for genetic engineering programs for enhancing abiotic stress tolerance.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00286

Supplementary Table 1 | List of all abiotic stress associated plant miRs.

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Differential expression of seven conserved microRNAs in response to abiotic stress and their regulatory network in *Helianthus annuus*

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Biotic and abiotic stresses affect plant development and production through alternation of the gene expression pattern. Gene expression itself is under the control of different regulators such as miRNAs and transcription factors (TFs). MiRNAs are known to play important roles in regulation of stress responses via interacting with their target mRNAs. Here, for the first time, seven conserved miRNAs, associated with drought, heat, salt and cadmium stresses were characterized in sunflower. The expression profiles of miRNAs and their targets were comparatively analyzed between leaves and roots of plants grown under the mentioned stress conditions. Gene ontology analysis of target genes revealed that they are involved in several important pathways such as auxin and ethylene signaling, RNA mediated silencing and DNA methylation processes. Gene regulatory network highlighted the existence of cross-talks between these stress-responsive miRNAs and the other stress responsive genes in sunflower. Based on network analysis, we suggest that some of these miRNAs in sunflower such as *miR172* and *miR403* may play critical roles in epigenetic responses to stress. It seems that depending on the stress type, theses miRNAs target several pathways and cellular processes to help sunflower to cope with drought, heat, salt and cadmium stress conditions in a tissue-associated manner.

Keywords: abiotic stress, miRNA, sunflower (*Helianthus annuus*), regulatory network

Introduction

Abiotic and biotic stresses impose challenging physiological hurdles to plants. As a response to adverse environmental conditions, plants re-program their cellular activities through multiple gene regulatory mechanisms including post-transcriptional regulation of gene expression. Transcription factors (TFs) and non-coding RNAs are the two major regulatory elements in functional genomics (Deihimi et al., 2012; Mahdi et al., 2013; Panahi et al., 2013; Chiasson et al., 2014).

Small RNAs, particularly microRNAs (miRNAs), have emerged as key post-transcriptional gene regulatory molecules in plants and animals (Trindade et al., 2010; Giacomelli et al., 2012; Sunkar et al., 2012). MiRNAs are 18–22 nucleotides in length and regulate expression of their target genes by degradation or translational repression (Voinnet, 2009; Sunkar et al., 2012). In particular, miRNAs play important roles in plant responses to abiotic and biotic stresses including cold, salt, heat, dehydration, oxidative and mechanical stresses (Jin et al., 2013; Panahi et al., 2013). For example, some of the genes in *ARF* transcription factor family are targeted by *miR160* and *miR167* through auxin regulation interference (Gutierrez et al., 2009), and seem to be important for the attenuation of plant growth and development under stress conditions (Sunkar et al., 2012). Another microRNA, *miR398*, is associated with response to abiotic and biotic stress condition (Zhu et al., 2011) and its expression is up-regulated in response to water deficit in *Medicago truncatula* (Trindade et al., 2010). *MiR403* controls the expression of *AGO2* (Allen et al., 2005), which is known to have an antiviral role (Harvey et al., 2011). Srivastava et al. (2013) reported that *miR426* is up-regulated in response to arsenic stress in *Brassica juncea*. In another study, *miR842* expression levels were observed to be up-regulated in *Arabidopsis* (Moldovan et al., 2010). These are handful examples of miRNAs that are differentially expressed in response to various stress conditions.

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops and is resistant to various abiotic stresses, due to its metabolic, physiological and morphological adaptation strategies. This crop is of special interest for its adaptation to high temperatures, limited water availability, high salinity and heavy metal concentrations in the soil (Merah et al., 2012). Using bioinformatics methods, previous studies have identified and reported several miRNAs in sunflower. A number of these were experimentally verified (Barozai et al., 2012; Monavar Feshani et al., 2012). However, their expression patterns under different stress conditions have not been studied.

We have examined the roles of seven of these sunflower miRNAs in response to different abiotic stresses including drought, high temperature, salinity and cadmium. Seven mentioned miRNAs, *miR160*, *miR167*, *miR172*, *miR398*, *miR403*, *miR426* and *miR842*, play crucial roles in plant growth and show different expression patterns under both biotic and abiotic stress conditions in several plant species. Simultaneously, we analyzed the expression levels of these miRNAs concurrently with their potential targets to corroborate the putative miRNA-target relationship. Based on our results, several gene regulatory networks were constructed to gain a comprehensive overview of the role of these miRNAs in stress responses.

Materials and Methods

Prediction of miRNA Targets

Most plant miRNAs bind to their targets with perfect or near-perfect complementarity (Jones-Rhoades and Bartel, 2004). We have used this characteristic for predicting potential miRNA targets. Sequences of pre-miRNAs were obtained from the public database, miRBase (Kozomara and Griffiths-Jones, 2011). The number of mismatches at complementary sites between miRNA sequence and potential mRNA target were kept below 4 and no gap at the complementary sites was tolerated. The miRNA targets were predicted using psRNAtarget online sever (http://plantgrn.noble.org/psRNATarget/).

Plant Material, Growth Conditions and Stress Treatments

Sunflower (*Helianthus annuus,* variety *Sirna*) seeds were surface sterilized and placed in petri dishes containing two layers of damp sterile filter paper. For germination, the seeds were incubated at room temperature for 4 days.

For applying drought stress treatment, five germinated seeds were sown in round pots (10.5 × 13.5 cm, D × H; five plants/pot) filled with soil (3/4 river sand and 1/4 soil). Plants were grown in the glasshouse under the controlled condition (16 h of light, 24°C; 8 h of dark, 15°C) and well watered.

For heat, salinity and cadmium stress treatments, five seedlings were grown in a hydroponic system. The system contained aerated Hoagland solutions, pH 5.8 (Hoagland and Arnon, 1950) in plastic pots (1.5 liters volume). One-week-old plantlets were planted in 1/4 strength Hoagland's nutrient for 1 more week to immerse the growing roots in the nutrient solution. Plantlets were then transferred to half-strength nutrient solution and grown until 6-leave stage (about 4 weeks). Four weeks old plantlets were then submitted to different stresses as specified in the following sections.

Drought Stress

Four weeks old plantlets (6 leaf stage) of sunflower were subjected to water stress by withholding water for 12, 24 and 48 h. Relative water content (RWC) was measured in leaves to determine the plant water status following Faatsky method (Čatský, 1960).

Heat Stress

Four weeks old plantlets in ½ strength Hoagland solution were transferred to humidity growth chamber (Memmert, Germany) with 70% relative humidity and maintained at 42 ± 1°C for 1.5, 3 and 6 h (Senthil-Kumar et al., 2003; Mashkina et al., 2010; Mangelsen et al., 2011). We selected stress-related *HSP70*-related protein gene (Gene Bank ID: AAB57695.1) to investigate the effect of heat stress in root tissue of sunflower.

Salt Stress

Plantlets were moved into solution contained either 75 or 150 mM of NaCl (Di Caterina et al., 2007). Leaf and root samples from stressed plants were collected 24 h later alongside control samples. Potassium to sodium ratio was measured as criteria for examining different salinity stress levels.

Cadmium Stress

Plantlets were transferred into containers with either 5 or 20 mg/L of $CdCl_2$ (Niu et al., 2007). Stress and control samples were collected after 24 h from leaf and root tissue and immediately frozen in liquid nitrogen before they stored at −80°C. Cadmium content was measured as an indicative of stress.

Total RNA Isolation from Leaves and Roots

Total RNAs were extracted from sunflower root and leaf tissues using TRIZOL Reagent (Invitrogen, USA) according to the manufacturer's instructions. The extractions were performed separately for two independent biological replicates. Equal amount of total RNA was subsequently pooled for each sample based on their concentration. The quantity and quality of purified RNAs were assessed with a Biophotometer spectrophotometer (Eppendorf, Germany) and the integrity was evaluated by conducting a denaturing agarose gel electrophoresis. All RNA samples were stored at −80°C until further processing.

Stem-loop Reverse Transcription

Stem-loop RT primers for *H. annuus miR160*, *miR167*, *miR172*, *miR398*, *miR403*, *miR426* and *miR842* were designed according to Varkonyi-Gasic et al. (2007) (Table S1). The miRNA specific RT reactions were carried out using prime script RT reagent kit (Cat. #RR037A, TAKARA, Japan). The miRNA stem–loop reverse transcription was accomplished according to Varkonyi-Gasic et al. (2007) with minor modifications at cDNA synthesis, using 200 ng of total RNA sample of treated leaf and root tissues (1 μL), 1 μL of 1 μM miRNA primer RT and 5.5 μL nuclease-free water. The mix was incubated at 65°C for 5 min followed by incubation on ice for 2 min. Two microliters of Primescript buffer (5×) and 0.5 μL Primescript RT enzyme mix were added to each tube and the RT reaction was performed for 30 min at 16°C followed by 60 cycles at 30°C for 30 s, 42°C for 30 s, 50°C for 1 s and terminated by incubation at 85°C for 5 min. Control reactions were performed with no RT primer and no RNA samples.

Quantitative Real-time PCR

To measure and compare the expression levels of the selected *H. annuus* miRNAs in root and leaf tissues under different stress treatments, RT-qPCR was conducted using SYBR *Premix Ex Taq* II (Cat. #RR820A, TAKARA) in a Rotor-GeneQ Real-Time PCR system (Qiagen).

For RT-qPCR analysis, 6 μL SYBR *Premix Ex Taq* II (2×), 1 μL (10 pmol) each of forward and reverse primers, 3 μL nuclease-free water and 1 μL RT stem-loop cDNA product were mixed. Forward primers were specifically designed for each miRNA, and 5′-GTGCAGGGTCCGAGGT-3′ sequence was used as the universal reverse primer (Varkonyi-Gasic et al., 2007) (Table S1). The RT-qPCR reactions were performed using following conditions; initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. The melting curves were generated during denaturation step at 95°C followed by the cooling of PCR products at 50°C and the fluorescence signals were collected continuously from 50 to 95°C as the temperature increased at 0.2°C per second. All reactions were repeated four times. For each condition, the RT-qPCR experiments were run as pooled biological duplicates and expression levels were normalized according to the previous studies (Schmittgen and Livak, 2008). Relative fold changes in miRNA expression were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method with *18S rRNA* as the endogenous control (Schmittgen and Livak, 2008). The miRNAs clustering analysis, based on their relative expression, and Pearson correlation between expression miRNAs and target genes in each stress were performed using Minitab 16.0. The heat map of expression was visualized using R 3.0 package.

Analysis of miRNAs Target Expression with RT-qPCR

To determine the expression of predicted miRNA targets and possible discovery of novel miRNA target genes under the drought, heat, salinity and cadmium stresses in sunflower, expression levels of miRNA-related target genes were measured with quantitative real-time PCR. ESTs for target quantification analysis were selected based on two criteria: (1) protein found in BLAST search should be a previously published target of the related miRNA in other plant species. (2) The EST should have a possible protein-coding ORF that can allow us to design RT-qPCR primers for the conserved 3′ UTR region. RT-qPCR primers for these selected genes (Table S1) were designed using Primer3 according to following criteria: (1) Primers 3′ self-complementary = 0. (2) Primer annealing temperatures = 62 ± 0–3°C. (3) Product size limit for primer pairs = 150 bp.

Total cDNAs were synthesized from 1 μg RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Further expression analysis was performed for all miRNA targets using the same batch of RNA samples for miRNA RT-qPCR assay. One microliter of this cDNA was amplified with 0.6 μM of specific primers in a total of 10 μl volume using SYBER *Premix Ex Taq* II (Cat. #RR820A, TAKARA) with Rotor-GeneQ Real-Time PCR system (Qiagen). The quantification was performed using *18S rRNA* (Gene ID: 18250984, forward: TTCAGACTGTGAAACTGCGAATGG /reverse: TCATCGCAGCAACGGGCAAA) as a normalizer and four independent PCR results with acceptable efficiency (1.8–2.2) were averaged. Specified RT-qPCR thermal setup was adjusted as follows: pre-denaturation step at 95°C for 1 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min. The melting curves were generated as mentioned for miRNAs.

Network Analysis of miRNAs

To construct miRNAs and target genes interaction network, RESNET Plant database of Pathway Studio software v.9 (Elsevier) was used. This database includes new aliases for genes in the model and non-model plant species including barely, corn, tomato, potato and tobacco and collects data through MedScan (text mining tool) to extract functional relationships between miRNAs, proteins, stresses and cellular processes (Nikitin et al., 2003; Alimohammadi et al., 2013; Ebrahimie et al., 2014). In addition to adding the result of this study to prediction database of RESNET database, this database was also updated by MedScan, especially from literature on miRNAs/target genes and drought, heat, salt and cadmium stress conditions before network construction. To predict the interaction networks, the software makes different groups of miRNAs and finds the relations between a protein and its group using algorithms such

as Fisher's Exact Test (Alanazi et al., 2013; Ebrahimie et al., 2014).

Network constructed by union selected and physical interaction algorithms were used to make statistical subnetworks based on miRNAs and their target genes. Also, GO (gene ontology) analysis was performed through DAVID Functional Annotation web-tool (https://david.ncifcrf.gov/) and separate tables were produced for biological process, molecular function and cellular component categories. MiRNAs and putative target genes are marked with yellow circles (**Figures 6**, **7**).

Results

In this study, expression levels of seven miRNAs including *miR160*, *miR167*, *miR172*, *miR398*, *miR403*, *miR426* and *miR842* and their targets were investigated in response to four abiotic stress conditions including drought, heat, salt and cadmium stresses. In addition, the impacts of these stresses on the physiological and molecular characteristics of plants were evaluated by measuring the changes in RWC, expression of *HSP70*-related protein, sodium, potassium and cadmium concentrations in different tissues of plant, grown under stress (Supplementary 1). The expression level of *HSP70*-related protein up-regulated over time compared to control in root tissue with the highest level at 6 h (Table S2). Moreover, alteration of sodium, potassium and cadmium concentration were remarkably higher in root tissue compared to leaf, in particular in later stage of stress (Table S3A). The average RWC of plants in response to drought stress showed slight decline at 12 and 24 h, and it started to reduce sharply subsequently (Table S3B). The results indicated that plants were significantly affected by stress exposure and were able to initiate stress related responses.

Prediction and Determination of *H. annuus* miRNA Targets

The mRNA targets of these miRNAs (**Table 1**) were predicted via psRNAtarget database using selected miRNAs as queries. Targets of *miR167* and *miR403* were previously reported in the other plant species (**Table 1**). We predicted and analyzed new targets for *miR160*, *miR398*, *miR426* and *miR842* for the first time in sunflower. Protein sequence analysis of the new putative *Helianthus miR160* target, *QHG18J04.yg.ab1* showed the presence of ARF, phosphorylase and kinase domains (data not shown) in this protein. Interestingly, *COX5b*, which was previously introduced as *miR398* target in *Arabidopsis*, was identified as a predicted target for *miR172* in sunflower.

Expression of miRNAs and Putative Targets under Drought Stress

MiRNAs expression levels were significantly down-regulated ($P < 0.05$) in leaves of plants grown under drought stress with the lowest expression levels were at 48 h (except *miR403*). *MiR172* expression modulation was not significant ($P < 0.05$) in all period of stress in root tissue. The expression patterns of *miR160*, *miR426* and *miR842* were similar in both tissues, except *miR160* which showed opposite pattern at 48 h after drought stress in root tissue. The miRNAs, *miR167* and *miR398* showed similar trend under drought stress in both tissues. They were down-regulated at all-time points within range of 2- to 19-fold change, whereas expression of *miR167* was slightly up-regulated at 48 h compared to moderate stress in root tissue. *MiR403* showed the opposite pattern in leaf and root tissues at 24 h. Its expression abruptly decreased (24-fold compared to control) in leaf tissue. It, however, exhibited an increasing trend in root tissue while its expression was still lower than control condition (**Table 2**; **Figure 1**).

The expression of *QHG18J04.yg.ab1* gene as target for the *miR160*, was down- regulated at 12 h and 24 h (2- to 8-fold), and up-regulated at 48 h in both tissues. Except at 48 h in leaf tissue, correlation of *miR160* and its target showed similar expression pattern over time and tissue. The expression of *ARF6* (target of *miR167*), *COX5b* (target of *miR172*), *AGO2* (target of *miR403*) and *TPP2* (target of *miR426*) displayed similar patterns in both tissues; their expression was induced only in the root tissue at 48 h of drought stress. Interestingly, the coherent trend was observed only at 48 h in the root tissue for *miR167*, *miR403* and *miR426* and their respective targets. The transcript of *NtGT5b* (target of *miR398*) was constantly decreased in both tissues with 2- to 10-fold under drought conditions where the minimum peak was at 48 h and 24 h after treatment in root and leaf tissue, respectively. The expression of *(R)-mandelonitrile lyase* (target of *miR842*) increased at 12 h and decreased at subsequent time points in leaf tissue whereas it showed opposite pattern in root. In general, most targets exhibited their highest expression level at 48 h after stress depending on the tissue (**Table 2**; **Figure 1**).

TABLE 1 | Putative *Helianthus annuus* stress-inducible miRNA target genes.

miRNA	miRNA sequence	Predicted targets	Accession number
miR160g	UGCCUGGCUCCCUGUAUGCCA	QHG18J04.yg.ab1	BU026935
miR167e	UGAAGCUGCCAGCAUGAUCU	Auxin Response Factors6 (ARF6)	839913
miR172d	AGAAUCCUGAUGAUGCUGC	Cytochrome-c oxidase activity (COX5B-2)	844363
miR398	GUGUUCUCAGGUCGCCCC	Glycosyltransferase (NtGT5b)	AB176524.1
miR403	UUAGAUUCACGCACAAACUCG	Argonaute 2 (AGO2)	840016
miR426	CUUUGGAAGUUUGUCCUUAGU	Probable trehalose-phosphate phosphatase 2 (OsTPP2)	AB277360.1
miR842	UCAUGGUCAGAUUCAUCAUCC	(R)-mandelonitrile lyase	AT1G73050

TABLE 2 | Expression of miRNAs and their target genes response to abiotic stress in *Helianthus annuus*.

miRNA/Target name	Drought stress						Heat stress						Salt stress				Cadmium stress			
Tissue	Leaf			Root			Leaf			Root			Leaf		Root		Leaf		Root	
	12 h	24 h	48 h	12 h	24 h	48 h	1.5 h	3 h	6 h	1.5 h	3 h	6 h	75 mM	150 mM	75 mM	150 mM	5 mg	20 mg	5 mg	20 mg
miR160	↓1.80**	↓2.87*	↓8.04*	↓1.41^{ns}	–	↑1.80**	↑2.22**	↓1.75**	↓1.54*	↑2.54*	↓10.02**	↓16.27**	↑2.25**	↓2.7**	↓1.81*	↑1.16^{ns}	↑2.21**	↓2.3538**	↑14.35**	↑14.85**
T160	↓8.57*	↓4.75*	↑2.54*	↓2.40^{ns}	↓2.92^{ns}	↑4.41**	↓6.49**	↑7.81**	↓1.09^{ns}	↓1.36^{ns}	↓1.23^{ns}	↑2.15**	↑4.26**	↑1.58*	↑1.14$^{n.s}$	↑1.67**	↑2.62**	↑5.15**	↑32.62**	↑47.42**
miR167	↓2.41*	↓10.19*	↓13.22*	↓4.28*	↓19.02*	↓9.67*	↑1.56*	↓1.39^{ns}	↓2.10**	↑4.04**	↓9.96**	↓3.97**	↓1.56*	↓2.0**	↑1.68*	↑2.1**	↑3.69**	↑6.96**	↑322.35**	↑25.24**
T167	↓13.45*	↓5.12*	↓8.13*	↓1.55*	↓2.10*	↑1.86**	↑1.34^{ns}	↓1.94**	↓2.33**	↓1.39*	↓39.39**	↓57.68**	↓1.67**	↓1.03**	↓1.03^{ns}	↑1.23^{ns}	↑1.46^{ns}	↓1.57^{ns}	↑22.51**	↑30.75**
miR172	↓6.38*	↓5.46*	↓6.64*	↑1.20^{ns}	↑1.56^{ns}	↑1.62^{ns}	–	↓2.43**	↓1.20^{ns}	↑2.37**	↓12.62**	↓2.16**	↓1.42^{ns}	↑1.22^{ns}	↓2.0^{ns}	↓1.07^{ns}	↑1.78**	↑1.005^{ns}	↑14.27**	↑57.38**
T172	↓5.85*	↓10.37*	↓18.06*	↓3.86*	↓2.0*	↑3.03*	↑1.36^{ns}	↓1.32^{ns}	↓1.47^{ns}	↑2.92**	↓9.18**	↓6.84**	↓2.44**	↓4.76**	↓1.70**	↓1.17^{ns}	↓3.58**	↓2.03**	↑5.34**	↑32.96**
miR398	↓1.65**	↓3.60*	↓16.85*	↓3.32*	↓2.08**	↓7.63*	↑3.42**	↑1.82^{ns}	↑2.68**	↑1.65*	↓8**	↓2.92**	↑11.31**	↑3.92*	↑20.40**	↑1.23^{ns}	↓1.62**	↑1.62**	↑385.34**	↑123.43**
T398	↓5.55*	↓5.65*	↓10.02*	↓2.25**	↓4.51*	↓2.63*	↓10.07**	↑3.23**	↑1.01^{ns}	↓2.28**	↑3.17**	↓1.28^{ns}	↑1.65*	↓2.08**	↑1.44^{ns}	↓2.27**	↓16.28**	↑1.37^{ns}	↓3.83**	↑10.93**
miR403	↓2.87*	↓24.67*	↓5.46*	↓8.77*	↓1.21^{ns}	↓10.99*	↑3.42**	↑1.72**	↑1.16^{ns}	↑1.20^{ns}	↓8.72**	↓2.96**	↑2.91**	↑1.18^{ns}	↓1.19^{ns}	↓1.41*	↑6.28**	↑1.02^{ns}	↑52.62**	↑35.94**
T403	↓3.60*	↓6.72*	↓2.29^{ns}	↓1.93**	↓2.33*	↑16.85*	↑2.63**	↑1.18^{ns}	↓1.12^{ns}	↑3.19**	↓1.31^{ns}	↓1.360^{ns}	↑2.83**	↓2.51**	↑2.46**	↑3.25**	↑1.07^{ns}	↓1.03^{ns}	↑22.12**	↑65.91**
miR426	↓3.19*	↓2.87*	↓5.06*	↓6.61*	↓1.41^{ns}	↓4.0*	↑1.36^{ns}	↓1.66^{ns}	↑1.28^{ns}	↑1.68**	↓38.71**	↓7.90**	↓1.46**	↓2.25**	↓1.11^{ns}	↑9.19**	↑1.80**	↑1.17^{ns}	↑11.65**	↑16.77**
T426	↓3.30*	↓3.08*	↓2.46*	↓1.83^{ns}	↓3.03*	↑4.83*	↓1.59**	↓1.52*	↓1.96**	↑1.46**	↓14.84**	↓13.29**	↓1.94**	↓3.38**	↑1.04^{ns}	↑2.11**	↓2.13**	↓2.03**	↑10.27**	↑14.75**
miR842	↓6.06*	↓10.02*	↓15.81*	↓5.37*	↑1.05^{ns}	↓3.86*	↑1.93^{ns}	↓1.47**	↓1.65^{ns}	↓1.49*	↓24.11**	↓4.72**	↑1.19^{ns}	↓10.7**	↓1.01^{ns}	↑3.34**	↑1.34**	↑2.26**	↑10.87**	↑4.65**
T842	↓1.71**	↓1.168^{ns}	↓3.30*	↓3.73*	↑1.53*	↑3.40*	↑1.09^{ns}	↓1.16^{ns}	↓5.99**	↓1.43^{ns}	↓2.37**	↓1.67^{ns}	↑1.65*	↑2.60**	↑4.50**	↑29.86**	↓2.34**	↓1.60^{ns}	↑7.96**	↑32.96**

*Sunflower seedling at the six leaf stages grew under normal conditions and leaves and roots of them were harvested as control then they treated by withholding the water for 12, 24 and 48 h. Plants treated at 42 ± 1°C for 1.5, 3, and 6 h. Plant subjected under NaCl treatment in two concentration, 75 and 150 mM. Plants treated with cadmium in 5 and 20 mg/L concentration. Number shows the fold change in relative to control. ↑ The expression is up-regulated. ↓ The expression is down-regulated. * Show that the t-test is significant as $p < 0.05$.** Show that the t-test is highly significant as $p < 0.01$. ns show that the t-test is not significant.*

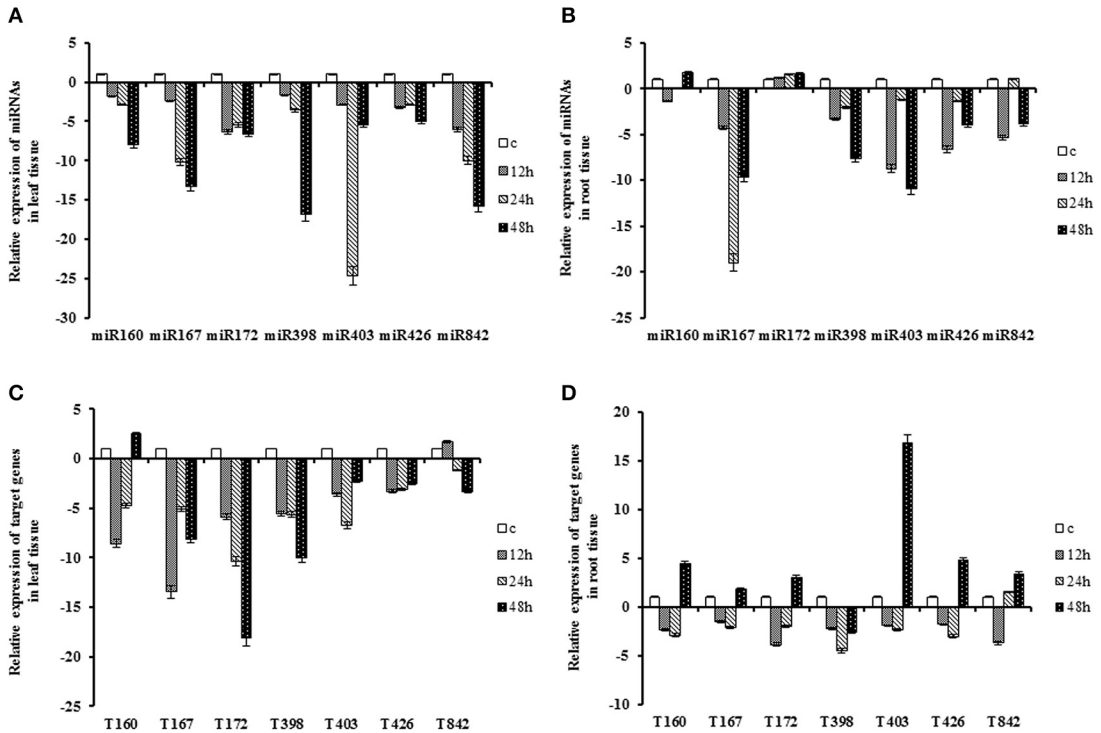


FIGURE 1 | Expression patterns of miRNAs and their target genes under drought stress. Sunflower seedling at the six leaf stages grew on soil (3/4 river sand and 1/4 soil) under normal conditions and leaves and roots of them were harvested as control then they treated by withholding the water for 12, 24 and 48 h. **(A)** Expression patterns of miRNAs in the leaf. **(B)** Expression patterns of miRNAs in the root. **(C)** Expression patterns of target gene in the leaf. **(D)** Expression patterns of target genes in the root.

Expression of miRNAs and Putative Targets under Heat Stress

The expression levels of all seven miRNAs in leaf tissue were slightly up-regulated after 1.5 h exposure to heat stress, except for *miR172* which had constant expression level. However, expression of miRNAs in leaves indicated a mixed pattern at 3 and 6 h after treatment. The expression levels of *miR167* and *miR172* were down-regulated within a range of 2- to 3-fold at 3 and 6 h after stress. But in root tissues, their reduction was between 2 and 12 fold at these two time points. *MiR398* and *miR403* showed similar trend in leaf and root tissues. Their expression was immediately up-regulated in leaf at the initial time point, although, their expressions were decreased at 3 and 6 h after stress. The levels of their expression were higher than control condition. However, in root tissue, they were up-regulated at 1.5 h but down-regulated subsequently compared to control with a 2- to 8-fold change at 3 and 6 h. The *miR160*, *miR426* and *miR842* exhibited similar trend under heat stress in leaf tissue. Their expressions were induced at 1.5 h and down-regulated at 3 h and again induced at severe stress. Nevertheless, their expressions were lower compared to the control. In root tissue, except 1.5 h after stress, *miR842* and *miR426* showed similar pattern within a range of 4- to 38-fold change after stress. The expression of *miR160* instantly decreased at 3 h (10-fold change compared to control) and its decrease continued constantly at 6 h (**Table 2**; **Figure 2**).

The expression of *QHG18J04.yg.ab1* was significantly down-regulated at 1.5 h and was reversed at 3 h, while its expression was only up-regulated at 6 h in root. The pattern of its transcript had negative correlation with *miR160*. Transcript of *ARF6* was down-regulated at all-time points with a sharp decrease in the root tissue at 3 h. The temporal variation of *COX5b* was significant only in the root tissue while it decreased sharply at 3 h after heat stress. *AGO2* expressed at a significant level at 1.5 h in both tissues where its expression was up-regulated with more than two-fold change, compared to the control. *NtGT5b* displayed similar pattern in both tissues. The expression level declined at an initial time point and was induced at 3 h after treatment, whereas it showed coherent type with *miR398*. In leaves, the expression of *(R)-mandelonitrile lyase* was abruptly down-regulated at 6 h, whereas its expression was declined slightly over time in the root. Expression of *TPP2* dropped in leaf tissue, but it decreased in root only at 3 h with 14-fold changes after stress. However, there was a slight increase in its expression at 6 h in the root. The expression was still lower compared with control (**Table 2**; **Figure 2**).

Expression of miRNAs and Putative Targets under Salt Stress

The expression of *miR167* showed opposite pattern in both tissues. In leaves, salt stress reduced the expression of *miR167* by two-fold while the expression level was up-regulated in the root tissues with a two-fold change. Interestingly, *miR403*

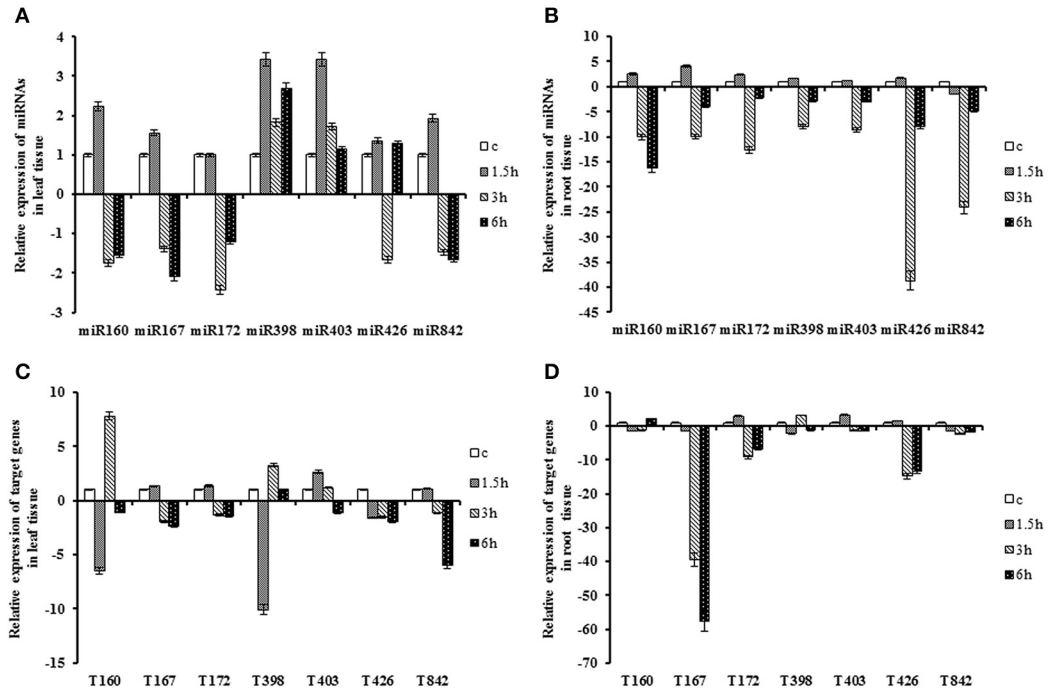


FIGURE 2 | Expression patterns of miRNAs and their target genes under heat stress. Plants treated at 42 ± 1°C for 1.5, 3, and 6 h. **(A)** Expression patterns of miRNAs in the leaf. **(B)** Expression patterns of miRNAs in the root. **(C)** Expression patterns of target gene in the leaf. **(D)** Expression patterns of target genes in the root.

exhibited opposite pattern of expression in leaf and root, whereas its expression had increasing and declining trend in leaf and root, respectively. The expression trends of *miR160*, *miR426* and *miR842* were similar in both tissues at severe stress. Their expression showed decreasing pattern in leaf and increasing pattern in root tissue. Interestingly, an abrupt gradient was observed for *miR842* in leaf tissue at 150 mM NaCl. None of the tissues disclosed a significant alteration for *miR172* expression in response to salt stress. Salt stress induced the *miR398* expression in both tissues with the sharp increase observed after 75 mM NaCl treatment in both tissues. In spite of this, there was a considerable reduction in its expression at 150 mM concentration in leaf and root, and the expression level was higher than control condition (**Table 2**; **Figure 3**).

Under salt stress, the transcripts of *QHG18J04.yg.ab1* slightly increased in the root. Although, there was a decline pattern at 150 mM of NaCl in comparison with an early stage of stress in the leaf tissue, its expression was higher compared to control. The expression of *ARF6* was not significant in the root. However, its expression showed down-regulation at both stages of treatment in leaf tissue. Its expression level revealed a slightly increasing trend at 150 mM compared with 75 mM of NaCl. *COX5b* expression was lower in both tissues at all stages of treatment with sharp reduction at 150 mM of NaCl in the leaf tissue. *NtGT5b* exhibited similar expression pattern in both tissues where its mRNA level was up-regulated at 75 mM concentration and was abruptly decreased at 150 mM. The *AGO2* transcript was highly accumulated at all stages in the root tissue, but in the leaf tissue, was induced at 75 mM of NaCl, and down-regulated subsequently compared with control. The expression pattern of *TPP2* was opposite in leaf and root tissue, as decreased in leaf and increased in root tissue with its peak of expression at severe stress. The expression of *(R)-mandelonitrile lyase* was induced in both tissues with its peak at 150 mM concentration (**Table 2**; **Figure 3**).

Expression of miRNAs and Putative Targets under Cadmium Stress

Cadmium treatment resulted in up-regulation of all miRNAs in roots, with fold-changes between 4 and 385. The lowest and highest peaks were for *miR842* in 20 mg/L and *miR398* in 5 mg/L concentration of cadmium, respectively. The expression of *miR426* was induced only at 5 mg/L in leaf, while in root induced at all stages of stress. The opposite pattern for *miR160* and *miR842* observed only at 20 mg/L in leaf tissue. The level of *miR160* was drastically decreased in leaf tissue, but the expression of *miR842* was slightly raised. In root tissue, their expression was increased although; the levels of *miR842* at 20 mg/L were declined in comparison to 5 mg/L, where its expression was still higher compared to control. *MiR403* expression was increased in both tissues whereas expression levels in root tissue were higher than leaf tissue. The highest expression of *miR403* was in 5 mg/L concentration with 52-fold change compared to control. The expression pattern of *miR172* was induced at all stages of treatment in both tissues with its peak at 20 mg/L concentration in root. Interestingly, the level of *miR398* was up-regulated at 5 mg/L, but down-regulated at 20 mg/L with 1.6-fold change in leaf tissue. Similar trend was observed for *miR167* and *miR398*

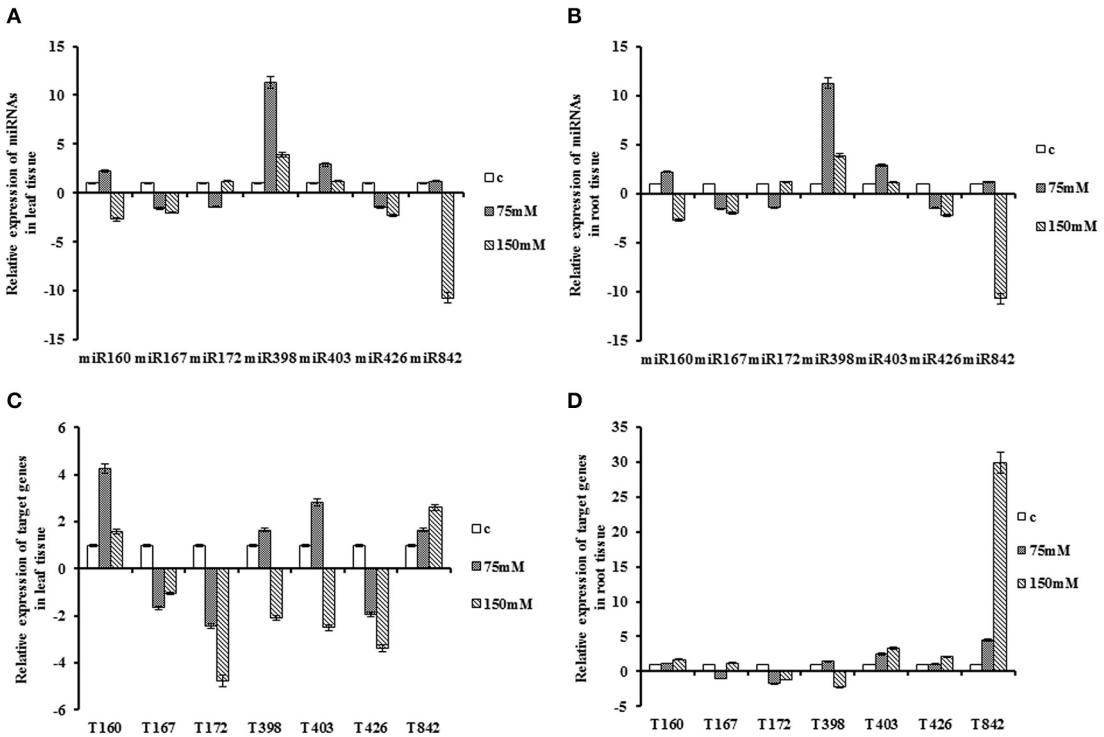


FIGURE 3 | Expression patterns of miRNAs and their target genes under salt stress. Plant subjected under NaCl treatment in two concentration, 75 and 150 mM. **(A)** Expression patterns of miRNAs in the leaf **(B)** Expression patterns of miRNAs in the root. **(C)** Expression patterns of target gene in the leaf. **(D)** Expression patterns of target genes in the root.

in root tissue, with the highest peak at 5 mg/L concentration (**Table 2**; **Figure 4**).

Transcript of *QHG18J04.yg.ab1* showed up-regulation trend in both tissues where in root, it was over 10-fold higher than leaf. The target of *miR167* and *miR403* exhibited similar trend in both tissues as their expressions were significantly induced in the root with their highest peak at 20 mg/L of $CdCl_2$. The expression of *COX5b* and *TPP2* was declined in leaf and was increased in root tissue in both concentrations of cadmium with its peak in 20 mg/L. *(R)-mandelonitrile lyase* highly accumulated in the root with abrupt increase at 20 mg/L. In contrast, its expression was decreased in leaf tissue after treatment. Expression of *NtGT5b* revealed temporal variation where it was down-regulated at 5 mg/L and up-regulated at 20 mg/L in both tissues. Interestingly, *miR172*, *miR398*, *miR426* and *miR842* showed inverse correlation with their targets in the leaf tissue at 5 mg/L (**Table 2**; **Figure 4**).

Cluster Analysis of miRNAs in *H. annuus* Root and Leaf Tissues based on their Expression Patterns

Cluster analysis was performed to further analyze the pattern of expression of these miRNAs under different stress conditions. A comparison of expression profiles of these seven miRNAs in both tissues was conducted to find their tissue-specific expression patterns, regardless of their response to different stress conditions. Three clusters were formed, when the expression pattern of the seven miRNAs were analyzed in response to stress (**Figure 5**). Cluster I included *miR167* and *miR398*. The expression pattern of leaf and root was opposite, but their trend was similar in each tissue over time and stress. In root tissue, these miRNAs revealed slight alteration in three treatments; the highest peak was in 5 mg/L cadmium stress. Cluster II contained *miR172* and *miR403* families and showed a fluctuated pattern in leaves and roots in all of stress conditions. Median value of expression of these miRNAs exhibited increasing pattern in four stresses. Due to similarity in expression patterns of *miR160*, *miR426* and *miR842*, they grouped in the cluster III. They displayed similar trend in each tissue during stress. The lowest peak of *miR842* was in severe stress through drought and salt condition in the leaf tissue, while it was at 3 h after heat treatment in the root tissue.

Relationship of miRNAs and Target Gene in Stress Response

Analysis of Pearson correlation showed positive and negative correlation between expression of miRNAs and target genes against stress in both tissues. The results indicated significant negative correlation between *miR172*, *miR398* and *miR403* and their putative targets in leaf tissue under cadmium stress. On the contrary, *miR398* presented significant negative correlation in root tissue after heat treatment. Weak correlation was observed between some miRNAs and their candidate targets in both tissues against specific stress such as *miR160* in drought stress

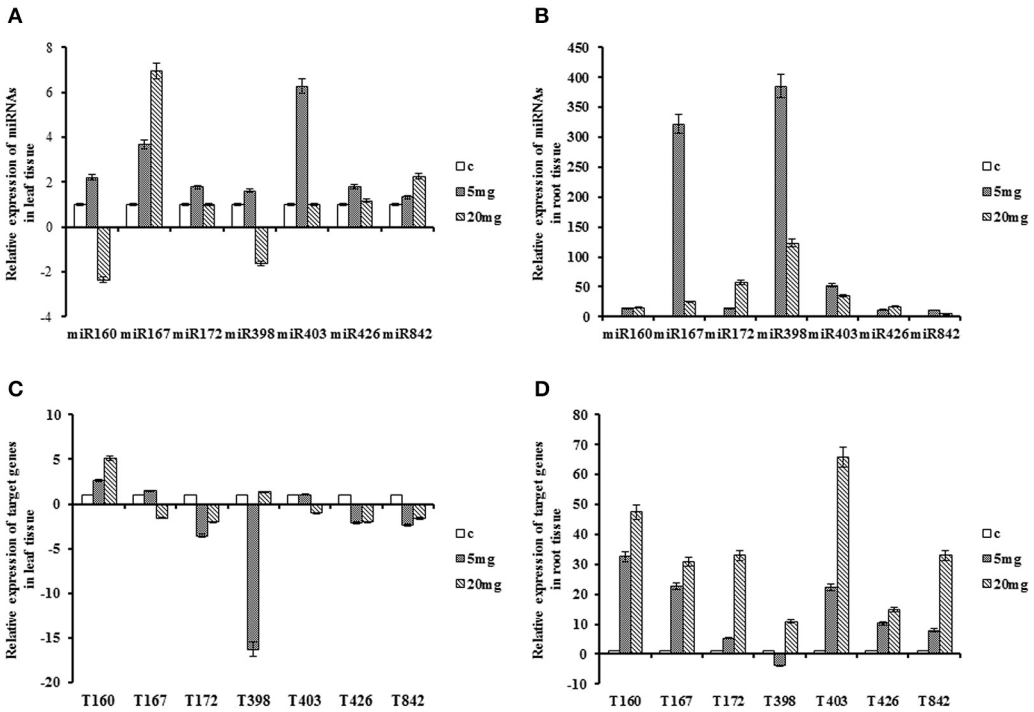


FIGURE 4 | Expression patterns of miRNAs and their target genes under cadmium stress. Plants treated with cadmium in 5 and 20 mg/L concentration. **(A)** Expression patterns of miRNAs in the leaf. **(B)** Expression patterns of miRNAs in the root. **(C)** Expression patterns of target gene in the leaf. **(D)** Expression patterns of target genes in the root.

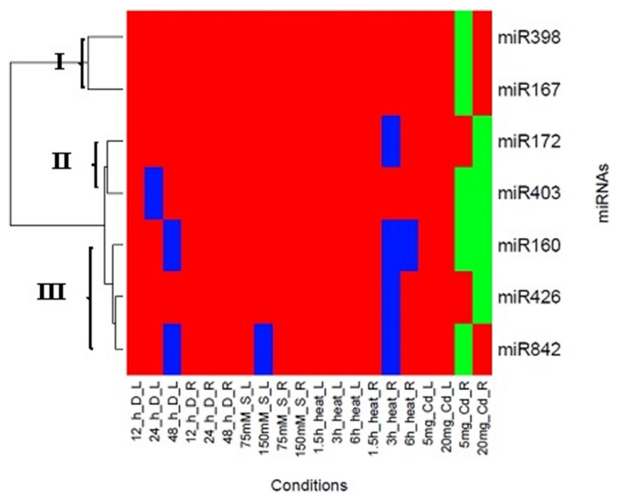


FIGURE 5 | Clustering of miRNAs expression profiles. Heat map diagram of miRNA expression prepared with two-way unsupervised hierarchical clustering of miRNAs expression under different abiotic stress. miRNAs are given in the rows and each columns represent a sample. The miRNA clustering tree is shown on the left (cluster I, II and III). Abbreviations: L, Leaf; R, Root; h, Hour; D, Drought stress; S, Salt Stress; Cd, Cadmium stress.

and *miR167* after heat treatment (Table S4). Some miRNAs displayed significantly positive correlation, which indicated other mechanisms are involved in target gene regulation.

Interaction between miRNAs and TFs-Mediated Gene Regulatory Subnetworks

The regulatory subnetworks that are constructed for each miRNA elucidated some of the intermingled miRNA and TF relationships as well as miRNA-miRNA relationships and the involvement of other miRNA families in miRNA specific post-transcriptional regulation pathways (**Figures 6**, **7**). References of interaction relations between miRNAs and genes in subnetworks and their correlated references are listed in Table S5.

GO analysis is a robust approach in understanding underlying molecular mechanisms of different cellular events and offer a reliable tool for GO based gene selection (Fruzangohar et al., 2013). GO classification showed that the target genes are involved in auxin signaling, RNA mediated silencing, ethylene signaling, DNA methylation and response to abiotic stress.

Subnetwork of *miR426* did not display any connectivity with other participants in the network. As *miR426* exhibits stress and tissue specific pattern, this miRNA is a suitable candidate for further studies in the context of miRNA mediated gene regulatory pathway. *MiR842* showed connectivity with *MBB18_8* and *(R)-mandelonitrile lyase* that drought, heat, salt and cadmium affected its expressions (**Figure 7B**).

MiR160 centered network exposed interaction between *QHG18J04.yg.ab1* and three TFs belonging to the *ARF* family (*ARF16*, *ARF17,* and *ARF18*), where four abiotic stress affect expression of *miR160* and *QHG18J04.yg.ab1* (**Figure 6A**). Interestingly, in this network, two other *ARF* family members; *ARF6* and *ARF8* regulate JA (Jasmonic acid) pathway and are

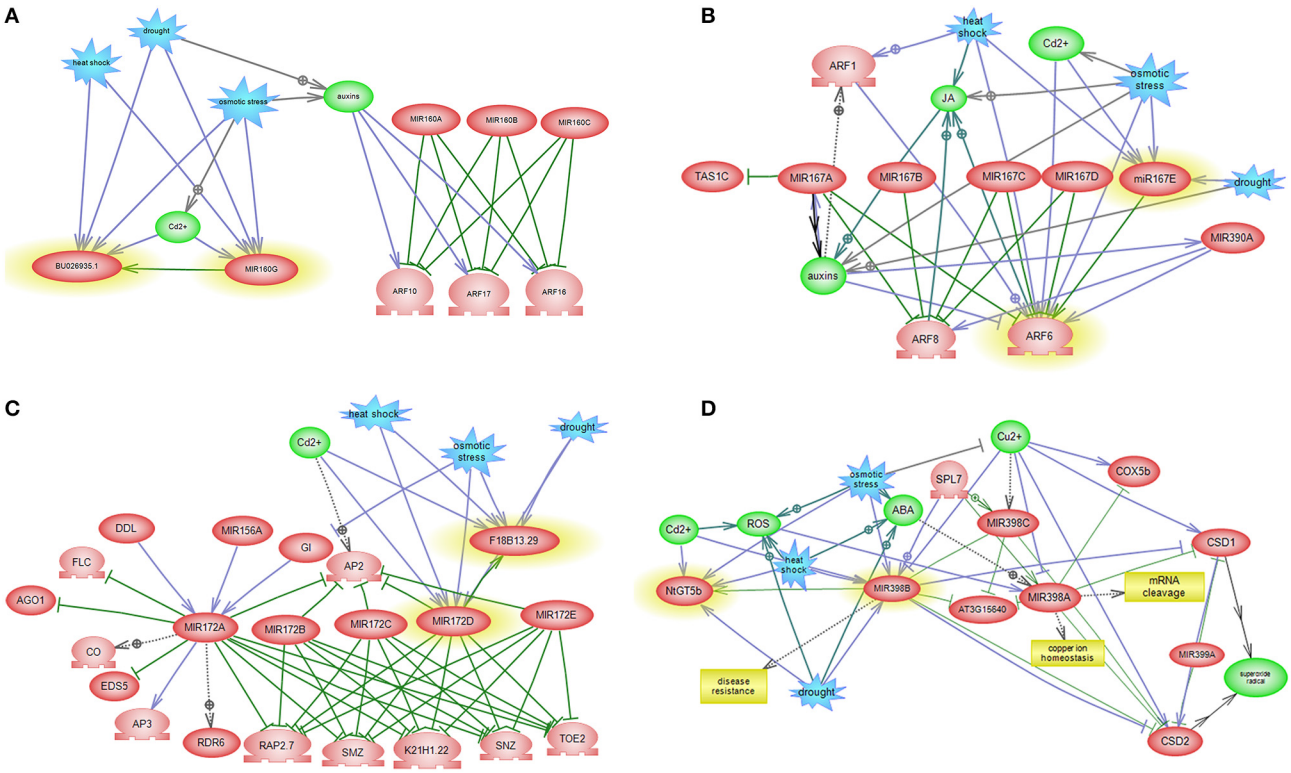


FIGURE 6 | miRNA-mediated gene regulatory sub networks in response to abiotic stress. miRNA-target-abiotic stress interaction are shown. **(A)** Feedback loop between miR160, its target and abiotic stress. **(B)** Feedback loop between miR167, its target and abiotic stress and abiotic stress. **(C)** Feedback loop between miR172, its target and abiotic stress. **(D)** Feedback loop between miR398, its target and abiotic stress.

in turn regulated by *miR167*. In addition, sub network of *miR167* includes regulation of *TAS1C*, which encodes a ta-siRNA (**Figure 6B**). Expression of TFs involved in ethylene signaling pathway and flower and seed development is regulated by the members of the *miR172* family. But *miR172* itself is regulated by *miR156* and *DDL* (a gene silencer) (**Figure 6C**). *MiR398* revealed cross talk with *SPL*, with central roles in many cellular processes such as auxin metabolism, root growth and cytokinesis; where *SPL* is regulated by *miR156*, *miR157* and *miR159*. Therefore, these three miRNAs are speculated to regulate the expression of *miR398* indirectly. *MiR156* have excelled as a key regulator in the regulation of other miRNAs. *MiR398* causes mRNA cleavage and disease resistance, which regulates expression of *CSDs* gene and *NtGT5b* (**Figure 6D**). Subnetwork of *miR403* revealed that *SE* and *miR402* regulate *AGO2*. It is known that *SE* has role in miRNA biogenesis, RNA splicing and chromatin modification. *MiR402* displayed connectivity with *DML1* and *DML3*, which are involved in DNA methylation and transcriptional control (**Figure 7A**).

Discussion

The role of seven miRNAs in response to several abiotic stresses was studied in *H. annuus* leaf and root tissues by RT-qPCR. Changes in RWC, expression of *HSP70*-related protein, sodium, potassium, and cadmium concentration revealed obviously various mechanisms stimulation to cope with stress. The measured alterations of metabolic and physiological status in *H. annuus* provide additional support for modification reactions toward stress conditions.

The temporal and spatial expression profiles of seven miRNAs in both tissues at altered time points were in agreement with previous studies (Sunkar et al., 2012; Zandkarimi et al., 2015; Zhang, 2015). For instance, high-throughput sequencing of *Brassica napus* showed up-regulation of *miR172* in the root tissue under cadmium excess, up-regulation of *miR167* from 2 to 24 h after exposure in 300 mM NaCl in *Arabidopsis* and down-regulation of *miR398* under drought stress in cotton which treated with PEG.

The different expression patterns of *miR167* observed in photosynthetic and non-photosynthetic tissues suggest that *miR167* may have a role in tissue-specific adaptation to stress. *MiR160* and *miR167* regulate *ARF* families and play roles in the auxin signaling pathway (Khraiwesh et al., 2012), and were differentially expressed under stresses compared to control conditions. In addition, ARF domain was found in the protein sequence of *QHG18J04.yg.ab1* (target of *miR160*). Therefore, this gene may be involved in auxin signaling pathway and have a role in promoting plant tolerance to stress. The alteration of *miR160* and *miR167* in *H. annuus* at early stage of treatments

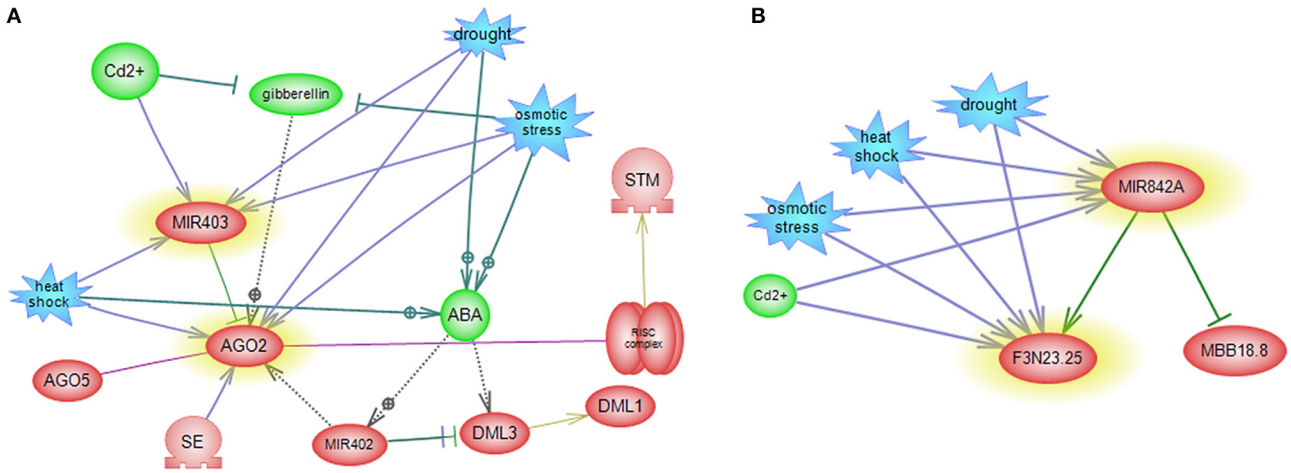


FIGURE 7 | miRNA-mediated gene regulatory sub networks in response to abiotic stress. miRNA-target-abiotic stress interaction are shown. **(A)** Feedback loop between miR403, its target and abiotic stress. **(B)** Feedback loop between miR842, its target and abiotic stress.

suggests that they are responsive to early stress response. Reduced expression of *miR160* and *miR167* in leaves under severe stress may alter basal level of auxin and consequently restrict plant growth through the antagonism between abscisic acid and auxin because of escape of tension. In turn, this could lead to attenuation of plant growth and development under stress to cope with the imposed stress (Sunkar et al., 2012). Even though they have common targets, in our study, *miR160* and *miR167* did not group together in one cluster. On the other hand, these miRNAs showed various expression patterns in other plant species under different stress (Khraiwesh et al., 2012; Sunkar et al., 2012). Besides, under normal conditions, their expression pattern was also variable in different plant genus and species (Zeng et al., 2010).

Some studies have suggested roles for *miR172* during cadmium, drought, cold and heat stress conditions (Sunkar et al., 2012; Zhou et al., 2012). In line with the previous studies, *miR172* revealed temporal up- or down-regulation in leaf and root in response to stress conditions except for the salt stress. Expression of *miR172* showed inverse correlation with its target *COX5b,* only in leaf tissue under cadmium stress. The post-transcriptional silencing of *COX5b* by *miR172* may reflect the providence of *Helianthus* plant from energy loss via avoiding respiration under excessive cadmium concentrations (Sunkar et al., 2012).

We found that *miR398* is especially up-regulated in leaf tissues during heat stress in line with the previous reports (Guan et al., 2013). Interestingly, *miR398* was down-regulated in root tissue while expression of *HSP70*-related was up-regulated, which may indicate that *miR398* has a tissue specific mode of action and localization during heat stress condition. Differential expression of *miR398* in response to drought, salt, heat and cadmium stress have been shown in several species such as wild emmer wheat, *Medicago*, *Nicotiana*, *Brassica* and *Arabidopsis* (Trindade et al., 2010; Frazier et al., 2011; Kantar et al., 2011; Zhou et al., 2012; Guan et al., 2013). In contrast, under severe drought and cadmium stress conditions, *miR398* was down-regulated in leaf tissue indicating a tissue-specific and stress-specific response orchestrated by this miRNA. In this study, the probable target of *miR398* was *NtGT5b* which is a microsomal enzyme responsible for glucuronidation reactions with a role in the storage of secondary metabolites and plants defense against stress (Miners et al., 2002; Ko et al., 2006). On the other hand, new target genes were predicted for *miR398* and *miR167* in *Phaseolus vulgaris* and *Malus hupehensis* in vegetative phase which involved in monogalactosyl diacylglycerol synthase, acyltransferase and dioxygenase, gluconeogenesis pathway and glycolytic process (Heyndrickx and Vandepoele, 2012; Han et al., 2014; Xing et al., 2014). Furthermore, in *Nicotiana tabacum* in response to TiO2 nanoparticles, these miRNAs were grouped in one cluster (Frazier et al., 2014). All of these results have led to the conclusion that *miR398* may be involved in the sugar biosynthesis pathway, associated with reduction in energy consumption for photosynthesis, and increase the tolerance of plant in abiotic stress conditions.

Surprisingly, the pattern of *miR403* was varying in each stress condition. Its expression was declined and was induced in both tissues in drought and cadmium stress, respectively. In contrast, *miR403* displayed increased expression in the leaf and decreased expression in the root during heat and salt stress. In the previous study, abundance of *miR403* was high in heat and salt libraries in *Raphanus sativus* (Wang et al., 2014; Sun et al., 2015). As a result, this discrepancy suggested that *miR403* was potentially expressed in stress-, tissue-, and species- specific manner during abiotic stress. Also, the expression of *AGO2* at all stages of treatment in both tissues was aberrant. In plants, *AGOs* are involved in various small RNA pathways from post-transcriptional gene silencing to epigenetic silencing phenomena such as RNA-directed DNA methylation (RdDM) pathway in *Arabidopsis* (Schraivogel and Meister, 2014). AGO1 and AGO2 proteins were regulated by *miR403* (**Figure 7A**). In addition, AGO2 has been shown to have an antiviral role (Harvey et al., 2011). Therefore, it is possible that this miRNA is involved in

the regulation of miRNA-mediated RNA cleavage carried out by other miRNAs during stress conditions. Furthermore, *AGO1* is also regulated by *miR172* family (Ronemus et al., 2006), which may designate a crucial role for *miR172* in general miRNA mediated gene silencing pathways (**Figure 6C**). This result, based on altered expression of *miR403* and its target under different abiotic stress and its subnetwork (**Figure 7A**) which showed *DML1* and *DML3*, involved in DNA methylation, may pave the ways for intricate control mechanisms for drought, heat, salt and cadmium stress tolerance in sunflower. Interestingly, *miR172* and *miR403* were grouped in one cluster (**Figure 5**) and they showed common targets; *AGO1* and *AGO2*, which may suggest a general role for these miRNAs in small RNA pathway and DNA methylation.

In this study, *miR426* and *miR842* show differential expression under abiotic stress. However, their expression exhibited stress-dependent manner during stress which was declined in root tissue under heat and drought stress and was reversed in salt and cadmium treatment. Also, inverse correlation of these miRNAs with their target was temporary in some stages. Their possible targets, *TPP2* and *(R)-mandelonitrile lyase*, were induced in response to some abiotic stresses and are reported to be involved in defense mechanism, oxidation-reduction process, cyanide biosynthetic process and alcohol metabolic process, which is indicated as a biocatalyst in organic chemistry (Hu and Poulton, 1999; Shima et al., 2007). *MiR842* revealed no significant change after waterlogging conditions (Moldovan et al., 2010), whereas it was repressed after ABA treatment of roots in *Arabidopsis* (Jia and Rock, 2013) which was similar with the expression of *miR842* under drought and heat stress in both tissues. In the earlier studies, *MBB18_8* a member of *Jacalin lectin* family (Gustafson et al., 2005) and a kinase-like protein (Barozai et al., 2012) were predicted as targets of *miR842*. As a result, *miR842* might have a role not only in sugar biosynthesis and sugar mediated signaling pathways but may also have a role as an osmoprotectant. Surprisingly, *miR842* gene was post-transcriptionally regulated by alternative splicing (Jia and Rock, 2013). Consequently, temporal expression in response to stress and regulation of its gene revealed that *miR842* might have a complicated function in plants. Our results suggest a novel function for *miR426* and *miR842* in the regulation of sunflower tolerance to abiotic stress. Interestingly, *miR160*, *miR426* and *miR842* showed similar pattern and were grouped in one cluster and according to their target, they are probably involved in carbohydrate signaling pathway.

In this study, expressions of miRNA targets are not consistent with the expression pattern of their related miRNAs at all times. The pattern of miRNAs and their target genes were semi-coherent, coherent or non-coherent type during stresses in leaf and root tissues. For example, expression of *miR842* and its target in leaf tissue showed inverse correlation during mild drought and cadmium stress. A recent study has revealed, the expression pattern of four miRNAs and their target have a semi-coherent fashion under salt stress in the halophyte smooth cordgrass (Zandkarimi et al., 2015). In addition, we did not analyze protein levels of target genes during stress, therefore we can neither confirm nor reject that these genes are direct targets for these miRNAs. It is possible that these miRNAs and their targets are expressed in a non-overlapping manner and regulate their targets in different cells, as reported for *miR395* and *AST68* in *Arabidopsis* (Kawashima et al., 2009). The coherent correlation between miRNA and mRNA is still under debate (Li et al., 2012), while the non-coherent type implicated the post transcriptional regulation mechanism by miRNA-directed cleavage for target mRNAs (Zandkarimi et al., 2015). Taken together, this information suggests that miRNAs play a versatile role for plant's acclimation to stress conditions. The kinetics of miRNAs and target regulation over time and in different tissues against tension and compression stresses imply complicated physiological and genetic mechanisms in *H. annuus* in order to deal with and adapt to harsh environment. Indeed, aberrant expression of many miRNAs during stress revealed that they respond to environmental stresses in a miRNA-, stress-, and tissue-dependent manner. Nevertheless, differential expression of certain miRNA rely on the specific stress condition, even in the same plant species (Zhang, 2015). As a consequence, aberrant expression of these miRNAs may reflect synergistic activities at the biochemical, physiological and molecular levels such as auxin signaling and sugar response, and finally at the organismal level to attenuate plant growth and development under stress. Interaction between miRNA and target gene is more flexible because of regulation of a mRNA target gene by multiple miRNAs or on the contrary regulation of numerous mRNA target by individual miRNA (Zandkarimi et al., 2015). As well, alternative splicing regulates miRNA biogenesis and expression of target genes to make different isoforms (Jia and Rock, 2013). This indicates that plants employ unrecognized regulatory loops to achieve tolerance via these regulatory small RNAs and suggests that they selectively regulate the expression of specific target genes under each condition. In conclusion this study adds to the growing body of literature on stress-responsive miRNAs in plants.

Author Contributions

BS conceived and designed the research. BS, EE, HF, FR, and HB conducted experiment. RE, SM, FK, and ZF carried out experiment and analyzed data. RE, BS, HF, and EE wrote the manuscript. All authors read and approved the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00741

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De novo transcriptome profiling of cold-stressed siliques during pod filling stages in Indian mustard (*Brassica juncea* L.)

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Low temperature is a major abiotic stress that impedes plant growth and development. *Brassica juncea* is an economically important oil seed crop and is sensitive to freezing stress during pod filling subsequently leading to abortion of seeds. To understand the cold stress mediated global perturbations in gene expression, whole transcriptome of *B. juncea* siliques that were exposed to sub-optimal temperature was sequenced. Manually self-pollinated siliques at different stages of development were subjected to either short (6 h) or long (12 h) durations of chilling stress followed by construction of RNA-seq libraries and deep sequencing using Illumina's NGS platform. *De-novo* assembly of *B. juncea* transcriptome resulted in 133,641 transcripts, whose combined length was 117 Mb and N50 value was 1428 bp. We identified 13,342 differentially regulated transcripts by pair-wise comparison of 18 transcriptome libraries. Hierarchical clustering along with Spearman correlation analysis identified that the differentially expressed genes segregated in two major clusters representing early (5–15 DAP) and late stages (20–30 DAP) of silique development. Further analysis led to the discovery of sub-clusters having similar patterns of gene expression. Two of the sub-clusters (one each from the early and late stages) comprised of genes that were inducible by both the durations of cold stress. Comparison of transcripts from these clusters led to identification of 283 transcripts that were commonly induced by cold stress, and were referred to as "core cold-inducible" transcripts. Additionally, we found that 689 and 100 transcripts were specifically up-regulated by cold stress in early and late stages, respectively. We further explored the expression patterns of gene families encoding for transcription factors (TFs), transcription regulators (TRs) and kinases, and found that cold stress induced protein kinases only during early silique development. We validated the digital gene expression profiles of selected transcripts by qPCR and found a high degree of concordance between the two analyses. To our knowledge this is the first report of transcriptome sequencing of cold-stressed *B. juncea* siliques. The data generated in this study would be a valuable resource for not only understanding the cold stress signaling pathway but also for introducing cold hardiness in *B. juncea*.

Keywords: *Brassica juncea*, transcriptome, cold stress, low temperature, silique, RNA-seq

INTRODUCTION

The Brassicaceae family, which includes nearly 3500 species and 350 genera is one of the 10 most economically important plant families (Warwick and Black, 1991). Within the family, species of the genus *Brassica* comprise multiple vegetables (cabbage, broccoli, brussels sprout, cauliflower, turnip—*B. oleracea*), oilseeds (*B. rapa*, *B. juncea, B. napus*), and condiments (*B. nigra*, *B. carinata, B. juncea;* Branca and Cartea, 2011). *B. juncea* ($n = 18$) is an amphidiploid species derived from interspecific crosses between two diploid progenitor parents, *B. nigra* ($n = 8$) and *B. rapa* ($n = 10$) (Prakash and Hinata, 1980). It is grown as an oilseed crop in India (brown or Indian mustard), as a leaf vegetable in China, and as a condiment in western countries (Rakow, 2004). India is the third largest producer of rapeseed-mustard in the world after China and Canada. This crop accounts for nearly one-third of the edible oil produced in India, making it the country's key edible oilseed crop. The major impediments in harnessing the true yield potential of mustard are biotic stresses such as blight, aphids, white rust and abiotic stresses such as frost, high temperature, salinity, and drought.

Low temperature is one of the most intimidating abiotic stresses that affect plant growth and development, thereby limiting the distribution of crop species. Based on its intensity, cold stress can be broadly classified into chilling and freezing stresses. Exposure to temperatures below 0°C results in freezing stress, whereas chilling stress occurs at temperatures ranging from 0 to 20°C. Plants such as rice, maize and tomato that grow in tropical and subtropical regions are chilling sensitive whereas the plants from temperate region are chilling tolerant (Solanke and Sharma, 2008; Chinnusamy et al., 2007). Plants have the ability to acquire tolerance to chilling and freezing conditions if they are pre-exposed to non-freezing temperatures, through a process known as cold acclimation (Levitt, 1980). Cold acclimation helps plants to fine tune their metabolism and improve freezing tolerance by initiating signaling cascades that leads to several biochemical and physiological changes, including modification of membrane lipid composition and changes in gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1998; Gilmour et al., 2000; Chinnusamy et al., 2003). The altered gene expression leads to accumulation of several protective proteins such as antifreeze proteins (Griffith et al., 1997), late embryogenesis abundant (LEA) proteins (Antikainen and Griffith, 1997), heat shock proteins (HSP) (Wisniewski et al., 1996), cold-regulated (COR) proteins and various metabolites such as amino acids, soluble sugars, organic acids, pigments (Krause et al., 1999), polyamines (Bouchereau et al., 1999), and antioxidants (Hausman et al., 2000). These metabolites and proteins help in protecting plant membranes and prevent cell disruption during cold stress by stabilizing membrane lipids, proteins, maintaining hydrophobic interactions, ion homeostasis and scavenging the reactive oxygen species (ROS) (Hare et al., 1998; Gusta et al., 2004; Chen and Murata, 2008; Janská et al., 2010).

Spatial and temporal gene expression changes have traditionally been studied by comparing levels of steady state transcripts. With advancements of molecular techniques, it is now possible to generate information on alterations in transcripts levels at whole genome level. Using cDNA microarrays or whole genome arrays, the expression pattern of genes in response to chilling stress has been analyzed in *Arabidopsis*, rice, sunflower and several other plants (Seki et al., 2002; Rabbani et al., 2003; Fernandez et al., 2008). Seki et al. (2001) used a full-length cDNA microarray of 1300 *Arabidopsis* genes, and identified 19 *COR* (cold-regulated) genes, among which the newly identified genes were ferritin, a nodulin-like protein, LEA protein and glyoxalase. In another study, Fowler and Thomashow (2002) reported 306 *COR* genes using microarray for 8000 genes. Of these 306 genes, 218 were up-regulated and 88 were down-regulated and 45 of these *COR* genes were found to be expressing under the control of CBF1. Different ecotypes of *Arabidopsis* exposed to non-freezing cold stress exhibited different transcriptome level signatures (Barah et al., 2013). Transcriptome profiling of cold stress subjected 3-week-old *B. rapa* plants resulted in identification of genes encoding CBF/DREB like transcription factor, *ERD10*, *RD29A/COR78*, *COR47/RD17* (Lee et al., 2008).

Low temperature has a negative impact on different stages of plant reproductive development leading to premature abortion of seeds and fruits (Thakur et al., 2010). Likewise pod-filling stages in *B. juncea* are highly susceptible to frost stress injury thereby resulting in significant reduction in crop yield. Rajasthan, being the largest producer of mustard in India, contributes to 47.2% of the total production. Injury caused by episodic incidents of frost in semi-arid plains of Rajasthan has caused huge financial losses to the farmers. Cold waves and frost stress affects mustard cultivation in Rajasthan where night temperature in winters fall below −4.4°C. The loss in crop production due to frost stress in 2006 in Rajasthan alone was 344,400 tons resulting in total economic loss of Rs 5906 million (Prasada Rao et al., 2010). Since low temperature stress is a major limiting factor in *B. juncea* crops, it is significant to study the response of plants to stress and the underlying mechanism of tolerance. The first step in achieving this goal is to understand the plant response to non-freezing cold stress at the molecular level. To gain an in-depth knowledge of the global gene expression changes in developing siliques of *B. juncea* that were exposed to chilling stress, RNA-seq was employed to generate the transcriptome profile. Manually, self-pollinated siliques (5 DAP-30 DAP) of *B. juncea* were subjected to either short (6 h) or longer (12 h) durations of cold stress followed by RNA extraction, library construction and sequencing using Illumina's next generation sequencing platform. This is the first genome wide report of transcriptional response in *B. juncea* siliques that were exposed to cold stress. Deciphering the global gene expression changes in cold-stressed developing siliques, would be useful in understanding of the molecular pathway of cold stress and devising future strategies for enhancing low temperature tolerance in Indian mustard.

MATERIALS AND METHODS

Plant Material and Growth Conditions

B. juncea var. Varuna seeds were obtained from National Seed Centre, IARI, New Delhi. Seeds were sown in the field at University of Delhi, South Campus during the growing season (October-March) of mustard. Plants were grown under field

conditions and self-pollination was initiated at the time of flowering.

Controlled Self-pollination and Stress Treatment of *B. juncea* var. Varuna

B. juncea var. Varuna was manually self-pollinated with pollen derived from the same cultivar. All the open flowers and siliques were removed from the flowering twigs followed by emasculation of unopened buds. Fresh pollen was dusted on the exposed stigma followed by bagging to avoid cross-pollination. The manually self-pollinated siliques were subjected to cold stress at 5, 10, 15, 20, 25, and 30 days after pollination (DAP). Self-pollinated branches were excised and placed in a beaker filled with water and subjected to cold treatment at 4°C for 6 h and 12 h followed by immediate harvesting. Light conditions were maintained during cold stress to simulate natural conditions. For control samples, self-pollinated branches were excised and placed in water under field conditions. Self-pollinated siliques of different stages of development (5–30 days) were frozen in liquid nitrogen and stored at −80°C until further use. The temperature range in the field on the day of stress and harvesting was between 17°C (minimum) and −24°C (maximum). The entire experiment was designed such that control samples corresponding to different DAPs, were harvested on the same day. To study the anatomical stages of embryo development in *B. juncea* var. Varuna, resin sectioning was performed. The detailed procedure for staging and the results obtained is given in Supplementary Datasheet 1.

Library Preparation and Data Analysis

Total RNA was extracted from stressed as well as control siliques using Qiagen's plant RNA extraction kit as per the manufacturer's protocol. RNA-seq libraries were prepared utilizing TruSeq RNA sample preparation kit v2 (Illumina Inc., USA) as per the manufacturer's protocol and sequenced on HiSeq 2000 (Illumina, USA). The raw reads from sequencing run of all stages was subjected to a quality filtering utilizing "NGS QC toolkit" (Patel and Jain, 2012). Low quality reads whose 70% of the read length had phred score less than 20 ($Q \leq 20$) were discarded followed by trimming of adapter sequences. Orphan reads remaining after applying these filtering criteria were also discarded. The QC filtered paired reads of all the samples were merged together into two files containing left and right reads, respectively. These merged files were used to reconstruct the transcripts using Trinity assembler with default parameters (Haas et al., 2013). The statistics of the assembled transcripts were measured utilizing TrinityStats.pl script of Trinity. QC filtered reads of each of the samples were aligned to the assembly using Bowtie2 aligner (Langmead and Salzberg, 2012) and quantification of each transcript was carried out with RSEM tool (Li and Dewey, 2011). Transcripts having less than 1 read count in all the samples were considered as misassembled or artifacts and hence discarded from the assembly. The statistics of the filtered assembly were again extracted using TrinityStats.pl script. Samples were normalized with the TMM normalization method (Robinson and Oshlack, 2010) of trinity and differentially expressed transcripts (with $p < 0.001$) were isolated using trinity differential expression module by applying a minimum cutoff of 2-fold change on log2 scale. Generation of heat map, Spearman correlation matrix, and cluster analysis of differentially expressed transcripts was performed using R-based tools. The reconstructed transcripts were annotated using FastAnnotator online server tool with default parameters (Chen et al., 2012a).

Real Time PCR Validation

Total RNA was extracted from three independent biological replicates from stressed as well as control siliques (5–30 DAP) using Qiagen's plant RNA extraction kit as per the manufacturer's protocol. 10 μg of total RNA was subjected to DNaseI treatment with 1 U DNaseI (NEB, USA). The reaction was carried out at 37°C for 10 min followed by heat inactivation at 65°C for 10 min. 2.5 μg of DNase-treated RNA was used for cDNA synthesis with iScript reverse transcriptase (BioRad, USA) according to the manufacturer's protocol. The expression of actin gene (*ACT7*) was found to be stable in our transcriptome database and hence was used as the normalization control in real time PCR. The primers used for *ACT7* were forward primer: 5′ TGGGTTTGCTGGTGACGAT 3′ and reverse primer: 5′ TGCCTAGGACGACCAACAATACT 3′. Primers were designed for selected transcripts from transcriptome database and real time PCR was performed using SYBR green I master mix (Roche, GmBH) on CFX-Connect™ Real time system (BioRad, USA). Details of the primers are presented in Supplementary Table 1. Relative expression of the transcripts was calculated using ΔΔCt method (Livak and Schmittgen, 2001). Scatter plot and Pearson correlation coefficient was calculated in MS-excel using log2 values of qPCR and digital expression data.

The RNA-seq data discussed in this publication has been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE73201 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73201).

RESULTS

De novo Assembly of *B. juncea* Silique Transcriptome Data

B. juncea var. Varuna plants were controlled self-pollinated at the time of flowering. Controlled self-pollinated siliques of *B. juncea* at different stages of development (5 DAP-30 DAP) were subjected to cold stress at 4°C for capturing the changes in gene expression. The siliques from control plants were also used to study the anatomical details of embryo development in *B. juncea* var. Varuna. Longitudinal sections of fertilized ovule showed first division of zygote at 5 DAP, first longitudinal division of embryo at 10 DAP, globular stage of embryo at 15 DAP, heart shape embryo at 20 DAP, torpedo stage of embryo at 25 DAP and cotyledonary immature embryo at 30 DAP (Supplementary Datasheet 1).

RNA-seq libraries were constructed from 18 different samples that encompassed six development stages (5, 10, 15, 20, 25, and 30 DAP) of *B. juncea* siliques, obtained by controlled self-pollination, and three different time points (0 h, also referred as control, 6 h and 12 h) of cold stress. Details of the experimental design and analysis pipeline are presented in **Figure 1**. As it was difficult to harvest enough embryos after controlled pollination,

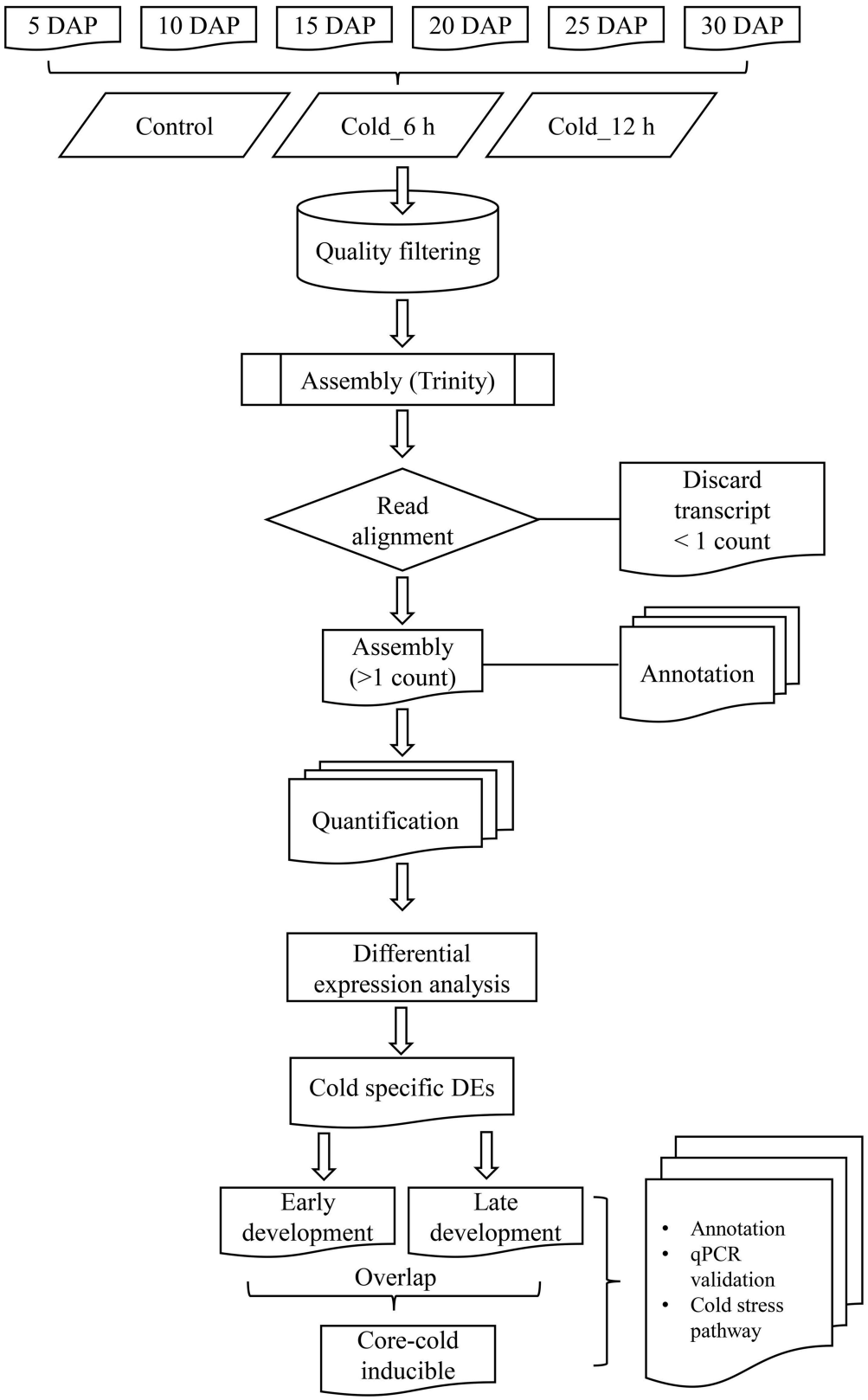


FIGURE 1 | Flowchart representing computational pipeline employed for analysis of transcriptome sequenced from *B. juncea* siliques.

TABLE 1 | Quality filtering and statistics of raw reads obtained in transcriptome libraries of *B. juncea* siliques exposed to low temperature for different durations.

S. No.	Sample	No. of raw reads	No. of HQ reads	Percentage (%)	Mapped reads	Percentage mapping (%)
1	5 DAP_C	34,996,934	33,098,872	94.58	23,672,240	71.52
2	5 DAP_6 h	46,283,995	43,352,879	93.67	31,094,528	71.72
3	5 DAP_12 h	27,407,889	21,593,463	78.79	15,463,603	71.61
4	10 DAP_C	31,916,211	30,033,245	94.10	21,664,687	72.14
5	10 DAP_6 h	38,077,842	35,335,917	92.80	25,477,680	72.10
6	10 DAP_12 h	20,851,323	16,264,475	78.00	11,803,985	72.58
7	15 DAP_C	36,410,242	34,111,247	93.69	24,585,074	72.07
8	15 DAP_6 h	40,661,329	37,646,457	92.59	27,322,541	72.58
9	15 DAP_12 h	44,470,147	36,420,384	81.90	26,589,923	73.01
10	20 DAP_C	54,387,436	51,174,780	94.09	37,227,245	72.75
11	20 DAP_6 h	55,097,716	51,238,446	93.00	37,445,913	73.08
12	20 DAP_12 h	48,736,907	38,808,054	79.63	28,375,618	73.12
13	25 DAP_C	34,719,115	28,553,791	82.24	20,805,257	72.86
14	25 DAP_6 h	46,760,019	37,279,174	79.72	26,906,383	72.18
15	25 DAP_12 h	92,720,961	78,646,125	84.82	58,031,931	73.79
16	30 DAP_C	45,741,906	37,042,168	80.98	26,990,691	72.86
17	30 DAP_6 h	63,185,861	50,328,733	79.65	37,024,361	73.57
18	30 DAP_12 h	58,774,016	50,083,797	85.21	36,735,973	73.35

Raw reads were filtered to remove adapter sequences and low quality reads using NGS QC tool kit. The percentage of reads retained after QC filtering is depicted in column 5.

entire siliques were harvested for tissue collection. Sequencing of 18 libraries generated a composite of approximately 820 million reads, which were subjected to quality filtering using "NGS QC toolkit" (Patel and Jain, 2012). The adapter contaminated reads as well as reads whose 70% bases had Phred score less than 20 ($Q \leq 20$) as well as orphan reads were discarded. This resulted in approximately 700 million high quality paired reads (**Table 1**).

The filtered HQ reads were subjected to assembly using the *de novo* assembler "Trinity" (Haas et al., 2013), which employs "de-Bruijn" graph approach (at a k-mer of 25 nucleotides) to assemble the reads into contigs. The preliminary assembly generated 212,124 contigs (hereafter referred to as "transcripts"). Combined length of these transcripts was 156 Mb and the N50 value of the assembly was 1202 bp. The obtained length of transcripts ranged between 201 and 14,199 bp and the GC content of assembled transcripts was found to be 41.92%. These transcripts also included isoforms and therefore represented 143,414 unigenes. The combined length of the unigenes was 83 Mb and had a N50 value of 822 bp. Average length of transcripts and unigenes (from the preliminary assembly) was found to be 737 and 577 bp, respectively. The preliminary assembly was subjected to assembly correction by aligning the HQ paired reads to the assembled transcripts with the help of Bowtie2 module of RSEM. More than 70% of the paired reads mapped to the transcripts of the preliminary assembly (**Table 1**). Quantification of each transcript was carried out using RSEM tool. Transcripts having expression value of less than 1 read count in all the 18 samples were removed from the assembled data. The final filtered assembly was reanalyzed for various parameters, including N50 value, transcript number, unigene number and average transcript length. Following assembly correction and filtering, 133,641 transcripts were retained, the combined length of which was 117 Mb. The average length of transcripts was 875 bp and the N50 value of the assembly was 1428 bp. While the maximum length of the transcript was found to be 16 kb, the minimum length was 200 bp. The distribution of transcript length is presented in **Figure 2A**. The 133,641 filtered transcripts represented 83,899 unigenes. The average length of the unigenes was 692 bp with a N50 value of 1225 bp (**Table 2**).

Annotation of Assembled *B. juncea* Siliques Transcripts

To gain an insight on probable functions of each transcript, assembly was subjected to annotation analysis utilizing web-based tool "FastAnnotator," which uses four different annotation protocols: (1) LAST (Local Alignment Search Tool) search to identify best hits in NCBI non-redundant (nr) database, (2) Blast2GO (https://www.blast2go.com/) to assign gene ontological (GO) terms, (3) PRIAM (http://priam.prabi.fr/) for identification of enzymes along with their EC numbers, and (4) RPS BLAST to predict functional domains in transcript sequences by searching the domain models (Pfamv26) from Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd; Chen et al., 2012a). The LAST search against NCBI nr database resulted in annotation of approximately 67% transcripts (90,223 out of 133,640 transcripts) with a minimum *e*-value of 10^{-5} (Supplementary Datasheet 2). In addition to the NCBI-based annotation, 438 transcripts were annotated by domain search and 10 were annotated with enzyme database. Out of the 90,671 annotated transcripts, 79,880 transcripts could be associated with at least one GO term. Nearly, 7237 transcripts had at least one enzyme hit and 45,584 transcripts had >50% identity with previously reported protein domains

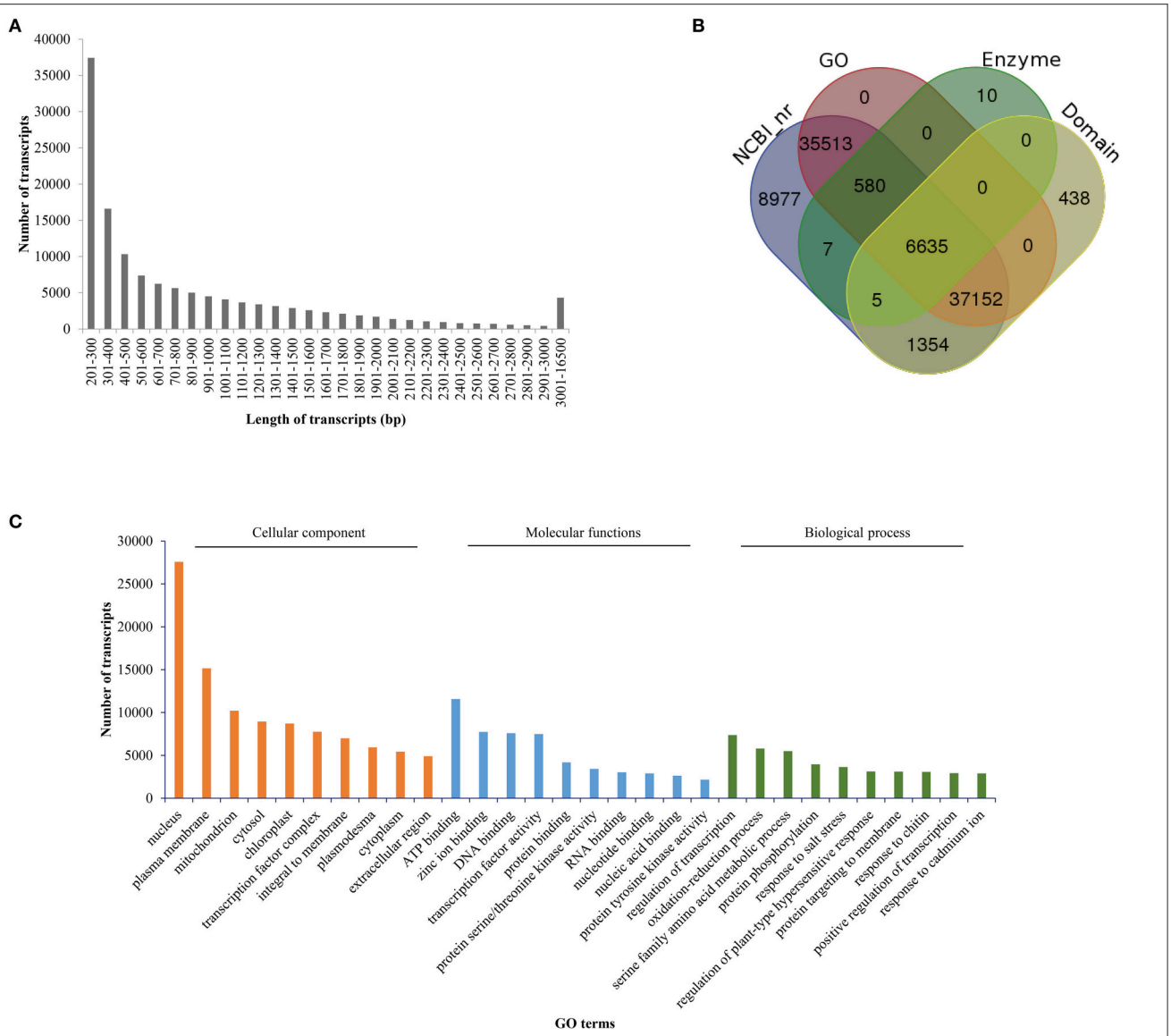


FIGURE 2 | Size distribution and gene ontology analysis of the assembled transcripts. (A) Assembled transcripts were evaluated for their size distribution and the number of transcripts in each size range is presented. **(B)** Overlap in the distribution of annotated transcripts based on their databases hits. The LAST search was used to identify transcripts in NCBI nr database whose output was further annotated with Blast2GO for assigning gene ontology terms. PRIAM database and RPS blast were employed for identification of enzymes and conserved domains, respectively. **(C)** Gene ontology categorization of annotated *B. juncea* transcripts on the basis of cellular components, molecular functions and biological processes. The numbers of transcripts in the ten most enriched GO terms for each of the categories are shown.

(**Figure 2B**). Similarly, 8977, 438, and 10 transcripts exhibited exclusive hits to NCBI nr, domain and enzyme databases, respectively. The top 10 GO terms (associated with the annotated transcripts) in various biological processes, molecular functions and cellular components are presented in **Figure 2C**. The top GO terms in biological processes were regulation of transcription, oxidation-reduction, protein phosphorylation and stress responses. On the basis of localization in cellular components, maximum number of transcripts localized to nucleus followed by plasma membrane and mitochondria. ATP binding, zinc ion binding and DNA binding were the most enriched GO terms in the molecular function category.

Identification of Differentially Expressed Transcripts (DETs) in Developing Siliques

Approximately, 13,342 transcripts exhibiting significant differential expression were identified by pair-wise comparisons (Supplementary Datasheet 3) in all possible combinations of the 18 samples. On the basis of expression profile, DETs were clustered to generate a heat map (**Figure 3A**) as well as Spearman correlation matrix (**Figure 3B**). Broadly, differentially

TABLE 2 | Assembly statistics of *B. juncea* transcriptome.

Parameters	Uncorrected	Corrected
Number of transcripts	212,124	133,641
Number of unigenes	143,414	83,899
Assembly length (Mb)	156	117
Minimum transcript length (bp)	201	201
Average transcript length (bp)	737	875
N50 (bp)	1202	1428

expressed transcripts of 5, 10, and 15 DAP clustered in one clade whereas those of 20, 25, and 30 DAP clustered together in another clade, thereby enabling us to categorize and identify gene expression changes occurring either during the early (occurring in 5–15 DAP) or late (occurring in 20–30 DAP) stages of silique development. Transcripts of controls corresponding to 10 and 15 DAP clustered together. Similarly, transcripts present in controls of 20 and 25 DAP clustered together. Nonetheless, in most of the cases 6 h and 12 h cold stressed differentially expressed transcripts clustered together. The only exception was at 5 DAP whose control samples clustered with the 12 h cold stress samples, whilst 6 h samples was placed in a different sub-clade (**Figures 3A,B**). Following clustering, transcripts displaying similar expression pattern in response to cold stress were cataloged for each developmental stages separately and their expression pattern is depicted in Supplementary Figures 1–6.

The samples (control and cold stress treated) from 5, 10, and 15 DAP were considered as early stages of silique development. Detailed analysis of early stage samples identified 5332 transcripts exhibiting $\geq$2-fold difference in expression levels (on a log2 scale), which were subsequently used for generation of a heat map. As evident in **Figure 4A**, the hierarchical clustering of samples resulted in two parent clades where 5 DAP samples clustered under one clade whereas 10 and 15 DAP samples were part of another clade. Heat map and the associated clusters of expression profile (**Figures 4A,B**) showed that expression of a large number of transcripts is modulated significantly in response to cold stress. Based on the expression patterns of transcripts, four major categories comprising of 972, 2152, 71, and 2137 transcripts in clusters 1, 2, 3, and 4, respectively were identified (**Figure 4B**). Clusters 1 and 2 contained transcripts that were up-regulated by cold stress, whereas clusters 3 and 4 had transcripts that were developmentally regulated, as their expression remained unchanged under cold stress. Expression of the transcripts belonging to cluster 1 increased in response to both 6 h and 12 h of cold stress. More importantly, this trend was observed in all the initial stages (i.e., 5, 10, and 15 DAP) of silique development. Transcripts belonging to cluster 2 were also up-regulated by cold stress. However, the expression of these transcripts increased only at 6 h of cold stress in 5 DAP samples. Their expression was largely stable in all the other stages and time points.

The stages of silique development at 20 DAP, 25 DAP, and 30 DAP were categorized as late stages and these stages compositely had 4522 differentially expressed transcripts. Hierarchical clustering revealed that control samples of 20 DAP and 25 DAP clustered together. However, cold stress samples of 6 h and 12 h (at all the stages) were placed in the same cluster (**Figure 4C**). From the hierarchical clustering, we identified six major patterns of gene expression in the late stages of silique development (**Figure 4D**). Out of the six clusters, only two clusters (clusters 3 and 5) had transcripts that were induced by cold stress. The expression of transcripts in cluster 3 increased (w.r.t. to the corresponding controls) after 6 h of cold stress in 20 DAP and after 12 h of cold stress in 25 DAP. Noticeably, expression of transcripts in cluster 5 had a similar pattern as cluster 1 of early stage as these transcripts were induced by both 6 h and 12 h of cold stress in all the later stages of silique development.

Identification and Characterization of Transcripts Inducible by Cold Stress in *B. juncea* Siliques

To identify the core group of genes that are induced in both the early and late stages of silique development, the transcripts from cluster 1 of the early stage (**Figure 4B**) was compared with those from cluster 5 of the late stage (**Figure 4D**). Comparison of 972 transcripts of cluster 1 with 383 transcripts of cluster 5 led to identification of 283 transcripts that were induced by cold stress in all the stages of embryo development. The 283 transcripts thereby constituted what is being referred to as the "core cold-inducible" transcripts. Additionally, this analysis revealed that 689 transcripts were specifically induced by cold stress in the early stages of silique development, whereas expression of 100 transcripts increased under cold stress, in the late stages of silique development. Results of this analysis are presented in **Figure 5A** and the list of the transcripts is provided in Supplementary Datasheets 4–6. To further assign relevance, transcripts from each of these subsets were annotated and their gene ontology terms in the biological processes category were identified (Supplementary Figure 7). Details of this analysis are presented below.

Annotation of Subsets

Functional annotations of the three subsets were fetched from the annotated assembly. More than 70% of the transcripts from each of the subsets were annotated with different databases. Out of 689 transcripts in early inducible subset, 492 were annotated with different databases. Similarly, annotations were possible for 231 and 72 transcripts of core-cold inducible and cold-inducible subsets at late stages of silique development, respectively (Supplementary Figure 7). Majority of the transcripts in all the three categories were annotated by GO search as well as by the domain structure databases. However, none of the transcripts were annotated specifically from enzyme database alone.

Considering only the top 20 GO terms, gene ontology analysis revealed that some of the biological processes were exclusively associated with specific categories. For example GO terms, "MAPKK" and "protein targeting to membrane" associated exclusively with cold-inducible subset of early silique development. Similarly, the other subset, containing cold-inducible transcripts in the later stages of silique development,

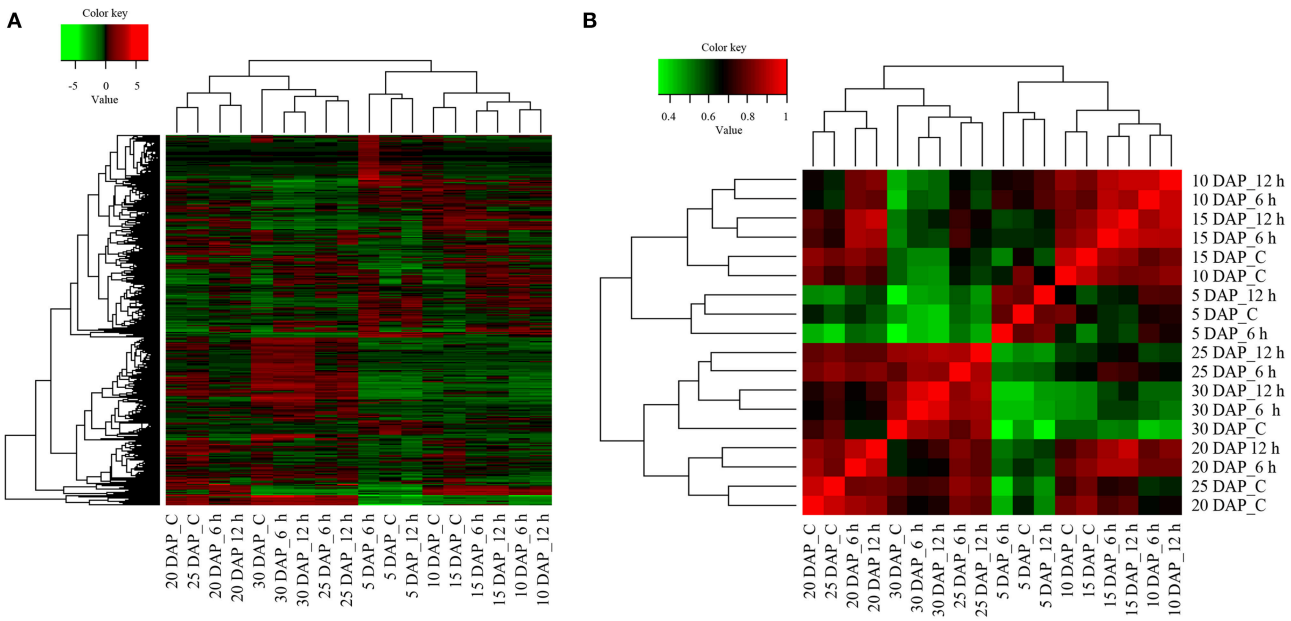


FIGURE 3 | Analysis of differentially expressed transcripts and their inter-relationships in *B. juncea* siliques. (A) Heat map of differentially expressed transcripts in control and cold stressed *B. juncea* siliques at different stages of development. Differentially expressed transcripts identified by pair-wise comparisons of all the 18 samples were clustered together on the basis of their expression values and heat map was plotted with TMM-normalized FPKM values. **(B)** Spearman correlation matrix of all the samples indicating extent of similarity between each possible pair. All the 13,342 transcripts exhibiting differential expression were clustered together and Spearman correlation matrix was plotted on TMM-normalized FPKM values. The key for sample labels should be interpreted as x DAP_y, where x denotes number of days; DAP represents days after pollination and y denotes either unstressed siliques –"C" or duration of cold stress in hours –"h".

associated specifically with GO terms like "oxidation-reduction process," "response to UV-B," and "stomatal complex morphogenesis." The GO terms "photoperiodic response," "photo-morphogenesis," "two-component signal transduction," and "myoinositol hexaphosphate processes" were linked to the core-group transcripts. GO terms representing transcripts involved in response to various abiotic and biotic stresses like cold, wounding, fungal infection etc., were present in all the three subsets.

Identification of TFs, TRs, and Kinases Inducible by Cold Stress in *B. juncea* Siliques

Transcription factors (TFs) are proteins, which directly bind to the DNA sequence and modulate transcription of a gene, whereas transcriptional regulators (TRs) are proteins, which interact with other proteins to regulate gene transcription. Apart from these proteins, kinases are known for their role in relaying stress signals to the downstream signaling components. Therefore, an in-depth analysis of the transcriptome data was performed to identify TFs, TRs, and kinases that are inducible by cold stress in the three subsets of cold-inducible transcripts.

Transcription Factors (TFs)

We identified 120 transcripts belonging to 22 TF families from the three subsets. These included members of various stress-responsive TFs such as WRKY, HSFs, MYBs, AP2-EREBs, NACs etc. Nineteen of the 22 families were detected in the early cold-inducible category and interestingly 12 of these were induced by cold exclusively during the early stages of silique development. Members belonging to 5 families of TFs were present in the later stages of development, however only 1 of these was exclusive to later stages. Maximum members (25) were detected for the Myb family; out of which 15 were cold-inducible exclusively in the early stages subset while another 10 were part of the core-group of transcripts. None of the Myb TF family member was inducible by low temperature in the later stages of silique development. The next major category of TF was AP2-EREBP type, which had 10 cold-inducible transcripts in the early group, 1 in late group and 4 in the core group (**Table 3**).

The core-cold inducible subset consisted of well-known positive regulators of cold stress pathway like *CBF*s, AP2-EREB, constans-like 1, *DREB2-2*, and members of Myb TF family whereas late stage cold-inducible subset included *HSFA6b* and AP2-EREB members. Transcription factors were maximally represented in early stages of silique development. Members of WRKY family have been implicated in abiotic as well as biotic stress (Kayum et al., 2015). *WRKY23* and *WRKY46* were expressed under cold stress in early stages of silique development. SPL family of TFs is a functionally diverse set of proteins and is known to be involved in regulating plant growth and development (Preston and Hileman, 2013). In early stages of development, *SPL13* was found to be cold-inducible. Similarly, zeaxanthin epoxidase the first enzyme in ABA synthesis pathway (Schwartz et al., 1997) was inducible by cold stress in the early

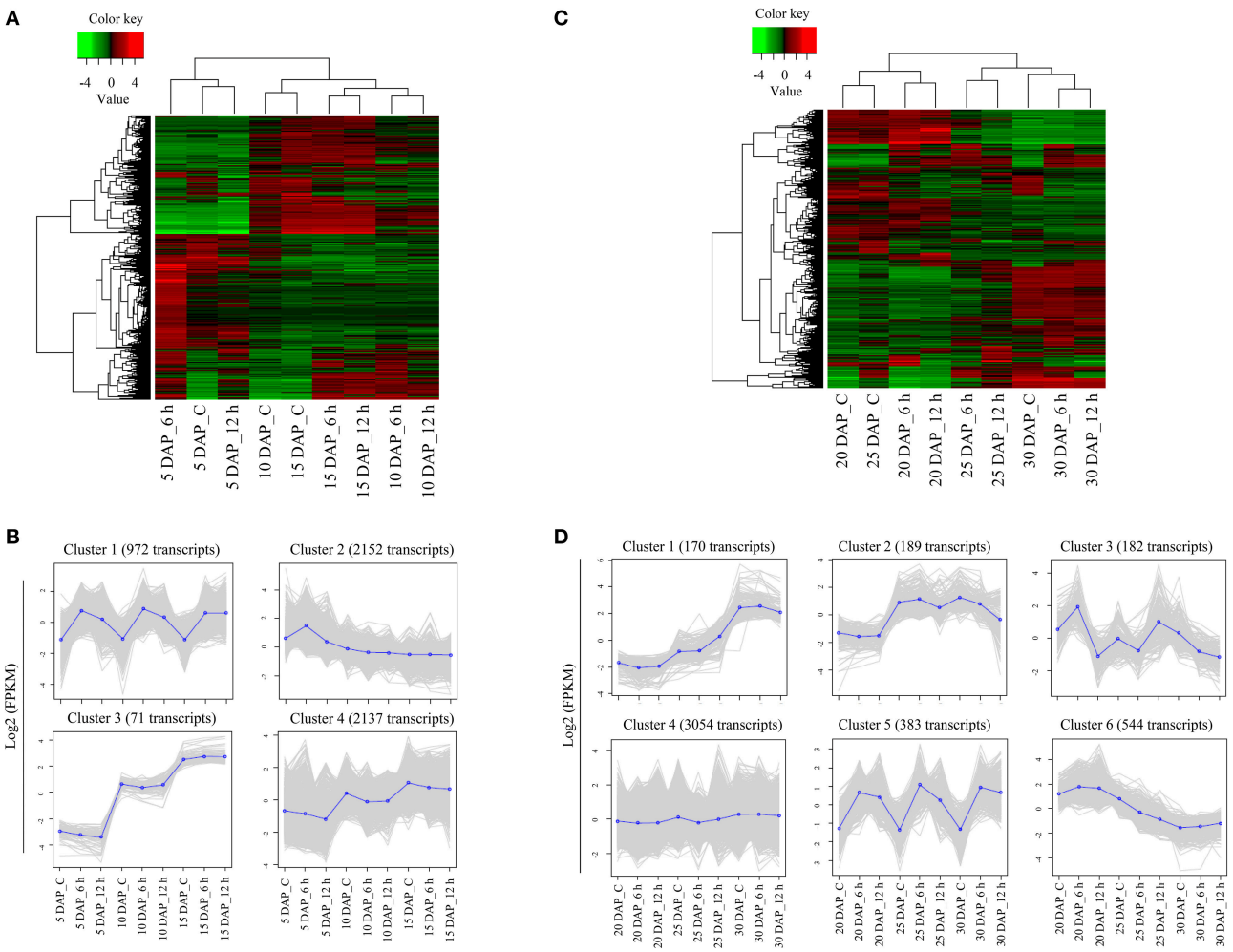


FIGURE 4 | Detailed analysis of differentially expressed genes in early and late stages of siliques exposed to cold stress. Heat maps of differentially expressed transcripts in control and cold stressed *B. juncea* siliques at early **(A)** and late **(C)** stages of development. Differentially expressed transcripts identified by pair-wise comparisons of 9 samples of each stage were clustered together on the basis of their expression values and heat map was plotted with TMM-normalized FPKM values. Clusters of transcripts showing similar expression profile in early **(B)** and late **(D)** stages of silique development under control and stress conditions. The average expression of each cluster was plotted as dark blue line whereas gray lines represent expression of individual transcripts. The key for sample labels should be interpreted as x DAP_y, where x denotes number of days; DAP represents days after pollination and y denotes either unstressed siliques –"C" or duration of cold stress in hours –"h".

stages of siliques development. ABA is a plant hormone well known for its involvement in seed development as well as abiotic stress response (Nakashima and Yamaguchi-Shinozaki, 2013). There is a direct evidence of light regulated expression of *CBF*s in cold stress. Expression of *CBF*s is regulated by circadian-rhythm and depends upon red to far-red ratio of light (Fowler et al., 2005). In our data sets, it was found that phytochrome interacting transcription factor was inducible by cold stress during early developmental stages whereas circadian-rhythm regulating factor was up-regulated in core-cold inducible datasets.

Transcriptional Regulators (TRs)

Transcriptional regulators are group of proteins, which interact with other proteins and affect transcription of genes. We were able to identify five families of cold-inducible TRs in developing siliques. Two families each were detected exclusively in early and core group of transcripts. None of the cold-inducible TR members were detected exclusively during late stages of silique development (**Table 3**). Members belonging to Orphan TRs, were abundantly represented in the core group of cold-inducible transcripts.

Kinases

Kinases play an important role in signal transduction by relaying the signal through protein phosphorylation. In our dataset, cold-inducible kinases were found only during the early phases of silique development. None of the kinases were up-regulated by cold stress either in later stages of development or were part of the core-cold inducible subsets. Families of kinases that are up-regulated by cold stress in the early development are presented

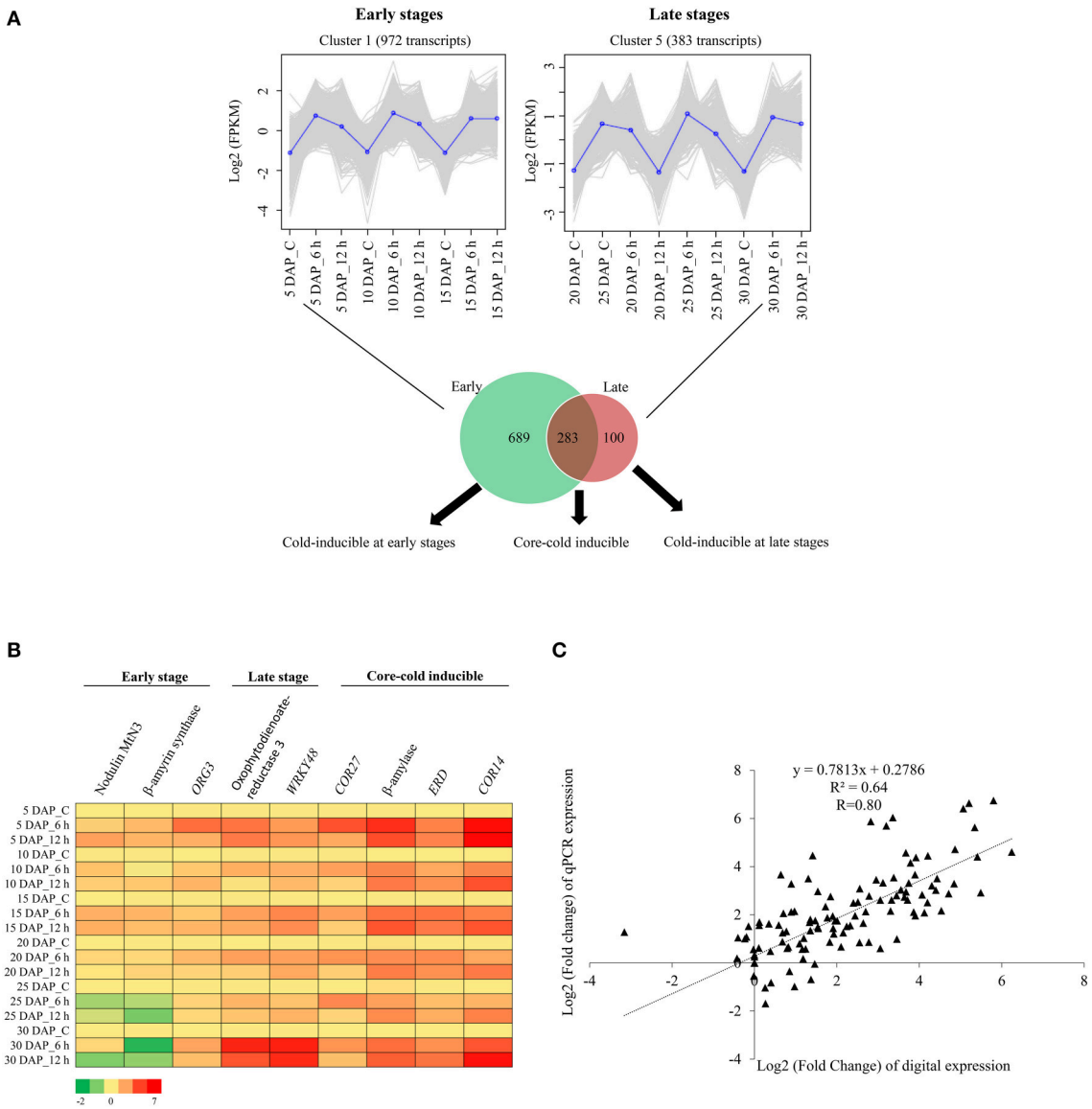


FIGURE 5 | Identification and validation of cold-inducible transcripts from early and late stages of silique development in *B. juncea*. (A) Transcripts of cluster 1 (early stage) and cluster 5 (late stage) were compared and candidates that were either specific or common to both the stages of silique development were identified. **(B)** Heat map showing relative abundance of selected cold-inducible transcripts belonging to the early, late and core subsets as determined by qPCR. **(C)** Scatter plot showing correlation between digital and qPCR expression profiles. Pearson correlation coefficient (R) was calculated between log2 fold change values of qPCR and digital expression. The key for sample labels should be interpreted as x DAP_y, where x denotes number of days; DAP represents days after pollination and y denotes either unstressed siliques –"C" or duration of cold stress in hours –"h".

in **Table 3**. A total of 16 transcripts encoding for kinases were identified out of which 7 belonged to the SNF1-related protein kinases.

Expression Profiling of Differentially Regulated Transcripts by Quantitative PCR (qPCR)

Nine transcripts representing all the three subsets (cold-inducible at early stage, core-cold inducible, and cold-inducible at late stage) were selected for validation of digital expression profile. Total RNA extracted from *B. juncea* siliques (5 DAP-30 DAP) that were subjected to 6 h and 12 h of cold stress, was used for quantification of the relative expression by qPCR. The heat map of relative expression for the 9 transcripts is depicted in **Figure 5B** and the corresponding bar graphs are given in Supplementary Figures 8–10. The expression pattern obtained by qPCR was in concordance with the expression pattern inferred by the RNA-seq data as shown by high Pearson correlation coefficient of 0.8 (**Figure 5C**). Three of the transcripts exclusive to early stages include β-amyrin synthase, ORG3-like transcription factor, and Nodulin MtN3 (*SWEET13*). The expression of

TABLE 3 | Major TF, TR and kinases family induced by low temperature belonging to three subsets (cold-inducible at early stage, cold-inducible at late stage, and core-cold inducible) of siliques development.

Type	Early stage specific	Late stage specific	Core-cold specific
TRANSCRIPTION FACTORS			
ABI3VP1	1	ND	ND
AP2-EREBP	10	1	4
bHLH	8	ND	ND
bZIP	2	ND	ND
C2C2-CO-like	ND	ND	12
C2C2-Dof	5	1	ND
C2C2-GATA	1	ND	ND
C2H2	5	ND	ND
C3H	5	2	5
FHA	1	ND	ND
HB	4	ND	1
HSF	ND	1	ND
MADS	1	ND	ND
MYB	15	ND	10
NAC	7	ND	ND
PLATZ	2	ND	1
Sigma70-like	ND	ND	2
SBP	1	ND	ND
TCP	1	ND	ND
Tify	2	ND	ND
Trihelix	2	ND	ND
WRKY	6	1	ND
TRANSCRIPTIONAL REGULATORS			
ARID	1	ND	ND
GNAT	1	ND	ND
Orphans	7	ND	31
Pseudo ARR-B	ND	ND	6
TRAF	ND	ND	1
KINASES			
Calcium dependent protein kinase	1		
Domain of unknown function 26 (DUF26) kinase	1		
Legume lectin domain kinase	1		
Leucine rich repeat kinase I	1		
Leucine rich repeat kinase XI & XII	1		
Other protein kinase	3		
SNF1-related protein kinase (SnRK)	7		
Unknown function kinase	1		

ND, not detected.

Nodulin MtN3 (*SWEET13*) was up-regulated by cold stress in both 5 and 15 DAP of silique development, whereas β-amyrin synthase was inducible in 5, 10, and 15 DAP. ORG3-like transcription factor was inducible in cold stress subjected 5 DAP. These transcripts were not inducible or had a reduced expression in response to cold stress during the late stages (20, 25, and 30 DAP) of silique development. The cold-induced expression of Oxophytodienoate reductase 3 and WRKY transcription factor 48 (genes whose digital expression were high only in the late stages) was found in early stages also, however, their induction levels at 30 DAP were much higher than early stages. Four core cold-inducible transcripts (*COR27*, *COR14*, putative β-amylase, and early response to dehydration) were found to exhibit high expression at all the stages of silique development in response to cold stress.

An additional set of seven transcripts, comprising known cold signaling pathway genes, were selected for expression analysis by qPCR. The heat map of relative expression profiles of the transcripts are depicted in **Figure 6A** and the bar graph of these genes are given in Supplementary Figure 11. *CBF1* and *COR47* were found to be inducible at all stages of silique development under cold stress, whereas *SnRK2.6* and *CAMTA3* were inducible at specific stages of silique development. The expression profiles of *ICE1* and *ICE2* were largely similar, and were induced after cold stress at 5 DAP and 30 DAP. However, increase in transcript level of *ICE2* was also observed in 15 and 25 DAP after cold stress. We failed to observe any significant increase in the expression of *SIZ1* in any of the developing stage on exposure to 6 h and 12 h of cold stress. The expression pattern obtained by qPCR was in agreement with the expression pattern inferred by the RNA-seq data. Pearson correlation coefficient (R) was 0.75 (**Figure 6B**).

DISCUSSION

The sowing period of mustard in India ranges from mid-October to first week of November (Shekhawat et al., 2012) and pod filling occurs at approximately 75–80 days after sowing (http://www.nuziveeduseeds.com/mustard-how-to-grow/). The minimum mean temperature during the pod filling stages ranges from 10 to 14°C (http://www.imd.gov.in/doc/Winter2010.pdf). As the early pod filling stages are sensitive to frost injury, it is important to understand the molecular response of developing siliques to low temperatures so that the gathered knowledge could be used for generating frost resilient *B. juncea* plants. To fully comprehend cold stress-mediated transcriptional response chilling stress was imposed to manually self-pollinated *B. juncea* siliques followed by RNA-seq. In several previous studies only vegetative parts of plants were exposed to cold stress and although it is likely that cold stress during the vegetative phase of plant growth affects the reproductive phases, we imposed cold stress to the developing siliques of *B. juncea* as cold and frost stress largely affects post-fertilization stages. Our study allowed us to focus on identification of specific changes in gene expression in post-fertilization stages, which define the yield under stress conditions. Nonetheless, it would be worthwhile to identify genes that impact developmental programming in plants exposed to cold stress during the vegetative phase. In the present study excised self-pollinated branches of field-grown mustard plants were subjected to low temperature in a cold chamber. To normalize the wounding response, self-pollinated branches were excised and placed in a beaker containing water under field conditions and this tissue was used as control. Cold stress was imposed for either 6 h or 12 h to identify gene expression changes in transcription factors and kinases, which are normally induced early as well as their downstream target genes that are up-regulated after a longer period of stress. With the help of transcriptome sequencing we were able to identify subsets of cold

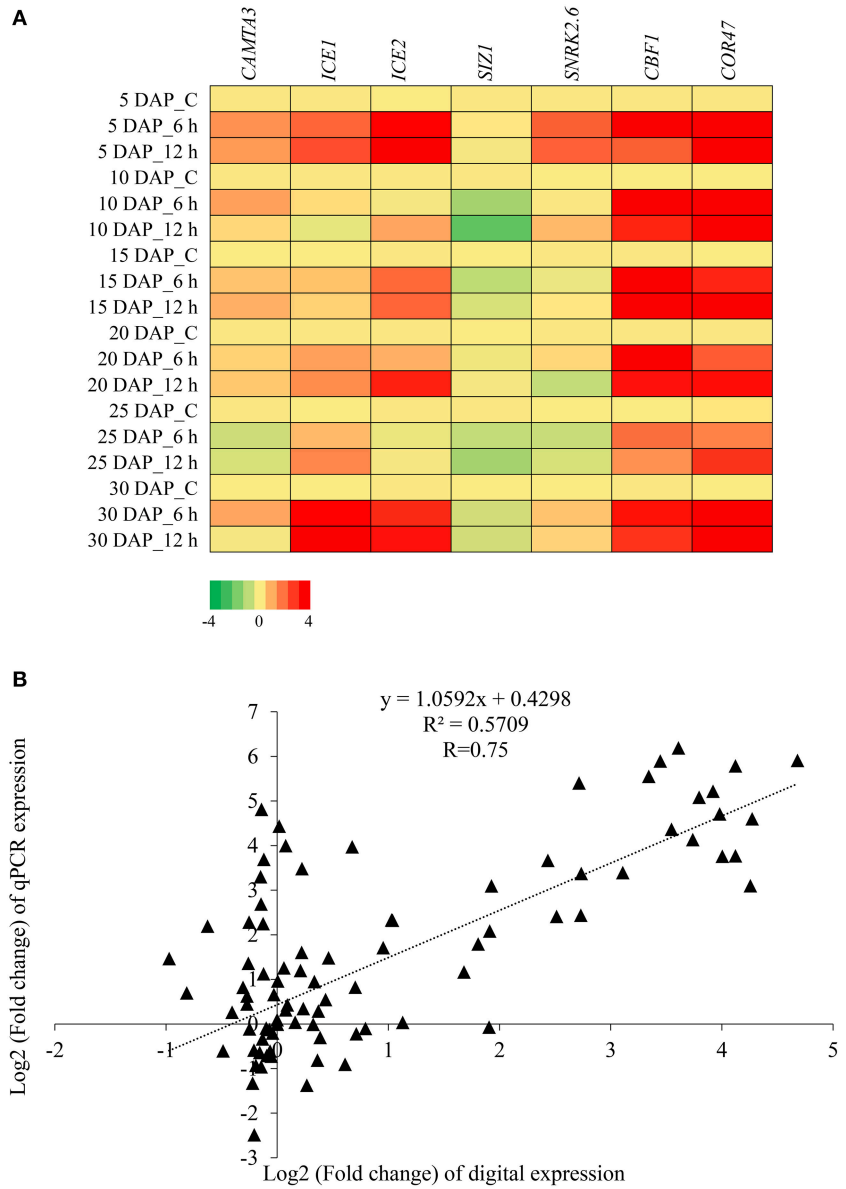


FIGURE 6 | Expression profiling of *B. juncea* transcripts homologous to cold-stress signaling pathway components. (A) Heat map showing relative abundance of genes belonging to cold signaling pathway as determined by qPCR. **(B)** Scatter plot showing correlation between digital and qPCR expression profile. Pearson correlation coefficient (R) was calculated between log2 fold change values of qPCR and digital expression.

responsive genes that are expressed in early and/or late stages of silique development. As reference genome for mapping the sequencing reads was not available, paired-end sequencing was performed to obtain an improved de *novo* assembly. In addition to the sequencing length, it is important that libraries should be sequenced with adequate depth so as to capture low abundance transcripts. As per the estimates published in a recent report, sequencing of >200 million paired-end reads can optimally discover rare transcripts and their isoforms in human genome (Sims et al., 2014). In the current study, approximately 700 million purity-filtered reads were generated, collectively from all the libraries in *B. juncea* (whose genome size is estimated to be one third of the human genome), and we believe that this depth is sufficient for discovering even the rare isoforms. Assembly, of transcripts was performed using trinity assembler, which resulted in 133,641 transcripts and composed 117 Mb of sequences.

Approximately 13,000 differentially regulated transcripts were identified by pair-wise comparison of all the 18 samples. Based on the expression profile of DETs, two distinct clades were observed- one comprising of 5, 10, and 15 DAP samples and another of 20, 25, and 30 DAP samples. Because of the clustering pattern, we categorized gene expression changes according to the early and late phases of silique development. It was also observed that cold stress subjected samples (i.e., 6 h and 12 h)

clustered together and were distinct from control samples. We identified multiple transcripts that were regulated by cold stress. Notably genes that code for proteins like dehydrin, *DREB1B*, early response to dehydration, low-temperature induced (Lti), glycine rich RNA binding protein, calcineurin B-like (CBLs), CBL-interacting protein kinases (CIPKs) etc. were up-regulated by low temperature stress. In several studies these proteins were shown to be involved in cellular response to cold stress (Gilmour et al., 1998; Fowler and Thomashow, 2002; Maruyama et al., 2004).

To identify the core components of cold stress signaling in developing siliques, we compared the transcripts that are cold-inducible of the early stages (5, 10, and 15 DAP) with those of late stages (20, 25, and 30 DAP). A total of 1072 transcripts were inducible by cold stress out of which 283 transcripts were detected from both early and late stages of silique development. This subset was therefore considered as the core group of cold-inducible transcripts. In addition, 689 transcripts and 100 transcripts were induced specifically in early and late stages of silique development respectively. These transcripts were therefore categorized as either early or late components of the cellular response to cold stress in developing siliques of *B. juncea*. To gain a better understanding about the role of these genes, we annotated them on the basis of their homology and more than 70% of the transcripts were annotated using existing databases. Multiple GO categories whose genes were involved in various abiotic and biotic stresses were highlighted in all the three subsets. Transcripts belonging to GO category "Circadian Rhythm" constituted the second largest number in the core group of transcripts. It is now well-known that circadian rhythmicity and cold stress mediated gene expression are interconnected (Espinoza et al., 2008; Dong et al., 2011; James et al., 2012; Maibam et al., 2013). Our results also show that transcripts belonging to circadian rhythmicity are cold-inducible throughout the silique development phases. Additionally, some of the transcripts of circadian rhythmicity were inducible by cold specifically in the later phases of silique development.

Another major GO category whose transcripts were inducible by low temperature was "long-day photoperiodism." Regulation of cold-inducible genes and freezing tolerance by light quality and duration have been previously reported in *Arabidopsis* (Franklin and Whitelam, 2007; Lee and Thomashow, 2012) and therefore, cold-induction of transcripts involved in photoperiodism is an additional evidence that these processes are intimately linked. A GO category whose transcripts were specifically up-regulated in the later stages of cold stress was "oxidation-reduction" process. One of the harmful effects that cold stress imposes is generation of ROS. Some of the genes that counteract the effects of ROS are cold-inducible (Lee et al., 2002; Zhu et al., 2007; Shi et al., 2014). Though generation of ROS is a function of stress, the enrichment of cold-inducible transcripts involved in oxidation-reduction process only in the later phases (which are exposed to similar cold stress as the early stages) is quite intriguing and requires further investigations.

A total of 120 transcripts belonging to 22 different TF (transcription factor) families were identified from the 1072 cold-induced transcripts. A large number of the TF transcripts (79) were cold-inducible specifically during early stages of silique development. These stages are vulnerable to the low temperature stress and therefore cold induction of kinases in these stages is possibly a mechanism to produce protective proteins by phosphorylation-mediated activation of the upstream TFs. During later stages of embryo development, LEA proteins start accumulating (Battaglia et al., 2008), and provide protection to the cells from ensuing desiccation. The core-cold-inducible subset has 4 members of AP2/EREBP family, which included one *CBF (DREB1)*, two *DREB2* members, and a single ERF. The core group also has 4 and 8 members of C2C2-constans like and C2C2-dof like TF family members, respectively, which are known to play regulatory roles in *Arabidopsis* in response to cold (Mikkelsen and Thomashow, 2009). The major family of TF enriched in core-cold (10 transcripts) as well as early stage cold-inducible (15 transcripts) subset is MYB TF family. The role of MYB genes in regulating genes of cold stress signaling pathway and in governing tolerance to cold stress is well documented (Vannini et al., 2004; Agarwal et al., 2006; Dai et al., 2007). CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), also belongs to MYB TF family and positively regulates expression of *CBFs* in *Arabidopsis* (Dong et al., 2011). Various transcripts coding for other TFs like NAC and WRKY were also identified from early stage cold-inducible subset. Transcript having homology to *AtHSFA6b* was induced by cold stress in later stages. These results are similar to the unpublished work from our lab where we found that *Arabidopsis HSFA6b* was inducible by low temperature.

Interestingly, it was found that a significant number of transcripts coding for transcriptional regulators (TRs) were inducible by low temperature. Some of the transcripts displayed inducibility in all the stages, whereas others were inducible only in the early stages. None of the TRs were cold-inducible exclusively in the later stages. One of the cold-inducible TR in early stages belonged to the GNAT (Gcn5-related N-acetyltransferase) family. Though it has been shown that CBF1 interacts with GCN5 (Stockinger et al., 2001; Mao et al., 2006), a direct role of GCN5 in promoting acetylation at COR promoters was not observed (Pavangadkar et al., 2010). Nonetheless, identification of a highly cold-inducible homolog of GNAT in *B. juncea* indicates that histone acetylation plays a critical role in modulating gene expression during cold stress. Involvement of histone modifications in cold stress is further supported by identification of a cold-inducible transcript of ARID (AT-rich interaction domain) family, members of which function as chromatin remodelers and histone demethylases (Tu et al., 2008; Lu and Tobin, 2011; Lin et al., 2014). Apart from TRs and TFs, multiple families of kinases induced by cold stress specifically during the early stages of embryo development were identified. The largest group of cold-inducible transcripts belonged to SnRK family. Members of SnRK families are involved in plant response to abiotic stresses (Ma et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Though, SnRK3 family members are routinely linked with abiotic stress response (Kim et al., 2003; Fujii and Zhu, 2009; Fujii et al., 2011), a recent study showed that the SnRK2 family member OST1 kinase phosphorylates ICE1, thereby increasing

its stability and freezing tolerance in *A. thaliana* (Ding et al., 2015).

Quantitative PCR was employed to validate the expression pattern congregated from RNA-seq data. Nine transcripts representing all the three stages (cold-inducible at early stage, core-cold inducible and cold-inducible at late stage) were selected for qPCR analysis. The 4 transcripts from core-cold inducible category included *COR27*, *COR14*, putative β-amylase, and early responsive to dehydration. Similar to the digital expression data, these genes were found to be inducible by cold stress in all the stages of silique development. Mikkelsen and Thomashow (2009) identified *COR27* along with *COL1* to be cold-inducible and as well as regulated by circadian clock. *COR14* is a cold stress regulated gene isolated in barley (Crosatti et al., 1995). Higher levels of *COR14* were observed in cold-tolerant variety during cold acclimation as compared to susceptible variety (Cattivelli et al., 1995). β-amylase hydrolyzes α-1,4 glycosidic linkages of polyglucan chains at the non-reducing end to produce maltose which in turn protects membranes, proteins, and photosynthetic electron transport chain during severe temperature stress (Kaplan and Guy, 2004). Cold stresses and dehydration result in the up-regulation of stress-induced genes such as RD (responsive to dehydration), ERD (early responsive to dehydration), COR (cold regulated), LTI (low-temperature induced), and KIN (cold-inducible). During cold acclimation, *DREB*s bind to the promoter region of the downstream target genes such as *RD29A*, *ERD10*, *COR15A*, *RD17,* and *Kin2*, subsequently resulting in low temperature tolerance (Seki et al., 2002). Enhanced expression of *COR*, *RD,* and *ERD* have been observed in transgenic *Arabidopsis* plants overexpressing *DREB1A* when exposed to low temperature (Liu et al., 1998; Kasuga et al., 1999). Transcripts selected from "cold-inducible at early stage" subset include Nodulin MtN3 family protein, β-amyrin synthase (bAS), and Transcription factor ORG3-like. Members of nodulin MtN3 family are polytopic membrane proteins having MtN3/saliva domain and some of the members of these family like *AtSWEET15* are cold-inducible, however their functional role in cold stress has not yet been characterized (Seo et al., 2011; Yuan and Wang, 2013). β-amyrin synthase is involved in biosynthesis of the secondary metabolite β-amyrin, which serves as an intermediate in the synthesis of triterpene glycosides associated with plant defense (Kemen et al., 2014). We found that bAS gene is mildly inducible by low temperature stress, which is in agreement with its previously reported stress-inducibility in *Bruguiera gymnorrhiza* and *Glycirrhiza glabra* (Basyuni et al., 2009; Nasrollahi et al., 2014).

Oxophytodienoate-reductase 3 and WRKY transcription factor 48 were shortlisted from "cold-inducible at late stage" subset. Oxophytodienoate-reductase 3 catalyzes reduction of double bonds in unsaturated aldehyde and ketone leading to production of jasmonic acid, which is involved in combating various abiotic and biotic stresses (Creelman and Mullet, 1995; Schaller et al., 2000). WRKY transcription factors are involved in plant responses to various biotic and abiotic stresses (Chen et al., 2012b). Specific members of *Arabidopsis WRKY* TF family are regulated during early stages of cold stress (Bakshi and Oelmuller, 2014). Several WRKY family genes were found to be responsive to cold stress in soyabean, *Vitis vinifera,* and barley (Marè et al., 2004; Zhou et al., 2008; Wang et al., 2014).

Attempts were made to study the expression of known cold signaling pathway genes. Primers, corresponding to sequences of *CBF1*, *CAMTA3*, *SIZ1*, *ICE1*, *ICE2*, *SnRK2.6,* and *COR47* were designed and expression of the above genes in cold stress subjected *B. juncea* siliques was quantified using real time PCR. *CBF*s bind to CRT/DRE cis-elements and induce the expression of downstream cold-regulated genes (Gilmour et al., 1998; Steponkus et al., 1998; Cook et al., 2004). Levels of *B. juncea CBF1* increased on exposure to both 6 h and 12 h of cold stress, however *CBF1* transcripts accumulated to lower levels at 12 h of cold treatment as compared to 6 h in all the development stages. This is in sync with previous reports where early induction of upstream transcription factors was observed after cold stress in *A. thaliana* (Chinnusamy et al., 2010). Targets of *CBF*s include member of Cold Regulated *G*enes (*COR*s). One of the cold-regulated genes, *COR47*, was validated and its transcript was up-regulated at all the stages of silique development after imposition of cold stress. Doherty et al. (2009) showed the dependence of *CBFs* expression on *CAMTA3* as cold-induced accumulation of *CBF*s was substantially reduced in *camta3* lines. *CAMTA3* was identified as a cold-induced gene in our transcriptome datasets, which was subsequently validated by qPCR. Moreover, a direct correlation in the levels of *CAMTA3* and *CBF1* support the previously proposed link between the two genes. At all the stages of silique development, expression of *CAMTA3* was induced by cold stress except at 25 DAP where lower levels with respect to control were observed. Similarly, cold-induced expression of *CBF1* was also considerably low at 25 DAP as compared to other stages. The decrease in *CAMTA3* levels at 25 DAP did not necessarily obliterate cold-inducibility of *CBF1*, thereby indicating that in addition to *CAMTA3* other TFs might also play a role in cold stress mediated regulation of *CBF1*. *ICE1* is the master regulator of cold stress which enhances the expression of *CBF*s and downstream *COR* genes, eventually conferring freezing tolerance (Chinnusamy et al., 2003). *ICE2*, a homolog of *ICE1,* also induces expression of *CBF1/DREB1B* and confers increased freezing tolerance in *Arabidopsis* (Fursova et al., 2009). We observed increased expression of *ICE1* and *ICE2* in *B. juncea* siliques exposed to low temperature stress. *SnRK2.6* also known as open stomata 1 (OST1) is a Ser/Thr protein kinase involved in ABA signaling. Recently, OST1 was shown to phosphorylate *ICE1* to increase its stability. The knockout lines of OST1 were found to be defective in freezing tolerance whereas overexpression lines exhibited enhanced tolerance (Ding et al., 2015). Though OST1 protein was not previously observed to be cold-inducible our results suggests that *SnRK2.6* is up-regulated on exposure to cold stress in *B. juncea* siliques. It will be interesting to study whether SnRK2.6 phosphorylates ICE1 and possibly ICE2 under cold stress in *B. juncea* siliques. Traditionally the genes involved in cold acclimation process have been used to impart both chilling as well as freezing tolerance in plants. With the help of RNA-seq we identified genes that are up-regulated by chilling stress and potentially these genes can be utilized to introduce frost tolerance during pod filling stages in *B. juncea*. In addition to

identifying homologs of CBF1-mediated cold stress signaling pathway genes, we also identified genes whose products have not yet been implicated in providing protection against low temperature stress. Further functional studies involving these genes will not only help us in extending our understanding on cold stress signaling but also pave way for designing frost hardy plants.

AUTHOR CONTRIBUTIONS

MA conceived, designed and supervised the research work. AJ and SK-A participated in regular discussions for designing the wet lab experiments and analysis of the sequencing data. SS and VKR contributed equally to the study. SS performed self-pollination, stress treatment, RNA isolation, prepared RNA-seq libraries, and qPCR based expression analysis. VKR performed high throughput sequencing and analyzed the transcriptome data. SS, VKR, and MA wrote the manuscript. BJ performed the anatomical staging. AJ and SK-A provided critical inputs for data presentation. AJ, SK-A, SG, and AK critically reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00932

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Transcriptomics profiling of Indian mustard (*Brassica juncea*) under arsenate stress identifies key candidate genes and regulatory pathways

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Arsenic (As) is a non-essential element, a groundwater pollutant, whose uptake by plants produces toxic effects. The use of As-contaminated groundwater for irrigation can affect the crop productivity. Realizing the importance of the *Brassica juncea* as a crop plant in terms of oil-yield, there is a need to unravel mechanistic details of response to As stress and identify key functional genes and pathways. In this research, we studied time-dependent (4–96 h) transcriptome changes in roots and shoots of *B. juncea* under arsenate [As(V)] stress using Agilent platform. Among the whole transcriptome profiled genes, a total of 1,285 genes showed significant change in expression pattern upon As(V) exposure. The differentially expressed genes were categorized to various signaling pathways including hormones (jasmonate, abscisic acid, auxin, and ethylene) and kinases. Significant effects were also noticed on genes related to sulfur, nitrogen, CHO, and lipid metabolisms along with photosynthesis. Biochemical assays were conducted using specific inhibitors of glutathione and jasmonate biosynthesis, and kinases. The inhibitor studies revealed interconnection among sulfur metabolism, jasmonate, and kinase signaling pathways. In addition, various transposons also constituted a part of the altered transcriptome. Lastly, we profiled a set of key functional up- and down-regulated genes using real-time RT-PCR, which could act as an early indicators of the As stress.

Keywords: arsenic, *Brassica juncea,* microarray, phytohormones, transporters, transposons

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Introduction

Crop productivity depends on factors like aeration, irrigation, and host–pathogen interactions, and also on presence/absence of abiotic and biotic stresses. Arsenic (As) is a highly toxic metalloid whose contamination is spread in large area of West Bengal, India, and Bangladesh. Such a widespread presence of As affects growth and yield of commonly cultivated crop plants in the area (Chaurasia et al., 2012; Kumar et al., 2015). In presence of As stress, an array of metabolic processes are affected and signs of As toxicity are often morphologically visible in terms of changes in root and shoot growth. Plants adapt several mechanisms of regulating the As tolerance viz., through altered expression of specific transporters or by modulating the epistatic interaction

of the interconnected genes in pathways (Yu et al., 2012). Important steps of As tolerance have been identified as reduction of arsenate [As(V)] to arsenite [As(III)], organ- and tissue-specific and subcellular distribution of As, complexation with sulfur-containing ligands and vacuolar sequestration (Srivastava et al., 2012; Kumar et al., 2015). A fine coordination of these mechanisms to avoid As toxicity is achieved through transcriptome and proteome changes (Ahsan et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009; Yu et al., 2012). However, plants suffer from toxicity when As accumulation surpasses a threshold level and particularly when its speciation dynamics are altered (Mishra et al., 2013).

An important step in As tolerance has been identified as "early sensing of the stress". Srivastava et al. (2009) compared responses of tolerant and sensitive varieties of *Brassica juncea* and suggested early perception of As stress to be the cause of variable stress tolerance among different varieties. They suggested a hypothesis that the perception of As stress could be mediated by various hormones, which may sense As indirectly through its impact on sulfur metabolism. Other studies suggest that As(V) acts as a phosphate mimic and misleads metabolic and regulatory perception of itself as an abundant supply of phosphate and thus represses genes normally induced under low phosphate conditions (Catarecha et al., 2007; Abercrombie et al., 2008). In lieu of the above studies, it can be concluded that plants avoid extreme As toxicity since repression of phosphate uptake systems leads to reduced As(V) uptake as well (Catarecha et al., 2007). Castrillo et al. (2013) found that As(V) stress induces a notable transposon burst in plants, in coordination with As(V)/phosphate transporter repression, which immediately restricts As(V) uptake. They identified WRKY6 as an As(V)-responsive transcription factor that mediates As(V)/phosphate transporter gene expression and restricts As(V)-induced transposon activation. Other microarray and transcriptomic analyses in rice under As stress (Chakrabarty et al., 2009; Yu et al., 2012) implicated the role of various signaling molecules like abscisic acid (ABA), ethylene, cytokinins, salicylic acid (SA), flavonoids, and gibberellic acid (GA) in As stress responses of plants. In addition, various transcription factors, and protein kinases were found to be up- and down-regulated in response to As(V) and As(III).

Brassica juncea belonging to the family Brassicaeae represents one of the major oil-yielding crops in India and contributes 28.6% in the total oilseeds production and ranks second after groundnut sharing 27.8% in the India's oilseed economy (Shekhawat et al., 2012). Srivastava et al. (2009) indicated an involvement of jasmonates in the signaling of As in *B. juncea.* Previous studies, conducted by our group on microRNA-specific microarray analysis of *B. juncea*, identified role of As-specific microRNAs in regulating sulfur metabolism, and metabolism and function of hormones like jasmonates, auxins, and ABA (Srivastava et al., 2013a). Taking into account all the studies, we understand that there is a need to reveal key candidate genes and pathways in *B. juncea* responsive to As stress that can also act as early As stress responsive markers in further studies. To identify such functional screening markers in root and shoot and to further enhance our understanding of As stress responses in *B. juncea*, we performed time-dependent transcriptome analysis of roots and shoots of *B. juncea* to understand the dynamic regulation of pathways involved in perception of and response to As stress and propose set of key genes and pathways.

Materials and Methods

Plant Material, As Treatment, and RNA Preparation

To study the response of the As stress, *B. juncea* (L.) Czern. var. TPM-1 was used as the plant material, which is an As tolerant variety. Seeds were sterilized and grown in a Plant Growth Chamber (Sanyo, Japan) as detailed previously (Srivastava et al., 2013a) having a daily cycle of a 14-h photoperiod with a light intensity of 150 μE $m^{-2}s^{-1}$, day/night temperature of 25 $\pm$ 2°C, and relative humidity of 65–75% for a week. After 12 days, seedlings were exposed to 500 μM arsenate [As(V); as Na_2HAsO_4] for 96 h. Seedlings were harvested for conducting microarray analysis at 4, 24, and 96 h and roots and shoots were separated and were used for RNA preparation. The quantity and purity of the RNA was determined by evaluating the absorbance at 260 nm and 260/280 nm absorbance ratio, respectively. Each of the total RNA preparations was individually assessed for RNA quality based on the 28S/18S ratio and RIN measured on an Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip Kit. With the use of Agilent's 1-Color Quick Amp Labeling Kit, 500 ng of high quality total RNA was denatured in the presence of a T7 promoter primer and a 1-Color RNA Spike-In Kit. Reverse transcriptase was used to retrotranscribe the mRNA. cDNA was used as a template for *in vitro* transcription where a T7 RNA polymerase simultaneously amplified target material and incorporated cyanine 3-labeled CTP. Labeled cRNA was purified using spin columns from the Qiagen RNeasy Mini Kit and the quantity and quality of the cRNA was determined by Nanodrop ND-1000 UV–VIS spectrophotometer.

Microarray Probe Design and Hybridization

For the design of the microarray probes, a total set of 53,939 sequences, which include expressed sequence tags (ESTs) and transcript sequences (mRNA) of *Brassica* sp., were downloaded from GenBank and clustered into unigenes using CAP3 (Huang and Madan, 1999). To avoid the formation of spurious assembly, the threshold value for the *–p* parameter, which represents the "overlap percent identity cutoff", was fixed to 97. For the probeset construction, a total of 26,881 non-redundant unigenes obtained after clustering were used, which includes 1,720 *B. juncea*, 15,259 *Brassica Napus*, and 5,075 *Brassica rapa* sequences; and 6,456 from other *Brassica* species using the Agilent eArray best probe composition algorithm with the option to design multiple probes for each sequence. The probes obtained were printed using Agilent's 4x44 array and a set of positive and negative controls were also added on to the microarray chip. To reduce the noise and the microarray hybridization bias, a set of replicate probes were also added for calculating the intra-array reproducibility.

For fragmentation, Agilent's Gene Expression Hybridization Kit was used. Briefly, 1.65 μg of cyanine 3-labeled linearly

amplified cRNA was added to hybridization cocktail and was then fragmented as per the manual of the Hybridization kit. The hybridization cocktail was then susbsequently dispensed into the wells of gasket slides and Brassica 4x44K Gene Expression Microarray was placed on the top of the gasket to allow for the hybridization. This microarray "sandwich" was then sealed in a hybridization chamber and was allowed to hybridize at 65°C by rotating at 10 RPM for 17 h. Following hybridization, slides were subsequently washed in Agilent's Gene Expression Wash Buffers according to manufacturer specifications. Hybridized microarrays were scanned using the Agilent Microarray Scanner and spot intensities were analyzed using the Agilent Technologies Feature Extraction software version 10.7.3.1. Microarray data quality was evaluated by reviewing ten standard QC metrics generated by the Feature Extraction Software (Consult the Agilent Technologies Feature Extraction Software version 10.7.3.1 reference guide for a full explanation of the QC metrics). To rule out the possibilities of having a large hybridization, staining, or wash artifacts, array images were loaded into Feature Extraction Software for manual inspections. Microarrays were determined to be free of any large artifacts ($\geq$10% of total surface area) that could have affected the quality of the data.

Microarray Data Analysis

The spot intensity values obtained from Agilent Feature Extraction software were background corrected, and were normalized using Quantile Normalization. Principal Component Analysis and Correlation Analysis were used to identify outlier samples and to check the correlations between the samples. Statistical comparisons using a fold change $\geq$2 were carried out to identify the regulation of the genes under the As stress and to identify the candidate genes. Probes that were found to be significantly up- or down-regulated using the threshold mentioned above in each comparison were classified as putative markers of As stress. For functional assignments, the differentially expressed (DE) genes were BLASTed against *Arabidopsis thaliana* TAIR 10[1] with an *E*-value of 1E-5, and in case of multiple hits, the corresponding hits were filtered based on e-values and % identity (See **Supplementary Data Sheet S1**). The genes showing up- and down-regulated profiles under the As treatment in root and shoot were assessed for the GeneSet Enrichment using assigned TAIR ids and PlantGSEA with cutoff value of 0.05 (Yi et al., 2013) Additionally, short time-series miner (STEM) analysis was done to cluster genes into specific profiles (Ernst and Bar-Joseph, 2006; **Supplementary Data Sheet S2**). The microarray data has been submitted to NCBI GEO (GSE66464). Further to develop network of interacting genes, methodology developed by Opgen-Rhein and Strimmer (2007) was used (**Supplementary Data Sheet S3**).

Validation of Key Candidate Targets for Developing As Stress Early Indicators

All the primers used for SyBr green real-time RT-PCR were obtained from the *A. thaliana* RT-PCR primer pair database. The details of the primers used are mentioned in **Supplementary Table S1**. Specificity of all the primers was confirmed by sequence analysis of RT-PCR amplicons derived from *B. juncea* as detailed earlier (Srivastava et al., 2009). The DNA free total RNA was isolated from root samples (100 mg) and then quantitative real-time PCR was performed as described previously using a Corbett rotor gene 6000 (Corbett Life Science; www.corbettlifescience.com). The PCR cycling conditions comprised of 94°C for 5 min and 40 cycles each comprising of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and final extension at 72°C for 10 min. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. Melting curves were generated and were analyzed using the dissociation curve software built into the Corbett rotor gene 6000. A relative expression ratio plot was generated using the software REST-MCS.

Inhibitor Treatment and Biochemical and Transcriptional Assays

To ascertain the role of signaling mediated by jasmonate and kinases, and their interconnections with glutathione (GSH) metabolism, several inhibitors were employed. These included L-Buthionine Sulfoximine (BSO: a potent inhibitor of γ-glutamylcysteine synthetase, a rate-limiting enzyme of GSH biosynthesis), Ibuprofen (IBP: an inhibitor of lipoxygenase and hence the jasmonate biosynthesis), and Staurosporine (STS: an inhibitor of phospholipid/calcium-dependent protein kinases). Twelve days old seedlings, grown in hydroponics, as mentioned above, were used for the study. Treatment conditions included 500 μM As(V) plus a inhibitor viz., BSO (1 mM), IBP (25 μM), and STS (200 nM). For each treatment, a separate control was also maintained. Seedlings were pretreated with inhibitors for 16 h and then subjected to As(V) treatment for 24 h. The shoot samples of seedlings subjected to each of the treatments were harvested and were then used for various assays. The biochemical analyses included activity assay of enzymes and level of metabolites of sulfur metabolism. The activities of cysteine synthase and γ-glutamylcysteine synthetase were assayed according to Seelig and Meister (1984) and Saito et al. (1994), respectively, and the levels of cysteine and GSH were estimated according to the protocol of Gaitonde (1967) and Hissin and Hilf (1976), respectively, as detailed previously (Srivastava et al., 2009). The expression analysis of MAPK-3 and 12-oxophytodienoate reductase 1 (OPR-1) genes was done using real-time RT-PCR as per the protocol mentioned above and primer details are summarized in **Supplementary Table S1**.

Results and Discussion

Overall Pattern of Up- and Down-regulated Genes in Root and Shoot

To identify the important candidate genes, microarray profiling was done at 24 and 96 h for both roots and shoot. In addition, for roots, 4-h time point was also chosen so as to capture early responsive genes with a special reference to signaling

[1]https://www.arabidopsis.org

related genes considering the fact that roots acts as the first contact point to As. At 4 h, a total of 359 genes showed significant change in expression with an up-regulation of 264 genes and down-regulation of 95 genes (**Figure 1A**). At 24 and 96 h, roots showed up-regulation of 302 and 265 genes, respectively, while down-regulation of 331 and 255 genes, respectively (**Figures 1B,C**). In shoots also, a large number of genes showed altered expression. A total of 349 and 196 genes were up-regulated and 272 and 299 genes were down-regulated at 24 and 96 h, respectively (**Figures 1F,G**). **Figures 1D,E,H,I** display the Venn diagram showing the amount of the co-expressed genes at each time point. Specifically, in roots, 111 genes were found to be up-regulated on all time points while 61 were found to be down-regulated. In shoots, commonly expressed up- and down-regulated genes were 115 and 180, respectively.

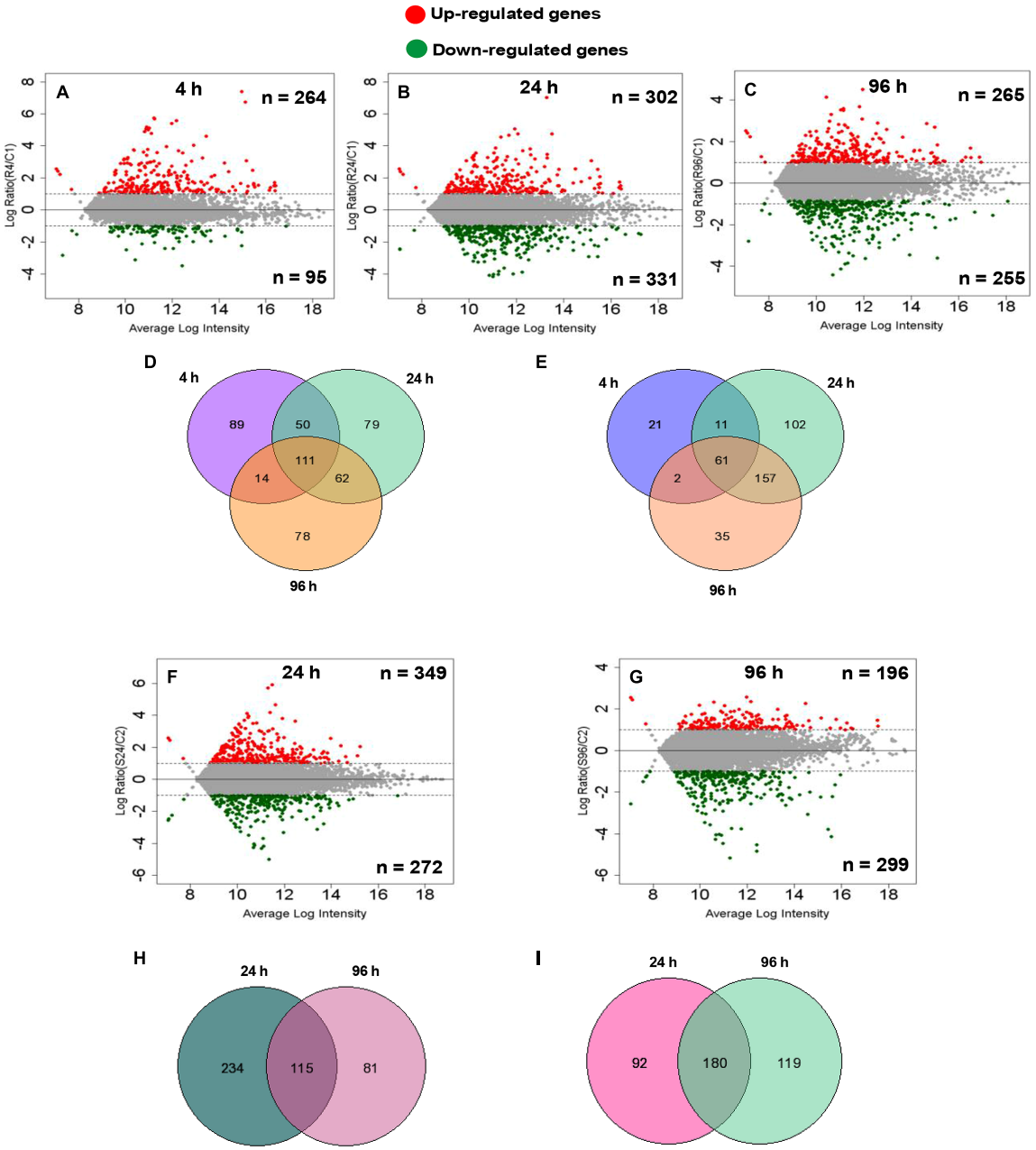


FIGURE 1 | Differentially expressed (DE) genes of root ($n = 3$) and shoot ($n = 2$) dataset at different time points compared with the control root and shoot samples calculated by difference (A–C,F,G) in arsenic-exposed *Brassica juncea* seedlings. Scatter plot represents average intensity (*x*-axis) vs. log2 ratio (*y*-axis). Red and green colors define the up-regulated and down-regulated genes at each time point. Venn analysis of common and exclusively differential (up-regulated and down-regulated) genes in arsenic-exposed *B. Juncea* root ($n = 3$) **(D,E)** and shoot ($n = 2$) **(H,I)** samples.

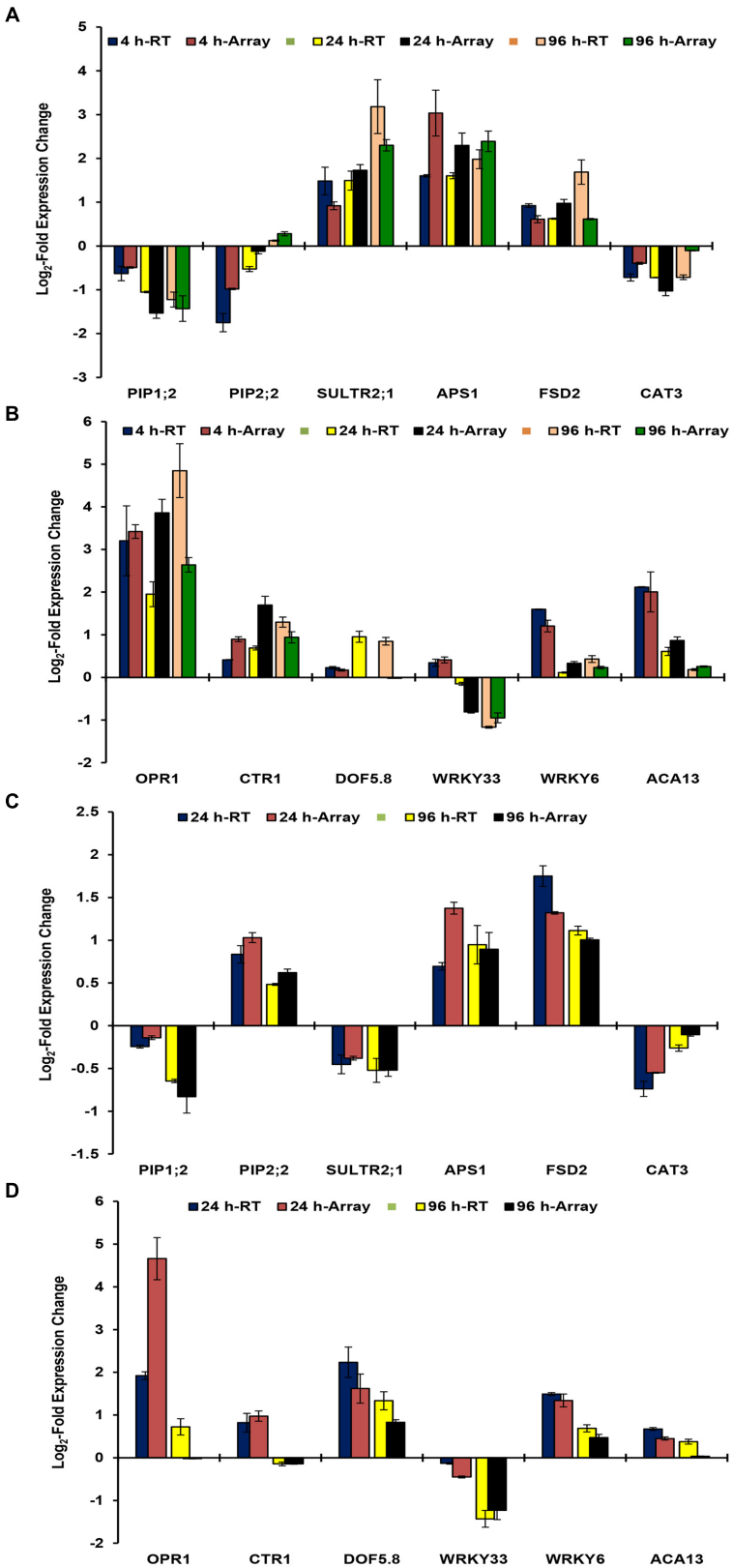


FIGURE 2 | Real time RT-PCR validation of microarray data of *B. juncea* roots at 4, 24, and 96 h (A,B); and of shoot at 24 and 96 h (C,D). The data of RT-PCR has been overlapped with that microarray to demonstrate expression patterns. The *x*-axis represents the expression of various genes in control conditions.

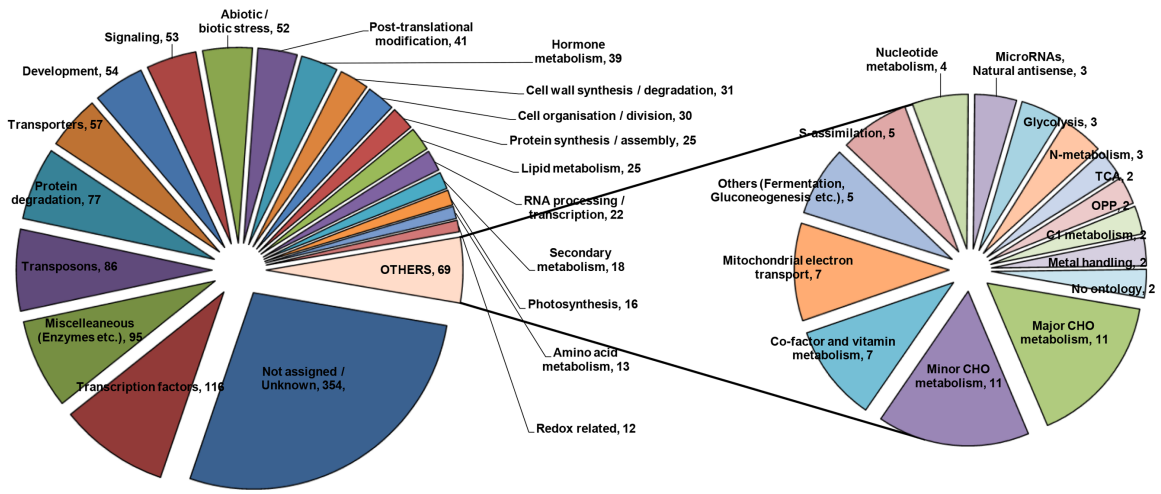


FIGURE 3 | Metabolic pathway analysis of the DE genes based on KEGG database using SuperViewer online tool.

To profile the genes into respective clusters, microarray data was clustered using STEM tool (Ernst and Bar-Joseph, 2006). In roots, STEM analysis clustered the genes into 20 clusters from which six clusters were identified as significantly ordered based on their *p*-values. Among these clusters, significantly down-regulated genes were clustered into three profiles as, P0, P4, and P10, while up-regulated genes were clustered into P17, P18, and P19 (**Supplementary Figure S1**; **Supplementary Data Sheet S2**). These clusters include either down-regulated or up-regulated genes showing similar change in their expression and include genes of all studied time points. In shoot, regulated genes were profiled into 20 clusters from which 5 clusters were identified as significant ordered based on their *p*-values. Significantly down-regulated genes were clustered into P0 and P4, while up-regulated genes were clustered into P11, P14, and P15 (**Supplementary Figure S2**; **Supplementary Data Sheet S2**). The profile gene datasets were further analyzed to obtain gene–gene network interactions on their functional co-regulation pattern. In roots, down-($n = 312$) and up-regulated ($n = 314$) profile gene datasets were analyzed that illustrated highly co-regulated 152 down-regulated (**Supplementary Figure S3**; **Supplementary Data Sheet S3**) and 145 up-regulated (**Supplementary Figure S4**; **Supplementary Data Sheet S3**) genes (Opgen-Rhein and Strimmer, 2007). On the same front, down- (216) and up-regulated (255) profile gene datasets of shoots revealed highly co-regulated 162 down-regulated and 99 up-regulated genes (**Supplementary Figures S5** and **S6**; **Supplementary Data Sheet S3**). The data of co-regulated genes depicts about biological, cellular, and molecular processes regulated in a coordinated manner.

Validation of the Set of Target Genes as Early Indicators

Microarray data was validated by analyzing the expression patterns of selected genes in roots and shoots (**Figures 2A–D**). These genes have known important functions in As stress responses ranging from water homeostasis (PIP1;2 and PIP2;2; Srivastava et al., 2013b), sulfur transport and assimilation (SULTR2;1 and APS1; Srivastava et al., 2014), jasmonate signaling (OPR1; Srivastava et al., 2009), Ca signaling (ACA13; Rai et al., 2012), As(V) uptake and response (WRKY6; Castrillo et al., 2013), and antioxidant responses (FSD2 and CAT3) and signaling (CTR1 and WRKY33). The expression patterns of selected genes were found to be similar in real time RT-PCR analysis and microarray analysis for all time points. However, fold change levels were a bit different in some cases, which may be due to differences in sensitivity of different instruments, fluorescent dyes, and methods. Correlation analysis between real time RT PCR data and microarray data depicted significant positive correlation at $p < 0.01$ for 4 h (0.919), 24 h (0.911), and 96 h (0.922) for roots; and for 24 h (0.796) and 96 (0.944) for shoot.

Gene Ontologies (GOs) and Enrichment Analysis of DE Genes

Transcriptomics offers an elusive way to look at the ubiquitous expression of genes and pathways specific to a particular stress conditions. To identify the genes, representative of particular pathways, SuperViewer[2] was used and genes were classified on the basis of Mapman data. In addition, the up- and down-regulated genes were BLASTed against the TAIR 10 database and the corresponding *A. thaliana* IDs were used for gene-set enrichment using PlantGSEA. A total of 35, 4, 158, and 9 enriched gene sets in biological processes were found using *A. thaliana* as a background with an FDR < 0.05 in root down-regulated, root up-regulated, shoot down-regulated and shoot up-regulated genes, respectively (**Supplementary Data Sheet S3**). In root up-regulated geneset, enrichment analysis identified a GO category (GO:0009605, *p*-value 1.22E-04, FDR = 0.0338), which is linked to the salicyclic acid-mediated signaling pathway suggesting the up-regulation of the genesets involved in salicyclic

[2]http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi

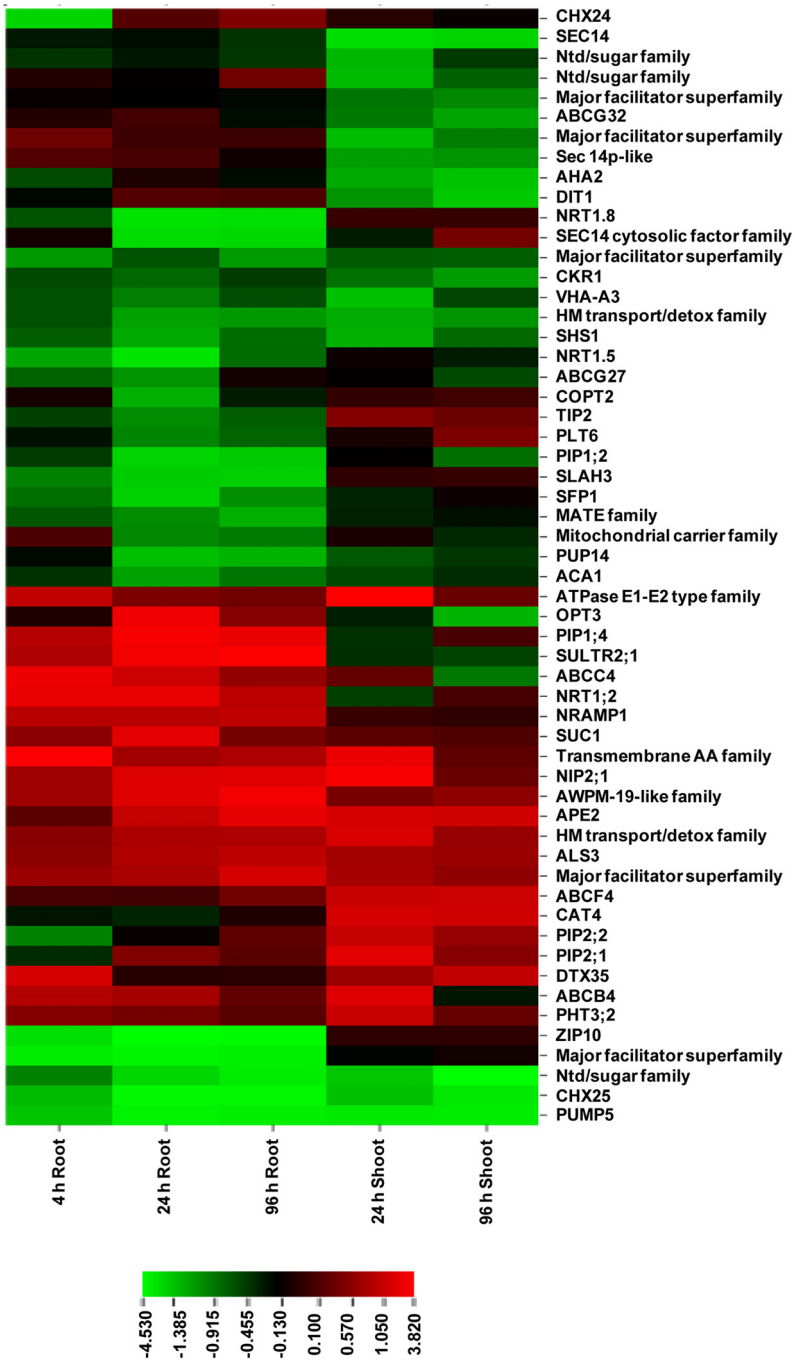


FIGURE 4 | Expression profile of DE genes encoding transporters at various time points in root and shoot of arsenic-exposed *B. juncea* seedlings. Red and green represent up-regulated and down-regulated genes, respectively.

acid singling. This observation of enriched GO of SA signaling correlates with the recent finding, where SA supplementation has been demonstrated to reduce the As toxicity by reducing the root to shoot translocation of As in *Oryza sativa* (Singh et al., 2015). Interestingly, less enriched genesets were observed in root down-regulated genes, which included GO:0034285 (Response to disaccharide stimulus, p-value = 1.61E-05, FDR = 0.0297), and GO:0009744 (Response to sucrose stimulus, p-value = 1.46E-05, FDR = 0.0297). The observed down-regulation of such genesets correlates with results of a previous study in *A. thaliana* showing down-regulation of the sugar transporters in response to As stress (Fu et al., 2014). In root up-regulated geneset, regulation of programmed cell death (GO:0043067, p-value = 1.00E-05, FDR = 9.47E-03) was also represented as the enriched GO

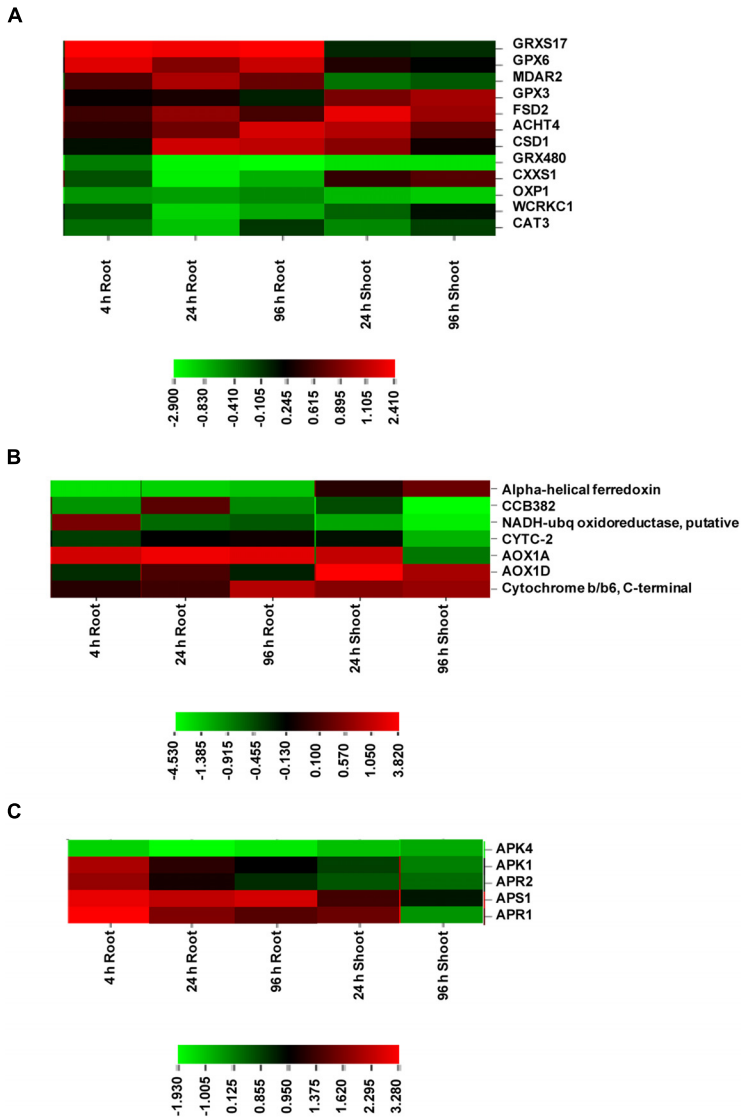


FIGURE 5 | Expression profile of DE genes encoding proteins of redox (A), electron transport (B), and S-assimilation (C) at various time points in root and shoot of arsenic-exposed *B. juncea* seedlings. Red and green represent up-regulated and down-regulated genes, respectively.

category. Proteomic characterization of the As stress induced roots revealed the accumulation of the lipid peroxidation, and *in vivo* H_2O_2 contents (Ahsan et al., 2008). The accumulation of these oxidation related metabolites reflects oxidative stress conditions, which might be lined to cell death. In shoots, geneset enrichment revealed a higher number of regulated GO terms in up-regulated genes as compared to root up-regulated genes. Among the GO terms the enriched were mainly associated with carbohydrate stimulus (GO:0009743, *p*-value = 5.85E-13, FDR = 4.58E-09), response to jasmonic acid (JA; GO:0009753, *p*-value = 2.29E-08, FDR = 1.28E-05), and oxylipin metabolic processes (GO:0031407, *p*-value = 2.78E-05, FDR = 3.63E-03). Previous studies on As stress using proteomics based characterization revealed the up-regulation of the JA pathway and is supported by several additional studies, which recently indicated the role of JA in response to the metal stress (Agrawal et al., 2003; Rodriguez-Serrano et al., 2006; Srivastava et al., 2009).

Regulatory Gene Categories Responsive to As Stress

To construct a picture from the transcriptome analysis, it was considered imperative to arrange genes into specific functional categories. SuperViewer software[3] was used for the purpose and genes were classified on the basis of Mapman data. Genes belonging to various major categories were plotted, which is presented in **Figure 3**. A few of the important categories are being discussed in the following sections.

[3]http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi

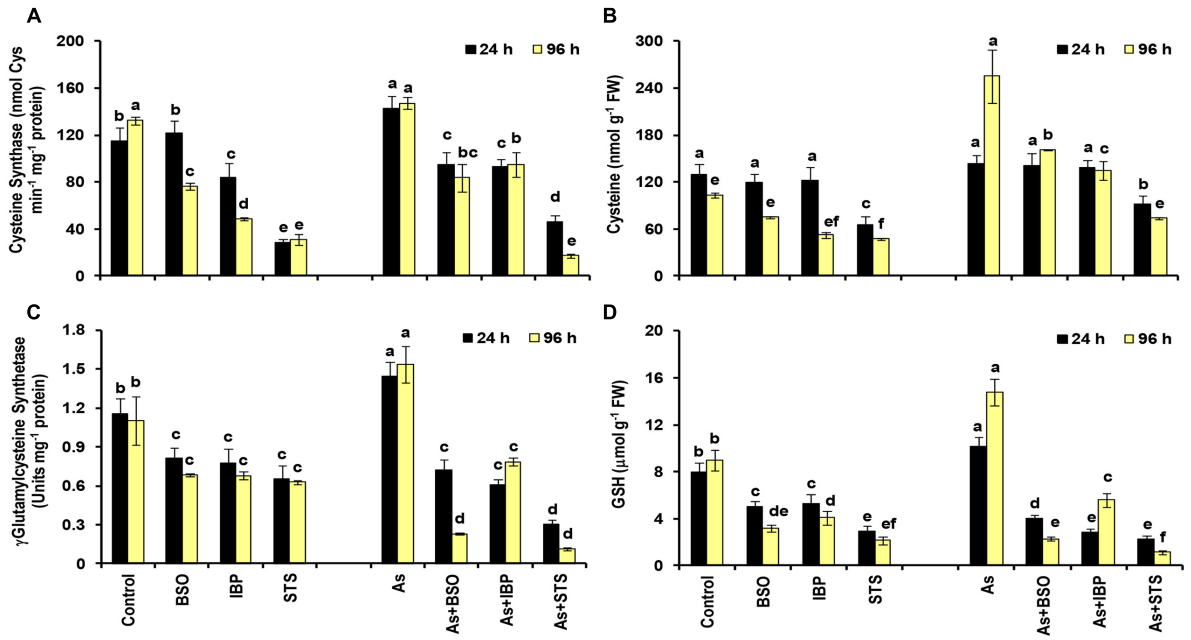


FIGURE 6 | Effect of Buthionine Sulfoximine (BSO), Ibuprofen (IBP), and Staurosporine (STS) on the activities of cysteine synthase (A) and γ-glutamylcysteine synthetase (C), and on the levels of cysteine (B) and glutathione (D) in control and arsenic-exposed *B. juncea* seedlings at 24 and 96 h. All values are means of triplicates ± SD. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values at a particular duration (DMRT, $p \leq 0.05$).

Transporters: Role of NIPs, PIPs, and Mitochondrial Transporters

Transporters play a key role in the regulation of uptake and transport of As and other metabolites. Superviewer analysis revealed a total of 57 up- and down-regulated transporters in As stress (**Supplementary Data Sheet S4**; **Figure 4**). Among these, we identified six transporters of major intrinsic proteins (MIP) superfamily comprising one nodulin 26-like intrinsic protein (NIP2;1), one tonoplast intrinsic protein (TIP2), and four plasma membrane intrinsic proteins (PIP1;2, PIP1;4, PIP2;1, and PIP2;2). The role of NIP2;1 in rice (OsNIP2;1, known as Lsi1; Low Silicon) in the uptake of As(III) and methylated As species has been previously demonstrated (Ma et al., 2008; Li et al., 2009). In *Arabidopsis* also, role of various NIPs viz., NIP1;1, NIP3;1, and NIP7;1 in AsIII transport has been experimentally demonstrated (Isayenkov and Maathuis, 2008; Kamiya et al., 2009; Xu et al., 2015). As responsiveness of PIPs has been earlier reported by Srivastava et al. (2013b) who suggested that they might regulate water uptake and transport under As stress. Mosa et al. (2012) also proposed a role of certain PIPs (OsPIP2;4, PIP2;6, PIP2;7) in As uptake. Additionally, five ATP-Binding Cassette (ABC) transporters were also found including ABCB4, ABCC4, ABCF4, ABCG27, and ABCG32. ABC transporters were found to be responsive to As(III) in rice also (Yu et al., 2012). Further, five members of mitochondrial transporters were found, which included one phosphate transporter (PHT3;2: up-regulated in shoot), dicarboxylate transporter 1 (DIT1; down-regulated in shoot) and dicarboxylate carrier 1 (DIC1; down-regulated in both shoot and root). Since, As(V) can act as a co-substrate for DIC1 (Palmieri et al., 2008), its down-regulation might be to prevent As(V) entry into mitochondrial matrix so as to avoid disturbance to energy and redox metabolism (Srivastava et al., 2013c).

Phytohormones: Early Indicators of As Stress

Among the hormone pathways, we specifically focused on JA and ABA pathway owing to their importance as regulators of abiotic stresses (**Supplementary Data Sheet S4**; **Supplementary Figure S7**). Jasmonates are important regulators of As stress perception and response mechanisms (Srivastava et al., 2009). In the present work, genes related to jasmonate biosynthesis and function included allene oxide cyclase 4 (AOC4, down-regulated in root), 12-oxophytodienoate reductases (OPR1, OPR2, and OPR3, up-regulated in both root and shoot), jasmonate resistance 1 (JAR1, down-regulated in shoot), and two jasmonate-zim-domain proteins (JAZ1 and JAZ5, down-regulated in roots). AOC4 and OPRs constitute enzymes of jasmonate biosynthesis pathways, while JAR1 is involved in synthesis of bioactive JA-Isoleucine from JA (Wasternack, 2007; Browse, 2009). JAZs are involved in repression of JA signaling and their degradation leads to jasmonate-dependent gene expression (Staswick, 2008; Ellinger and Kubigsteltig, 2010). Another important gene, sulfotransferase 2 A, which encodes a hydroxyjasmonate sulfotransferase and shows induction upon treatment with methyljasmonate and 12-hydroxyjasmonate, was significantly up-regulated. This gene is supposed to regulate excess JA or biological activity of 12-hydroxyjasmonic acid

(Gidda et al., 2003). Among ABA related genes, three important ones were highly ABA-induced PP2C1 (HAI1), ABA insensitive 1 (ABI1), and ABA interacting protein 2 (AIP2). With respect to auxins, genes mostly included those of auxin responsive proteins from shoots and roots, which were up-regulated. In addition, there were two auxin efflux carrier family proteins (PIN3 and PIN6), both of which were down-regulated in shoot. Down-regulation of PIN3 and PIN6 appears to be related to inhibition of root growth (Friml et al., 2002; Cazzonelli et al., 2013) under As stress. In addition, SA biosynthesis was also altered as there was consistent up-regulation of an important gene, farnesoic acid carboxyl-*O*-methyltransferase (FAMT) in both roots and shoot. Therefore, various hormones appeared to play important functions in response to As stress in plants with JA and SA being probably the major facilitators as indicated by our pathway enrichment analysis. This is in contrast to earlier study of Yu et al. (2012) where JA was considered to be the major player in signaling of As stress.

Transcription Factors: Role and Regulation in As Stress

A total of 116 transcription factors showed change in expression upon As stress. These included Myb, WRKY, GATA, AP2/EREBP, heat shock, G2-like, basis helix-loop-helix, homeobox, C2H2 zinc fingers, constants-like, DOF zinc finger etc (**Supplementary Data Sheet S4**; **Supplementary Figures S8A,B**). Two heat shock TFs, HSFA2 and HSFB2A showed significant up-regulation in roots on all studied time points, while HSFA2 also demonstrated significant up-regulation in shoot. HSFA2 is known to act as a key regulator in inducing the defense system under a number of environmental stress as well as against H_2O_2 treatment (Nishizawa et al., 2006). One transcription factor, plant U-box 23 (PUB23) was down-regulated in both root and shoot on all time points. PUB23 plays a role in drought stress and negatively regulates water stress response (Cho et al., 2008). Hence, down-regulation of PUB23 might be achieved to balance As-mediated disturbance to water status (Srivastava et al., 2013b).

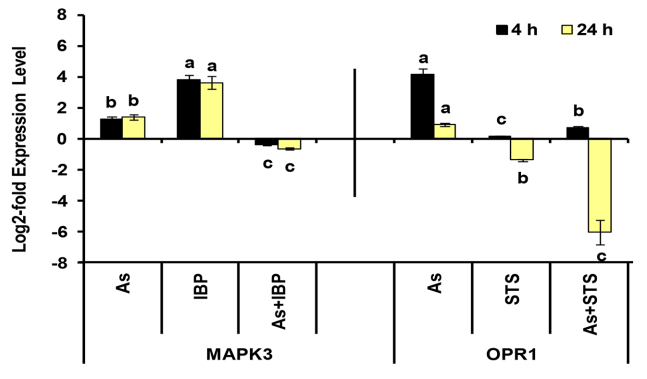


FIGURE 7 | Effect of IBP and STS on the log2-fold expression level of MAPK3 and OPR1 genes in arsenic-exposed *B. juncea* seedlings at 4 and 24 h. All values are means of triplicates ± SD.ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values at a particular duration for a gene (DMRT, $p \leq 0.05$).

Two important C2H2 zinc finger TFs included zinc finger of *A. thaliana*, ZAT6 and ZAT12, which were significantly down-regulated in root and shoot, respectively. ZAT6 is a repressor of primary root length and regulator of phosphate homeostasis (Devaiah et al., 2007a). ZAT12 forms oxidative stress signal transduction network along with ZAT7 and WRKY25 for the expression of ascorbate peroxidase 1 (APX1; Rizhsky et al., 2004) and plays a central role in ROS homeostasis (Davletova et al., 2005). WRKY family comprised six TFs including WRKY6, WRKY18, WRKY33, WRKY40, WRKY48, and WRKY75. Of these, WRKY75 showed significant up-regulation at 4 h in roots and WRKY6 was up-regulated significantly at 4 h in roots and at 24 h in shoot. In recent analysis of Castrillo et al. (2013) As(V) stress was found to induce burst of transposons and to repress As(V)/Pi transporter PHT1;1 and these responses were mediated by WRKY6. Further, WRKY75 is also known as a modulator of Pi starvation response and root development (Devaiah et al., 2007b). An early induction of WRKY6 and WRKY75 at 4 h thus confirms to the earlier reports that As(V) acts as a Pi mimic and affects expression of genes induced by Pi starvation (Catarecha et al., 2007). Myb family included a total of 13 transcriptional factors showing up or down-regulation at various time points in either root or shoot. Three TFs, Myb15, Myb39, and Myb77 were down-regulated on all time points in both root and shoot. Two other Mybs, Myb28, and Myb73 were both down-regulated in shoots only and have functions in the regulation of glucosinolate metabolism (Augustine et al., 2013) and SA and JA-signaling pathways, respectively (Jia et al., 2011). Another important TF was oxidation-related zinc finger 1 (OZF1; up-regulated in shoot only). OZF1 transcripts get induced in response to H_2O_2 and ABA treatments and OZF1 overexpressing plants are relatively resistant to oxidative stress than wild type plants (Huang et al., 2011).

Signaling Kinetics in As Tolerance

A total of 53 genes were grouped into signaling-related. Signaling related genes included a number of kinases including mitogen-activated protein kinases, calcium and light-mediated genes (**Supplementary Data Sheet S4**; **Supplementary Figure S9A,B**). One leucine-rich repeat receptor like kinase family gene, root hair specific 6 (RHS6) showed an induction of greater than fivefold. This gene is not yet fully characterized; however a study suggested that it might mediate specific external signals for root hair development (Won et al., 2009). Its early induction indicates toward a possible role of RHS6 in perceiving and delivering the As stress signal. A total of 14 genes belonged to calcium signaling category. Calreticulin 3 (CRT3) was found to be up-regulated until 24 h in roots. This is a high affinity Ca^{2+} binding molecular chaperone regulating Ca^{2+} homeostasis in ER lumen (Qiu et al., 2012). Other important known genes included calmodulin-like 38 (CML38; down-regulated in both root and shoot), calcium-dependent protein kinase 4 (CIPK4, up-regulated in both root and shoot), CML42 (up-regulated at 4 h in root and at 24 h in shoot), calcineurin B-like protein 4 (CBL4 or salt overly sensitive 3, SOS3, up-regulated in root and shoot on early time points) and CML1 (up-regulated at 24 h in shoot). CML38 and CML42 play important roles as sensors

TABLE 1 | List of selected genes, which are functionally important in the context of arsenic response of *Brassica juncea* plants and which can be utilized as early markers of arsenic stress.

Arabidopsis Gene ID	Gene Name	Function	Log2-fold expression (Root)			Log2-fold expression (Shoot)	
			4 h	24 h	96 h	24 h	96 h
At1g32350	AOX1D_alternative oxidase 1D	To maintain metabolic homeostasis during abiotic stress	**1.00**	**1.15**	**1.09**	**2.83**	**1.48**
At3g09350	Fes1A	Cytosolic Hsp70 stability and abiotic stress tolerance	**3.69**	**3.68**	**2.06**	**3.00**	**1.23**
At3g22890	APS1__ATP sulfurylase 1	Sulfur metabolism	**3.03**	**2.29**	**2.39**	**1.37**	0.89
At4g04610	APR1_APS reductase 1	Sulfur metabolism	**3.28**	**1.62**	**1.46**	**1.49**	0.03
At1g76680	OPR1_12-oxophytodienoate reductase 1	Jasmonate biosynthesis	**3.42**	**3.86**	**2.64**	**4.66**	−0.02
At2g26150	HSFA2_heat shock transcription factor A2	Regulator of several environmental stresses	**2.66**	**3.03**	**1.38**	**2.94**	1.65
At4g09570	CPK4_calcium-dependent protein kinase 4	ABA signal transduction	**2.18**	**2.51**	**2.39**	**3.16**	0.31
At5g54160	OMT1_O-methyltransferase 1	Phenylpropanoid metabolism	**4.04**	**3.43**	**1.62**	**2.46**	0.44
At3g09270	GSTU8_glutathione S-transferase TAU 8	Glutathione conjugation	**2.73**	**2.48**	**1.86**	**2.86**	0.27
At1g62300	WRKY6	Regulation of As and phosphate uptake	**1.21**	0.33	0.23	**1.34**	0.47
At4g04950	GRXS17_Monothiol glutaredoxin 17	Involved in ROS regulation and auxin signaling	**2.41**	**2.39**	**2.41**	0.01	−0.02
At5g48850	SDI1_Sulfur-deficiency induced 1	Sulfur deficiency inducible, indicator of sulphur nutritional status	**2.93**	**1.68**	**2.48**	2.01	0.9
At5g59880	ADF3__actin depolymerizing factor 3	Plant abiotic response and tolerance	**3.60**	**1.90**	**2.05**	0.17	0.03
At5g10180	SULTR2;1__slufate transporter 2;1	Sulfur uptake and transport	0.92	**1.73**	**2.3**	−0.38	−0.52
At5g51100	FSD2__Fe superoxide dismutase 2	Superoxide radical dismutation	0.61	0.975	0.615	**1.32**	**1.00**
At2g22500	DIC1_Dicarboxylate carrier 1	Mitochondrial phosphate transport	**−1.38**	**−2.55**	**−2.53**	**−2.29**	**−2.51**

Values in bold represent significant change in expression.

in Ca^{2+}-mediated signaling (Vanderbeld and Snedden, 2007; Vadassery et al., 2013). CML42 acts as a linker connecting Ca(2+) and JA signaling and affects expression of JA responsive genes negatively (Vadassery et al., 2013). CIPK4 is a positive regulator of Ca-mediated ABA signaling through phosphorylation of ABA responsive transcription factors (Zhu et al., 2007). CBL4 along with its interacting kinase CIPK6 (which was also found among responsive genes in this study) modulates the activity of Na^+ and K^+ channels to regulate ion homeostasis (Held et al., 2011). Among MAP kinase signaling pathway, MAPKKK3 (down-regulated in shoots), MKK4, and MAPK3 (down-regulated in roots) were altered in response to As. MAPKs functions in a cascade to transducer extracellular signals to the nucleus for cellular adjustments and consists of three components (Sinha et al., 2011). In rice, As(III) stress was found to induce transcripts of MPK3 and MKK4, which were also demonstrated to interact with each other (Rao et al., 2011). In *Brassica,* As stress response appears to involve MAPKKK3–MKK4–MPK3 cascade but its exact nature requires to be analyzed in further studies. FYPP3, a serine threonine protein phosphatase, showed up-regulation of 6.7-fold in 4 h As treated roots and was expressed at control levels on other time points. Such a high level of up-regulation within 4 h of As treatment warrants its important role in As stress perception and signaling, which needs to be evaluated in future work.

Redox-Related Profiled Genes in As Stress

Redox related genes included a total of 12 genes, of which 7 were up-regulated and 5 were down-regulated (**Supplementary Data Sheet S4**; **Figure 5A**). The up-regulated genes included monothiol glutaredoxin 17 (GRXS17), glutathione peroxidase 6 (GPX6), monodehydroascorbate reductase (MDAR2), copper/zinc superoxide dismutase 1 (CSD1) in roots and GPX3 and Fe SOD 2 (FSD2) in shoot. Glutaredoxins (Grxs) are small ubiquitous proteins of the thioredoxin (Trx) family and mediate reversible reduction of disulphide bonds of their substrate proteins in the presence of GSH via a dithiol or monothiol mechanism (Rouhier et al., 2008). GPX6 encodes cytosolic and mitochondrial isoforms and was found to be strongly up-regulated under various abiotic stresses including copper (Milla et al., 2003). JA has been found to induce specifically the GPX6 expression. GPX3 was found to show a slow increase under various stresses. This is in corroboration with the present transcriptome results. GPX3 has been found to play a dual role in plants: H_2O_2 homeostasis and relaying of H_2O_2 signal in guard cells. This signal mediates stomatal regulation in response to ABA (Miao et al., 2006). Thus, GPX6 and GPX3 overexpression at different time points appeared to regulate ROS levels and stomatal opening under As stress. SODs constitute the first line of defense against ROS and dismutate superoxide radicals to H_2O_2. MDAR is a component enzyme of ascorbate-glutathione pathway, which is involved in regulating H_2O_2. Catalase is another important enzyme for H_2O_2 regulation. The up-regulation of SODs and MDAR under As stress suggests stimulation of antioxidant machinery to protect against oxidative damage. CAT3 down-regulation appears to be due to differential regulation of three CAT genes (CAT1, CAT2, and CAT3) under various abiotic stress and developmental stages (Du et al., 2008).

Mitochondrial Electron Transport

All the genes belonging to mitochondrial electron transport chain showed significant up-regulation in both root and shoot including alternative oxidase (AOX1D and AOX1A), cytochrome b/b6 and cytochrome c-2, indicating that ETC was under stress in presence of As due to altered energy requirements (**Supplementary Data Sheet S4**; **Figure 5B**). A significant up-regulation of AOX1 isoforms in this study suggests their critical involvement in the regulation of ROS levels, redox and energy status under As stress (Giraud et al., 2008; Srivastava et al., 2013c). This is in sharp contrast to earlier studies where As treatment failed to induce AOX transcripts during microarray studies in *Arabidopsis* and rice (Abercrombie et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009).

Transposons

Recent study of Castrillo et al. (2013) elucidated the role of the transposon burst in As stress response, with as many as 869 genes up-regulated in *A. thaliana* plants after 1.5 h exposure to As(V) (Castrillo et al., 2013). Transposon activation in response to stress can lead to deleterious effects, such as gene deletion or insertion, chromosome rearrangement, and alterations in gene expression (Ma and Bennetzen, 2006; Ito, 2012). As proposed by Castrillo et al. (2013) change in transposon expression might be linked to epigenetic alterations and be part of survival mechanisms of plants. A large number of transposons were also found among the responsive genes in this work. This included 37 transposons, which were down-regulated and 38 transposons that were up-regulated on various time points in roots and shoot (**Supplementary Data Sheet S4**; **Supplementary Figure S10**). Only two transposons showed both up-regulation and down-regulation in tissue specific manner. A few of the As-responsive transposons identified by Castrillo et al. (2013) were also present in our study; however, they showed both down- and up-regulation. Further, we observed an up-regulation of WRKY6 gene, which was suggested to be a regulator of transposon expression, at 4 h in roots and at 24 h in shoots in the present study. It thus appears more appropriate to denote these transposons as As(V)-responsive rather than As(V)-inducible. Castrillo et al. (2013) also found that As(V) induction of transposons was transient, with the highest expression at 1.5 h after As(V) exposure. Hence, transposons do appear to play some important role in As stress response of plants but their exact functions and its time dependency is not known, which needs to be delineated in future studies using the profiled genes in ours study as a platform.

Sulfur Metabolism in As Stress and Inhibitor Treatments to Understand its Interconnections

Among sulfur assimilation pathway, ATP sulfurylase 1 (APS1), adenosine-5′-phosphosulfate reductase (APR1 and APR2) and adenosine-5′-phosphosulfate kinase (APK1) were up-regulated, while APK4 was down-regulated. Further, one sulfate transporter, SULTR2;1 was significantly up-regulated in roots. Hence, sulfur assimilation toward cysteine biosynthesis was more stimulated under As stress, which is in concurrence to earlier biochemical studies (Srivastava et al., 2009; **Supplementary Data Sheet S4**; **Figure 5C**). We further used inhibitors (BSO, IBP, and STS) to understand the interconnection of sulfur metabolism with JA and kinases. The inhibitor treatments were found to have significant impacts on cysteine synthase and γECS activities and on the levels of cysteine and GSH when control and control + inhibitor or As and As + inhibitor treatments were compared (**Figures 6A–D**). Kinase inhibitor produced most significant negative impact on studied parameters. Further, the expression of MAPK3 was affected significantly in presence of jasmonate inhibitor, IBP while the expression of OPR1 was altered in presence of kinase inhibitor STS when As and As+inhibitor treatments were compared (**Figure 7**). Thus, the inhibition of jasmonate or kinase signaling affected the sulfur metabolism. It further affected the expression of important gene of each other. Therefore, sulfur metabolism, jasmonate, and kinase signaling pathways appear to be interconnected and their mutual relationships affects the plants' responses to As exposure.

Conclusion

To conclude, we present a genome-wide transcriptional screening to highlight the genes regulated during the As stress and propose candidate genes, which could act as tools for monitoring the early indications of the As stress (**Table 1**). The present study also highlighted the role of the signaling and metabolic pathways in As stress and their dynamic nature with time. We would also like to emphasize that in such a large dataset with several time points and different tissues, there are several genes, which show consistent change that may not, sometimes, be above a threshold, but those genes can certainly carry important functions. Further, there were several unknown genes, which showed significant up-regulation or down-regulation on all time points and in both roots and shoot. Such unknown genes can give new insights into As metabolism in plants.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00646

Supplementary File S1 | Provides information of primers used for Real-time RT PCR analysis (Supplementary Table S1) and contains all supplementary figures (Supplementary Figures S1–S10) of clustering and networking analyses.

Supplementary Data Sheet S1 | List of *Arabidopsis* gene IDs for Brassica custom IDs.

Supplementary Data Sheet S2 | List of profile genesets clustered using STEM tool for root and shoot.

Supplementary Data Sheet S3 | List of co-regulated network genesets analyzed using ReviGo and Gene ontologies and pathway enrichment analyses conducted using PlantGSEA.

Supplementary Data Sheet S4 | List of all genes showing differential expression in microarray analysis and their categorization in various pathways/functional categories.

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Comparative analysis of root transcriptomes from two contrasting drought-responsive Williams 82 and DT2008 soybean cultivars under normal and dehydration conditions

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The economically important DT2008 and the model Williams 82 (W82) soybean cultivars were reported to have differential drought-tolerant degree to dehydration and drought, which was associated with root trait. Here, we used 66K Affymetrix Soybean Array GeneChip to compare the root transcriptomes of DT2008 and W82 seedlings under normal, as well as mild (2 h treatment) and severe (10 h treatment) dehydration conditions. Out of the 38172 soybean genes annotated with high confidence, 822 (2.15%) and 632 (1.66%) genes showed altered expression by dehydration in W82 and DT2008 roots, respectively, suggesting that a larger machinery is required to be activated in the drought-sensitive W82 cultivar to cope with the stress. We also observed that long-term dehydration period induced expression change of more genes in soybean roots than the short-term one, independently of the genotypes. Furthermore, our data suggest that the higher drought tolerability of DT2008 might be attributed to the higher number of genes induced in DT2008 roots than in W82 roots by early dehydration, and to the expression changes of more genes triggered by short-term dehydration than those by prolonged dehydration in DT2008 roots vs. W82 roots. Differentially expressed genes (DEGs) that could be predicted to have a known function were further analyzed to gain a basic understanding on how soybean plants respond to dehydration for their survival. The higher drought tolerability of DT2008 vs. W82 might be attributed to differential expression in genes encoding osmoprotectant biosynthesis-, detoxification- or cell wall-related proteins, kinases, transcription factors and phosphatase 2C proteins. This research allowed us to identify genetic components that contribute to the improved drought tolerance of DT2008, as well as provide a useful genetic resource for in-depth functional analyses that ultimately leads to development of soybean cultivars with improved tolerance to drought.

Keywords: soybean, dehydration, root microarray, differential expression, differential drought tolerability

Introduction

Soybean (*Glycine max* L.) has been regarded as one of the major legume crops worldwide with multibillion dollars in value. Its seed product provides a substantial source of vegetable protein and oil, micronutrients and minerals for animal feed and human consumption (Tran and Nguyen, 2009; Choudhary and Tran, 2011). In the last several years, soybean has also shown its increasing importance in industry by supplying materials for production of biodiesel, plastics, lubricants, and hydraulic fluids (Hsien, 2015). Unfortunately, like many other crops, soybean's growth and development, and thus its productivity, are severely affected by various environmental stresses, especially drought that can cause yield loss by approximately 11–50% in various countries, including Vietnam (Vinh et al., 2010; Sadeghipour and Abbasi, 2012; Ferreira Neto et al., 2013; Ku et al., 2013). Thus, in recent years, scientific community has paid a great attention to research toward understanding of mechanisms underlying soybean responses to drought, ultimately leading to development of improved drought-tolerant soybean cultivars (Tran and Mochida, 2010; Thao and Tran, 2012; Hossain et al., 2013; Deshmukh et al., 2014).

In general, to cope with drought, a number of adaptive mechanisms are activated in plants, including soybean, through various signal transduction pathways which lead to the activation of various molecular, biochemical, and physiological responses (Hadiarto and Tran, 2011; Ha et al., 2012; Hossain et al., 2013; Deshmukh et al., 2014; Karan and Subudhi, 2014; Khan et al., 2014). Studies of the mechanisms regulating these adaptive responses, as well as identification of genes involved in these mechanisms have become a great interest of the research community. Recent advances in omics technologies, especially transcriptomics, have enabled us to identify genes, gene families and pathways associated with plant responses to stresses in a systematic manner (Ma et al., 2012; Jogaiah et al., 2013; Deshmukh et al., 2014). Taking advantage of the available soybean genomic sequences and recent progress in microarray technologies (Schmutz et al., 2010; Mochida and Shinozaki, 2011), the 66K Affymetrix soybean array platform has been designed by a US consortium, which allows us to study the expression of all the putatively annotated genes in soybean at different developmental stages, under normal, abiotic, and biotic stress conditions in a relatively reliable manner (Valdes-Lopez et al., 2011; Le et al., 2012b; Wan et al., 2015).

Root development and plasticity have been identified as a key trait in plant adaptation to drought as they determine plant access to soil water. For instance, longer primary root and/or larger xylem diameters in deep roots and/or larger lateral root system are desirable root traits which help plants adapt better to drought by acquiring water from lower soil layers or foraging subsoil surface moisture (Manavalan et al., 2009; Comas et al., 2013). Thus, identification of quantitative trait loci and genes involved in determination of root traits has been regarded as an important task of research community that has interest in elucidation of molecular mechanisms regulating plant responses to drought (Manavalan et al., 2009; Comas et al., 2013; Thao et al., 2013; Satbhai et al., 2015).

In this report, we used the 66K Affymetrix soybean GeneChip to study (i) the transcriptome-wide changes in soybean dehydrated roots vs. non-dehydrated roots and (ii) analyze the genome-wide differential gene expression in the root tissues of Williams 82 (W82) and DT2008, which have differential dehydration/drought-responsive phenotype (Ha et al., 2013), under normal and dehydration conditions. W82 is a model cultivar whose genome was sequenced several years ago (Schmutz et al., 2010), while DT2008 is an economically important cultivar grown in many regions of Vietnam (Vinh et al., 2010). DT2008 was reported to display stronger tolerance to drought than W82 in a comparative analysis, which might be associated with a better root trait (Ha et al., 2013). The results of this study will enable us to identify dehydration-responsive genes in soybean roots and understand the genetic network underlying the differential drought tolerability of W82 and DT2008, as well as provide us with a list of promising candidate genes that hold potential application in development of improved drought-tolerant transgenic soybean varieties through genetic engineering.

Results

Microarray Analysis of W82 and DT2008 Root Transcriptomes under Normal and Dehydration Conditions

In our experimental design, root transcriptomes of drought-sensitive W82 and drought-tolerant DT2008 were compared at 0 (unstressed), 2 (early stress), and 10 h (late stress) of dehydration (**Figure 1A**) by microarray analysis using the 66 K soybean GeneChip (Supplementary Table S1). The relative water content (RWC) of dehydrated plants was measured during dehydration treatment, and the values were 70.2 and 75.8% for W82 and DT2008, respectively, at 2 h, whereas the respective values at 10 h of dehydration were 18.1 and 40% (**Figure 1B**), indicating the mild and severe stress intensities. This experimental design thus allowed us to identify (i) dehydration-responsive genes in each cultivar in a time-course manner (W-D2/W-C and W-D10/W-C; DT-D2/DT-C and DT-D10/DT-C), as well as (ii) genes involved in regulatory network that regulates differential root trait (DT-C/W-C, DT-D2/ W-D2, and DT-D10/W-D10), thereby potentially contributing to higher drought tolerance of DT2008 relative to W82.

Recently, the soybean genome sequence and its annotation have been substantially improved in the newest version Glyma v2.0 [Glyma.Wm82.a2.v1 (genome assembly 2 annotation version 1)] released by Phytozome 10.1 (http://phytozome.jgi.doe.gov/pz/portal.html). Using this latest Glyma v2.0 annotation, the 66K soybean GeneChip allowed us to study the expression of 38172 genes with high confidence. These genes were subjected to a search for differentially expressed genes (DEGs) using the criterion of two-fold expression change ($q < 0.05$) (Supplementary Table S2). We found that 105 and 526 genes were upregulated and 47 and 215 were downregulated in W82 roots treated with dehydration for 2 and 10 h, respectively

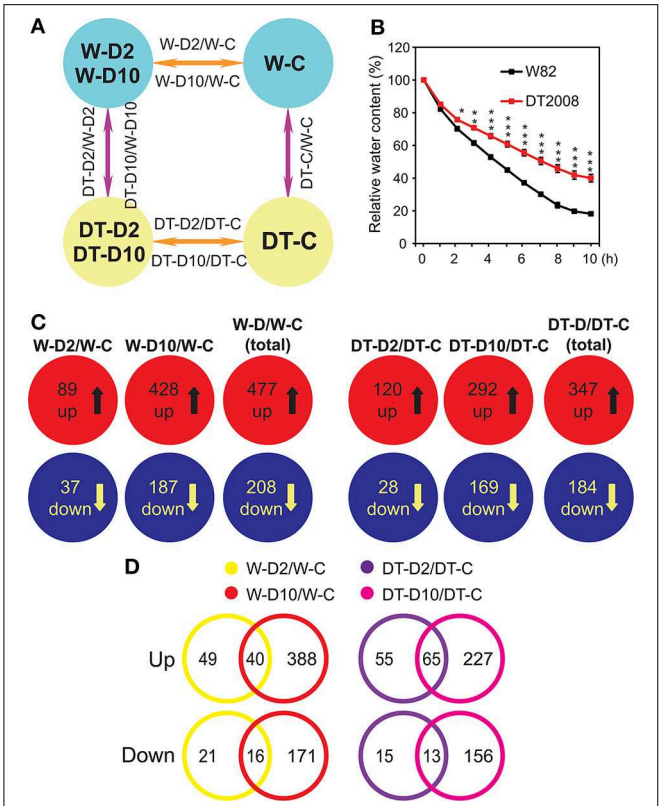


FIGURE 1 | Experimental design and summary of the results of the microarray analysis. (A) Diagrams showing experimental design and comparisons. **(B)** Relative water content of W82 and DT2008 plants exposed to a dehydration treatment. Data represent the mean and SE ($n = 5$). Asterisks indicate significant differences as determined by a Student's *t*-test (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$). **(C)** Upregulated and downregulated genes identified in each comparison from 34097 genes that were assigned with a putative function. Data were obtained from the results of three independent microarray experiments of three biological repeats. **(D)** Effect of stress intensity on gene expression in roots of W82 and DT2008 as indicated by Venn analysis of differentially expressed gene sets identified in **(C)**. W-D2/W-C, W82-dehydrated-2 h vs. W82-well-watered control-0 h; W-D10/W-C, W82-dehydrated-10 h vs. W82-well-watered control-0 h; W-D/W-C represents W-D2/W-C and/or W-D10/W-C (W82-dehydrated-2 h and/or 10 h vs. W82-well-watered control-0 h); DT-D2/DT-C, DT2008-dehydrated-2 h vs. DT2008-well-watered control-0 h; DT-D10/DT-C, DT2008-dehydrated-10 h vs. DT2008-well-watered control-0 h; DT-D/DT-C represents DT-D2/DT-C and/or DT-D10/DT-C (DT2008-dehydrated-2 h and/or 10 h vs. DT2008-well-watered control-0 h).

(Supplementary Figure S1A, comparisons W-D2/W-C and W-D10/W-C; Supplementary Tables S3A–D), whereas 131 and 355 genes were upregulated and 34 and 199 were downregulated in 2 and 10 h-dehydrated DT2008 roots vs. control, respectively (Supplementary Figure S1A, comparisons DT-D2/DT-C and DT-D10/DT-C; Supplementary Tables S4A–D). A Venn analysis indicated that 50 genes were upregulated in both 2 h- and 10 h-dehydrated W82 roots, whereas 55 were upregulated by 2 h dehydration and 476 genes by 10 h dehydration only (Supplementary Figures S1A,B; Supplementary Table S3E), making a total of 581 unique genes upregulated by at least one dehydration treatment (Supplementary Figure S1A, W-D/W-C). Similarly, we found an overlap of 21 downregulated genes in roots of W82 treated with dehydration for 2 and 10 h, and a list of 241 unique genes downregulated in dehydrated W82 roots under these two treatment conditions (Supplementary Figures S1A,B; Supplementary Table S3F). As for the drought-tolerant DT2008, we noted from the Venn diagrams that 71 upregulated and 16 downregulated genes were overlapped between DT-D2/DT-C and DT-D10/DT-C comparisons, while totally 415 and 217 unique genes were upregulated and downregulated, respectively, in 2 and/or 10 h-dehydrated DT2008 roots (Supplementary Figures S1A,B, comparison DT-D/DT-C; Supplementary Tables S4E,F).

Identification of Dehydration-responsive Genes with Putative Function in W82 and DT2008 Roots

Next, to identify genes modulated by dehydration in roots of W82 and/or DT2008, which have a predicted function for subsequent comparative analyses, we removed the genes with "no original description," which are a total of 4075 genes, and examined only 34097 genes that could be assigned with a putative function (Supplementary Table S5). This approach allowed us to link the expression change by stress treatment with gene function, thereby enabling us to explain the differential root responses of W82 and DT2008 to drought. We noted 89 and 428 upregulated genes and 37 and 187 downregulated genes in W-D2/W-C and W-D10/W-C comparisons, respectively (**Figure 1C**; Supplementary Tables S6A–D). At the same time, we were able to detect 120 and 292 upregulated genes and 28 and 169 downregulated genes in DT-D2/DT-C and DT-D10/DT-C comparisons, respectively (**Figure 1C**; Supplementary Tables S7A–D). As shown by Venn analysis, 40 upregulated and 16 downregulated genes were overlapped between W-D2/W-C and W-D10/W-C comparisons, while a total of 477 and 208 unique genes were found to be upregulated and downregulated, respectively, in dehydrated W82 roots (**Figure 1D**; Supplementary Tables S6E,F). In case of DT2008, we detected 65 and 13 overlapped genes in the upregulated and downregulated gene sets obtained from DT-D2/DT-C and DT-D10/DT-C comparisons. Removing the overlapped genes made the lists of unique genes upregulated (347) or downregulated (184) by at least one dehydration treatment in DT2008 roots vs. control for further analyses (**Figures 1C,D**; Supplementary Tables S7E,F). Several genes showing various degrees of induction and repression by dehydration were selected for verification of the microarray data using real-time quantitative PCR (RT-qPCR) (Supplementary Table S8). Results shown in **Figure 2** clearly demonstrated the reliability of the microarray data.

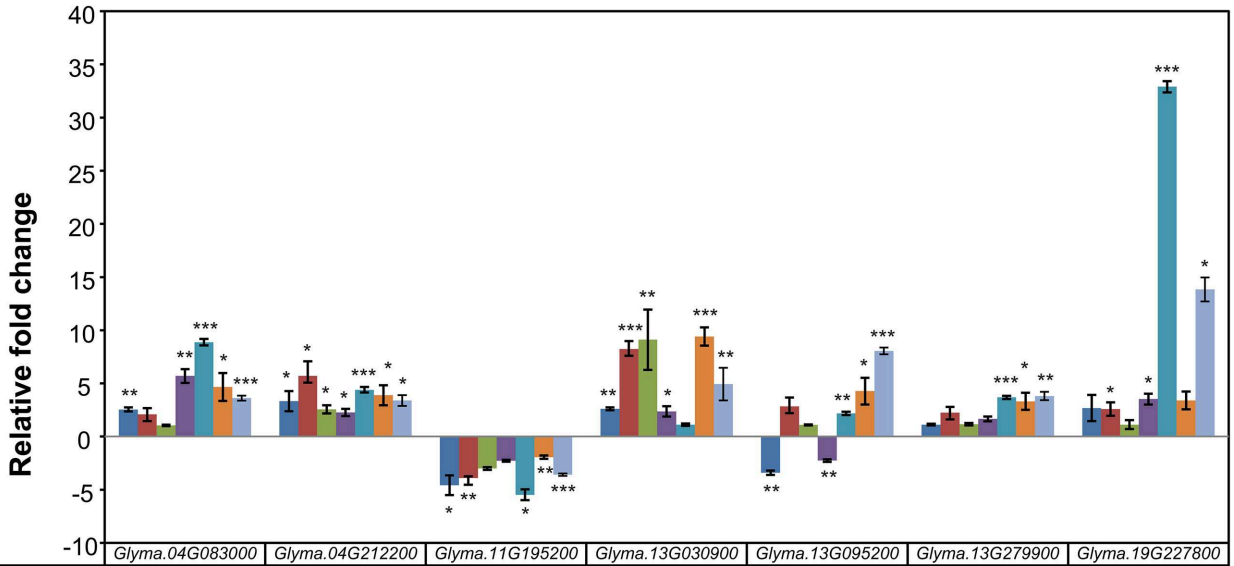
Distribution of the Dehydration-responsive Gene Sets Identified in W82 and DT2008 Roots into Functional Categories

As a means to understand the molecular mechanisms underlying root responses that soybean plants develop to increase their adaptation to drought, we used MapMan to classify the dehydration-responsive genes detected in W82 and DT2008 into various functional categories. Lists of unique genes with

A

Glyma 2 ID	Microarray data													
	DT-C vs W-C		DT-D2 vs W-D2		DT-D10 vs W-D10		W-D2 vs W-C		W-D10 vs W-C		DT-D2 vs DT-C		DT-D10 vs DT-C	
	Fold change	q-value	Fold change	q-value	Fold change	q-value	Fold change	q-value	Fold change	q-value	Fold change	q-value	Fold change	q-value
Glyma.04G083000	2.614555	0.042432	3.258701	0.050831	1.17867	0.595939	6.755826	0.039775	14.97512	0.015301	8.420253	0.037245	6.750947	0.018568
Glyma.04G212200	5.388354	0.041824	8.15626	0.037069	2.222395	0.19264	2.316769	0.088876	7.598483	0.024694	3.506853	0.097159	3.133949	0.064747
Glyma.11G195200	-4.28059	0.101151	-2.9485	0.034435	-1.93853	0.169071	-1.6523	0.103328	-3.32594	0.03359	-1.13811	0.851662	-1.5062	0.47248
Glyma.13G030900	3.141957	0.025417	2.730304	0.05332	2.092875	0.149397	1.729662	0.132444	2.142148	0.071613	1.503046	0.114282	1.426897	0.066656
Glyma.13G095200	-1.91738	0.205791	2.155244	0.040768	1.032759	0.884863	-1.81058	0.221835	2.091222	0.129926	2.282378	0.057367	4.141017	0.02328
Glyma.13G279900	1.248811	0.395151	1.521326	0.073822	1.124983	0.643191	1.964096	0.069524	3.092159	0.028103	2.3927	0.070246	2.785551	0.045294
Glyma.19G227800	3.071611	0.140748	3.704389	0.031099	1.096094	0.910573	4.933751	0.064345	46.86791	0.018625	5.950145	0.06752	16.72466	0.041343

B



	Glyma.04G083000	*Glyma.04G212200*	*Glyma.11G195200*	*Glyma.13G030900*	*Glyma.13G095200*	*Glyma.13G279900*	*Glyma.19G227800*
DT-C/W-C	2.549	3.333	-4.584	2.609	-3.403	1.125	2.676
DT-D2/W-D2	2.085	5.709	-3.900	8.225	2.835	2.250	2.579
DT-D10/W-D10	1.038	2.567	-3.004	9.105	1.084	1.165	1.125
W-D2/W-C	5.696	2.271	-2.282	2.367	-2.261	1.659	3.523
W-D10/W-C	8.877	4.397	-5.477	1.120	2.184	3.681	32.900
DT-D2/DT-C	4.659	3.891	-1.941	9.404	4.267	3.317	3.395
DT-D10/DT-C	3.613	3.387	-3.588	4.925	8.056	3.811	13.833

FIGURE 2 | Validation of microarray data by real-time quantitative PCR (RT-qPCR). Seven genes were selected for RT-qPCR verification of the microarray data. **(A)** Fold changes were obtained from microarray analysis. Red- and blue-color letters indicate absolute fold-changes ≥2 with $q < 0.05$. **(B)** Fold changes obtained by RT-qPCR of three independent biological replicates. The *Fbox* was used as reference gene. Data represent the mean plus SE ($n = 3$). Asterisks indicate significant differences as determined by a Student's *t*-test (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$). Red- and blue-color letters indicate absolute fold-changes ≥2 with $P < 0.05$. W-D2/W-C, W82-dehydrated-2 h vs. W82-well-watered control-0 h; W-D10/W-C, W82-dehydrated-10 h vs. W82-well-watered control-0 h; DT-D2/DT-C, DT2008-dehydrated-2 h vs. DT2008-well-watered control-0 h; DT-D10/DT-C, DT2008-dehydrated-10 h vs. DT2008-well-watered control-0 h.

putatively predicted function (Supplementary Tables S6E,F, S7E,F), which were found to be upregulated or downregulated in W82 or DT2008 roots by at least one dehydration treatment, either 2 or 10 h treatment, were assembled and subjected to MapMan analyses for assignment of each gene into functional category (**Figure 3**). Our data indicated that among the 20 most abundant categories, in both W82 and DT2008 roots, the upregulated genes of the TF category were the most highly enriched genes, whereas the downregulated genes were enriched in "protein synthesis, targeting, modification, etc" category.

Brief Description of the Dehydration-responsive Gene Sets Identified in W82 and DT2008 Roots

A closer look at the sets of the DEG sets identified in W82 and DT2008 roots under dehydration revealed a number of common phenomena between their up- and downregulated gene sets, respectively (comparisons W-D/W-C and DT-D/DT-C) (Supplementary Tables S6E,F, S7E,F). Many genes belonging to different TF families, such as the AP2_EREBP-, bZIP-, MYB- and NAC-type TF families, exhibited transcriptional changes by dehydration in both W82 and DT2008 roots, of which more dehydration-inducible genes were found than

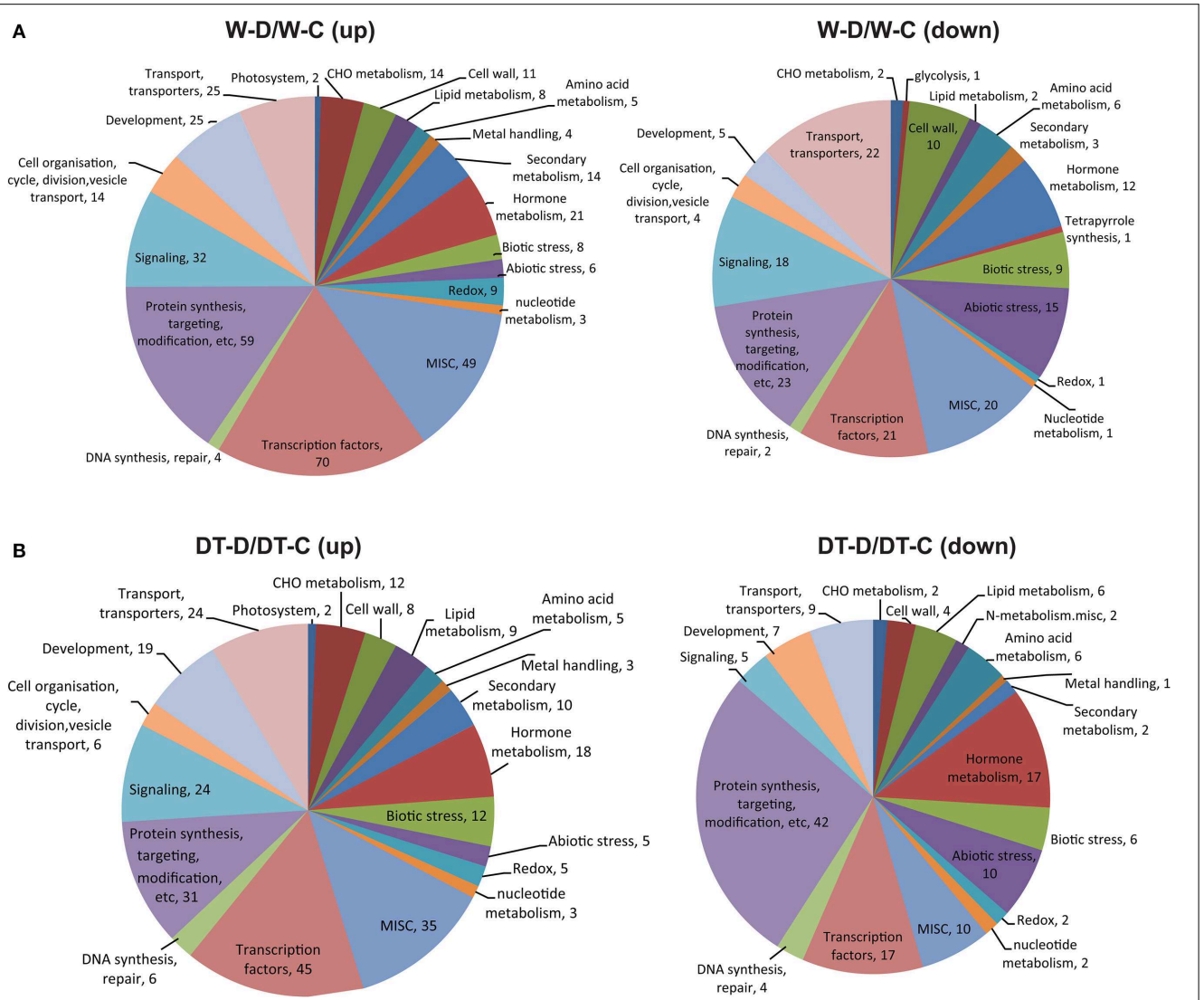


FIGURE 3 | Distribution of genes differentially expressed in roots of W82 and DT2008 by dehydration treatment into functional categories. Genes upregulated or downregulated in W82 **(A)** or DT2008 **(B)** roots by at least one dehydration treatment were classified into functional categories using MapMan. W-D/W-C represents W-D2/W-C and/or W-D10/W-C (W82-dehydrated-2 and/or 10 h vs. W82-well-watered control-0 h); DT-D/DT-C represents DT-D2/DT-C and/or DT-D10/DT-C (DT2008-dehydrated-2 and/or 10 h vs. DT2008-well-watered control-0 h).

dehydration-repressible genes (W-D2/W-C, W-D10/W-C, DT-D2/DT-C, and DT-D10/DT-C in **Figure 4**; Supplementary Figure S2; Supplementary Table S9). For instance, there were 13 and 5 upregulated *GmNAC* genes, in dehydrated W82 and DT2008 roots, respectively, while there were only 0 and 1 downregulated *GmNAC* genes detected in the respective root samples (Supplementary Table S9). Another example is that among the AP2_EREBP-type members, 13 and 11 dehydration-induced genes were found in W82 and DT2008 roots, respectively, in comparison with 3 and 4 dehydration-repressed genes in the respective dehydrated roots (Supplementary Table S9). Under our stringently set criteria of the fold change and *q*-values, the majority of the TF genes of these representative TF families were observed to be induced in either W82 or DT2008 roots by the prolonged 10 h rather than the short 2 h dehydration treatment (**Figure 4**; Supplementary Table S9).

Apart from the regulatory TFs, a number of DEGs encoding other types of regulatory proteins, such as kinases and hormone signaling-related proteins, were found in the signaling and protein modification categories. Some of them were predicted to be SnRK (sucrose non-fermenting-related), RLK (receptor-like), and MAP (mitogen-activated protein) kinases and PP2C (protein phosphatase 2C) proteins based on sequence homology with their *Arabidopsis* counterparts (Supplementary Figure S2; Supplementary Tables S6E,F, S7E,F). These proteins have been shown to be involved in regulation of plant responses to various stresses, including drought (Umezawa, 2011; Osakabe et al., 2013). Among many dehydration-inducible genes coding

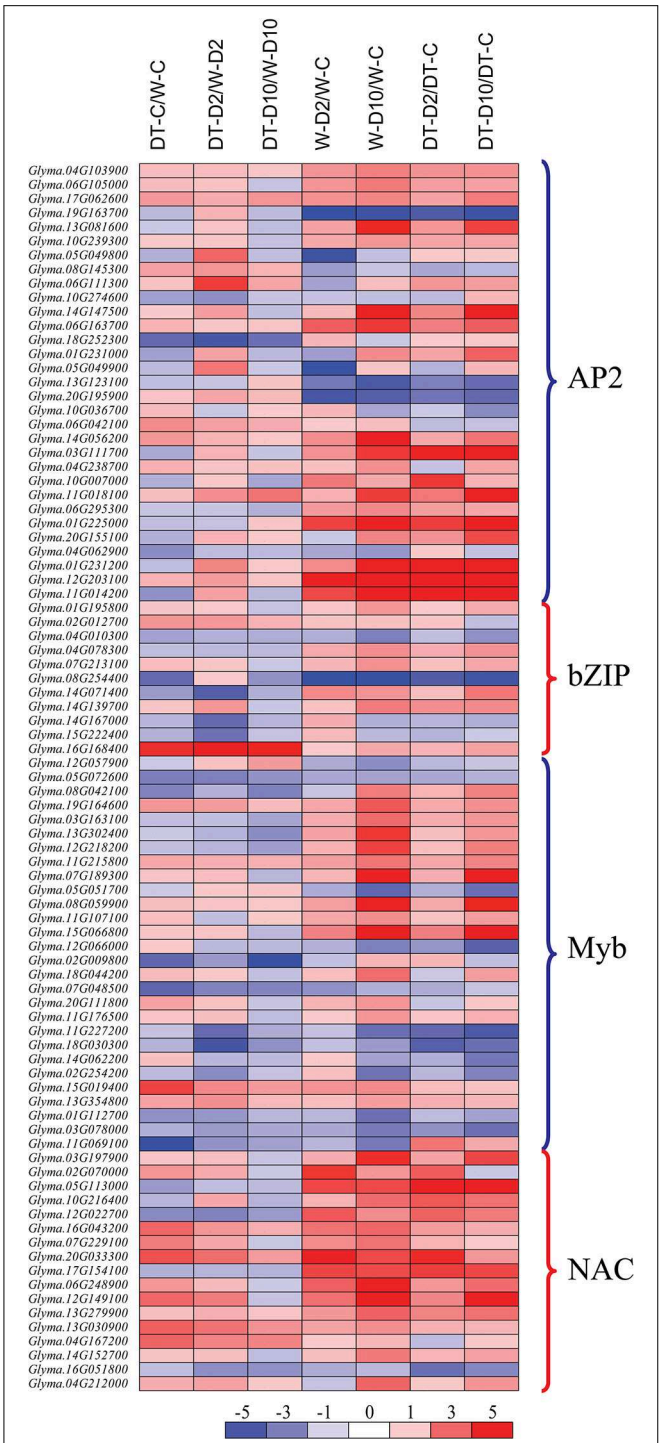


FIGURE 4 | Heatmap analysis of genes from well-known stress-related transcription factor families showing differential expression in various comparisons under well-watered and/or dehydration conditions.
DT-C/W-C, DT2008-well-watered control-0 h vs. W82-well-watered control-0 h; DT-D2/W-D2, DT2008-dehydrated-2 h vs. W82-dehydrated-2 h; DT-D10/W-D10, DT2008-dehydrated-10 h vs. W82-dehydrated-10 h; W-D2/W-C, W82-dehydrated-2 h vs. W82-well-watered control-0 h; W-D10/W-C, W82-dehydrated-10 h vs. W82-well-watered control-0 h; DT-D2/DT-C, DT2008-dehydrated-2 h vs. DT2008-well-watered control-0 h; DT-D10/DT-C, DT2008-dehydrated-10 h vs. DT2008-well-watered control-0 h.

for non-regulatory proteins are those encoding proteins of transporters, osmoprotectant biosynthesis-related proteins, and detoxification enzymes. Some are deserved to be mentioned, such as ABC (ATP-binding cassette) transporters, the ABA-importing transporter 1 (AIT1)-like proteins that might have ABA importer activity (Kanno et al., 2012), aquaporins, galactinol synthases, and polyamine oxidases (Supplementary Tables S6E, S7E). An appropriate change of their levels during stress may lead to a better adaptation of the plants (Osakabe et al., 2013; Himuro et al., 2014; Minocha et al., 2014; Rangan et al., 2014; Srivastava et al., 2014).

Differential Expression between W82 and DT2008 Roots under Normal and Dehydration Conditions—the Upregulated Gene Sets

To study the correlation between the differential gene expression in roots of W82 and DT2008 and their differential drought tolerance, we first compared their root transcriptomes under both normal and dehydration conditions. With regard to the upregulated gene sets derived from DT2008 vs. W82 comparison, we found that under well-watered conditions, 82 genes were upregulated in DT-C/W-C comparison, whereas under dehydration, a total of 147 genes were upregulated in DT-D/W-D comparison, with more induced genes being identified during earlier stress (**Figure 5A**). Namely, 143 upregulated genes were found in DT-D2/W-D2, while only nine upregulated genes in DT-D10/W-D10 (**Figure 5A**; Supplementary Tables S10A–D). A number of upregulated genes identified in DT-C/W-C and DT-D/W-D comparisons possess putative regulatory functions, as they encode transcription factor, kinase and hormone-related proteins (Supplementary Figure S3).

Next, to identify genes that might contribute to higher drought-tolerant level of DT2008, we first searched for genes that are more highly expressed in drought-tolerant DT2008 than drought-sensitive W82 under normal conditions and are dehydration-inducible in W82 and/or DT2008 roots. We, therefore, subjected the upregulated gene sets obtained from the following comparisons DT-C/W-C, W-D/W-C, and DT-D/DT-C to a Venn analysis (DT-C/W-C vs. W-D/W-C, DT-C/W-C vs. DT-D/DT-C, DT-C/W-C vs. W-D/W-C vs. DT-D/DT-C) (Supplementary Tables S11A–C). As shown in **Figure 5B**, out of 82 genes displaying higher expression in DT2008 roots than in W82 roots (DT-C/W-C comparison, Supplementary Table S10A), six and two genes were found to be inducible by dehydration in W82 and DT2008 roots, respectively, with one gene, *Glyma.04G083000*, was upregulated in both dehydrated W82 and DT2008 roots (Supplementary Tables S11A–C). Furthermore, genes showing higher expression in DT2008 roots than in W82 roots under dehydration conditions, and being dehydration-inducible in W82 and/or DT2008 roots, might also have impact on improved drought-tolerant level of DT2008 vs. W82. Thus, the upregulated gene sets of DT-D/W-D, W-D/W-C, and DT-D/DT-C comparisons were also evaluated by a Venn analysis. Among 147 genes with more abundant transcripts in DT2008 roots than in W82 roots (DT-D/W-D, Supplementary Table S10D), 10 and 14 genes were detected to be upregulated in dehydrated W82 and DT2008 roots, respectively, of which five

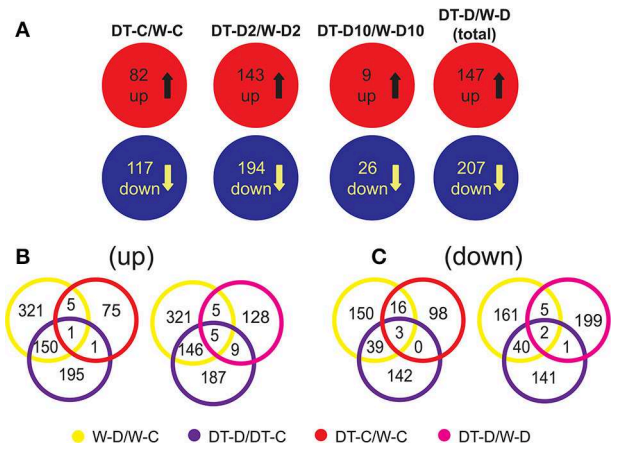


FIGURE 5 | Comparison of root transcriptomes of W82 or DT2008 under well-watered or dehydration conditions. (A) Differentially express gene sets between W82 or DT2008 roots under well-watered or dehydration conditions. **(B,C)** Identification of dehydration-responsive genes in differentially expressed gene sets that are derived from comparison of root transcriptomes of W82 or DT2008 under well-watered or dehydration conditions. DT-C/W-C, DT2008-well-watered control-0 h vs. W82-well-watered control-0 h; DT-D2/W-D2, DT2008-dehydrated-2 h vs. W82-dehydrated-2 h; DT-D10/W-D10, DT2008-dehydrated-10 h vs. W82-dehydrated-10 h; W-D/W-C represents W-D2/W-C and/or W-D10/W-C (W82-dehydrated-2 h and/or 10 h vs. W82-well-watered control-0 h); DT-D/DT-C represents DT-D2/DT-C and/or DT-D10/DT-C (DT2008-dehydrated-2 h and/or 10 h vs. DT2008-well-watered control-0 h); DT-D/W-D represents DT-D2/W-D2 (DT2008-dehydrated-2 h vs. W82-dehydrated-2 h) and/or DT-D10/W-D10 (DT2008-dehydrated-10 h vs. W82-dehydrated-10 h).

genes were upregulated in roots of both cultivars by dehydration (**Figure 5B**, Supplementary Tables S11D–F).

Differential Expression between W82 and DT2008 Roots under Normal and Dehydration Conditions—the Downregulated Gene Sets

As for the downregulated gene sets obtained from comparative analysis of W82 and DT2008 root transcriptomes, we detected 117 and 207 downregulated genes in DT-C/W-C (normal conditions) and DT-D/W-D (dehydration conditions) comparisons, respectively. We also observed that more genes (194 vs. 26 genes) were downregulated in DT-D/W-D comparison by the short-term 2 h (DT-D2/W-D2) rather than the prolonged 10 h (DT-D10/W-D10) dehydration treatment (**Figure 5A**; Supplementary Tables S10E–H).

In a similar manner, the downregulated gene sets obtained from the comparative analysis of root transcriptomes of W82 and DT2008 under non-stressed and stressed conditions (DT-C/W-C and DT-D/W-D) were also analyzed to identify dehydration-repressible genes exhibiting lower expression in drought-tolerant DT2008 as these genes would also be responsible for better performance of DT2008 relative to W82 under drought. Thus, downregulated gene sets of DT-C/W-C, DT-D/W-D, W-D/W-C, and DT-D/DT-C comparisons were evaluated by a Venn analysis as well (Supplementary Table S12). Venn diagrams shown in **Figure 5C** indicated that a total of 19 genes had lower expression in DT2008 roots than in W82 roots under well-watered conditions. All these 19 genes were repressed by dehydration in W82 roots, of which three genes were also downregulated in DT2008 roots (Supplementary Tables S12A–C). As for genes showing lower expression levels in DT2008 roots than W82 roots under stress conditions, we found a total of eight genes of which five and one genes were repressed by dehydration in W82 or DT2008 roots only, while two genes were dehydration-repressed in roots of both cultivars (**Figure 5C**, Supplementary Tables S12D–F).

Discussion

Large-scale transcriptome analysis is one of the most comprehensive approaches used to identify gene repertoire whose members are responsible to certain stressors (Mochida and Shinozaki, 2011). The completion of soybean genomic sequence has enabled us to carry out high-throughput transcriptomic studies in this important legume crop under various stress conditions in different organs (Schmutz et al., 2010; Le et al., 2012b; Ferreira Neto et al., 2013; Wan et al., 2015). Genes identified through the large-scale expression profiling studies, not only in soybean but also in other crops, have significantly accumulated in the past decade, providing a valuable resource for further functional genomics and comparative analyses (Ma et al., 2012).

DT2008 is an elite soybean cultivar cultivated in many regions in Vietnam, owing to its strong tolerance to drought and dehydration in comparison with many other cultivars (Vinh et al., 2010; Ha et al., 2013; Sulieman et al., 2015). In a previous study, we compared the drought tolerability of DT2008 and the W82 model cultivar, and found that the higher drought-tolerant degree of DT2008 relative to W82 might be attributed, at least, to its better root development in comparison with W82 (Ha et al., 2013). To explain this phenomenon at molecular level, in the current study we carried out a microarray analysis of root transcriptomes of both DT2008 and W82 under normal, as well as mild (2 h-treated) and severe (10 h-treated) dehydration stress conditions using the 66K soybean GeneChip (**Figures 1A,B**, Supplementary Table S1). This custom 66K Affymetrix GeneChip has been shown to be a reliable tool for large-scale gene expression analysis in different organs under different types of stress, such as leaves (Le et al., 2012b) and roots (this work) under drought/dehydration stress, and in the same organs under biotic stress (Valdes-Lopez et al., 2011; Wan et al., 2015).

With the release of the newest annotation version Glyma v2.0 (http://phytozome.jgi.doe.gov/pz/portal.html), we were able to examine the expression of 38172 genes with high confidence through our transcriptome analysis (Supplementary Table S2). In general, we found more DEGs in roots of drought-sensitive W82 than in that of drought-tolerant DT2008 under dehydration in both upregulated and downregulated categories. Specifically, 2.15% (822/38172 genes) of the 38172 examined genes, which were annotated with high confidence, showed altered expression by dehydration in W82 roots, whereas 1.66% (632/38172 genes) of the analyzed genes exhibited differential

expression in DT2008 roots under the same treatment conditions (Supplementary Figure S1A). On the other hand, in another independent study using DeepSuperSAGE (26 bp tags) for comparative root transcriptome analysis of 15-day-old drought-tolerant Embrapa 48 and drought-sensitive BR 16 seedlings at early stage of dehydration stress (between 0 and 150 min with 25 min interval), the authors in total found more differentially expressed soybean unitags in drought-tolerant Embrapa 48 roots than in drought-sensitive BR 16 roots in both upregulated and downregulated categories (Ferreira Neto et al., 2013). These findings suggest that different varieties might transcriptionally respond to dehydration/drought in different ways to activate root-related mechanisms for higher tolerability when compared with a specific drought-sensitive genotype. Alternatively, the different growth conditions might be a reason for the different observations of the two studies, as we grew the soybean plants in soil, whereas Ferreira Neto and colleagues hydroponically cultivated their soybean plants in nutrient solution (Ferreira Neto et al., 2013). It is worthy to notice that we also detected more upregulated genes in drought-tolerant DT2008 roots than drought-sensitive W82 roots by early 2 h dehydration treatment (131 vs. 105), although a reverse tendency was observed in case of downregulated genes (34 vs. 47) (**Figure 1C**, Supplementary Figure S1A). These results together suggest that induction of more dehydration/drought-responsive genes in roots of drought-tolerant cultivars, as compared with that in drought-sensitive cultivar, at early stage of stress exposure might contribute to its higher drought tolerability (Vinh et al., 2010; Ferreira Neto et al., 2013; Ha et al., 2013).

In addition, we recorded more DEGs in roots of both DT2008 (DT-D10/DT-C vs. DT-D2/DT-C) and W82 (W-D10/W-C vs. W-D2/W-C) by 10 h than 2 h dehydration treatment (**Figure 1C**, Supplementary Figure S1A). These data indicated that the long-term dehydration stress triggered change in expression of more genes in soybean roots than the short-term one, independently of the genotype. Furthermore, the MAPMAN analysis showed that TF encoding genes were the most highly enriched upregulated genes, whereas those classified to "protein synthesis, targeting, modification, etc" category were the most highly enriched downregulated genes in both W82 and DT2008 roots under dehydration (**Figure 3**). This finding suggested that genes belonging to these categories were those whose expression in roots is the most responsive to dehydration to aid the plants in adapting to the stress. Interestingly, a previous microarray analysis using the same GeneChip found a reverse trend in V6 and R2 leaves of the W82 cultivar. The authors reported that in these W82 leaf tissues, TF encoding genes were enriched among the downregulated genes; while, for the upregulated gene sets, "protein synthesis, targeting, modification, etc" was the most significantly enriched category (Le et al., 2012b).

With respect to the TF encoding genes, many members of the major TF families, such as AP2_EREBP, bZIP, MYB, and NAC, showed differential expression by dehydration in both W82 and DT2008 roots (**Figure 4**). Moreover, the heatmap analysis also indicated that the majority of the dehydration-inducible TF genes, such as *NAC* genes, exhibited higher expression level in DT2008 roots than W82 roots, especially under well-watered and early dehydration treatment (**Figure 4**). Increasing evidence has shown that members of these TF families play important roles in plant responses to water deficit by controlling transcription of downstream genes through their specific binding to the so-called *cis*-acting elements located in the promoters of target genes (Yamaguchi-Shinozaki and Shinozaki, 2006; Hadiarto and Tran, 2011; Jogaiah et al., 2013). A number of published reports have shown positive correlation between *NAC* gene expression levels, specifically in roots or leaves or whole plants, and drought tolerability of various crops, including soybean (Nakashima et al., 2007; Zheng et al., 2009; Xue et al., 2011; Thao et al., 2013; Thu et al., 2014; Zhu et al., 2014; Nguyen et al., 2015; Yang et al., 2015), further supporting that NAC TFs, at least in part, might contribute to the higher drought tolerance of DT2008 vs. W82. Molecular tailoring of the TF encoding genes has provided a promising approach for improvement of tolerance of a number of crops to various types of environmental stresses, including drought (Yang et al., 2010; Thao and Tran, 2012).

Apart from the TF genes, many other dehydration-inducible genes also displayed higher expression levels in DT2008 roots than W82 roots under normal or dehydration conditions (Supplementary Table S11), which might contribute to differential drought tolerance of DT2008 and W82. Results summarized in **Figure 5** indicated that the short-term dehydration-induced expression changes might be more highly required for enhanced drought tolerance of DT2008 vs. W82 than the prolonged dehydration-induced ones, as significantly higher number of DEGs were identified in DT-D2/WT-D2 comparison than DT-D10/WT-D10 comparison. With regard to dehydration-upregulated genes with higher expression levels in DT2008 roots vs. W82 roots under well-watered conditions (Supplementary Tables S11A–C), *Glyma.14G216500* encodes an ortholog STH2 (salt tolerance homolog2) (**Table 1**), a B-box TF that can act as a positive regulator of photomorphogenesis and anthocyanin biosynthesis (Datta et al., 2007). This gene might play a role in enhanced tolerance of DT2008 as anthocyanins are known to protect plants against various environmental stresses, including drought (Pourcel et al., 2007). Modulation of the signaling molecule phospholipids, which involved in regulation of plant response to environmental stimuli, through *Glyma.04G083000* (induced by dehydration in both DT2008 and W82 background, **Table 1**) and *Glyma.20G189100* that encode putative proteins with function in phosphoinositide signaling, might also be responsible for increased tolerance of DT2008 to drought (Liu et al., 2013). As for the dehydration-inducible genes showing higher transcription levels in DT2008 roots vs. W82 roots under dehydration (Supplementary Tables S11D–F), *Glyma.19G227800*, *Glyma.16G003500,* and *Glyma.13G095200* encoding osmoprotectant biosynthesis-, detoxification- or cell wall-related proteins (**Table 1**), such as the orthologs of *Arabidopsis* AtGOLS2 (*Arabidopsis thaliana* galactinol synthase 2), glyoxalase I family protein and xyloglucan endotransglycosylase, may play important roles in better adaptation of DT2008 to drought relative to W82 as supported

TABLE 1 | List of several candidate genes that might contribute to higher drought tolerance of DT2008 vs. W82.

Glyma 2 ID	*Arabidopsis* ortholog	Description	W-D/W-C	DT-D/DT-C	DT-C/W-C	DT-D2/W-D2	DT-D10/W-D10
			Responsiveness	Responsiveness	Fold change ($q < 0.05$)	Fold change ($q < 0.05$)	Fold change ($q < 0.05$)
Glyma.04G083000	*AT3G22810*	Putative phosphoinositide binding protein	Induced	Induced	2.61	Unchanged	Unchanged
Glyma.20G189100	*AT3G10550*	Putative inositol or phosphatidylinositol phosphatase	Induced	None	2.03	Unchanged	Unchanged
Glyma.14G216500	*AT1G75540*	Similar to STH2 (salt tolerance homolog2)	Induced	None	4.40	Unchanged	Unchanged
Glyma.13G095200	*AT4G25810*	Similar to XTR6 (xyloglucan endotransglycosylase 6)	None	Induced	Unchanged	2.16	Unchanged
Glyma.16G003500	*AT1G80160*	Similar to GLYI7 (glyoxylase I 7)	Induced	None	Unchanged	2.91	Unchanged
Glyma.19G227800	*AT1G56600*	Similar to AtGOLS2 (*A.thaliana* galactinol synthase 2)	Induced	Induced	Unchanged	3.70	Unchanged
Glyma.02G069400	*AT4G18710*	Similar to BIN2 (brassinosteroid-insensitive 2)	Repressed	Repressed	−3.56	Unchanged	Unchanged
Glyma.06G050900	*AT5G36250*	Similar to protein phosphatase 2C 74	Repressed	Repressed	−3.23	−2.30	Unchanged
Glyma.08G254400	*AT3G30530*	Similar to bZIP42	Repressed	Repressed	Unchanged	Unchanged	−2.05
Glyma.12G202400	*AT4G03415*	Similar to protein phosphatase 2C 52	Repressed	None	−2.11	Unchanged	Unchanged

W-D/W-C represents W-D2/W-C and/or W-D10/W-C (W82-dehydrated-2 h and/or 10 h vs. W82-well-watered control-0 h); DT-D/DT-C represents DT-D2/DT-C and/or DT-D10/DT-C (DT2008-dehydrated-2 h and/or 10 h vs. DT2008-well-watered control-0 h); DT-D2/W-D2, DT2008-dehydrated-2 h vs. W82-dehydrated-2 h; DT-D10/W-D10, DT2008-dehydrated-10 h vs. W82-dehydrated-10 h.

by previous studies (Xu et al., 1995; Taji et al., 2002; Kaur et al., 2014).

Additionally, dehydration-repressible genes with lower expression level in drought-tolerant DT2008 roots than drought-sensitive W82 might also contribute to the better performance of DT2008 vs. W82 under drought. Among the dehydration-repressible genes that had lower expression levels in DT2008 than W82 under well-watered conditions (Supplementary Tables S12A–C), *Glyma.02G069400* codes for an ortholog of *Arabidopsis* BIN2 (brassinosteroid-insensitive 2) (**Table 1**). It was reported that the rice ortholog of BIN2, the OsGSK1 (glycogen synthase 3-like protein kinase), acts as a negative regulator of plant responses to multiple stresses, including drought (Koh et al., 2007). Repression of *Glyma.02G069400* might therefore contribute to increased drought tolerance of DT2008. Another example is *Glyma.12G202400* encoding a protein with high homology to an *Arabidopsis* phosphatase 2C protein (AT4G03415) (**Table 1**) that might be involved in drought response perhaps through its interaction with CPK16 of Ca^{2+}-dependent protein kinase/sucrose non-fermenting related kinase (CPK/SnRK) superfamily (Curran et al., 2011). CPK16 has been known to be implicated in regulation of root gravitropism that is a trait important for plant response to water stress (Kirkham, 2008; Huang et al., 2013). With regard to the dehydration-repressible genes that displayed lower expression levels in DT2008 roots than W82 roots under dehydration (**Figure 5C**; Supplementary Tables S12D–F), *Glyma.06G050900* and *Glyma.08G254400* encoding phosphatase 2C and bZIP orthologs of *Arabidopsis*, respectively, caught our attention (**Table 1**). *Glyma.06G050900* exhibited lower expression in DT2008 roots than W82 roots under both normal and dehydration conditions (**Table 1**). It is well established that many members of the phosphatase 2C family are involved in stress signaling (Schweighofer et al., 2004). Several phosphatase 2C proteins have been found to act as negative regulators of ABA signaling (Umezawa et al., 2010). As for *Glyma.08G254400*, although there is no specific information either for this gene or its highest *Arabidopsis* homolog (AT3G30530/AtbZIP42), there have been published reports that several bZIP TFs act as negative regulators of drought tolerance. For instance, overexpression of *OsbZIP52* in rice significantly enhanced sensitivity of transgenic plants to cold and drought stresses (Liu et al., 2012). Thus, downregulation of such a gene would allow plants to adapt better to adverse environmental conditions.

In summary, our comparative analysis of root transcriptomes of DT2008 and W82 under both well-watered and dehydration conditions have allowed us to identify genetic components that might contribute to the improved drought tolerance of DT2008. Our study also provides a useful genetic resource for scientists with interests in basic and/or applied research to carry out further in-depth gene characterization and functional analyses. This in turn will contribute to deeper understanding of mechanisms regulating drought responses and adaptation in soybean, which ultimately leads to development of soybean cultivars with improved tolerance to drought.

Materials and Methods

Plant Growth and Dehydration Treatment

W82 and DT2008 soybean plants were separately grown in pots containing vermiculite (6 plants per 6-liter pot) under

well-watered conditions in a controlled greenhouse (continuous 30°C temperature, photoperiod of 12/12 h, 150 μmol m^{-2} s^{-1} photon flux density). For the collection of well-watered and dehydrated root tissues, 14-d-old soybean plants with two trifoliate leaves (V2 stage) were carefully removed from pots, then gently washed to remove soil from the roots. Subsequently, the W82 and DT2008 plants were dried on a filter paper for different time periods under the condition of 44% relative humidity, 23°C room temperature and 10 μmol m^{-2} s^{-1} photon flux light intensity. The severity of the stress level was measured by determination of RWC of the aerial parts of dehydrated plants. After the dehydration treatment, plants dehydrated for 0, 2, and 10 h were collected and the roots were separated from the shoots. Root samples were quickly frozen in liquid nitrogen and stored at −80°C until RNA purification. Accordingly, the following root samples were collected in three biological replicates from W82 and DT2008 plants for microarray analysis: W82 well-watered control 0 h (W-C), W82 dehydrated 2 h (W-D2), W82 dehydrated 10 h (W-D10), DT2008 well-watered control 0 h (DT-C), DT2008 dehydrated 2 h (DT-D2); DT2008 dehydrated 10 h (DT-D10).

Microarray Analysis of the Root Samples using 61K Affymetrix Microarray

RNAs were extracted from root samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer's protocol. Purified total RNA was subsequently subjected to a DNase I treatment prior to the quality assessment by an Agilent 2100 Bioanalyzer (Le et al., 2011a). For microarray analysis, cDNA synthesis, cRNA amplification, and synthesis of sense strand cDNAs were carried out using the Ambion WT expression kit. cDNA labeling was carried out using Affymetrix GeneChip WT Terminal Labeling Kit according to the supplier's instructions. Hybridization and scanning of hybridized arrays (G2505B microarray scanner, Agilent Technologies) were performed as described previously (Nishiyama et al., 2012). Three biological replicates collected from each treatment were subjected to the microarray experiment. Microarray data were analyzed using Affymetrix Expression Console with library supplied from Affymetrix and GeneSpring (Ver. 11) as essentially described (Le et al., 2012b). Statistical significance of each gene in each treatment (p-value) was estimated by a Student's t-test, while its certainty level (the corrected p-values, i.e., q-values) was assessed using Benjamini and Hochberg False Discovery Rate. Genes with expression change ≥2-fold ($q < 0.05$) were regarded to be differentially expressed. The obtained microarray data have been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/browse/?view=series) (accession number GSE65553)[1].

MapMan Analysis of the Root Transcriptomes

MapMan (http://mapman.gabipd.org) was used to annotate and analyze the microarray data as according to previously published methods (Thimm et al., 2004; Le et al., 2012b; Nishiyama et al., 2012; Ha et al., 2014). The lists containing DEGs obtained from corresponding comparisons were supplied to MapMan for classification of DEGs into functional groups.

Validation of Microarray Data by RT-qPCR

Several genes were randomly selected for verification of the microarray data using RT-qPCR.

The specific primer pairs used in RT-qPCR were listed in Supplementary Table S8. The *Fbox* gene was used as a reference gene in the RT-qPCR analysis of RNA samples from three biological replicates (Le et al., 2012a). Preparation of cDNAs from DNase I-treated RNA samples for RT-qPCR was performed as previously described (Le et al., 2011b).

Author Contributions

L-SPT conceived research and wrote the manuscript. CVH, YW, UTT, DTL, MT, and KHN performed the experiments and analyzed the data. MS and DVN contributed research materials.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00551

[1]Accession number to microarray data deposited at Gene Expression Omnibus database: GSE65553.

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Genome-Wide Transcriptional Profiling and Metabolic Analysis Uncover Multiple Molecular Responses of the Grass Species *Lolium perenne* Under Low-Intensity Xenobiotic Stress

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Lolium perenne, which is a major component of pastures, lawns, and grass strips, can be exposed to xenobiotic stresses due to diffuse and residual contaminations of soil. *L. perenne* was recently shown to undergo metabolic adjustments in response to sub-toxic levels of xenobiotics. To gain insight in such chemical stress responses, a *de novo* transcriptome analysis was carried out on leaves from plants subjected at the root level to low levels of xenobiotics, glyphosate, tebuconazole, and a combination of the two, leading to no adverse physiological effect. Chemical treatments influenced significantly the relative proportions of functional categories and of transcripts related to carbohydrate processes, to signaling, to protein-kinase cascades, such as Serine/Threonine-protein kinases, to transcriptional regulations, to responses to abiotic or biotic stimuli and to responses to phytohormones. Transcriptomics-based expressions of genes encoding different types of SNF1 (sucrose non-fermenting 1)-related kinases involved in sugar and stress signaling or encoding key metabolic enzymes were in line with specific qRT-PCR analysis or with the important metabolic and regulatory changes revealed by metabolomic analysis. The effects of pesticide treatments on metabolites and gene expression strongly suggest that pesticides at low levels, as single molecule or as mixture, affect cell signaling and functioning even in the absence of major physiological impact. This global analysis of *L. perenne* therefore highlighted the interactions between molecular regulation of responses to xenobiotics, and also carbohydrate dynamics, energy dysfunction, phytohormones and calcium signaling.

Keywords: RNA-Seq, glyphosate, tebuconazole, no observable adverse effect, residual pollution, perennial ryegrass, SnRKs

Abbreviations: G, glyphosate; T, tebuconazole; GT, combination of glyphosate and tebuconazole.

INTRODUCTION

Modern agriculture uses large amounts of phytosanitary products to maximize crop production. These products are mainly pesticides or mixtures of pesticides, which have been developed to repel, attenuate or kill pests and competitive plants (Arias-Estévez et al., 2008). However, runoff, leaching, or spray drift lead to the entry of a large fraction of pesticides into environmental compartments (soil, water, sediment, or atmosphere). These diffuse and residual contaminations are composed of mixtures of parent compounds, of their degradation products, and also of associated adjuvants (Helander et al., 2012) and induce many environmental impacts (Patty et al., 1997; Köhler and Triebskorn, 2013). Pesticides and related-degradation products chemically stress many non-target organisms in natural ecosystems, among which plants are particularly affected as sessile organisms (Child et al., 1993; Serra et al., 2013, 2015).

Mechanisms and regulations of chemical stress responses may differ between species as a result of micro- and macroevolutionary processes (Medina et al., 2007) and may influence the sensitivity or tolerance of plants and thereby their capabilities to maintain growth and development in polluted areas. Moreover, herbicide efficiency is increasingly affected by the emergence of resistance processes (Délye, 2013). Target-site resistance (TSR) results from gene mutations that alter herbicide targets, whereas processes of non-target-site resistance (NTSR) can result from multiple mechanisms of detoxification, tolerance or regulation (Délye, 2013). In the last decades, large-scale pesticide use on agricultural lands, resulting in long-term contamination (Singh et al., 2004), has put strong ecological and evolutionary pressure on the dynamics of plant communities, leading to resistance emergence (Délye, 2013). Widespread agricultural weeds are controlled by herbicide applications, which result in recurrent exposition for the surrounding vegetation generally composed of a majority of grasses. Among these species, several populations of *Lolium perenne* have been described as resistant to herbicides. Recently, it has been demonstrated that, in a population that displays glyphosate resistance, other mechanisms than mutation in the target site of glyphosate, the plastidic enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), were involved (Salas et al., 2015). Similarly, resistance to the acetolactate-synthase (ALS) inhibiting herbicide pyroxsulam in *Lolium* sp. populations involves a NTSR response implying differential gene expression and different mechanisms that remain to be elucidated (Duhoux et al., 2015). Multiple-herbicide resistance has also been described in a specific population of *Lolium perenne* spp. *multiflorum*, but although some resistances to chemical groups of ALS inhibitors and triazine have been explained by the presence of mutations in target genes, none is responsible for the resistance of this population to glyphosate (Liu et al., 2013a), showing the complexity of NTSR and underlying mechanisms.

The mechanisms of plant responses to chemical stress induced by xenobiotics are not fully understood. Such mechanisms are often studied under conditions of high exposures corresponding to application levels in the field. High levels of xenobiotics strongly impact organism physiology and defense responses, by inducing molecular injury and damages (Teixeira et al., 2007; Ramel et al., 2009; Das et al., 2010; Nobels et al., 2011; Gomes et al., 2014), mainly related to oxidative stress, membrane disruption, lipid peroxidation, protein damage, or DNA damage. In contrast, few studies deal with the responses to conditions of low exposure to pesticides in a context of runoff or residual contaminations. It has been shown that long-term exposure to sub-lethal pesticide level impacts plant community at the plant development level without causing mortality (Pfleeger et al., 2012). Londo et al. (2014) demonstrated that biomass, flowering phenology, and reproductive functions are affected by sub-lethal glyphosate exposure in *Brassica* spp. Moreover, Ivanov et al. (2013) observed that although sub-lethal concentrations of atrazine did not cause immediate negative and visible effect, long-term exposition impacted the redox homeostasis through an oxidative stress. Long and low herbicide exposure results also in rapid herbicide resistance evolution for exposed populations as demonstrated by Yu et al. (2013) for *Lolium rigidum* in presence of diclofop-methyl. At the molecular level, Das et al. (2010) demonstrated by genome-wide expression profiling that five commercial herbicide formulations at concentration producing a 50% reduction in shoot dry weight (EC50, sub-lethal levels) specifically affected the expression of genes related to ribosome biogenesis and translation, secondary metabolism, cell wall modification and growth. A very recent study demonstrated that subtoxic levels of herbicides acted as chemical hybridization agents, leading to male sterility for the production of hybrid seeds. Their effects were related to reprogramming of gene expression and metabolism in response to low-level herbicide treatments (Li et al., 2015). This study thus showed that complex mechanisms of low-intensity herbicide stress responses may exist. ^{1}H NMR fingerprinting was also undertaken to analyse substantial metabolic changes in *Lemna minor*'s metabolome after short exposure to different pesticide treatments leading to no phytotoxicity symptom in attempt to develop novel ecotoxicological biomarkers. The discrimination between treatments was mostly based on metabolic variations of substances containing methylene, methine, hydroxy, amine, thiol, olefin, and aldimine groups without mechanistic conclusions (Aliferis et al., 2009). Complex mixtures of xenobiotics at realistic environmental levels produced different effects than those caused by compounds alone or by simple addition of effects (Lydy et al., 2004). Depending on the chemical properties and modes of toxic action of each compound, mixtures can result in higher (synergism) or weaker (antagonism) toxicity, as highlighted for various organisms, as humans, invertebrates or plants (Hertzberg and MacDonell, 2002; Hernández et al., 2012; Cedergreen, 2014). Frankart et al. (2002) have thus shown synergistic effect of a mixture of herbicide (flumioxazin) and copper on photosynthesis inhibition in *L. minor* whereas a mixture of fungicides (fludioxonil or procymidone) and copper produced an antagonism effect. Mixture effects are difficult to analyse and to predict (Dévier et al., 2011; Serra et al., 2013, 2015), and interactions between compounds can alter bioavailability or uptake rate and transport, metabolic activities, target site binding and/or compound excretion (Cedergreen, 2014). Their

study remains however of interest, in particular in the case of no observed effect individual concentrations (Walter et al., 2002).

Hormetic effects and safener effects indicate that xenobiotics can also affect plants under conditions of no adverse effect (NOAE situation: No Observable Adverse Effect) through mechanisms that have seldom been investigated. Hormetic effects that induce beneficial impacts by exposure to low doses of a potentially toxic stressor are achieved through the activation of signal and regulation pathways independently of cellular damage (Velini et al., 2008; Costantini et al., 2010; Belz and Duke, 2014). In that context Nadar et al. (1975) described in Sorghum the growth-promoting effect of atrazine at sub-lethal concentrations in relation with cytokinin-like activity. Stamm et al. (2014) demonstrated in soybean that, even though a thiamethoxam seed treatment did not significantly impacted shoot height and plant biomass, the expression of genes related to plant defense and stress response was altered. Thus, the use of Cruiser® 5FS induces unexpected effects, regarded as cryptic, on a non-target organism. Such cryptic effects were observed in *Arabidopsis thaliana* by Serra et al. (2013) who analyzed the effects of low doses of pesticides, of pesticide degradation products and of their mixtures. In this study, AMPA and hydroxyatrazine, the main degradation products of glyphosate and atrazine, respectively, led to NOAE situations, and nevertheless had significant effects on the expression of genes already known to be affected by high pesticide exposure and on metabolic profiles (Serra et al., 2013). Some chemical treatments induced extensive metabolic changes, such as accumulation of stress-related metabolites (ascorbate) and decrease of carbohydrate levels. Moreover, these chemical stresses effects occurred in parallel with modifications of hormone-related and transcription regulation-related gene expression, thus suggesting underlying regulatory actions of xenobiotic compounds (Serra et al., 2013). Strong interaction effects between chemicals at the molecular, metabolic and physiological levels confirmed that pesticide-related products may act on regulation pathways (Serra et al., 2013).

An another integrative study, focusing on the physiological and metabolic responses of *L. perenne* to diverse subtoxic conditions of chemical stresses, showed primary effects of chemical stressors on seedling metabolism, physiology and growth (Serra et al., 2015). A short exposure to low doses of glyphosate, tebuconazole and their mixture, which consisted of transfer exposure on chemical stressors containing medium during 4 days, did not have any negative effect on root length (NOAE situation), root growth being the most sensitive physiological parameter, but caused cryptic effects on metabolic, regulatory, and signaling processes (Serra et al., 2015). These effects did not however translate into long-term loss of fitness, thus indicating a situation of tolerance to low-level chemical stress. Short exposure was associated with unexpected metabolic changes, as for example significant decrease of Suc and Glc, corresponding to major reorientation of central carbon metabolism. The global analysis in *L. perenne*, combining leaves and root metabolites after short exposure and direct and long exposure, allowed to demonstrate that responses to low chemical stress were associated through a complex network of metabolic correlations converging on Asn, Leu, Ser, and glucose-6-phosphate (Glc-6-P), which could potentially be modulated by differential dynamics and interconversion of soluble sugars (Suc, trehalose, and Glc; Serra et al., 2015). Such complex metabolic changes reflected chemical stress adjustment rather than deregulation of homeostasis, then leading to root growth maintenance, even under long-term exposure, and thus suggesting the implication of primary mechanisms and molecular regulations (Serra et al., 2015). More importantly, these analyses suggested that complex signaling networks may directly participate in chemical stress responses to rapidly adjust plant metabolism and to counteract mild damaging stress. Moreover, the discovery of major cryptic effects on metabolic, regulatory, and signaling mechanisms under such NOAE conditions raises the issue of the stress concept in plants, as outlined by other authors (Kranner et al., 2010). Within this context, the varying levels of plant stress responses to varying stress intensity may be adaptive. In the absence of harmful effects, a "mild" stress can induce an "alarm response" characterized by post-translational and stress signaling resulting in transcriptomic modifications. The characterization of such low-intensity adaptive mechanisms is therefore of utmost importance, with a number of potential agronomical and ecological applications (stress shield concept).

L. perenne is a perennial species of high ecological and agronomic interest. It is one of the predominant forage grasses of high quality in temperate areas (Casler and Duncan, 2003; Barbehenn et al., 2004). Carbon sequestration, soil formation and nutrient cycling are improved by *L. perenne* cover crops (Pouyat et al., 2009). Its value also resides in its relative tolerance to various pollutants of different chemical nature (Dear et al., 2006), which explains why it has been used for phytoremediation strategies (Bidar et al., 2009; D'Orazio et al., 2013).

The present work analyses the molecular responses of *L. perenne,* using a transcriptomic approach, in order to characterize the mechanisms underlying responses to mild chemical stresses induced by no-adverse-effect doses of pesticides. In order to decipher primary mechanisms and pathways involved in adjustments, stress treatments consisted in transfer experiments of non-stressed plants to xenobiotic-containing medium during short exposure. Pesticide treatments consisting of glyphosate, tebuconazole and their combination were applied at NOAE levels to the roots of *L. perenne* seedlings. Both pesticides have been found at residual levels in soils of field margins (Serra et al., 2013) and are frequently detected in runoff water (Potter et al., 2014; Sasal et al., 2015). They are representative examples of agricultural pollution. The impact of root exposure on the whole plant is therefore a primordial process during plant/xenobiotic interactions under conditions of edaphic xenobiotic pollution. Glyphosate is a broad spectrum herbicide (Duke and Powles, 2008) which disrupts the synthesis of aromatic amino acids by inhibiting EPSPS, a key enzyme in the shikimate pathway (Steinrücken and Amrhein, 1980). Tebuconazole, besides acting and being used as a fungicide, can inhibit sterol 14α-demethylase enzymes (Lamb et al., 2001) and limit the rate of gibberellin biosynthesis (Child et al., 1993) in plants. Transcriptomic analysis of *L. perenne* responses to

chemical stress was carried out by RNA-Seq approach involving the pyrosequencing of leaf cDNA libraries (Huang et al., 2012; Ward et al., 2012). A *de novo* transcriptome of *L. perenne* was obtained by the assembly of cDNA reads. A *de novo* assembly was performed using model species data as there is no reference genome available for *L. perenne*. Differential expression of genes and functions were analyzed in parallel with metabolic data in order to obtain a functional insight of molecular regulations induced by low-intensity chemical stress. Our present study shows that, taken together, the effects of pesticide treatments on metabolites and gene expression strongly suggest that low levels of pesticides, whether as single molecule treatment or as mixture, interact, even in the absence of major physiological impact, with plant cell functions as carbohydrate regulations and signaling. The characterization of plant/xenobiotic direct interactions with signaling and hormone cross-talk effects, which is a major field of research in animal toxicology (Frye et al., 2011), should provide novel insights into the environmental impact of low-level runoff or persistent pesticides on plant communities.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *L. perenne* (Brio cultivar) were briefly washed in ethanol and surface-sterilized in bayrochlore (20 g L^{-1} in water) containing 0.05% tween (v/v) for 20 min and rinsed five times in sterilized water. Moistened seeds were placed in Petri dishes in the dark at 4°C for 7 days in order to break dormancy and homogenize germination. Seeds were sown on pieces of gauze and placed at the top of sterile culture tubes containing liquid growth medium. Gauze pieces were continuously moistened by soaking gauze edges into culture medium, in order to maintain humidity for germination. Germination and growth were carried out under axenic conditions in a control growth chamber at 22°C/20°C under a 16 h light (6000 lux)/8 h dark regime. Growth medium consisted of Hoagland basal salt mix (N°2, Caisson Laboratories, North Logan, UT, USA) adjusted to pH 6. Transfer experiments consisted in xenobiotic exposure of young plants at the same stage of photosynthetic development. After 7 days of growth under control conditions, seedlings were transferred to fresh growth medium containing chemical stressors, thus resulting in xenobiotic exposure at root level. Shoots were harvested 4 days later, corresponding to 11 days of total growth. Different chemical treatments were applied: the broad-spectrum herbicide glyphosate (G, 1 μM), the fungicide tebuconazole (T, 4 μM) and a combination of glyphosate and tebuconazole (GT, 1 μM and 4 μM, respectively). Leaves of seedlings were collected just before the start of the daylight period, ground in liquid nitrogen and stored at −80°C until use.

Transcriptome Sequencing

Five independent biological replicates, consisting in aerial parts (50 mg fresh weight) of 10 plantlets each, were harvested after transfer experiment and used for transcriptome sequencing. RNA was extracted using TRI Reagent® (Sigma) with an additional DNase treatment. Total RNA samples from each replicate were pooled per treatment condition [Control (C), glyphosate (G), tebuconazole (T) and glyphosate and tebuconazole mixture (GT)] and resulting samples were polyA-enriched with the Oligotex mRNA kit (Qiagen Cat. N°. 70042). Then, 250 ng of mRNA-enriched RNA were fragmented ($ZnCl_2$), and reverse-transcribed to cDNA using cDNA Synthesis System kit (Roche Cat. N°. 11117831001). For each treatment, one Roche 454 library was prepared and sequenced twice on a quarter of plate on a Roche 454 GS-FLX, using titanium chemistry (titanium chemistry, Sequencing Kit XL+) at the Biogenouest core facility (Rennes, France). The sequencing data are available in the NCBI Sequence Read Archive (SRA) database under the accession reference PRJNA287779 (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi).

Assembly and Read Processing

Read filtering and final assembly were performed using the Galaxy instance (Goecks et al., 2010). The 454 reads were processed to remove sequences below 250-bp and above 1000-bp. Reads were improved according to quality and by removing adapter sequences, undetermined bases ("N") and poly-A/T tails. For generation of the reference transcriptome, selected clean reads from all libraries were pooled and assembled to improve mRNA lengths and produce a global library. Reads were assembled in contigs using the *de novo* assembly program Trinity (Haas et al., 2013). Trinity is an Illumina/Solexa-specialized transcriptomic assembler, which is suitable for non-strand-specific and single-end-read data. According to Ren et al. (2012), it gives the best performance among the multiple de Bruijn graph assemblers, and represents an alternative solution for reconstructing full-length transcripts from 454 reads. The assembly was conducted using the default parameters. Reads were ascribed to contigs using RSEM (RNA-seq by expectation-maximization) software and filtered according to the reads per kilobase of target transcript length per million reads mapped (RPKM) values equal to 1 or greater. After a first Trinity assembly, the unmapped (172,634 total unmapped sequences), and unused reads, were submitted again to the Trinity program, leading to a new assembly. Reads that again did not fit into contigs (42,351 unmapped reads) were defined as singletons. These unique sequences were added to the 14,811 unique contigs to constitute the reference transcriptome. The resulting singletons and contigs represented the candidate unigene set.

Annotation and Functional Analyses

After assembling, tBLASTx alignments ($e < 10e\text{-}5$) against The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org.gate1.inist.fr) databases were undertaken and unigenes with the highest sequence similarity were functionally annotated using Blast2GO (Conesa et al., 2005) with cut-off e-values of 10e-5 (Blastx) and 10e-6 (mapping). Blast-based annotations were complemented with domain-based annotations using the Inter-ProScan tool (v5). Functional classification of unigenes was based on Blast2GO analysis. Pathway assignments were carried out according to KEGG database (Kyoto Encyclopedia of Genes and Genomes (KEGG) resource; http://www.kegg.jp/ or http://www.genome.jp/kegg/).

The statistical assessment of Gene Ontology (GO) term enrichments after xenobiotic treatments in comparison to control condition was performed [Fisher's Exact Test with multiple testing correction; false discovery rate (FDR) < 0.05; $p < 0.005$] as implemented in Blast2GO (Conesa et al., 2005). Comparison of conserved amino acids was performed using Clustal Omega for alignment (Sievers et al., 2011).

Differential Expression Analysis

In order to determine differentially expressed (DE) genes between the 4 conditions, the DESeq method was used. This *in silico* normalization method is included in the DESeq Bioconductor package (Anders et al., 2013). As each condition was represented by a single sample of pooled individuals, the differential expression for each gene was estimated by using the variance of expression for this gene across the 4 conditions. Unigenes were considered as DE at $p < 0.05$.

qRT-PCR validation

Quantitative RT-PCR was used to confirm *in silico* differential expression and analyse the expression of genes potentially involved in chemical stress response in *L. perenne*. qRT-PCR experiments were carried out using, for each condition, five new independent biological replicates of pooled aerial parts (50 mg fresh weight) from 10 plantlets harvested after transfer experiments. RNA from aerial parts was extracted using TRI Reagent® (Sigma) with an additional DNase treatment. RNA was used for cDNA synthesis (Iscript™ cDNA Synthesis kit, Bio-Rad, Hercules, CA, USA). Resulting cDNAs were used to determine expression profiles according to the different treatments. Quantitative PCR was performed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Conditions were as follows: 95°C 3 min, and 40 (95°C 15 s, 60°C 30 s, 72°C 30 s) cycles. All samples were run in duplicate for each primer set. Specific primers for each gene selected for analysis were designed according to 454 sequences using Primer3 software (Rozen and Skaletsky, 2000; Supplemental Table 1). The results of the analysis were treated with Gene Expression version 1.1 software. Relative expressions were assessed in relation to the stable expression level of the GAPDH housekeeping gene (Furet et al., 2012).

Metabolic Profiling

Five new independent biological replicates of aerial part samples, each consisting in 10 pooled plantlets, were harvested after a transfer experiment, freeze-dried and used for metabolomic profiling. Samples were extracted and analyzed using gas chromatography mass spectrometry (GC/MS) as described by Serra et al. (2015). Metabolite levels were quantified using XCalibur v2.0.7 software (Thermo Fisher Scientific Inc., Waltham, MA, USA) and expressed as $nmol.mg^{-1}$ of dry weight (DW).

Statistical Analysis

Metabolic parameters were measured on at least five independent replicates of at least 10 individual plantlets. Gene expression quantification was carried out on at least five other independent biological replicates of 10 pooled plantlets. Statistical analyses were carried out with version 3.0.1 of R software, analysis between means was carried out using the non-parametric Mann-Whitney-Wilcoxon test.

RESULTS

De novo sequence Assembly of the *L. perenne* Shoot Reference Transcriptome

A *de novo* transcriptome analysis of *L. perenne* was carried out on leaves from plants subjected to a transfer experiment involving NOAE levels of chemical stressors and short periods of exposure. *L. perenne* seedlings were submitted to 3 conditions of short-term low-level xenobiotic exposure [glyphosate (G), tebuconazole (T), and a combination of the two (GT)] in comparison to control condition (C). Leaf cDNA libraries, corresponding to these 4 conditions, were prepared and sequenced using 454 mass sequencing. Before preprocessing, sequencing of cDNA libraries resulted in approximately 0.7 Gbp of sequence data with a GC content of 54%. The genome size of *L. perenne* is estimated to be 2.7 Gb (Fiil et al., 2011). Each quarter plate run was preprocessed, and the runs of each treatment were pooled, thus leading to 300,795, 249,254, 338,011, and 307,033 high-quality reads for, respectively, control, glyphosate, tebuconazole and glyphosate + tebuconazole treatments (**Table 1**). The reference transcriptome, which was derived from the complete pool of reads from the 4 conditions (C, G, T, GT), contained 1,195,093 reads (**Table 1**).

Read assembly resulted in an initial reference transcriptome of 14,492 contigs with length ranging from 250 to 6437 bp (**Table 2A**) and an average contig size of 890 bp. The unmapped reads (172,634 reads) of 250–1000 bp length range were used for a second round of assembly, which resulted in 3564 new contigs. Among the 172,634 reads used, 42,351 remained unmapped and were defined as resulting singletons. Among the 3564 new contigs, 3245 were very similar, if not identical, to contigs built in the first assembly. The 42,351 singletons and the 319 new contigs were added to the 14,492 contigs leading to 57,162 sequences or unigenes (**Table 2B**) which were used further for whole transcriptome annotation.

The quality of the assembly was checked by comparing the resulting transcriptome to SRA data of *L. perenne* (43,049 sequences, E-MTAB-1556 in ArrayExpress, Vigeland et al., 2013). A tblastx analysis using an $e < 10e\text{-}6$ revealed that 45,962 sequences (13,782 contigs and 32,180 singletons) from the present transcriptome matched similar sequences from the deposited SRA data. The reference transcriptome was also compared to the complete mitochondrial (GenBank: JX999996.1) and chloroplastic (NCBI Reference Sequence: NC_009950.1) genomes of *L. perenne* using tblastx program. The mitochondrial genome contains genes corresponding to 14 tRNA, 3 rRNA and 34 proteins (Islam et al., 2013), among which 27 genes matched with high identities ($e < 10e\text{-}6$) with unigenes from the present reference transcriptome. Furthermore, 80 genes from the complete chloroplastic genome, which contains genes encoding 76 unique proteins, 30 tRNAs and four rRNAs (Diekmann et al., 2009), were identical or very close ($e < 10e\text{-}6$) to the transcriptome unigenes.

TABLE 1 | Summary of 454 sequencing data.

Number of high-quality (HQ) reads		Control	Glyphosate	Tebuconazole	Glyphosate + Tebuconazole
Sequencing reads before preprocessing (Average bp length of HQ reads)	Run 1	224,814 (481 ± 184)	207,483 (450 ± 180)	234,687 (488 ± 176)	227,001 (483 ± 183)
	Run 2	171,316 (401 ± 232)	143,737 (351 ± 218)	193,881 (431 ± 224)	173,328 (411 ± 233)
Reads after 250–1000 bp trimming	Run 1	190,551	169,605	202,685	193,374
	Run 2	112,834	84,325	139,427	116,918
Reads after trimming and preprocessing (poly-A/T, N, Quality)	Run 1	189,143	167,578	200,697	191,833
	Run 2	111,652	81,676	137,314	115,200
Total HQ reads in pools of run 1 and run 2	Pool	300,795	249,254	338,011	307,033
Reference transcriptome	Pool	1,195,093 reads			

TABLE 2 | Assembly results.

	Reference	Control	Glyphosate	Tebuconazole	Glyphosate + Tebuconazole
(A) FIRST ASSEMBLY					
Number of contigs	14,492	10,155	5773	9109	9408
Total length of contigs (bp)	12,905,107	10,070,689	6,098,760	9,166,250	9,611,071
Number of contig ≥ 1000 bp	4342	3937	2549	3661	3921
Mean contig length (bp)	890 ± 466	991 ± 498	1056 ± 537	1006 ± 508	1031 ± 503
(B) FINAL ASSEMBLY					
First assembly	14,492	8089	3646	7005	7256
Second assembly	319	130	50	94	103
Singletons	42,351	8089	6218	12,553	11,025
Total	57,162	22,087	9914	19,652	18,384

In order to validate further the assembled unigenes, sequence-based alignments were performed against the TAIR (*Arabidopsis thaliana*) database by using the tblastx algorithm (Altschul et al., 1997). According to tblastx data, 55.44% (31,696 unigenes) of the matched sequences showed strong homology with TAIR data ($e < 10e\text{-}50$), and 84.9% (48,550 unigenes) of the top hits exhibited lower but significant homology (e-value over 10e-6).

Functional Annotation of the *L. perenne* Shoot Reference Transcriptome

Functional annotation of the reference transcriptome was undertaken using the Blast2GO tool and TAIR database. 9311 (16% of all unigenes) unigenes showed no blast hit in the TAIR (*Arabidopsis thaliana*) database. Among the unigenes yielding blast results, 204 (0.32%) were not mapped and 1296 (2.2%) mapped unigenes were not annotated, thus leading to 46,330 (81%) annotated unigenes. In many cases, multiple GO terms were assigned to the same unigene.

GO terms were classified into functional groups according to biological process, molecular function, and cellular component classes. Distribution of the various GO terms identified for the *L. perenne* reference transcriptome is presented in **Figure 1** for the major GO classes. The proportions of biological activities were similar to those described in other *L. perenne* transcriptomes (Farrell et al., 2014; Duhoux et al., 2015). GO terms linked to metabolism and biosynthetic processes represented more than 50% of the biological processes (**Figure 1A**). GO terms related to stress responses, signaling and regulations (response to stress, response to external/abiotic/chemical stimulus, detection of stimulus, single organism signaling, regulation of biological processes) were also well represented (16.6%). Concerning molecular functions (**Figure 1B**), GO terms linked to various binding activities, as well as activities related to translation (structural constituent of ribosomes), were highly represented. Functional categories also covered enzyme activities such as hydrolase, transferase, oxidoreductase, or lyase activities. The subcellular localization of these processes and functions (**Figure 1C**) showed the importance of gene products associated with intracellular organelles, chloroplasts (thylakoids, light-harvesting complexes), cytoplasm and ribosomal complexes. Functional distribution therefore demonstrated that this *L. perenne* reference transcriptome exhibited a good coverage of essential types of plant biological activities.

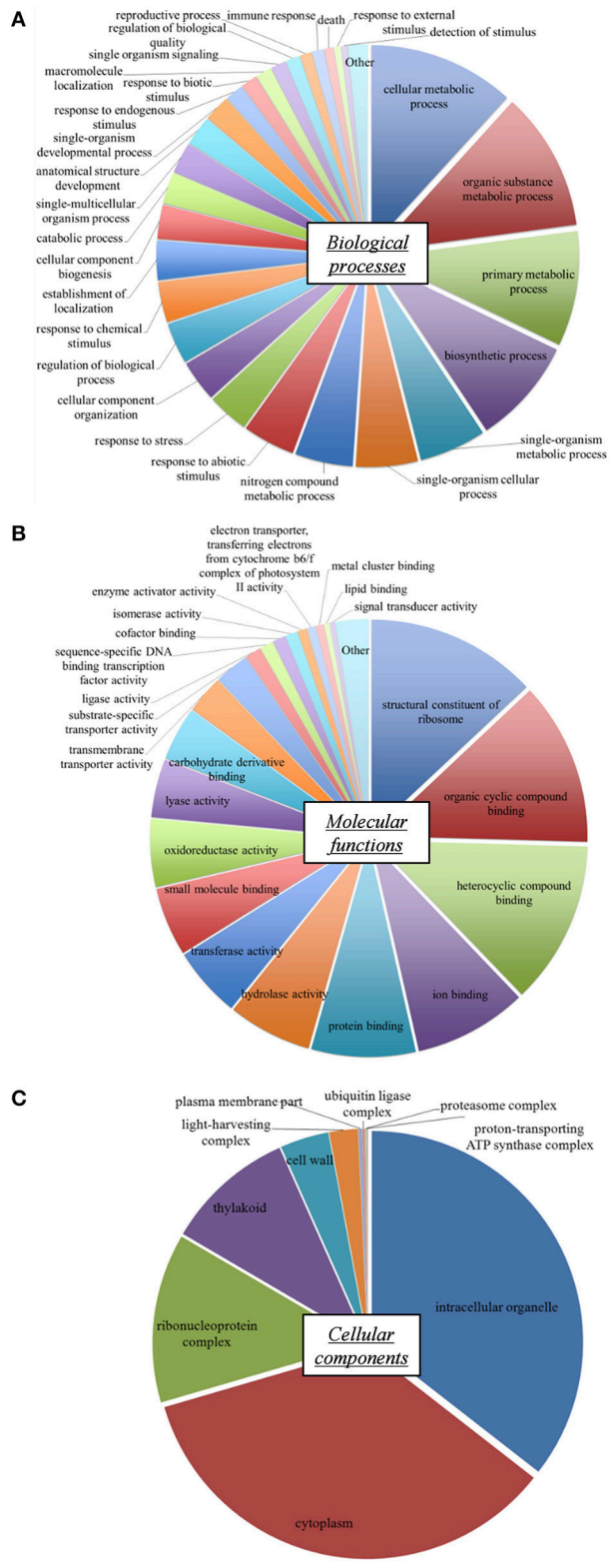


FIGURE 1 | Distribution of Gene Ontology (GO) terms in the reference shoot transcriptome of *L. perenne*. Biological processes **(A)**, Molecular functions **(B)** and Cellular components **(C)** classes are shown. The relative size of pie chart sectors reflects the percentage of annotated unigenes of each GO term category.

The relative importance of various metabolic pathways was assessed from the assignment of unigene annotations in the KEGG database (**Figure 2**). Most of the predominant pathways were found to belong to carbon metabolism (Glyoxylate and dicarboxylate metabolism, Glycolysis/Gluconeogenesis, Starch and sucrose metabolism, Pentose phosphate pathway, Fructose and mannose metabolism, Amino sugar and nucleotide sugar metabolism, Pyruvate metabolism, TCA cycle, Galactose metabolism, Ascorbate, and aldarate metabolism) and to energy metabolism (Carbon fixation in photosynthetic organisms, Methane metabolism, Oxidative phosphorylation, Nitrogen metabolism, Photosynthesis). Five pathways related to amino acid metabolism were also represented.

Effects of Short-Term Root-Level Exposure to NOAE Levels of Chemical Stressors on the Relative Regulation of Functional Categories in *L. perenne* leaves

Assessment of Xenobiotic-Related Molecular Effects

The relative proportions of functional categories, as defined by GO terms, were analyzed relative to the different conditions of chemical stress. GO enrichment analysis for each class (biological processes, cellular components, and molecular functions) revealed the GO terms for which the quantity of annotated unigenes (contigs and singletons) presented significant differences (FDR < 0.05 and $p < 0.005$) after xenobiotic treatments in comparison to control condition. Results were expressed as the percentage of unigenes in each GO term category in a given condition relative to the total number of annotated unigenes in the same class. The distribution of significantly-enriched GO term categories is given in **Figure 3**. The percentage of annotated reads in each GO term category in a given condition was also calculated relative to the total number of annotated reads in the same class. In this analysis of annotated read levels, only GO term categories for which at least 50 reads had been counted in at least one condition were selected (Supplemental Table 2). Changes in proportions of GO term categories in terms of unigene enrichment were likely to reflect the effects of xenobiotic treatments on global regulatory processes of genome expression affecting each category. Modifications of proportions of GO term categories in terms of read proportion gave further information on induction or repression.

In a number of cases, the relative proportions of GO term categories were decreased by xenobiotic treatments in terms of percentages relative to annotated unigenes (**Figure 3**), and also in terms of percentages relatively to annotated reads (Supplemental Table 2). Nevertheless, among all the significant differences induced by treatments, G and T treatments mainly negatively affected the proportion of GO terms relative to control. By contrast, GT treatment induced as much enrichment as depletion in the proportions of GO terms relative to control (**Figure 3**). Such differences strongly suggested that the mixed GT treatment induced specific effects in comparison with those of single xenobiotic treatments as "response to monosaccharide stimulus", "sugar mediated signaling pathway", "starch catabolic process", "negative regulation of translation", "pentose phosphate shunt",

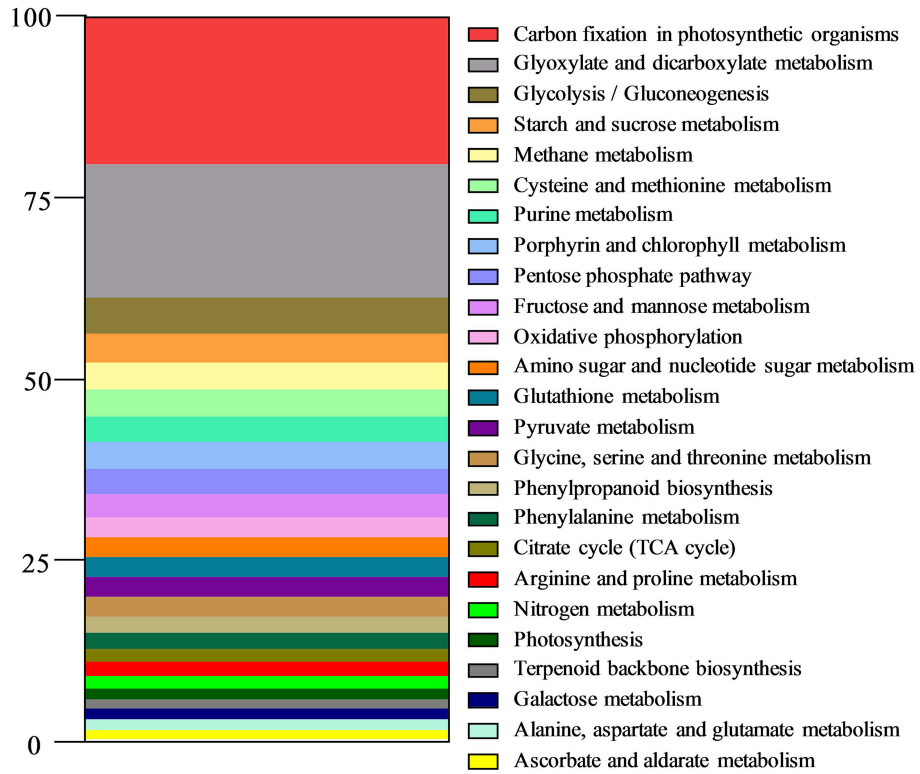


FIGURE 2 | Distribution of metabolic pathways in the reference shoot transcriptome of *L. perenne*. Assignment of annotated unigenes to metabolic pathways was carried out with the KEGG database (Kyoto Encyclopedia of Genes and Genomes (KEGG) resource; http://www.kegg.jp/ or http://www.genome.jp/kegg/). Results are expressed as percentages of annotated unigenes of each metabolic pathway.

"pyrophosphatase activity", "NADP metabolic process", "ATP binding", "Golgi apparatus part". Moreover G treatment often led to more pronounced effects than those of T and GT treatments (**Figure 3**).

Effects on Gene Expression and Protein Translation Processes

The "structural constituent of ribosome" category (**Figure 3B**) showed increased proportions in the presence of G. The potential involvement of ribosome biogenesis in xenobiotic responses was also reflected by the significant increase of "ribosomal subunit" category in the presence of xenobiotics and particularly in presence of G (**Figure 3C**). Other aspects of the biogenesis of functional ribosomes seemed to be sensitive to chemical stresses, since categories of linked GO terms such as "ribonucleoprotein complex biogenesis", "rRNA processing" and "ribosome biogenesis" were negatively affected by chemical stress treatments. G and GT treatments also induced a slight decrease of unigene proportion for "DNA-dependent transcription" (**Figure 3A**). The stability of unigene proportion related to "negative regulation of translation" in the presence of T and G contrasted with a strong decrease of unigene proportion in presence of GT, thus suggesting combination-specific regulations (**Figure 3A**). For upper-level biological process classes, such as "translation", overrepresented in presence of G and T, and "gene expression", overrepresented in presence of G and slightly underrepresented in presence of GT, the increase of unigene proportion in the presence of G (**Figure 3A**) was associated with an increase in read number (Supplemental Table 2), thus indicating the induction of specific genes. This increase of "gene expression" category occurred in parallel with a decrease in the "gene silencing" category (**Figure 3A**). Finally, negative effects of G on "histone modification"-related unigenes were observed in upper levels of classification such as "chromatin organization" and "macromolecule modification" (**Figure 3A**).

Effects on Photosynthesis and ATP Dynamics Processes

Unigenes linked to the "ribulose-1,5-bisphosphate carboxylase/oxygenase activator activity" or "Rubisco activator" showed decreased proportions in response to xenobiotics, particularly in response to G (**Figure 3B**). The associated "ribulose-bisphosphate carboxylase activity" category was significantly more represented in response to T and underrepresented in the presence of G and GT. G and GT treatments reduced unigene proportions corresponding to biological processes associated with "carbon fixation", "photosynthesis", "photosystem II assembly", and "photosynthetic electron transport chain" (**Figure 3A**). Glyphosate application also reduced the number of unigenes involved in "chlorophyll biosynthetic process" and in related pathways such as "porphyrin-containing compound metabolic

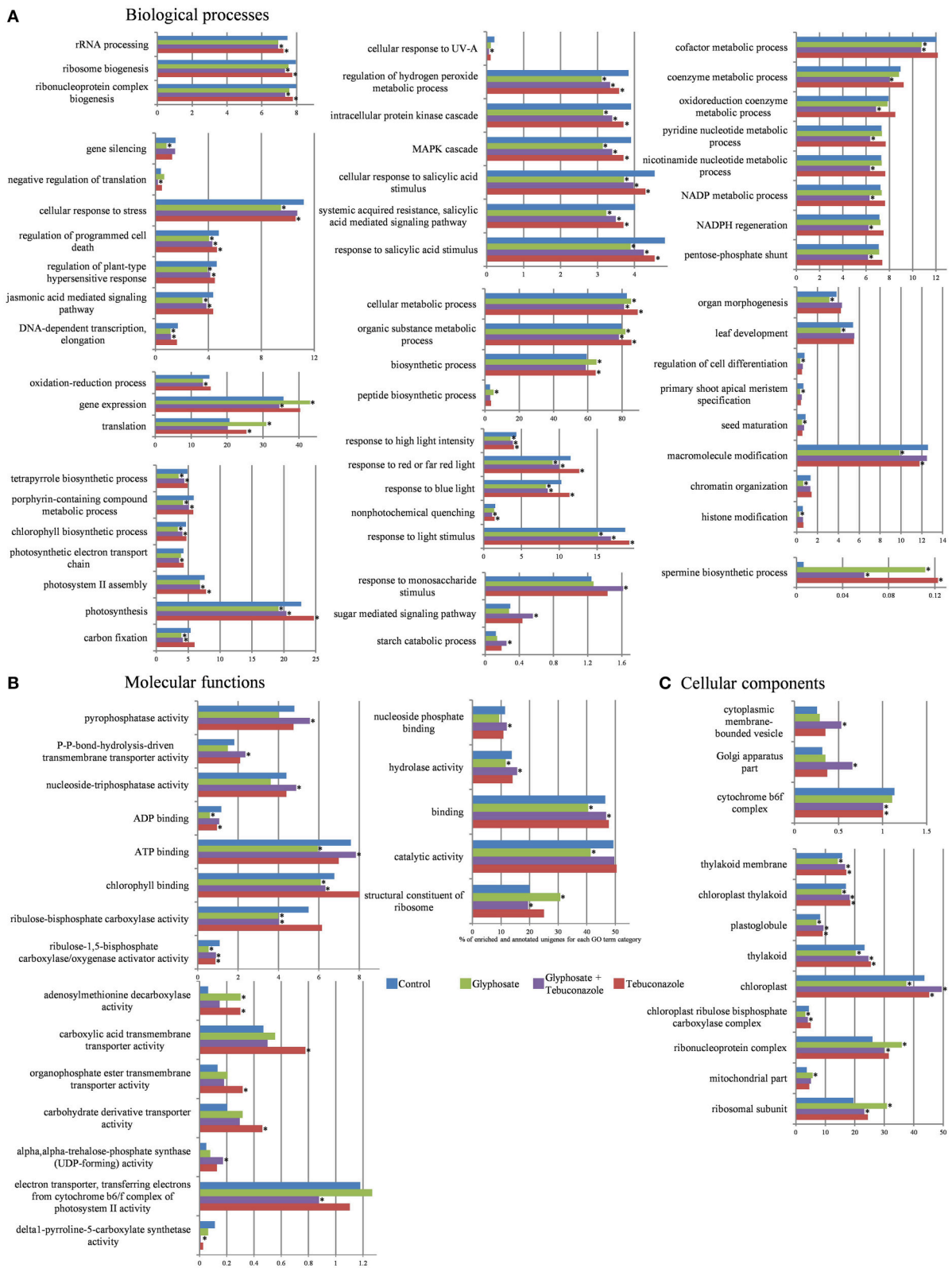


FIGURE 3 | Effects of xenobiotics on the distribution of representatively-enriched Gene Ontology (GO) terms in the shoot transcriptomes of *L. perenne*. Biological processes **(A)**, Molecular functions **(B)**, and Cellular components **(C)** classes are shown. Results are expressed as percentages of enriched and annotated unigenes for each GO term category and for each condition (control, glyphosate, tebuconazole, glyphosate plus tebuconazole) relatively to the total number of annotated unigenes in that class. Stars indicated FDR < 0.05 and $p < 0.005$ after Fisher's Exact Test with robust FDR (false discovery rate) correction.

process" and "tetrapyrrole biosynthetic process" (**Figure 3A**). While no unigene enrichment of "magnesium chelatase activity" category was observed, there was an increase in read numbers in the presence of the 3 xenobiotic treatments, for genes whose functions were related to these chlorophyll biosynthetic pathway enzymes (Supplemental Table 2). Xenobiotic-related perturbations in chloroplastic and photosynthetic pathways were also identified for cellular component GO terms (**Figure 3C**). Numerous unigenes were ascribed to GO terms related to "chloroplast", "thylakoid", "plastoglobule", "chloroplast thylakoid", and "thylakoid membrane". These categories generally showed higher proportion of unigenes in the presence of T and GT, while these xenobiotics tended to decrease slightly the proportion of unigenes related to "cytochrome b6f complex" categories (**Figure 3C**).

The "binding" GO term category was well represented (**Figure 3B**), in particular with regard to "nucleoside phosphate binding", "ATP binding", and "ADP binding". Proportions of annotated unigenes for these molecular functions decreased in the presence of G, and slightly increased in the presence of GT. However, concerning the "ATP binding" annotation, which was highly represented among GO terms, an increase of the number of corresponding reads was observed in the presence of G, suggesting strong expression of specific and constitutive unigenes (Supplemental Table 2). Likewise, other functions related to nucleoside and nucleotide dynamics, which showed frequent occurrences and high percentages, were responsive to xenobiotic treatments. Thus, the GO term category "nucleoside-triphosphatase activity" showed decreased proportion in the presence of G, whereas GT treatment increased it (**Figure 3B**). This metabolic activity was characterized by an increase of related read numbers in the presence of G, T, and GT, suggesting strong expression of specific unigenes (Supplemental Table 2). This pattern of modifications was reflected in the changes affecting the corresponding upper-level GO term, "hydrolase activity". Nucleoside-triphosphatase activity is an essential provider of energy for active transport and is related to the "P-P-bond-hydrolysis-driven transmembrane transporter activity" category, which showed higher proportions of unigenes in the presence of GT (**Figure 3B**).

Effects on Environmental Response Processes

G and GT treatments induced slight decreases of unigene proportions for many biological processes related to abiotic and biotic stress signaling processes, such as "jasmonic acid mediated signaling pathway", "regulation of plant-type hypersensitive response", "regulation of programmed cell death", and "cellular response to stress" (**Figure 3A**). Moreover, the proportions of unigenes and reads related to "response to salicylic acid", "MAPK cascade", "intracellular protein kinase cascade", and "regulation of hydrogen peroxide metabolic process" were negatively affected by all the chemical stressors (**Figure 3A**). Most of the biological processes linked to light responses showed a decrease for the proportions of related unigenes in the presence of G and GT. GO term categories such as "response to light stimulus", and related sublevels such as "non-photochemical quenching", "response to blue light", "response to red or far red light", and "response to high light intensity", followed the same tendency (**Figure 3A**).

Effects on Metabolic Functions

The proportions of unigene categories linked to carbohydrates and their derivatives were significantly influenced by chemical treatments. Unigenes annotated in "starch catabolic process", "sugar mediated signaling pathway", and "response to monosaccharide stimulus" in biological processes were induced by GT treatments (**Figure 3A**). GT condition reduced the number of unigenes related to "pentose-phosphate shunt" (**Figure 3A**). The effects of chemical treatments on the "pentose-phosphate shunt" category were reflected in the responses of closely related categories such as "NADPH regeneration", "NADP metabolic process", "nicotinamide nucleotide metabolic process", "pyridine nucleotide metabolic process", "oxidoreduction coenzyme metabolic process", "coenzyme metabolic process", and "cofactor metabolic process" (**Figure 3A**).

A number of transport activities in the molecular function category were found to be potentially induced by xenobiotics, particularly by T. This was the case for the "organophosphate ester transmembrane transporter" and "carboxylic acid transmembrane transporter activity" categories (**Figure 3B**). There was also an increase in the unigene proportion of "cytoplasmic membrane-bound vesicle" category in response to GT (**Figure 3C**).

Finally, GT treatment decreased the unigene proportion of "delta1-pyrroline-5-carboxylate synthetase (P5CS) activity" (**Figure 3B**) implicated in the proline biosynthesis pathway.

Effects on Growth and Development Processes

All chemical treatments, and more particularly T and G, increased the number of unigenes annotated in the "adenosylmethionine decarboxylase (SAMDC) activity" molecular function category (**Figure 3B**), which is related to spermidine and spermine biosynthesis from putrescine. In contrast, for the "polyamine oxidase activity" category, which catalyzes the oxidative degradation of polyamines, the related unigenes were not differentially enriched while the number of reads decreased in response to T, G, and GT (Supplemental Table 2). Such maintenance of polyamine levels, which are essential for growth and development in plants (Galston and Sawhney, 1990), may be, at least in part, related to the variations of categories at a higher level of organization. Indeed, T and GT treatments enhanced the number of unigenes correlated to developmental processes such as "seed maturation", "primary shoot apical meristem specification" and more generally "regulation of cell differentiation", while G treatment had opposite effects on proportions of unigenes and reads (**Figure 3A**, Supplemental Table 2). The presence of G also induced a decrease of unigene and read proportions linked to "leaf development" and "organ morphogenesis".

Identification and Characterization of Xenobiotic-Responsive Genes in *L. perenne*

Using a $p < 0.05$, DESeq analysis revealed that 69 unigenes were significantly DE in at least one of the four conditions (**Table 3**). These gene expression profiling data obtained from

TABLE 3 | Significantly differentially expressed transcripts (DESeq analysis).

Unigene ID	Description (TAIR)	DEseq analysis Log_2 [ratio]		
		T/C	G/C	GT/C
comp7278_c0_seq1	Magnesium-chelatase subunit chlH	Repressed	Repressed	0.304
comp7493_c0_seq1	Protein FLUORESCENT IN BLUE LIGHT (FLU)	−2.456	Repressed	−1.124
comp7590_c0_seq3	Plastid transcriptionally active (PTAC16)	Induced	Induced	Induced
comp7590_c0_seq4	Plastid transcriptionally active (PTAC16)	Repressed	Repressed	−1.124
comp7427_c0_seq4	Light harvesting complex photosystem II	Repressed	Repressed	−1.149
comp7634_c0_seq2	Photosystem I, PsaA/PsaB protein	0.939	Repressed	0.069
comp7693_c0_seq11	Photosystem II reaction center protein D	3.129	4.874	Repressed
comp7726_c0_seq27	Photosystem II reaction center protein B	−1.363	Repressed	−2.879
comp7726_c0_seq5	Photosystem II reaction center protein B	−1.704	1.167	Repressed
comp7726_c0_seq1	Photosystem II reaction center protein B	−0.434	Repressed	−0.654
comp6560_c0_seq1	Photosystem I P subunit	1.222	Repressed	−1.372
comp6663_c0_seq5	Isoleucyl-tRNA synthetase-like protein	Repressed	Repressed	1.706
comp7674_c0_seq3	Phosphoenolpyruvate carboxylase	Induced	NDE	NDE
comp6943_c0_seq1	Uncharacterized protein	−0.380	Repressed	−0.366
comp6984_c0_seq1	Rubredoxin family protein	Induced	NDE	Induced
comp6746_c0_seq2	Actin 7	Repressed	2.586	3.379
comp7483_c1_seq2	Phosphoglycerate kinase family protein	−0.723	Repressed	−2.239
comp7355_c0_seq2	CIPK9, SnRK3.12, PKS6, CBL-interacting protein kinase	1.097	Repressed	1.794
comp7489_c0_seq2	Calcium sensing receptor	Repressed	2.415	0.654
comp7362_c0_seq6	Aldehyde dehydrogenase 3F1	−2.379	2.737	0.291
comp7713_c0_seq4	Protein kinase superfamily protein	Induced	NDE	Induced
comp7705_c0_seq3	Phototropin serine-threonine kinase	Repressed	Repressed	0.645
comp7527_c0_seq1	Protein phosphatase 2C family protein	2.737	Repressed	−0.516
comp7476_c0_seq2	Ferredoxin-NADP(+)-oxidoreductase 1 (FNR)	−1.100	Repressed	0.670
comp7633_c0_seq4	4-hydroxy-3-methylbut-2-enyl diphosphate synthase	Induced	NDE	Induced
comp7402_c0_seq2	F-type H+-transporting ATPase subunit delta	Repressed	4.265	1.863
comp7682_c1_seq3	H(+)-ATPase 2	Repressed	−1.322	0.192
comp7721_c0_seq19	ATP synthase subunit alpha	Induced	NDE	Induced
comp7721_c0_seq17	ATP synthase subunit alpha	Repressed	1.415	−1.294
comp7700_c0_seq2	ABC transporter F family	−0.214	Repressed	0.016
comp7571_c0_seq5	Major facilitator superfamily protein	0.959	Repressed	0.564
comp7726_c0_seq35	RNA polymerase subunit alpha	Induced	NDE	NDE
comp7697_c0_seq14	Homeodomain-like superfamily protein	−0.029	Repressed	0.232
comp7697_c0_seq11	Homeodomain-like superfamily protein	0.222	Repressed	Repressed
comp7688_c0_seq2	NAD(P)-binding Rossmann-fold superfamily protein	Repressed	0.023	−0.101
comp7592_c0_seq2	Translation initiation factor IF	−0.778	Repressed	1.128
comp6892_c0_seq2	Gametogenesis related family protein	0.824	2.314	0.914
comp7302_c0_seq1	Uncharacterized protein	0.740	1.816	0.545
comp7589_c0_seq4	Chloroplast stem-loop binding protein	3.392	4.265	Repressed
comp7452_c0_seq3	Indoleacetic acid-induced protein	Induced	NDE	Induced
comp7604_c0_seq2	Tubulin alpha-5	−2.100	Repressed	0.429
comp7501_c0_seq5	DNA photolyase	−0.314	Repressed	−0.982
comp7711_c0_seq1	Presequence protease 1	−1.397	−2.426	Repressed
comp7711_c0_seq5	Presequence protease 1	NDE	Induced	Induced
comp7711_c0_seq3	Presequence protease 1	Induced	NDE	Induced
comp7458_c0_seq2	Peroxidase superfamily protein	Induced	NDE	Induced
comp7458_c0_seq1	Peroxidase superfamily protein	Repressed	−0.285	−1.409
comp7628_c1_seq8	Senescence-associated protein	−0.555	1.415	Repressed
comp7309_c0_seq3	Glycolate oxidase 1 (GOX1)	2.410	Repressed	−1.294
comp7324_c0_seq3	Phenylalanine ammonia-lyase	Repressed	0.337	−0.957

(Continued)

TABLE 3 | Continued

Unigene ID	Description (TAIR)	DEseq analysis Log_2 [ratio]		
		T/C	G/C	GT/C
comp7722_c0_seq7	Phenylalanine ammonia-lyase	Repressed	1.678	Repressed
comp7722_c0_seq5	Phenylalanine ammonia-lyase	−0.710	−2.977	Repressed
comp7722_c0_seq3	Phenylalanine ammonia-lyase	NDE	NDE	Induced
comp6929_c0_seq4	O-methyltransferase	Repressed	0.093	−1.031
comp7591_c1_seq3	ACC oxidase 5 (ACO5)	Induced	Induced	Induced
comp7591_c1_seq4	ACC oxidase 5 (ACO5)	Repressed	−0.700	Repressed
comp6832_c0_seq2	β-1,3-endoglucanase	2.485	Repressed	0.969
comp7382_c0_seq3	Glutamate synthase 1 [NADH]	0.753	Repressed	−0.294
comp7386_c0_seq10	Polyamine oxidase	Induced	NDE	Induced
comp7536_c0_seq5	Sulfite reductase	Induced	NDE	Induced
comp7497_c0_seq1	ATP sulfurylase 2	Repressed	1.830	−0.071
comp7497_c0_seq2	ATP sulfurylase 2	0.222	Repressed	Repressed
comp6892_c0_seq1	Uncharacterized protein	0.850	2.148	0.842
comp7454_c0_seq1	Uncharacterized protein	−0.915	1.126	Repressed
comp7706_c0_seq8	Dentin sialophosphoprotein-related	Repressed	0.699	Repressed
comp7528_c0_seq1	Uncharacterized protein	2.170	1.608	−0.346
comp7706_c0_seq6	Dentin sialophosphoprotein-related	Induced	Induced	Induced
comp7188_c0_seq3	Unknown protein	Induced	Induced	Induced
comp6657_c0_seq2	Pentapeptide repeat-containing protein	Induced	Induced	Induced

C, control; G, glyphosate; T, tebuconazole; GT, glyphosate + tebuconazole. The log_2 [ratio] values could not be calculated when no expression was detected for a given transcript. Such cases were indicated by "Induced" when no expression was detected in the control, "Repressed" when no expression was detected in the treatment conditions. Unigenes were considered as differentially expressed at $p < 0.05$. "NDE" indicated cases for which transcripts were non-differentially expressed. Unigene descriptions were established according to best blast hits in TAIR. The intensities of red or green colors respectively indicate increase or decrease of expression levels in the presence of G, T, and GT compared to the control condition.

RNA-seq were complemented with qRT-PCR analysis of the relative expression of candidate genes (**Figure 4**). The alignments of corresponding protein sequences of candidate unigenes with *Arabidopsis thaliana, Oryza sativa*, and *Brachypodium distachyon* orthologs (Supplemental Figure 1) highlighted high degree of sequence conservation between *L. perenne* and these monocot and dicot model species, and strengthened the automatic *in silico* annotation.

Most of the observed differential expressions (**Table 3**) validated the analysis of GO term category variations under conditions of xenobiotic treatment (**Figure 3**). Among the DE unigenes, a high proportion of xenobiotic-repressed genes were associated with photosynthesis or chlorophyll biosynthetic processes. This was particularly the case for the transcripts of genes related to the tetrapyrrole pathway, such as those encoding Magnesium-chelatase subunit chlH and Fluorescent In Blue Light (FLU), a tetratricopeptide repeat (TPR)-containing protein, highly conserved between *A. thaliana*, *O. sativa,* and *B. distachyon* (Supplemental Figure 1) that were decreased by xenobiotic treatments (**Table 3**). This decrease was confirmed by qRT-PCR, when glyphosate or tebuconazole were applied alone (**Figure 4**). Similar expression profiles were observed for all photosynthesis- and chlorophyll-related unigenes, except for one isoform encoding the plastid transcriptionally active 16 (*PTAC16*) gene that was induced by all conditions (**Table 3**). Induction of expression under G and T treatments was detected by DESeq for the transcript annotated "photosystem II reaction centre protein D". This was also the case for some genes related to photosystems, which exhibited significant induction under either G or T treatments (**Table 3**).

The DESeq analysis revealed that a high proportion of xenobiotic-responsive genes are involved in signaling, and more particularly, correspond to signal transduction through protein-kinases and protein-phosphatases (**Table 3**). This was the case for the CIPK9-annotated gene, which encodes a Calcineurin B-Like Protein (CBL)-interacting protein kinase highly conserved between monocot and dicot species (Supplemental Figure 1). This Serine/Threonine protein kinase annotated unigene, also known as sucrose non-fermenting (SNF)-related kinase family of serine/threonine kinase 3.12 (SNRK3.12), as well as a calcium sensing receptor-annotated transcript, were found to be DE (**Table 3**). DESeq showed a repression of *CIPK9* by G and an induction by T and GT (**Table 3**). qRT-PCR analysis confirmed this repression by G (**Figure 4**).

Transcripts related to protein phosphorylation and dephosphorylation were highly represented in the different datasets. The effects of xenobiotic stresses on highly conserved Serine/Threonine protein kinases (Supplemental Figure 1), such as comp7296_c0_seq6, annotated as a Serine/Threonine protein kinase, and a unigene comp2769_c0_seq1, annotated as a SNRK2 protein (Kertesz et al., 2002), were analyzed by

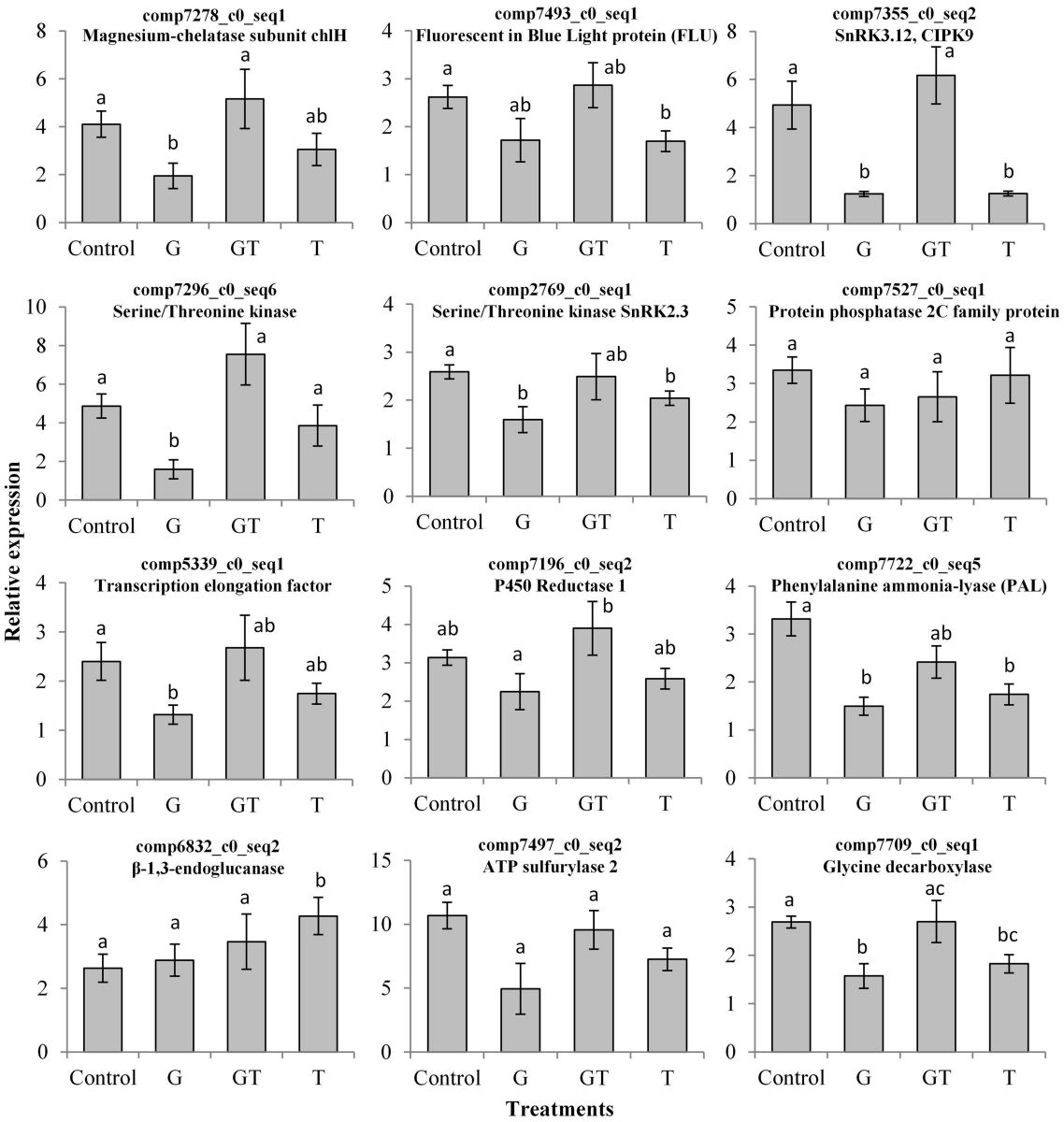


FIGURE 4 | Effects of xenobiotics on the transcript levels of candidate xenobiotic-responsive genes in leaves of *L. perenne*. *L. perenne* plants were grown for 7 days in the absence of pesticide and then transferred to fresh growth medium containing the different xenobiotic treatments [glyphosate (G, 1 μM), tebuconazole (T, 4 μM) and a combination of glyphosate and tebuconazole (GT, 1 μM and 4 μM, respectively)]. Transcript levels (mean ± SEM) were quantified by qRT-PCR after 4 days of treatment. Unigene descriptions were established according to best blast hits in TAIR. Statistical analysis between means was carried out using the Mann-Whitney-Wilcoxon test. Statistical significance of differences ($p < 0.05$) between treatments is indicated by different letters above bars.

qRT-PCR (**Figure 4**). Glyphosate, like for the CIPK9-annotated gene, repressed expression of these genes (**Figure 4**). As observed for SnRK2-type unigenes, glyphosate tended to repress a PP2C-annotated gene, comp7527_c0_seq1 (**Table 3**, **Figure 4**). Different types of expression profile were found, such as the high upregulation of F-type H^+-transporting ATPase subunit delta-encoding gene by G, the specific repression of the unigene related to H^+-ATPase 2 by G or T, or the induction by T or GT of one of the isoforms encoding an ATP synthase subunit alpha (**Table 3**).

The expression of unigenes associated with the regulation of translation or transcription also differed depending on the chemical stressor. Glyphosate repressed the expression of genes associated with Homeodomain-like superfamily proteins and involved in the regulation of transcription (**Table 3**). Another transcription elongation factor encoded by comp5339_c0_seq1 presented the same kind of expression profile (**Figure 4**). On the contrary, the expression of 3 unigenes, whose function was linked to translation (comp6892_c0_seq2, comp7302_c0_seq1, and comp7589_c0_seq4), was significantly

upregulated by glyphosate and GT (**Table 3**). A *GOX1* gene encoding a Glycolate oxidase was differentially repressed in the presence of glyphosate and induced in presence of tebuconazole (**Table 3**). Genes encoding Cytochrome P450 reductase proteins tended to be repressed by glyphosate, whereas the GT mixture had opposite effects (**Figure 4**).

The qRT-PCR analysis of one of genes annotated as Phenylalanine ammonia-lyases (PAL) demonstrated the repressive effect of xenobiotics on its expression (**Figure 4**). A gene homologous to a gene encoding a caffeate *O*-methyltransferase was repressed by T and GT (**Table 3**). 1-aminocyclopropane-1-carboxylic acid (ACC) oxidases were characterized in the shoot transcriptome of *L. perenne* by two isoforms that were differentially regulated, with one being induced by chemical stresses, while the other was repressed (**Table 3**). In parallel, the gene annotated as β-1,3-endoglucanase was induced by T and GT, as confirmed by qRT-PCR trends for T treatment (**Table 3**, **Figure 4**). The sulfur assimilation pathway, which leads to cysteine biosynthesis and *in fine* to glutathione (GSH) biosynthesis, involves, *inter alia*, ATP sulfurylase and sulfite reductase (Anjum et al., 2015). In *L. perenne*, one of the 2 isoforms annotated as ATP sulfurylase showed repression by T and induction by G, while the other was repressed by G and GT (**Table 3**). The qRT-PCR analysis also showed that G tended to repress this latter isoform (**Figure 4**). In contrast, T and GT had a positive impact on the transcription of the sulfite reductase-annotated gene (**Table 3**). Finally, differential expression analysis revealed several uncharacterized transcripts whose expression, for most of them, was induced in the presence of glyphosate and with contrasting effects of the other treatments (**Table 3**).

Metabolic Modifications in *L. perenne* leaves Under Conditions of Short-Term Root-Level Exposure to NOAE Levels of Chemical Stressors

The effects of the present xenobiotic stress conditions (low intensity, short-term exposure, root exposure) were also analyzed in terms of metabolic changes in *L. perenne* leaves (**Figure 5**). Moreover, as previously described in *A. thaliana* (Serra et al., 2013), major metabolic changes were found to occur despite the absence of major stress perturbations. All of the subtoxic conditions analyzed here (G, T, GT) induced or tended to induce a decrease of soluble sugar contents (Fru, Glc, Suc) whereas Tre levels were no affected (**Figure 5**). In contrast, G and T acted differently on the levels of Fru-6-P compared to the GT mixture, with an increase of this phosphorylated sugar with G and T and no effect with GT. Glc-6-P remained relatively stable in response to the three conditions.

Some metabolic changes could be related to the patterns of differential expression described above (**Figures 3**, **4**, **Table 3**). Glyphosate or tebuconazole exposure induced a decrease of glycine levels (**Figure 5**) and also affected the expression of glycine-related genes such as glycine decarboxylase (**Figure 4**). The decrease in putrescine levels in *L. perenne* leaves in response to xenobiotic treatments (**Figure 5**) was in line with the induction by T and GT of the unigene encoding a polyamine oxidase (**Table 3**) and with significant variations of related GO term categories, as "adenosylmethionine decarboxylase (SAMDC) activity" and "spermine biosynthetic process" (**Figure 3**). The decrease of glutamate levels in *L. perenne* leaves in response to G (**Figure 5**) was reminiscent of the decrease of glutamate synthase expression (**Table 3**).

All treatments tended to increase the levels of arabinose, Phe, Lys, Leu, Ile, Val, Tyr, citrate and lactate and to decrease the levels of putrescine and Pro. In particular, glyphosate treatment did not decrease levels of aromatic amino acids (Trp, Tyr, Phe). For Ala, Asp, Fru-6-P, glutamate and ornithine, effects of G or T alone were mostly comparable, and GT mixture effects were not additive (**Figure 5**). In the case of stress metabolites, pipecolate, putrescine, inositol and Pro decreased or were not affected, while lactate increased by low chemical stress (**Figure 5**). This decrease of Pro levels of plants subjected to GT (**Figure 5**) could be related to the reduction of proportion of genes related to "P5CS activity" category (**Figure 3B**).

DISCUSSION

Xenobiotic-Regulated RNA-Seq and qRT-PCR Markers Reveal Novel Potential Pathways of Chemical Stress Responses

The present *de novo* RNA-Seq approach yielded a *L. perenne* xenobiotic-dependent genomic database (**Figures 1**, **2**) that was coherent with recent transcriptomic analyses of *L. perenne* (Farrell et al., 2014; Duhoux et al., 2015) and that was very useful to explore relationships between metabolic changes, stress adjustments, regulation of metabolism genes and signaling pathways under conditions of xenobiotic stress. The experimental conditions of short-term exposure and low levels of xenobiotics led, in all of the cases (glyphosate, tebuconazole, GT mixture), to no observable adverse effects, especially on root growth, which is particularly xenobiotic-sensitive (Serra et al., 2013, 2015). However, these conditions were sufficient to induce significant molecular and metabolic changes (**Figures 3–6**), which could be expected to reflect pre-damage, and probably primary events in the xenobiotic response. The strong impact of these various xenobiotic treatments on the proportions of transcript functional categories and on individual gene expression established that low-level xenobiotic exposure strongly interacted with molecular regulations of gene expression. Moreover, differential effects of the different xenobiotic treatments showed that there were some xenobiotic-dependent specificities in the potential molecular mechanisms of xenobiotic action and of plant responses to xenobiotics, and also that there were specific responses for mixture (**Figures 3–5**). However, this global analysis also revealed the involvement of common mechanisms that are interdependent with each other and closely connected. Finally, detection of major metabolic and molecular rearrangements in leaves, which were not the primary site of exposure, demonstrated that root exposure

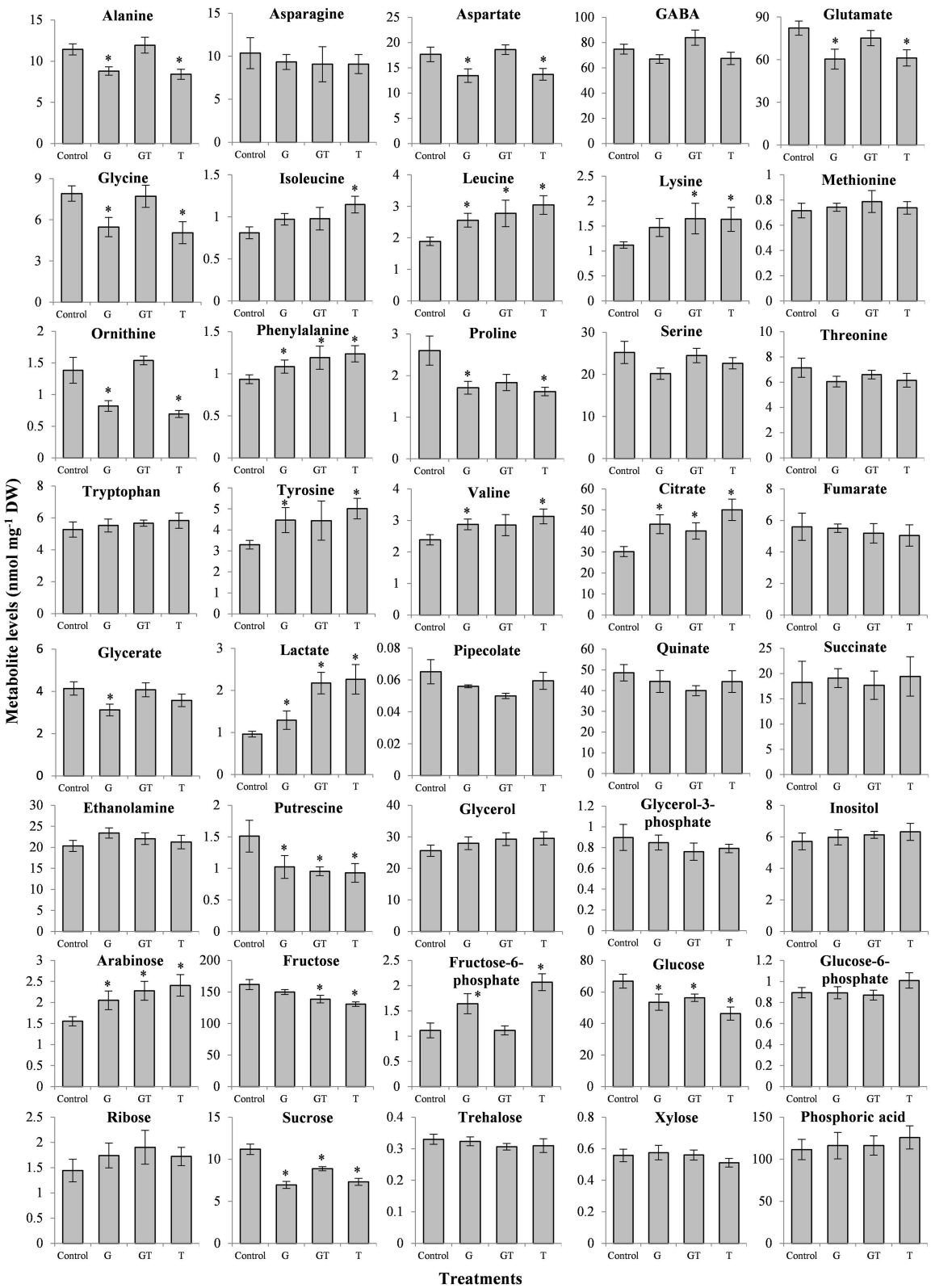


FIGURE 5 | Effects of xenobiotics on metabolite levels in *L. perenne* leaves. *L. perenne* plants were grown for 7 days in the absence of pesticide and then transferred to fresh growth medium containing the different xenobiotic treatments [glyphosate (G, 1 μM), tebuconazole (T, 4 μM) and a combination of glyphosate and tebuconazole (GT, 1 μM and 4 μM, respectively)]. Metabolite levels (mean value ± SEM) were quantified after 4 days of treatment. Stars indicated statistical significance of differences between treatment and Control ($p < 0.05$) carried out using the Mann-Whitney-Wilcoxon test.

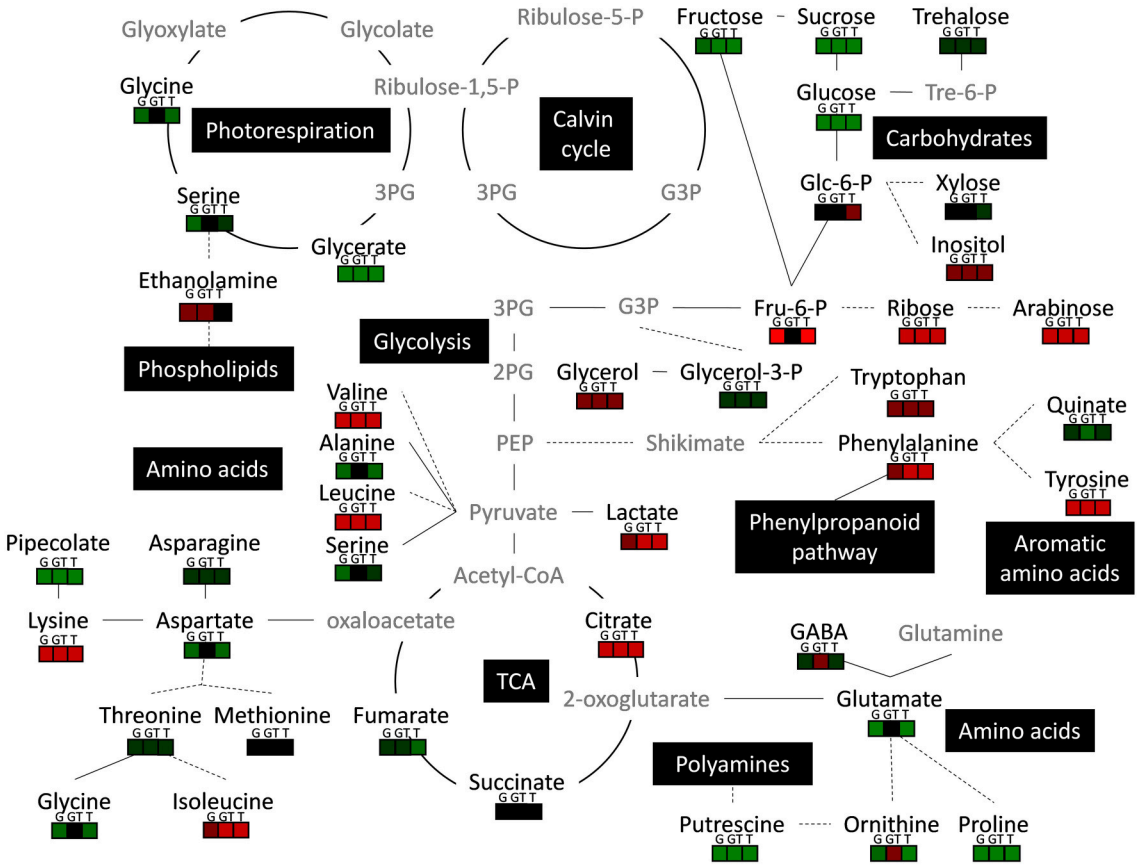


FIGURE 6 | Metabolic integration of low-intensity xenobiotic stress in *L. perenne*. Black names represent quantified metabolite and gray names represent non-quantified metabolites. 2PG, 2-Phosphoglycerate; 3PG, 3-Phosphoglycerate; APS, adenosine 5′-phosphosulfate; G3P, glyceraldehyde-3-phosphate; PEP, Phosphoenolpyruvate; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine. The red or green colors respectively indicate increase or decrease of metabolite levels in the presence of glyphosate (G), tebuconazole (T) and a combination of glyphosate and tebuconazole (GT) compared to the control condition. Solid line indicate direct relation between metabolites, and dotted line show indirect reaction.

involved strong xenobiotic-related root-shoot communication, through xenobiotic, metabolite or signal transport, underlining the impact of such exposure.

Major genes that were found to be DE under xenobiotic exposure did not belong to classical xenobiotic or herbicide stress response pathways such as detoxification pathways involving glutathione S-transferase, cytochrome P450 or glycosyltransferase enzyme activities (Duhoux and Délye, 2013; Délye, 2013), thus suggesting that novel molecular mechanisms leading to xenobiotic-induced metabolic rearrangements have been identified. Most of the 69 DE genes, underlined without *a priori* by the DESeq analysis, surprisingly formed a coherent set as shown by the several associated pathways described below. A significant number of xenobiotic-regulated genes were linked to signal metabolisms and to signal transduction pathways, thus underlining the importance of homeostatic mechanisms and crosstalks between metabolites, carbohydrates, phytohormones, plastid-to-nucleus and light signaling under conditions of xenobiotic stress. The DESeq analysis also suggested that activities of phosphorylation linked to signal transduction could be affected by differential expression of genes related to MAPK cascade and to ATP synthesis or hydrolysis (**Table 3**). Increase of phenylalanine levels in leaves in response to GT and T treatments was associated with repression of genes related to phenylalanine ammonia lyase (PAL) activity (**Figures 4–6**). PALs catalyze the first step of the phenylpropanoid pathway and have overlapping roles in plant growth, development, and responses to environmental stresses (Huang et al., 2010). When added at high and toxic levels, the herbicides paraquat and glyphosate induce PAL activity in plants, thus suggesting involvement of PAL in regulation of the phenylpropanoid pathway in response to oxidative stress produced by lethal herbicide levels (Duke et al., 1980; Lee et al., 2003). In *L. perenne*, low-intensity xenobiotic stress also had significant gene expression effects (**Table 3**, **Figure 4**) on the gene annotated as Cytochrome P450 reductase protein, which functions in electron transfer from NADPH to cytochrome P450 oxidases, and on a caffeate *O*-methyltransferase-homologous gene. These enzyme activities may be both involved in the general phenylpropanoid pathway, as described by Sundin et al. (2014) and Do et al.

(2007) for Arabidopsis, strengthening the involvement of PAL in chemical stress responses. PAL, ACC synthase, which catalyzes the synthesis of ACC for ethylene production, and auxin biosynthesis are thought to be co-regulated (Duke et al., 1980; Soeno et al., 2010). Tebuconazole treatment increased the expression of the gene annotated as β-1,3-endoglucanase and of an ACC-oxidase-annotated gene (**Table 3**), which functions are both related to ethylene action through regulation or metabolism (Zhong and Burns, 2003; Soeno et al., 2010; Jafari et al., 2013). These molecular markers confirmed the relationships between xenobiotic effects, phytohormone, and more particularly ethylene, dynamics and signaling, as previously reported in studies coupling hormone-signaling mutants and xenobiotic-induced stress (Sulmon et al., 2007; Weisman et al., 2010).

GO term enrichments allowed to detect by reads counting the overexpression of unigenes whose functions were related to "magnesium chelatase activity" (Supplemental Table 2). Nevertheless, comp7278_c0_seq1, whose direct function, according to its annotation, is a magnesium chelatase activity (Magnesium-chelatase subunit ChlH), presented an expression significantly affected by glyphosate and tebuconazole (**Table 3**, **Figure 4**), suggesting that overexpressed unigenes may be involved in the negative regulation of expression of the magnesium chelatase activity. The Fluorescent In Blue Light (FLU)-annotated gene was also significantly affected by glyphosate and tebuconazole single treatments (**Table 3**, **Figure 4**). FLU and ChlH are involved in regulation of tetrapyrrole biosynthesis (Kauss et al., 2012; Apitz et al., 2014) and in one of the multiple signaling pathways which mediate plastid signals controlling expression of Photosynthesis-Associated Nuclear Genes (PhANG; Mochizuki et al., 2008; Moulin et al., 2008; Zhang et al., 2013). Moreover, tetrapyrrole gene expression and ABA signaling are interconnected in chloroplast-to-nucleus signaling pathways (Voigt et al., 2010). Zhang et al. (2011) showed that resistance to concomitant high-light and herbicide stress requires the activity of ABI4, an ABA-regulated Apetala 2 (AP2)-type transcription factor, which is also involved in the coordination of several pathways such as sugar, redox, and hormonal [ABA, jasmonate (JA) and salicylic acid] signaling during stress response (Foyer et al., 2012). ABA catabolism markers were also reported to be affected in response to chemical stress treatments as glyphosate (Serra et al., 2013) and triazole including tebuconazole (Saito et al., 2006; Serra et al., 2013). Moreover, triazoles have also been shown to be inhibitors of brassinosteroid synthesis (Kaschani and van der Hoorn, 2007), of the plant sterol pathway in Sorghum (Lamb et al., 2001) and of gibberellin accumulation in rapeseed (Child et al., 1993). Thus, different hormone pathways and signaling seem to be important in numerous xenobiotic responses (Couée et al., 2013). As an example, promoter-level induction by glyphosate and hormones has been described in rice for a glutathione S-transferase involved in xenobiotic detoxification (Hu et al., 2011). However, further experimental studies need to be carried out in order to clearly establish the role of these hormones in plant chemical stress responses.

Low Levels of Xenobiotics Have Significant Effects on Multiple Low Energy and Low Carbohydrate Regulations

Genes related to components of photosynthetic reaction centers presented patterns of expression that were largely influenced by xenobiotics. The gene annotated as photosystem II reaction center protein D presented the highest observed induction by G and T and repression by GT. More generally, differential regulation of genes related to photosynthesis in the presence of sub-lethal concentrations of xenobiotics was specific of the xenobiotic involved (**Table 3**). Some herbicides, such as atrazine, act through binding to D1 protein of photosystem II (PSII) reaction center, thus blocking electron transfer to the plastoquinone pool (Rutherford and Krieger-Liszkay, 2001; Sulmon et al., 2004). However, neither glyphosate nor tebuconazole are known to affect photosynthesis through this mechanism of action. These variations may thus originate from non-target effects, in accordance with the maintenance of aromatic amino acid levels under G treatment (**Figure 5**), suggesting that the canonical EPSPS target of glyphosate was not affected. These differential regulations of photosynthesis-related genes by chemical treatments did not however result in modifications of photosynthetic parameters (Serra et al., 2015), as also reported by Ramel et al. (2007) and Faus et al. (2015), underlining adjustment processes to maintain, at least temporarily, efficient carbon assimilation.

Variations of carbohydrate-related and energy-related GO term categories (**Figure 3**) and, more directly, the variations of soluble sugars (**Figure 5**) strongly indicated that carbohydrate and energy metabolisms were involved in the responses to low-intensity xenobiotic stress. The level of water-soluble carbohydrates is particularly important as a primary source of energy in *L. perenne* (Smith et al., 1998). Some of the metabolic changes induced by xenobiotics, especially soluble sugar depletion and lactate accumulation (**Figures 5**, **6**), therefore suggested a situation of energy imbalance. All of this strongly suggested that low-intensity xenobiotic stress could be perceived as a central energy perturbation. It must be highlighted that such a relationship has been described in other plant-xenobiotic experimental systems (Qian et al., 2014; Serra et al., 2015). Stress-induced energy deficit typically leads to the activation of genes involved in catabolism (proteolysis, amino acid catabolism, sugar degradation, lipid mobilization) and repression of genes involved in protein synthesis (Valluru and Van den Ende, 2011; Ramel et al., 2012; Serra et al., 2013). The strong variations of amino acid levels (**Figure 5**) and of amino acid-related genes (**Figure 4**, **Table 3**) may reflect the mobilization of amino acid substrates (Valluru and Van den Ende, 2011; Ramel et al., 2012; Serra et al., 2013; Duhoux et al., 2015). Further studies are needed to characterize the relations between amino acid and carbohydrate metabolisms, energy perturbation and this particular NOAE situation produced by short exposure to low doses of xenobiotics.

The potential variations of spermine and spermidine levels, that could be deduced from the strong increase in unigenes related to the "spermine biosynthetic process" category (**Figure 3A**), and from the decrease of putrescine levels in

shoots from xenobiotic-treated plants (**Figure 5**), also suggested that low-level xenobiotic exposure caused rearrangements of N-related metabolisms.

Situations of low carbohydrate and low energy can be strongly associated with oxidative stress processes (Couée et al., 2006; Baena-González et al., 2007; Dietrich et al., 2011; Valluru and Van den Ende, 2011). The differential expression of genes linked to the pentose phosphate shunt (**Table 3**) could thus be related to variations of carbohydrate levels and to the supply of reducing equivalents for antioxidant defenses (Couée et al., 2006). Low-intensity xenobiotic stress modified the expression of other genes related to ROS defense. A Glycolate oxidase (GOX1)-annotated gene was found to be regulated in a contrasted manner by glyphosate, tebuconazole and GT treatments (**Table 3**). Glycolate oxidase is involved in the oxidation of glycolate leading to the production of glyoxylate and H_2O_2 during photorespiration (Fahnenstich et al., 2008). In parallel, the levels of photorespiration metabolites (**Figures 5**, **6**) and the expression of a Glycine Decarboxylase-annotated gene (**Figure 4**) were strongly affected by xenobiotic treatments. These results suggested that these conditions of mild chemical stress, and in relation with carbohydrate, energy and ROS processes, could affect photorespiration.

Various Molecular Markers Point Out to the Importance of SnRKs in Xenobiotic Stress Responses

Sugar and energy limitation induced by abiotic stresses are regulated in plants by an array of metabolic sensors (Baena-González and Sheen, 2008; Dietrich et al., 2011). One of these sensors, SnRK1, is involved in responses to a range of stresses that limit photosynthesis and respiration, including the PSII-inhibiting herbicide, DCMU (Baena-González et al., 2007). Sugars, and more particularly Glc-6-P produced by hexokinase (HXK) and Tre-6-P, can block SnRK1 activity (Baena-González and Sheen, 2008; Ramon et al., 2008; Valluru and Van den Ende, 2011; Nunes et al., 2013). Our previous global analysis in *L. perenne*, combining leaves and root metabolites after short exposure and direct and long exposure, showed that various xenobiotic treatments mostly increased Glc-6-P and its correlated co-metabolite, Fru-6-P, levels (Serra et al., 2015). Under the present conditions of short exposure, Glc-6-P remained relatively stable in leaves, suggesting that SnRK1 may not be inhibited by the phosphorylated sugar.

In the present case of *L. perenne*-xenobiotic interactions, several genes, annotated as F-type H^+-transporting ATPase, H^+-ATPase, and ATP synthase, were DE in the presence of xenobiotics (**Table 3**). Moreover, repression of a gene potentially involved in ATP hydrolysis and induction of ATP synthesis genes suggested some kind of cellular mobilization for energy restoration under conditions of xenobiotic exposure (**Table 3**). Decrease of, or threat to, ATP levels occurring under environmental stresses, including xenobiotic stress, were also related to the involvement of mammalian homolog of SnRK1, the AMP-activated kinase (AMPK) energy sensor (Blättler et al., 2007; Hardie et al., 2012).

SnRK1 and several other serine/threonine kinases, especially SnRK2 and SnRK3, have activities that link stress and ABA signaling with metabolic signaling (Halford and Hey, 2009; Yu et al., 2014). In Arabidopsis, 38 members of the SnRK family have been described. The different SnRK subfamilies, SnRK1, SnRK2 and SnRK3, have respectively 3, 10 and 25 representatives (Halford and Hey, 2009). All of these SnRKs are associated with metabolic regulations and stress responses. The present transcriptome reveals that *L. perenne* possesses 146 unigenes with high homologies ($e < 10e\text{-}6$) with these SnRK subfamilies. In comparison, a recent study has identified 44 SnRKs in *Brachypodium distachyon* that respond to multiple stresses and stress-related signal molecules like ABA, ethylene and H_2O_2 (Wang et al., 2015). At least some of *L. perenne* SnRK genes were strongly modulated by low-intensity xenobiotic stress (**Table 3**, **Figure 4**, Supplemental Figure 1), thus strengthening the above-discussed link with the involvement of ABA signaling in xenobiotic responses. ABA regulates the activity of SnRK2 and SnRK3 subfamilies which are part of signaling pathways involved in responses to abiotic stresses such as nutrient limitation, drought, cold, salt, and osmotic stresses (Coello et al., 2011). It has also been shown that SnRK2 could be activated by Ca^{2+} (Coello et al., 2012). Among SnRK2-interacting proteins, Protein Phosphatase 2Cs (PP2Cs) act as negative modulators in relation with ABA signaling (Fujita et al., 2013; Danquah et al., 2014). Interestingly, a PP2C-annotated gene was found to be regulated by xenobiotic treatments, at least in the DESeq analysis (**Table 3**). Moreover, the SnRK2.3-annotated unigene was DE after xenobiotic treatments (**Figure 4**). DESeq and qRT-PCR analysis also showed that a transcript related to SnRK3.12 (CIPK9) was repressed by G treatment, which, in contrast, induced the expression of a calcium sensing receptor gene and of an aldehyde dehydrogenase gene. Such aldehyde dehydrogenases have been involved in calcium-mediated signaling and stress-regulated detoxification in young plants (Stiti et al., 2011). SnRK3s interact with calcineurin B-like (CBL) calcium-binding proteins, hence their description as CBL-interacting kinases (CIPKs; Coello et al., 2011), and these CIPKs carry out crosstalks between Ca^{2+} and ABA signaling in the responses to abiotic stresses (Yu et al., 2014). Despite some contradictory studies about the sensitivity of plants lacking *CIPK9* on low potassium media, a function in potassium homeostasis has also been suggested (Pandey et al., 2007; Liu et al., 2013b; Yu et al., 2014). All of these data therefore suggest that SnRKs and interacting proteins could be implicated in xenobiotic responses, cell homeostasis under xenobiotic stress and xenobiotic stress adaptation through complex regulations that involve hormone signaling, calcium signaling and potassium homeostasis. Further studies, using hormone inhibitors or antagonists and mutants related to SnRK or hormone signaling, would allow to understand the role of SnRK-related signaling in plant responses to xenobiotics. Interestingly, herbicide resistance in the agricultural weed *Ipomoea purpurea* has been associated with the differential expression of genes involved in such cellular signaling, with high glyphosate treatment inducing an AtPK7-like serine/threonine-protein kinase and repressing genes encoding receptor-like kinases (Leslie and Baucom, 2014).

CONCLUSION

The present results are in line with the involvement of genes linked to SnRKs and other protein kinases and to hormonal regulation in response to low-intensity chemical stresses in *L. perenne*. Our results emphasize that responses to xenobiotic stresses require signal transduction mechanisms involving protein-kinases and protein-phosphatases, in accordance with other studies (Sulmon et al., 2007; Fukudome et al., 2014). Our parallel metabolic and molecular approaches establish a vision of responses to mild chemical stress potentially involving complex and interconnected regulation processes. Energy dysfunction is likely to be related to carbohydrate dynamics and regulation. The present results therefore suggested the existence of complex relations between sugar signaling, the signaling pathways associated with phytohormones and calcium (León and Sheen, 2003; Rolland et al., 2006; Kudla et al., 2010; Biswal et al., 2011) and the molecular responses to organic xenobiotics (Ramel et al., 2012; Serra et al., 2013). Finally the close relations between protein-kinase regulations and ABA signaling suggest hormonal actions in response to mild chemical stress. The exact role of these processes in xenobiotic responses remains to be elucidated. This role is likely to be connected to molecular and physiological processes involved in plant growth and development. However, the nature of the primary events leading to such signaling is still completely unknown. Interestingly, herbicide resistance in the agricultural weed *Ipomoea purpurea* has been associated with the differential expression of genes involved in such cellular signaling, with high glyphosate treatment inducing an AtPK7-like serine/threonine-protein kinase and repressing genes encoding receptor-like kinases (Leslie and Baucom, 2014). Situations of NTSR observed in rye-grasses have been correlated with mechanisms of gene expression regulation (Duhoux et al., 2015; Salas et al., 2015). Identification and characterization of key steps of the corresponding signal transduction processes should therefore be of particular interest for herbicide management in crop systems and for restoration-phytoremediation activities in ecological engineering.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01124

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Comprehensive Expression Profiling of Rice Tetraspanin Genes Reveals Diverse Roles During Development and Abiotic Stress

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Tetraspanin family is comprised of evolutionarily conserved integral membrane proteins. The incredible ability of tetraspanins to form 'micro domain complexes' and their preferential targeting to membranes emphasizes their active association with signal recognition and communication with neighboring cells, thus acting as key modulators of signaling cascades. In animals, tetraspanins are associated with multitude of cellular processes. Unlike animals, the biological relevance of tetraspanins in plants has not been well investigated. In *Arabidopsis* tetraspanins are known to contribute in important plant development processes such as leaf morphogenesis, root, and floral organ formation. In the present study we investigated the genomic organization, chromosomal distribution, phylogeny and domain structure of 15 rice tetraspanin proteins (OsTETs). OsTET proteins had similar domain structure and signature 'GCCK/R' motif as reported in *Arabidopsis*. Comprehensive expression profiling of *OsTET* genes suggested their possible involvement during rice development. While *OsTET9* and *10* accumulated predominantly in flowers, *OsTET5*, *8,* and *12* were preferentially expressed in root tissues. Noticeably, seven *OsTETs* exhibited more than twofold up regulation at early stages of flag leaf senescence in rice. Furthermore, several *OsTETs* were differentially regulated in rice seedlings exposed to abiotic stresses, exogenous treatment of hormones and nutrient deprivation. Transient subcellular localization studies of eight OsTET proteins in tobacco epidermal cells showed that these proteins localized in plasma membrane. The present study provides valuable insights into the possible roles of tetraspanins in regulating development and defining response to abiotic stresses in rice. Targeted proteomic studies would be useful in identification of their interacting partners under different conditions and ultimately their biological function in plants.

Keywords: rice, tetraspanin, abiotic stress, hormone, nutrient deprivation, gene expression

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INTRODUCTION

Tetraspanins belong to a superfamily of highly evolutionarily conserved integral membrane proteins with typical arrangement of four transmembrane (TM) domains (TM1-4), two extracellular loops (EC1 and EC2) of unequal sizes, small intracellular loop (IL), short N-, C-terminal cytoplasmic tails and a signature motif ('GCCK/RP' in plants and 'CCG' in animals) in

EC2 (Huang et al., 2005; Boavida et al., 2013). They are conspicuously present in all multicellular organisms and unicellular protozoan amoeba, but are remarkably absent in yeast. Till date, 33 tetraspanins in humans, 37 in *Drosophila melanogaster,* 20 in C*aenorhabditis elegans* (Huang et al., 2005), 4 in fungi (Gourgues et al., 2002), and 17 in *Arabidopsis thaliana* (Garcia-Espana et al., 2008) have been reported. Majority of the tetraspanin proteins are targeted to plasma membrane (PM) where they are believed to recognize extracellular signals, which may cause conformational changes in their cytoplasmic domain resulting in the activation of specific signaling cascades (Levy and Shoham, 2005; Andreu and Yanez-Mo, 2014). The remarkable ability of tetraspanins to form multi-molecular complexes with each other (secondary interactions) and other partner proteins (primary interactions) enables them to form 'tetraspanin-enriched microdomain' (TEM) or 'tetraspanin web'. They are believed to act as 'molecular organizers or facilitators' and they actively participate in the coordination of intracellular signaling pathways with cytoskeleton as they interact with various proteins, including integrins, immunoglobulin superfamily, major histocompatibility complex, growth factor receptors, signaling molecules and receptor proteins (Hemler, 2005). In animals tetraspanins are crucial in regulating cell adhesion, proliferation, motility, cell-to-cell interaction, fusion, and intracellular trafficking (Yunta and Lazo, 2003; Hemler, 2005; Berditchevski and Odintsova, 2007). Functionally tetraspanin proteins are associated with multitude of physiological processes such as pathogenesis, fertilization, induction of immune responses, and tumor progression and suppression (Hemler, 2005; Zoller, 2009).

The dynamic nature of tetraspanins to interact with multiple molecules and their diverse biological roles enthused plant biologists to look into the diversity in structure and function of plant tetraspanins. However, plant tetraspanins have not been investigated in much details and very little information is available on the function of these proteins in plants. Survey of several plant genomes for tetraspanin proteins revealed that they are ubiquitously found in multicellular plant species, but not in unicellular plant forms (Van Bel et al., 2012; Wang et al., 2012). Owing to redundancy in their function, mutations for loss-of-function phenotypes have generated limited information on the specific function of tetraspanin proteins in plants. The first evidence for the involvement of tetraspanin protein in plant development was provided by studies on *ekeko* mutant harboring a T-DNA insertion in *Arabidopsis TET1* gene (Olmos et al., 2003). Severe developmental defects in leaf patterning, root growth and floral organ formation, probably due to dysregulated cell differentiation, suggested their role in regulating key developmental processes in plants (Olmos et al., 2003). Another mutant allele of *TET1*, which was named as *TORNADO2 (trn2)*, exhibited defects in early leaf development (Cnops et al., 2006). A study by Chiu et al. (2007) on *trn2* mutant implicated tetraspanin in defining cellular decisions at the periphery of shoot apical meristem (SAM). It was found that *TRN2* could partially compensate for the loss of shoot meristemless gene, *STM*, which contributes to establishment and maintenance of SAM in *Arabidopsis*. Lieber et al. (2011) demonstrated that *Arabidopsis WINDHOSE* (*WIN*) gene that encodes for small GYPP-repeat proteins functions with *TRN2* in promoting megasporogenesis. However, the molecular role of *TRN2* in regulating phase transition from somatic to reproductive cell fate needs to be explored. Based on the expression studies in different cell types in reproductive tissues, Boavida et al. (2013) proposed involvement of TET proteins in reproductive development of *Arabidopsis*. Split ubiquitin assays in yeast showed that several members of *Arabidopsis* tetraspanin family associate strongly as homomers or heteromers, which could explain the functional redundancy due to dynamicity and diversity in the molecular interactions among these proteins. Recently Wang et al. (2015) performed a more detailed study advocating the role of tetraspanin in different aspects of *Arabidopsis* development and further proposed transcription factor (TF)-TET regulatory network for prediction of molecular function of TETs in various plant pathways.

In the present study efforts were made to perform a genome-wide analysis of tetraspanin protein family in rice with respect to the identification of true tetraspanin protein encoding genes, their genomic organization, phylogenetic analysis and motif analysis of predicted tetraspanin proteins followed by identification of *cis*-regulatory elements in putative gene promoters. We generated expression pattern information of tetraspanin genes in vegetative and reproductive tissues of rice. Detailed expression profiling during progression of senescence of flag leaf showed that several of these genes were induced at early stage of senescence, pointing toward their role in regulating senescence in plants. Additionally, rice tetraspanin genes were regulated by abiotic stresses, nutrient deprivation, and exogenous application of phytohormones in rice seedlings indicating that these proteins may act versatile as signal relays and participate in mediating multitude of biological processes. We generated a comprehensive expression atlas of rice tetraspanin genes along with the information on their subcellular localization, all of which would be useful in understanding the biological function of this multifunctional protein family in plants.

MATERIALS AND METHODS

Identification of Tetraspanin Gene Family Members in Rice

To identify members of rice tetraspanin (*OsTET*) gene family keyword search was performed using 'TETRASPANIN' in RGAP v7[1] and Phytozome v10.3[2] databases. The second strategy involved exploring the complete rice proteome available at RGAP pseudo molecules v7.0 for the identification of transmembrane (TM) containing proteins using TMHMM v2.0 (TM Helices Hidden Markov Model[3]). Further shortlisting was done for the proteins containing four TM proteins. Subsequently these proteins were screened for the presence of characteristic features of tetraspanin proteins: 4TM, 2ECL of unequal sizes, 1ICL, 9

[1]http://rice.plantbiology.msu.edu/

[2]http://www.phytozome.net/

[3]www.cbs.dtu.dk/services/TMHMM/

cysteine residues in EC2, 'GCCK' motif, short N-, C-terminal tails. Perl scripts were designed for analyzing the output obtained at each step. The outputs obtained by two strategies were compared and members commonly present in both sets were identified as 'true tetraspanin proteins'. All predicted protein sequences were subjected to SMART (Sequence Modular Architecture Research Tool), Pfam and InterPro analysis to confirm the presence of TM domains and tetraspanin-specific 'GCCK' motif. All the *in silico* analyses were carried out using available nucleotide or protein sequences of rice variety, Nipponbare.

Nomenclature, Chromosomal Distribution, and Gene Duplication of Rice Tetraspanins

The *OsTET* genes were mapped on 12 chromosomes of rice by using chromosome map tool in Oryzabase database[4] and a map of was drawn on the basis of output generated. Tetraspanins were sequentially numbered on the basis of their location on rice chromosomes. Gene duplication analyses were performed using PGDD (Plant Genome Duplication Database[5]). Segmental duplication of *OsTET* genes was determined with the maximal length distance allowed between colinear gene pairs of 500 kb using data available at RGAP[6]. Genes were considered as tandemly duplicated if they belong to the same family, located on the same chromosome and not separated by a maximum of 10 unrelated genes (Du et al., 2013; Jiang et al., 2013).

Phylogenetic Analysis of Rice and *Arabidopsis* Tetraspanin Proteins

Arabidopsis (*A. thaliana*) tetraspanin protein sequences were obtained from The *Arabidopsis* Information Resource (TAIR[7]). All predicted full-length rice *(Oryza sativa)* tetraspanin protein sequences were downloaded from Rice Genome Annotation Project (RGAP) database. Multiple sequence alignment of these proteins was carried out with ClustalX 2.1. The sequence alignment was imported to Molecular Evolutionary Genetic Analysis (MEGA) v6.0 for generating an un-rooted neighbor-joining phylogenetic tree with 1000 bootstrap value. The proteins were clustered together in respective clades based on significant bootstrap value ($\geq$50%).

Analysis of Conserved Motifs in Rice Tetraspanin Proteins

To identify characteristic structural components and the divergence in tetraspanin proteins in rice, corresponding predicted protein sequences were aligned by ClustalX 2.1. TM domains were predicted by SMART (Sequence Modular Architecture Structure Tool[8]). Potential palmitoylation sites were predicted with Palmitoylation CSS-Palm 2.0 (Ren et al., 2008) and NetNGlyc 1.0 server[9] was employed to identify potential *N*-glycosylation sites. OsTET proteins identity and similarity matrix was generated by using online tool Sequence Identity And Similarity (SIAS[10]). All sequences were edited with GeneDoc software 2.7.

In Silico Analysis of Putative Promoter Sequences of Rice Tetraspanins

Nucleotide sequence 1 kb upstream of translational start site of rice tetraspanin genes were extracted from RGAP v7.0. The putative promoter sequences were analyzed for various *cis*-acting regulatory elements using New PLACE (A Database of Plant *Cis*-acting Regulatory DNA Elements[11]) and PlantCARE (Plant *Cis*-Acting Regulatory Element[12]) databases. Different classes of regulatory elements involved in tissue specificity and stress responsiveness were identified and their positions were marked.

Plant Material, Growth Conditions and Stress Treatments

Oryza sativa L. sp. *indica* var. Pusa Basmati 1 (PB1) seeds were surface sterilized with 70% ethanol for 1 min, followed by 2% sodium hypochlorite for 20 min. After overnight soaking in water, seeds were grown hydroponically on rice growth media or RGM (Yoshida et al., 1976) for 7 days at 28 $\pm$ 2°C under 16 h light/8 h dark photoperiodic conditions. Seven-day-old rice seedlings were exposed to different abiotic stresses such as heat stress (42°C for $^1/_2$, 1, 4, and 8 h), salinity stress (200 mM NaCl for 2, 6, 12, and 24 h), cold stress (4°C for 2, 6, 12, 24, and 48 h), water-deficit stress (imposed by 15% PEG-6000 for 2, 6, 12, and 24 h) and oxidative stress (10 mM H_2O_2 for 1, 4, 8, and 12 h). Similarly, several hormones such as ABA (100 μM), brassinosteroid (1 μM epibrassinolide), methyl jasmonate (100 μM), and gibberellic acid (100 μM) were also applied exogenously for 1, 3, 6, and 12 h. For simulating nutrient deprivation conditions 7-day-old rice seedlings grown in RGM [1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μM H_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$, 40.5 μM citric acid, pH 4.5-5.0] were deprived of nitrogen (−N) or phosphorous (−P) or potassium (−K) and sulfur (−S) for 12, 24, 48, and 72 h.

For tissue-specific expression profiling, different tissues such as shoots and roots of 7-day-old seedlings, leaves at young stage (15 Days After Transplanting or DAT), active tillering phase (40 DAT) and stem elongation phase (60 DAT), spikelets (80 DAT), young flag leaf (YFL; 70 DAT), mature flag leaf (MFL; 85 DAT). Fully expanded mature flag leaves were also collected at different stages of senescence, i.e., early stage or S1 (105 DAT), mid stage or S2 (120 DAT) and late stage or S3 (135 DAT) with 95–100%, 60–80%, and 40–60% total chlorophyll content, respectively. For all the treatments and stages appropriate controls were

[4]http://viewer.shigen.info/oryzavw/maptool/MapTool.do

[5]http://chibba.agtec.uga.edu/duplication/index/locus

[6]http://rice.plantbiology.msu.edu/

[7]http://www.arabidopsis.org

[8]http://smart.embl-heidelberg.de/

[9]www.cbs.dtu.dk/services/NetNGlyc/

[10]http://imed.med.ucm.es/Tools/sias.html

[11]www.dna.affrc.go.jp/PLACE/

[12]http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

kept and the tissues were harvested, quickly frozen and stored at −80°C.

RNA Isolation, cDNA Synthesis and Quantitative PCR

Total RNA was isolated following the modified protocol by Chomczynski and Sacchi (1987). The quantitative and qualitative analysis was carried out using spectrophotometer (Bio-Rad, USA) and 1.2% formaldehyde agarose gel, respectively. 2.5 μg of total RNA treated with DNaseI (New England Biolabs, USA) was reverse transcribed using iScript cDNA Synthesis Kit as per manufacturer's instructions (Bio-Rad, USA). Quantitative PCR (qPCR) was performed with three biological replicates and two technical replicates using Sso fast Evagreen supermix (Bio-Rad, USA), appropriate primers and Master cycler RealPlex2 (Eppendorf, Germany). Melting curve analysis was performed to check the specificity of amplification with a particular set of primers. *eEF1α* (eukaryotic translation elongation factor 1 α; GenBank Accession #: AK061464) was employed as internal control for normalization. Relative fold change was calculated using ΔΔCT method as proposed by Livak and Schmittgen (2001). Hierarchical clustering analysis of relative fold change was performed to prepare a dendrogram and a heat map using Hierarchical Clustering Explorer v3.5 software. The nucleotide sequences of primers employed for gene expression analyses are provided in Supplementary Table S2.

For compiling expression profile of *OsTET* and *AtTET* genes using already available microarray datasets, heat maps were constructed. The datasets, employed for preparing rice *TET* genes expression profile, were retrieved from Genevestigator-https://genevestigator.com/gv/ (abiotic stress-specific profile) and RiceXpro-http://ricexpro.dna.affrc.go.jp/index.html (tissue-specific expression profile). Expression data on *Arabidopsis* plants treated with abiotic stresses was extracted from *Arabidopsis* eFP browser- http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi.

Subcellular Localization of Rice Tetraspanins

Nicotiana benthamiana (tobacco) transient assays were employed for determining the subcellular localization of OsTET proteins. For this cDNAs corresponding to *OsTET* genes were amplified using Phusion high-fidelity DNA polymerase (Thermo Scientific, USA) and cloned into pENTR-D/TOPO (Invitrogen, USA) followed by their mobilization into destination vector, pGWB541, to obtain OsTET-YFP fusion under the control of constitutive CaMV 35S promoter. All entry and expression clones were confirmed by DNA sequencing. For co-localization studies binary vector containing PM marker (PIP2A-CFP; ABRC catalog-pm-ck CD3-1001) was employed (Nelson et al., 2007). OsTET-YFP and PM marker constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101. For transient assays, leaves from 4 to 6 week-old wild-type tobacco plants were infiltrated with the agrobacterial suspension harboring OsTET-YFP and PM marker as described by Voinnet et al. (2003). The infiltrated plants were marked and kept in a growth room at 25 ± 2°C. Fluorescence was visualized after 48–72 h of infiltration using TCS SP5 laser scanning confocal microscope (Leica, Germany). CFP and YFP signals were detected at 470–500 and 520–550 nm laser band width range with excitation at 433 and 514 nm lasers, respectively. All the images were further processed using Leica LAS AF Lite software. The nucleotide sequences of primers used for amplification of full length *OsTET* cDNAs are provided in Supplementary Table S3.

RESULTS

Genome-wide Identification of Tetraspanin Gene Family Members in Rice Genome

To explore tetraspanin gene family in rice genome we performed a systematic analysis by employing two different approaches. First approach involved searching for the keyword 'tetraspanin' in two rice databases, Phytozome v10.3 and RGAP v7, which resulted in identification of 17 members belonging to tetraspanin family. The second approach was based on the prediction of TM helices in proteins using TMHMM v2.0 by which we were able to identify 11,404 membrane proteins in the RGAP rice database. Further, Perl scripts were designed to screen for the presence of four TM domains resulting in shortlisting of 569 proteins, which were further analyzed for the presence of canonical features of plant tetraspanins: two extracellular loops of unequal sizes, one intracellular loop, nine cysteine residues in EC2, presence of conserved 'GCCK' motif, short N- and C-terminal tails. The second approach identified 15 tetraspanin (*OsTET*) genes in rice. Comparison of the output from two approaches revealed that 15 members were commonly represented and were therefore considered as true tetraspanins, which was further confirmed by detailed analysis of protein sequence and domains. Two additional members identified by the first approach displayed presence of four TM, two ECL, one ICL, small N-, C- cytoplasmic tails but lacked highly conserved 'GCCK' motif in EC2. Moreover EC2 was found to be smaller in size than EC1 and it lacked conserved cysteine residues. For the abovementioned reasons, therefore these proteins were not considered as bonafide members of rice tetraspanin family. The 15 tetraspanins were subsequently named according to their locations on 12 rice chromosomes. The information on these 15 tetraspanin genes such as gene names, locus ID, number of introns, and details about the deduced protein are compiled and presented as **Table 1**.

Chromosomal Distribution and Duplication Events Among Rice Tetraspanin Genes

With an aim to gain insight into the genomic organization of tetraspanin genes, their locations were mapped onto rice chromosome sequences. While 15 *OsTET* genes were unevenly distributed among 9 chromosomes, none of these genes could be mapped on chromosome 4, 7 and 11 (Supplementary Figure S1). Whereas chromosome 3 contained three *OsTET* genes, chromosomes 1, 9, 10, and 12 had only one *OsTET* gene each.

TABLE 1 | Details of rice tetraspanin genes.

Gene name	TIGR ID	RAP ID	Full-length cDNA accession ID	Full-length CDS (bp)	Number of introns	Accession number of rice T-DNA mutant	Predicted protein		
							Length (aa)	MW(kDa)	Putative localization
OsTET1	LOC_Os01g74570	Os01g0977100	–	866	1	–	321	35	PM
OsTET2	LOC_Os02g12750	Os02g0219800	AK242431	813	1	–	272	29.45	PM
OsTET3	LOC_Os02g49630	Os02g0728800	–	1143	4	–	381	41.43	PM
OsTET4	LOC_Os03g01012	Os03g0100020	AK073982	825	0	–	275	29.53	PM
OsTET5	LOC_Os03g63600	Os03g0853000	–	894	1	PFG_3A-15631.R	298	31.85	PM
OsTET6	LOC_Os03g63620	Os03g0853200	AK109128	858	1	PFG_4A-04052.L	286	30.86	Chloroplast
OsTET7	LOC_Os05g03140	Os05g0122800	AK065567	885	1	PFG_2D-11070.R	295	32.88	ER
OsTET8	LOC_Os05g03530	Os05g0126100	AK099070	867	1	PFG_1C-07632.R	289	31.14	PM
OsTET9	LOC_Os06g37510	Os06g0572400	AK070273	819	1	PFG_2A-30186.L	273	29.8	PM
OsTET10	LOC_Os06g44310	Os06g0653100	AK242956	810	1	PFG_2D-10796.L	270	29.49	PM
OsTET11	LOC_Os08g16050	Os08g0260600	AK108529	822	1	PFG_2B-60309.L	274	29.12	PM
OsTET12	LOC_Os08g34460	Os08g0443800	AK110630	834	0	PFG_K-00453.L	278	29.96	PM
OsTET13	LOC_Os09g25760	Os09g0425900	AK061801	831	1	FL054449	277	30.75	PM
OsTET14	LOC_Os10g35980	Os10g0503600	AK103857	813	10	PFG_2D-21433.R	271	30.62	PM
OsTET15	LOC_Os12g14580	Os12g0249100	–	933	1	–	311	34.13	PM

Gene ID of each tetraspanin was retrieved from TIGR (The Institute for Genomic Research) and RGAP (Rice Genome Annotation Project) database. Length of coding sequence (CDS) of rice tetraspanin genes is presented along with the number of introns. Rice T-DNA mutants were identified from SIGNAL-RiceGE database v6.1. Molecular weights of tetraspanin proteins were predicted by using Geneious v6.0. Putative localizations of TET proteins were predicted with pSORT. Aa, amino acids; kDa, kiloDaltons; PM, plasma membrane; ER, endoplasmic reticulum; bp, base pairs; MW, molecular weight.

To examine the inter-relationship of *OsTET* family genes we investigated for tandem and segmental gene duplications events. Only one pair of genes, *OsTET5* and *OsTET*6, separated by a genomic region of 7530 bp on chromosome 3, appeared to have arisen because of probable tandem duplication. *OsTET2* and *OsTET9* located on chromosome 2 and 6, respectively, were segmentally duplicated. *OsTET7* and *OsTET8* present on chromosome 5 were separated by a distance of 242 kb. *OsTET1* and *OsTET4* were located at the terminal portion of chromosome 1 and 3, respectively. Whereas, only six genes were present between *OsTET1* loci and the chromosome terminal, a single gene separated *OsTET4* from the end of chromosome 3 (Supplementary Figure S1).

Gene Structure of Rice Tetraspanins

Understanding the gene structure of different members of a gene family provides information on the evolution of these genes. In fact tetraspanin superfamily in different organisms has been employed as an excellent system for studying intron evolution using phylogenomics approach (Garcia-Espana et al., 2009; Huang et al., 2010). Ten out of 15 rice tetraspanins exhibited conserved gene structure with two exons separated by a single intron and presence of short UTRs (**Figure 1**). *OsTET8* exhibited three splice forms, of which only *OsTET8.3* had conserved gene structure similar to that seen for most of the other tetraspanin genes, while the other two forms (*OsTET8.1* and *8.2*) contained two introns each. However, one of the spliced isoform, *OsTET8.1,* lacked 'GCCK' motif and other characteristic features of tetraspanin proteins (**Figures 1** and **2**). This analysis also suggests that rice tetraspanins have fewer introns relative to the average number of introns (3.9 introns/gene) reported in this species[13]. *OsTET14* contained a maximum of 10 introns followed by *OsTET3* that consisted of four introns. The sizes of introns in rice tetraspanin genes ranged from 95 to 4200 bp. *OsTET4* and *OsTET12* were predicted to be intron less genes (**Figure 1**).

The number and position of introns is generally conserved within members of a gene family (Fedorov et al., 1992). We found that in addition to the presence of a single intron in most of the rice tetraspanins position of the intron was also conserved. These introns were invariably present after the first second conserved cysteine residues in variable region of EC2. In a previous study, non-uniform distribution of intron phase has been reported in several plants, with phase 0 being highly represented and phase 2 being the least represented (Nguyen et al., 2006). After determining the gene architecture of 15 *OsTET* genes, we investigated distribution of the intron phase in these genes and found that majority of the *OsTET* genes contained phase 0 ancestral introns (introns that are present between two codons) followed by presence of phase 2 introns (introns present between the second and third bases of the codon). Among the 15 *OsTET* genes, only one (*OsTET3*) had a phase 1 intron (intron located between the first and second bases of the codon), along with two phase 0 and one phase 2 introns (**Figure 1**). All these observations support the earlier findings that phase 0 introns are

[13]http://rice.plantbiology.msu.edu/analysesfacts.shtml

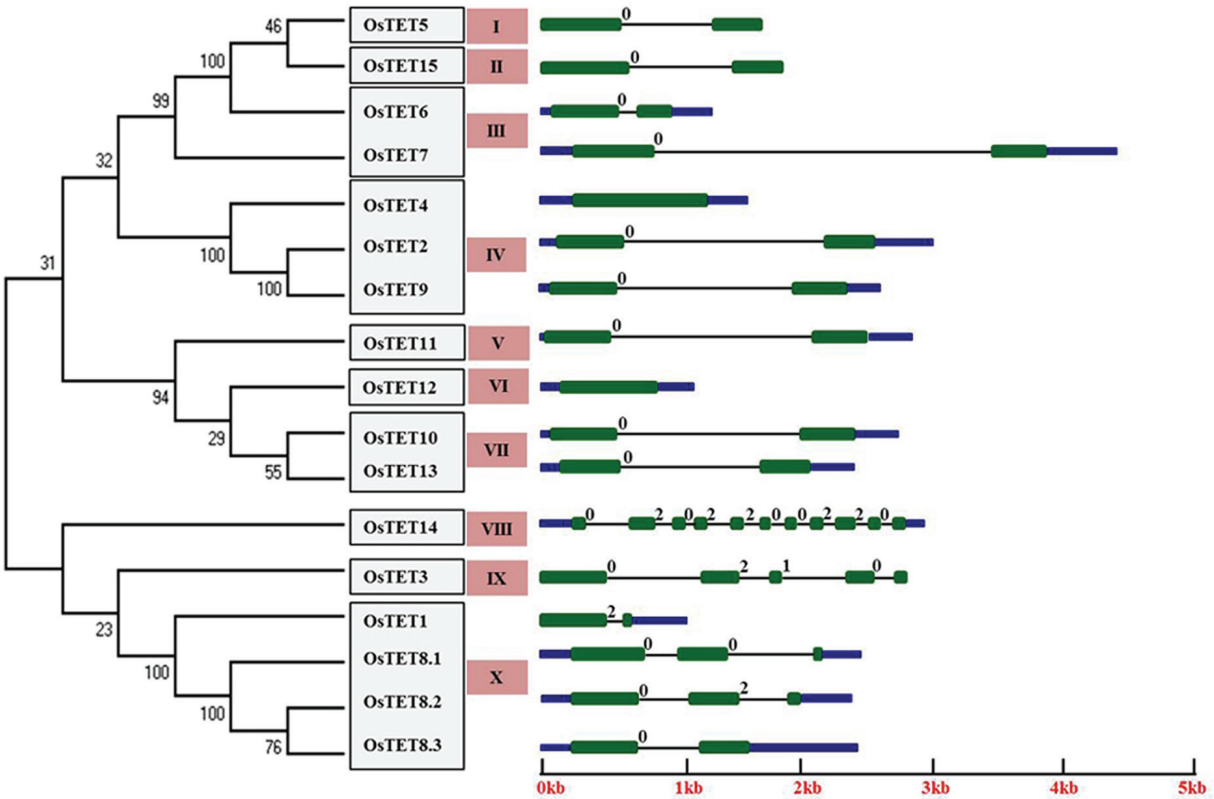


FIGURE 1 | Structure of rice tetraspanin genes and phylogenetic tree of predicted proteins. An online tool, Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/), was used to draw tetraspanin gene structure. Green boxes indicate exons, black lines depict introns, upstream/downstream sequences are shown by blue boxes. Intron phases are indicated at exon-intron junctions. An unrooted neighbor-joining phylogenetic tree of rice tetraspanin proteins is shown on the left side. Numbers above branches indicate bootstrap percentage values. Clade numbers are indicated by salmon colored boxes. OsTET proteins were clustered based on significant bootstrap value (≥50%).

most abundantly present in tetraspanin proteins (Garcia-Espana et al., 2009). Variation in the length of introns within family members is already reported and is believed to contribute to functional diversification of gene family members (He et al., 2012; Zhao et al., 2014). Considerable variation in the length of introns of 15 *OsTET* genes was found, with 95 bp in *OsTET14* as the shortest and 4200 bp in *OsTET7* as the longest intron, which suggests that members of *OsTET* family are functionally diverse.

Phylogenetic Analysis and Protein Structure of Rice Tetraspanin Proteins

To determine the level of conservation and similarity among rice tetraspanin proteins, the predicted amino acid sequences of 15 OsTETs were aligned using ClustalX 2.1. On the basis of pairwise comparison of rice tetraspanins it was concluded that these proteins share an average of 34% identity and 48% similarity (Supplementary Figures S2–S4), which is comparable to that observed for *Arabidopsis* tetraspanin family of proteins (Boavida et al., 2013). An un-rooted phylogenetic tree was constructed using neighbor-joining method for members of rice and *Arabidopsis* tetraspanin protein family and the proteins were clustered together based on the significant bootstrap value of ≥50%. Among 15 OsTET proteins, only 10 proteins clustered with *Arabidopsis* proteins in orthologous clades. Our analyses revealed that OsTET5-OsTET7 and OsTET15 formed a cluster with AtTET3/AtTET4. While OsTET2/4/9 clade contained AtTET1/AtTET2 and OsTET1/OsTET8 clade consisted of AtTET5/AtTET6 (Supplementary Figure S5). Boavida et al. (2013) proposed that AtTET10 and AtTET13 are the founding members of *Arabidopsis* tetraspanin protein family. OsTET14 clustered closely with AtTET10, which signifies its ancient nature of origin, but a member corresponding to AtTET13 was not found in rice. AtTET7-AtTET9, AtTET11-AtTET17 formed separate clades, which indicated that these tetraspanins were specific to *Arabidopsis*. Similarly, OsTET3, OsTET10-OsTET13 did not cluster with *Arabidopsis* tetraspanins, which suggested that these tetraspanins were probably rice-specific (Supplementary Figure S5). We further constructed a phylogenetic tree for members of rice tetraspanin proteins only, which resulted in a total of 10 paralogous clades, of which clade IV consisted of a maximum of three OsTET proteins followed by clades III, VII, and X containing two members each (**Figure 1**). The tandemly duplicated tetraspanins, OsTET5 and OsTET6, were clustered in two

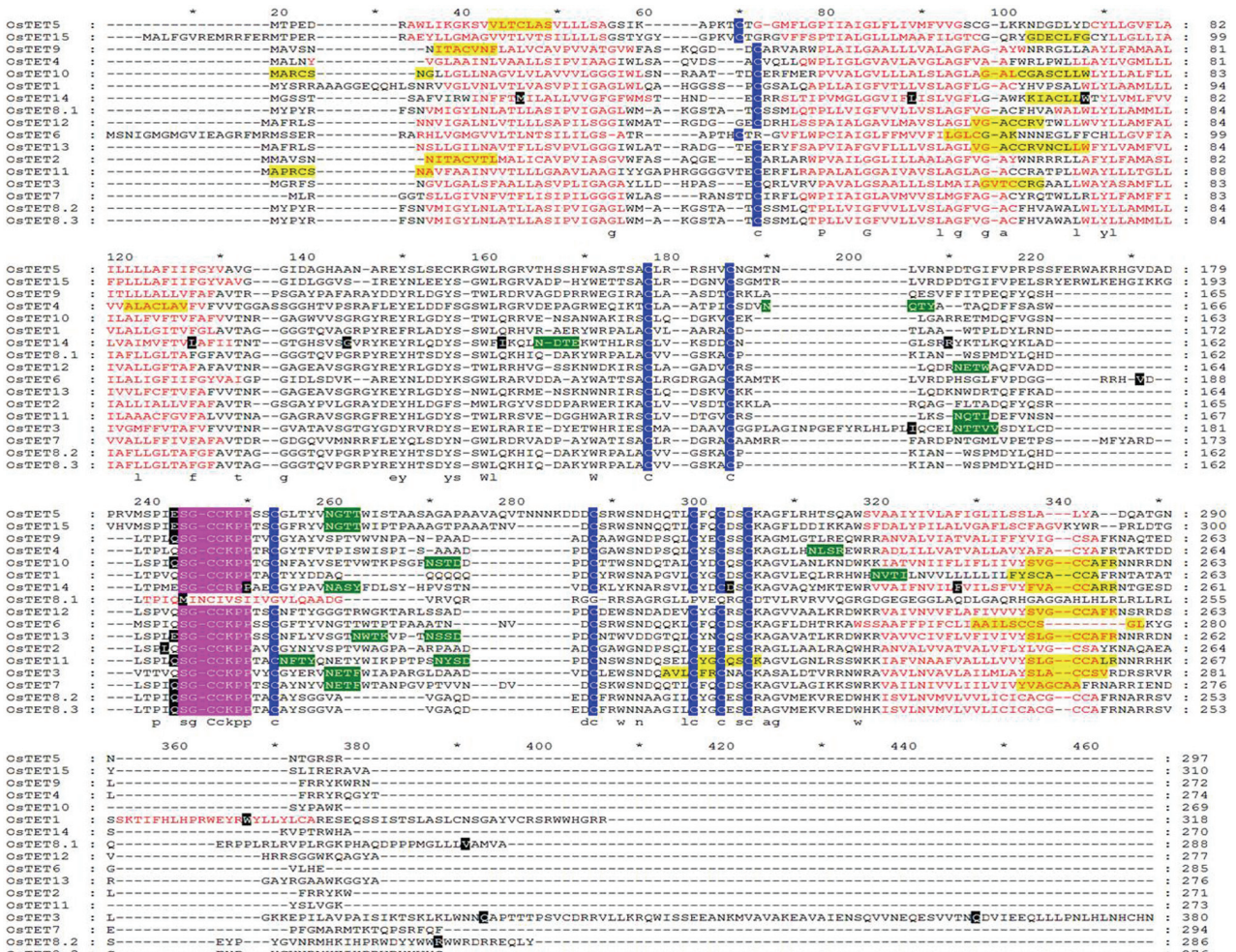


FIGURE 2 | Multiple sequence alignment of predicted rice tetraspanin proteins. Protein sequences of rice tetraspanin proteins were retrieved from RGAP and were aligned using ClustalX 2.1. Red amino acids indicate transmembrane (TM) domains. Predicted palmitoylation and glycosylation sites are highlighted in yellow and green color, respectively. Highly conserved cysteine residues are shaded by blue color. Tetraspanin signature 'SGCC(K/R)PP' motif is shaded in pink color. Intron positions are shaded by black color. Sequences were edited with Genedoc software (http://www.nrbsc.org/gfx/genedoc/).

different clades, I and III, respectively. The segmental duplicated members, OsTET2 and OsTET9, exhibited 100% bootstrap value indicating high level of conservation in their protein sequences (**Figure 1**).

The conserved domains of OsTET proteins were predicted so as to evaluate the extent of conservation among 15 members. Multiple sequence alignment of full length OsTET proteins revealed presence of four TM domains (predominantly 22 amino acids each), two extracellular loops of unequal sizes (EC1 and EC2) and highly conserved 'GCC(K/R)' motif, short N- and C- terminal cytoplasmic tails and small intracellular loop (ICL) as depicted in **Figure 2** and Supplementary Table S1. All OsTET proteins were found to contain one and nine conserved cysteine residues in EC1 and EC2, respectively. Animal and metazoan tetraspanins are known to possess conserved 'CCG' motif in EC2 domain (DeSalle et al., 2010). On the other hand plant tetraspanins are characterized by the presence of 'GCCK/RP' motif and few variants as seen in *Arabidopsis*. Interestingly we did not find any variant of 'GCCK/RP' motif in rice tetraspanin members. In fact we observed a relatively longer motif with sequence "SGCCK/RPP" in all the rice tetraspanin proteins. Most of the tetraspanins were predicted to contain potential post-translation modifications sites such *N*-glycosylation in EC2 region and palmitoylation of cysteine residues proximal to TMs, which are believed to play crucial role in protein–protein interactions. The TMs of all rice tetraspanins showed presence of conserved polar residues, which is another distinct feature of animal, metazoan and plant tetraspanins (**Figure 2**). The multiple sequence alignment and motif analyses revealed that rice tetraspanins possess characteristic features of plant tetraspanins and there exists high conservation in amino acid residues as well as motifs in these proteins, which is similar to that found in previously discovered plant tetraspanins.

Expression Profiling of Rice Tetraspanin Genes in Different Tissues and during Flag Leaf Senescence

Two studies using *Arabidopsis* have implicated tetraspanin proteins in controlling developmental processes such as leaf venation, leaf pattering, root pattering, and floral organ development (Cnops et al., 2000, 2006; Olmos et al., 2003). On the basis of tissue- and domain-specific expression in *Arabidopsis*, Boavida et al. (2013) predicted involvement of tetraspanins in reproductive processes. With an aim to obtain the spatial-temporal expression profile of *OsTET* genes, we determined the expression levels of *OsTET* genes in various tissues: shoot and root tissues of 7-day-old seedlings, young leaf (YL), leaves at active tillering phase (AT-phase), leaves at stem elongation phase (SE-phase), spikelets (Spks), young (YFL) and mature fully-expanded flag leaf (MFL). Quantitative PCR was employed for determining the relative levels of *OsTET* transcripts in different tissues. However, only 14 out of the 15 predicted *OsTET* genes were amplifiable (Supplementary Figures S6 and S7) and therefore expression profiling studies were carried out for 14 out of 15 *OsTET* genes. The genes that exhibited ≥2-fold change in $\log_2$ scale in root tissue relative to the levels detected in shoot tissue of 7-day-old seedlings were considered as significantly regulated (**Figure 3A**). As compared to shoot tissue, three *OsTETs* (*OsTET5*, *8*, *12*) and four *OsTETs* (*OsTET3*, *4*, *7*, *13*) were significantly up regulated and down regulated in root tissue, respectively. We also compared the relative levels of *OsTET* genes in field-grown young leaves (YL) with respect to the 7-day-old shoots. Notably, 10 out of 14 *OsTET* genes (*OsTET2-7*, *OsTET11-14*) were up regulated in YLs, of which *OsTET2* showed 21-fold change in its steady state levels (Supplementary Figure S8). For all other stages (AT-phase, SE-phase, Spks, YFL, and MFL) the transcript levels of *OsTET* genes were quantified relative to the levels present in YL. Expression of *OsTET6* declined in most of the tissues as compared to YLs. *OsTET2, 3, 5, 9, 11,* and *12* accumulated to higher levels in leaves at active tillering phase and their levels (except *OsTET11*) were sustained even during stem elongation phase (**Figure 3A**). Only three *OsTETs* (*OsTET1, 9,* and *10*) were highly expressed in the spikelets as compared to young leaf tissues. On the other hand six *OsTETs* (*OsTET3, 4, 6, 11, 12,* and *13*) exhibited significant decline in their transcript

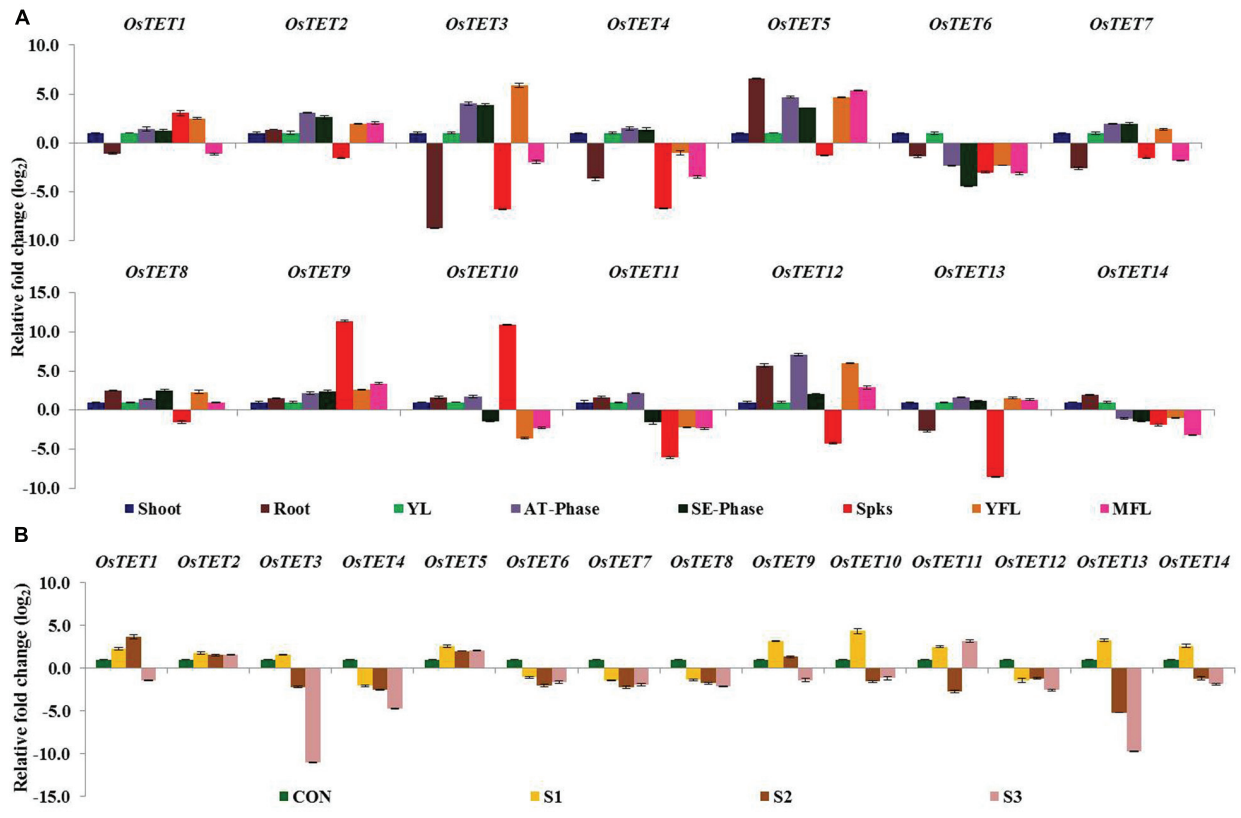


FIGURE 3 | Expression profiling of rice tetraspanin genes in different tissues of rice. (A) Quantitative PCR analysis of transcript levels of rice tetraspanins in tissues namely shoot, root, young leaf (YL), active tillering phase (AT-Phase), stem elongation phase (SE-Phase), spikelets (Spks), young flag leaf (YFL), and mature flag leaf (MFL). For root tissue normalized fold change ($\log_2$ scale) was calculated relative to that in shoot tissue of 7-day-old seedlings. For tissues obtained from field grown plants fold change ($\log_2$ scale) was calculated relative to that in young leaf. **(B)** Quantitative PCR analysis of transcript levels of rice tetraspanins during progression of senescence in flag leaf of field-grown rice plants. CON: fully expanded MFL; 100% chlorophyll, S1: 80–90% chlorophyll; S2: 60–80% chlorophyll; S3: 40–60% chlorophyll. Normalized fold change ($\log_2$ scale) was calculated relative to that in MFL. For normalization *eEF-1α* was used as internal control. Three biological replicates and two technical replicates were included in the study. Error bars represent standard error (SE) of three independent biological replicates.

levels in spikelet tissue. Flag leaves harvested at both young and mature stages were used to investigate the expression pattern of *OsTETs*. It was observed that six *OsTETs* (*OsTET1, 3, 5, 8, 9,* and *12*) were highly expressive in young flag leaves, of which only *OsTET5, 9,* and *12* maintained their higher levels even in the MFL (**Figure 3A**).

Several tetraspanin genes in plants have been annotated as either senescence-associated genes (SAGs) or SAG-like, therefore it was reasonable to investigate the expression pattern of rice tetraspanins during flag leaf senescence. Three stages of senescence of fully expanded mature flag leaves of rice were included: early senescence (S1: total chlorophyll content was 80–90% of that in unsenesced flag leaf), mid senescence (S2: total chlorophyll content was 60–80%), and late senescence (S3: total chlorophyll content was 40–60%). Nearly 50% of rice tetraspanin genes, that included *OsTET1*, *5*, *9*, *10*, *11*, *13,* and *14* exhibited high expression at S1 stage when compared to their expression in mature flag leaves (**Figure 3B**). With progression of senescence (stages S2 and S3), expression of several *OsTET* genes declined. However, *OsTET5* exhibited high expression levels ($\geq$2-fold) at all the three stages of flag leaf senescence (**Figure 3B**). All these observations advocate involvement of tetraspanins in regulating development, including flag leaf senescence, in rice.

Gene Expression Profiling of Tetraspanins under Different Abiotic Stresses, Nutrient Deprivation and Exogenous Treatment of Hormones

To assess the role of tetraspanin genes in abiotic stresses, we initially performed a survey of publicly available gene expression atlas of rice seedlings exposed to different abiotic stresses[14]. Several of *OsTET* genes were found to be differentially expressed in seedlings challenged with abiotic stress conditions. However, as the expression data was available only for single duration of stress, therefore we designed experiments to determine the kinetics of *OsTET* gene expression. On exposure to supra-optimal temperature *OsTET1*, *2*, *4,* and *5* were substantially up regulated. The increase in their levels started as early as 30 min after heat stress and continued till 4 h of stress. Beyond this time-point, excluding *OsTET4*, expression of other genes either attained levels comparable to control conditions or declined (**Figure 4** and Supplementary Figure S9). On the other hand several *OsTET* genes (*OsTET6*, *12, 13,* and *14*) were significantly down regulated during heat stress. On exposure of seedlings to high levels of salt, *OsTET2*, *3*, *4*, *12*, *13,* and *14* were highly induced. However, the expression levels of three *OsTETs* (*OsTET5*, *6,* and *9*) declined considerably in high saline conditions.

On exposure of rice seedlings to low temperature conditions, *OsTET3*, *12,* and *13* consistently exhibited high accumulation of their corresponding transcript, with the highest induction shown by *OsTET13* at 48 h (**Figure 4** and Supplementary Figure S9). Although levels of *OsTET6* transcript were low in cold stressed

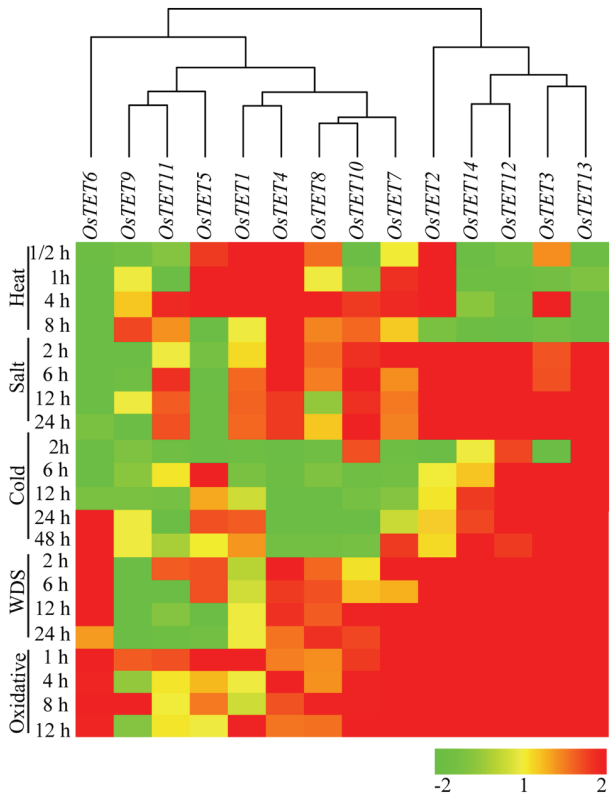


FIGURE 4 | Heat map representing expression profile of rice tetraspanin genes in rice seedlings exposed to different abiotic stresses. Seven-day-old rice seedlings were exposed to different abiotic stresses such as heat stress at 42°C; salinity stress with 200 mM NaCl; water deficit stress (WDS) imposed by 15% PEG; cold stress at 4°C; oxidative stress with 10 mM H_2O_2 for different time durations as indicated on the left (duration in h). Expression levels of tetraspanin genes were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in unstressed seedlings. For normalization *eEF-1α* was used as internal control. Three biological replicates and two technical replicates were included in the study. Hierarchical clustering analysis of relative fold change was performed to prepare a dendrogram and a heat map using Hierarchical Clustering Explorer v3.5 software.

seedlings at earlier time points, there was significant up regulation at later time points of 24 and 48 h. Water-deficit conditions were imposed by transferring the rice seedlings to 15% PEG solution and the expression levels of *OsTET* genes were examined during stress. It was found that eight *OsTET* genes were upregulated as *OsTET2*, *3*, *4, 6*, *7*, *12*, *13,* and *14* exhibited more than twofold change (on a log_2 scale) in expression levels as compared to that in control conditions (**Figure 4** and Supplementary Figure S9). Oxidative stress imposed by H_2O_2 treatment resulted in up regulation of eight genes (*OsTET2, 3, 6, 7, 10, 12, 13,* and *14*), of which five (*OsTET2, 3, 12, 13,* and *14*) were also induced by salt treatment (**Figure 4** and Supplementary Figure S9). Noticeably these seven (*OsTET2, 3, 6, 7, 12, 13,* and *14*) out of eight genes exhibited consistent up regulation in their transcript accumulation at all time-points of oxidative stress tested in this study.

[14]https://genevestigator.com/gv/

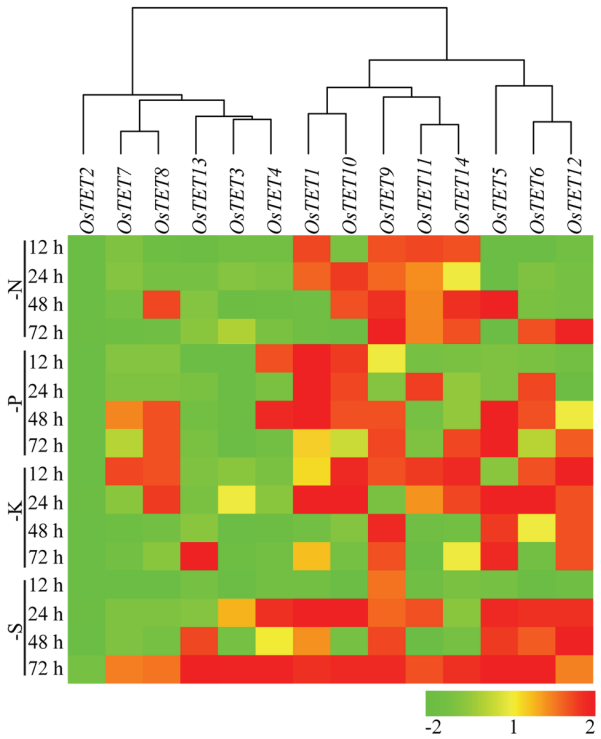


FIGURE 5 | Heat map representing expression profile of rice tetraspanin genes in rice seedlings exposed to nutrient deprivation. Seven-day-old rice seedlings were grown in different nutrient deprivation media such as nitrogen deprivation (N), phosphorous deprivation (P), potassium deprivation (K), sulfur deprivation (S) for different time durations as indicated on the left (in h). Expression levels of tetraspanin genes were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in unstressed seedlings. For normalization *eEF-1α* was used as internal control. Three biological replicates and two technical replicates were included in the study. Hierarchical clustering analysis of relative fold change was performed to prepare a dendrogram and a heat map using Hierarchical Clustering Explorer v3.5 software.

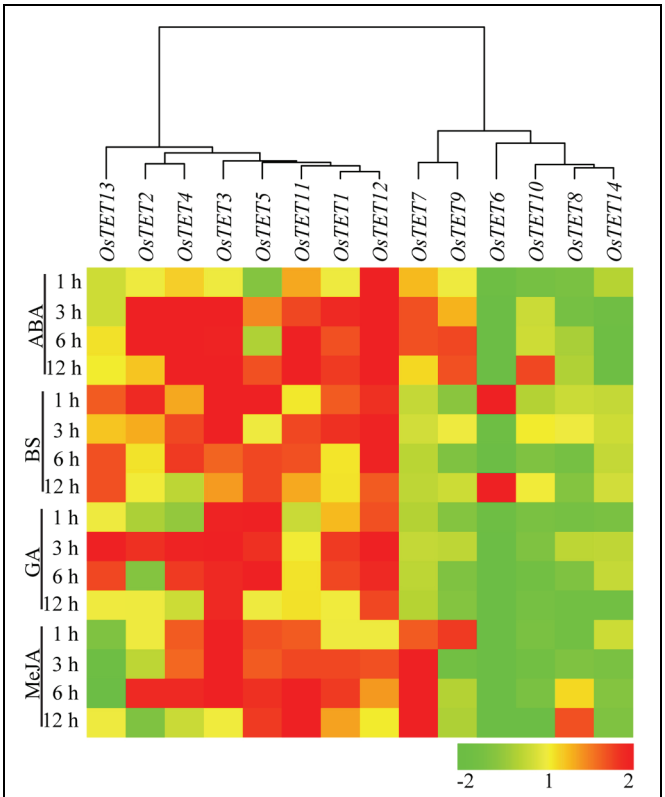


FIGURE 6 | Heat map representing expression profile of rice tetraspanin genes in rice seedlings exposed to different hormones. Seven-day-old seedlings were exposed to exogenous hormones namely abscisic acid (ABA), brassinosteroids (BS), gibberellic acid (GA), and methyl jasmonate (MeJA) for different time durations (in h) as indicated on the left. Expression levels of tetraspanin genes were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in untreated seedlings. For normalization *eEF-1α* was used as internal control. Three biological replicates and two technical replicates were included in the study. Hierarchical clustering analysis of relative fold change was performed to prepare a dendrogram and a heat map using Hierarchical Clustering Explorer v3.5 software.

Nutrient deprivation conditions were imposed by depleting growth medium with different essential elements that are normally required for optimal growth and development of rice (Qiu et al., 2009; Hur et al., 2012; Ma et al., 2012). Unlike the expression kinetics of *OsTET* genes under abiotic stress, very few genes were induced under nutrient deprivation conditions. While *OsTET1* was slightly induced in early time-points of nitrogen deprivation, *OsTET5* showed up regulation at later time-points only. *OsTET2* was significantly down regulated in all the nutrient deprivation conditions tested in this study. *OsTET1*, *OsTET5,* and *6* exhibited up regulation in sulfur-deprived seedlings (**Figure 5** and Supplementary Figure S10). Phytohormones are known to regulate developmental processes in plants and therefore it was worthwhile to examine whether exogenous application of these hormones affects the expression levels of *OsTET* genes in rice. Among 14 *OsTET* genes, seven *OsTET* genes (*OsTET2, 3, 4, 5, 7, 11*, and *12*) showed significant increase in their expression levels on exogenous application of plant hormones (**Figure 6** and Supplementary Figure S11). Out of 7 upregulated genes, *OsTET3* was noticeably induced by all the four hormone treatments. On the other hand *OsTET6* exhibited decline in expression when seedlings were treated with ABA, GA and MeJA (**Figure 6** and Supplementary Figure S11). Based on these observations it was concluded that while several *OsTET* genes were responsive to abiotic stress conditions, very few showed alteration in expression under nutrient deprivation or by hormone treatments.

With an aim to characterize the overlaps in the expression pattern of members of *OsTET* gene family, we constructed Venn diagrams. Only the genes that exhibited consistent change in expression (twofold or more on log_2 scale) at several time-points tested in our time kinetics study (**Figures 3** and **4**) were included in this analysis. We compared the differentially expressing *OsTET* genes in eleven different tissues (shoot, root, young leaf, active tillering phase, stem elongation phase, spikelets, YFL, MFL, S1, S2, and S3 stages of flag leaf senescence) versus those in five abiotic stresses (heat, cold, salinity, water-deficit, and oxidative stress) tested in this study. It was found that three (*OsTET8,*

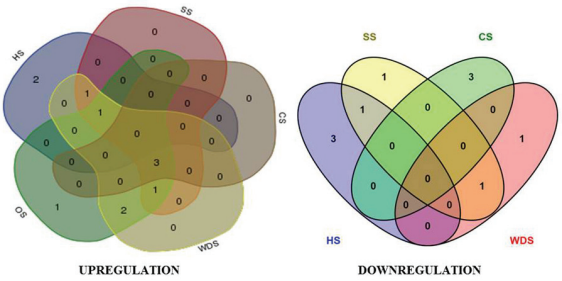


FIGURE 7 | Overlap in expression pattern of rice tetraspanin genes in various abiotic stresses. Venn diagram representing overlap of *OsTET* genes exhibiting significant change (≥2-fold change in $\log_2$ scale) in expression among different abiotic stresses. Both upregulated and downregulated genes are shown. Five abiotic stresses: heat stress (HS), salinity stress (SS), cold stress (CS), water deficit stress (WDS), and oxidative stress (OS) were included in this study.

9, and *11*) and two (*OsTET3* and *OsTET7*) *OsTET* genes were specifically induced and repressed in tissues, respectively (Supplementary Figure S12). A similar number of TET genes were either up regulated (*OsTET4, 6*, and *7*) or down regulated (*OsTET5* and *OsTET9*) in all the abiotic stress conditions. Eight rice tetraspanin genes were found to be co-regulated in tissue as well as during abiotic stress (Supplementary Figure S12). Among five abiotic stress treatments only two (*OsTET1, 5*) and three *OsTET* (*OsTET12-14*) genes were specifically up regulated and down regulated during heat stress, respectively (Supplementary Figure S12). Interestingly the expression levels of three *OsTET* genes (*OsTET3, 12,* and *13*) were commonly induced by four abiotic stress treatments (SS, CS, WDS, and OS; **Figure 7**). During cold stress three *OsTET* genes (*OsTET4, 8,* and *10*) were specifically repressed followed by one each in salinity stress (*OsTET5*) and water-deficit stress (*OsTET11*). It is evidently clear that very few *OsTET* gene members are specifically regulated in either tissues or stress-treated seedlings. Nevertheless, several members of tetraspanin family in rice have evolved to function redundantly in development as well as during stress, pointing toward the biological significance of these proteins in plants.

In Silico Analysis of Rice Tetraspanin Promoters for Putative *cis*-acting Elements

In the present study it is evidently clear that several of *OsTET* genes are spatio-temporally regulated and it is therefore pertinent to identify *cis*-acting regulatory elements within the promoter regions of these genes and correlate with their expression/function. The nucleotide sequence 1 kb upstream of translation initiation site was extracted and scanned for sequences of *cis*-elements using New PLACE and PlantCARE databases. The analysis resulted in identification of large number of putative *cis*-elements in the promoters of 15 *OsTET* genes. The data pertaining to six *cis*-acting elements, which are mainly associated with abiotic stress response or tissue specificity or hormone response (ABRE, HSE, MeJA response, LTR, POLLEN1LELAT52, and root motif-containing elements), is presented as Supplementary Figure S13. We found two temperature-responsive motifs in the promoters of *OsTET* genes: HSEs (heat shock elements) which are abundantly present in the promoters of majority of heat shock induced genes (Nover et al., 2001) and LTRs (low temperature response elements) which are associated with induction of cold-regulated genes (Dunn et al., 1998). The promoters of *OsTET1*, 2, *3, 9, 10,* and *12* were found to contain HSE, however, out of these six genes only *OsTET1* and two displayed induction by high temperatures in our expression studies (**Figure 4**). Similarly, though LTRs could be identified in the promoters of eight *OsTET* genes (*OsTET1, 2, 3, 7, 11, 12, 13,* and *14*), only three (*OsTET3, OsTET 12,* and *13*) were found to be responsive to cold stress (**Figure 4**).

ABRE or ABA-responsive elements are known to confer ABA-responsiveness to minimal plant promoters and we found that at least 4 *OsTET* genes (*OsTET4, 7, 9,* and *11*) contained ≥3 ABREs in their promoter regions. Furthermore we found that all these four genes exhibited significant transcript accumulation in response to exogenous application of ABA (**Figure 6**). It is known that multiple ABREs or ABRE in combination with coupling elements (CEs) or DREs (dehydration responsive elements) are involved in triggering induction by ABA (Narusaka et al., 2003; Zhang et al., 2005; Gomez-Porras et al., 2007). ABA is crucial in regulating several physiological, developmental and abiotic stress responses in plants (Yamaguchi-Shinozaki and Shinozaki, 1994; Fujita et al., 2011). Several of the ABA-responsive *OsTET* genes were found to be induced by various abiotic stresses such as salinity, cold, water-deficit, and oxidative stress in the present study (**Figure 4**). It would be interesting to look for other CEs and DREs in the promoter regions of *OsTET* genes, analyze the orientation and combination of these motifs and subsequently correlate with gene expression analyses. Although we found methyl jasmonate (MeJA)-responsive elements in upstream regions of several *OsTET* genes (*OsTET2, 4, 6, 7, 9, 10–12, and 14*) but among these only two genes (*OsTET7* and *11*) were significantly up regulated by application of methyl jasmonate (**Figure 6**).

Two tissue-specific motifs were also identified: root motif that confers specific expression in roots (Elmayan and Tepfer, 1995) and POLLEN1LELAT52 which is responsible for pollen-specific activation of genes (Filichkin et al., 2004). Root-specific elements were found in several *OsTET* genes (*OsTET1-5, 8–12, and 14*), with the maximum copies in *OsTET5* (10 root motifs) and this corroborates with high expression of *OsTET5* in root tissue. Although *OsTET12* promoter contained only one root motif the corresponding gene exhibited increased expression in root tissue when compared with shoot tissue. POLLEN1LELAT52 element was also identified in eleven OsTET gene promoters (*OsTET1-4, 6, 8–12,* and *15*), of which *OsTET1, OsTET9* and *10*, exhibited spikelet-specific expression (**Figure 3A** and Supplementary Figure S13). Boavida et al. (2013) have shown that tetraspanin genes are discretely expressed in male and female gametophytes of *Arabidopsis*. It would be worthwhile to study the expression levels of *OsTET* genes in male and female reproductive parts of rice. All these analyses confirm the presence of multiple

cis-elements in the promoter regions of *OsTET* genes that could explain the complexity in their expression profile in different tissues, under various abiotic stress conditions and by hormone treatments.

Subcellular Localization of OsTET Proteins in Transient Tobacco Assays

Subcellular localization studies are helpful in understanding the biological function as well as predicting molecular interactions of proteins in plants. *In silico* prediction of subcellular localization suggested that majority of OsTET proteins are targeted to plasma membrane, with two exceptions, of *OsTET6* and *OsTET7* genes that were predicted to reside in chloroplast, and endoplasmic reticulum, respectively. Transient expression of genes is a reliable, simple, and rapid approach for determining the subcellular localization of several plant proteins (Kokkirala et al., 2010). We employed model plant system *N. benthamiana* (tobacco) leaves for agroinfiltration of several OsTET-YFP fusion constructs. The PCR-amplified cDNAs corresponding to several *OsTET* genes were fused in-frame to YFP cDNAs under the control of constitutive cauliflower mosaic virus 35S (CaMV35S) promoter. These constructs were mobilized into *Agrobacterium* and were transiently expressed in tobacco leaves. Visualization of infiltrated tobacco leaves showed that all the OsTET proteins tested in this study distinctly accumulated at the periphery of tobacco epidermal cells which overlapped with the plasma-membrane marker protein, PIP2-CFP (**Figure 8B**). In the leaf tissues infiltrated with YFP alone (vector only), fluorescence was observed throughout the cell, i.e., in the nucleus as well as cytoplasm (**Figure 8A**). Based on the co-localization studies it is concluded that several OsTET proteins are specifically targeted to plasma membrane, which is also in agreement with prediction studies as well as previous studies on *Arabidopsis* TET proteins.

DISCUSSION

Tetraspanin genes encode for proteins that are components of large molecular complexes at the surface of the plasma membrane. These proteins together with their interacting partners form microdomains that modulate signaling cascades, thereby affecting diverse biological processes. With an exception of yeast, TET proteins are present ubiquitously in eukaryotes. With an aim to comprehend their biological roles in plants, we performed characterization of the rice tetraspanin family members.

Rice Genome Encodes for 15 Tetraspanin Proteins

The availability of complete and annotated rice genome sequence was seemingly an appropriate starting point for identification of tetraspanin genes in rice. We devised a stringent strategy based on presence of all the canonical features in plant tetraspanin proteins and recognized 15 true tetraspanin members in rice genome. The *OsTET* loci were unevenly distributed throughout the rice genome as evident by their mapping onto only nine chromosomes. In contrast the 17 *Arabidopsis TET* (*AtTET*) loci reside on all five chromosomes, of which four members were present on chromosomes 1, 2, and 5 each (Boavida et al., 2013). With an exception of *OsTET5/6* that were clustered on chromosome 3, no other clusters of *OsTET* loci were found. Predictions revealed that *OsTET5/6* were present as tandemly duplicated loci, whereas *OsTET2/9* were segmentally duplicated. Gene duplications have acted as the fundamental force for the functional diversification and providing evolutionary novelty in plant genomes. Divergence occurs mainly due to mutations or conversions in coding sequence or regulatory region (Fawcett and Innan, 2011; Wittkopp and Kalay, 2012). We therefore aligned coding sequences and the respective upstream *cis*-regulatory regions of the two duplicated pairs (*OsTET2* vs. *OsTET9* and *OsTET5* vs. *OsTET6*). Interestingly no significant homology was found in the regulatory regions within members of the two pair (data not shown). However, we found 71 and 44% identity in the protein sequences of OsTET2/9 and OsTET5/6 pairs, respectively. Similarly, analysis of *AtTET* genes indicated that *AtTET11/16* residing on chromosome 1 were organized in tandem (analysis based on Du et al., 2013; Jiang et al., 2013). It would be interesting to correlate the level of sequence conservation with the spatio-temporal expression within members of duplicated pairs.

Gene architecture studies showed that majority of the *OsTET* genes contained a single 'phase 0' intron, with a conserved intron/exon junction. Abundance of phase 0 introns in rice tetraspanin gene family is in agreement with earlier findings showing phase 0 introns as the most abundant and phase 2 as the least abundant in tetraspanin genes of several organisms (Fedorov et al., 1992). Due to presence of 10 introns (a feature considered to be present in ancient genes) and its homology with *AtTET10* (considered as one of the founding members of *TET* gene family in *Arabidopsis*), *OsTET14* appears to be the founding member of rice tetraspanins. Detailed phylogenetic analysis of tetraspanin evolutionary origin and organization performed with genomes of metazoans, fungal, animal, and plants suggested that tetraspanins are derived from single or few ancestral gene(s) through sequence divergence (Garcia-Espana et al., 2008). Biasness in position of intron with respect to the protein sequence has been reported (Fedorov et al., 2002) in tetraspanin genes and our analysis also revealed that majority of rice tetraspanin family members had insertion of intron in the region coding for EC2 domain. Our results are in concordance with Garcia-Espana et al. (2009) who studied intron evolution in tetraspanins and found that EC1 and EC2 domains are the hotspots for insertion of new introns.

Rice Tetraspanin Proteins Contain Conserved Domains

The availability of complete protein sequences of *Arabidopsis* and rice tetraspanins enabled us to perform a detailed phylogenetic analysis of OsTET with AtTET proteins. Notably, AtTET10, one of the *Arabidopsis* founding tetraspanin protein clustered with OsTET14. Several AtTET members were clustered as an outgroup because they did not group with any OsTET protein, which indicated that they could be specific to *Arabidopsis*. Similarly, OsTET3, OsTET10-13 formed separate clades from AtTET

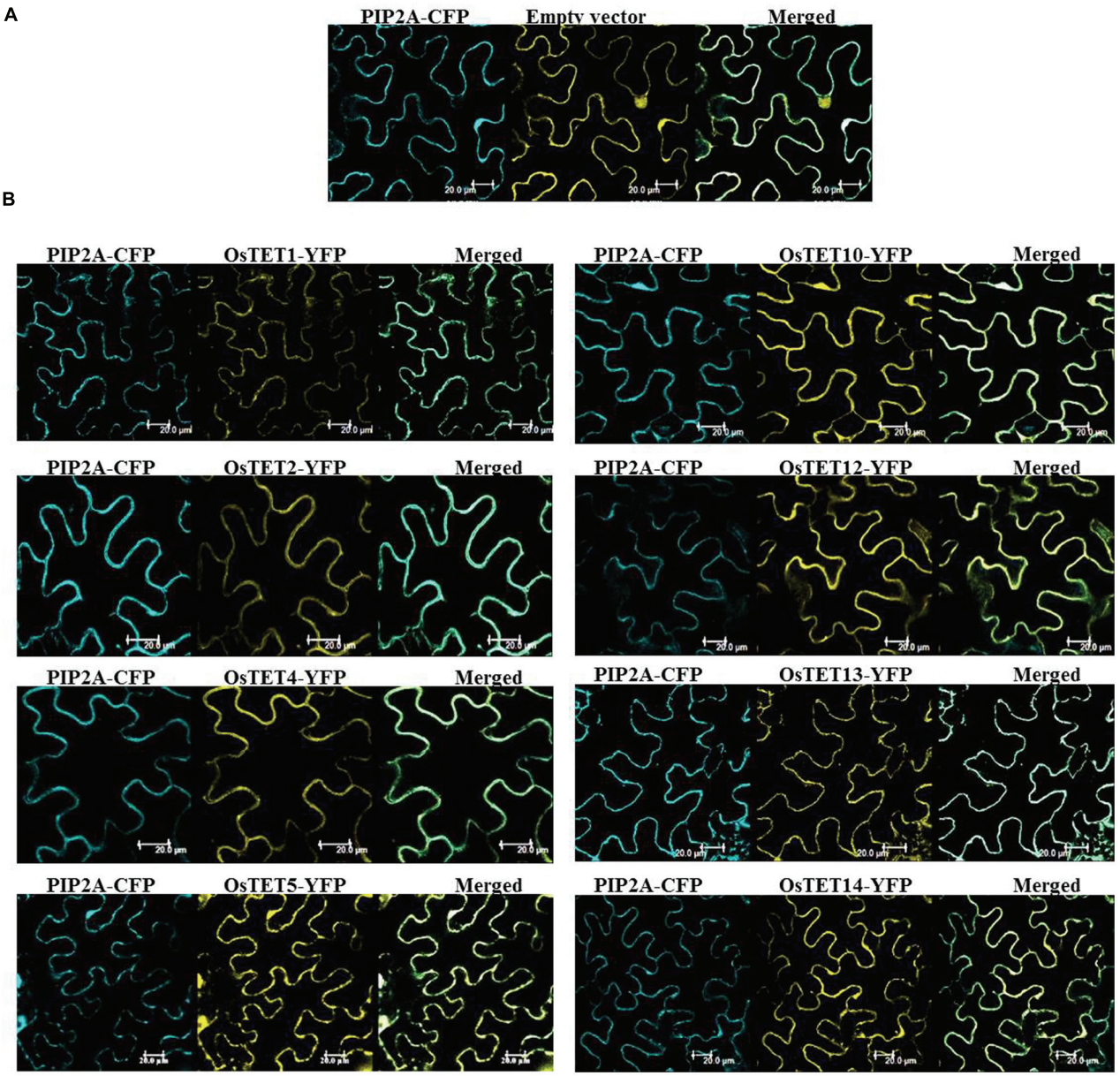


FIGURE 8 | Subcellular localization of rice tetraspanin proteins in tobacco epidermal cells. The OsTETs fused to YFP were transiently expressed in tobacco (*Nicotiana benthamiana*) leaves. Localization in plasma membrane was confirmed by coexpression of PIP2A-CFP, a plasma membrane (PM) marker. **(A)** Colocalization of pGWB542 (empty vector) with PM marker. **(B)** Colocalization of OsTET-YFP proteins with PM marker. The merged fluorescence of marker and OsTET-YFP is shown at the right. Scale bar = 20 μm.

members suggesting that these members are specifically present in rice or possibly only in monocots. To confirm this, we further analyzed the phylogenetic relationship of rice tetraspanins with other plant tetraspanins presented by Boavida et al. (2013) and found that most of the *Sorghum bicolor* TET proteins clustered with OsTET proteins. Two SbiTETs (Sb04g029140, Sb10g009940) clustered with OsTET3, which hinted that OsTET3 is a monocot-specific tetraspanin. However, OsTET10-13 clustered with TET proteins from other dicots such as *Glycine max*, *A. lyrata*, *Vitis vinifera,* and *Populus trichocarpa* and are therefore not rice specific. Phylogenetic analysis of only OsTETs members resulted in 10 different clusters, wherein two segmental duplicates clustered closely in clade IV with 100% bootstrap value. The two tandem duplicated members, OsTET5 and 6, were separately placed in clades I and III, respectively. OsTET3, 5, 11, 12, 14, and 15 were represented as singletons in their respective clades. Rice tetraspanins share an average of 34% identity and 48% similarity in their amino acid sequences. The segmentally duplicated pair of OsTET2 and OSTET9, showed highest level of identity (83.4%) and similarity (90.5%) and were therefore adjacently placed in

clade IV. On the other hand, OsTET3 and 6 pair exhibited 22.2% identity and 30.71% similarity, and were placed in clade IX and III, respectively.

A highly conserved 'CCG' motif within the EC2 domain is the hallmark of TET family members in animals and metazoans (Stipp et al., 2003). Plant TETs differ from animal and metazoan TETs as they contain signature 'GCCK/RP' motif (Wang et al., 2012). In fact 'GCCK/RP' motif is present in tetraspanins of an ancient vascular plant *Selaginella moellendorffii*, which suggests early appearance of this motif in plant ancestry followed by its reorganization (Garcia-Espana and Desalle, 2009). Variants of 'GCCK/RP' motif, such as 'YCCAO' or 'GCCM/NR/P,' are present in two members each of *Arabidopsis* TET family (Boavida et al., 2013). Noticeably a slightly longer conserved motif 'SGCCK/RPP' was present in EC2 domain of rice. Closer examination of 17 AtTET proteins revealed that at least eight of these contained conserved motif identical to that found in rice. The cysteine residues present in conserved motif mediate correct folding and stabilization of EC2. It has also been proposed that the conserved region of EC2 mediates homodimerization of TETs in membrane complexes, whereas variable region is responsible for specific binding with interacting protein partners (Stipp et al., 2003).

Amongst different domains of OsTET proteins, the two TM domains, TM2, and TM3, showed highest identity with an average 43.5 and 42.6%, respectively. The high level of identity between TM domains observed in tetraspanin proteins of rice and other organisms is crucial for the hydrophobic interactions, which are required for assembly of the tetraspanin web. While EC2 domain within OsTET proteins was more conserved (average 37.6% identity) as compared to EC1 (average 22.4% identity), the amino acid sequence of their short cytoplasmic C-terminal tail (average 14.2% identity) was highly variable. It is likely that the variability in C-terminus tail provide additional functional diversification by either directing subcellular localization or by its interaction with different partner proteins. OsTETs also consisted of a single conserved cysteine residue in EC1 as found in other plant tetraspanin members. Little is known about the molecular function of EC1 except a study by Masciopinto et al. (2001), which demonstrated that EC1 is required for optimal expression of EC2 on cell surface in a human tetraspanin protein, CD81. It will be worthwhile to investigate whether a similar role of EC1 exists in plants and whether the conserved cysteine residue is contributing to its function. Several potential palmitoylation and glycosylation sites predicted in majority of OsTET proteins, are likely to specify primary and secondary interactions within tetraspanin web. Based on these findings it is evident that OsTET proteins contain conserved domains and their further analysis will be helpful in linking the structural features of OsTET proteins with specific functions.

Spatio-temporal Changes in Expression Suggest Functional Diversification of Rice Tetraspanin Family Members

Survey of the already available microarray-based expression data in *Arabidopsis* showed that few *AtTET* genes are differentially regulated by abiotic stress treatments in shoot and root tissues (Supplementary Figure S14). Similar analyses of rice microarray datasets indicated tissue- and abiotic stress-specific expression pattern of few *OsTET* genes (Supplementary Figure S15). With an aim to predict the biological function of OsTETs, detailed spatio-temporal expression analysis was carried out. Transcripts of three genes accumulated to higher levels in root tissue whereas transcripts of four genes were specifically present in active tillering phase. At the same time steady state levels of three genes were high in the spikelets. Flag leaf in cereals is the one of the most important leaves, which contributes to at least 50% of photosynthesis required for grain filling and yield (Sylvester-Bradley et al., 1990). Therefore studying the molecular changes that take place during its development and senescence would provide useful insights into mechanisms that control grain production. Six *OsTETs* were detected at higher levels in YFL, of which only four maintained their levels in MFL. The onset of senescence in flag leaves resulted in upregulation of seven *OsTETs*, of which *OsTET5* was conspicuously detected even at later stages of senescence. Except spikelets *OsTET5* and *OsTET12* were expressed at higher levels in all tissues indicating that might play a more generalized role in plant development. Members of segmentally duplicated gene pair, *OsTET2* and *9* co-expressed in several tissues whereas tandemly duplicated genes, *OsTET5* and *6*, exhibited inverse expression profile in most of the tissues. In addition to the sequence alignment that showed OsTET5 and 6 to be structurally diverse, their distribution in tissues indicates that they are functionally diverse as well. Overall *OsTETs* are widely expressed in different tissues of rice at different developmental stages, which may reflect their fundamental requirement in defining plant growth and development.

Perception of signals at the cell surface is one of the important steps in defining abiotic stress responses in plants. We studied the expression pattern of *OsTET* genes during various abiotic stress, nutrient deprivation, and hormone treatments. Among the five abiotic stresses studied *OsTET2* was induced by all stresses, except low temperature conditions. Similarly, *OsTET12* and *13* were upregulated by all, except high temperature stress. While *OsTET1* and *5* were specific to heat stress, *OsTET10* was slightly induced by oxidative stress only. Expression of *OsTET5, 8,* and *12* was specific to roots and it was conceivable that these genes are responsive to salinity or water deficit stress. *OsTET12*, but not *OsTET5* and *8*, was responsive to both the stresses in whole seedlings. As we pooled root and shoot tissue, wherein shoot tissue was predominantly represented, it is likely that stress mediated induction of *OsTET5* and *8* would be observed if their expression is analyzed in root tissue only. It will be interesting to study the expression profile of these *OsTET* genes separately in root and shoot tissue after stress imposition. In contrast to the coexpression of *OsTET2* and *9*, observed in a majority of tissues the transcripts of these genes were inversely regulated in almost all the examined abiotic stresses. On the other hand tandemly duplicated genes *OsTET5* and *6*, were seemingly coexpressed in salinity, oxidative stress, and sulfur deprivation. Future investigations on the factors regulating these genes in different tissues and stress conditions will be beneficial in delineating the mechanisms responsible for their conditional

coexpression. Based on the expression studies it can be concluded that *TET* genes in rice underwent functional diversification with respect to development and abiotic stress conditions.

Several Rice Tetraspanin Proteins Preferentially Associate with Plasma Membrane

To gain insights into the function of rice tetraspanins we performed subcellular localization of several OsTETs using tobacco transient assays. All the eight OsTETs (OsTET1, 2, 4, 5, 10, 12, 13, and 14) tested in this study were localized to the plasma membrane. This is in agreement with *Arabidopsis* TETs and mammalian TETs, which are preferentially associated with plasma membrane. However, few *Arabidopsis* TETs are known to accumulate in cytoplasmic organelles such as endoplasmic reticulum or ER (Boavida et al., 2013). The subcellular targeting prediction of OsTET7 suggested its localization to ER, however, it needs to be validated experimentally. It is possible that OsTET7 is localized in membranes of the ER and play a role in protein trafficking. Localization at cell surface is necessary for organizing tetraspanin microdomains and mediating cell-to-cell interactions. Boavida et al. (2013) demonstrated cell-specific accumulation of TETs in reproductive tissues of *Arabidopsis*, which provided evidence for their crucial role in reproductive development. Similar studies for rice tetraspanins would provide valuable information on their probable biological function.

TET proteins are dynamic in nature with respect to their molecular interactions and biological functions. The importance of TET proteins is highlighted by the functional redundancy among these proteins. Yeast split ubiquitin interaction assays confirmed AtTET proteins interact physically leading to formation of homomers or heteromers (Boavida et al., 2013). The functional redundancy could thus be attributed to multiplicity in TET–TET interactions, wherein association with specific interacting partners in microdomain imparts discrete biological functions to these proteins. Human CD151 is tightly associated with the integrins to mediate integrin-dependent cell adhesion activities (Levy et al., 1998; Berditchevski, 2001; Boucheix and Rubinstein, 2001). Potential association of tetraspanin with other partners such as immunoglobulin superfamily proteins, proteases, signaling enzymes, GPCRs, cadherins, and proteoglycans has been hypothesized (Hemler, 2005). Proteomic approaches for purification of tetraspanin microdomains complexes under different conditions or in different tissues followed by the identification of the components would contribute to the elucidation of molecular and functions of these proteins.

Collectively, the results obtained in the present study provide valuable insights into the genomic organization, phylogenetic relationship, protein structure, and functional significance of rice tetraspanin proteins. Comprehensive expression profiling of OsTETs suggests that they are crucial in regulating plant development and defining plant's response to environmental challenges. Based on our findings it is can be envisaged that these genes are potential candidates for manipulating stress tolerance in plants.

AUTHOR CONTRIBUTIONS

SK-A conceptualized, designed and supervised the project. BM carried out all the experiments and *in silico* analyses. BM and SK-A wrote the manuscript. MA regularly discussed the experiments, analyzed the results, provided useful suggestions during the project and critically revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01088

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Natural variations in expression of regulatory and detoxification related genes under limiting phosphate and arsenate stress in *Arabidopsis thaliana*

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Abiotic stress including nutrient deficiency and heavy metal toxicity severely affects plant growth, development, and productivity. Genetic variations within and in between species are one of the important factors in establishing interactions and responses of plants with the environment. In the recent past, natural variations in *Arabidopsis thaliana* have been used to understand plant development and response toward different stresses at genetic level. Phosphorus deficiency negatively affects plant growth and metabolism and modulates expression of the genes involved in Pi homeostasis. Arsenate, As(V), a chemical analog of Pi, is taken up by the plants via phosphate transport system. Studies suggest that during Pi deficiency, enhanced As(V) uptake leads to increased toxicity in plants. Here, the natural variations in *Arabidopsis* have been utilized to study the As(V) stress response under limiting Pi condition. The primary root length was compared to identify differential response of three *Arabidopsis* accessions (Col-0, Sij-1, and Slavi-1) under limiting Pi and As(V) stress. To study the molecular mechanisms responsible for the differential response, comprehensive expression profiling of the genes involved in uptake, detoxification, and regulatory mechanisms was carried out. Analysis suggests genetic variation-dependent regulatory mechanisms may affect differential response of *Arabidopsis* natural variants toward As(V) stress under limiting Pi condition. Therefore, it is hypothesized that detailed analysis of the natural variations under multiple stress conditions might help in the better understanding of the biological processes involved in stress tolerance and adaptation.

Keywords: ***Arabidopsis*, arsenic, gene expression, natural variations, phosphate, transcription factors**

INTRODUCTION

Diverse spectrum of environmental stresses severely affects plant growth and development and thus reduces productivity and yield. Several studies have reported that the genetic variations within and in between the species play role in establishing interactions and responses of plants with the environment. In recent years, natural variations in different plant species such as *Arabidopsis*, maize, and rice have been used to understand the genetic impact on plant development and

physiology (Alonso-Blanco et al., 2009; Li et al., 2012; Weigel, 2012; Yadav et al., 2013). Apart from the developmental studies, natural variations have also been used to study the effect and response of different accessions under stress conditions (Stein and Waters, 2012). Advanced studies using genetically divergent populations within the species have helped in the elucidation of the genomic variations and their associations with various traits and adaptability. However, among the other plant species *Arabidopsis thaliana* has easily established itself as a tool for the evolutionary and ecological studies due to its number of features (Turck and Coupland, 2013) such as a small genome size and the ease with, which it can be manipulated (Koornneef and Meinke, 2010). In addition, *Arabidopsis* natural variations have been used to elucidate the molecular mechanisms and processes involved in various stresses including salt (Wang et al., 2013), drought (Bouchabke et al., 2008), temperature (Degenkolbe et al., 2012; Barah et al., 2013), and flooding (Vashisht et al., 2011).

Natural and human-induced factors like industrialization; mining, agricultural practices have resulted in the release of detrimental pollutants including toxic heavy metals in the environment. Toxic heavy metals cause drastic changes in the growth, physiology, and metabolism of plants (Finnegan and Chen, 2012). Heavy metals not only hamper plant growth and productivity but also cause severe human health hazards due to the food chain contamination. One such ubiquitous pollutant is arsenic (As), which is widely distributed in the environment. Arsenic occurs in two inorganic forms, arsenite [As(III)] and arsenate [As(V)] of which As(V) can be readily reduced to As(III) after entering into the plant cell. Both these inorganic forms disrupt plant metabolism but through distinct mechanisms (Finnegan and Chen, 2012). As(V) is chemically analogous to inorganic phosphate (Pi) and therefore, is taken up by the plant roots from soil via Pi transport system (Raghothama, 1999; Catarecha et al., 2007; Wu et al., 2011; Castrillo et al., 2013). Inside the plant cell, it replaces PO_4^- from ATP, resulting in the inhibition of ATP synthesis and phosphorylation due to disturbance in the Pi metabolism (Tripathi et al., 2007; Zhao et al., 2010). The other inorganic form, As(III), which is more toxic, is a predominant species under anaerobic conditions, and enters the root via nodulin 26-like intrinsic protein (NIP) aquaporin channels (Meharg and Jardine, 2003; Bienert et al., 2008; Ma et al., 2008). It perturbs protein functioning due to the interaction with -SH group present in many proteins (Tripathi et al., 2007; Finnegan and Chen, 2012). Thus, it is necessary to study the biological processes involved in the uptake, transport, and detoxification of such heavy metals so that effective strategies can be developed for developing plants with tolerance as well as low accumulation in plant parts (Song et al., 2010). In the past, various studies have been initiated to understand the molecular networks and processes involved in As stress response. Recently, utilizing natural variations in *Arabidopsis,* several genes and components involved in As stress tolerance and physiological responses (Chao et al., 2014; Fu et al., 2014; Sánchez-Bermejo et al., 2014) have been identified.

Phosphorous is an essential macronutrient and is critical for the plant growth and development. Phosphorous deficiency negatively affects plant growth and metabolism, and induces the expression of genes involved in inorganic Pi acquisition (Raghothama, 1999; Karthikeyan et al., 2002). In *Arabidopsis*, the high affinity Pi transporters, PHOSPHATE TRANSPORTER 1;1 (PHT1;1) and PHOSPHATE TRANSPORTER 1;4 (PHT1;4), have shown to play important role in As(V) uptake (Shin et al., 2004). Various studies suggest that the Pi starvation responses are under strict transcriptional control through various transcription factors. These transcription factors have been shown to regulate expression of PHTs and thus Pi uptake from the medium. Apart from PHT1;1 and PHT1;4 modulation, As(V) exposure also induces a notable transposon burst in the plants, which is restricted by WRKY6, thus emphasizing the importance of regulatory genes in Pi homeostasis under As(V) stress (Castrillo et al., 2013).

The general detoxification mechanism for As comprises reduced As uptake, extrusion out of the cells or sequestration of As-PC (phytochelatins) complexes inside the vacuole (Shukla et al., 2013; Shri et al., 2014). In recent years, studies have utilized *Arabidopsis* natural variations to understand the differential effect of Pi starvation on the accessions (Narang et al., 2000; Chevalier et al., 2003; Reymond et al., 2006) or their response toward As stress (Chao et al., 2014; Fu et al., 2014; Sánchez-Bermejo et al., 2014). In addition, studies suggest that Pi starvation during As exposure plays important role in its uptake and stress response (Remy et al., 2012). However, no study have been carried out to understand As(V) stress under Pi starvation using these natural variations. In the present study, the natural variations in *Arabidopsis* have been utilized to study growth response toward Pi availability and As(V) uptake at the molecular level. Study suggests differential response of selected *Arabidopsis* accessions (Col-0, Sij-1, and Slavi-1) in terms of root length under different Pi and As(V) concentrations. To get an insight into the extent of biodiversity and the identification of underlying plausible mechanisms in providing differential stress response in *Arabidopsis* natural variants, expression profiling of the genes involved in Pi/As uptake, detoxification mechanism as well as regulatory factors have been carried out. Analysis suggests differential expression of a set of genes, which might lead to differential response in *Arabidopsis* natural variations.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The seeds of three *Arabidopsis thaliana* accessions Columbia-0 (Col-0, CS60000), Sijak-1 (Sij-1, CS76379), and Slavianka-1 (Slavi-1, CS76419) were obtained from *Arabidopsis* Biological Resource Center (https://abrc.osu.edu/). The seeds were surface sterilized with 70% ethanol (v/v) for 1 min, 4% NaOCl (v/v) for 4 min, followed by washing with distilled water and were placed on 0.5X Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.5 % (w/v) sucrose and 0.8 % (w/v) agar. For the Pi sufficient condition, 1.25 mM KH_2PO_4 was added to the medium, while for the Pi deficient condition, 15 μM KH_2PO_4 was used. For the Pi deficient media, KH_2PO_4 was replaced with KCl. For As(V) treatment, 50 μM Na_2HAsO_4 (Stock Solution 50 mM; Na_2HAsO_4, ICN, USA) was added in the

media and pH was adjusted to 5.5 using 0.1 M KOH or HCl. After stratification at 4°C for 2 days, plates were transferred to a growth chamber set at 16/8 h light-dark cycle, 250 μmol m^{-2} s^{-1} light intensity and 22°C temperature.

Evans Blue Staining

Evans blue is a non-permeating dye and is used to determine the dead cells in plant samples. *Arabidopsis* accessions were grown on Pi sufficient, Pi sufficient + As(V), Pi deficient and Pi deficient + As(V) medium for 10 days. Seedlings were incubated in Evans blue solution [0.15% (w/v) Evans blue in water] for 1 h (Dubey et al., 2014). After washing for 10 min with water, seedlings were observed for the tissue damage under Stereoscope Zoom binocular microscope (Leica S8AP0, Germany).

RNA Isolation, cDNA Preparation, and Gene Expression Analysis

Total RNA from 10 days old seedlings was isolated using Spectrum Plant Total RNA Kit (Sigma–Aldrich, USA) as per manufacturer's instructions. RNA was quantified using NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and the quality was assessed using 1.2% agarose gel electrophoresis. Genomic DNA contamination was removed using RNase-free-DNase-I (Fermentas, Life Sciences, ON, Canada). Approximately, 1 μg total RNA was reverse transcribed using RevertAid First Strand cDNA synthesis kit (Fermentas, Life Sciences, ON, Canada) according to the manufacturer's instructions. Quantitative real time-PCR was performed using SYBR Green Supermix (ABI Biosystems, USA) in an ABI 7500 instrument (ABI Biosystems, USA). Tubulin gene was used as an internal control to estimate the relative transcript level of the genes analyzed. The list of oligonucleotides used in the study is provided in the Supplementary Table S1. The PCR was performed in a final volume of 10 μL containing 1 μL of each of the forward and reverse primers (5 pM), 5 μL of the SYBR green master mix and 1 μL of cDNA (1:10 dilution), and 2 μL of nuclease free water. All PCR reactions were performed in the triplicate. The PCR conditions were 50°C for 2 min, 95°C for 2 min for initial denaturation followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. Data was analyzed using comparative Ct ($2^{-\Delta\Delta ct}$) method (Schmittgen and Livak, 2008).

Nucleotide Sequence Analysis

To analyze variations in nucleotide and deduced amino acid sequences in ACR2 gene (At5g03455) in different accessions, full-length cDNA was amplified using oligonucleotides spanning complete open reading frame (Supplementary Table S1). Amplicons were sequenced from both the ends using 96 capillary automated sequencing systems (ABI 3730 DNA Analyzer, UK).

Arsenic Estimation

Ten days old seedlings of *Arabidopsis* accessions; Col-0, Sij-1, and Slavi-1 were thoroughly washed with distilled water and air dried for 4–5 days followed by overnight drying in an oven at 80°C. Dried samples (∼100 mg) were digested in HNO_3 and H_2O_2 (3:1 v/v) at 80°C on a hot plate till the samples were converted into fine residue. The residue from digested samples was dissolved in 5 ml distilled water and filtered using filter paper (Whatman™ 125 mm). The samples were used for the total As determination through an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500 USA) as per the standard protocol (Dubey et al., 2014). The standard reference metals (E-Merck, Germany) were used for the calibration and quality assurance for each analytical batch.

Statistical Analysis

Each experiment was carried out under completely randomized design with three replicates repeated at least thrice. The data were analyzed by Student's unpaired *t*-test, and the treatment mean values were compared at $P \leq 0.05$–0.001.

RESULTS AND DISCUSSION

Natural Variation in Response to Pi and As(V) Exposure

Phosphate deficiency causes a profound effect on the root morphology of the plants (Chevalier et al., 2003). Various studies have reported natural variations among *Arabidopsis* accessions in response to Pi deficiency (Narang et al., 2000; Chevalier et al., 2003; Reymond et al., 2006). Recently, natural variations for As(V) stress response has also been shown in different plant species including *Arabidopsis* (Chao et al., 2014; Fu et al., 2014; Sánchez-Bermejo et al., 2014) and rice (Rai et al., 2010; Wu et al., 2011; Sharma et al., 2015). However, no information is available for the response of these natural variants toward combined stress of Pi deficiency and As(V). Therefore, to understand the effect of genetic variations on plant growth and development, different accessions were analyzed for their response toward As(V) stress. Among different accessions Col-0, Sij-1, and Slavi-1 were identified as tolerant, moderate and sensitive, respectively, toward As(V) stress. In order to understand the interaction between As(V) and Pi uptake, these accessions were grown on optimum Pi concentration (Control; Pi-sufficient; 1.25 mM) and low Pi concentration (Pi-deficient; 15 μM). No significant change in the primary root length was observed in the three ecotypes under limiting Pi as compared to optimum Pi condition (**Figures 1A,B**).

Since it is well known that As(V) is an analog of Pi and it competes with the Pi uptake system (Raghothama, 1999; Catarecha et al., 2007; Wu et al., 2011), the root morphology was compared in the three accessions under Pi-sufficient medium supplemented with As(V) (50 μM). No significant effect on the root length was observed in the three accessions grown on Pi sufficient medium under As(V) stress (**Figures 1A,B**). However, continuous growth of *Arabidopsis* natural variants on the medium containing As(V) and Pi limiting condition caused a significant decrease in the root length. This suggests differential interaction and competition between Pi and As(V) uptake in these selected *Arabidopsis* natural variants (**Figures 1A,B**). Differential reduction in the root length was observed in the three accessions. Lesser reduction in the root length was observed in Col-0 (60%) as compared to Sij-1 and Slavi-1(>80%),

(**Figures 1A,B**) suggesting better tolerance and adaptation of Col-0 under combined stress of limiting Pi and As(V). Hence, it was inferred that Col-0 is more tolerant toward Pi deficient + As(V) stress in comparison to other natural variants.

Natural Variation in As(V) Induced Tissue Damage

As(V) induces the production of reactive oxygen species (ROS) inside the plant cell leading to lipid peroxidation and damage to proteins and nucleic acids (Halliwell, 2006; Møller et al., 2007). The induced oxidative stress during As(V) stress is combated by antioxidant enzymes in concert with non-enzymatic antioxidants such as Non-Protein Thiols (NPTs) and glutathione (GSH; Shri et al., 2009; Rai et al., 2010). It has been reported that the increased production of ROS results into cellular damage and ultimately cell death (Breusegem and Dat, 2006). Therefore, As(V) induced cellular damage was analyzed in the accessions using Evans Blue staining. It was observed that in comparison to control (sufficient Pi) condition, under Pi sufficient + As(V) and Pi deficient + As(V) conditions, Sij-1 and Slavi-1 were severely affected by As(V) stress as compared to Col-0 (**Figure 2A**). The cellular damage was indicated by deeply stained tissues in these natural variants. This is in corroboration with the phenotypic analysis, which showed that the impact of As(V) stress was more pronounced in Sij-1 and Slavi-1 as compared to Col-0 (**Figures 1A,B**). Also, our study is in corroboration with the studies carried out on the rice root, where differential staining pattern was observed for different heavy metals, which is an indicator of varying degree of toxicity caused by heavy metals upon their accumulation (Dubey et al., 2014). Thus, Evans blue staining suggests that the toxicity and cell death due to As(V) exposure varies substantially among *Arabidopsis* accessions. Therefore, it can be inferred that *Arabidopsis* natural variants possess distinct molecular mechanisms for the acquisition of Pi as well as to sustain growth and development under As(V) stress conditions.

Natural Variation in Arsenic Accumulation in *Arabidopsis*

Investigation of the molecular function of the genes responsible for As uptake, accumulation and metabolism is prerequisite to minimize As stress in plants (Kumar et al., 2015). Studies have reported that rice accessions differ in their As accumulation potential and are categorized as high and low As accumulating germplasms (Rai et al., 2010; Sharma et al., 2015). Therefore, to investigate the effect of Pi deficiency on As(V) uptake and accumulation, Col-0, Sij-1, and Slavi-1 were grown on Pi sufficient and deficient medium supplemented with As(V). It was observed that As accumulation potential was equivalent in all the accessions under both Pi sufficient and deficient conditions. However, under Pi deficient + As(V) condition, accessions accumulate many folds higher As compared to Pi sufficient + As(V) condition due to enhanced As uptake

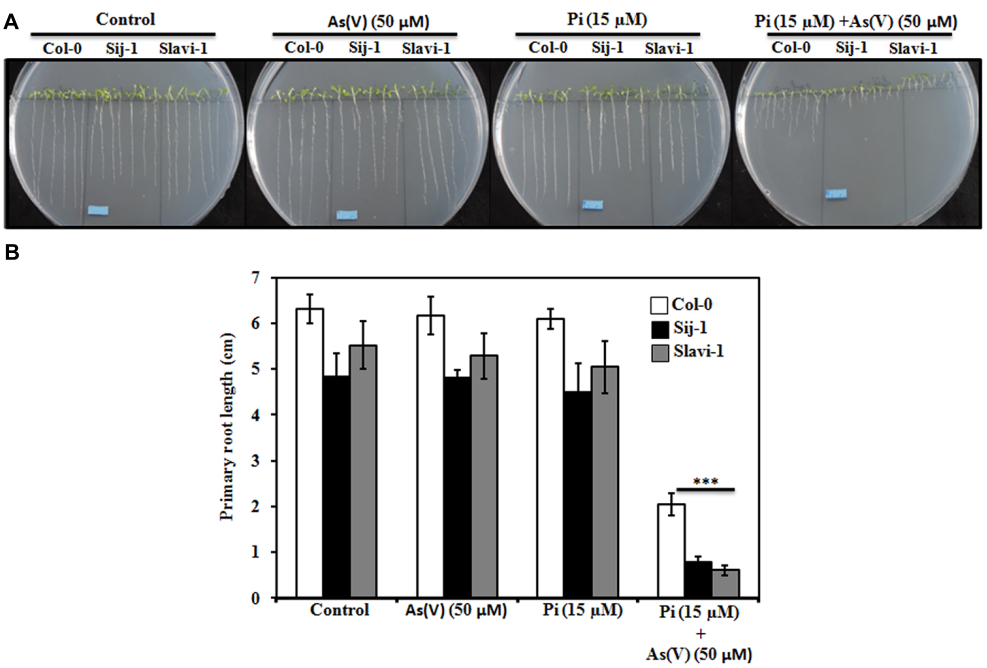


FIGURE 1 | Effect of limiting phosphate (Pi) and As(V) on the growth of *Arabidopsis* accessions. Seeds were grown on 0.5X MS medium and the root length was evaluated after 10 days of growth. **(A)** Phenotype of *Arabidopsis* accessions on 0.5X Murashige and Skoog (MS) media (control), As(V) (50 μM), Pi (15 μM; Pi limitation), and Pi (15 μM) supplemented with As(V) (50 μM). Scale bar = 1cm. **(B)** Primary Root length of the three accessions grown under different treatments. Data are mean ± SD calculated from three biological replicates per treatment. Experiments repeated thrice with similar results. *** indicate values that differ significantly from control at $P < 0.001$, according to student's unpaired *t*-test.

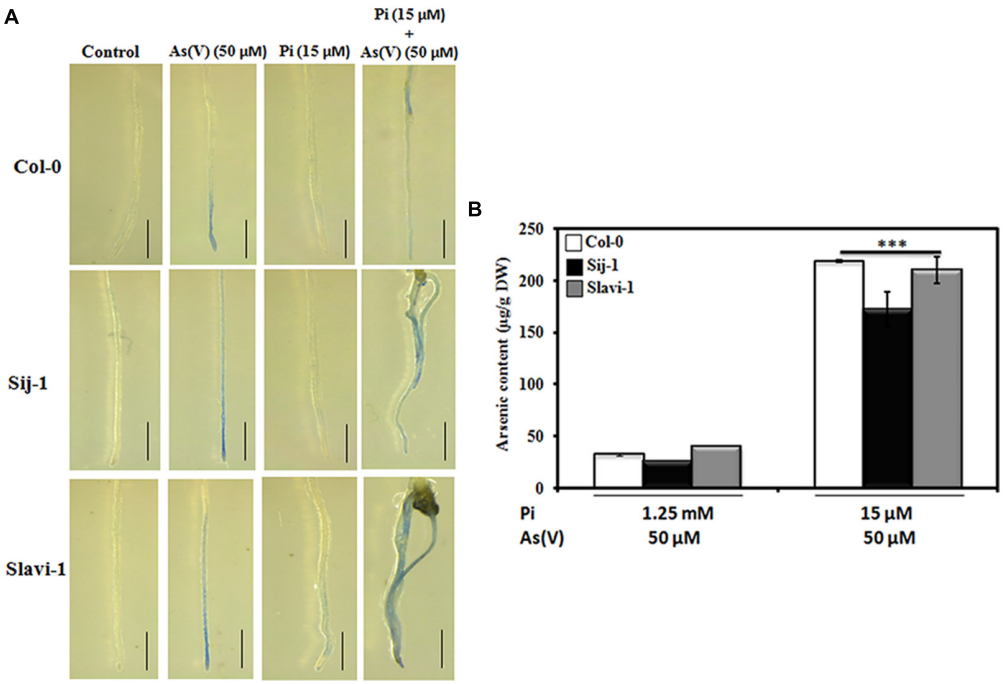


FIGURE 2 | Cell viability/death during limiting Pi and As(V) stress using Evan's blue staining and metal accumulation by *Arabidopsis* accessions. **(A)** Seeds of *Arabidopsis* accessions were grown for 10 days on medium containing sufficient Pi (1.25 mM) (control), As(V) (50 μM), deficient Pi (15 μM) and deficient Pi (15 μM) supplemented with As(V) (50 μM). The seedlings were incubated with Evan's blue and observed under Stereoscope Zoom binocular microscope (Leica S8AP0, Germany) Scale bar = 1 mm. **(B)** The seeds were grown on Pi sufficient medium containing As(V) (50 μM) and deficient Pi medium (15 μM) supplemented with As(V) (50 μM) for 10 days and arsenic content was estimated using ICP-MS. Data are mean ± SD calculated from three biological replicates per treatment.*** indicate values that differ significantly from control at $P < 0.001$, according to student's unpaired *t*-test.

(**Figure 2B**). Similar observation with enhanced As uptake under Pi deficiency has been observed in *Arabidopsis* and rice (Catarecha et al., 2007; Dubey et al., 2014). Thus, it can be inferred that with decreasing Pi concentration As accumulation increases, however, accumulation potential does not differ significantly between different natural variants in these accessions. This suggests that differential As(V) response in these natural variants might be dependent on detoxification mechanism involving transport, accumulation or regulatory mechanisms.

Differential Expression of Genes Related to Transport System

Phosphate enters into the plant cell via a set of Pi transporters both under Pi sufficient and deficient conditions (Shin et al., 2004). PHT1;1 and PHT1;4 are high affinity Pi transporters, which expresses in the root epidermis and root hair and have maximum transcript abundance among all the nine putative members of phosphate transporter (PHT) family (Shin et al., 2004; Lapis-Gaza et al., 2014). In *Arabidopsis*, among different members of Pi transporters, PHT1;1 and PHT1;4 are known to play an important role in As(V) uptake (Shin et al., 2004). To understand the genetic variations with respect to expression of PHTs, the transcript abundance of these two Pi transporters was analyzed in the seedlings of Col-0, Sij-1, and Slavi-1. Differential expression pattern of PHT1;1 was observed in all the accessions with enhanced expression of PHT1;1 under Pi deficiency in comparison to control (Pi sufficient condition; **Figure 3**). Under limiting Pi, PHT1;1 expression was highest in Col-0 followed by Sij-1 and Slavi-1 (**Figure 3**). The expression pattern of PHT1;1 in the presence of As(V) decreased significantly as compared to limiting Pi condition in the natural variants. This decrease in the expression of PHT1;1 was similar in Col-0 and Sij-1 (∼50%); however, lesser change in expression was observed in Slavi-1 (∼25%). Similar to PHT1;1, the expression of PHT1;4 was higher under Pi deficiency in comparison to control in all the *Arabidopsis* natural variants. Conversely, the expression of PHT1;4 was lower in Col-0 in comparison to other accessions in both Pi deficient and Pi deficient + As(V) conditions (**Figure 3**). This suggests differential regulation of these PHTs during limiting Pi and in the presence of As(V). As it is already known that regulatory factors responsible for the expression of both these PHTs during Pi deficient conditions are different (Devaiah et al., 2007a,b; Nilsson et al., 2007; Duan et al., 2008; Karthikeyan et al., 2009; Castrillo et al., 2013; Wang et al., 2014), it seems that differential regulation by transcription factors might be responsible for the natural variation in *Arabidopsis* in response to nutrient deficiency and As(V) stress.

Further, in *Arabidopsis*, Phosphate transporter 1 (PHO1) has been identified to be involved in the loading of inorganic Pi into the xylem of roots (Hamburger et al., 2002). PHO1 mainly

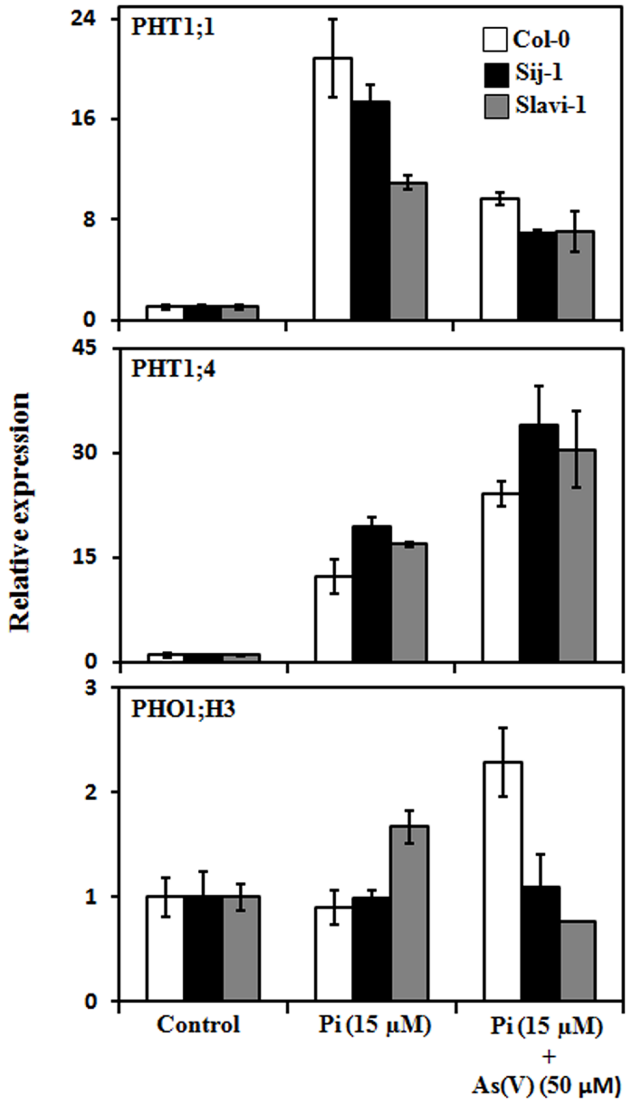


FIGURE 3 | Expression analysis of genes involved in Pi and As(V) uptake under sufficient Pi (1.25 mM) (control), deficient Pi (15 μM), and deficient Pi (15 μM) supplemented with As(V) (50 μM) using real time PCR. Error bars represent ±SD from three technical replicates of 30 seedlings each.

expresses in the root cells and helps in the maintenance of Pi homeostasis (Hamburger et al., 2002). PHO1 homolog, PHO1;H3 is up regulated under Zn-deficiency and negatively regulates Pi loading into the xylem of root tissues (Kisko et al., 2014). Since PHO1;H3 is known to have a major role in crosstalk between heavy metal Zn and Pi under Zn deficiency conditions (Khan et al., 2014), it was analyzed that whether the expression pattern of PHO1;H3 was also modulated under Pi-deficient and Pi-deficient + As(V) stress in *Arabidopsis* natural variants. Differential expression of PHO1;H3 was observed in Col-0 and Slavi-1, whereas no modulation in the expression was observed in Sij-1 under both Pi-deficient and Pi-deficient + As(V) conditions (**Figure 3**). Under Pi deficiency, increased PHO1;H3 expression in Slavi-1 suggests less Pi mobilization in comparison to Col-0 and Sij-1 as it is a negative regulator of Pi movement via xylem. The decreased expression of PHO1;H3 in Slavi-1 under Pi deficient + As(V) condition results into increased Pi movement, which might lead to enhanced As(V) translocation causing hampered growth and sensitivity. Under Pi deficiency, no modulation in the PHO1;H3 expression was observed in Col-0 suggesting better Pi mobilization toward the shoot via xylem, whereas, enhanced expression of PHO1;H3 was observed in Col-0 under Pi deficient + As(V) condition (**Figure 3**) further suggests restricted Pi movement and so as that of As(V). Intriguingly, in spite of severe growth retardation, no significant modulation in the expression of PHO1;H3 was observed in Sij-1. Therefore, the differential expression pattern of PHTs and PHO1;H3 under Pi deficient and Pi deficient + As(V) conditions suggests that although these transporters are involved in Pi acquisition from soil and its homeostasis, their expression may be differentially regulated in *Arabidopsis* accessions.

Transcription Factors and Natural Variations under Low Pi and As(V)

Previous reports have accounted the role of transcription factors in regulating Pi starvation responses in plants (Wu et al., 2003; Misson et al., 2005). Therefore, we analyzed the expression pattern of WRKY and other Pi and As(V) responsive transcription factors in *Arabidopsis* accessions under Pi deficiency and Pi deficient + As(V) conditions.

Modulation in the Expression of WRKY Transcription Factors

WRKY6 is an As(V)-responsive transcription factor, which negatively regulates PHT1;1 expression (Castrillo et al., 2013) and has role in defense against other stresses (Robatzek and Somssich, 2002). Expression analysis suggests differential expression pattern of WRKY6 in the *Arabidopsis* accessions; with a maximum expression in Col-0 (fivefold) followed by Sij-1 (>3-fold) and Slavi-1 (>2-fold), under Pi-deficient + As(V) condition in comparison to Pi deficient condition (**Figure 4A**). As WRKY6 is known to repress the expression of PHT1;1, in the presence of As(V) as described by Castrillo et al. (2013), this can be easily correlated with the increased WRKY6 expression (**Figure 4A**) and decreased PHT1;1 expression (**Figure 3**) in all the accessions under Pi deficient + As(V) stress. Earlier report by Catarecha et al. (2007) has shown that expression of PHT1;1 is significantly down regulated under As(V) stress and this is in correlation with our data that activation of WRKY6 in response to As(V) stress might reduce the expression of PHT1;1 and thus As(V) toxicity (**Figures 3** and **4A**).

WRKY45 is known to be induced under Pi starvation, and recently, WRKY45 was reported to positively regulate the expression of PHT1;1 but not PHT1;4 (Wang et al., 2014). Interestingly, the expression of WRKY45 was not significantly induced upon Pi deficiency in all the three accessions (**Figure 4A**), but the expression of PHT1;1 significantly increased in Col-0 (∼20-fold), Sij-1 (∼15-fold) and Slavi-1 (∼10-fold), (**Figure 3**). Our analysis showed that WRKY45 expression is also induced under Pi-deficient + As(V) condition (**Figure 4A**)

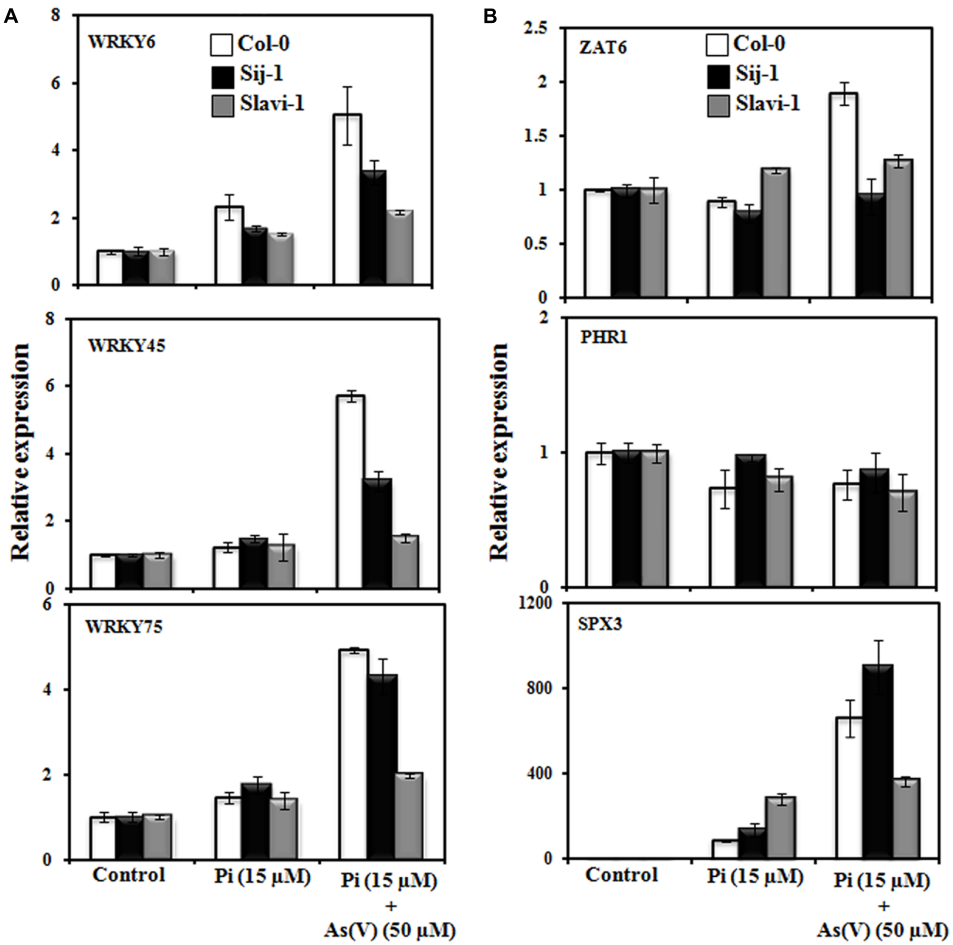


FIGURE 4 | Transcription factors mediated response of *Arabidopsis* accessions under Pi deficient and As(V) stress. (A) Expression analysis of members of WRKY transcription factor gene family and **(B)** Other transcription factors known to regulate Pi homeostasis. Error bars represent ±SD from three technical replicates of 30 seedlings each.

but this induction did not modulate the expression of PHT1;1 (**Figure 3**). This suggests a different regulatory mechanism of PHT1;1 regulation under Pi-deficient + As(V) condition, which might be mediated by transcription factors other than WRKY45.

In a recent study, WRKY75 was demonstrated to regulate Pi homeostasis by controlling both the Pi acquisition and modulation in the root architecture (Devaiah et al., 2007a). It was observed that the expression of WRKY75 is induced under Pi-deficiency (**Figure 4A**) which might have resulted into strong induction of PHT1;1 expression under the same condition (**Figure 3**). Enhanced expression of WRKY75 was also observed in all the accessions in Pi deficient + As(V) stress with a maximum increase in Col-0 (fivefold) followed by Sij-1 (4.4-fold) and Slavi-1 (twofold) as compared to that in Pi deficient condition (**Figure 4A**). But this induction in WRKY75 expression had no effect on modulating the expression of PHT1;1 under Pi deficient + As(V) stress condition, similar to that of WRKY45 (**Figures 3** and **4A**). Altogether, the analysis suggests that WRKY45 and WRKY75 positively regulate the expression of PHT1;1, which might lead to increased expression of PHT1;1 under Pi-deficiency, in spite of the similar metal accumulation potential of the accessions (**Figure 2B**). The increased expression in Col-0 in comparison to Sij-1 and Slavi-1 suggested better Pi acquisition potential of Col-0 from the external medium.

The expression analysis under Pi-deficient + As(V) stress revealed that while the expression of WRKY45 and WRKY75 was induced in the presence of As(V), the expression of PHT1;1 decreased (**Figures 3** and **4A**). This down regulation in PHT1;1 expression might be due to WRKY6, which plays important role in rescue mechanism of plants to avoid As toxicity (Castrillo et al., 2013). In spite of the down regulation of PHT1;1 under Pi-deficient + As(V) condition and similar metal accumulation potential as that of Col-0, the accessions Sij-1 and Slavi-1 were severely affected by As(V) toxicity, the probable reason could be the functional redundancy of PHT transporters. Expression of most of these transporters are regulated by different transcription factors, therefore, although PHT1;1 is sufficiently repressed putatively by WRKY6; these accessions still suffered severe toxicity.

Modulation in Expression of ZAT6 Transcription Factor

Expression analysis has revealed that significant enhanced expression of ZAT6 is observed only in Col-0 under Pi deficient + As(V) condition as compared to that in Pi deficient condition (**Figure 4B**). Previous study by Devaiah et al. (2007b) suggested that ZAT6 negatively regulates the expression of PHT1;1, therefore, we analyzed the correlation between ZAT6 expression and PHT1;1 repression under Pi deficient and Pi deficient + As(V) condition in *Arabidopsis* natural variants (**Figure 4B**). Results demonstrated that ZAT6 expression is not significantly modulated, however; PHT1;1 expression was differentially enhanced under Pi-deficient condition as compared to control (**Figures 3** and **4B**). It was observed that PHT1;1 expression decreased in all the accessions under Pi deficient + As(V) condition (**Figure 3**) but no significant change in ZAT6 expression was observed under same condition (**Figure 4B**) except in Col-0. Differential modulation in ZAT6 expression in natural variants and specific enhancement in Col-0 might be regulating PHT1;1 as well as metal stress response. However, the exact role of ZAT6 in As(V) stress response needs to be functionally validated.

Modulation in Expression of PHR1 Transcription Factor

Studies on phr1 mutants and overexpressing lines emphasized that PHOSPHATE STARVATION RESPONSE 1 (PHR1) is a central regulator of Pi starvation responses (Nilsson et al., 2007; Bustos et al., 2010). It play important role in plant development under different stress conditions (Rubio et al., 2001; Rouached et al., 2011; Nilsson et al., 2012) and also participates in long distance Pi signaling in plants (Bari et al., 2006; Lin and Chiou, 2008). It regulates Pi homeostasis by binding to P1BS motifs present in the promoter region of the genes, which are regulated (Rubio et al., 2001; Franco-Zorrilla et al., 2004; Stefanovic et al., 2007). Thus, we analyzed its expression under Pi deficiency and Pi deficient + As(V) condition. Result suggests that expression of PHR1 did not significantly modulate in response to Pi deficiency and Pi deficiency + As(V) condition in any of the accessions, which was in accord with the other studies (**Figure 4B**), (Rubio et al., 2001). It seems that post-translational modifications of PHR1 as demonstrated earlier (Miura et al., 2005) might be responsible for the differential Pi and As(V) response in *Arabidopsis* natural variants.

Modulation in the Expression of SPX Transcription Factor

It has been reported that Pi deficiency induces the expression of AtSPX3 (Shi et al., 2014) which plays important role in restoring Pi balance following Pi starvation (Duan et al., 2008). Therefore, the expression pattern of *AtSPX3* was investigated in natural variations in response to different treatments including Pi sufficient, Pi deficient and Pi deficient + As(V) stress conditions. In response to Pi deficiency, enhanced expression of AtSPX3 was observed in Slavi-1 (300-fold) and Sij-1 (>100-fold) in comparison to Col-0 (∼100-fold) as compared to control condition (**Figure 4B**). Previous studies suggest that AtSPX3 is induced by PHR1 and exerts negative feedback control over AtSPX1, which is involved in the regulation of various genes encoding regulatory enzymes such as RNS1 (Pi remobilization), PAP2 (anthocyanin biosynthesis), IPS1; At4 (Pi allocation), PHT1;4 and PHT1;5 (Pi transport) to circumvent Pi induced hypersensitive responses during prolonged Pi starvation (Duan et al., 2008). Our result also demonstrated that in Col-0 the expression of PHT1;4 was lower as compared to Sij-1 and Slavi-1 (**Figure 3**) which reflect that these accessions might require more Pi through PHT1;4 under Pi deficient condition. The expression of AtSPX3 was also evaluated under Pi-deficient + As(V) stress. Interestingly, significantly enhanced expression was observed in Sij-1 (>900-fold), Col-0 (>600-fold) and Slavi-1 (>300-fold) as compared to Pi deficient condition. The observed expression pattern during Pi deficiency was altered with the supplementation of As(V) and least expression was observed in Slavi-1 whilst Sij-1 showed highest expression (**Figure 4B**). Similar expression pattern was observed for PHT1;4 under Pi-deficient + As(V) stress as compared to Pi deficient condition (**Figure 3**) suggesting that though SPX3 exerts a negative regulation over PHT1;4 under Pi starvation, its repression is altered in the presence of As(V) which might result in the enhanced expression of PHT1;4 in Sij-1 and Slavi-1 causing increased toxicity in these accessions in comparison to Col-0.

Natural Variation in the Expression of Genes Involved in Detoxification System

In order to combat As stress, plant should possess an efficient detoxification system (Tripathi et al., 2012; Kumar et al., 2013a,b). It is well documented that As(V) after entering into the plant cell via high affinity Pi transporters is converted to As(III) by arsenate reductase, which is another inorganic and more toxic form of As (Dhankher et al., 2006; Chao et al., 2014; Sánchez-Bermejo et al., 2014). Recently, natural variation in As(V) tolerance identified a quantitative trait locus encoding arsenate reductase (ACR2; Sánchez-Bermejo et al., 2014). In a different study, GWA mapping identified the same locus involved in controlling variation in As accumulation in plants termed as High Arsenic Content 1 (HAC1), which is an arsenate reductase required to reduce As(V) to As(III) (Chao et al., 2014). However, in our study, modulation in the expression of ACR2 was not observed in the three accessions (Supplementary Figure S1). In addition, the nucleotide sequencing of ACR2 in the three accessions was carried out, which showed no difference in the nucleotide sequence and thus in the protein encoded by ACR2 in Col-0, Sij-1, and Slavi-1 (Supplementary Figure S2). As(V) tolerance is usually linked with the slow As(V) uptake (Meharg and Macnair, 1991, 1992), and increase As accumulation (Catarecha et al., 2007; Castrillo et al., 2013). As a detoxification mechanism, As(V) is reduced to As(III), which is subsequently sequestered inside the vacuole as phytochelatins (PCs)-metal complex through tonoplast localized AtABCC1 and AtABCC2 transporters (Briat, 2010; Song et al., 2010).

In order to have an insight into the modulation in the expression of these transporters and the genetic variations occurring in response to As stress and Pi deficiency, the

expression pattern was analyzed in all the three selected accessions grown under different experimental conditions. Significant up-regulation of the genes encoding AtABCC1 and AtABCC2 were observed in Sij-1 and Slavi-1 in comparison to Col-0 under Pi deficient + As(V) stress as compared to Pi sufficient condition (**Figure 5A**). This suggests that Sij-1 and Slavi-1 may accumulate more As, resulting increased sensitivity toward As stress. However, metal accumulation in Col-0, Sij-1, and Slavi-1 under Pi sufficient + As(V) stress demonstrated no significant variation in As accumulation in all the accessions. Increased level of As accumulation was observed in Col-0 (∼sevenfold) in comparison to Slavi-1 and Sij-1 (∼fivefold) under Pi deficient + As(V) as compared to Pi sufficient + As(V) stress (**Figure 2B**). Therefore, the analysis suggested that As accumulation increased under Pi starvation in three accessions at varying level. Among the three accessions, increased tolerance of Col-0 under Pi deficient + As(V) stress and also enhanced As accumulation suggests the presence of a different mechanism of detoxification conferring tolerance in Col-0 as compared to other accessions.

To understand better tolerance and adaptability of Col-0 with respect to other accessions, expression pattern of the three members of Lambda class glutathione S-transferse (GST) gene family was analyzed in natural variants exposed to different growth conditions. GSTs are a superfamily of enzymes that have a role in detoxification of xenobiotics (Dixon et al., 2002; Theodoulou et al., 2003). Recently, the role of rice Lambda class of GSTs was explored in heavy metal stress tolerance (Kumar et al., 2013a,b). The Lambda class of AtGSTs comprises of three members and out of the these members, differential expression pattern of only one member (AtGSTL1) was observed in all the accessions under Pi deficient + As(V) stress. Interestingly, a most remarkable increase in the expression of AtGSTL1 was observed in Col-0 (>80-fold) followed by Sij-1 (>10-fold), and Slavi-1 (>5-fold), (**Figure 5B**). This suggests strong detoxification machinery of Col-0 in comparison to Sij-1 and Slavi-1 and one of the possible reasons for providing tolerance to Col-0 against stress conditions in comparison to other natural variants.

Through expression analysis of genes in *Arabidopsis* accessions in response to low Pi and low Pi + As(V), we propose a model, which depicts the putative sequence of events occurring under these conditions (**Figure 6**). In low Pi condition, WRKY45, ZAT6, and WRKY75 positively induces the expression of PHT1;1 to acquire Pi from the medium. The expression of PHT1;4 is regulated by PHR1, which is a central regulator of Pi starvation response. During Pi

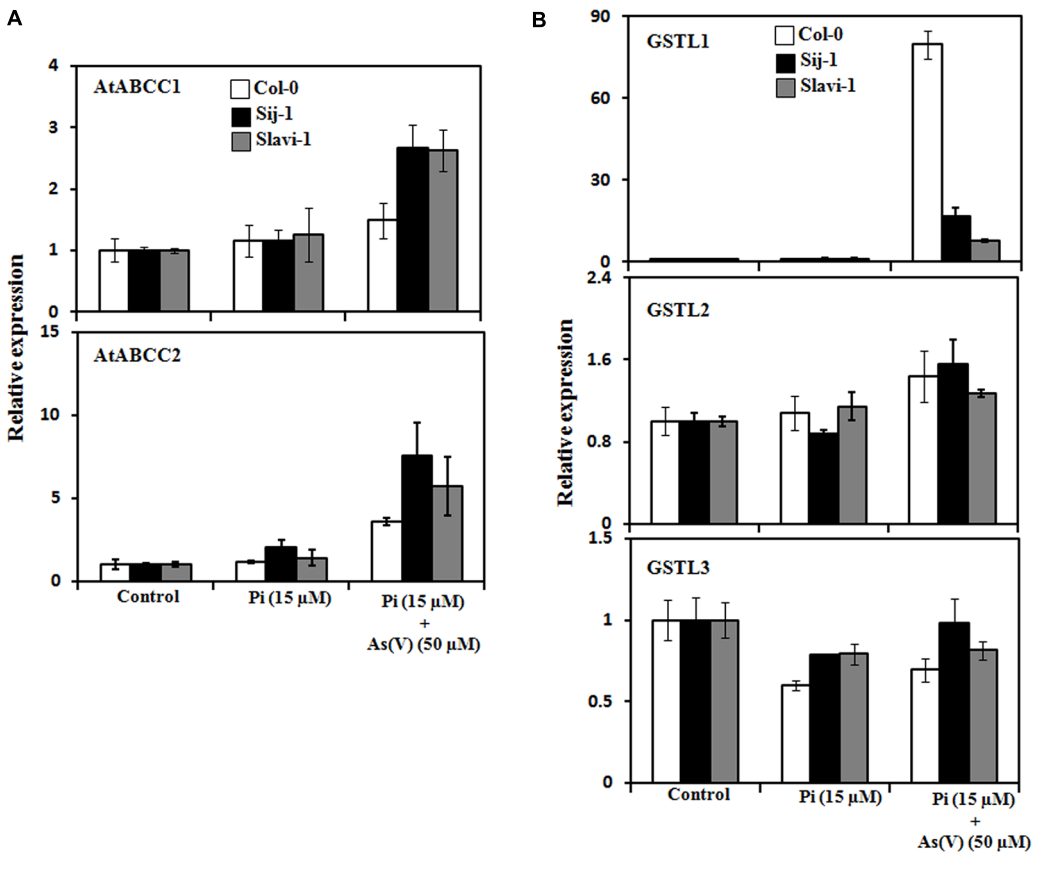


FIGURE 5 | Modulation in the expression of genes involved in detoxification mechanism in *Arabidopsis* accessions under limiting Pi and As(V) stress. **(A)** qRT-PCR analysis of transporters involved in sequestration of PC-As(III) complexes inside the vacuole. **(B)** qRT-PCR of AtGSTL members involved in As detoxification. Error bars represent ±SD from three technical replicates of 30 seedlings each.

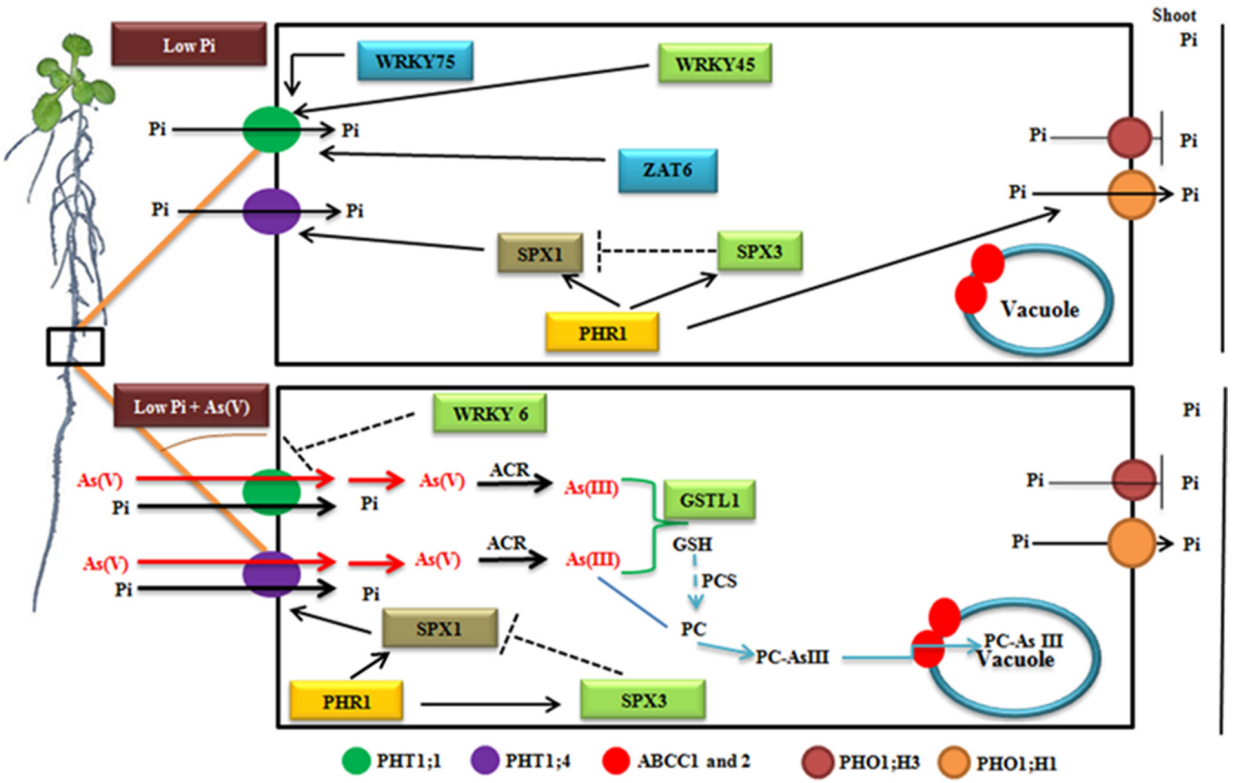


FIGURE 6 | Schematic model representing the events occurring inside the plant under low Pi and low Pi + As(V) growth conditions. Solid lines indicate positive regulation whereas negative regulation is indicated via flattened lines.

starvation, PHR1 is needed for inducing the expression of SPX1 and SPX3. SPX1 positively modulates the expression of PHT1;4 whereas SPX3 exerts a negative feedback regulation over SPX1, which is prerequisite to avoid hypersensitive response during prolonged Pi starvation. After being taken up by the Pi transporters, Pi is mobilized to shoot via PHR1 induced PHO1;H1 whereas PHO1;H3 negatively regulate Pi movement. Under low Pi + As(V) stress, As(V) and Pi competes to enter inside the plant via PHT transporters. WRKY6, an As(V) responsive transcription factor, negatively regulate the expression of PHT1;1, restricting As(V) and Pi movement. PHR1 strongly induces the expression of SPX1 and SPX3 but the negative regulation of SPX3 over SPX1 is diminished resulting in increased expression of PHT1;4 and As(V) movement inside the plant. Further, As(V) is reduced to As(III) by ACR2, which is further detoxified by GSTL1 or gets sequestered inside the vacuole via ABCC1 and ABCC2 transporters (**Figure 6**).

CONCLUSION

Plants evolve and adapt to plethora of environmental stresses and leads to intraspecific variations. Despite the considerable variation, little is known about the genetic basis of *Arabidopsis* response to nutrient deficiency and heavy metal stress. Our study demonstrate natural variation in *Arabidopsis* under Pi deficiency and Pi deficient + As(V) stress. Result suggest substantial contrast in the accessions (Col-0, Sij-1, and Slavi-1) toward low Pi and As(V) stress. The phenotypic data and the expression profiling of the genes involved in Pi/As(V) uptake, Pi mobilization, As detoxification and the members of different transcription factors gene family was evaluated. Out of the three accessions studied, Col-0 showed least reduction in the primary root length in comparison to other natural variants under Pi deficient + As(V) stress. In spite of the difference in the response to As(V) stress, no significant change in the capacity of metal accumulation was observed in the accessions. Expression analysis suggested a significant differential expression of PHT1;1 and PHT1;4 in three accessions, which might be the possible reason of tolerance of Col-0 toward As(V) stress in comparison to other accessions. In addition, the increased expression of AtGSTL1 and decreased expression of AtABCC1 and AtABCC2 in Col-0 as compared to Sij-1 and Slavi-1 might be responsible for better detoxification system to combat As(V) stress under Pi deficient condition. In addition, modulated expression of regulatory genes such as WRKY6 and SPX3 in different natural variants might be involved in different response of accessions to As(V) stress under Pi deficient condition. Further, the detailed analysis under combined stress conditions utilizing natural variations will help in understanding the biological processes involved in heavy metal uptake, transport and detoxification.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00898

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frontiers in Plant Science

HYPOTHESIS AND THEORY
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Hypothesis: NDL proteins function in stress responses by regulating microtubule organization

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N-MYC DOWNREGULATED-LIKE proteins (NDL), members of the alpha/beta hydrolase superfamily were recently rediscovered as interactors of G-protein signaling in *Arabidopsis thaliana*. Although the precise molecular function of NDL proteins is still elusive, in animals these proteins play protective role in hypoxia and expression is induced by hypoxia and nickel, indicating role in stress. Homology of NDL1 with animal counterpart N-MYC DOWNREGULATED GENE (NDRG) suggests similar functions in animals and plants. It is well established that stress responses leads to the microtubule depolymerization and reorganization which is crucial for stress tolerance. NDRG is a microtubule-associated protein which mediates the microtubule organization in animals by causing acetylation and increases the stability of α-tubulin. As NDL1 is highly homologous to NDRG, involvement of NDL1 in the microtubule organization during plant stress can also be expected. Discovery of interaction of NDL with protein kinesin light chain- related 1, enodomembrane family protein 70, syntaxin-23, tubulin alpha-2 chain, as a part of G protein interactome initiative encourages us to postulate microtubule stabilizing functions for NDL family in plants. Our search for NDL interactors in G protein interactome also predicts the role of NDL proteins in abiotic stress tolerance management. Based on published report in animals and predicted interacting partners for NDL in G protein interactome lead us to hypothesize involvement of NDL in the microtubule organization during abiotic stress management in plants.

Keywords: N-MYC DOWNREGULATED GENE, N-MYC DOWNREGULATED-LIKE, phospholipase D, phosphatidic acid, microtubule assembly, microtubule-associated protein, abiotic stress

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INTRODUCTION

An average estimated yield loss by abiotic stress is more than 50% across the world, caused mainly by salinity, drought and temperatures (Boyer, 1982). Matter of concern is that global population is likely to reach 10 billion by 2050 (almost doubled) (Tilman et al., 2002). So the generation of stress tolerant plants is the need of the hour (Smedema et al., 2000). Salinity is the most destructive and complex stress, affects more than 45 million hectares of irrigated land worldwide, in INDIA about 8.6 million hectare area is affected by salinity (Pathak, 2000).

Right from the beginning of seed germination till crop yield, salt stress affects plant adversely via ionic imbalance leading to toxicity, nutritional disorder, hampering metabolic processes, osmotic stress leading to membrane disorganization, reduction of cell divisionand expansion, and oxidative stress (Hasegawa et al., 2000; Duan et al., 2015; Khare et al., 2015).

Although, the role of lipids in salt stress is not well understood, it has been indicated that expression of several *phospholipase-D* (*PLD*) genes is induced by salt stress (Katagiri et al., 2001; Hong et al., 2010). Hydrolysis product of PLD, phosphatidic acid (PA) is shown to bind and activate mitogen-activated protein kinase 6 (MPK6), which in turn phosphorylates salt overly sensitive 1 (SOS1) transporter *in vitro* (**Figure 1**; Yu et al., 2010). The SOS1 gene encodes a plasma membrane Na+/H+ antiporter, playing protective role in saline environment. These findings have indicated a link between lipid signaling, MAPK cascades, and salt stress tolerance in plants (Morris, 2010). Plant responses to salt stress include osmolyte biosynthesis, water flux control, and transport of ions for re-establishment of homeostasis and microtubule depolymerization and reorganization (Wang and Nick, 2001; Lü et al., 2007; Wang et al., 2007, 2010). Although all of the events are equally important for cell survival, microtubule depolymerization and reorganization are believed to be essential for plant survival under abiotic stress.

NDRG AS A MICROTUBULE-ASSOCIATED PROTEIN (MAP)

Microtubule organization is regulated by MAPs (Dixit and Cyr, 2004; Sedbrook, 2004). In animals, several MAPs have been identified and characterized. Detailed analysis of human N-MYC DOWNREGULATED GENE (*NDRG*) gene family showed that the family comprises of four members (*NDRG1-4*), each sharing 57–60% amino acid sequence similarity (Qu et al., 2002). Among these, only NDRG1 has been reported to be a MAP which participates in the spindle checkpoint in animals (Kim et al., 2004).

Microtubule dynamics is affected by an array of reversible post-translational modifications including acetylation, phosphorylation, and palmitoylation (Piperno et al., 1987; Westermann and Weber, 2003; Zhang et al., 2003). Acetylated tubulin is one of the major characteristics of stabilized microtubule structure and may contribute to regulating microtubule dynamics (Westermann and Weber, 2003; Parrotta et al., 2014). Mammalian NDRG1 knockdown cell line have decreased accumulation of acetylated -tubulin and disrupted spindle fiber formation (**Figure 1**; Kim et al., 2004). Moreover, growing body of evidences also show that NDRG1 recruits on recycling endosomes in the Trans Golgi Network by binding to phosphatidylinositol 4-phosphate and interacts with membrane bound Rab4aGTPase (Kachhap et al., 2007). Kachhap et al. (2007) used a prostate cancer cell line to show that NDRG1 is a novel effector for the small GTPase, Rab4a, and is important in recycling E-cadherin in proliferating cells.

STRUCTURAL SIMILARITIES BETWEEN NDRG1 AND NDL1

In plants, NDL proteins were first reported in sunflower (SF21) as stigma and transmitting tissue cell specific proteins (Kräuter-Canham et al., 1997). Thereafter, studies on SF21

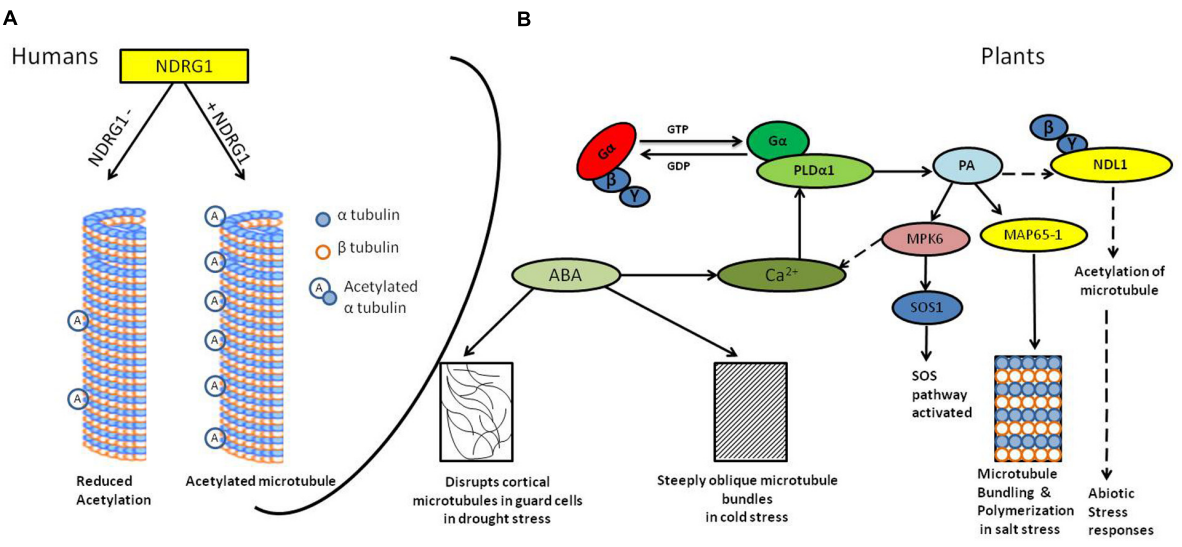


FIGURE 1 | Diagrammaticrepresentation of the signaling in abiotic stress and microtubule responses. (A) N-MYC DOWNREGULATED GENE (NDRG1) knock-down human mammary epithelial cells (hNMECs) shows decrease in tubulin acetylation as compared to cells having wild type levels of NDRG1(Kim et al., 2004). **(B)** Abiotic stress activates G-protein-coupled receptors (GPCRs; Yadav and Tuteja, 2011), *phospholipase-Dα*1 (PLDα1) interacts with activated Gα subunit (Zhao and Wang, 2004); PLD hydrolyzes membrane lipids to generate phosphatidic acid (PA). PA binds to MAP65-1, resulting into microtubule bundling and polymerization which helps in salt tolerance (Zhang et al., 2012) it also activates MPK6, which further phosphorylates SOS1 resulting into activation of SOS pathway (Yu et al., 2010). PA may interact with NDL1, interactor of Gβγ dimer and possible downstream regulator of microtubules. ABA production during cold and drought stress results into steeply oblique and disrupted microtubules, respectively, (Wang and Nick, 2001; Pollock and Pickett-Heaps, 2005). Solid lines depicts confirmed interactions, dotted line depicts hypothesized interactions

proteins identified it as a small gene family with putative role as a signaling molecules in pollen-pistil interaction. Across plant species, *SF21* gene has been reported in dicots (*Lycopersicon esculentum*, *Arabidopsis thaliana*) monocots (*Oryza sativa*) (Lazarescu et al., 2006), gymnosperms as well as in the moss, *physcomitrella patens* (Lazarescu et al., 2010). *Arabidopsis NDL* gene family has three members *NDL1, NDL2,* and *NDL3*. All family members contain NDR domain, an alpha/beta hydrolase fold, a conserved hydrophobic patch of 23 amino acids and a conserved Asp. All these mentioned features strongly suggest that NDL proteins belong to NDR protein family. NDL proteins in *A. thaliana* are novel effectors of G-protein signaling playing important role in root and shoot development (Mudgil et al., 2009, 2013). G-protein core complex relay signal intracellularly with the help of downstream effectors or secondary messengers.

We previously observed that Mouse NDRG1 interacts with *Arabidopsis* AGB1/AGG1 and AGB1/AGG2, suggesting that this interaction is evolutionarily conserved (Mudgil et al., 2009). Human NDRG1 is 93% similar to mouse NDRG1 (Mudgil et al., 2009), so we can postulate similar interaction of human NDRG1 with plant's G protein components. Also, NDL in *Arabidopsis* and NDRG1 of mouse were shown to interact with the C-terminal domain of regulator of G-protein signaling (RGS1), a candidate seven-transmembrane receptor in AGB1/NDL-mediated signaling via yeast two-hybrid (Mudgil et al., 2009).

N-MYC DOWNREGULATED GENE1 functions as a MAP and acetylates microtubules in human. NDRG1 also act as novel effector for the small GTPase. In plants, protein domains search revealed that all α tubulin family subunits contain GTPase domain as the tubulin C terminal domain so NDL might also interact with α tubulin in plants.

MICROTUBULES DYNAMICS-ROLE IN ABIOTIC STRESS TOLERANCE

Microtubules are the polymers of heterodimeric protein αβ-tubulin, which provides shape to cells and maintains tracks for vesicle transport and segregation of chromosome. Microtubule organization is regulated by microtubule-associated proteins (MAPs; Dixit and Cyr, 2004; Sedbrook, 2004). A variety of MAPs have been reported in higher plants. The MAP65 family and some of kinesin family are important in bundling and

TABLE 1 | N-MYC DOWNREGULATED GENE (NDRG1) and N-MYC DOWNREGULATED-LIKE (NDL1) shared interactors which are involved in common pathways/ processes.

	NDRG1 a	NDL1 b	Reference
Cyclin-dependent kinases	Cyclin-dependent kinase 15	Cyclin-dependent kinase – G1 Cyclin-dependent kinase regulatory subunit 2	**a** (Huttlin et al., 2015) **b** (Klopffleisch et al., 2011)
Calcium-dependent phospholipid binding proteins	Annexin A5	Annexin 1	**a** (Havugimana et al., 2012) **b** (Klopffleisch et al., 2011)
Heat shock protein	HSPA4 HSPA5 HSP90AA1	BOBBER 1	**a** (Tu et al., 2007; Ambrosini et al., 2009) **b** (Klopffleisch et al., 2011)
Eukaryotic translation initiation factor	Eukaryotic translation initiation factor 2 Eukaryotic translation initiation factor 3 Eukaryotic translation initiation factor 4H DEAD (Asp-Glu-Ala-Asp) box helicase 1 DEAD (Asp-Glu-Ala-Asp) box helicase 5 DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	Eukaryotic initiation factor 4A-III DEAD-box ATP-dependent RNA helicase 2	**a** (Tu et al., 2007; Kristensen et al., 2012) **b** (Klopffleisch et al., 2011)
Protein phosphatases	Protein phosphatase 2, regulatory subunit B, alpha	protein phosphatase 2A subunit A2	**a** (Tu et al., 2007) **b** (Klopffleisch et al., 2011)
Components of cytoskeleton machinery	ACTG1, Actin, gamma 1 kinesin family member 5B	TUA2, Tubulin alpha-2 chain KINESIN LIGHT CHAIN-RELATED 1	**a** (Tu et al., 2007) **b** (Klopffleisch et al., 2011)
Glutathione reductases	Glutathione reductase HEL-75	HOT5, S-nitrosoglutathione reductase	**a** (Kristensen et al., 2012) **b** (Klopffleisch et al., 2011)
Fatty acid pathway	Fatty acid synthase (FASN) Acyl-CoA synthetase long-chain family member 3 (ACSL-3) Acyl-CoA thioesterase 7 (ACOT7)	KCS9 (3-KETOACYL-COA SYNTHASE 9); acyltransferase/ catalytic/ transferase, transferring acyl groups other than amino-acyl groups Lipoxygenase (LOX2)	**a** (Tu et al., 2007; Kristensen et al., 2012) **b** (Klopffleisch et al., 2011)
Salinity response	ATPase, Na+/K+ transporting, alpha 1 polypeptide	SLT1 (sodium- and lithium-tolerant 1)	**a** (Tu et al., 2007) **b** (Klopffleisch et al., 2011)

polymerization of the microtubules (Smertenko et al., 2004; Van Damme et al., 2004; Mao et al., 2005; Hamada, 2007) *A. thaliana* genome contains nine *MAP65*-related genes with different functions (Hussey et al., 2002).

Calcium is a well-known second messenger which participates in the stress signaling in plants (Knight, 2000; Xiong et al., 2002; Chinnusamy et al., 2005). Cortical microtubules have been suggested to regulate the calcium levels in the cells by regulating the activity of calcium channels (Thion et al., 1996; Himschoot et al., 2015). Treatment of microtubule-destabilizing drug improved the survival and growth of *A. thaliana* seedlings under salt stress while treatments with microtubule-stabilizing drug caused salt stress hypersensitivity (Wang et al., 2007). Moreover, reorientation of microtubules was also observed in maize roots and tobacco BY-2 cells upon short term exposure to salt stress (Blancaflor and Hasenstein, 1995; Dhonukshe et al., 2003). In *A. thaliana*, long term salt stress affected the cortical microtubule organization. *spr1* mutant, [*SPIRAL1*(*SPR1*), a plant-specific MT-localizing protein] has right-handed helical root growth phenotype, salt stress suppresses this phenotype (Shoji et al., 2006). Directional cell expansion (anisotropic growth) is necessary for plant morphogenesis which is achieved by well-organized interphase, cortical microtubule and SPR1 is thought to control anisotropic cell expansion through MT arrangements (Nakajima et al., 2004, 2006). Mutation in critical amino acids of tubulin gene family (mainly located at longitudinal interface of the α and β tubulins), in lateral contact region and in GTPase-activating region in α tubulin (Ishida et al., 2007) disrupts the proper organization and hence functions of microtubules (Hashimoto, 2013). Tubulin mutations affect cortical microtubule arrays in interphase resulting into altered directional growth. Mutation in TUA genes, α tubulin 6 and α tubulin 4 results into right handed helical array of cortical microtubules producing left handed helical growth phenotype, lefty 1 and lefty 2, semi dominant skewing mutants (Thitamadee et al., 2002). These results indicated that the proper organization of microtubule is one of the critical factors for growth and development.

In addition, abscisic acid (ABA), which is produced in response to salt stress, also affects the organization of cortical microtubules (Sakiyama and Shibaoka, 1990; Shibaoka, 1994). In drought stress accumulation of ABA is one of the most pronounced ways to cope up with water deficit stress. ABA leads to stomata closure thereby decrease the water loss and also enhances water uptake by root (Boudsocq and Laurière, 2005). Dehydration triggers plasmolysis of cells and it consequently destroys microtubule (Pollock and Pickett-Heaps, 2005), ABA also disrupts cortical microtubules in guard cells, but not in epidermal cells (Jiang et al., 1996). During cold stress in wheat (Chinese winter wheat) ABA produced steeply oblique microtubule bundles (**Figure 1**; Wang and Nick, 2001).

Phospholipase D is involved in the rearrangement of cortical microtubules (Dhonukshe et al., 2003). In *A. thaliana pldα1* salt-sensitive mutant cortical microtubule showed massive depolymerization patterns (Bargmann et al., 2009; Yu et al., 2010) compared to wild type control. However, upon salt removal from the growth medium organization was recovered in wild-type plants but not in *pldα1* plants indicating involvement of PLDα1 in reorganizing microtubules after depolymerization induced by salt stress (Zhang et al., 2012).

Phosphatidic acid, the end product of PLDα reaction, is a key regulator of microtubule polymerization; exogenous application of PA lead to recovery in salt-disrupted microtubule arrays in *pldα1* mutant (Zhang et al., 2012). PA regulates microtubule bundling and polymerization together with MAP65-1 and their interaction is important for salt tolerance. PA could not bind or bundle microtubules and rescue microtubule disruption caused by salt in the *map65-1* mutant, suggesting that MAP65-1 is necessary for PA-mediated stabilization of microtubules (Zhang et al., 2012). There are two contradictory reports regarding interaction of tubulin and PA. In the first report, a mass spectrometry based approach was used to identify the PA binding proteins which showed that TUA2 is PA binding protein (Testerink et al., 2004). However, in the second report, it was found that neither PLDα1 nor PA species bound to either α- nor β- tubulins. MAP65-1, a microtubule associated protein, was shown to bind to PA but not to other phospholipids like diacylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, or Phosphatidylcholines. These results indicate that PA requires other MAP to interact with microtubules (Zhang et al., 2012), further experimentation to confirm involvement/role of other MAPs is awaited.

Our analysis of existing information on NDL1 interactome shows interaction with Annexin 1 (ANNAT1) which has role in drought stress (Konopka-Postupolska et al., 2009), sodium and lithium-tolerant 1 (SLT1) which is involved in salt stress (Matsumoto et al., 2001) whereas lesion stimulating disease 1(LSD1) regulates cell death trigged by cold stress (Huang et al., 2010), O-Acetylserine (THIOL) Lyase (OAS-TL) Isoform A1 (OASA1) shows increased cadmium tolerance (Domínguez-Solís et al., 2001) and *Arabidopsis* Ribosomal Protein S27 (ARS27A) is involved in genotoxic stress (Revenkova et al., 1999). Also, comparative analysis shows overlap of NDRG1 and NDL1 interactors involved in similar pathways (**Table 1**).

Our proposed hypothesis that NDL might be playing role in stress mediated processes by regulating microtubule organization (**Figure 1**) can be easily tested by checking NDL1 effect on microtubules bundling and polymerization *in vitro* using purified NDL1 and tubulin proteins. Already available *ndl* loss of function mutants can be used for checking and comparing status of acetylated tubulin in the absence and presence of *NDL*. Effects of various stress responses on tubulin pattern in relation to *NDL* levels can be further studied by analyzing GFP-tagged α tubulin (35S: GFP-TUA2) patterns in *NDL* up and downregulated backgrounds.

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REVIEW
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Staying Alive or Going to Die During Terminal Senescence—An Enigma Surrounding Yield Stability

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Breeding programs with the aim to enhance yield productivity under abiotic stress conditions during the reproductive stage of crops is a top priority in the era of climate change. However, the choice of exploring stay-green or senescence phenotypes, which represent an opposing physiological bearing, are explored in cereal breeding programs for enhanced yield stability to a different extent. Thus, the consideration of stay-green or senescence phenotypes is still an ongoing debate and has not been comprehensively addressed. In this review, we provide arguments for designing a target phenotype to mitigate abiotic stresses during pre- and post-anthesis in cereals with a focus on hormonal balances regulating stay-green phenotype versus remobilization. The two major hypothesis for grain yield improvement are (i) the importance of the stay-green trait to elevate grain number under pre-anthesis and anthesis stress and (ii) fine tuning the regulatory and molecular physiological mechanisms to accelerate nutrient remobilization to optimize grain quality and seed weight under post-anthesis stress. We highlight why a cautious balance in the phenotype design is essential. While stay-green phenotypes promise to be ideal for developing stress-tolerant lines during pre-anthesis and fertilization to enhance grain number and yield *per se*, fine-tuning efficient remobilizing behavior during seed filling might optimize grain weight, grain quality and nutrient efficiency. The proposed model provides novel and focused directions for cereal stress breeding programs to ensure better seed-set and efficient grain-filling in cereals under terminal drought and heat stress exposure.

Keywords: carbohydrate remobilization, cereals, drought stress, heat stress, photosynthesis, senescence, stay-green

INTRODUCTION

The World's major cereal crops, i.e., rice (*Oryza sativa*), wheat (*Triticum aestivum*), and maize (*Zea mays*) account for more than 70% of the total production, provides a major share of the world's caloric demand to help sustain global food security (Swaminathan, 2010). Global grain yield productivity of the above cereals more than doubled during the past six decades, but the rate of increase has slowed down considerably due to lack of genetic gain (Swaminathan, 2010; McKersie, 2015). The pressure to further improve germplasm is enhanced with the predicted increase in intensity and

magnitude of drought and heat stress events under changing climate (Sreenivasulu et al., 2007; Dolferus, 2014). In particular over the last decade, erratic rainfall patterns and an enhanced frequency of heat waves have significantly reduced cereal production across the world and affected global agricultural production (Peng et al., 2004; Welch et al., 2010; Lobell and Gourdji, 2012). Using 9 years of satellite data, a +2°C scenario resulted in a 20% reduction in wheat yield losses along the Indo Gangetic Plain of Northern India due to significant acceleration of senescence and reduction in crop growing duration (Lobell and Gourdji, 2012; Lobell et al., 2012). Drought and heat stress occurrence, particularly during the terminal stages of plant growth cycle limits crop productivity world-wide by drastically decreasing grain number and altering seed filling events (Seiler et al., 2011, 2014; De Storme and Geelen, 2014; Raorane et al., 2015; Stratonovitch and Semenov, 2015). In addition, high temperature is also known to affect key grain quality traits, such as reduced head rice recovery, higher chalk percentage and impaired starch accumulation (Shi et al., 2013; Sreenivasulu et al., 2015). Hence, there is a need to breed varieties that can withstand such harsh environmental conditions providing options to extend cultivation to areas that are vulnerable to these stresses for sustaining food security under changing climate.

Drought and heat stress reduces photosynthesis and induces the onset of leaf senescence through the induction of a series of complex metabolic changes. Major physiological reprogramming events occur under severe stress exposure leading to chlorophyll degradation, production of reactive oxygen species (ROS), oxidation of proteins and lipids affecting source strength. ROS generated in chloroplasts and mitochondria during drought and heat may ultimately cause senescence of leaves and affect yield potential (Hui et al., 2012; Khanna-Chopra, 2012; Chen et al., 2013; Semenov et al., 2014). With the onset of senescence chloroplasts are dismantled and stromal enzymes are degraded leading to reduced photosynthesis, while mitochondria remain functional (Sakuraba et al., 2014). Rubisco degradation is required to meet the N demand of sink organs under senescence or accelerated senescence due to abiotic stresses (Gotz et al., 2007; Gomes de Oliveira Dal'Molin et al., 2015). Glutamine synthetase (GS) and Rubisco are the key enzymes for N and C assimilation, with the plastidial form of GS being degraded quicker than the cytosolic GS. A link has been proposed between chloroplasts, cytosol and vacuoles in the form of Rubisco vesicular bodies to be involved in the autophagocytosis of cytosolic and chloroplastic proteins (Prins et al., 2008). However, there is an ongoing debate on the location, the rate of Rubisco degradation, as well as the need for identifying autophagy-related genes involved in regulating Rubisco-containing bodies under natural and stress induced senescence.

Carbon and nitrogen are important resources which are liberated and recycled or remobilized for re-use in other growing parts of the plants during the senescence process. During stress and in naturally senescing leaves, sugars (glucose, fructose) are accumulated (Wingler et al., 2006). But, how sugars are accumulating despite a decline in photosynthesis in senescing leaves is still unknown. There are two possibilities for sugar accumulation under senescence: one is cleavage of starch, which was accumulated during pre-anthesis, and the other possibility is a higher availability of carbon from decreased amino acid synthesis (Jongebloed et al., 2004). In addition, low nitrogen and high light results in senescence of leaves and accumulation of sugars. These findings suggest that the balance between sugar and nitrogen during the sink/source transition of leaves can play a critical role in the induction of leaf senescence (Masclaux-Daubresse et al., 2014). *SAG12*, a senescence-specific gene was induced over 900-fold by glucose (Pourtau et al., 2006). In addition, trehalose 6-phosphate (considered to be a signal for high carbon availability) is required for the onset of leaf senescence associated with high carbon availability in *Arabidopsis* (Wingler et al., 2012). Though accumulation of hexoses in aging leaves is hypothesized to initiate or accelerate senescence, this alone may not trigger senescence, rather, it is the complex network of other metabolites (nitrogen) and environmental factors.

While nitrogen deficiency induces leaf senescence (nitrogen deficiency induced senescence, NDI senescence) and increases N recycling and remobilization, higher or optimal N concentrations promote leaf growth and greenness (Diaz et al., 2008). Therefore, improving N use efficiency (NUE) of crop plants is important under water deficit conditions. It is a well known fact that water deficit enhanced senescence in wheat by accelerating loss of leaf nitrogen and leaf chlorophyll and increasing lipid peroxidation and therefore phloem loading is crucial for efficient N remobilization. If N uptake during grain set is too low, the plant's N demand cannot be met. This situation reduces cytokinin (CK) biosynthesis which induces leaf protein degradation. The amino acids that are released due to protein degradation are exported to the grains via the phloem. Up to 95% of seed proteins consist of amino acids that have been exported to the seed after protein degradation in rosette leaves (Fait et al., 2011). This illustrates that senescence is an important pre-requisite for remobilizing not just nitrogen but also other important nutrients. The chloroplast harbors a major pool of reduced leaf nitrogen. Hence, remobilization of nitrogen essentially includes the degradation of chloroplast proteins to various transportable forms of nitrogen (Hortensteiner and Feller, 2002). Different classes of proteases are activated during senescence to ensure that leaf proteins are degraded into amino acids which are eventually transported to the developing grains (Distelfeld et al., 2014). In conjunction, several findings have shown that sugars in combination with low nitrogen supply can induce senescence (Guiboileau et al., 2013; Avila-Ospina et al., 2015). Other internal cues that can induce senescence under natural or stress environments include hormones, transcription factors, the cellular redox state, and the sink strength which triggers nutrient remobilization.

A stay-green phenotype relies more on current photosynthesis and retains more functional leaf chlorophyll that enables them to synthesize carbohydrates and to provide assimilates during anthesis as well during seed development. The delayed onset or slower rate of senescence has been described to be advantageous in *Sorghum bicolor* (Borrell et al., 2014), *Triticum aestivum* (Spano et al., 2003), *Hordeum vulgare* (Seiler et al., 2014), *Pennisetum glaucum* (Sehgal et al., 2015), *Oryza sativa* (Fu et al., 2011b), and *Zea mays* (Cairns et al., 2012; Almeida et al., 2014), showing

positive correlations between water use efficiency and final yields to combat terminal drought stress (Condon et al., 2004). The impact of a stay-green phenotype and the contribution of its photosynthetically active tissue in the spike under terminal drought is of high relevance in tribe Triticeae, but these mechanisms have not been explored (Tambussi et al., 2005; Raven and Griffiths, 2015). The lemma-derived awns usually grow long as bristle-like structures, possessing a smooth or rough (with minute barbed hooks) surface with stomata, which contribute toward production of photo-assimilates (Toriba and Hirano, 2014). Recent research re-emphasized the importance of spike photosynthesis and assimilate supply in optimizing grain yield under stressful conditions (Maydup et al., 2010; Reynolds et al., 2012; Zhou et al., 2014; Kohl et al., 2015).

On the contrary, in such adverse conditions, senescence triggers the remobilization of carbon and nitrogen from vegetative tissues (leaf canopy and stems) to the grains and accelerates the grain-filling rate. These events alter carbon and nitrogen metabolism and impair translocation mechanisms leading to source-sink disturbances which are regulated through the action of a complex web of hormones and a multilayered regulatory network of genes (Gregersen et al., 2013; Albacete et al., 2014; Thomas and Ougham, 2014). In principle, a combination of faster remobilization and enhanced grain-filling rate could outweigh the loss of reduced photosynthesis and the shortened grain-filling period which ultimately ensures improved grain weight and grain quality in cereals. In this scenario, unfavorably delayed leaf senescence is becoming a concern for poor grain filling in rice and wheat which leaves large amounts of water soluble carbohydrates unused in stems (Yang and Zhang, 2006; Li et al., 2015a; Liu et al., 2015; Zhang et al., 2015). The role of transporters activated during remobilization process plays an important role in fine tuning source-sink dynamics has been discussed elsewhere (Gregersen et al., 2008; Braun et al., 2014; Distelfeld et al., 2014) and therefore we will not discuss this topic in the present review.

From these circumstantial evidence, several intriguing questions arise: (i) Do we need to search for stay-green genotypes that are characterized by perseverance in photosynthesis during the time of seed set and fertilization and thus avoid seed abortion? (ii) Are target genotypes more advantageous when they possess a higher remobilization capacity during the later seed filling phase? and (iii) Is it possible to combine both phenomena to achieve drought and heat tolerant lines for anthesis and post-anthesis stress, and if so, what are the contributing molecular mechanisms?

Summarizing the outcome of the vast number of studies reporting on the complex link between yield potential and phenotypes related to staying alive (stay-green) or choosing cell death of the canopy (senescence) rather increases confusion than providing resolution (Gregersen et al., 2013). This is most likely due to the fact that major abiotic stresses induce imbalances in source (impairment in photosynthesis and/or induction of remobilization) and sink tissues (sterility and inefficiency in seed filling) to a different extent depending on the developmental stage (anthesis or post-anthesis). Also the intensity and duration of a stress and the ability to cope with stress based on the plasticity of a given genotype contribute to yield stability varies between cultivars and species. This review focuses on the progress achieved in addressing the mechanisms related to source-sink imbalance, weighs the key findings of stay-green and remobilization impact and provides future research direction in safeguarding yield stability and improving grain quality under pre- and post-anthesis drought and heat stress in cereals.

TABLE 1 | List of stay-green mutants and QTLs identified in different cereal species.

Species	Role of mutant or QTL	Reference
Oryza sativa L.	*nyc1* mutant cosmetic, chlorophyll *b* reductase	Kusaba et al. (2007)
Oryza sativa L.	*sgr* mutant, cosmetic	Sato et al. (2009)
Oryza sativa L.	SNU-SG1, functional stay-green	You et al. (2007)
Sorghum bicolor	Stg1, 2, 3, 4, Stg A and B QTLs; Stay-green for higher tiller grain yield QL41, cosmetic	Thomas and Howarth (2000)
Triticum aestivum	Functional stay-green QTLs	Spano et al. (2003), Naruoka et al. (2012)
Triticum aestivum	Functional stay-green XN901 QTL	Gong et al. (2005)
Zea mays	Functional stay-green FS854 QTL	Thomas and Howarth (2000), Almeida et al. (2014)

IMPORTANCE OF THE STAY-GREEN TRAIT TO ELEVATE GRAIN NUMBER UNDER DROUGHT AND HEAT STRESS

Stay-green phenotypes have been reported in several crops, like sorghum, barley, wheat, pearl millet, maize and rice, to confer crop yield improvement under terminal drought and heat stress (Spano et al., 2003; Fu et al., 2011b; Chen et al., 2013; Borrell et al., 2014; Seiler et al., 2014; Sehgal et al., 2015). Depending on the dynamics of accelerating or delaying senescence, "functional" stay-green types, characteristically possess active or extended photosynthesis under drought resulting in higher yield stability (Gregersen et al., 2013), mainly due to a reduction in reproductive organ sterility and improvement in seed set (Ji et al., 2010; Dolferus et al., 2011; Sreenivasulu and Schnurbusch, 2012; Dolferus, 2014). There are at least four types of stay-green phenotypes described depending on the dynamics of senescence (Thomas and Howarth, 2000; Thomas and Ougham, 2014). If senescence is initiated late and then proceeds at a normal rate, it is type A. In contrast, type B represents genotypes, in which senescence is initiated on schedule, but the rate of senescence proceeds comparatively slowly. In type C, though chlorophyll is retained indefinitely, senescence proceeds normally beneath the chlorophyll layer, and in type D, leaves remain stay-green with active photosynthesis and senescence onset is very slow (Thomas and Howarth, 2000). Stay-green mutants have been identified in a number of plant species (**Table 1**), and the pathway which distinguishes from "functional stay-green" with type C encompassing "cosmetic" mutants is being unraveled, where plants retain chlorophyll and remain green while their photosynthetic capacity is severely impaired (Sato et al., 2007; Hortensteiner, 2009; Zhou et al.,

2011; Grassl et al., 2012; Luo et al., 2013; Thomas and Ougham, 2014). Stay-green mutants as *nyc1/nol* retained 10 times more chlorophyll in the seeds than the wild type due to lack of chlorophyll *b* reductase, seriously affecting seed development, maturation, viability and finally impairing their germination (Nakajima et al., 2012). Hence, distinguishing such cosmetic phenotypes from true stay-green lines requires physiological and biochemical markers to assess source-sink strength, when stress-tolerant lines are developed in breeding programs. Several attempts have been made to develop stay-green phenotypes in cereals using QTL mapping (**Table 1**) and molecular markers are derived (Almeida et al., 2014; Borrell et al., 2014; Rama Reddy et al., 2014; Sehgal et al., 2015). The significance of stay-green for drought tolerance is also evident from a number of transgenic plants over-accumulating CK relative to abscisic acid (ABA) through the overexpression of isopentenyl transferase (IPT; Werner et al., 2010) or fine-regulating the catabolism of ABA under terminal drought (Seiler et al., 2014). The enhanced drought tolerance of such transgenic plants was the result of an extended photosynthetic capacity and maintenance of green leaf area (Ma, 2008; Peleg et al., 2011; Merewitz et al., 2012; Seiler et al., 2014).

Several stress factors, in particular drought and heat stress, induced seed yield penalties that were conferred by different phenological alterations in the sink tissue, i.e., during the young microspore stage and subsequently during the anthesis and grain filling stage (Ji et al., 2010). Though crop yield reductions under abiotic stress are mainly a consequence of reduced grain number (Dolferus et al., 2011), the mechanisms of how stay-green phenotypes improve grain number under stress remain poorly understood. A highly flexible and dynamic adjustment due to premature abortion of developing florets under drought or heat stress exposure, can potentially occur throughout the spike development starting from floral meristem differentiation due to sugar starvation (Guo et al., 2015; Li et al., 2015b), which could be averted by reducing ABA level and by elevating brassinosteroids (Ji et al., 2011; Zhu et al., 2015). Under high night temperatures exposure from panicle initiation onwards, a significant floret degeneration was attributed to a competition for assimilates between the growing stem and developing ear (Shi et al., 2013). Moreover, a significant drop in peduncle elongation further increased floret sterility under drought stress mainly due to a shortage of assimilates and competition between stem and ear for the depleted assimilate pool (Jagadish et al., 2010). Two key processes that could determine the viability of reproductive organs and thereby grain numbers are the availability of sufficient amounts of sugars through maintained photosynthesis and efficient sucrose cleavage pathway to channel gradients of hexoses to the developing reproductive tissues (Ji et al., 2010; Dolferus et al., 2011; Suneja et al., 2015). Hence, mechanisms that impact grain numbers through key physiological events including altered male and female gametophyte development, pollen and ovule viability, fertilization events and optimum seed filling are key drivers for maintaining yield under stress (**Figure 1**).

Heat stress (39°C) during the sensitive microspore stage in rice, led to a failure of tapetal degeneration affecting pollen grain wall composition and causing poor adherence of pollen onto the stigma (Endo et al., 2009). Similar phenomena were observed during barley meiosis leading to aborted tapetal and pollen mother cell development leading to dramatic transcriptome reprogramming (Oshino et al., 2007). Physiological mechanisms including tapetal dysfunction, microspore collapse, loss of pollen and ovule viability, anther indehiscence, lack of pollen adhesion on stigma and poor pollen germination and fertilization are a series of cascading effects that may follow during anthesis and post-anthesis stress. In the male gametophyte development, the meiotic cell division is affected by stress factors, thereby disturbing the events connected to DNA replication followed by two rounds of chromosome segregation (MI and MII), crossover formation and recombination. The overall rate of recombination has been found to increase substantially mostly due to elevated ABA concentrations under stress. ABA positively regulates meiotic recombination 11 (MRE 11), an exo/endonuclease, leads to enhanced crossover (Prado and Aguilera, 2003) and also ABA, which is known to downregulate RAD51 causing chromosome fragmentation (Dray et al., 2006). The plasmadesmatal connections become undetectable and apoplastic transport is impaired under stress leading to a disturbance in sugar transport from the tetrad to the mature pollen stage, which is also known to be influenced by ABA (Oliver et al., 2007; Nguyen et al., 2010). The active portrayal of the tapetum and the functional role of invertases in determining pollen viability and seed set is well documented (Proels et al., 2003; Oliver et al., 2007; Ji et al., 2011). Key tapetal cell wall invertase genes *IVR1* in wheat and *OsINV4* in rice repressed by water stress led to increased starch accumulation on anther walls (Koonjul et al., 2005; Oliver et al., 2007). The reduced cell wall invertase under short and long term heat stress during microspore meiosis led to irreversibly altered carbohydrate metabolism inducing starch deficiency and pollen abortion in rice, sorghum, and tomato (Jain et al., 2007, 2010; Li et al., 2012a, 2015b). With heat stress exposure, *Mha1* (plasma membrane H^+-ATPase), *SUT3* and *MST7* (sucrose and monosaccharide transporter proteins), transcripts were highly abundant despite poor pollen viability and low seed set with an irreversible decline in ICW (*SbIncw1*) in sorghum microspores (Jain et al., 2010). Similarly, drought-stressed rice anthers accumulated high amounts of sucrose due to regulated expression of sucrose (*OsSUT5*) and monosaccharide transporter (*OsMTS7*) with repressed expression of cell wall invertase (*OsCIN4*, Nguyen et al., 2010). Further, the spatial expression of *OsSUT5* and *OsMTS7* were mainly detected in young microspores, the tapetum and the anther middle layer and thereby indicated no restriction of sugar flow from anther walls to the developing microspores with invertases being the major bottleneck. Looking at a wider genetic pool and contrasting entries, drought-tolerant wheat germplasm maintained carbohydrate accumulation in the reproductive organs throughout stress duration by virtue of their ability to control and maintain sink strength and carbohydrate supply to the anthers (Ji et al., 2010). Invertase *IVR1* located in the wheat anther tapetum and around the vascular bundles was not repressed in drought-tolerant lines but strongly inhibited in the susceptible entries (Ji et al., 2010). Similarly, expression of the

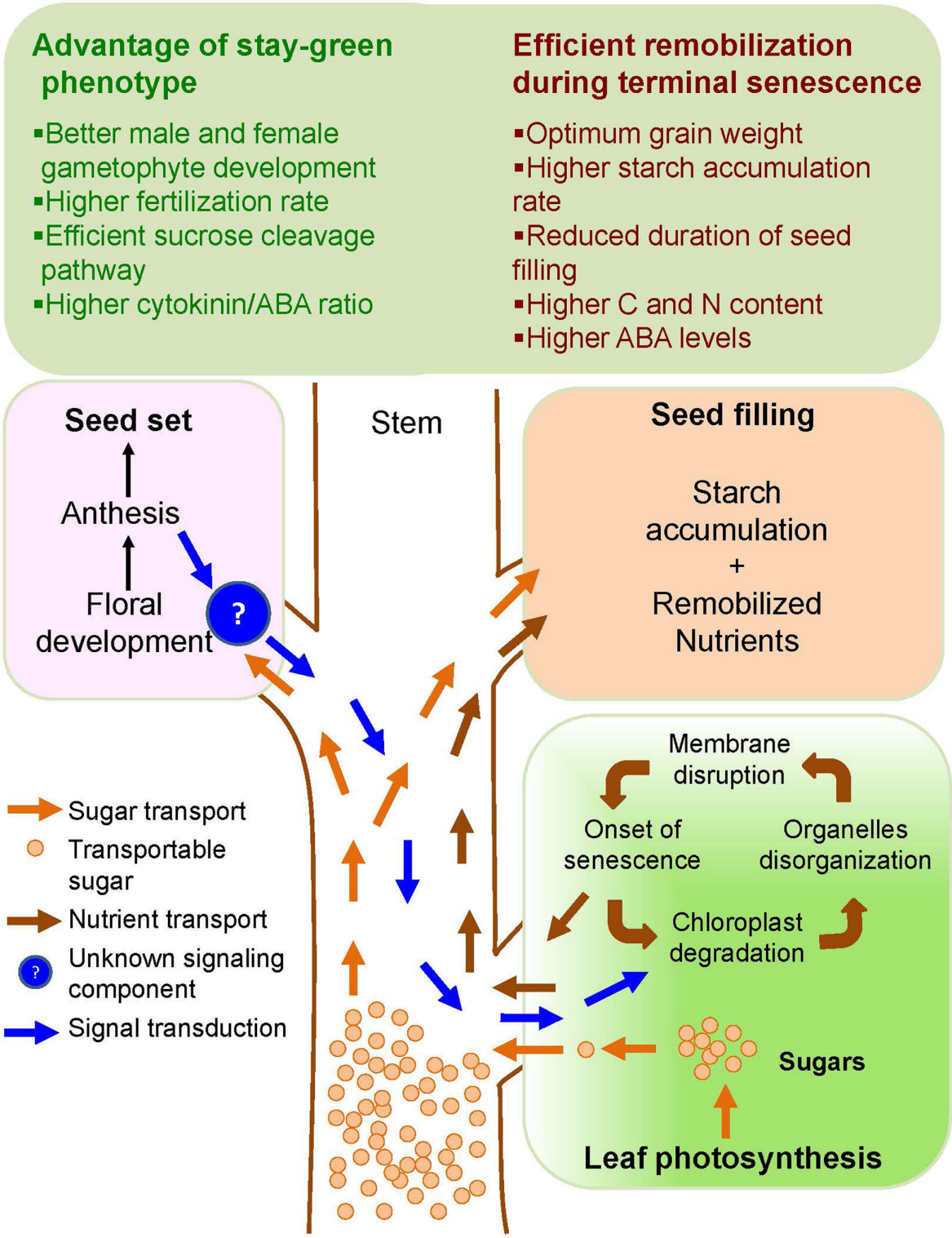


FIGURE 1 | Schematic diagram showing integrative effect of stay-green and terminal senescence traits in plants. Extended stay-green trait provides sufficient photosynthate available as transportable sugar (orange arrows) for floral development and higher starch accumulation during grain filling. Conversely, initiation of terminal senescence after seed set provides additional nutrient supply (brown arrows) to the developing grains to improve optimum grain weight and quality. An unknown signaling component (?) from the floral organ (blue arrows) is thought to initiate the senescence process in leaves.

fructan biosynthesis genes *I-SST* and *6-FST* was reduced only in the susceptible wheat germplasm (Ji et al., 2010).

The regulatory pathway that controls anther sink strength and cell wall invertase activity remains elusive (Dolferus et al., 2011). Moreover, pollen sterility can be induced without reduction in spikelet water potential, and the ABA signal from the leaves is considered to trigger pollen collapse (Oliver et al., 2007). On the other hand, during the extremely metabolically active young microspore stage in the rice anther tapetum, mitochondrial numbers have been shown to increase by 20- to 40-fold (Dunwell and Sunderland, 1976; Mamun et al., 2005), to meet the high energy demand (Dolferus et al., 2011). In addition, poor or disturbed mitochondrial metabolism in the anther tapetum led to premature tapetal death, resulting in pollen abortion (Liu and Fan, 2013). Hence, in anthers a continuous supply of sugars is essential to maintain the pollen and ovule viability by meeting their hugely enhanced energy demand, and hence only a functional stay-green phenotype safeguards reproductive organ energy demand under stress.

Ovaries of cereals are normally loaded with glucose and starch on the day of pollination under control conditions (McLaughlin and Boyer, 2004). The sink strength of the ovary increases and gets to the highest point after fertilization and during grain filling where ovary growth cessation has been correlated with reduced sugars and depletion of starch (Zinselmeier et al., 1995). When the delivery of photosynthates is curtailed at low water potentials during drought, enzymes that metabolize sucrose, in particular the cell wall and soluble invertases loose activity (Zinselmeier et al., 1995; McLaughlin and Boyer, 2004). Under these conditions, previously accumulated starch is consumed through activation of amylases (Ruan, 2014), resulting often in seed abortion. The starch depletion in the wheat ovary is reversible, while that of the pollen is irreversible (Ji et al., 2010). The placento-chalazal cell wall invertase activity in ovules of open-pollinating maize is substantially reduced under drought stress restricting sugar delivery to the pedicel phloem. This resulted in a decrease of the sugar gradient between the pedicel and the nucellus surrounding the ovary sac and in ovary abortion (McLaughlin and Boyer, 2004; Makela et al., 2005). An initial down-regulation of invertases (*Incw 1-4*, *Ivr1-2*) and sucrose synthases (*SS1*, *SS2*) in maize ovaries following drought stress triggered a ribosomal inactivating protein (*RIP2*) and phospholipase D (*PLD1*), an indicator of membrane damage and irreversible loss of ovary viability (Andersen et al., 2002; McLaughlin and Boyer, 2004; Boyer and McLaughlin, 2007). ABA is known to repress cell wall invertases. The overexpression of ABA catabolism gene ABA-8′-hydroxylase maintained sink strength in wheat under cold stress (Ji et al., 2011). Increased accumulation of ABA in ovaries and reduced endogenous auxin levels in the anthers resulted in female flower and anther sterility, respectively (Vriezen et al., 2008; McAtee et al., 2013). The implications discussed in improving seed set lies in improved carbohydrate availability, transport and utilization. Hence, a stay-green phenotype meets the huge energy demand mentioned above and allows to reduce the heat- and drought-induced pre-anthesis at anthesis, fertilization and early embryo formation losses in grain number.

IMPORTANCE OF ACCELERATED NUTRIENT REMOBILIZATION AS A TRAIT TO OPTIMIZE GRAIN QUALITY AND SEED WEIGHT UNDER POST-ANTHESIS DROUGHT AND HEAT STRESS

An alternative source of assimilates are pre-anthesis stem reserves in the form of sugars, starch or fructans, which constitute a buffer in case that source capacities are reduced as a result of drought-induced senescence. These reserves are readily utilized for grain filling, which may become a critical factor in sustaining grain filling when drought occurs during the peak of seed filling in wheat, rice, and barley (Yang and Zhang, 2006; Govind et al., 2011; Distelfeld et al., 2014; Zhang et al., 2015). Grain size determination is only initiated shortly after anthesis and during grain filling (Ji et al., 2010). Interestingly, with temperatures above 30°C assimilate transport from flag leaf to grain was substantially reduced but the stem transport was not affected even up to 50°C (Miyazaki et al., 2013). Stem reserves contribute ≥70% final grain mass (Rebetzke et al., 2008; Chochois et al., 2015; Zhang et al., 2015). Hence, breeding approaches should focus on increasing the stem sink potential to overcome heat and drought stress-induced yield and grain quality losses in cereals (**Figure 1**). A greater contribution of stem reserves play a critical role in maintaining yields under terminal drought stress. A wide genetic diversity in wheat stem WSC has been documented (Li et al., 2015a) and the introduction of the *Rht1* and *Rht2* dwarfing genes in wheat driving the green revolution is associated with a reduced WSC stem storage due to the shorter peduncles (Ellis et al., 2002; Wu et al., 2010), and the same could be the case with the introduction of semi dwarfing gene (*sd1*) in rice. Therefore plant height manipulation has been a crucial factor to readjust source-sink relationships and to improve yield stability.

Cereal crops store excess carbohydrates in the form of soluble sugars or sugar polymers within the vegetative tissues (Wehner et al., 2015). They are also capable of storing non-structural carbohydrates in the parenchyma cells of stems surrounding the vascular bundles located within internodes. Stem carbohydrates stored as sucrose, fructans (as in barley, wheat), or starch will be a good alternative source of assimilates when photosynthesis is impaired under post-anthesis stress (Joudi et al., 2012; Li et al., 2015a; Zhang et al., 2015). These studies suggested that one way to increase sink strength in the developing seed is through readjustment of non-structural carbohydrates in stems, which help to optimize carbon partitioning to increase kernel weight. Whole-plant carbon partitioning plays a vital role to buffer the source-sink interactions which may ultimately support yield stability by providing an alternative source when photosynthetic capacity is low during the period of drought stress. Accumulation of sugars in the stems may also help the plants to pull water from the soil into the vegetative parts of the plants through adjustment of turgor (Fu et al., 2011a). Such a readjustment is based on many interconnecting factors such as photosynthetic efficiency. Assimilate competition between organs (newly formed tillers, stem reserve accumulation versus

seed biomass) and environmental influences such as water and nutrient availability, photoperiod and temperature. Genetic factors controlling assimilate partitioning eventually decide over seed filling.

Grain yield in cereals is a result of coordinated activities between source and sink tissues. Under optimal conditions, grain growth or seed yield are generally sink limited where as under stress treatments it will undergo source-limited sink dramatic readjustment. Therefore under terminal drought, yield losses in cereals are a result of both source and sink limitations. Yield reduction in barley and other crops even with adequate assimilates made available through artificial feeding to developing grains clearly highlights the importance of sink activity in determining yield under terminal drought (Boyer and Westgate, 2004). Sink strength plays a primary role in grain filling of cereals. But how pre-anthesis WSC reserves are related to the generation of sink strength especially under stress has not yet been explored in detail. Nitrogen (N) application at the spikelet differentiation stage improved pre-anthesis WSC reserves and sink strength in plants. Besides the lower number of endosperm cells being the limiting factor of sink strength, the rate of storage product accumulation and duration of seed filling has also been identified as another important stepping stone to increase grain weight under drought (Sreenivasulu et al., 2012).

As starch is the predominant storage form of carbohydrates in cereal grains, activities of enzymes involved in the conversion of sucrose to starch are major factors determining sink activity and hence crop yield (Sreenivasulu and Wobus, 2013; Wang et al., 2015). Among various enzymes involved in starch synthesis, sucrose synthase (SuSy), which catalyzes the conversion of sucrose to fructose and UDP-glucose is considered to be one of the important marker enzymes for sink strength in several crops including cereals (Worch et al., 2011; Hou et al., 2014). Its activity was found to be a major determinant of the duration of seed filling in barley and other cereals under both optimal and water-deficit conditions (Worch et al., 2011; Sreenivasulu and Wobus, 2013). On the other hand, reduction in the activity of acid invertase, another enzyme involved in the breakdown of sucrose especially during early stages of seed development in barley (Sreenivasulu et al., 2004) was pronouncedly inhibited under water limited conditions in wheat as well as in maize (Zinselmeier et al., 1995; Li et al., 2012a). Therefore, fine tuning of different sucrose cleavage pathways in a stage-dependent fashion is an important criterion for regulating seed metabolism under post-anthesis stress is essential.

ADP-glucose pyrophosphorylase (AGPase), an important rate-limiting enzyme of starch synthesis catalyzing the production of ADP-glucose was found to be negatively affected by severe drought stress in barley, wheat, and rice but moderate drying results show added advantage with increased rate of starch accumulation (Yang and Zhang, 2006; Seiler et al., 2011; Ruan, 2014; Wang et al., 2015). A notable exception to all the above results was reported in a controlled soil drying experiment carried out in rice and wheat during the grain filling (Yang and Zhang, 2006). These authors found that activities of SuSase, SSS, SBE (starch branching enzyme) and AGPase were significantly enhanced under moderate drought and were positively correlated with an increased rate of seed starch accumulation resulting in better seed weight compared to control but with reduced seed filling duration. Enhanced seed filling under mild drying was attributed to the accumulation of ABA which enhanced sink strength and remobilization of stem reserves (Seiler et al., 2011; Wang et al., 2015).

Starch rapidly accumulates in the central endosperm from early to mid grain filling and later at the periphery, whereas a shortage of assimilates during heat stress led to chalky rice grains due to lose packaging of amyloplasts (Wada et al., 2014; Sreenivasulu et al., 2015). Source-sink manipulation studies in rice have shown a close relationship between assimilate supply and white core chalk formation. With higher temperature a generally large-celled thick aleurone layer with irregular starch granules were formed leading to the trigger of chalk phenotype (Kim et al., 2012). Moreover, a 1°C increase above the optimal growing temperature of 25°C, the grain filling duration could be reduced by 2.8 days. Hence a line sufficiently equipped with stem reserves to overcome the reduced duration of grain filling induced with faster senescence induced either through heat stress or drought stress will be instrumental for matching optimum grain weight (Lobell et al., 2012; Reynolds et al., 2012).

REGULATORY MECHANISMS UNDERLYING THE INITIATION OF SENESCENCE AND NUTRIENT REMOBILIZATION

Hormonal Complexes Regulating the Initiation of Senescence

Among various phytohormones, ABA and CK are two major plant hormones having antagonistic effects on plant senescence under abiotic stress (Peleg and Blumwald, 2011). ABA mediated signaling cascade is known to promote senescence (Lee et al., 2015). Evidence has been presented for CK to inhibit leaf senescence, by expressing *IPT*, a key member of CK biosynthesis, under control of the senescence-associated genes *SAG12* or *SAG13*. This substantially delayed the initiation of senescence (Swartzberg et al., 2011). Further, it has been proved that CK inhibits senescence via an apoplastic invertase that produces extracellular hexoses. It appears that intracellular sugar sensing via hexokinase is dominant over extracellular sugar sensing with regard to leaf senescence (Swartzberg et al., 2011). *Arabidopsis* hexokinase (*AtHXK1*), an intracellular mitochondrial associated enzyme accelerates leaf senescence, while CK inhibits it (Cho et al., 2010). However, recent evidence suggested that apart from these two, there are also other hormones involved in a coordinated regulation of leaf senescence (Jaillais and Chory, 2010). In addition to their role in senescence, ABA and CK are also implicated in grain filling in different cereals, influencing endoreduplication, onset of seed storage and desiccation-related events (Sreenivasulu et al., 2010; Seiler et al., 2011) and thus seed yield (Tamaki et al., 2015). In a partial soil drying experiment during grain filling in wheat, the ABA content in the grain was found to positively correlate with enzymes involved in

grain filling (Yang et al., 2003). ABA is also a well-known plant hormone which accumulates under stress and mediates transpirational loss through stomatal closure and thus ABA homeostasis is an important element in achieving water use efficiency (Seiler et al., 2014). Transgenic plants overexpressing 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), an important enzyme in the ABA biosynthetic pathway under a drought-inducible promoter exhibited enhanced drought tolerance and maintained a better leaf water status and more green leaf area, whereas ubiquitous overexpression triggered senescence-related events (Thompson et al., 2000). Hence, for studying the role of hormones in plant development, it is necessary to use conditional promoters driving gene expression at a specific developmental stage or in response to specific environmental stimuli.

Ethylene is a gaseous plant hormone, associated primarily with fruit ripening, which promotes leaf senescence (Kim et al., 2015). The antisense suppression of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, a key ethylene biosynthesis gene caused delayed leaf senescence (John et al., 1995). It is also known that ethylene alone may not be sufficient to initiate senescence and most likely that age-dependent factors are perhaps necessary for ethylene-regulated senescence. Characterizing mutants deficient in ethylene perception and signal transduction showed an enhanced leaf longevity in *ethylene-resistant 1* (*etr1-1*) and (*ein2/ore3*, Yang et al., 2008). Characterization of a large number of *onset of leaf death* (*old*) mutants confirmed the notion that the effect of ethylene on leaf senescence depends on age-related changes through *OLD* genes (Jing et al., 2005). These studies also proved that multiple genetic loci are required to regulate the action of ethylene in leaf senescence. In the *old1etr1* double mutant, in which ethylene perception was blocked, an age-dependent earlier onset of senescence occurred. Altogether, these experiments suggest that *OLD1* negatively regulates the integration of ethylene signaling into leaf senescence. Ethylene-induced *SAGs* together with physiological studies revealed extensive cross talks between ethylene and other hormones that are associated with the progression of leaf senescence.

Transcription Factor Cascades Regulating Senescence

Genetic analysis revealed that senescence is controlled by *senescence associated genes (SAG)* acting in the loop as various negative and positive regulators (Gregersen et al., 2008; Masclaux-Daubresse et al., 2014; Huang et al., 2015). However, the complete transcription factor and signaling cascades involved in regulating leaf senescence are still unknown. Notably, several regulatory elements, such as signal transduction-related proteins and transcription factors were identified among the SAGs in cereals (Hollmann et al., 2014), and some of their functions were validated utilizing *Arabidopsis* T-DNA insertion lines (Lim et al., 2007). Interesting observations revealed that the transcription factors *WRKY53* and *AtNAP* (a member of NAC TFs family) act as positive regulators of leaf senescence coordinating the progression through the final stages of leaf development (Guo and Gan, 2006; Ay et al., 2009). The importance of the senescence-induced remobilization of nitrogen in crop plants is exemplified by the map-based cloning of the grain protein concentration (*GPC*) locus, *NAM-B1* (encoding a NAC transcription factor) originally identified in wheat chromosome 6B. The presence of a functional *NAC* gene was found to increase the grain protein content as a result of an earlier induction of post-anthesis senescence (Uauy et al., 2006). A similar gene was also identified on chromosome 6H of barley (*HvNAM-1*) through QTL analysis, that explained 45% of the heritable variance in protein content of a mapping population (Jamar et al., 2010). Recently, using near isogenic lines developed for the 6H locus, it was found that, in addition to acceleration of post-anthesis flag leaf senescence, the *GPC* locus also accelerated the pre-anthesis development from transition of the shoot apical meristem (SAM) onwards (Jukanti and Fischer, 2008; Parrott et al., 2012). Transgenic wheat lines, in which expression of *NAM-B1* and its homeologous genes were down-regulated using RNAi, were characterized by delayed leaf senescence and lower grain protein, Fe and Zn concentrations (Waters et al., 2009). The authors concluded that wild wheat encodes a NAC transcription factor (*NAM-B1*) which accelerates senescence along with the remobilization of protein and nutrients such as iron and zinc from leaves to grains. Transgenics overexpressing *NAP* showed premature senescence. Moreover, a *NAP* homolog of rice restored delayed leaf senescence in the *AtNAP*-deficient *Arabidopsis* mutant (Guo and Gan, 2006).

The *WRKY* family (zinc finger type) of TFs is another important family (the second largest group of TFs) associated with senescence as well as disease resistance (Zentgraf et al., 2010). In dark-induced senescence, 21 *WRKY* TFs out of the 59 known WRKYs were differentially expressed (Friedel et al., 2012; Li et al., 2012b). However, the function of the individual WRKY factors that are expressed during senescence is not yet very clear. Several reports pointed out that WRKY factors act in a regulatory network, in which the transcription of other WRKY factors is influenced, rather than in a linear signal transduction pathway. *WRKY53* has been identified in *Arabidopsis* as an important factor in controlling leaf senescence (Zentgraf et al., 2010). Inhibiting the function of *WRKY53* using RNA interference retarded leaf senescence (Miao et al., 2004). These authors have identified more than 60 targets of WRKY53 including six other members of the WRKY gene family. It appears that WRKYs act as an upstream element in the signaling pathway. Though WRKY TFs have been shown to be involved in the regulation of leaf senescence, not much is known about the upstream regulation of the senescence-specific expression of *WRKY* factors. DNA-binding protein with an unknown function that contains a transcriptional activation domain and a kinase domain regulates WRKY53 transcription factor (Miao et al., 2008). *In vitro* studies revealed that this activation domain protein (AD protein) can phosphorylate itself, and that phosphorylation increased its DNA-binding activity to the WRKY53 promoter region. Moreover, the AD protein interacted with a mitogen-activated protein kinase kinase 1 (MEKK1). These studies revealed that that there may be competition between WRKY53 and the AD protein for binding of *MEKK1* at the *WRKY53* promoter, and that

the AD protein is a positive regulator of *WRKY53* expression. The transcriptional factor GATA4 and an orthologous gene product S40-3 have been reported to regulate the expression of *WRKY53* and positively regulate the senescence process (Fischer-Kilbienski et al., 2010). The *s40-3a* mutant, carrying a T-DNA insertion in the promoter region, exhibited a stay-green phenotype. Thus, both GATA4 and S40-3 are supposed to play a significant role in senescence regulation. Besides the central role of WRKY53 in senescence regulation, WRKY6 and WRKY22 have been reported as positive regulators of senescence (Lim et al., 2007).

Kinases that are involved in senescence regulation by hormones are members of the ORE (oresara) family, a member ORE12-AHK3 is a histidine kinase-type CK receptor (Kim et al., 2006). AHK3 is mainly involved in CK signaling, as a loss-of-function *ahk3* mutant displayed an early senescence phenotype. As discussed above, sugars exert a regulatory influence over senescence. Certain protein kinases sense the sugar level and regulate sugar-mediated signaling. One such protein kinase is SnRK1 (Snf1-Related Kinase), which acts as a translational inhibitor and a transcriptional inducer for a wide range of proteins or genes influencing development and environmental responses (Robaglia et al., 2012). SnRK-1 can be activated by darkness, nutrient starvation and high sucrose or low glucose concentrations in the cell. Down-regulation of *SnRK1* led to a number of developmental irregularities including premature senescence. Extracellular invertase, by hydrolyzing sucrose to hexoses, counteracts the influence of SnRK1. Other sugar-sensing senescence regulators include hexose kinase (Lee et al., 2015). In *Arabidopsis*, several HXKs act as sensors of the glucose level and are associated with mitochondria. However, other HXKs can also be found in the nucleus in higher-molecular weight complexes, which repress the expression of photosynthetic genes. The *Arabidopsis gin2-1* mutant carries a non-sense mutation in the *HXK1* gene exhibited delayed senescence due to alteration in glucose sensing. Moreover, this mutant showed a reduced senescence response to glucose feeding and reduced sugar accumulation (Pourtau et al., 2006). These experiments indicated that senescence is associated with *HXK1*-dependent signaling.

CONCLUSION

Through empirical means physiologists have identified "stay-green"—defined by the extended lifespan of photosynthetic activity under challenging environments, as an antagonist to senescence—defined by the breakdown of chlorophyll. Crop yield under water deficit or heat stress strongly depends on photosynthates provided either through current photosynthesis or through the remobilization of stored carbohydrates from the stem. An alternative assimilate source to reduced photosynthesis under drought stress consists of carbohydrates (sugar, starch, and fructans) that were built up during pre-anthesis and stored in the stem. These reserves may be utilized during grain filling, especially if current photosynthesis is reduced due to drought in cereals. However, this mechanism is effective only during seed filling, but not necessarily advantageous during the critical stages encompassing gametogenesis, anthesis or fertilization (−10 days before fertilization until 8 days after fertilization). The relative importance of these two plant strategies (stay-green versus remobilization) for efficient seed set and seed filling under terminal drought depends on genotype plasticity and its ability to cope with the severity of stress. Thus, what underpins the crop productivity ultimately under drought stress therefore depends on how the crop (i) captures assimilates to maintain pollen viability and safeguard fertilization and (ii) undertakes effective assimilate partitioning to sink organs.

Several studies have proposed to use the starch content per seed as a measure for a drought tolerance index, but other studies have demonstrated an increase in seed number with only a marginal impact on seed weight. Thus, there is a need to dissect how stay-green and remobilization phenotypes impact on yield stability by influencing grain number and grain weight, as both components determine yield stability. Occurrence of drought stress during gametogenesis, anthesis and onset of seed development is critical, resulting in impaired grain set, reduced grain weight and yield loss. This is thought to be at least partly due to a decrease in photosynthetic efficiency and to changes in sucrose cleavage processes in reproductive organs. Although invertases and their activity are negatively affected by stress and further deteriorated under elevated ABA levels, resilient sources of invertases have lost attention. The recent identification of such resilient invertases and sucrose synthase in wheat supports molecular marker development and promises to overcome the identified bottle-neck in sugar conversion to facilitate an undisturbed supply of sugars to the sensitive reproductive organs. Assessing the population for yield stability under heat, drought and combined drought and heat stress with defined stable QTLs for yield from wheat mapping populations in field trials help to develop climate resilient varieties through breeding (Bennett et al., 2012; Bonneau et al., 2013). The switch between stay-greenness and senescence and its influence on seed yield stability is still elusive. Several studies employing the NDVI (normalized difference vegetation index) technique, stay-green during physiological maturity and rate of senescence were shown to positively and negatively correlate with yield, respectively, under terminal stresses. Hence, the use of a high-throughput chlorophyll fluorescence or alternative techniques with more specific spectral indices to estimate the senescence pattern and to identify the elusive switch from stay-green to senescence, is needed. With the unraveled molecular mechanisms it is possible to breed for functional stay-greenness in cereals by optimizing assimilates, modify photorespiration and enhance sink strength to attain yield stability by defining distinct strategies during anthesis under post-anthesis abiotic stresses. Moreover, drought-tolerant wheat germplasm that is able to maintain source strength when stressed at the young microspore stage failed to maintain sink strength when stressed at anthesis and grain-filling (Ji et al., 2010). This provides an excellent example of a pre-anthesis "stay-green" phenotype and for an enhanced remobilization efficiency phenotype that operates independently (**Figure 1**). Hence, we propose future cereal stress breeding programs to exploit these unique phenomena by identifying lines or accessions possessing these features. Such

contrasting lines may subsequently be used to develop lines with a pre-anthesis stay-green and a post-anthesis remobilization phenotype.

AUTHOR CONTRIBUTIONS

Overall concept of the review has been designed and written by NS. All other authors of the manuscript meet the essential criteria of the publication by contributing to individual sections and figure. All authors have read and approved the manuscript.

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REVIEW
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Multi-Level Interactions Between Heat Shock Factors, Heat Shock Proteins, and the Redox System Regulate Acclimation to Heat

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High temperature has become a global concern because it seriously affects the growth and reproduction of plants. Exposure of plant cells to high temperatures result in cellular damage and can even lead to cell death. Part of the damage can be ascribed to the action of reactive oxygen species (ROS), which accumulate during abiotic stresses such as heat stress. ROS are toxic and can modify other biomacromolecules including membrane lipids, DNA, and proteins. In order to protect the cells, ROS scavenging is essential. In contrast with their inherent harms, ROS also function as signaling molecules, inducing stress tolerance mechanisms. This review examines the evidence for crosstalk between the classical heat stress response, which consists of heat shock factors (HSFs) and heat shock proteins (HSPs), with the ROS network at multiple levels in the heat response process. Heat stimulates HSF activity directly, but also indirectly via ROS. HSFs in turn stimulate the expression of HSP chaperones and also affect ROS scavenger gene expression. In the short term, HSFs repress expression of superoxide dismutase scavenger genes via induction of *miRNA398*, while they also activate scavenger gene expression and stabilize scavenger protein activity via HSP induction. We propose that these contrasting effects allow for the boosting of the heat stress response at the very onset of the stress, while preventing subsequent oxidative damage. The described model on HSFs, HSPs, ROS, and ROS scavenger interactions seems applicable to responses to stresses other than heat and may explain the phenomenon of cross-acclimation.

Keywords: heat response, heat shock factor, heat shock protein, reactive oxygen species, ROS scavenging, signaling, interaction, cross-talk

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THE HEAT RESPONSE

Plants are continuously exposed to biotic and abiotic stress factors, such as herbivory, pathogen attack, drought, salinity and extreme temperatures. These challenges pose a serious threat to their growth and reproduction and as such affect agricultural yields. With considerable advances in pest and disease management, abiotic factors are now thought to be the primary cause for crop losses worldwide (Wang et al., 2003; Suzuki et al., 2014). In case plants cannot prevent an abiotic stress factor from affecting organismal homeostasis (i.e., escape or avoid internal stress), they may adapt their metabolism to acquire a certain level of tolerance (Larkindale and Knight, 2002; Valliyodan and Nguyen, 2006; Munns and Tester, 2008; Krasensky and Jonak, 2012).

Heat stress can be defined as a rise in temperature beyond a threshold level for a period of time, sufficient to cause irreversible damage to plant growth and development (Wahid et al., 2007). Sudden rises in temperature to high levels may lead to cell death within a few minutes as a consequence of extensive protein denaturation and aggregation and loss of membrane integrity (Schöffl et al., 1999; Wahid et al., 2007). Furthermore, prolonged exposure to moderately high temperatures can lead to reduced cellular function and overall plant fitness (Bokszczanin et al., 2013). An important process in this respect is the accumulation of reactive oxygen species (ROS), formed as a by-product in various aerobic metabolic pathways in different cellular compartments such as chloroplasts, mitochondria and peroxisomes (del Rio et al., 2006; Navrot et al., 2007) and probably also in the apoplast through the activation of NADPH oxidases (Gechev and Hille, 2005; Torres and Dangl, 2005; Miller et al., 2009; Wang et al., 2014a). Under steady state conditions, ROS molecules are formed as quickly as they are scavenged by anti-oxidative defense mechanisms, but this equilibrium is perturbed by abiotic stress factors such as heat (Foyer and Noctor, 2005). There is ample evidence that, when plants are exposed to heat, ROS production rapidly becomes excessive (Morgan et al., 1986; Dat et al., 1998; Vacca et al., 2004; Volkov et al., 2006; Bhattacharjee, 2012, 2013; Chou et al., 2012; Hasanuzzaman et al., 2012, 2013; Wu et al., 2012; Hossain et al., 2013; Das and Roychoudhury, 2014; Mostofa et al., 2014). This causes cellular damage to membranes, proteins, lipids, organelles, and DNA (Baker and Orlandi, 1995; O'Kane et al., 1996; Giardi et al., 1997; Larkindale and Knight, 2002; Volkov et al., 2006; Wu et al., 2012; Bokszczanin et al., 2013). In order to prevent cell damage and regain redox homeostasis, one of the responses to heat is the hyper-activation of the ROS scavenging machinery. The expression and protein level of genes responsible for ROS scavenging are induced under heat stress in many different plant species (Chao et al., 2009; Chou et al., 2012; Mittal et al., 2012; Suzuki et al., 2013) and has been associated to basal heat tolerance (Rui et al., 1990; Badiani et al., 1993; Gupta et al., 1993; Sairam et al., 2000; Almeselmani et al., 2006; Kang et al., 2009; Bhattacharjee, 2012; Wang et al., 2014c). Furthermore, the induction of scavenging genes was significantly stronger in heat tolerant genotypes than that of sensitive ones (Rainwater et al., 1996), and improvement of plant heat stress tolerance has been achieved by increasing antioxidant enzymes activities (Rui et al., 1990; Badiani et al., 1993; Gupta et al., 1993; Sairam et al., 2000; Almeselmani et al., 2006; Wu et al., 2012; Chen et al., 2013). Taken together, this shows the importance of ROS scavenging in the heat-stress response.

In contrast to their harmful character, however, ROS are also considered as important signal molecules. Cells are capable of rapid and dynamic production and control of several forms of ROS, enabling a tight local control in the cell as well as more holistic control of the entire plant (Vranová et al., 2002; Mittler et al., 2011; Petrov and Van Breusegem, 2012). Therefore, they are thought to be involved in the transduction of intracellular and intercellular signals controlling gene expression and activity of anti-stress systems (Desikan et al., 2001, 2004; Apel and Hirt, 2004; Foyer and Noctor, 2005; Torres and Dangl, 2005; Miller et al., 2009; Galvez-Valdivieso and Mullineaux, 2010; Mittler et al., 2011; Kreslavski et al., 2012). Indeed, NADPH oxidase activity is rapidly induced upon heat (Miller et al., 2009) and the mutation of *RBOHB* makes *Arabidopsis* seedlings more sensitive to heat (Larkindale et al., 2005; Wang et al., 2014a).

One of the best studied anti-stress mechanisms is the production of heat shock proteins (HSPs) upon exposure to high temperatures (Wang et al., 2004). By acting as molecular chaperones, HSPs prevent deleterious protein conformations and eliminate non-native aggregations, which are formed during stress (Vierling, 1991; Boston et al., 1996; Morimoto, 1998). The expression of HSPs and other heat-responsive genes is regulated by heat shock factors (HSFs; Kotak et al., 2007) through their association to a palindromic binding motif (5′-nAGAAnnTTCTn-3′) in the promoter region of the heat-responsive genes: the heat shock element (HSE; Pelham, 1982; Scharf et al., 2012). Activation of HSFs upon stress occurs via a multistep process involving homotrimer formation and acquisition of transcriptional competence for target gene induction (Liu et al., 2013).

Clearly, both the activation and production of HSFs/HSPs and the increase in ROS/scavenging activity belong to the major responses of plants to heat stress and play important roles in acclimation. A number of recent genetic and biochemical studies, however, indicate that there are complex interactions between these responses. This review describes the evidence for crosstalk between HSFs, HSPs, ROS, and ROS scavenging enzymes at various points in the heat stress response pathway and presents a model with a timing component.

ACTIVATION OF HSFs BY ROS

In non-stressed situations, the HSFs are located in the cytoplasm for most eukaryotes, in an inactive monomeric form due to association with HSP70, HSP90, and potentially other proteins (Morimoto, 1998; Schöffl et al., 1998). According to the chaperone titration model, heat results in a higher load of denatured proteins, which pulls HSPs away from HSF complexes through competitions to act as molecular chaperones. This then leads to the release of HSFs, which form trimers and relocate to the nucleus to activate expression of *HSP* and other heat-responsive genes (Zou et al., 1998; Volkov et al., 2006).

A number of studies, however, report that expression of heat-responsive genes is also increased upon application of the ROS H_2O_2 (Uchida et al., 2002; Wahid et al., 2007; Banti et al., 2008). For example, *AtHSP17.6* and *AtHSP18.6* achieved similar expression levels through heat treatment as they do through H_2O_2 application at room temperature (Volkov et al., 2006). Several hypotheses have been formulated that suggest that heat can indirectly activate HSFs via the action of ROS.

Firstly, damaging amounts of heat-induced ROS also induce protein denaturation. In this way ROS enhances dissociation of the HSP–HSF complex, as described by the titration model (Schöffl et al., 1998). Secondly, and similar to what was found for mammalian and *Drosophila* HSFs, it has been proposed that certain plant HSFs act as H_2O_2 sensors (Ahn and Thiele, 2003; Miller and Mittler, 2006). Among all the ROS molecules, H_2O_2 plays a key role in signaling due to its moderate reactivity and

thus relatively long lifetime (Vranová et al., 2002). In addition, H_2O_2 can bypass membranes easily, making it a good candidate to function as a signaling molecule (Petrov and Van Breusegem, 2012). Miller and Mittler (2006) suggested that H_2O_2 might directly modify HSFs and induce HSF trimerization. Indeed, both heat and oxidative stresses result in the formation of high molecular weight HSE-binding complexes and the formation of these complexes has been shown to be a signature of early HSFA1a/A1b-dependent gene expression in heat-stressed leaves of *Arabidopsis* (Lohmann et al., 2004; Volkov et al., 2006). *In vitro* and *in vivo* studies confirmed activation of AtHSFA1a via trimerization in response to heat and H_2O_2 stress but also via pH alterations (Liu et al., 2013). HSFA1a, purified from *E. coli*, sensed the different stresses directly in a redox dependent fashion. *In vitro* stress treatments caused monomer-to-trimer transitions of HSFA1a, while the presence of the reducing agent dithiothreitol reversed this action. Although the study suggested a redox dependent fashion for HSF trimerization for all three stresses, the exact mechanism of action is still unclear. There is empirical evidence that the transcription factors may be sensitive to H_2O_2 via "single-Cys" or "two-Cys" redox sensory mechanisms (Mittler et al., 2011). These cysteine residues are typically responsive to oxidative stress. HSFA1a contains one Cys residue located at the *N*-terminal portion of the trimerization domain (Hübel and Schöffl, 1994). *N*-terminal deletions of HSFA1a negatively affected the sensing of H_2O_2 and pH changes, which suggests that trimerizations were induced by HSF conformational changes (Liu et al., 2013). In addition, Giesguth et al. (2015) recently showed that an HSFA8 Cys residue is responsible for translocation to the nucleus upon oxidative stress: H_2O_2 treated protoplasts showed cytosol-to-nucleus translocations of the wild-type HSFA8, but not of the HSFA8C24S mutant variant (Giesguth et al., 2015). Interestingly, however, the *N*-terminal deletion of HSFA1a did not inhibit heat sensing. This shows that activation of this particular transcription factor is stress-specifically regulated despite a common dependency on oxidative activity (Mittler et al., 2011). Notably, all stress treatments induced of HSFA1a binding to the *HSP18.2* and *HSP70* promoter, as detected by both formaldehyde cross-linking and chromatin immunoprecipitation, which paralleled the mRNA expression of these HSFA1a target genes (Volkov et al., 2006; Liu et al., 2013).

In addition to the above two processes, cellular communication between ROS and HSFs may involve mitogen-activated protein kinases (MAPK). HSF phosphorylation has been observed in yeasts and mammals (Chu et al., 1996; Knauf et al., 1996; Kim et al., 1999) and might thus occur in plants as well (Link et al., 2002). Indeed, *Arabidopsis* HSFA2 was found to be phosphorylated by MPK6 on T249 after heat treatment, and this was associated with subsequent intracellular localization changes (Evrard et al., 2013). Furthermore, MPK3- and MPK6-dependent phosphorylation of AtHSFA4A Ser309 and physical interaction between the proteins was reported recently (Pérez-Salamó et al., 2014). Activated HSFA4A in turn controlled the transcription of *HSP17.6A* (Pérez-Salamó et al., 2014). In tomato, heat-induced MAPKs were shown to transduce heat stress signals via HSFA3 (Link et al., 2002). In Arabidopsis, the same MAPKs that phosphorylate HSFs, namely MAPK3 and MAPK6, have been shown to be activated by H_2O_2 (Kovtun et al., 2000; Moon et al., 2003; Rentel et al., 2004). However, despite the presence of putative phosphorylation sites in tomato HSFA1, no heat-induced phosphorylation of this HSF was observed. Also, the phosphorylation site in AtHSFA4 was not conserved in HSFA4A proteins of citrus, grapevine and poplar (Pérez-Salamó et al., 2014). Taken together, this implies that both HSF oxidation and ROS-dependent phosphorylation can play a role in HSF activation, but that the latter is not a general signaling mechanism.

HSF–ROS SCAVENGING GENE INTERACTIONS

In addition to activation of HSFs by ROS signaling, evidence for interaction between HSFs and ROS scavenging genes has also been obtained. The expression of *APX1* was found to be regulated by *HSFA2*: overexpression of *HSFA2* resulted in increased expression of *APX1*, while *AthsfA2* knock out mutants showed a reduced expression of *APX1* (Li et al., 2005). In agreement with this, AtHSFA2 overexpression lines showed increased heat and oxidative stress tolerance (Li et al., 2005). Expression of a dominant-negative construct for *AtHSFA4a* prevented the accumulation of *APX1* transcripts (Pnueli et al., 2003; Apel and Hirt, 2004; Mittler et al., 2004; Davletova et al., 2005). Interestingly, the *AtHSFA4a* dominant-negative construct also prevented accumulation of the H_2O_2-responsive zinc-finger protein ZAT12, which is required for *APX1* expression during oxidative stress. The *ZAT12* promoter contains HSE binding sites (Rizhsky et al., 2004) and therefore, HSFA4a might directly interact with the *ZAT12* promoter (Davletova et al., 2005). However, HSEs are also present in the promoter region of the *APX1* gene itself, suggesting that direct activation via HSFs is also possible (Storozhenko et al., 1998; Panchuk et al., 2002). Using *Pennisetum glaucum APX1* and a *PgHSFA*, a specific binding interaction between the *APX1* HSE and HSF was confirmed, via *in vitro* gel shift assays as well as their expression patterns over time (Reddy et al., 2009).

Although *APX1* has been shown to be a central component of the *Arabidopsis* ROS network (Davletova et al., 2005), *APX2*, another isoform also localized in the cytosol, revealed a stronger induction by heat stress (Panchuk et al., 2002). AtHSFA2 has also been found to act as an *APX2* activator (Schramm et al., 2006; Nishizawa et al., 2008). Transcription level comparison between wild-type and *athsfa2* knock out plants revealed that transcripts of *APX2* were absent in heat shock induced leaves of the knock out background, but present in the wild-type plants (Schramm et al., 2006). Deletion analyses of the promoter region of *APX2* functionally mapped the HSFA2 binding sites to HSEs near the transcription start site (Schramm et al., 2006).

In addition, Nishizawa et al. (2006) and Banti et al. (2010) found strongly enhanced expression of galactinol synthase (*GolS1* and *GolS2*) ROS scavenging genes in an *HSFA2* overexpressing line.

Combining these results, HSFA2 seems to play a central role in ROS scavenger expression and thus constitute an important link between heat shock and oxidative stress responses.

HSP CHAPERONES SUPPORT ROS SCAVENGING ACTIVITY

Heat shock proteins function as molecular chaperones and play an important role in stress tolerance. In tomato, overexpression of the *LeCDJ1* DnaJ protein coding gene (also known as J-protein or HSP40; Qiu et al., 2006) resulted in improved thermotolerance, accompanied by increased APX and superoxide dismutase (SOD) activity after heat stress and reduced accumulation of O_2^- and H_2O_2. Despite the higher APX and SOD activity, transcription of the corresponding genes was not enhanced in the transgenic plants. Therefore, the influence of DnaJ proteins on APX and SOD activity was proposed to be post-transcriptional, due to their functionality as chaperones. Other studies have found similar effects of HSPs on ROS scavenging proteins upon heat stress. In *Arabidopsis*, overexpression of *RcHSP17.8* enhanced SOD activity (Jiang et al., 2009) whereas overexpression of *ZmHSP16.9* in tobacco enhanced POD, CAT, and SOD activity (Sun et al., 2012). Altogether, it may be hypothesized that the HSP proteins positively affect thermotolerance by protecting ROS scavenging protein conformation and activity, resulting in a lower ROS concentration (Kong et al., 2014a).

An alternative link between DnaJ proteins and ROS scavenging was suggested by Zhou et al. (2012). They showed that *Arabidopsis* AtDjB1 knockout plants (*atj1-1*) were more sensitive to heat stress than wild-type plants. After heat shock, the knockout plants showed an increased concentration of H_2O_2 and other oxidative products as well as a decreased concentration of the antioxidant ascorbate (ASC; Mittler et al., 2004; Zhou et al., 2012). The viability of *atj1-1* knockout seedlings after heat stress was rescued by exogenous ASC application. This suggests that lower concentrations of the antioxidant in *atj1-1* knockout plants resulted in increased H_2O_2 concentrations leading to a decreased thermotolerance (Zhou et al., 2012). As the underlying cause, the authors hypothesize a link with the electron transport chain (ETC). AtDjB1 directly interacts with a mitochondrial HSP70 and stimulates ATPase activity (Zhou et al., 2012), a mechanism which is conserved among several kingdoms (Qiu et al., 2006). AtDjB1 knockout potentially leads to the accumulation of cellular ATP, which feedback inhibits ETC. Because the last step of ASC synthesis is linked to the ETC (Bartoli et al., 2000), decreased ETC results in decreased ASC concentration and, consequently, the accumulation of H_2O_2 (Zhou et al., 2012).

Although it is unclear whether there is a specific interaction between HSPs and the ROS scavenging machinery or that HSPs generally maintain protein functions, these results indicate that upon heat stress, accumulation of ROS is reduced via HSP-supported ROS scavenger activity.

A POSITIVE FEEDBACK LOOP INCLUDING HSFs, ROS SCAVENGING GENES, AND MIRNA398

In contrast to the positive effects of ROS reducing mechanisms on heat stress tolerance, an *Arabidopsis* study provided evidence linking enhanced ROS accumulation to higher stress tolerance (Guan et al., 2013). The research indicated the existence of a positive a feedback loop, whereby heat and ROS allow for further ROS accumulation, depending on the actions of microRNA398 (*miRNA398*). *miRNA398* expression was found to be induced within 1 h and reach its peak 2 h after heat stress. The *miRNA398* promoter region contains a putative HSE, and chromatin immune-precipitation assays revealed direct binding of HSFA1b and HSFA7b to the HSE promoter region under heat stress. Thus, association of these HSFs to the promoter region seems to be responsible for the induction of this miRNA upon heat stress (Guan et al., 2013). miRNA398 negatively regulates the expression of three target genes: *CSD1*, *CSD2*, and *CCS* (Guan et al., 2013). *CSD1* and *CSD2* genes are isoforms of copper/zinc-SOD scavenging genes which are located in the cytoplasm and chloroplasts, respectively (Bowler et al., 1992; Kliebenstein et al., 1998) and *CCS* is a copper chaperone encoding gene, which delivers copper to both *CSD* genes (Cohu et al., 2009). Consequently, *CSD1*, *CSD2*, and *CCS* are down-regulated during heat stress, allowing further ROS accumulation. This pathway acts in an autocatalytic manner, as H_2O_2 in turn promotes expression of various HSFs, including *HSFA7b* (Guan et al., 2013). Accumulation of ROS seems to be an unfavorable response for the plant to survive heat stress. However, comparison of wild-type and *csd1*, *csd2*, and *ccs* mutants plants revealed higher heat tolerance in mutant plants while transgenic plants over-expressing *miR398*-resistant versions of *CSD1*, *CSD2*, or *CCS* were hypersensitive to heat stress (Guan et al., 2013).

These unexpected outcomes may be explained by the increases in oxidative power for helping activate the primary set of HSFs at the start of the heat response. In contrast to Guan et al. (2013); Sunkar et al. (2006) found that lack of *miRNA398* enhances tolerance to some other stress factors, high light and chemically induced ROS, via enhanced expression of *CSD1* and *CSD2* (Sunkar et al., 2006). Therefore, it seems that the benefit of reduced SOD activity is heat-specific, potentially due to the importance of high HSF activity in the first hours of the response to this stress.

A MULTI-LEVEL INTERACTION MODEL

A number of recent studies have provided evidence for connections between HSFs, HSPs, ROS, and ROS scavengers upon heat stress. Here, we propose a comprehensive model on the relations between the various components to explain a large proportion of the observations (**Figure 1**). Through contrasting effects on ROS scavenging activity, heat shock induces a short-term positive (roughly, within the first few hours of heat stress) and a long-term negative feedback loop (after the first few hours of heat stress) on the HSF signaling pathway. The proposed complexity of the heat-stress response network is mirrored in some of the counter-intuitive observations, such as enhanced heat tolerance in certain scavenger mutants (Rizhsky et al., 2002; Vanderauwera et al., 2011). However, analogous to the proposed *miRNA398* mechanism, constitutively, slightly elevated ROS levels in such mutants may result in a primed

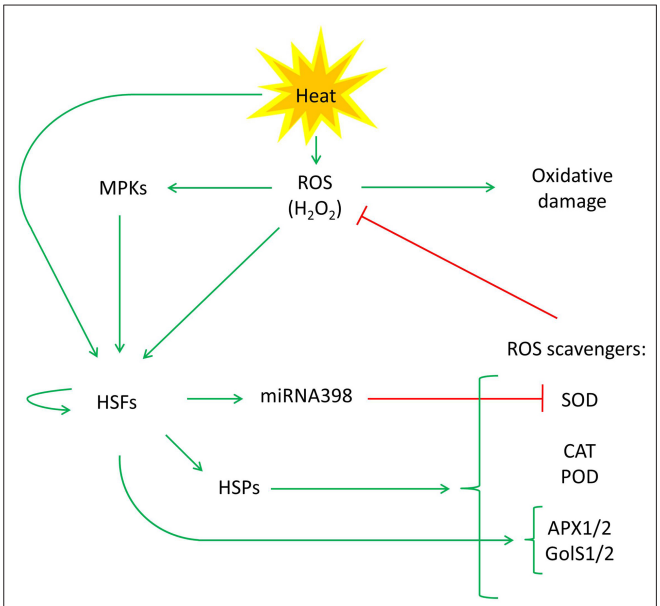


FIGURE 1 | Model describing crosstalk between the HSF/HSP and ROS pathways in the heat stress response. In addition to directly activating HSFs, high temperature leads to the accumulation of ROS. This, in turn, leads to a further activation of HSFs, either directly or indirectly via activation of a MAPK pathway. The HSFs bind to HSE in the promoter region of *HSF*, *HSP*, *miRNA398*, and ROS scavenging genes. The miRNA398-dependent down-regulation of a subset of SOD scavengers might play a role in the rapid ROS accumulation upon exposure to heat. This would support the activation of HSFs and thereby boost the induction of the heat-stress response in the short term. In the longer term, the induction and stabilization of other scavengers would start to suppress ROS levels to avoid excessive cellular damage.

state and, as a consequence, a stronger and/or faster response to a heat treatment. Guan et al. (2013) indeed showed that knockout mutations in *CSD1* and *CSD2* were accompanied by constitutively higher levels of HSF and HSP transcripts. In accordance, the importance of ROS at early heat response was shown by Volkov et al. (2006): a rapid oxidative burst of ROS during the first 15 min of the heat shock stimulates HSF DNA-binding and is essential for the induction of heat responsive gene expression, e.g., of *HSPs* and *APX2* (Volkov et al., 2006). The typical "late" high mobility HSE-binding complexes, formed after 2 h, were shown to be ROS independent (Lohmann et al., 2004; Volkov et al., 2006), which is in accordance with the production of anti-oxidants and ROS scavengers reducing the ROS overload after the early onset of the HSR (Chaitanya et al., 2002; Wahid et al., 2007; Frank et al., 2009; Dong et al., 2015). Nevertheless, if plants are continuously exposed to heat stress, the activity of some antioxidants and scavengers, e.g., APX and CAT, decreased after 3 days of heat stress in tomato, alfalfa and tobacco cell cultures (Wu et al., 2012; Li et al., 2013; Sgobba et al., 2015). The changes of these components of the antioxidant system were ascribed to the impaired health and growth of plants under long term heat stress and are different from short term heat stress (de Pinto et al., 2015).

Importantly, the model described here refers specifically to the complexity of events after a short term heat shock; its applicability to other types of heat stress, e.g., mild levels of heat stress, which only affect plant physiology in the long term, is not evident and more research will be necessary in order to clarify how the HSF/HSP and ROS systems behave under those circumstances. Also, it should be noted that the proposed model is not stand-alone and will interact with other factors, such as phytohormones. Abscisic acid (ABA), salicylic acid (SA) and ethylene have all been implicated in the heat response and can induce the production of ROS (Kwak et al., 2006; Foyer and Noctor, 2009). While a number of phytohormone-related mutants show impaired tolerance to heat (Larkindale et al., 2005), application of these hormones may enhance thermotolerance via an effect of ROS. SA application, for example, enhanced SOD activity and *HSP* expression during heat stress (Clarke et al., 2004; He et al., 2005). Dedicated analysis of the role of hormones during the first hours of heat treatment should clarify their putative positions in the response model.

The model may well have broader applicability then to the heat response only (Jiang and Zhang, 2002; Jammes et al., 2009; Bartoli et al., 2013; Wang et al., 2014b; Hossain et al., 2015). Not only are ROS accumulation, signaling and scavenging thought to occur and play a role in myriad other stress responses (Mittler, 2002; Hossain et al., 2015), but so is HSP activity (Pastori and Foyer, 2002; Banti et al., 2008; Pucciariello et al., 2012). HSPs are also induced upon water stress, salinity and osmotic stress, cold, anoxia, UV-B light, and oxidative stress (Vierling, 1991; Waters et al., 1996; Wang et al., 2004; Loreti et al., 2005; Swindell et al., 2007). Furthermore, overexpression of various HSFs enhanced tolerance to abiotic stresses other than heat, including salt, drought, osmotic, and anoxic stress (Bechtold et al., 2013; Chauhan et al., 2013; Shen et al., 2013; Pérez-Salamó et al., 2014). Also, tomato plants overexpressing the DnaJ/HSP40 *LeCDJ1* showed both higher heat and chilling tolerance (Kong et al., 2014a,b) and overexpression of BRZ-INSENSITIVE-LONG HYPOCOTYLS 2 (BIL2), a mitochondrial-localized DnaJ/HSP40 family member, enhanced resistance against salinity and high light stress (Bekh-Ochir et al., 2013). The role of both the oxidative stress and HSF/HSP systems in multiple stress responses might explain the phenomenon of cross-acclimation, where exposure to a certain stress factor improves tolerance to a subsequent different stress factor (Banti et al., 2008, 2010; Chou et al., 2012; Byth-Illing and Bornman, 2013; Hossain et al., 2015).

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REVIEW
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Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms

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In field conditions, plants are often simultaneously exposed to multiple biotic and abiotic stresses resulting in substantial yield loss. Plants have evolved various physiological and molecular adaptations to protect themselves under stress combinations. Emerging evidences suggest that plant responses to a combination of stresses are unique from individual stress responses. In addition, plants exhibit shared responses which are common to individual stresses and stress combination. In this review, we provide an update on the current understanding of both unique and shared responses. Specific focus of this review is on heat–drought stress as a major abiotic stress combination and, drought–pathogen and heat–pathogen as examples of abiotic–biotic stress combinations. We also comprehend the current understanding of molecular mechanisms of cross talk in relation to shared and unique molecular responses for plant survival under stress combinations. Thus, the knowledge of shared responses of plants from individual stress studies and stress combinations can be utilized to develop varieties with broad spectrum stress tolerance.

Keywords: tailored response, unique adaptation mechanisms, drought, heat, pathogen infection, concurrent stress

Introduction

Under field conditions, plants are concurrently exposed to a number of abiotic and biotic stresses. Stress combinations instead of individual stresses have been recognized as realistic threats faced by plants (Rizhsky et al., 2004; Mittler, 2006; Kissoudis et al., 2014; Suzuki et al., 2014; Mahalingam, 2015; Ramegowda and Senthil-kumar, 2015). Therefore, for development of plants with better adaptation under field conditions, focus should now be diverted toward understanding plant responses under combined stress conditions. Simultaneous occurrence of different biotic and abiotic stresses results in deployment of stress-adaptation strategies which are different and sometimes contrasting to those seen under individual stresses. For example, under combined drought and heat stress, *Arabidopsis thaliana* plants accumulate sucrose instead of proline (Rizhsky et al., 2004). The enhanced transpiration to cool leaf surface during heat stress aggravate the effects of concurrent drought and salinity because increased transpiration rate leads to more water loss and increased uptake of salts (Rizhsky et al., 2002; Mittler, 2006). Concurrent occurrence of an abiotic

stress with a biotic stress either aggravates or inhibits the effect of latter leading to either enhanced or reduced susceptibility to pathogens (Audenaert et al., 2002; Mohr and Cahill, 2003; Ton and Mauch-Mani, 2004; Melotto et al., 2006; Adie et al., 2007; Asselbergh et al., 2008; Ramegowda and Senthil-kumar, 2015). Thus, abiotic stresses can strongly modulate plants tolerance or susceptibility toward pathogen by different mechanisms which include trade-off between biotic and abiotic stress responses, and lead to modification in plant–pathogen interactions. This presents the need to study physiological and molecular responses of plants under abiotic and biotic stress combinations in order to understand plants tolerance against stress combinations.

The abiotic and biotic stress signaling networks of plants consist of several interacting pathways (Knight and Knight, 2001; Smekalova et al., 2014). Different abiotic and biotic stress conditions lead to some common physiological and molecular processes in plants apart from the unique responses. Plant adaptation strategy to a combination of two stresses consists of both "shared" and "unique" response. Shared responses refer to the molecular and physiological responses which are common to the two different stresses and unique responses are the ones which are specific to the individual stresses or the stress combinations (Rizhsky et al., 2002, 2004; Atkinson et al., 2013; Narsai et al., 2013; Prasch and Sonnewald, 2013; Sewelam et al., 2014; Supplementary Figure 1). Shared mechanisms constitute a considerable portion of plants response to both individual and combined stresses. These mechanisms include production and detoxification of reactive oxygen species (ROS), calcium-, phytohormone-, and MAPK-signaling pathway (Xiong and Yang, 2003; Li et al., 2008; Atkinson and Urwin, 2012; Suzuki et al., 2012, 2014; Rejeb et al., 2014). The shared responses are general physiological adaptation of plants and can guard them against multiple individual stresses. Some unique adaptation strategies tailored for stress combinations have been identified in the recent reports (Mittler, 2006; Atkinson et al., 2013; Choi et al., 2013; Prasch and Sonnewald, 2013). For example, combination of heat stress and virus infection led to up-regulation of cytosolic invertases instead of cell wall bound invertases (Prasch and Sonnewald, 2013).

Among different stress combinations that occur in field conditions, heat and drought stress and their interaction with pathogens are the most studied (Rizhsky et al., 2002, 2004; Mittler, 2006; Prasad et al., 2011; Bostock et al., 2014; Rejeb et al., 2014; Suzuki et al., 2014; Pandey et al., 2015; Ramegowda and Senthil-kumar, 2015). Therefore, taking these stresses as representatives of abiotic–abiotic and abiotic–biotic stress combinations, we enumerate the unique and shared responses exhibited by plants under drought–heat, drought–pathogen, and heat–pathogen combinations. We also provide a comparison of the overlap between cross talk of signaling pathways identified from multiple individual stress studies and the shared responses identified from combined stress transcriptome studies. Such overlapping responses can be a source of potential stress tolerance traits that can be engineered into crops to confer multiple stress resistance into plants.

Delineation of Shared and Unique Responses in Abiotic Stress Combinations

When two stresses occur concurrently, the adaptation strategy of plants to stress combination is governed by the interaction of two stresses which is conceived by plants as a new state of stress (Mittler, 2006). Thus, adaptation strategies of plants to combined stress may be different from that of two individual stresses. The overall effect of stress combination on plants depends largely on the age of plant, the inherent stress-resistant or susceptible nature of plant and severity of two stresses involved. Plant responses to stress combination are majorly determined by the more severe stress (dominant stressor, **Figures 1B**, **2B**) such that the physiological and molecular processes of plants subjected to combined stress resemble with those observed under more severe individual stress.

The shared responses under combined stresses constitute the generic morpho-physiological and molecular events evoked by both stresses constituting stress combination (Supplementary Table 1). For example, drought, salinity, and chilling induce osmotic effect on plants resulting in induction of common physiological processes, one of which is accumulation of osmoprotectants (Chinnusamy et al., 2004). The other stress induced response shared by almost all abiotic stress conditions is the production of ROS. Heat and salt stress are known to commonly affect the transport and compartmentation of ions in plants (Munns, 2002). Drought and salinity stress evoke the generic response of creating a physiological water deficit in plants. Additionally, both stresses cause decreased CO_2 diffusion into chloroplast due to reduced stomatal opening leading to reduced carbon metabolism.

In addition, some physiological traits are unique to individual drought, heat, salinity and chilling stress (Supplementary Table 1). For example, in salinity and chilling stress, ion-compartmentation and regulation of ice nuclei formation, respectively, are the unique responses (Chinnusamy et al., 2004). Salinity stress specifically disturbs the ion homeostasis by increased Na^+ and reduced K^+ uptake. Similarly, heat stress causes changes in membrane fluidity, affecting ion transporters, and pumps thereby disrupting ion transport (Plieth et al., 1999; Conde and Chaves, 2011).

The interaction between two stresses can either be additive or antagonistic to each other. The combination of drought and salinity is an example of additive interaction between the two stress conditions (Supplementary Table 1). Concurrent drought and salinity affected the growth of *Hordeum spontaneum* (wild variety of barley) more severely than the individual stresses (Ahmed et al., 2013). The individual and combined drought and salt stress led to drastic inhibition of net photosynthetic rate, stomatal conductance, and enhanced oxidative damage. Combined stress also resulted in enhanced reduction of chlorophyll b as compared to that observed under individual stresses. Overall, interaction between the two stress conditions was found to be additive for almost all the physiological parameters resulting in enhanced damage to plants under stress combination. Some differences in responses to individual and

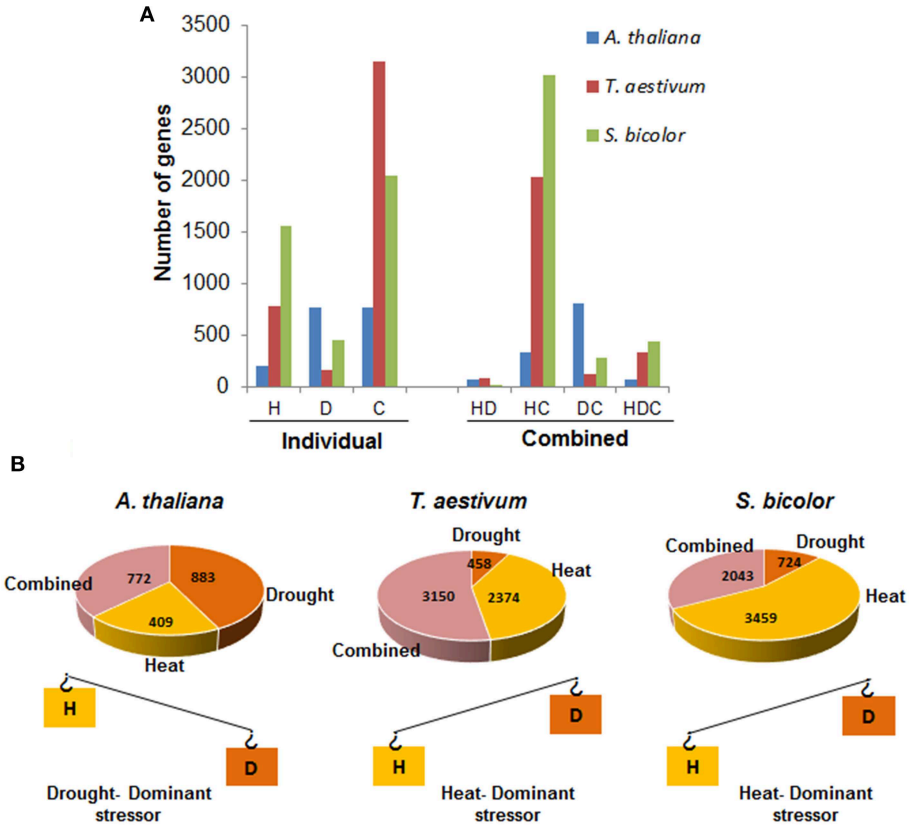


FIGURE 1 | Representation of unique and shared responses and the "dominant stressor" concept in *A. thaliana*, *T. aestivum*, and *S. bicolor* under combined heat and drought stress. (A) H, D, and C denote the number of genes modulated (refer to both up- and down-regulated) exclusively under heat, drought, and combined heat and drought stress, respectively. HD, HC, DC, and HDC represent the commonly regulated genes under heat and drought stress, heat and combined, drought and combined stresses, and all the three stresses, respectively. The figure is a graphical representation of the data (number of genes modulated under the different stress condition) provided in three independent cDNA array studies in *A. thaliana*, *T. aestivum,* and *S. bicolor* by Rizhsky et al. (2004), Aprile et al. (2013), and Johnson et al. (2014), respectively. **(B)** Representation of the "dominant stressor concept" under combined stress. The rectangles represent heat and drought stress. In a given stress combination, two stresses involved differ in severity of impact on plants. The severity of the two stresses is represented by "see saw." In case of *A. thaliana*, the molecular responses seen are drought specific with a maximum overlap between genes modulated under drought and combined stress. In *T. aestivum*, the number of heat stress specific genes outweighs the number of drought specific genes. However, the number of combined stress-specific genes is far greater than the individual stress specific genes and molecular response to the combined stress conditions is mostly unique in this plant. In case of *S. bicolor*, the number of genes specific to heat stress outweighs the number of drought stress specific genes. The genes commonly modulated (both up- and down-regulated) under heat and combined stress forms the maximum share in the combined stress response. H, heat; D, drought; C, combined stress. The pie chart represents the molecular response of plants to the combined stress and the area denotes the number of genes modulated under each category.

combined stress have also been noticed. For example, combined drought and salt stress led to enhanced Na^+ accumulation in roots as compared to leaves and stems whereas under salinity stress Na^+ preferably accumulated in shoots (Ahmed et al., 2013).

The combined stress mitigation strategy of plants also constitutes some unique morpho-physiological processes which makes the overall response of plants to stress combination different from that seen under individual stresses (Supplementary Table 1). For example, although both heat and salt stress are damaging to plants, concurrent salinity with heat stress enhanced salt tolerance of *Solanum lycopersicum* (Rivero et al., 2014). The combined heat and salt stress led to Na^+ accumulation in roots rather than in leaves and shoots. Thus, heat stress resulted in salinity tolerance by inhibiting uptake of Na^+ ions and by directing the accumulation of Na^+ to roots rather than shoots (Rivero et al., 2014). *S. lycopersicum* plants treated with combined heat and salinity stress accumulated the osmoprotectants glycine betaine and trehalose in large amounts instead of proline which is a predominant osmoprotectant accumulated in plants challenged with salinity stress only. Under individual salt stress, activity of the enzyme 1-pyrroline-5-carboxylate synthase (P5CS) increased indicating the synthesis of proline from glutamate. However, under combined stress, a decrease in the activity of P5CS and increase in the activity of ornithine aminotransferase (OAT) was observed suggesting that under the combined stress, proline synthesis occurred from ornithine through ornithine aminotransferase (OAT). The occurrence of proline synthesis through OAT has been observed in plants under some conditions (Krell et al., 2007, reviewed in Verslues and Sharma, 2010). Taken together, enhanced accumulation of glycine, betaine, and

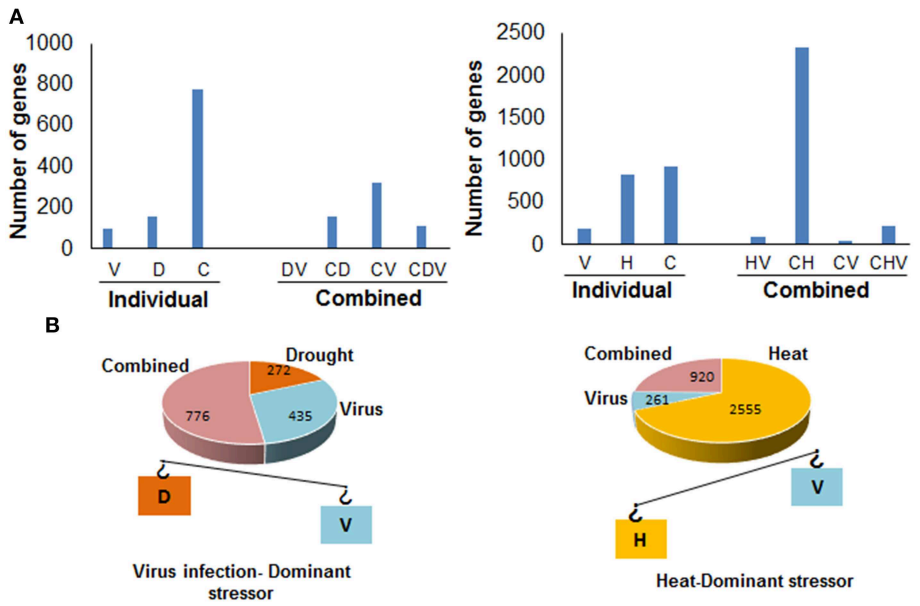


FIGURE 2 | Unique and shared responses and the "dominant stressor" concept in *A. thaliana* under combined heat, virus, and drought stress. **(A)** Representation of unique and shared responses of *A. thaliana* under drought, virus infection, and their combination(left) and heat, virus, and their combination (right). The bar diagram (left) represents the number of genes modulated exclusively under virus (V), drought (D), combined heat and drought stress (C) as well as the number of commonly regulated genes under virus infection and drought stress (DV), drought and combined (DC), virus and combined stresses (VC), and all the three stresses (CDV). The bar diagram at the right represents the number of unique genes modulated exclusively under virus (V), heat (H), combined heat and virus stress (C) as well as the number of genes commonly regulated under heat and virus infection (HV), heat and combined (CH), virus and combined stresses (CV), and all the three stresses (CHV). **(B)** The figure represents the dominant stressor concept. Drought and virus stress are represented by orange and blue rectangles. In this case, virus infection has more effect on the gene expression of *A. thaliana* plants. The number of genes unique to combined stress is far greater than that of individual stress genes and molecular response to the combined stress conditions is mostly unique. Heat and virus stress are represented by yellow and blue rectangles. In this case, heat stress has more effect on the gene expression. The number of heat and combined stress genes are nearly same and molecular response to the combined stress conditions mostly consists of genes commonly modulated under heat and combined stress. The figure is a graphical representation of the data provided in microarray study by Prasch and Sonnewald (2013). H, heat; D, drought; C, combined stress.

trehalose improved tolerance of plants exposed to combined stress (Rivero et al., 2014).

To further explicate the distinct and shared mechanisms of plants response to individual and combined abiotic stress conditions, we selected drought and heat stress combination as an example and hereby describe their effects on physiological and molecular processes. The enhanced damage incurred by heat and drought stress combination as compared to individual stresses is due to the fact that heat and drought share a number of physiological traits and the overall effect of the two stresses on plants is additive and leads to aggravated stress effects. However, the two stress conditions also evoke unique responses as outlined in sections below. In a study conducted by Rollins et al. (2013) on two genotypes (Arta and Keel) of *Hordeum vulgare*, drought stress was found to have stronger effect on traits like plant height, biomass and spike number whereas reproductive traits like number of aborted spikes and kernel weight were more affected by heat stress (Rollins et al., 2013).

Morpho-physiological Responses of Plants to Drought and Heat Stress Combination

The mechanism of adaptation to drought and heat stress varies considerably which results in unique morphological responses under these stresses. Plants adapt to drought stress by minimizing water loss and increasing water uptake. This is achieved by reducing leaf number, area, and increasing root growth by plants. On the other hand, long term adaptive strategies for heat tolerance encompass decreasing the leaf canopy temperature through increased transpiration by increasing leaf number and area. Drought and heat stress have contrasting effect on some morphological processes. For example, leaf expansion, leaf number, and size were reduced due to drought stress (Alves and Setter, 2004) while heat stress led to increase in leaf number and leaf elongation (Bos et al., 2000; Prasad et al., 2006). Heat stress was shown to decrease number, length, and diameter of roots but moderate drought stress increased root growth which is required for water uptake from deeper layers of soil (Prasad et al., 2008). Drought stress reduced the leaf area (Poorter et al., 2009) whereas heat stress led to production of thinner leaves with higher specific leaf area (Luomala et al., 2005; Poorter et al., 2009). On the other hand, biomass allocation to roots increased in response to drought while heat stress enhanced reproductive allocation. During combined stress, some of the responses were shared with drought and some with heat stress. For example, leaf size was found to increase, leaf number was decreased, and biomass allocation was seen to occur preferably in roots and

reproductive parts under combined stress in *A. thaliana* (Vile et al., 2012).

Unlike the contrasting effect of drought and heat stresses on vegetative growth of plants, drought, heat and their combination had similar effects on the reproductive development of plants. These stresses have been shown to delay flowering, reduce grain weight and yield of *Triticum aestivum* (Savin and Nicolas, 1996; Prasad et al., 2006; Pradhan et al., 2012). The combined stress conditions were found to be more detrimental than the individual stresses in reducing yield of *H. vulgare* (Rollins et al., 2013). Drought and drought–heat combination reduced the spike number, which was not affected by heat stress. Similarly heat and combined stress increased the number of aborted kernels (Rollins et al., 2013). However, drought stress did not cause any change in size and nutrient accumulation in plant endosperm while combined heat and drought stressed plants produced enlarged endosperm with higher accumulation of starch and protein (Szucs et al., 2010).

Heat and drought stress differentially affect stomatal characteristics. Under combined heat and drought stress, stomata remained closed leading to increased leaf temperature of *Nicotiana tabacum* plants (Rizhsky et al., 2002). Plants under combined stress minimize leaf temperature in a unique way. Vile et al. (2012) reported that *A. thaliana* plants exposed to combined heat and drought stress adapt to heat stress by adjusting leaf orientation through increasing their leaf insertion angle. *A. thaliana* plants exposed to individual and combined heat and drought stresses showed increased stomatal density in response to drought stress which was reduced in response to heat stress. Under combined stress, however, stomatal density decreased (Vile et al., 2012). This suggests that in case of a stress combination constituting of two stresses differing in their severity, plant's physiological processes are apparently determined by the more severe stress. The combined heat and drought stress led to higher leaf temperature in two genotypes of *T. aestivum*, Ofanto and Cappelli which differ in water use efficiency (WUE). Cappelli is characterized by higher WUE and lower stomatal conductance compared to Ofanto. The combined stress led to a higher leaf temperature in Cappelli as compared to Ofanto (Aprile et al., 2013). This indicates that the effect of combined stress also varies among the genotypes of a particular plant species.

The combined heat and drought stress have been shown to affect a number of physiological processes more severely than the individual stresses. Rizhsky et al. (2002) reported that *N. tabacum* plants exposed to simultaneous heat and drought stress led to greater suppression of photosynthetic activity as compared to individual stresses. Similarly, as compared to individual stresses, combined heat and drought stress lead to enhanced lipid peroxidation in *Lotus japonicus* (Sainz et al., 2010) and severe abnormalities in the ultra-structure of chloroplasts and mitochondria in *T. aestivum* (Szucs et al., 2010; Grigorova et al., 2012). The combined stress also led to greater reduction in photosynthetic activity and enhanced production of ROS in *Populus yunnanensis* (Li et al., 2014) and greater diminution in root viability and photochemical efficiency of photosystem II (PS-II) in *Festuca arundinacea* (Jiang and Huang, 2001). The reduction in photosynthetic activity is a response shared between the individual heat and drought stresses. However, photosynthesis is less affected by heat stress and only high temperatures (>40°C) are known to be detrimental. Heat stress mediated reduction in photosynthesis mainly occurs due to enhanced photorespiration (Prasad et al., 2008), reduced RuBisCO activity (Salvucci and Crafts-Brandner, 2004), and reduced PS-II activity (Yang et al., 2007). Heat stress did not reduce photosynthetic activity of tobacco plants, but drought stress and combined heat and drought stress led to more than 80% reduction in photosynthetic activity (Rizhsky et al., 2002). The RuBisCO activity in *Cicer arietinum* leaves was increased with heat stress and decreased with drought stress and combined stress (Awasthi et al., 2014). Similarly, Sainz et al. (2010) reported significant disruption in PSII function when *L. japonicas* plants were subjected to combined heat and drought stress. Jiang and Huang (2001) compared the response of *F. arundinacea* and *Lolium perenne* to combined heat and drought stress and observed that stress combination led to enhanced reduction in photochemical efficiency of PS-II, as compared to individual stresses.

The modulation of mitochondrial respiration is also a shared response under drought and heat stress (Prasad et al., 2008). The rate of dark respiration increased with increasing temperatures whereas drought stress reduced plant respiration rates (Bryla et al., 2001). Similar observations were made by Rizhsky et al. (2002) who found that drought stress led to suppression of respiration but heat and combined drought and heat stress led to enhancement of respiration in *N. tabacum* leaves.

Molecular Response of Plants to Heat and Drought Stress Combination

The transcriptomic analysis of combined heat and drought stressed *A. thaliana*, *N. tabacum*, *H. vulgare*, and *T. aestivum* by different groups have revealed a combination of shared and unique transcriptomic changes (Rizhsky et al., 2002, 2004; Rampino et al., 2012; Johnson et al., 2014). However, the transcriptomic changes are dependent on the plant type, duration and severity of stresses. In *A. thaliana* plants subjected to combined drought and heat stress (Supplementary Table 3), the molecular response under combined stress was dominated by drought specific transcriptomic changes and consisted of 208, 765, and 772 genes specifically modulated (refer to both up- and down-regulated) under heat, drought and combined stress, respectively. Furthermore, 77, 806, and 332 genes were commonly regulated under drought and heat, drought and combined stress and heat and combined stress, respectively (Rizhsky et al., 2004). In case of *T. aestivum* (var. Ofanto) plants heat stress response was found to be the most dominating (Supplementary Table 3). The combined stress led to modulation of 5645 transcripts out of which, 2037 and 121 were common with heat and drought stress, respectively, and 3150 transcripts were unique to combined stress. The transcripts modulated specifically under heat and drought stress totaled 159 and

779, respectively, with 90 transcripts commonly regulated under heat and drought stress response (Aprile et al., 2013; **Figure 1**). Rampino et al. (2012) studied gene expression profile of *T. aestivum* plants by cDNA amplified fragment length polymorphism (cDNA-AFLP). The study revealed that 380 genes were modulated in all the three stress conditions. Out of 242 up-regulated genes, 44, 15, and 90 genes were specifically induced by heat, drought and combined stress, respectively. While 18 genes were commonly up-regulated in heat and drought stress, 51 and 24 up-regulated genes were common among individual heat, drought and the combined heat and drought stress, respectively. Therefore, in case of *T. aestivum*, the response under combined stress constituted more of unique than shared response. Similarly, transcriptomic analysis of individual and combined stressed *Sorghum bicolor* plants (Supplementary Table 3) using DNA microarray revealed that 1554, 448, and 2043 genes were specifically modulated under heat, drought and combined stress whereas 18, 3021, and 286 of genes were found to be common under heat and drought, heat and combined stress and drought and combined stress, respectively (**Figure 1**). A total of 438 genes were commonly regulated under individual drought, heat and combined drought and heat stress conditions (Johnson et al., 2014). Thus, it is evident that the number of heat stress specific genes outweighs the number of drought specific genes in *T. aestivum* and *S. bicolor*. This may be due to the severe nature of the heat treatment. In case of *A. thaliana*, the molecular responses seen are drought specific. Moreover, in all the three plants, the number of combined stress-specific genes is more than the individual stress-specific genes showing thereby that the molecular response of these plants to the combined stress conditions is mostly unique. Plants have to maintain a balance between energy and resource allocation toward growth and stress adaptation. Thus, when simultaneously exposed to multiple stress conditions, they respond to the more damaging stress condition. This is evident from the gene expression studies which show that the molecular responses are more tuned toward heat stress in the above mentioned instances of *T. aestivum* and *S. bicolor* (**Figure 1B**).

The shared response under combined drought and heat stress constituted the induction of heat shock proteins (HSPs), ROS detoxification enzymes, and enzymes involved in photosynthesis and glycolysis (Rizhsky et al., 2002, 2004; Rampino et al., 2012; Johnson et al., 2014). Rizhsky et al. (2002) reported induction of genes encoding small HSPs (sHSPs), HSP70, HSP90, and HSP100 under individual as well as combined stress in *N. tabacum*. Apart from HSPs, the other genes constituting shared response under individual and combined stress include late embryogenesis 7 (*LEA7*) genes, dehydrin, photosynthesis related genes, and genes encoding enzymes involved in pentose pathway and anthocyanin biosynthesis (Rizhsky et al., 2004). Functional classification of genes commonly regulated under individual and combined drought and heat stress response in *A. thaliana* revealed that the largest class of commonly regulated genes was constituted by those involved in different metabolic processes (Supplementary Figure 2A). Chaperones formed the largest class of proteins commonly regulated under heat and combined stress. Transferases, oxidoreductases, and hydrolases encoding genes comprised the largest class of commonly regulated genes between drought stress and combined stress response (Supplementary Figure 2B; Rizhsky et al., 2004).

Although combined and individual stress response of plants constituted a number of commonly regulated genes, differences were observed in their expression levels in individual and combined stress conditions i.e., the expression was tailored to combined stress condition. For example, when compared to individual drought and heat stressed plants, combined stressed plants exhibited higher induction of HSP coding genes (Rizhsky et al., 2002). Differences were also seen in the type of ROS detoxification genes induced under the three stress conditions reflecting stress dependent ROS-detoxification mechanisms. For example, heat stress induced cytosolic ascorbate peroxidase (*APX*) and thioredoxin peroxidase (*TPX*). Drought stress led to the induction of catalase (*CAT*) and glutathione peroxidase (*GPX*). However, under combined stress, genes encoding alternative oxidase (AOX), GPX, glutathione reductase (GR), copper–zinc superoxide dismutase (CuZnSOD), and glutathione S transferase (GST) were found to be specifically induced.

Some unique genes were also found in the individual and combined stress conditions. For example, Sb02g038425 [homologous to resistance to *Pseudomonas syringae* pv. maculicola 1 (RPM1) protein] was found to be up-regulated exclusively under heat stress. Sb01g021320 (homologous to LEA D34 protein) and Sb05g017950 (*H. vulgare* aleurone 22 [HVA22] like protein) were found to be up-regulated exclusively under drought and combined stress (Johnson et al., 2014). Similarly, combined drought and heat stress led to the induction of various stress related genes which were not induced under individual stresses. These included genes encoding pathogenesis related (PR) and phenylalanine ammonia lyase (PAL) proteins. Induction of transcript encoding WRKY transcription factors and ethylene response transcriptional co-activator (ERTCA) were also unique to combined stress (Rizhsky et al., 2002). Other genes specifically elevated under combined stress are receptor-like kinases, protein kinases (*MAP3K*), small GTP-binding proteins, MYB transcription factors, transporters, aquaporin membrane intrinsic protein (*MIP*, Rizhsky et al., 2004), and genes encoding transcription factor WRKY8, calcium transporter ATPase9, heat shock protein cognate 70, and a disease resistance related protein (Rampino et al., 2012). Another unique response seen under combined stress was the down-regulation of gene encoding glycolate oxidase, which was otherwise induced under drought stress (Rizhsky et al., 2002). The metabolic overview map generated through MapMan (Supplementary Figure 3) revealed the induction of genes involved in carbohydrate and lipid metabolism under combined drought and heat stress in *A. thaliana* plants (Rizhsky et al., 2004).

Plant Responses to Combined Biotic and Abiotic Stresses

Occurrence of abiotic stresses such as drought, heat, cold, salinity, ozone, ultraviolet (UV) radiation, and nutrient stress

dramatically alters the response of plants to biotic stresses. Similarly, interaction of plants with pathogens affects their responses to abiotic stresses. The outcomes of these interactions can either provide resistance or susceptibility toward any of the two stresses depending on the plant species, pathogen and stress intensity.

Abiotic stresses generally reduce some of obligate or biotrophic pathogen infection and severity of diseases (Schoeneweiss, 1975). For example, in *L. esculentum*, drought stress reduced infection of necrotrophic fungus *Botrytis cinerea* (causal agent of gray mold in tomato) by 50% and suppressed the biotrophic fungus *Oidium neolycopersici* (causal agent of powdery mildew in tomato) infection with concomitant two-fold increase in ABA compared to well-watered infected plants (Achuo et al., 2006). Conversely, hemibiotrophic pathogens can cause severe disease during drought stress even from low level of inoculum. For example, in *Carthamus tinctorius* drought stress increased the root rot caused by *Phytophthora cryptogea* (causal agent of root rot in safflower; Duniway, 1977). Long-term abiotic stress weakens plant defenses and causes enhanced susceptibility to pathogens (Amtmann et al., 2008; Goel et al., 2008; Mittler and Blumwald, 2010). This can also happen with increased colonization of pathogens in presence of abiotic stresses. For example, salinity increased colonization of roots by *Phytophthora cryptogea* in *Chrysanthemum morifolium* resulting in increased susceptibility of plants to the root rot (MacDonald, 1982). These evidences suggest that the outcome of combined abiotic stress and pathogen interactions may lead to increased severity of disease in the host plant.

Abiotic stresses can enhance disease resistance of plants through primed physiological adaptations (Kuwabara and Imai, 2009). For example, salicylic acid (SA) biosynthesis under cold temperatures with a corresponding induction of PR proteins minimized the impact of pathogens (Kim et al., 2013). Similarly, pathogen infection can also bring physiological adaptations in plants resulting in enhanced tolerance of plants to abiotic stresses. For example, in *A. thaliana*, *P. syringae* infection caused stomatal closure and prevented pathogen entry which resulted in reduced water loss from the infected plant, hence increasing the tolerance of plants to drought stress (Goel et al., 2008; Beattie, 2011). Similarly, infection of *A. thaliana* plants with soil borne fungal pathogen *Verticillium longisporum* (causal agent of wilt in thale cress) resulted in increased plant tolerance to drought stress due to *de novo* xylem formation resulting in enhanced water flow (Reusche et al., 2012). These evidences suggest that physiological adaptations caused by the prior stress can enhance tolerance of plants to the subsequent stress when plants are challenged with combination of biotic and abiotic stresses.

Presence of abiotic stress can arrest the infection ability of some pathogens. For example, salt-induced osmotic stress increased tolerance of *H. vulgare* plants to *Blumeria graminis* (causal agent of powdery mildew in barley) in a concentration dependent manner (Wiese et al., 2004). During this osmotic stress, papilla-mediated resistance resulted in callose deposition and this prevented fungal growth and infection. Similarly, presence of abiotic stress can increase the infection ability of some of the pathogens causing severe disease. For example, chilling increases susceptibility of *Gossypium* spp. to *Alternaria alternate* (causal agent of leaf spot in cotton), increasing leaf senescence and resulting in premature defoliation (Zhao et al., 2012). In *Oryza sativa*, low temperatures decreased resistance of plants to blast pathogen *Magnaporthe oryzae* (causal agent of blast in rice; Koga et al., 2004). Thus, the concurrent abiotic stress may directly modulate the plant-pathogen interactions leading either to enhanced or reduced disease in plants.

Among the abiotic and biotic stress combinations, drought-pathogen and heat-pathogen are the most studied stress combinations. In the following sections, physiological and molecular response of plants to these stress combinations are discussed.

Physiological Response of Plants to Combined Drought and Pathogen Stress

Interaction of drought and pathogens is mainly influenced by changes in the water potential of plants (Mattson and Haack, 1987; Boyer, 1995). Altered water potential by one stress can increase either the susceptibility or tolerance of plants to the subsequent stress. Drought induced reduction in plant water potential has negative effect on plant interaction with root pathogens. For example, drought led to reduced plant water status in *Phaseolus vulgaris* resulting in more susceptibility of plants to *Macrophomina phaseolina* (causal agent of charcoal rot disease in common bean, Mayek-Perez et al., 2002). It has been shown that *Nicotiana benthamiana* plants challenged with *Sclerotinia sclerotiorum* (causal agent of white mold in beans), exhibit severe cell death, whereas in the drought acclimated plants the extent of cell death was much reduced (Ramegowda et al., 2013). Evidences show the accumulation of abscisic acid (ABA) under combined drought and pathogen stress. For example, drought-stressed *S. lycopersicum* plants that exhibited enhanced resistance against *B. cinerea* also showed the accumulation of ABA (Achuo et al., 2006).

Pathogens can also lower the water potential of plant influencing its responses to drought stress. For example, *Xylella fastidiosa* (causal agent of Pierce's disease in grapes) causes pathogen-induced drought in *Vitis vinifera* by reducing water potential (Choi et al., 2013). One major defense response common to drought and pathogen infection is stomatal closure. Therefore, drought and pathogen-induced stomatal closure can have positive effect on plants under combined drought and pathogen infection (Sawinski et al., 2013). Similarly, drought tolerance of *A. thaliana* plants infected with vascular pathogen *V. longisporum* increased due to increased *de novo* xylem formation resulting in increased water flow (Reusche et al., 2012). The interactive effects of drought and pathogen on plants are discussed in detail by Ramegowda and Senthil-kumar (2015) and Pandey et al. (2015). The effect of concurrent drought on plant pathogen interaction has been discussed in detail by Pandey et al. (2015). Also, Ramegowda and Senthil-kumar (2015) have reviewed the tailored molecular strategies adopted by plants to deal with the stress combination.

Physiological Response of Plants to Combined Heat and Pathogen Stress

Similar to drought, heat stress can also lead to resistance or susceptibility of plants to pathogen depending on the stress intensity and duration. Heat stress facilitates pathogen spread and cause susceptibility to the diseases (Bale et al., 2002; Luck et al., 2011; Madgwick et al., 2011; Nicol et al., 2011). In wheat, higher mean temperatures observed over a 6 year experimental period correlated with heightened susceptibility to the fungus *Cochliobolus sativus* (causal agent of root rot in wheat, Sharma et al., 2007). In *N. tabacum* and *A. thaliana*, hypersensitive response (HR)—and resistance (*R*)—gene mediated defense responses to *P. syringae* pathovars (causal agent of brown spot in thale cress) and viral elicitors were compromised at high temperatures, allowing increased growth of these pathogens (Wang et al., 2009). Non-acclimation to heat stress causes more susceptibility of plants to pathogen. For example, ornamental plant roots directly exposed to 45°C soil temperatures increased severity of *Phytophthora infestans* (causal agent of root rot in ornamentals, MacDonald, 1991). Heat stress also imparts pathogen resistance. For example, *Cucumis sativus* seedlings exposed to brief heat shock of 50°C resulted in increased resistance to the fungal pathogen *Cladosporium cucumerinum* (causal agent of scab in cucumber; Stermer and Hammerschmidt, 1987). Temperature-dependent suppression of host resistance has been reported for *Tobacco mosaic virus* (TMV; causal agent of mosaic disease in tobacco) and *Tomato spotted wilt virus* (TSWV; causal agent of spotted wilt in tomato). TMV is able to overcome the *N*-gene mediated resistance at temperatures above 28°C in *N. tabacum* (Király et al., 2008), while TSWV is able to suppress TSW, a dominant gene-mediated resistance in *Capsicum chinense* plants at high temperatures (Moury et al., 1998). Thus, heat stress generally leads to suppression of host defense responses along with the other metabolic processes, thereby increasing their susceptibility to pathogens.

Molecular Responses of Plants under Combined Drought and Pathogen Stress

The transcriptome analysis of *A. thaliana* plants exposed to individual drought, *Turnip mosaic virus* (TuMV, causal agent of mosaic disease in crucifers) and combined drought and TuMV indicated the presence of both shared and unique molecular response in combined stressed plants (Prasch and Sonnewald, 2013). A total of 98 and 157 genes were unique to virus and drought stress whereas 776 genes were unique to combined stress indicating a major reprogramming of plants' defense response under combined stress. Only six genes were common in individual virus and drought stress modulated transcriptome. A total of 160 and 323 genes were commonly regulated under combined stress–drought and combined stress–virus treatment. Totally 112 genes were commonly modulated under all the three stress conditions (**Figure 2A**). Majority of the commonly regulated genes under combined drought–virus treatment and individual stresses were metabolism related genes (Supplementary Figure 4A). Functional categorization of these commonly regulated genes on the basis of protein classes revealed that the shared response was dominated by protein class constituting oxidoreductases and membrane transporters (Supplementary Figure 4B).

Down-regulation of photosynthetic genes and up-regulation of stress responsive genes constituted the major shared molecular response between individual and combined stressed plants. The combined stress treatment led to up-regulation of 72 stress specific genes as compared to 16 and 29 genes specifically up-regulated under individual drought and virus infection. Virus infection and combined stress treatment lead to up-regulation of *PR* genes. However, *PR* genes were down-regulated under individual drought treatment (Prasch and Sonnewald, 2013). The overview of the expression changes related to metabolic pathways in *A. thaliana* plants during the combined drought and virus infection using MapMan software (Supplementary Figure 5) revealed the up regulation of genes involved in carbohydrate and lipid metabolism. The genes involved in flavonoid metabolism were found to be strongly up-regulated (Prasch and Sonnewald, 2013).

The analysis of *Vitis vinifera* plants subjected to individual drought stress, *X. fastidiosa* infection and combined drought and *X. fastidiosa* infection also revealed down regulation of transcripts involved in photosynthesis, nutrient assimilation, and cellular homeostasis (Choi et al., 2013). The transcriptome analysis of plants in this case largely reflects the exacerbation of disease development by drought stress. Transcript analysis of individual and combined stress treated plants showed a time dependent transcriptional modulation. Whereas early response did not show major changes in the transcriptome, increased stress exposure led to modulation of nearly 700 genes. *X. fastidiosa* infection and drought stress led to some common changes in transcriptome which included up-regulation of ABA and JA synthesis-, pathogenesis related-, and phenylpropanoid and flavonoid biosynthesis-related genes (Choi et al., 2013).

The genes specifically up-regulated by bacterial infection included the ones encoding PR proteins, chitinases, thaumatin like proteins, and lipid-transfer proteins. A characteristic response to *X. fastidiosa* infection was the up-regulation of aquaporin gene which was not observed under drought only treatment. The bacterial infection also led to up-regulation of gene encoding galactinol synthase (GOLS) which is responsible for synthesis of osmoprotectants galactinol and raffinose. The response characteristically seen under combined stresses in *V. vinifera* plants consisted of early induction of ABA biosynthesis gene, 9-cis epoxycarotenoid dioxygenase (*NCED*), and calceneurin B like interacting protein kinase (*CIPK*). Overall, drought stress and bacterial infection in this case led to activation of ABA mediated drought response that led to enhanced development of disease (Choi et al., 2013).

Thus, in both the above instances, transcriptome of plants challenged with combination of drought and viral infection was more affected by the pathogen signifying the dominant effect of biotic over drought stress. In both cases, abiotic stress enhanced the susceptibility of plants to pathogen infection. In the latter case, however, pathogen produced effects similar to

drought stress as was reflected by up-regulation of ABA related genes and accumulation of osmoprotectants under individual bacterial treatment. In both instances, transcripts specifically modulated under combined stress treatment outnumbered the drought and pathogen specific as well as commonly regulated transcripts between the two stress conditions. This clearly shows that combined stress is perceived by plants as a new stress leading to major redirection of gene expression in the combined stressed plants.

Molecular Responses of Plants under Combined Heat and Pathogen Stress

Although physiological effects of combined heat and pathogen on plants has been studied in a number of cases (Bale et al., 2002; Sharma et al., 2007; Wang et al., 2009; Luck et al., 2011; Madgwick et al., 2011; Nicol et al., 2011), molecular response of plants exposed to combined heat and pathogen has been discussed only in a study by Prasch and Sonnewald (2013). In coherence with earlier reports (MacDonald, 1991; Wang et al., 2009), Prasch and Sonnewald (2013) reported that *A. thaliana* plants subjected to combination of heat and *Turnip mosaic virus* (TuMV) infection were more susceptible to viral infection. The authors observed that combination of heat and viral infection led to enhanced transcript accumulation of P3 gene, which is a marker for viral replication (Kim et al., 2010) suggesting more viral replication in combined stressed plants. Microarray analysis of individually and combined stressed *A. thaliana* plants revealed the presence of 190, 920, and 823 unique genes in the transcriptome of heat alone, virus alone, and combined stress treated plants, respectively. Out of the total modulated genes, 88 were commonly regulated under combined stressed and individual virus infected plants and 46 transcripts were common in combined stressed and individual heat stressed plants. The number of transcripts common between heat and combined stressed plants was far higher and was estimated to be 2340. This shows that molecular response of combined stressed plants was majorly governed by heat stress (**Figure 2B**). A total of 215 transcripts were commonly modulated under all the three stress conditions. Functional classification of transcripts commonly modulated under virus alone and combined stress treated plants revealed that the majority of commonly modulated genes belonged to class of metabolism related genes (Supplementary Figure 6). The virus alone, heat alone and combined heat and virus infection led to up-regulation of 29, 110, and 108 stress responsive transcripts, respectively (Prasch and Sonnewald, 2013). The individual and combined stressed plants shared molecular responses like down-regulation of photosynthetic genes, and differential expression of toll/interleukin1 receptor-nucleotide binding site and leucine rich repeat (TIR-NBS-LRR) genes (Prasch and Sonnewald, 2013).

The individually and combined stressed plants also showed some contrasting molecular responses. For example, virus infection led to up-regulation of *PR1*, *PR2*, *PR5*, whereas these genes were down-regulated under combined heat and virus infection further substantiating the heat mediated suppression of basal defense mechanism. The virus alone treatment also led to up-regulation of cell wall bound invertases. However, under heat and combined heat and virus infection, the expression of cell wall bound invertases was down-regulated and that of vacuolar and cytosolic invertases was up-regulated pointing toward the intracellular hydrolysis of sugars in heat stressed and combined stressed plants (Prasch and Sonnewald, 2013). The metabolic overview map generated through MapMan (Supplementary Figure 7) revealed the slight down regulation of genes involved in carbohydrate and lipid metabolism, photosynthesis and mitochondrial electron transport under combined heat and virus infection in *A. thaliana* plants (Prasch and Sonnewald, 2013).

Cross Talk between Abiotic and Biotic Stress Defense Response and Its Extrapolation to Combined Stress Response

Apart from the unique gene expression mediated by different stress conditions, there can be various points of cross talk between the stress signaling pathways (**Figures 3A,B**). As defined by Knight and Knight (2001) cross talk refers to "any instance of two signaling pathways from different stressors that converge." The signaling pathways for abiotic and biotic stresses share common elements including ROS (Møller et al., 2007; Wong and Shimamoto, 2009), calcium ions (Galon et al., 2010), transcription factors (Walley and Dehesh, 2010), hormones (Fonseca et al., 2009; Ton et al., 2009), and mitogen-activated protein kinase (MAPK) cascades (Pitzschke et al., 2009). Identification of cross talk between signaling pathways has been crucial in envisaging and strengthening our understanding on regulation of plants response to a particular stress condition. In recent years, the studies dealing with cross talk between abiotic and biotic stress signaling pathways have shed light on genes or gene products that are involved in two different stress conditions and thus are a part of shared response. The transgenic overexpression or down-regulation of these genes showed that they play crucial role in conferring tolerance to more than one abiotic or biotic stress conditions (Supplementary Table 2). Thus, these genes can be significant in providing resistance to plants against combined biotic and abiotic stresses.

Ca^{2+} and ROS are ubiquitous components of both abiotic and biotic stress signaling pathways. Genes involved in ROS and Ca signaling constitutes an important part of the shared molecular response of the combined stress plants (Rizhsky et al., 2004; Johnson et al., 2014). Analysis of different calcium dependent protein kinase (CDPK) genes in *T. aestivum* showed that out of 12 CDPKs which were responsive to *Blumeria graminis* pv. *tritici* (causal agent of powdery mildew in wheat) infection, eight also responded to abiotic stresses substantiating them as an important point of cross talk (Li et al., 2008). Similarly genes involved in ROS scavenging pathway like *APX,* have been shown to impart tolerance against various abiotic and biotic stresses (Sarowar et al., 2005; Choi and Hwang, 2012).

A number of transcription factors belonging to myeloblastosis (MYB) transcription factors family e.g., OsMYB4, ethylene responsive factors (ERF) like GmERF and botrytis-susceptible1

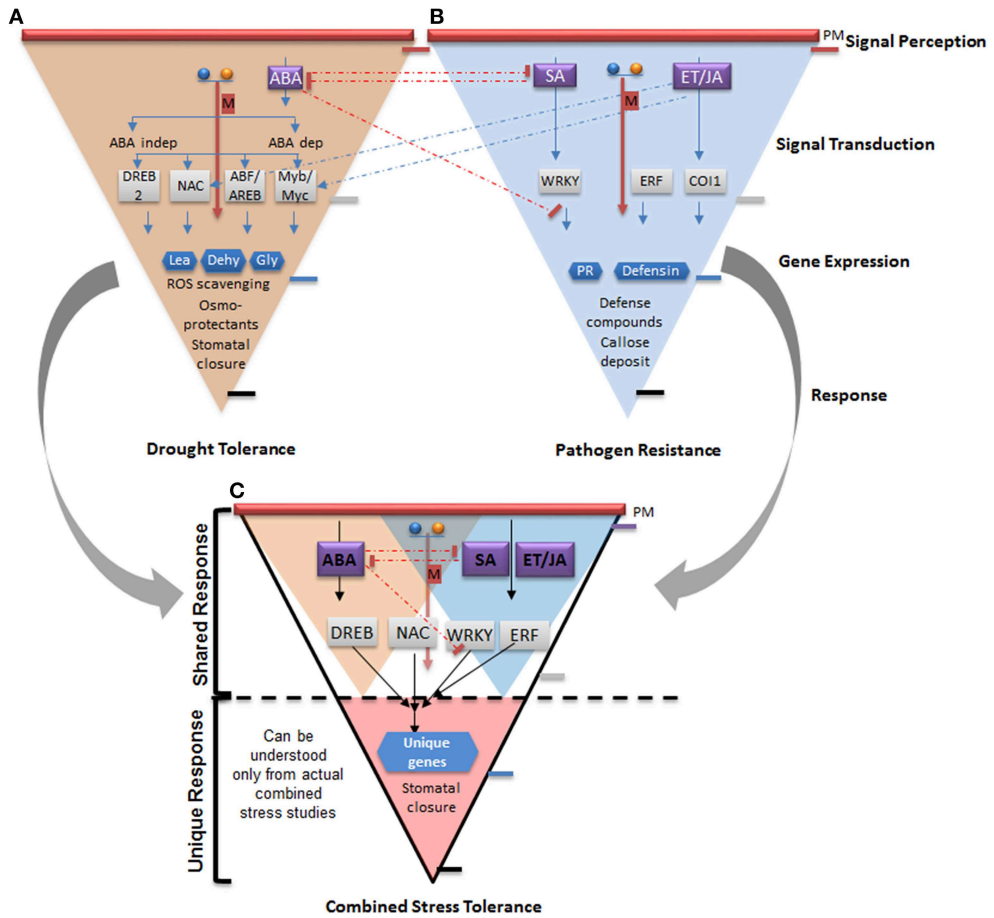


FIGURE 3 | Model representing cross talk between drought and pathogen stress signaling pathway from individual stress studies and its relation to shared and unique response pathway provoked under combined stress. The two inverted triangles in the upper panel represents the drought stress **(A)** and biotic stress signaling pathway **(B)** consisting of signal perception, signal transduction, gene expression and response generation steps with the representative gene products. The inverted shape represents the general response (wide upper side) at the stress perception and signal transduction stage culminating to a specific response (tapering lower end) contributing to tolerance to a particular stress. The response of the plants to combined drought and pathogen stress consist of both the shared (responses common to drought and pathogen stress) and unique responses as represented by the triangle **(C)**. The overall response of plants to the combined stress is governed by the key players involving ROS, Ca^{2+}, MAPKs, and the different transcription factors as well as some unique genes regulating the tailored responses. The shared responses can be deciphered by understanding the cross talk between the drought and the pathogen stress tolerance signaling networks whereas dedicated studies are required to understand the unique responses. The yellow colored small triangle represents the responses shared between drought and combined stress whereas the blue triangle represents the responses shared between pathogen and combined stress. The overlapping area between the two small triangles represents the responses shared by the drought and pathogen stress. The red colored triangle at the apex represents the unique response under the combined stress. The blue and yellow color spheres represent the calcium and ROS molecules. The red color arrow represents the MAPK pathway. The dashed arrows indicate the cross talk wherein the red colored dashed arrows represent the suppression and blue dashed arrows represent the activation of the respective stress responsive genes. PM, plasma membrane; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; M, MAPK pathway; ABA dep, ABA dependent pathway; ABA indep, ABA independent pathway; DREB, dehydration responsive element binding; NAC, NAM-ATAF and CUC 6 transcription factor; ABF, ABA binding factor; AREB, ABA responsive element binding; Myb, myeloblastosis; Myc, myelocytomatosis; ERF, ethylene responsive factor; WRKY stands for the first four amino acids (tryptophan [W], arginine [R], lysine [K], and tyrosine [Y] of the heptapeptide WRKYGQK, which is the hall mark of WRKY proteins; COI1, coronatine insensitive 1; LEA, late embryogenesis; Gly, glyoxylase; dehyd, Dehydrin; PR, pathogenesis related; ROS, reactive oxygen species.

(BOS1) are important regulators of different hormone signaling pathways and have a role in imparting biotic and abiotic stress resistance to plants (Mengiste et al., 2003; Iriti et al., 2007; Zhang et al., 2009). A number of WRKY genes like *O. sativa* WRKY89 (OsWRKY89), *Capsicum annum* WRKY40 (CaWRKY40), MAPK like *Gossypium hirsutum* MPK16 (GhMPK16), and OsNAC6 have been successfully used to impart biotic and abiotic stress resistance to plants (Supplementary Table 2). Similarly MAPK and NAM-ATAF and CUC 6 (NAC) transcription factors also play a crucial role in regulating biotic and abiotic stress response of plants. The phytohormone ABA has also been known to be an important modulator of plants responses to various abiotic and biotic stress conditions (Tuteja, 2007; Ton et al., 2009). The fact that these "cross talk" genes regulate both biotic and abiotic stress response of plants points toward their probable importance in conferring

combined stress tolerance to plants. However, this needs to be validated by actual combined stress studies wherein expression of these genes under combined stress needs to be investigated.

The transcriptome analysis of *A. thaliana* plants subjected to combined drought and TuMV infection has revealed the presence of some of the genes involved in cross talk between individual stresses as a part of shared response under combined stress. For example, genes like *AtERF1b*, *AtERF1a*, *WRKY38*-related (WRKY transcription factor 38), glutathione-S-transferase F12 (*GSTF12*), mitogen activated protein kinase 9 (*AtMAPKK9*), *MAPKK16* are common to drought and combined virus and drought stress response of *A. thaliana* plants subjected to combined drought and TuMV infection (Prasch and Sonnewald, 2013). Thus, individual stress studies which give an indication about molecules involved in the cross talk can be important to gain insights into the shared response under combined stress treatment (**Figure 3C**).

Conclusion and Future Perspectives

The stress response mechanism of plants against the abiotic and biotic stress combinations is governed by interaction between responses evoked by individual stresses at both physiological and molecular level. As already stated, the interaction is governed by factors like severity of stresses, age of plant, and whether the plant is tolerant or susceptible to any one of the individual stress. Even plants belonging to same genus may show different molecular response to a stress combination (Aprile et al., 2013). If the two stresses under a stress combination lead to same kind of physiological changes in plants, the overall effect of stress combination becomes additive causing enhanced damage to plants. However, stress combinations can have entirely different effects on physiological and molecular processes of plants. The overall response of plants to stress combination is apparently governed by the more severe stress. The interaction between two stresses sometimes leads to a completely unique response that ensures best utilization of plant energy resources. Thus, adaptation mechanism to combined stresses consists of both shared and unique responses. The identification of genes involved in shared and unique response under combined stresses would be an important step toward developing a comprehensive understanding on the mechanism of combined stress tolerance in plants. Further analysis and characterization of these genes would help in choosing the right candidates among shared and unique response genes which can be potential targets for conferring combined stress tolerance to plants.

The increasing demand for food, deteriorating environmental conditions as well as emergence of newly evolved pathogens have necessitated the development of crops which are better equipped to deal with biotic and abiotic stresses and produce better yields. The fact that occurrence of combination of stresses instead of individual stress are important challenges for crop production demands thorough and intensive studies to understand plants response to stress combinations. A couple of studies in this direction throwing light on shared and unique responses of plants under combined stresses have already been published. It is now required to extend these studies to major crop plants. For proper understanding of plants responses to combined stress which occur under field conditions, the experiments should be carefully designed so that they nearly mimic the field conditions. It is also pertinent to identify the stages vulnerable to the combined stresses by studying the stage specific effect of combined stresses on the transcriptome of different plants. The transcriptomic analysis of plants under combined stresses can generate useful and substantial information regarding common and unique genes modulated under combined stresses. The advances in NGS and high throughput sequence analysis platforms for precise detection and accurate quantification of even small changes in the transcriptome as well as the recently developed genetic engineering tools can be useful in exploring the molecular responses of plants under combined stresses.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00723

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OsCYP21-4, a novel Golgi-resident cyclophilin, increases oxidative stress tolerance in rice

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OsCYP21-4 is a rice cyclophilin protein that binds to cyclosporine A, an immunosuppressant drug. CYP21-4s in Arabidopsis and rice were previously shown to function as mitochondrial cyclophilins, as determined by TargetP analysis. In the current study, we found that OsCYP21-4-GFP localized to the Golgi, rather than mitochondria, in *Nicotiana benthamiana* leaves, which was confirmed based on its co-localization with *cis* Golgi α-ManI-mCherry protein. *OsCYP21-4* transcript levels increased in response to treatments with various abiotic stresses and the phytohormone abscisic acid, revealing its stress-responsiveness. CYP21-4 homologs do not possess key peptidyl prolyl cis/trans isomerase (PPIase) activity/cyclosporine A (CsA) binding residues, and recombinant OsCYP21-4 protein did not convert the synthetic substrate Suc-AAPF-pNA via *cis- trans-* isomerization *in vitro*. In addition, transgenic plants overexpressing *OsCYP21-4* exhibited increased tolerance to salinity and hydrogen peroxide treatment, along with increased peroxidase activity. These results demonstrate that OsCYP21-4 is a novel Golgi-localized cyclophilin that plays a role in oxidative stress tolerance, possibly by regulating peroxidase activity.

Keywords: cyclophilin, golgi-resident protein, oxidative stress, peroxidase activity, PPIase, salinity tolerance

Introduction

The Golgi is a highly dynamic organelle that serves as the major site for post-translational protein modification and synthesis of various polysaccharides and glycolipids destined for the cell wall and plasma membrane, respectively (Lerouxel et al., 2006; Nilsson et al., 2009). The Golgi also plays a defining role in the processing and sorting of transport (cargo) proteins, lipids, and complex carbohydrates to various destinations within most eukaryotic cells (Matheson et al., 2006; Nanjo et al., 2006).

Unlike in animal cells, the Golgi apparatus in plant cells is located close to the nucleus in a rather stationary state; the Golgi takes the form of numerous individual Golgi stacks, which are mostly regarded as functional features required for the synthesis and trafficking of complex carbohydrates to the cell wall and transport of proteins to organelles (Radhamony and Theg, 2006). Furthermore, plant Golgi stacks do not disassemble at any stage during mitosis, whereas mammalian Golgi stacks remain intact and increase somewhat in number throughout the cell cycle (Nebenführ et al., 2000; Faso et al., 2009; Ito et al., 2014). Plant cells contain between several and hundreds of distinct Golgi

stacks (cisternae; six on average) and the functional subdivision of Golgi stacks into cis-, medial-, and trans-cisternae is based on enzyme activity (Dupree and Sherrier, 1998).

Nevertheless, despite numerous studies over the past 30 years, no consensus amino acid sequence that serves as a Golgi retention signal has been identified, but the transmembrane domain and the cytosolic tail appear to be involved in this process (Saint-Jore-Dupas et al., 2004). Numerous Golgi-resident proteins have been identified in human and mouse (1183), whereas only approximately 400 plant Golgi proteins have been experimentally verified (Parsons et al., 2012). Since plant Golgi proteins do not possess obvious target signals that help proteins localize to other subcellular compartments, the Golgi-resident prediction computational programs are less than adequate for determining their localization (Sprenger et al., 2006). Recently, Chou et al. developed a novel Golgi-prediction server, GolgiP, which predicts both transmembrane- and non-transmembrane-associated Golgi-resident proteins in plants (Chou et al., 2010). GolgiP was used to predict Golgi proteins in 18 fully sequenced plant genomes based on their functional domains, revealing that similar percentages of Golgi proteins are found among lower to higher plant species. GolgiP currently supplies multiple models for predicting plant Golgi proteins.

A growing body of evidence indicates that the Golgi apparatus participates in stress signaling sensing, although little is known about this process in plants. The Golgi apparatus is involved in oxidative stress-mediated pathogenesis (Hu et al., 2007; Fan et al., 2008), apoptosis (Hicks and Machamer, 2005), and endoplasmic reticulum (ER) stress (Xu et al., 2010) in mammalian cells. Some studies elucidated the relationship between the Golgi apparatus and signal transduction under oxidative stress (Zhou et al., 2007; Braga et al., 2008). Maturation of complex N-glycan is required for plant adaptation to salinity stress (Kang et al., 2008; Von Schaewen et al., 2008; Zhang et al., 2009), heat stress (Shiraya et al., 2015), and pathogen immunity (Häweker et al., 2010). However, although our knowledge of the importance of the Golgi apparatus under oxidative stress is advancing, the molecular mechanisms underlying this process are mostly unknown. Further investigation is essential for elucidating these underlying mechanisms.

Cyclophilns (CYPs), the target of the immunosuppressive drug CsA, belong to the peptidyl prolyl *cis/trans* isomerase superfamily and play central roles in various biological processes in living cells, including splicesome assembly (Horowitz et al., 2002; Mesa et al., 2008), RNA processing (Gullerova et al., 2006), protein trafficking (Freskgård et al., 1992; Ferreira et al., 1996), miRNA activity (Smith et al., 2009), complex assembly and stabilization (Iki et al., 2012), signal transduction (Brazin et al., 2002), cell division (Faure et al., 1998), and detoxification of reactive oxygen species (ROS) (Hong et al., 2002). Arabidopsis CYPs have been functionally well-characterized compared to other plant CYPs, playing roles in assembly and maintenance of PSII supercomplex (Fu et al., 2007), effector activation (Coaker et al., 2005), organogenesis (Li et al., 2007), transcription and pre-mRNA processing (Leverson and Ness, 1998), plastid cysteine biosynthesis (Dominguez-Solis et al., 2008), cellular redox homeostasis (Kopriva, 2013; Park et al., 2013b), and phytochrome and cryptochrome signaling (Kang et al., 2008; Trupkin et al., 2012; Ma et al., 2013). By contrast, in monocot rice, only a few cyclophilins have been characterized (Ruan et al., 2011; Kim et al., 2012; Kang et al., 2013). In a previous study, we analyzed stress-responsive CYPs in rice (Ahn et al., 2010) and characterized the Os CYPs involved in environmental stress defense (Kim et al., 2012; Park et al., 2013a; Seok et al., 2014; Lee et al., 2015). Nevertheless, much work on CYPs remains to be conducted, and there have been no previous reports on the functional analysis of Golgi-localized CYPs in different plants.

This study is the first to attempt the functional characterization of Golgi-localized CYP and the results may serve as a starting point for further studies concerning its role within the Golgi apparatus under cellular stress conditions.

Materials and Methods

Bioinformatics Prediction

The *OsCYP21-4* sequence was used as a query to search for OsCYP21-4 homologs from the NCBI database through BLAST analysis. The amino acid sequences from OsCYP21-4 and its homologs were aligned using ClustalW2 and GeneDoc2.7. The phylogenetic tree of CYP21-4 homologs was constructed using the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA; version 5). The accession numbers are as follows: OsCYP21-4, NP_001059626.1 (*Oryza sativa*); XP_003563133.1 (*Brachypodium distachyon*); BAJ94163.1 (*Hordeum vulgare*); NP_001146433.1 (*Zea mays*); AtCYP21-4, NP_187319.1 (*Arabidopsis thaliana*); XP_002882485.1 (*Arabidopsis lyrata*); XP_010464248.1 (*Camelina sativa*); XP_009147140.1 (*Brassica rapa*); CDY05284.1 (*Brassica napus*); XP_002277818.1 (*Vitis vinifera*); and XP_004246609.1 (*Solanum lycopersicum*). The OsCYP21-4 sequence was analyzed using WoLF PSORT (http://wolfpsort.org/), PSORT (http://psort.ims.u-tokyo.ac.jp/), TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/), Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html), MitoProtII (http://ihg.gsf.de/ihg/mitoprot.html), SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), GolgiP (http://csbl1.bmb.uga.edu/GolgiP/), and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) programs.

Plant Materials, Growth Conditions, and Stress Treatments

Sterilized rice (*Oryza sativa* L. cv Dong Jin) seeds were embedded in 1/2MS medium and grown at 28°C for 1–2 weeks under a 12 h light/12 h dark cycle with 100 μE $m^{-2}s^{-1}$ light intensity, and several stresses treatments were performed as described previously (Lee et al., 2015). The seedlings were desiccated for drought stress treatment or treated with 100 μM ABA, 200 mM NaCl, 10 mM H_2O_2, and 10 μM MV and harvested at 0, 1, 3, 6, 12, and 24 h. Heat stress involved treatment at 42°C, followed by harvesting at 0, 0.1, 0.5, 1, 2, 3, and 4 h. Three experiments were performed per treatment, with at least three replicated measurements for each parameter assayed.

Gene Expression Analysis

Total RNA was extracted from plants grown under normal or stress conditions using RNAiso Plus (TaKaRa, Tokyo, Japan). Total RNA treated with RNase-free DNase I (Fermentas, Burlington, Canada) was used for cDNA synthesis (RevertAid First-strand cDNA Synthesis Kit; Fermentas). Quantitative reverse transcription PCR (qRT-PCR) was performed in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Premix Ex-Taq (TaKaRa), according to the manufacturer's instructions. Relative expression levels are presented after normalization with *OsACT1* expression levels. All RT-PCR experiments were performed in at least three biological replicates, each with three technical repeats, under the same conditions.

Expression and Purification of OsCYP21-4-His-tagged Protein

Expression and purification of recombinant OsCYP21-4 were carried out using the Novagen pET28a vector according to the supplier's protocols (EMD Millipore, Darmstadt, Germany). *OsCYP21-4* was cloned into pET28a and sequenced. The *OsCYP21-4* construct was transformed into *Escherichia coli* BL21 (DE3) for expression of His-tagged OsCYP21-4, and recombinant protein was purified on nickel-NTA agarose columns. Finally, the concentration and purity of OsCYP21-4-His protein were determined using the Bradford assay (Bio-Rad) and SDS-PAGE analysis.

Protease-coupled Assay for PPIase Activity

The PPIase activity of recombinant OsCYP21-4 was measured *in vitro* against a synthetic tetrapeptide with the composition N-succinyl-Ala-Ala-Pro-Phe-NA (Suc-AAPF-pNA; Sigma-Aldrich, St. Louis, USA) in a chymotrypsin-coupled assay (Fischer et al., 1984) with some modifications. A 6 mM Suc-AAPF-pNA substrate stock was prepared in trifluoroethanol containing 0.47 M LiCl. Assay blanks (1 mL total) contained 60 μL of chymotrypsin (10 mg/mL) and 20 μL of substrate stock in assay buffer (50 mM HEPES and 100 mM NaCl, pH 8.0). PPIase assays were identical to the blank assays, except that they included purified OsCYP21-4-His protein. Chymotrypsin and OsCYP21-4-His protein were mixed with assay buffer and transferred to a quartz cuvette. The substrate was then introduced into the cuvette and mixed. The absorbance of the solution at 390 nm was recorded immediately after mixing and monitored at 10°C for 300 s in a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) with a thermostatically controlled cuvette holder. All assays were carried out in triplicate.

Subcellular Localization of OsCYP21-4 Proteins

The subcellular localization of OsCYP21-4 was determined by creating fluorescent fusion proteins. *OsCYP21-4* and *OsCYP21-4* deletion fragments were inserted into binary vector pCAMBIA1302 containing the CaMV 35S promoter and GFP gene. To obtain *OsCYP21-4* and *OsCYP21-4* deletion fragments (OsCYP21-4TM, 1-57 amino acids; OsCYP21-4ΔTM, 58-235 amino acids) containing coding sequence without the stop codon, *OsCYP21-4* cDNAs were ligated into the Nde1/Spe1 sites upstream of the N-terminal end of the GFP (Supplementary Table S1). The constructs were transiently expressed in *N. benthamiana* leaves using agro-infiltration. Two days after infiltration, the leaves were examined by fluorescence or confocal microscopy (Park et al., 2013a). The OsCYP21-4TM-GFP construct was co-expressed with the RFP-labeled endoplasmic reticulum (ER) marker, BiP. Brefeldin A (BFA) was used to block secretion of OsCYP21-4-GFP to the Golgi apparatus. OsCYP21-4-GFP was transiently expressed in *N. benthamiana* treated with 50 μM BFA for 3 h.

Generation and Stress Treatment of *OsCYP21-4* Overexpressing Plants

The full-length cDNA sequence of *OsCYP21-4* was cloned into pCAMBIA1300 under the control of the 35S promoter (Supplementary Table S1). Rice was transformed with the construct by *Agrobacterium*-mediated transformation (Hiei et al., 1994). Genomic DNA was isolated from the leaves of wild-type (WT) and transgenic plants. To identify positive transgenic plants, one primer was designed to bind to the promoter region and another was designed to bind to the *OsCYP21-4* cDNA region. WT plants were used as a control. The expression level of the *OsCYP21-4* transgene was determined by RT-PCR. Actin was used as a reference for normalization. For various stress treatments, transgenic and WT rice seeds were germinated on 1/2MS medium plates with (for transgenic lines) or without (for WT) hygromycin. After 3 days, the rice seedlings were transferred onto 1/2MS medium containing 150 mM NaCl or 5 μM ABA and grown for 5 days. The roots of 3-day-old rice seedlings, which were selected using the method described above (for salt treatment), were incubated in a solution of H_2O_2 and NaCl for 3 and 5 days, respectively. The fresh weight, and root or shoot length of each seedlings were measured in triple independent experiments.

H_2O_2 Detection

Plant leaves were excised and immersed in a solution of 1 mg/mL 3′,3′-diamino benzidine (DAB) in Tris-HCl buffer (pH 6.5) containing 0.01% Triton X-100. After vacuum-infiltration for 60 min, the samples were incubated at room temperature for 20 h in the dark. To remove chlorophyll, the leaves were bleached by boiling in ethanol for 20 min. Brown spots indicated the presence of H_2O_2 *in situ* (Thordal-Christensen et al., 1997). Two independent experiments were carried out.

Peroxidase and Catalase Activity

To determine peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) activities, 1 g of frozen power from leaf samples was homogenized in ice-cold 100 mM potassium phosphate pH 6.0 (POD), 50 mM pH7.5 sodium phosphate buffer (APX), or 50 mM pH7.0 potassium phosphate buffer (CAT). The homogenate was centrifuged at 12,000 g for 20 min at 4°C, and the supernatant was used for enzyme activity determinations. POD, APX, and CAT activities were analyzed by monitoring the increase in absorbance at 420, 290, and 240 nm, respectively. POD and APX activities were assayed according to the method described

by Kwak et al. using pyrogallol and ascorbate as substrates, respectively (Kwak et al., 1995). CAT activity was determined by monitoring the consumption of H_2O_2 (Aebi, 1984). All measurements of activity were performed in triplicate.

Statistical Analysis

Statistical differences between treatments on different samples were analyzed following the Student's *t*-test using Excel. Differences were considered significant at a probability level of $p \leq$ Diff, 0.1, 0.05, or 0.01.

Results

OsCYP21-4 Is a CYP Protein That Is Conserved in Different Plant Species

OsCYP21-4 (Os07g29390) is a rice cyclophilin protein containing 235 amino acids with a molecular mass of 26.4 kDa. It contains a single CYP domain (amino acids 80–230). By searching NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) using the OsCYP21-4 sequence as a query, we identified OsCYP21-4 homologs in three monocot and seven dicot plants (**Figure 1A**). Multiple amino acid sequence alignments showed that OsCYP21-4 shares high similarity with its homologs from monocots (84% similarity with *B. distachyon*, 81% with *H. vulgare*, and 81% with *Z. mays*) and less conserved sequence homology with CYP21-4 homologs from dicots (76% similarity with *V. vinifera*, 70% with Arabidopsis, 69% with *C. sativa,* and *Brassica,* and 67% with *S. lycopersicum*). Moreover, the results of phylogenetic tree analysis based on the full-length sequences of these homologs are good in agreement with the evolutionary relationships among these species (**Figure 1B**): OsCYP21-4 has a closer evolutionary relationship with CYP21-4 homologs from monocots than from dicots.

OsCYP21-4 Is Localized to the Golgi Apparatus and BFA Inhibits Its Localization

Previous studies revealed that two CYP21-4s from Arabidopsis and rice are mitochondrial cyclophilins, as predicted using the TargetP program (He et al., 2004; Ahn et al., 2010). In the current study, we analyzed OsCYP21-4 using WoLF PSORT, MitoProtII, and Predotar as well as TargetP. The results of these predictions are summarized in **Table 1**. All of these programs except PSORT predicted that OsCYP21-4 is also localized to the mitochondria. To examine the intracellular localization of this protein, we cloned *OsCYP21-4* into plant expression vector pCAMBIA1302 between the 35S promoter and *GFP* gene (Supplementary Figure S1A), and transiently expressed the OsCYP21-4-GFP fusion protein in *N. benthamiana* leaves, followed by confocal laser scanning microscopy (CLSM) to observe its localization. OsCYP21-4-GFP was not clearly localized to the mitochondria, instead showing inconsistent localization patterns using the mitochondria marker MitoTracker (**Figure 2A**). We also found that GFP fused to OsCYP21-4 did not co-localize with the peroxisomal marker, RFP-SKL (three amino acids fused to red fluorescent protein) or with RFP-BiP ER-Tracker (Supplementary Figures S1B,C). Interestingly, a few punctuate OsCYP21-4-GFP signals were closely linked to or merged with the peroxisomal marker, and numerous punctuate signals were merged or closely linked to RFP-BiP (Supplementary Figures S1B,C: closed white arrowheads). Therefore, we cannot rule out ER-mediated localization of OsCYP21-4 in the cell.

Prediction by another program, GolgiP, which predicts Golgi-localized proteins in plants, supported the possibility that OsCYP21-4-GFP is localized to the Golgi, although the accuracy of the prediction is not high (**Table 1**). To verify that the fluorescent signals were indeed coming from the Golgi, we employed α-Mannosidase I fused to mCherry fluorescent protein (α-ManI-mCherry) (Nelson et al., 2007), which labels *cis* Golgi (Saint-Jore-Dupas et al., 2006). When OsCYP21-4 fused to GFP (OsCYP21-4-GFP) was co-expressed with α-ManI-mCherry, OsCYP21-4-GFP clearly co-localized with the α-ManI-mCherry fluorescence (**Figure 2B**). As determined by SignalP, a signal peptide predictor, OsCYP21-4 lacks a signal peptide. On the other hand, a traditional transmembrane topology predictor, TMHMM, predicted that an N-terminal transmembrane (TM) segment of OsCYP21-4 protein is a signal peptide (Supplementary Figure S1D). To analyze the signal peptide of Golgi-localized OsCYP21-4, we generated two OsCYP21-4 deletion constructs based on the results of TMHMM prediction. One construct contained the N-terminal TM segment (1-57 amino acid residues) of OsCYP21-4 fused to GFP (OsCYP21-4TM-GFP), and the other contained TM deleted-OsCYP21-4 fused to GFP (OsCYP21-4ΔTM-GFP) (Supplementary Figure S1E). OsCYP21-4TM-GFP localized to the Golgi, indicating that the N-terminal region of OsCYP21-4 is critical for its localization. By contrast, the OsCYP21-4ΔTM-GFP produced fluorescence only in the cytosol (**Figures 2C,D**).

Since OsCYP21-4 appears to be a Golgi-resident protein, trafficking of OsCYP21-4 should be inhibited by BFA, an inhibitor of the Golgi apparatus. To determine whether OsCYP21-4 trafficking to the Golgi is blocked by BFA, epidermal cells infiltrated with a mixture of *Agrobacterium* suspension harboring the OsCYP21-4-GFP construct and the silencing suppressor p19 were examined after 2 days of infiltration under a confocal microscope. Solutions containing infiltration buffer (as a negative control) or 50 μg/mL BFA in infiltration buffer were injected into leaves to reveal the Golgi localization of the fusion protein. The leaves were visualized 3 h after BFA injection using confocal microscopy (**Figure 2E**). Treatment with BFA resulted in enhanced intracellular GFP fluorescence in a pattern resembling the ER network, which was merged with the signal from red fluorescent protein fused with chaperone binding protein (BiP) (Kim et al., 2001), implying that BFA treatment prevented further translocation of OsCYP21-4 from the ER to the Golgi or caused the protein to relocate from the Golgi to the ER.

Gene Expression Analysis of *OsCYP21-4*

To characterize the expression pattern of *OsCYP21-4* in rice, we analyzed the transcript levels of this gene at different developmental stages and in different tissues by semi-quantitative RT-PCR. *OsCYP21-4* was expressed differentially in various tissues at different stages during plant development. In 1-week-old seedlings, *OsCYP21-4* was expressed at higher levels in the roots than in the endosperm and sheath tissues. Two-week-old

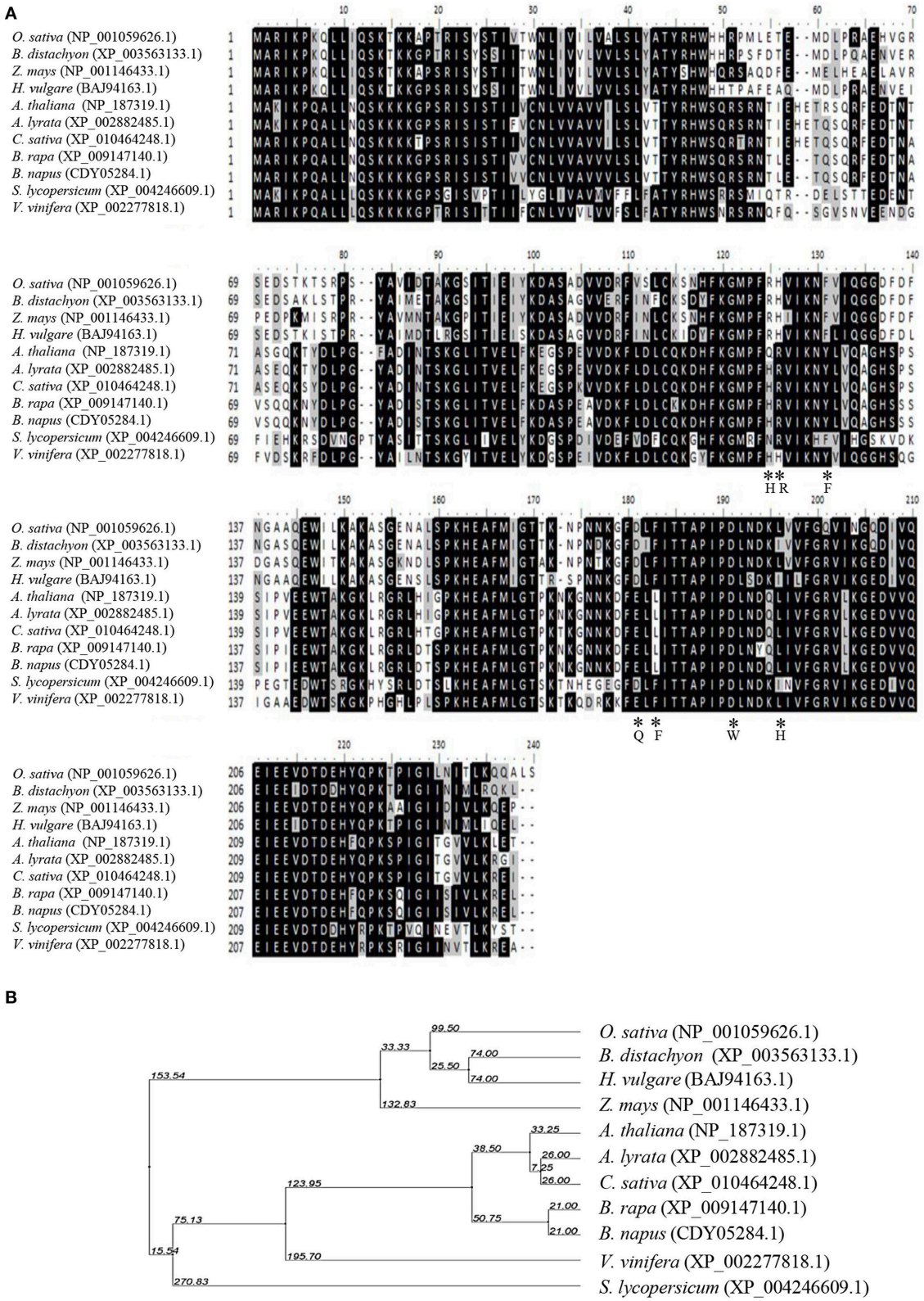


FIGURE 1 | Multiple sequence alignment and phylogenetic relationship between OsCYP21-4 and CYP21-4 homologs from various plants. (A) Sequence comparison of OsCYP21-4 protein with selected CYP21-4 homologs from various plant species. The amino acids necessary for PPIase activity/CsA binding and are marked by asterisks. The degree of background shading indicates amino acid identity and similarity (*black: identity* >50%, *gray: similarity* > 50%). **(B)** Phylogenetic distance between OsCYP21-4 and other homologs.

TABLE 1 | Prediction of OsCYP21-4 subcellular localization by website programs.

WoLFPSORT		PSORT		TargetP	MitoProtII	Predotar		GolgiP
Mito	8	PM	0.790	mTP (0.754)	Y (0.775)	Mito	(0.67)	Golgi (0.917)
Chlo	3	Chlo	0.399	47 residues		Else	(0.33)	Accuracy (34.2%)
Cyto	1	Golgi	0.300					
Pero	1	ER						

For WoLF PSORT and PSORT predictions, the four most favorable localizations are reported with corresponding scores. For TargetP prediction, the scores of transit peptide presence and transit peptide length are given. For MitoProtII, Predotar and GolgiP predictions, the scores are given. cTP, chloroplast transit peptide; Mito, mitochondria; Chlo, chloroplast; Cyto, cytoplasm; Pero, peroxisome; mTP, mitochondria transit peptide; PM, plasma membrane. GolgiP prediction indicates the accuracy of prediction.

plants exhibited ubiquitous expression in the endosperm, roots, and leaves, except in stem tissue. On the other hand, in 6-week-old mature plants, *OsCYP21-4* was more highly expressed in leaves than in other tissues (**Figure 3A**).

To investigate the effects of abiotic stress on *OsCYP21-4* expression, we examined the expression patterns of this gene by qRT-PCR in rice seedlings subjected to salt, drought, H_2O_2, MV, heat, and ABA treatment. As shown in **Figure 3B**, *OsCYP21-4* expression increased 4–12-fold under various abiotic stress conditions. The expression of *OsCYP21-4* increased approximately 10-fold under high salinity, drought, and ABA stress conditions. Interestingly, the expression of *OsCYP21-4* was rapidly induced by MV, H_2O_2, high salinity and heat treatment within 1–3 h and gradually decreased thereafter. However, *OsCYP21-4* expression under drought and ABA treatment continued to increase until 24 h of treatment (**Figure 3B**). The up-regulated expression patterns of *OsCYP21-4* suggest that it may be involved in responses to such stresses.

OsCYP21-4 Does Not Have PPIase Activity *In vitro*

The structure of OsCYP21-4 resembles that of other members of the CYP family in rice, and it contains a single CYP domain (80–230 amino acids). However, multiple amino acid sequence alignments revealed that the seven core amino acids necessary for CsA binding and PPIase activity, as determined for human CyPA (Zydowsky et al., 1992), are not conserved in CYP21-4 homologs (**Figure 1A**). In particular, OsCYP21-4 and its homologs from monocots contain only two conserved amino acid residues among the seven key amino acid residues, suggesting that these proteins lack PPIase activity. To determine whether OsCYP21-4 has PPIase activity, we expressed recombinant OsCYP21-4-His in *E. coli* and purified OsCYP21-4-His using nickel-affinity purification (**Figure 4A**). We measured the PPIase activity of OsCYP21-4, which is rate-limited by *cis-trans* isomerization of the Ala-Pro peptide bond of the synthetic peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, using a chymotrypsin-coupled PPIase assay. Kinetic data were obtained in the presence of increasing amounts of OsCYP21-4-His. The isomerization of the peptide substrate was not accelerated in the presence of 50, 100, or 200 nM OsCYP21-4-His, showing O.D. values identical to that of the blank, a negative control. Recombinant HsCypD protein (Giorgio et al., 2010) was used as a positive control (**Figure 4B**). These results suggest that OsCYP21-4 is not an active PPIase and that it may function as a PPIase-independent protein (like a chaperone) in the Golgi.

Overexpression of *OsCYP21-4* Increases Salt Tolerance and Peroxidase Activity in Transgenic Rice

We generated OsCYP21-4-overexpressing transgenic plants containing the full-length ORF of *OsCYP21-4* under the control of the CaMV 35S promoter (for the constitutive expression) (Supplementary Figure 2A). We analyzed *OsCYP21-4* expression in WT and OsCYP21-4-overexpressing (OE: OE1, OE2, and OE3) transgenic plants via semi-quantitative RT-PCR. *OsCYP21-4* expression was higher in OsCYP21-4 OE plants than in WT (Supplementary Figure 2B). To investigate the phenotypes of OsCYP21-4 OE plants under abiotic stress conditions, we exposed 3-day-old WT and OsCYP21-4 OE seedlings to 1/2MS medium containing 0, 100, and 1500 mM NaCl for 5 days. As shown in **Figure 5A**, OsCYP21-4 OE transgenic plants were more tolerant to salt stress than WT plants. Although OsCYP21-4 OE2 and OE3 plants had slightly increased root lengths even under normal conditions, after 100 or 150 mM NaCl treatment, all OE plants had obviously different root lengths and fresh weights from those of WT plants. Under salt stress conditions, both the root lengths and fresh weights of the OE lines were approximately 10–20% higher than those of WT plants (**Figures 5B,C**).

Abiotic stresses such as salinity induce the accumulation of ROS, which are toxic molecules that induce oxidative injury in plants (Apel and Hirt, 2004). To determine whether OsCYP21-4 plays an important role in ROS homeostasis under salt stress, we investigated the accumulation of H_2O_2 by examining the precipitation of polymerized 3,3′-diaminobenzidine (DAB) in OsCYP21-4 OE and WT plants grown under high salinity conditions (**Figures 5D,E**). Three-day-old WT and OsCYP21-4 OE seedlings were treated with 200 mM NaCl in sterilized distilled water for 5 days. The salt tolerance phenotypes of OsCYP21-4 OE plants grown in salt solution were similar to those of OsCYP21-4 OE plants grown on the salt-containing 1/2MS medium (**Figure 5D**). Detached leaf fragments from salt stress-treated or untreated seedlings were incubated in DAB staining solution. An intense brown precipitate was observed in the leaves of WT plants stained with DAB after 5 days of exposure to high salinity. Under high salinity conditions, the intensity of DAB staining was markedly lower in the leaves of OsCYP21-4 OE plants than in the leaves of WT plants (**Figure 5E**). Under mock

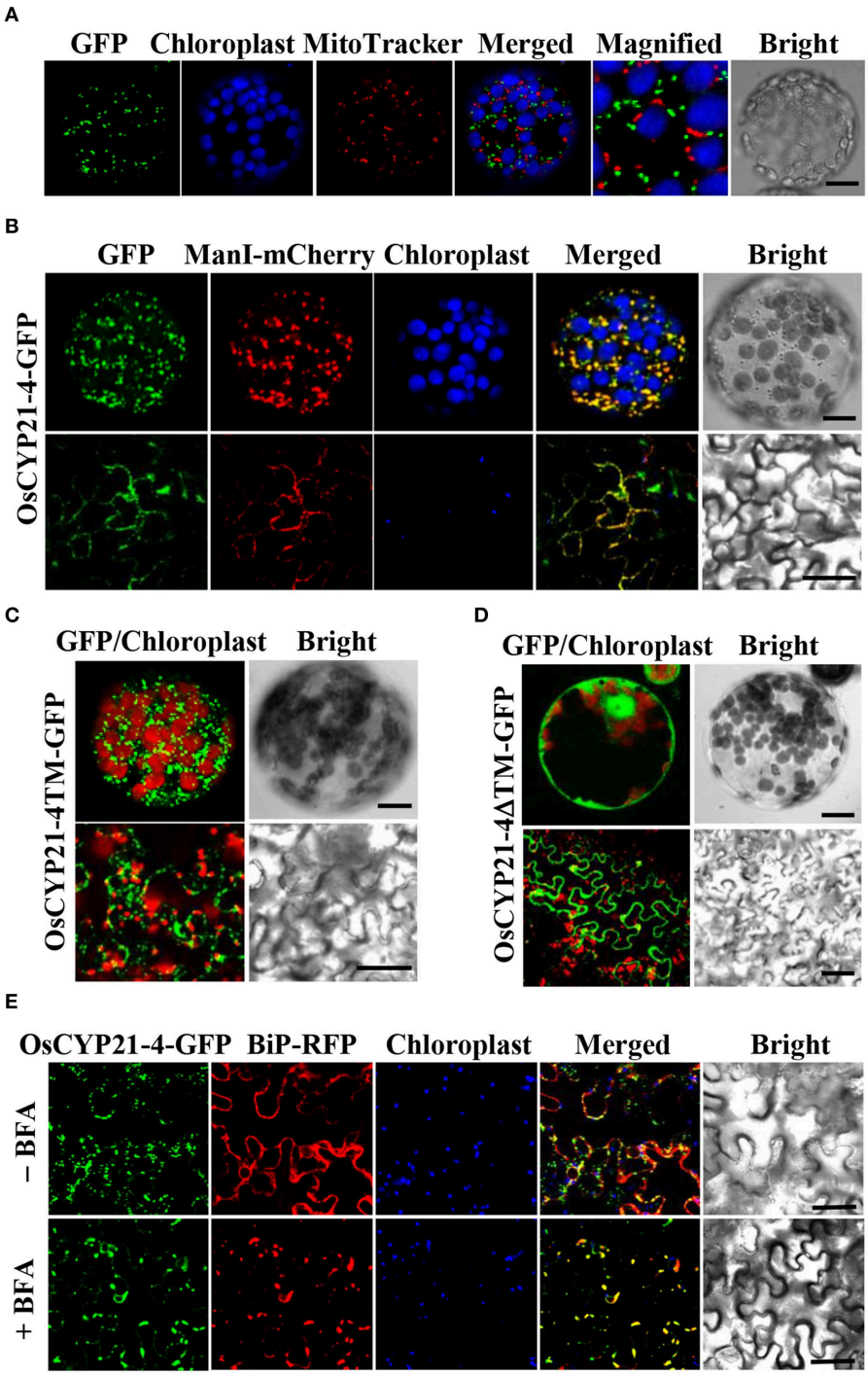


FIGURE 2 | Localization of OsCYP21-4-GFP in *N. benthamiana*. (A) OsCYP21-4-GFP did not co-localize with the mitochondrial marker, MitoTracker. **(B)** OsCYP21-4-GFP co-localized with the Golgi Apparatus marker α-ManI-mCherry. **(C,D)** Subcellular localizations of OsCYP21-4TM-GFP and OsCYP21-4ΔTM-GFP proteins. **(E)** Co-localization of OsCYP21-4-GFP with the ER marker, BiP-RFP under treatment with or without 50 μM BFA for 3 h. Bars = 20 μm.

conditions, no difference was observed between DAB-stained leaf fragments of WT and OE plants. DAB *in vivo* staining showed that the salt stress tolerant OsCYP21-4 OE plants accumulated less H_2O_2 than the WT control plants. To investigate the possibility that the oxidative tolerance of the transgenic plants was associated with an increase in antioxidant enzyme activity, we conducted experiments to determine the activities of major H_2O_2 scavenging enzymes, POD, APX, and CAT, in transgenic

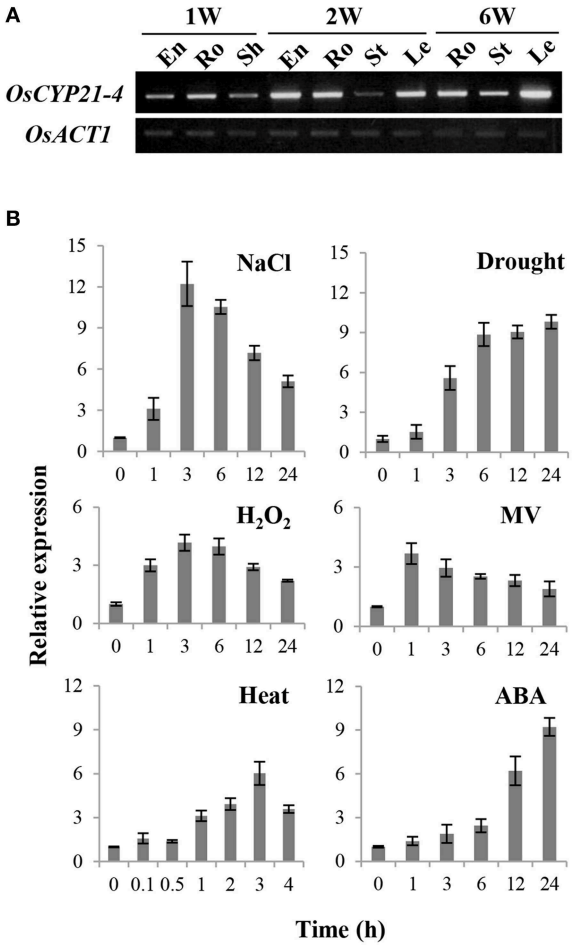


FIGURE 3 | Expression of *OsCYP21-4* in rice. (A) Expression levels of *OsCYP21-4* in different tissues and stages by semi-quantitative RT-PCR analysis. 1 W, 1-week-old; 2 W, 2-week-old; 6 W, 6-week-old; En, endosperm; Ro, root; Sh, sheath; St, stem; Le, leaf; *OsACT1*, rice actin1. *OsACT1,* rice *actin1. OsACT1* was used as an internal control for mRNA quantification. **(B)** Expression levels of *OsCYP21-4* in 2-week-old rice seedlings in response to salt (200 mM), drought (desiccation), H_2O_2 (10 mM), MV (10 μM), heat (42°C), and ABA (100 μM) treatment. Relative expression levels of *OsCYP21-4* (revealed by qRT-PCR) were normalized to that of *OsACT1*. MV, methyl viologen; H_2O_2, hydrogen peroxide; ABA, abscisic acid.

plants. POD and APX activity levels were clearly higher in OsCYP21-4 OE than in WT plants (**Figures 5F,G**), whereas CAT activity was not shown any difference between OsCYP21-4 OE and WT plants (Supplementary Figure 3). The higher POD and APX enzyme activities of OsCYP21-4 OE plants under high salt stress conditions could lead to less H_2O_2 accumulation, and therefore to greater salt tolerance.

OsCYP21-4 OE Plants are Tolerant of H_2O_2 Treatment and Have Increased Peroxidase Activity

As shown in **Figure 5**, OE plants exhibited salt tolerance and lower H_2O_2accumulation compared to WT plants. Therefore, we investigated whether exogenous treatment with H_2O_2 affects the growth of OsCYP21-4 OE and WT plants. Three-day-old WT and OsCYP21-4 OE seedlings were cultured in distilled water containing 0 (Mock), 10, and 30 mM H_2O_2 for 5 days. Under normal conditions (**Figure 6A**; Mock), there was no significant difference between WT and OsCYP21-4 OE plants. However, the overexpressing lines exhibited much better growth than WT seedlings under oxidative stress conditions (**Figure 6A**; 10 and 30 mM H_2O_2). To quantify the phenotypic differences under H_2O_2 stress conditions, we measured the fresh weights, root lengths, and shoot lengths of the plants, revealing significant increases in shoot lengths (**Figure 6B**) as well as fresh weights (data not shown) in all three OE lines. However, we did not detect clear differences in root length between WT and OE plants under H_2O_2 stress conditions (**Figure 6A**).

Like in the case of salt stress, we next conducted experiments to determine the activities of major H_2O_2 scavenging enzymes, POD, APX, and CAT, under H_2O_2 stress conditions. Even under normal growth conditions (**Figure 6A**; Mock), POD activity levels were 1.2–1.5-fold higher in the OsCYP21-4 OE plants than in the WT plants, suggesting that the increased *OsCYP21-4* expression of OE plants resulted in higher POD activity. Furthermore, the OE plants had 1.5–2-fold higher POD and APX activities than WT plants after H_2O_2 treatment (**Figures 6C,D**), whereas, there was no significant difference in CAT enzyme activity between OsCYP21-4 OE and WT plants under the same conditions (Supplementary Figure 3). These results suggest that the increased oxidative stress toleranace of the OsCYP21-4 OE plants may be due to their increased POD and APX activities.

Plants perceive and respond adaptively to abiotic stresses via pathways primarily controlled by the phytohormone, ABA. We therefore evaluated the response of OsCYP21-4 OE lines to ABA treatment. WT and OsCYP21-4 OE plants were grown on 1/2 medium supplemented with 5 μM ABA. The overexpressing lines exhibited much better growth than WT under ABA treatment conditions (Supplementary Figure 4A). We measured the fresh weights, root lengths, and shoot lengths of the plants, revealing noticeable differences in fresh weights in all three OE lines (Supplementary Figure 4B), as well as obvious differences in shoot and root lengths under ABA treatment conditions (data not shown). These results suggest that artificially up-regulating *OsCYP21-4* expression may improve the productivity of crops under environmental stress conditions, such as salt and oxidative stress.

Discussion

Cyclophilins are ubiquitous proteins present in all organisms that are involved in a wide range of crucial cellular processes. Rice CYPs consist of 27 structurally distinct family members. Among these, OsCYP21-4 and OsCYP21-3 are putative mitochondria-localized cyclophilins, as previously revealed using prediction programs (Ahn et al., 2010). The current alignment results show that CYP21-4 homologs exist only in certain land plant species. Interestingly, *CYP21-4* is not present in photosynthetic cyanobacteria or Chlamydomonas, which supports the notion that *CYP21-4* genes originated after the divergence of Chlamydomonas from land plants (**Figure 1**).

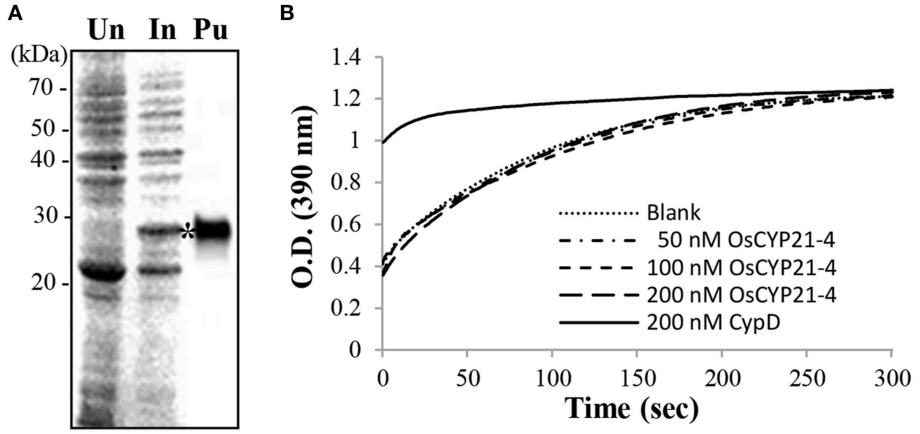


FIGURE 4 | Recombinant OsCYP21-4 lacks PPIase activity *in vitro*. (A) Recombinant OsCYP21-4 expression was induced with IPTG in *E. coli* and purified on nickel-NTA agarose columns. Samples were separated by 12% SDS-PAGE and stained with Coomassie blue. Un/In: Total protein un-induced/induced with isopropyl β-D-1-thiogalactopyranoside in *E. coli;* Pu: Purified OsCYP21-4 protein (star). **(B)** Traces showing isomerization of the Suc-AAPF-pNA substrate in the absence of OsCYP21-4 (Blank; …) and in the presence of 50 nM (50 nM OsCYP21-4;– -), 100 nM (100 nM OsCTP21-4;– -), and 200 nM (200 nM OsCTY21-4; _ _) recombinant OsCYP21-4 protein. CypD (200 nM CypD;) as a positive control. A representative recording from one of three independent experiments is shown.

Sequence analyses also suggested that CYP21-4 homologs lack essential amino acid residues for PPIase activity/CsA binding. In practice, recombinant OsCYP21-4 protein exhibited nearly no PPIase activity in an *in vitro* assay (**Figure 4**). Not all cyclophilin proteins possess PPIase activity, indicating that their PPIase activity may have been lost during the course of evolution and gain of function independent of their PPIase activity (Kumari et al., 2013). Moreover, numerous studies revealed that multiple immunophilins have retained a chaperone function independent of their PPIase activity (Chakraborty et al., 2002; Mok et al., 2006). In agreement with the *in vitro* results, OsCYP21-4 might have a PPIase-independent chaperone-like function for preventing aggregation or maintaining the stability of the target protein in plant cells.

In the current study, the results of analysis using various programs to predict the subcellular localization of OsCYP21-4 (like AtCYP21-4) were conclusive, revealing its mitochondrial localization, although PSORT (employed before GolgiP) predicted that this protein is localized to the plasma membrane (**Table 1**). We co-expressed OsCYP21-4-GFP and the peroxisomal marker RFP-SKL, and probed the material with mitochondrial (MitoTrack) and ER (RFP-BiP) markers to confirm the localization of OsCYP21-4 *in vivo*. Contrary to expectations, OsCYP21-4 did not completely co-localize with MitoTracker, but the peroxisomal marker RFP-SKL was co-expressed with OsCYP21-4-GFP; in rare cases, the signals merged in particular regions. Furthermore, signals from the RFP-labeled ER marker, BiP (RFP-BiP) merged with those of OsCYP21-4-GFP in quite a few regions (**Figure 2** and Supplementary Figures 1B,C). Meanwhile, GolgiP predicted that OsCYP21-4 is a Golgi-resident protein with a transmembrane domain. As a result, OsCYP21-4-GFP co-localized with cis α-GolgiManI-mCherry in most cases, depending on the transmembrane domain at the N-terminus (**Table 1** and Supplementary Figure 1D). The fluorescence that did not co-localize with the α-ManI-mCherry signal may have resulted from trafficking of OsCYP21-4-GFP to another type of Golgi, such as the trans- Golgi network or post-Golgi compartments (**Figure 2**). The BFA treatment assay revealed that OsCYP21-4 localizes to the Golgi through ER transport. However, further experiments are needed to elucidate and verify the precise localization of OsCYP21-4 based on external stress and growth/developmental conditions.

Although an increasing body of evidence suggests that CYPs play an important role in diverse cellular processes, little is known about their physiological relevance and the molecular basis of their stress-responsive expression. *OsCYP21-4* is also responsive to multiple environmental stresses and to the representative stress-related phytohormone, ABA. Among the stresses examined, salt stress produced the strongest increase in *OsCYP21-4* expression, with transcript levels up to 10-fold higher than control levels, which suggests that OsCYP21-4 is involved in the salinity stress response (**Figure 3**). Soil salinity is a critical environmental constraint to crop production, and extensive research and biotechnological developments to facilitate the production of crops with improved salt tolerance are currently underway. OsCYP21-4 OE plants exhibited enhanced salinity tolerance and reduced H_2O_2 accumulation in response to treatment with high levels of salt (**Figures 5A–E**), indicating that OsCYP21-4 plays an important role in removing sources of ROS production in plant cells, which may explain why OsCYP21-4 OE seedlings exhibited significantly better growth than WT seedlings under high salinity conditions (**Figure 5D**). Glycans mature in the Golgi, and mutants defective in N-glycan modification are more salt-sensitive than WT plants (Kang et al., 2008). Therefore, it is also possible that the OsCYP21-4 OE plants are salt tolerant due to enhanced N-glycan maturation arising from *OsCYP21-4* overexpression.

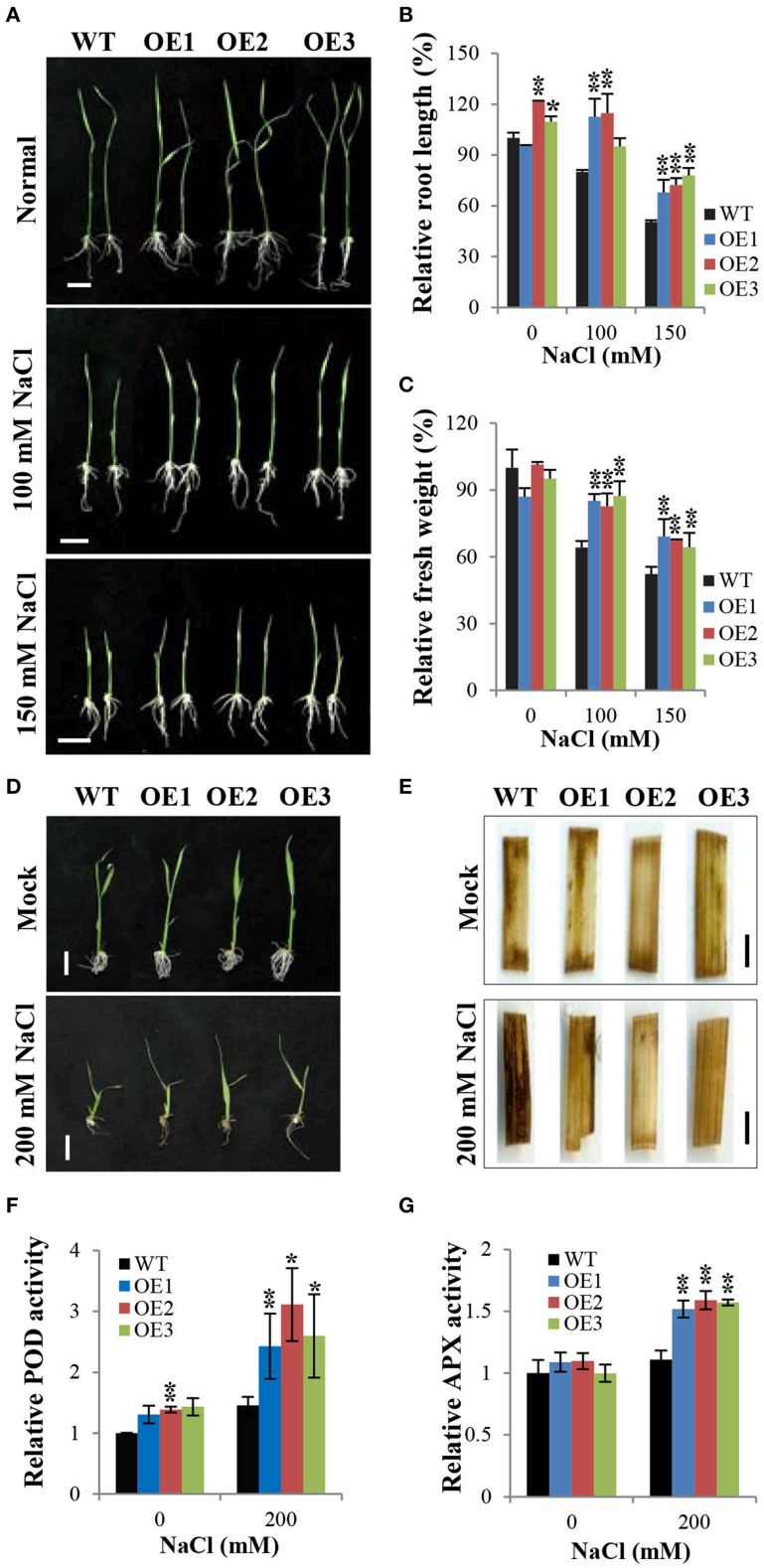


FIGURE 5 | Overexpression of *OsCYP21-4* increases salt stress tolerance in rice. (A,D) Comparison of rice seedlings under NaCl stress. Transgenic plants (OE) and wild-type (WT) seeds were germinated on MS medium with or without hygromycin (50 mg/L), respectively, and after 3 days, they were transferred to fresh MS medium plates **(A)** or to distilled water containing 0, 150 or 200 mM NaCl **(D)** and grown for 5 days. **(B,C)** Relative root length and fresh weight of transgenic and

(Continued)

FIGURE 5 | Continued
WT control seedlings grown on MS medium plates. Error bars mean SE of three biological replicates. Asterisks indicate statistically significant differences between WT control and OsCYP21-4 OE transgenic plants (Student's *t*-test: $*p < 0.1$, $**p < 0.01$). **(E)** *In situ* H_2O_2 accumulation in plants shown in **(D)** was detected by 3,3′-diaminobenzidine (DAB) staining. The experiments were repeated twice with similar results obtained in each experiment. **(F,G)** Relative peroxidase and ascorbate peroxidase activities in WT and OsCYP21-4 OE plants. Error bars denote SE of three biological replicates. Asterisks indicate statistically significant difference between WT control and OsCYP21-4 OE transgenic plants (Student's *t*-test: $*p < 0.1$, $**p < 0.05$). Bars = 2 cm **(A,D)** and 2 mm **(E)**.

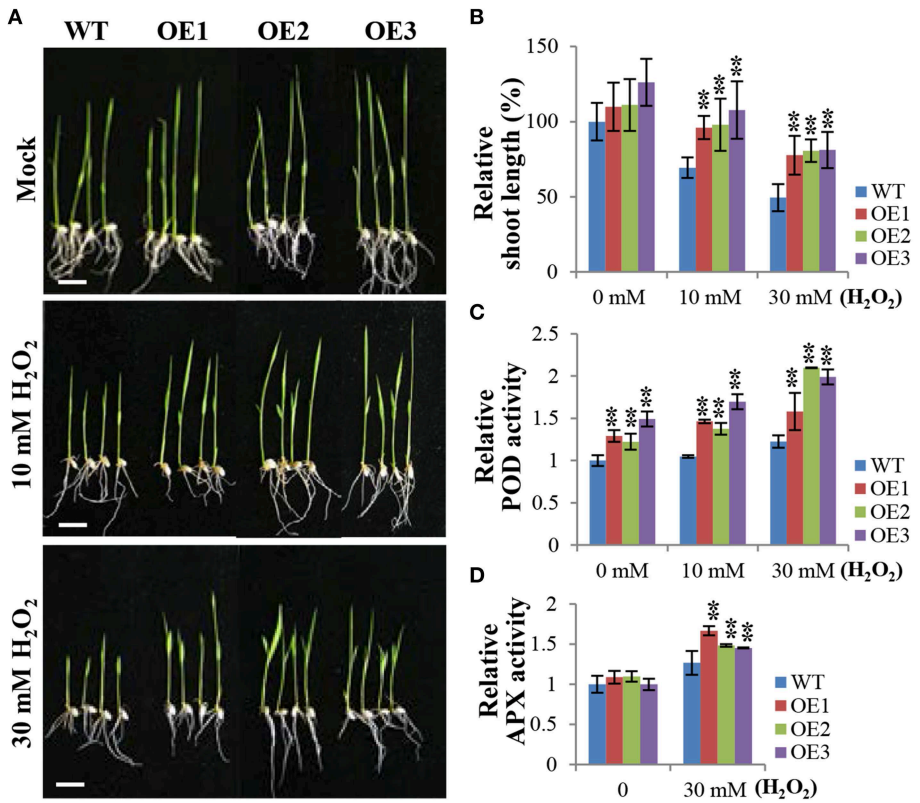


FIGURE 6 | *OsCYP21-4* OE plants exhibit enhanced tolerance to H_2O_2 treatment and increased peroxidase activity. (A) Comparison of rice seedlings under H_2O_2 treatment. Transgenic plants (OE) and wild-type (WT) seeds were germinated on MS medium with or without hygromycin (50 mg/L), respectively, and after 3 days, they were transferred to distilled water containing 10 mM or 30 mM H_2O_2 and grown for 5 days. **(B)** Relative shoot lengths of transgenic and WT control seedlings. **(C,D)** Relative peroxidase and ascorbate peroxidase activities in WT and OsCYP21-4 OE plants. Error bars represent SE of three biological replicates. Asterisks indicate significant differences between WT control and OsCYP21-4 OE transgenic plants (Student's *t*-test: $**p < 0.05$). Bars = 2 cm.

As previous DAB staining results (**Figure 5E**) support the notion that OsCYP21-4 functions in ROS scavenging, we were interested in examining the behavior of OsCYP21-4 OE plants in response to direct treatment with H_2O_2. OsCYP21-4 OE plants exhibited less H_2O_2-induced damage than WT, suggesting that the transgenic plants were more tolerant to oxidative stress than WT plants (**Figures 6A,B**). To uncover the cause of this oxidative tolerance, we examined the activities of major H_2O_2 scavenging enzymes POD, APX and CAT under high salt and H_2O_2 treatment conditions. POD and APX enzyme activities were higher in the OsCYP21-4 OE plants than in the WT plants under both stress conditions (**Figures 5F,G**, **6C,D**), suggesting that the transgenic plants possess a more efficient antioxidant network than the WT plants. This notion is corroborated by the accumulation of lower amounts of H_2O_2 in the transgenic plants. Therefore, the enhanced oxidative stress tolerance of the OsCYP21-4 OE plants may be attributed, at least in part, to their maintenance of low intracellular ROS pools, which is probably regulated by high peroxidase (POD and APX) activity. By contrast, CAT activity was not different between transgenic and WT plants (Supplementary Figure 3). Elucidating the relationship between peroxidase and CAT activities may provide important insights into the reciprocal physiological and biochemical effects of OsCYP21-4 under oxidative stress.

Plant CATs are important peroxisomal enzymes possessing a carboxyl terminal consensus sequence that primarily functions in peroxisomal import (Anderson et al., 1995; Mhamdi et al., 2012) and contributes to oxidative signaling. Plants possess large multigene families encoding secreted class III POD proteins (Passardi et al., 2004) and that play various physiological roles,

such as the salt stress response, lignification, and defense against pathogens (Kawano, 2003; Passardi et al., 2005). Class III PODs are extensively glycosylated in the Golgi apparatus and are involved in the biosynthesis of lignin (Deepa and Arumughan, 2002). Therefore, we speculate that Golgi-resident OsCYP21-4 partly assist in the glycosylation of peroxidases, although the molecular mechanism underlying this process is currently unknown. The current results indicate that OsCYP21-4 confers oxidative stress resistance by regulating POD and APX enzyme activities in OsCYP21-4 OE plants. Further exploration of the enhanced stress tolerance of these plants may help shed light on the pivotal roles played by the Golgi under oxidative stress conditions.

Author Contributions

HC conceived and designed the study and wrote the manuscript, SL, HP, WJ, and AL performed the experiments and wrote the manuscript, DY, YY, HK, BK, and JA contributed research materials.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00797

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Differentially expressed seed aging responsive heat shock protein OsHSP18.2 implicates in seed vigor, longevity and improves germination and seedling establishment under abiotic stress

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Small heat shock proteins (sHSPs) are a diverse group of proteins and are highly abundant in plant species. Although majority of these sHSPs were shown to express specifically in seed, their potential function in seed physiology remains to be fully explored. Our proteomic analysis revealed that OsHSP18.2, a class II cytosolic HSP is an aging responsive protein as its abundance significantly increased after artificial aging in rice seeds. *OsHSP18.2* transcript was found to markedly increase at the late maturation stage being highly abundant in dry seeds and sharply decreased after germination. Our biochemical study clearly demonstrated that OsHSP18.2 forms homooligomeric complex and is dodecameric in nature and functions as a molecular chaperone. OsHSP18.2 displayed chaperone activity as it was effective in preventing thermal inactivation of Citrate Synthase. Further, to analyze the function of this protein in seed physiology, seed specific *Arabidopsis* overexpression lines for *OsHSP18.2* were generated. Our subsequent functional analysis clearly demonstrated that OsHSP18.2 has ability to improve seed vigor and longevity by reducing deleterious ROS accumulation in seeds. In addition, transformed *Arabidopsis* seeds also displayed better performance in germination and cotyledon emergence under adverse conditions. Collectively, our work demonstrates that OsHSP18.2 is an aging responsive protein which functions as a molecular chaperone and possibly protect and stabilize the cellular proteins from irreversible damage particularly during maturation drying, desiccation and aging in seeds by restricting ROS accumulation and thereby improves seed vigor, longevity and seedling establishment.

Keywords: sHSP, chaperone, seed vigor, CDT, stress

Abbreviations: ABA, abscisic acid; ACD, α-crystallin domain; CDT, controlled deterioration treatment; CS, citrate synthase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); PEG, poly ethylene glycol; sHSP, small heat shock Protein; TZ, 2,3,5-triphenyltetrazolium chloride; VC, vector control; WT, wild type.

Introduction

Seeds, during development acquire remarkable protective mechanisms that allow them to survive desiccation to extremely low water content and to maintain their germinability even after many years of storage. Current evidence indicate that acquisition of desiccation tolerance and seed longevity is a multifunctional trait and diverse mechanisms have been proposed to be involved in acquiring such traits in seeds. As for example, presence and abundance of specific mono, di and oligosaccharides, soluble sugars and specific proteins along with the mechanisms related to ROS removal were shown to play important roles in the acquisition of desiccation tolerance and seed longevity in orthodox seeds (Blackman et al., 1995; Wehmeyer and Vierling, 2000). Previous studies also suggest that orthodox seeds essentially acquire desiccation tolerance during seed development particularly during maturation drying when most of these protective molecules are highly abundant (Roberts, 1973). Among these protective molecules, sHSPs have recently been suggested to play an important role in seed desiccation tolerance and longevity (Wehmeyer and Vierling, 2000; Sun et al., 2002; Tsvetkova et al., 2002; He and Yang, 2013). sHSPs are known to function as molecular chaperones that facilitate protein folding and prevent irreversible protein aggregation during developmental and adverse environmental conditions. Plants synthesize at least 21 different types of sHSPs and are grouped into six different classes on the basis of their subcellular localization and sequence alignments. This sHSP family has members with molecular size ranging from 16 to 42 kDa and invariably contains a conserved ACD in the C-terminal. These sHSPs are believed to play diverse role in plant biology (de Jong et al., 1998). Even though, sHSPs are known more for their extensive role in plant defense against abiotic stresses, their role in seed physiology has recently came into light (Wehmeyer and Vierling, 2000; Sun et al., 2001; Guo et al., 2007; Perez et al., 2009). Certain sHSPs have been reported as preferentially expressed in seeds particularly during development, maturation, and germination, highlighting their role in desiccation tolerance and longevity of seeds (Wehmeyer and Vierling, 2000; Scharf et al., 2001; Sun et al., 2002; Tsvetkova et al., 2002; Neta-Sharir et al., 2005; Volkov et al., 2005; Sarkar et al., 2009; Zhou et al., 2012). A heat shock transcription factor from sunflower (HaHSFA9) overexpressed in tobacco shows an increase in the accumulation of certain sHSPs and consequent improvement of seed vigor and longevity (Prieto-Dapena et al., 2006). Recently, a sHSP from *Nelumbo nucifera* was shown to increase seed germination vigor and seedling thermo tolerance in transgenic *Arabidopsis* (Zhou et al., 2012). In rice, 23 sHSP genes have been identified and majority of these genes are expressed in seed indicating their potential role in seed physiology. However, the role of sHSPs particularly in seed desiccation tolerance and longevity has not been well elucidated (Sarkar et al., 2009).

Rice (*Oryza sativa*) is a monocot model crop which feeds nearly half of the world population. Most of the cultivated rice varieties have a low or null dormancy and variable periods of storage life. Even under best storage conditions, rice seeds lose their viability and germination vigor (He and Yang, 2013). Therefore, a focused effort is required to elucidate and identify the proteins and underlying mechanisms implicated in seed vigor, viability, and longevity. More so, since the production of high quality seeds for food and germplasm preservation is also dependent on seed vigor and longevity. To identify such aging responsive proteins in rice seeds, a proteomic approach has been adopted in this study and eventually a small HSP (OsHSP18.2, a class II heat shock protein, LOC_Os01g08860) has been identified. Subsequently, the *OsHSP18.2* cDNA has been cloned from rice. Further, to get detailed insight of the involvement and participation of this sHSP protein in seed desiccation tolerance and longevity, transcript accumulation was analyzed in different organs, during seed development, aging, germination, and stress conditions. Further, *OsHSP18.2* was bacterially expressed, purified, and biochemically characterized. Finally the ability of this gene to enhance seed vigor and longevity has been analyzed through seed specific overexpression in *Arabidopsis*.

Materials and Methods

Plant Materials and Stress Treatments

Rice seeds (*O. sativa* indica cv. PB-1) were imbibed overnight in dark and sown in glass bottles over moist cotton bed and a layer of germination paper. The bottles were then kept for normal growth in growth room 28 ± 1°C and 65% humidity levels. For different stress treatments, 7 day-old seedlings were subjected to various stresses as described by Liu et al. (2009). For time course heat stress, 10 day-old seedlings were transferred to 42°C for 15 min, 30 min, 1, 2, 3, 5, and 24 h.

Controlled Deterioration Treatment

Seeds harvested on the same day from plants grown under identical conditions were used for all comparisons. Rice and *Arabidopsis* seeds were imbibed for 1 h and then blot dried. The seeds were treated with a combination of high temperature (45°C) and high humidity (100% Relative humidity) for 6 days (for rice) and 4 days (for *Arabidopsis*) to induce artificial aging (Delouche and Baskin, 1973; Verma et al., 2013). CDT treated rice seeds were used for protein extraction for 2 D electrophoresis. Transgenic *Arabidopsis* seeds subjected to CDT were used to score seed viability via germination percentage, tetrazolium assay and H_2O_2 accumulation via DAB staining.

Protein Extraction, 2D Electrophoresis, and Protein Identification

Artificially aged rice seeds (250 mg) were ground to fine powder and homogenized with 2 ml of extraction buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 1 mM PMSF, 1 mM DTT, and 1% Triton X-100). Homogenate was centrifuged at 15000 g for 15 min at 4°C and supernatant was collected as total soluble protein. Protein (500 μg) was precipitated with ice cold 100% acetone and pellets were solubilised in 250 μl of DeStreak Rehydration solution (GE) with 2% IPG buffer pH 3-10 NL. The protein sample was

used to rehydrate IPG strips 13 cm, pH 3-10NL overnight. Isoelectric focussing (IEF) was performed with rehydrated strips on Ettan IPGphor 3 (GE) at 100V–2 h; 500V–1 h; 1000V–1 h; 8000V–30 min and finally at 8000V–3 h. After IEF, the strips were equilibrated first with 1% (w/v) DTT in equilibration buffer (10 ml of 50 mM Tris-Cl (pH 8.8), 2% SDS, 6 M urea and 30% glycerol) followed by 4% (w/v) iodoacetamide in equilibration buffer. The strips were then loaded on 12.5% polyacrylamide gels for SDS-PAGE. Gels were stained with PlusOne Silver Staining Kit (GE) and analyzed with Image Master Platinum seven software (GE). The protein spots were excised manually and in-gel digestion was performed using trypsin (Sigma). Peptide analysis was carried out on 4800 MALDI-TOF/TOF (Applied Biosystems/MDS SCIEX) and peptides were identified using Mascot search engine run on GPS explorer version 3.6 (Applied Biosystems) using the following parameters: 800–4000 m/z interval MS peak filtering, monoisotopic, MSDB version 20060831 (3239079 sequences; 1079594700 residues), enzyme trypsin with maximum allowance of one missed cleavage, taxonomy Viridiplantae (Green Plants; 247347 sequences), ±100 ppm peptide tolerance, ±0.3 Da fragment mass tolerance, with fixed modification as carbamidomethyl (C) and variable modification as oxidation (M). Protein samples with probability score above default threshold level ($p < 0.05$) as determined by Mascot were considered for further analysis.

Quantitative Real-time PCR

For real time quantification of the transcripts, total RNA was isolated from rice seeds and seedlings using modified guanidine hydrochloride protocol and TRI reagent, respectively (Singh et al., 2003). Total RNA was treated with DNaseI from Ambion. First-strand cDNA was synthesized from the Dnase treated RNA using the Verso cDNA Synthesis Kit (Thermo). cDNA was then quantified by Nanodrop and dilutions were adjusted for 50 ng/μl. Real time quantification was performed as described in Kaur et al. (2008). Primers used for real time are described in Supplementary Table S1.

Cloning and Transformation of OsHSP18.2

Full length CDS and 5′ upstream sequence of *OsHSP18.2* (LOC_Os01g08860) was obtained from Ensembl plant *O. sativa* indica group database available at http://ensembl.gramene.org and GenomeIndia's manually curated database of rice proteins respectively (Gour et al., 2014). Complete ORF was amplified based on the primers designed from these sequences (Supplementary Table S1) from *O. sativa* indica var. PB-1. The coding region was cloned into pJET1.2 vector and sequence was confirmed. Finally the coding region was subcloned into modified pCAMBIA2301 vector under the control of napin promoter for seed specific expression. The construct was then transferred to Agrobacterium strain GV3101 and finally *Arabidopsis* plants were transformed by floral dipping (Clough and Bent, 1998). The 5′ upstream promoter sequence of *OsHSP18.2* was scanned for the presence of *cis*–acting regulatory elements with online available softwares PLACE (Higo et al., 1999) and PlantCARE database (Lescot et al., 2002).

Bacterial Overexpression and Purification of Recombinant OsHSP18.2

CDS of OsHSP18.2 was subcloned into bacterial expression vector pET23b (Novagen) and transformed into *Escherichia coli* host strain BL21DE3. Transformed cells were grown in LB medium with appropriate antibiotic till A_{600} reached 0.5 and then induced by adding 0.5 mM IPTG. After 6 h growth at 37°C, cells were harvested. Finally, 6X His tagged recombinant HSPs were purified from soluble fraction using nickel-charged affinity columns (GE) following the manufacturer's protocol.

Thermal Inactivation Assay

Chaperone activity of OsHSP18.2 was assayed by the method of Lee et al. (1995). CS and purified OsHSP18.2 were combined in equimolar concentration (150 nM) in 50 mM HEPES-KOH buffer, pH-8 with total reaction volume of 500 μl. Controls were made without HSP and with Lysozyme as the control protein. The tubes were incubated in a water bath set at 38°C for 60 min after which the tubes were shifted to 22°C to allow refolding of CS for another 60 min. Twenty micro liter aliquots were removed and CS activity was assayed every 20 min starting at zero minute.

Citrate Synthase Assay

Citrate synthase activity was measured according to Eigentler et al. (2012). Reaction was set up in a cuvette, 25 μl each of 12.2 mM acetyl coA and 10% triton X-100 was added with 100 μl of 1.01 mM DTNB solution (prepared in 1 M Tris-Cl, pH-8.1). Twenty micro liter of sample was added from the refolding reaction and the final volume was brought to 1 ml. Finally 50 μl of 10 mM Oxaloacetate was added to start the reaction. The absorbance was recorded in a Biorad spectrophotometer set at 412 nm for 2 min at an interval of 20 s. The initial rate of reaction was compared with non-denatured CS and the data was presented as percentage reactivation relative to this activity.

Tetrazolium Staining

Seeds were stained with 1% solution of 2,3,5-triphenyltetrazolium chloride (Sigma) to differentiate between viable and non-viable seeds according to Verma et al. (2013). Seeds were photographed using Zeiss SteREO Discovery V12 microscope fitted with Axiocam ICc 3 camera.

DAB Staining

Seeds treated with or without CDT were stained with 3,3′- DAB according to Yi et al. (2010). Seeds were incubated overnight in DAB staining solution (0.1 mg/ml DAB in 50 mM tris actetate buffer, pH 5.0) at 25°C in dark. Next day seeds were bleached in 80% ethanol for 10 min at 70°C. Seeds were photographed using Zeiss SteREO Discovery V12 microscope fitted with Axiocam ICc 3 camera.

Germination Assay Under Stress Treatments

Seeds from WT, VC, and transgenic lines were harvested at the same time and stored at room temperature till further use. Germination assays were carried out as described in Saxena et al. (2013). Three biological replicates with $n = 50$ seeds were used. Seeds were surface sterilized and plated on to either

½ MS or ½ MS supplemented with NaCl (150 mM), PEG (−0.4 MPa). For heat stress, sterilized seeds were incubated at 45°C for 1 h and then plated on to ½ MS. Seeds were considered germinated when radicle protruded beyond the testa and seedling establishment was considered positive with the emergence of green cotyledonary leaves (Silva-Correia et al., 2014).

Statistical Analysis

Statistical analysis was performed with one way ANOVA using Duncan's multiple comparison test to determine significant differences among samples. Differences were taken as significant when $P < 0.01$.

Results

OsHSP18.2 is an Aging and Seed Vigor Associated Protein

Controlled deterioration treatment has been used widely for quick evaluation of seed vigor, longevity and also to artificially induce aging related changes in seeds (Delouche and Baskin, 1973; TeKrony, 1995; Lanteri et al., 1996; Prieto-Dapena et al., 2006; Oge et al., 2008). Therefore, to identify the seed vigor and aging responsive proteins in rice seed, changes in protein expression after CDT were analyzed using proteomic approach. For CDT, dry seeds were subjected to a combination of high temperature and humidity as described in Section "Materials and Methods." Subsequently total soluble proteins were extracted from control and deteriorated seeds and were separated by two dimensional polyacrylamide gel electrophoresis (2 D-PAGE). Following silver staining, protein spot showing apparent variation indicated in **Figure 1** was excised from the gel. Excised spot was subjected to trypsin digestion and peptides were extracted and analyzed by MALDI-TOF/TOF. Subsequently peptides were identified using Mascot search engine run on GPS explorer 3.6. Only the best matches with high confidence level were selected. The protein was identified as OsHSP18.2 (LOC_Os01g08860). *OsHSP18.2* is an intronless gene which encodes a protein of 166 aa with an isoelectric point of 5.61 and a predicted molecular mass of 18.2 kDa. Sequence analysis revealed a conserved 90 amino acid alpha crystalline domain, a characteristic feature of sHSP family (Supplementary Figure S1). Pairwise alignment of OsHSP18.2 with its *Arabidopsis* homolog, AtHSP17.0 (AT5G12020.1) shows 59% sequence identity between them (Supplementary Figure S2).

OsHSP18.2 Upregulates in Seed, During Seed Maturation, and Upon Accelerated Aging

To elucidate the function and mechanism of *OsHSP18.2* in seed maturation and desiccation tolerance, we initially investigated the transcript accumulation of *OsHSP18.2* in seed along with other organs. As shown in **Figure 2A**, *OsHSP18.2* transcript was highly abundant in seed and more specifically in embryo than endosperm. Subsequently, to get the refined view of transcript accumulation during seed development, flowers were tagged according to the day after pollination (DAP) as described by Agarwal et al. (2011). Accumulation of *OsHSP18.2* transcript was found to be relatively low during initial stages of development, i.e., S1 till S4 stage (0–20 DAP) then strikingly increased in the late maturation stage S5 (21–29 DAP) and reached highest levels in dry mature seed (**Figure 2B**). This data essentially revealed that *OsHSP18.2* markedly increased at the later stages of seed development consistent with the time when seed actually acquires desiccation tolerance and longevity.

Transcript accumulation was also studied during germination and data showed a sharp decline in transcript level as germination proceeds (**Figure 2C**). Next, we investigated the correlation of transcript accumulation of OsHSP18.2 and aging in rice seeds. For this, rice seeds were subjected to CDT treatment for 6 days and transcript accumulation pattern was studied. Results clearly revealed a significant increase in transcript accumulation due to artificial aging even after 1 day of CDT which was further increased to more than 60-fold after 6 days of CDT (**Figure 2D**).

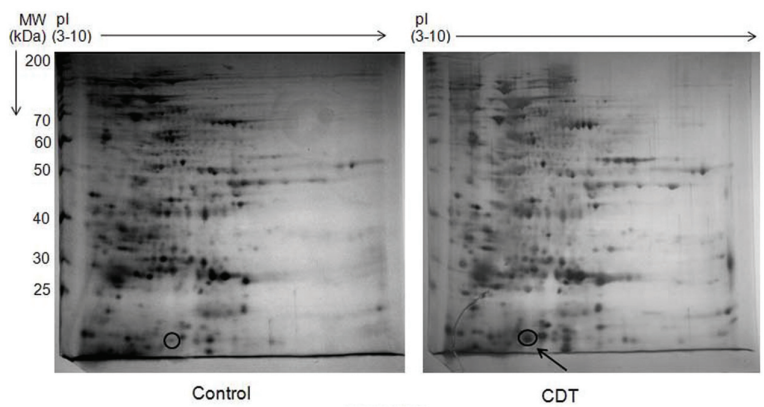


FIGURE 1 | 2D profile of rice seed proteins under control and CDT. Total 500 μg of protein from Control and CDT treated seeds (6 days) was used for IEF using a non-linear IPG strip of pH 3–10. Second dimension was run on 12.5% SDS-PAGE and gels were stained with silver stain. Gel images were analyzed with Imagemaster 2D Platinum 7.0 (GE Healthcare) and differentially expressed spots were identified. Encircled spot was eluted and sequenced using MALDI-TOF/TOF.

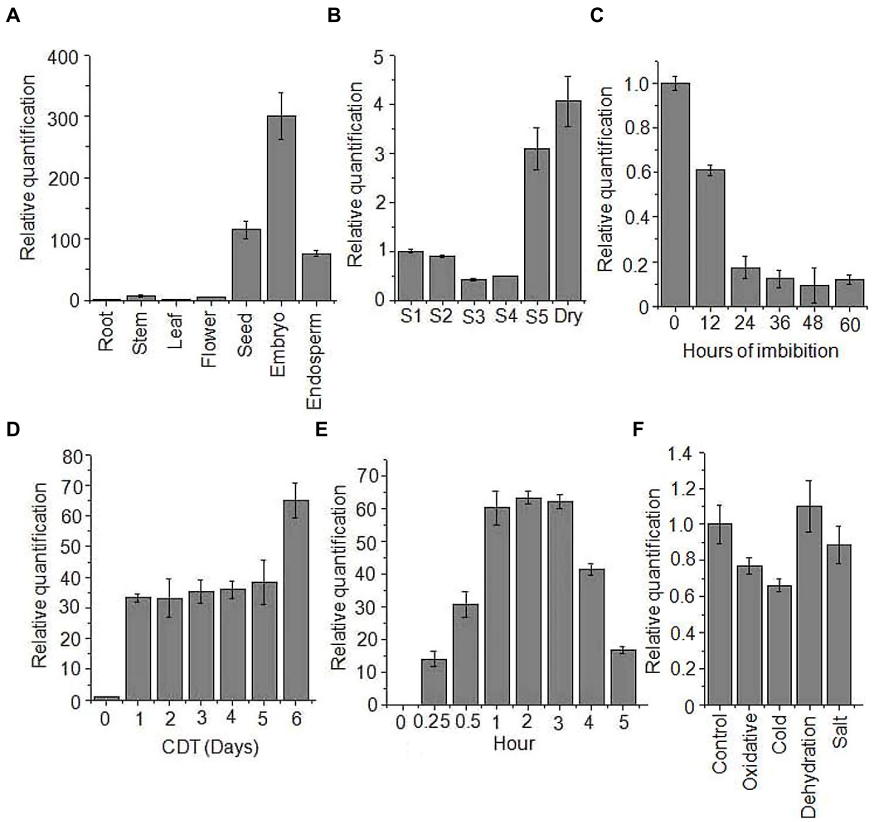


FIGURE 2 | Expression profiling of *OsHSP18.2*. Transcript accumulation was analyzed by quantitative real time PCR **(A)** in different organs, **(B)** during seed development, **(C)** during seed germination, **(D)** under CDT, **(E)** under heat stress, and **(F)** under various abiotic stresses. Actin was used as an endogenous control. The relative expression value of each gene was normalized to an endogenous control and calculated using the $\Delta\Delta$CT method (Applied Biosystems). Expression values are the mean ± SD of three independent biological measurements.

To examine the possible involvement of *OsHSP18.2* in abiotic stress tolerance, transcript accumulation was checked in rice seedlings challenged with various stresses (**Figures 2E,F**). As shown in **Figure 2E**, heat stress triggered maximum induction of *OsHSP18.2* transcript as expected while other abiotic stresses had minor changes in transcript levels (**Figure 2F**). This analysis clearly revealed that *OsHSP18.2* is upregulated during seed maturation and upon aging and indicates its participation in maturation drying and seed longevity. Transcriptional induction of *OsHSP18.2* in rice seedlings challenged with heat stress also indicates its participation in thermal stress tolerance. Investigation of the 5′ upstream sequence of OsHSP18.2 revealed the presence of many interesting *cis*-acting regulatory elements which are possibly responsible for abiotic stress responsiveness and seed and embryo specific expression. A list of some of these *cis*-acting elements is shown in **Table 1**.

Purified Recombinant OsHSP18.2 Forms Higher Order Oligomers

sHSPs are known to form multimers with varying number of subunits, most commonly between 2 and 48 subunits (Basha et al., 2012). In order to explore this, we isolated and cloned OsHSP18.2 coding region into bacterial expression vector pET23b and transformed into *E. coli* expression host BL21DE3. The recombinant protein was induced by IPTG and was found to be expressed in soluble phase (**Figure 3A**). Subsequently, 6X His tagged OsHSP18.2 protein was purified using nickel charged affinity chromatography. Size exclusion chromatography was used to check the oligomeric state of OsHSP18.2 and the purified protein was shown to elute as an oligomer of ∼202 kDa thus having approximately 12 subunits which agrees to the dodecameric nature of wheat sHSP16.9 (van Montfort et al., 2001; **Figure 3B**). The gel exclusion fractions were run on SDS-PAGE and revealed the presence of a single band in the 202 kDa peak fraction corresponding to the single subunit size of 18.2 kDa (**Figure 3C**). In order to confirm this oligomeric association of OsHSP18.2, the purified protein was run on native PAGE (**Figure 3D**) which revealed a band across 240 kDa marker band thus endorsing the gel filtration results.

OsHSP18.2 is an Active Chaperone and Prevents Thermal Denaturation of CS

Many small HSPs are known to have *in vitro* chaperone activity. Therefore, to assess the chaperone activity of OsHSP18.2, we used CS as a substrate for refolding experiments (Collada

TABLE 1 | Summary of key *cis* regulatory elements in the 5′-upstream sequence of *OsHSP18.2*.

S. No.	*Cis* regulatory element	Position	Sequence	Description	Reference
1	2SSEEDPROTBANAPA	−325 (+)	CAAACAC	Seed specific expression element	Stalberg et al., 1996
2	CANBNNAPA	−325 (+)	CNAACAC	Embryo and endosperm-specific Expression element	Ellerstrom et al., 1996
3	CBFHV	−89 (+)	RYCGAC	Dehydration responsive element	Xue, 2002
4	DPBFCOREDCDC3	−322, −333, −1461 (+)	ACACNNG	ABA and embryo specific element	Kim et al., 1997
5	DRE2COREZMRAB17	−89 (+)	ACCGAC	ABA and drought responsive element	Busk et al., 1997; Kizis and Pages, 2002
6	EBOXBNNAPA	−236, −308, −332, −723, −1460, −1715, −1842 (+)	CANNTG	Seed specific expression element	Stalberg et al., 1996
7	HSE	−422 (+)	GAANNTTCNNGAA	Heat stress responsive element	Amin et al., 1988
8	LTRE1HVBLT49	−247 (+)	CCGAAA	Low-temperature-responsive element	Dunn et al., 1998
9	LTRECOREATCOR15	−88 (+)	CCGAC	Low temperature responsive element	Baker et al., 1994; Jiang et al., 1996
10	MYBCORE	−551, −1074 (+)	CNGTTR	Dehydration responsive element	Urao et al., 1993
11	MYCCONSENSUSAT	−236, −308, −332, −724, −1460, −1715, −1842 (+)	CANNTG	Dehydration and cold responsive element	Abe et al., 2003; Chinnusamy et al., 2003
12	NAPINMOTIFBN	−1001 (+)	TACACAT	Seed specific expression element	Ericson et al., 1991
13	POLLEN1LELAT52	−64, −353, −640, −917, −1281, −1297, −1414, −1434, −1562, −1934, −1938 (+)	AGAAA	Pollen specific expression element	Bate and Twell, 1998; Filichkin et al., 2004
14	SEF4MOTIFGM7S	−648, −1091, −1652 (+)	RTTTTTR	Seed specific expression element	Allen et al., 1989
15	UPRMOTIFIAT	−124 (+)	CCACGTCA	Unfolded protein response	Martinez and Chrispeels, 2003; Oh et al., 2003
16	UPRMOTIFIIAT	−138 (+)	CCNNNNNNNNNN NNCCACG	Unfolded protein response	Martinez and Chrispeels, 2003; Oh et al., 2003

et al., 1997; Lee et al., 1997). CS monomers (150 nM) were incubated with or without HSP and with lysozyme as control at 38°C for 60 min and a quick decrease in CS activity was observed (**Figure 3E**). Control without HSP and with lysozyme showed 20% remaining activity after just 20 min of heat stress which came down to almost zero after 60 min of heat stress whereas CS with OsHSP18.2 retained 30% activity even after 60 min of heat. After 60 min, all the combinations were shifted to 22°C, temperature permissive for refolding. Even at 22°C, control lacking HSP and lysozyme control did not display any regain in CS activity but CS with OsHSP18.2 showed a regain of 60% of native CS activity. This result clearly demonstrated that OsHSP18.2 functions as a molecular chaperone that facilitates protein folding and prevents thermal denaturation.

Seed Specific Overexpression of *OsHSP18.2* in *Arabidopsis thaliana* Improves Seed Vigor and Longevity

To examine the functional implication of sHSP in seed vigor and longevity, *OsHSP18.2* was overexpressed in seeds in *Arabidopsis thaliana* using the napin promoter. Seed specific *OsHSP18.2* transcript accumulation was examined through quantitative RT PCR and significant levels of transcripts were observed in seeds of transgenic lines. Based on this analysis, three independent homozygous T3 lines were selected and subsequently used to assess their germination vigor and longevity. To evaluate seed vigor and longevity, seeds were subjected to CDT and germination performance was analyzed. Under normal conditions, transformed and control seeds (empty vector transformed or WT) exhibited 100% germination (**Figure 4A**), however, after CDT, control seeds showed <20% germination as opposed to *OsHSP18.2* transformed seeds where remarkably >50% germination was observed in each line (**Figure 4B**). In addition, TZ staining was carried out to examine the potential viability of these seeds (Berridge et al., 1996). TZ precipitates to red colored 2,3,5 triphenyl formazan by the activity of dehydrogenases present in the living cells thus staining them red. As expected under normal condition, *OsHSP18.2* expressing and control seeds were stained dark red indicating their viability while after CDT, only *OsHSP18.2* transformed seeds exhibited dark red staining (i.e., viable) in contrast to control seeds which remain unstained or were stained pale red (non-viable; **Figure 4C**). Aging in seeds is also accompanied with ROS

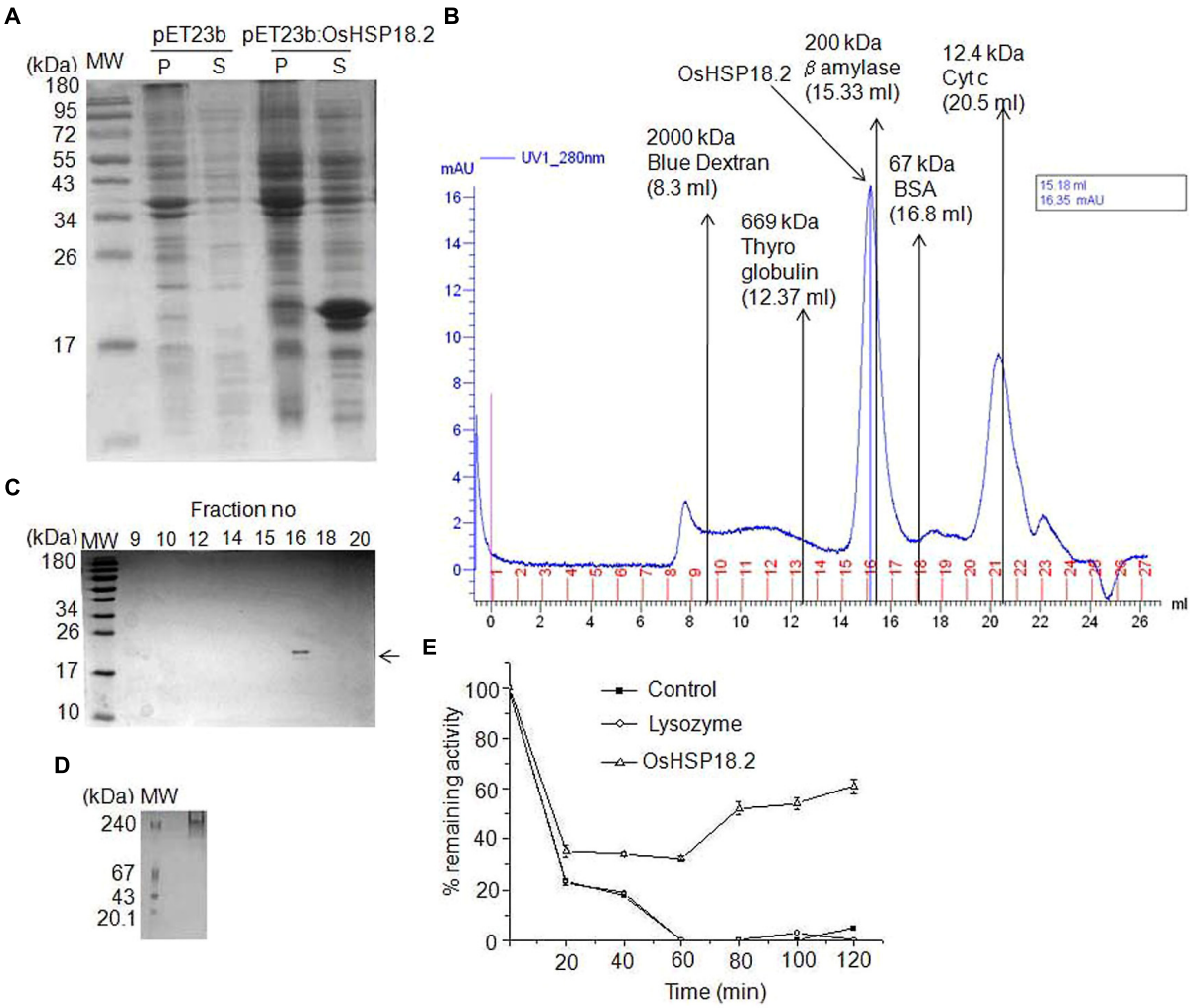


FIGURE 3 | Characterization of recombinant OsHSP18.2. (A) Bacterial overexpression of OsHSP18.2 in *Escherichia coli*. Proteins were separated on a 15% SDS-PAGE gel. pET23b empty vector transformed cells were used as a control. Pellet (P) and Supernatant (S) were run for control and *OsHSP18.2* transformed cells along with the molecular weight marker. **(B)** Size exclusion chromatography on Superose 6 column revealed the homooligomeric nature of OsHSP18.2. Purified protein and appropriate molecular weight markers were run and their elution time (V_e) to void volume (V_o) ratio (V_e/V_o) was plotted against their molecular weight in log scale and fitted in an exponential function where $y = 512304e^{-4.306x}$. Approximate molecular weight of OsHSP18.2 was calculated from the above equation as ~202 kDa which reflects dodecameric conformation. **(C)** Gel filtration fractions as run on 15% SDS-PAGE show a single band of OsHSP18.2 in fraction number 16 corresponding to the gel filtration peak. **(D)** Five percent native polyacrylamide gel shows a protein band of OsHSP18.2 across the 240 kDa marker band. **(E)** OsHSP18.2 prevents irreversible thermal inactivation of CS at 38°C. CS was incubated without HSP (—■—), with lysozyme (—○—) and with OsHSP18.2 (—Δ—) at 38°C for 60 min and then shifted to 22°C for refolding. CS enzyme activity was assayed at the mentioned time points. Values are the mean ± SE of three independent experiments.

accumulation (Bailly, 2004). ROS adversely affects cellular proteins and enzymes and renders them inactive. Therefore, we wanted to check if the overexpression of *OsHSP18.2* protects the seed against ROS mediated damage. DAB staining was performed for this purpose as it stains the areas of H_2O_2 production (Mao and Sun, 2015). Results clearly demonstrated that WT and VC seeds accumulated more H_2O_2 after CDT in comparison to *OsHSP18.2* overexpressing lines (**Figure 4D**). These data clearly indicate that WT or empty vector transformed seeds subjected to controlled deterioration exhibited higher mortality, reduced germination and more H_2O_2 accumulation while seeds from transgenic lines overexpressing *OsHSP18.2* exhibited less mortality, better germination and less H_2O_2, thus demonstrating the role of *OsHSP18.2* toward maintaining seed viability and longevity during aging.

OsHSP18.2 Transformed *Arabidopsis* Seeds Display Improved Seed Germination and Seedling Establishment Under Abiotic Stress Conditions

Germination performance of these transgenic seeds under various stress situations were also evaluated, since seed vigor also implies the ability to complete germination under widely

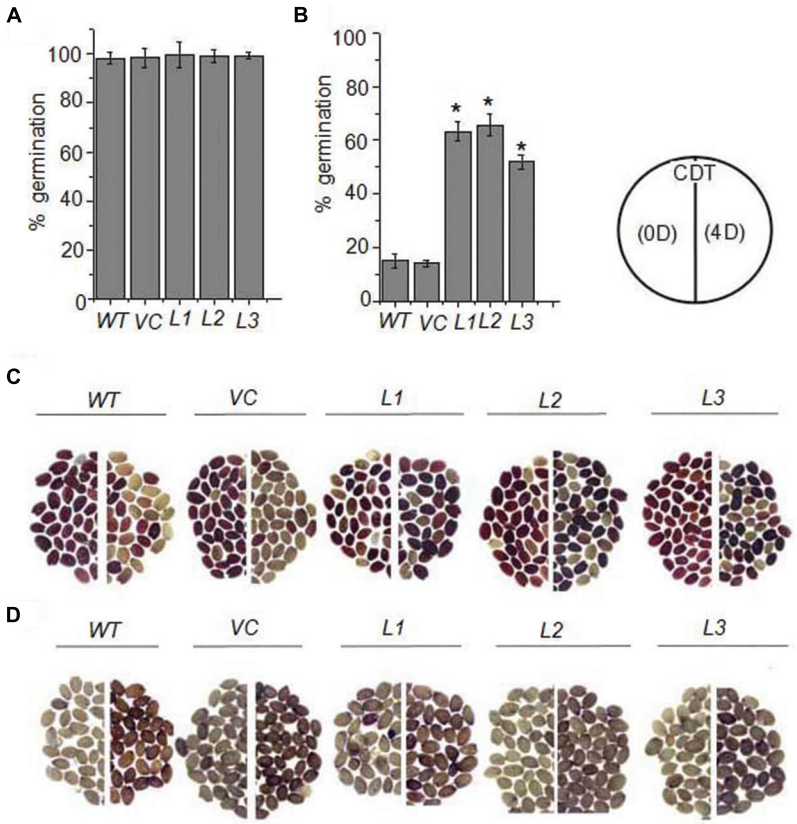


FIGURE 4 | Germination and viability of *OsHSP18.2* overexpressing seeds after CDT. Six week-old seeds were subjected to 4 days of CDT as described in Materials and Methods. Germination analysis of WT, empty vector VC, and three *OsHSP18.2* transgenic lines (L1, L2, and L3) before **(A)** and after **(B)** 4 days of CDT. **(C)** Tetrazolium assay for seed viability and **(D)** DAB staining for H_2O_2 production of WT, empty vector (VC), and three *OsHSP18.2* transgenic lines (L1, L2, and L3) before (left) and after 4 days of CDT (right). Age matched seeds were used for all germination experiments. In graph the values are mean ± SE of three independent sets ($n = 50$). Asterisk indicate significant difference between WT and test genotypes ($^*P < 0.01$).

variable environmental conditions. Germination of WT, VC, and transgenic seeds was assessed under heat, dehydration, and salt stress and was considered complete when radicle emerged beyond testa. While moderate level of these stresses (i.e., 37°C heat, 100 mM NaCl and −0.25 MPa PEG) did not much affect the germination (data not shown) but slightly elevated stresses revealed a significant difference in germination pattern among control and overexpressing seeds (**Figure 5**). In normal conditions, *OsHSP18.2* transformed and control seeds showed 100% germination. For heat stress, seeds were treated at 45°C for 1 h and then plated on ½ MS, results revealed that >90% of transgenic seeds could complete germination in all lines by the end of 5 days whereas only 70% seeds of WT and VC could germinate by this time (**Figure 5C**). At 150 mM NaCl concentration, >90% seeds of transgenic lines could germinate compared to 80% of WT and VC (**Figure 5E**). Dehydration stress was provided by −0.4 MPa PEG and as expected overexpression lines performed better than control lines (**Figure 5G**). All seeds were monitored for 7 days and seedling establishment was checked which was taken as the emergence of green cotyledons over this period as indicated in previous study (Silva-Correia et al., 2014). Results suggest that in addition to seed germination a significant difference was also observed for seedling establishment among control and *OsHSP18.2* overexpressing seeds in adverse conditions (**Figure 5**).

Overall results conclude with strong evidence that *OsHSP18.2* is an aging responsive protein which plays an important role in maintaining seed vigor and longevity as well as seedling emergence by protecting structural damage to proteins and restricting ROS accumulation during prolonged storage.

Discussion

Research on small HSPs has been more emphasized toward plant stress tolerance, however, their role in seed physiology has not been elucidated properly, particularly in rice where majority of the sHSPs are specifically expressed in seed (Sarkar et al., 2009). In this study, our proteomic analysis identified OsHSP18.2 as an aging responsive protein whose abundance significantly increases after artificial aging, indicating its involvement in rice seed vigor and longevity. Previous studies clearly revealed that similar molecular events accompany both in artificial and

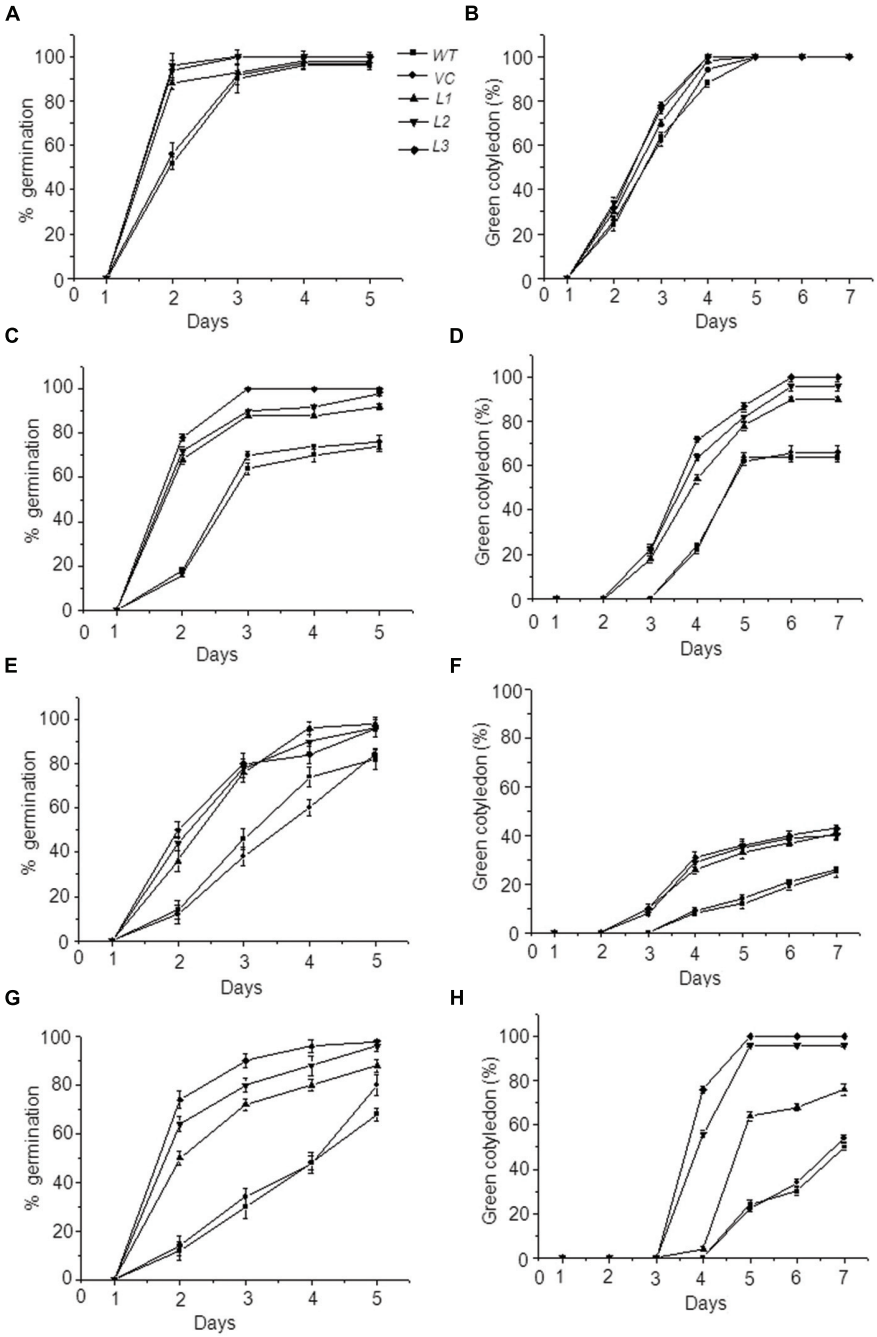


FIGURE 5 | Germination and seedling establishment of *OsHSP18.2* overexpressing seeds under abiotic stresses. Germination percentage **(A,C,E,G)** and green cotyledon emergence **(B,D,F,H)** of WT, VC, and transgenic seeds overexpressing *OsHSP18.2* scored under **(A,B)** Control conditions, **(C,D)** Heat (45°C), **(E,F)** Salt (150 mM NaCl), and **(G,H)** Dehydration (−0.4 MPa PEG). Age matched seeds were surface sterilized and plated on either ½ MS or ½ MS supplemented for various stresses. Plates were stratified at 4°C for 3 days and transferred to growth chamber at 22 ± 2°C. Values are mean ± SE of three independent sets ($n = 50$).

natural seed aging and thus artificial aging induced protein is also likely to be induced by natural aging (Catusse et al., 2008; Rajjou et al., 2008). Subsequently, we showed that *OsHSP18.2* transcript is markedly increased at the late maturation stage and is highly abundant in dry seeds and sharply decreases after germination. This expression pattern of *OsHSP18.2* also supports its involvement in desiccation tolerance and longevity as other protective molecules that are generally associated with seed desiccation tolerance and longevity in orthodox seeds are also accumulated in maturation phase and are highly abundant in dry seeds. High transcript accumulations of various sHSPs during seed maturation and in dry seed were also reported in several

plant species like pea, sunflower, *Arabidopsis* and rice (Coca et al., 1994; DeRocher and Vierling, 1994; Wehmeyer et al., 1996; Sarkar et al., 2009; Omar et al., 2011; Zhou et al., 2012). Accelerated aging induced increase in *OsHSP18.2* transcript level in rice seed also strengthens our hypothesis that OsHSP18.2 indeed participates in seed vigor and longevity. Notably, a high abundance of sHSPs was reported in beech seeds stored for 8 years (Kalemba and Pukacka, 2008). In addition, studies also suggest that induction of certain sHSPs in embryo is a routine part of seed development and seed desiccation tolerance program (DeRocher and Vierling, 1994).

Moreover, analysis of *OsHSP18.2* promoter also revealed motifs like 2SSEEDPROTBANAPA, CANBNNAPA, DPBFCOREDCDC3, EBOXBNNAPA, NAPINMOTIFBN, and SEF4MOTIFGM7S which are responsible for seed and embryo specific expression (**Table 1**). In addition to this there are many abiotic stress and hormone responsive elements as well which include the heat stress response element HSE (GAANNTTCNNGAA) and well known dehydration responsive element CBFHV (RYCGAC), MYBCORE and MYCCONSENSUSAT thus explaining a higher expression of the transcript during heat and dehydration stress. Surprisingly, few low temperature responsive sequences were also present but our real time experiments revealed not much induction of OsHSP18.2 transcript during cold stress. DPBFCOREDCDC3 (ACACNNG) and DRE2COREZMRAB17 (ACCGAC) responsible for ABA inducible expression are also present. Expression data available on GENEVESTIGATOR also reveals slightly up regulation in response to ABA and Salicylic acid treatment (Hruz et al., 2008). Multiple repeats of pollen specific expression element POLLEN1LELAT52 (AGAAA) present in the promoter suggests that developmentally regulated expression of sHSPs during desiccation associated plant stages such as pollen formation, sporulation, and seed development emphasizes their crucial role during the acquisition of desiccation tolerance (DeRocher and Vierling, 1994). Interestingly, some highly conserved sequence motifs like UPRMOTIFIAT (CCACGTCA) and UPRMOTIFIIAT (CCNNNNNNNNNNNNCCACG) for unfolded protein response are also present as these elements have been also shown to occur in the promoters of other heat shock protein (HSP90) in *Arabidopsis*. Thus, these sequences might drive the expression of OsHSP18.2 gene during the accumulation of unfolded polypeptides in the cell.

Subsequently, our biochemical study clearly demonstrated that OsHSP18.2 forms oligomeric complex, is dodecameric in nature and function as a molecular chaperone (**Figure 3**). Studies on wheat sHSP16.9 also demonstrated a dodecameric structure of this heat shock protein although homologs from other species have been shown to have structures with subunits ranging between 2 and >48 subunits (van Montfort et al., 2001; Basha et al., 2012). This is well known that sHSPs function in plant stress adaptation by binding to vulnerable cellular proteins during stress conditions, prevent their aggregation and thus hold them in a competent state for refolding by other chaperones (Ehrnsperger et al., 1997; Veinger et al., 1998; Murakami et al., 2004). Our data clearly establishes that OsHSP18.2 works as a chaperone at high temperature and prevents irreversible thermal inactivation of CS. Comparative studies have shown that addition of pea HSP18.1 significantly increased the refolding of Luciferase even though DnaK system was alone sufficient for stabilization and refolding thus emphasizing its role in restricting denaturation and assisting refolding (Lee and Vierling, 2000). Taken together the increased accumulation of *OsHSP18.2* in later stages of seed maturation and aging and its function as a molecular chaperones, suggests that OsHSP18.2 might protect vulnerable cellular proteins during maturation drying, desiccation and aging in seeds. This hypothesis fits well as during seed maturation, desiccation, and storage, seed, particularly embryo faces severe dehydration and oxidative stress that can potentially damage seed proteins (Tamarit et al., 1998; McDonald, 1999; Ingrosso et al., 2000; Rajjou and Debeaujon, 2008). In addition, *OsHSP18.2* was shown to be highly induced in rice seedlings exposed to thermal stress, indicating its participation also in thermal stress tolerance.

Our subsequent functional studies essentially demonstrated that *OsHSP18.2* has the ability to improve seed vigor and longevity (**Figure 4**). This improved seed vigor and longevity correlates well with the reduced deleterious ROS accumulation in transformed seeds. Similar to our observation, heterologous expression of a sHSP from sacred lotus in *Arabidopsis* also lead to enhanced germination of transgenic seeds after accelerated aging treatment (CDT; Zhou et al., 2012). In addition, certain members of the sHSP family have been known to be involved in scavenging of these reactive oxygen species (Härndahl et al., 1999; Fedoroff, 2006). Our results also demonstrated faster and better germination and seedling establishment under adverse situations in *OsHSP18.2* overexpressing lines. Collectively, our findings clearly establish that OsHSP18.2 is seed vigor and aging responsive protein which functions as a molecular chaperone in stabilizing the cellular proteins from irreversible damage and containing ROS accumulation during seed development particularly during seed maturation, drying and storage. Finally, our results also propose that this gene can be a good candidate to improve seed vigor and longevity in terms of better seed germination and seedling establishment in wide environmental stress conditions.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00713

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A Novel Soybean Intrinsic Protein Gene, *GmTIP2;3,* Involved in Responding to Osmotic Stress

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Water is essential for plant growth and development. Water deficiency leads to loss of yield and decreased crop quality. To understand water transport mechanisms in plants, we cloned and characterized a novel tonoplast intrinsic protein (TIP) gene from soybean with the highest similarity to TIP2-type from other plants, and thus designated *GmTIP2;3*. The protein sequence contains two conserved NPA motifs and six transmembrane domains. The expression analysis indicated that this gene was constitutively expressed in all detected tissues, with higher levels in the root, stem and pod, and the accumulation of *GmTIP2;3* transcript showed a significant response to osmotic stresses, including 20% PEG6000 (polyethylene glycol) and 100 μM ABA (abscisic acid) treatments. The promoter-GUS (glucuronidase) activity analysis suggested that *GmTIP2;3* was also expressed in the root, stem, and leaf, and preferentially expressed in the stele of root and stem, and the core promoter region was 1000 bp in length, located upstream of the ATG start codon. The GUS tissue and induced expression observations were consistent with the findings in soybean. In addition, subcellular localization showed that *GmTIP2;3* was a plasma membrane-localized protein. Yeast heterologous expression revealed that *GmTIP2;3* could improve tolerance to osmotic stress in yeast cells. Integrating these results, *GmTIP2;3* might play an important role in response to osmotic stress in plants.

Keywords: soybean, *GmTIP2;3*, heterologous expression, promoter, osmotic stress

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INTRODUCTION

Lack of water resources is an important factor restricting the development of agriculture. Plant growth and development depend on water uptake and transport regulation across cellular membranes and tissues. In the past, it was thought that water moved across cell membranes by free diffusion through the lipid bilayer. However, its transport is now thought to be highly and selectively regulated by aquaporins. Aquaporins (AQPs) belong to the ancient major intrinsic protein (MIP) family found in animals, microbes, and plants. Since the discovery of AQP1

Abbreviations: GFP, Green Fluorescent Protein; GUS, Glucuronidase; TIP, Tonoplast Intrinsic Protein; QRT-PCR, Quantitative reverse transcriptase Chain Reaction; CDS, Coding sequence; PEG, Polyethylene glycol; ABA, Abscisic acid.

(Denker et al., 1988), many aquaporin genes have been found in plants including 35 AQPs in *Arabidopsis* (Quigley et al., 2002; Boursiac et al., 2005), 31 in *Zea mays* (Chaumont et al., 2001), and 33 in *Oryza sativa* (Sakurai et al., 2005). Guo et al. (2006) further analyzed the expression and function of the rice plasma membrane intrinsic protein (PIP) gene family. Other scholars found 23 AQPs in *Physcomitrella patens* (Danielson and Johanson, 2008), 37 in *Solanum lycopersicum* (Sade et al., 2009), 66 in soybean (Zhang et al., 2013), 47 in tomato (Reuscher et al., 2013), 71 in *Gossypium hirsutum* (Park et al., 2010), and 53 in Chinese cabbage (Tao et al., 2014). Plant AQPs can be categorized into major four subfamilies based on localization and expression patterns: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small and basic intrinsic proteins (SIPs) (Chaumont et al., 2001; Kaldenhoff and Fischer, 2006), and uncategorized X intrinsic proteins (XIPs) (Danielson and Johanson, 2008).

AQPS play important roles in various physiological processes in plants, such as growth, development, and response to biotic and abiotic stresses. Srivastava et al. (2015) also reviewed the versatile functions of aquaporins as molecular conduits in the plant response to abiotic stresses. For example, Guenther and Roberts (2000) isolated two major intrinsic membrane proteins from *Lotus japonicus*, named *LIMP1* and *LIMP2*. Functional analysis using the *Xenopus oocytes* system indicated that LIMP1 appeared to be a member of the TIP subfamily and LIMP2 was a nodulin 26 ortholog protein. Rodrigues et al. (2013) investigated a gene encoding a root-specific tonoplast intrinsic aquaporin (TIP) from *Eucalyptus grandis* named *EgTIP2*, whose expression was induced by PEG and mannitol treatments but was downregulated by abscisic acid, suggesting that *EgTIP2* might be involved in the eucalyptus response to drought. Wang et al. (2014) cloned and characterized a tonoplast AQP gene (*TsTIP1;2)* from the halophyte *Thellungiella salsuginea* and reported that it mediated the transduction of both H_2O and H_2O_2 across the membranes and might contribute to the survival of *T. salsuginea* under multiple stresses. Ligaba et al. (2011) studied the expression patterns of 7 *MIP* genes from barley under different abiotic stresses using quantitative real-time PCR (RT-PCR), indicating that abiotic stress modulates the expression of major intrinsic proteins in barley. Zelazny et al. (2007) by using FRET imaging analysis showed that plasma membrane aquaporins could interact to regulate their subcellular localization in living maize cells. Tomato *SiTIP2;2* expressing in transgenic *Arabidopsis* could enhance the plant's tolerance to salt stress and interact with its homologous proteins SiTIP1;1 and SiTIP2;1 (Xin et al., 2014). Gao et al. (2010) overexpressed *TaNIP*, a putative aquaporin gene from wheat, and found that it could enhance salt tolerance in transgenic *Arabidopsis*. Wang et al. (2011) cloned the novel *Glycine soja* tonoplast intrinsic protein gene *GsTIP2;1*, and the overexpression of *GsTIP2;1* in *Arabidopsis* repressed/reduced tolerance to salt and dehydration stress, suggesting that *GsTIP2;1* might mediate stress sensitivity by enhancing water loss in plants.

In this study, a novel tonoplast intrinsic aquaporin from soybean, *GmTIP2;3*, was cloned and characterized. Protein structure analysis showed that *GmTIP2;3* possesses typical aquaporin characteristics, such as six transmembrane domains and NPA motifs. The expression analysis indicated that it was constitutively expressed in all tissues tested, especially in the root, stem, and pod, and exhibited responses to ABA and PEG treatments at certain time points. Subcellular localization showed it to be localized in the cell plasma membrane. The promoter activity assay demonstrated that the core sequence for this gene was 1000 bp upstream from the ATG start codon. Yeast heterologous expression revealed that *GmTIP2;3* could improve osmotic tolerance in yeast cells. Integrating these results, *GmTIP2;3* plays an important role in response to osmotic stress in plants.

MATERIALS AND METHODS

Plant Materials

Glycine max var. Willimas 82 was selected for the experiments, which included growth of seedlings, flowering, podding, extracting total RNA for *GmTIP2;3* cloning, and tissue expression and induced expression analysis. *Lotus japonicus* was used to transfer the promoter sequence for activity testing and Arabidopsis ecotype Col-0 was used for transformation. Protoplasts were grown in a 7:2:1 (v/v/v) mixture of vermiculite:soirite:perlite under a 16-h light/8-h dark regime, and the day and night temperatures were 23°C / 20°C, respectively. The plants were watered every week.

Gene Cloning and Sequence Analysis

The gene primers were designed based on the full-length coding sequences, and RT-PCR (reverse transcriptase-polymerase chain reaction) was performed to isolate the genes from soybean tissues. The neighbor-joining (NJ) tree was constructed from soybean *GmTIP2;3* and from other plant TIPs based on alignment using the Clustalx and MEGA 5.0 software, and used to explore the evolutionary relationships of soybean and other plant TIPs.

qRT-PCR Analysis

Soybean samples from the seedling, flowering, and podding stages (root, stem, leaf at young seedling stage; root, stem, leaf, and flower at flowering stage; and root, stem, leaf, and pod at podding stage) were harvested and frozen in liquid nitrogen for RNA extraction. Soybean roots were collected from plants treated with PEG6000 (polyethylene glycol) for 0, 2, 4, 12, and 48 h and with 100 μ M ABA (abscisic acid) for 10, 20, 30, 45, 60, 90, and 120 min. The total RNA for all samples used in this study was isolated using TRIzol® reagent (Invitrogen) following the manufacturer's instructions and used for qRT-PCR analysis. The qRT-PCR analysis was conducted according to the method described by Zhang et al. (2013) and was repeated three times. The primers used are given in **Table 1**.

Subcellular Localization

PCR-generated *Hind III-BamH1* fragments containing the open reading frame of *GmTIP2;3* were subcloned upstream of the GFP gene in plasmid pJIT166GFP. All constructs were validated by sequencing. The primers used are listed in **Table 1**.

Arabidopsis protoplasts were isolated according to Yoo et al. (2007). The CDS of *GmTIP2;3* without stop codons was used to create an in-frame fusion with GFP gene inserted into the pJIT166-GFP vector. The resulting fusion construct and an empty vector as a control (p35S::GFP) were introduced into *Arabidopsis* protoplasts by the PEG4000-mediated method (Abel and Theologis, 1994). After incubation of transformed *Arabidopsis* protoplasts for 18–24 h at room temperature, GFP signal was detected by confocal fluorescence microscopy (Zeiss, LSM510 Meta, Carl Zeiss AG).

Promoter Analysis

A 2081 bp-long region (named P1) located upstream of the ATG start codon was cloned from soybean genome DNA using primers described in **Table 1**, and sequence analysis was performed using the PlantCARE online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). To find the core promoter region of *GmTIP2;3,* seven truncated fragments (P2-P8) were cloned from P1 and transformed into *A. rhizogenes* strain K599 for GUS activity detection. Soybean hairy root transformation was performed according to the method given by Subramanian et al. (2005). The primer pairs are listed in **Table 1**.

Histochemical and Fluorometric GUS Assay

For histochemical staining of GUS, fresh tissue samples including whole transgenic lotus plants, soybean hairy roots, and dissected leaves from positive plants that had undergone osmotic stress (20% PEG6000 and 100 μMABA), salinity (200 mM NaCl solution), and wounding for 2 h were immediately dipped into X-Gluc solution, as previously described (Jefferson et al., 1987). After overnight incubation at 37°C in the growth chamber, stained samples were bleached with 70% (v/v) ethanol, washed several times with ddH_2O, and observed under a Zeiss Stemi 2000-C microscope, Germany.

A quantitative fluorometric GUS assay was conducted as described by Jefferson et al. (1987). The protein concentrations from a series of truncated constructs pGUSP1-P4 in transgenic soybean hairy roots were assessed by Bradford method, using bovine serum albumin (BSA) as a standard. GUS activity was normalized to the protein concentration of each sample and calculated as nmol of 4-MU per milligram of soluble protein per minute.

Generation of Transgenic *Lotus japonicus* Plants

The resulting pGUS-*GmTIP2;3* promoter(p3):GUS plasmid was introduced into *Agrobacterium rhizogenes* strain K599 and used

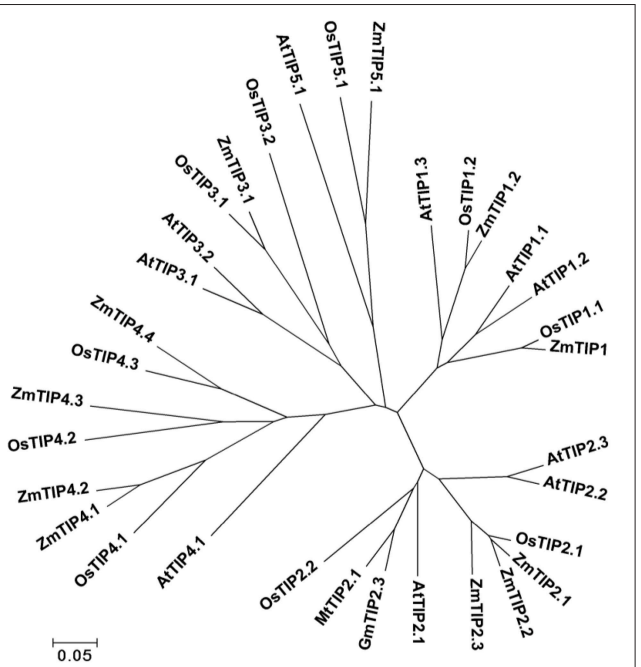


FIGURE 1 | Phylogenetic tree of *GmTIP2;3* and reported TIP proteins from *Arabidopsis thaliana* TIPs (AtTIPs), *Oryza saliva* TIPs (OsTIPs), *Zea mays* TIPs (ZmTIPs), and *Medicago sativa* TIPs (MtTIPs). The *Glycine max TIP* cloned in this paper showed the highest similarity to TIP2-Type proteins from other plants. Therefore, *TIP* was designated as *GmTIP2;3*.

TABLE 1 | Primers for this study.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′
GmTIP2;3 qRT-PCR	CCTTATCTATCTTCACCTCCATCT	GCCACCAGAGATGTTGGCACCA
GmTIP2;3 GFP	CGCAAGCTTATGGGTGGCATTGCAT	CGCGGATCCAAATTCACTGGAAAGA
GmTIP2;3 Yeast	ATGCGGCCGCATGGGTGGCATTGCAT	CGCGGATCCAAATTCACTGGAAAGA
P1	ATGTGCAGGATGATGACCAG	CATCTTCAGAAGTTTCGAG
P2	GACTCCTCCTGCGGCTGGCATTA	CATCTTCAGAAGTTTCGAG
P3	GAAATATCATAATCTTGCTTCTTGT	CATCTTCAGAAGTTTCGAG
P4	AGGAATCATTCATTAGCTTCCGGA	CATCTTCAGAAGTTTCGAG
P5	CATAGACGTAAACAACCAATGAGT	CATCTTCAGAAGTTTCGAG
P6	GTTTACTTCTTAAAATAAACAG	CATCTTCAGAAGTTTCGAG
P7	AATATTTTTTTTAACAAAACCG	CATCTTCAGAAGTTTCGAG
P8	ATTTTGAAATTCCACAACCTCTT	CATCTTCAGAAGTTTCGAG
GmActin	CGGTGGTTCTATCTTGGCATC	GTCTTTCGCTTCAATAACCCTA

to transform small *Lotus japonicus* seedling to produce hairy roots, as in the soybean hairy root system. The hairy roots were transferred to MS medium with 0.5 mg/L 6-BA for 20 days to generate adventive buds, and 1–2 cm high regeneration seedlings without roots were transferred to 1/2 MS medium to produce roots. Finally, the whole seedlings were transferred to pots.

Yeast Transformation

The novel pYES2-GFP was reconstructed via the recombination of pYES2 and pJIT166-GFP using the same double-digestion by *Hind III* and *EcoR I*. The CDS with *Hind III* and *BamH I* digestion for the forward primer and reverse primer, respectively (**Table 1**), was inserted into the pYES2-GFP vector digested with the same enzymes.

The resulting pYES2-*GmTIP2;3*:GFP plasmid was introduced into *S. cerevisiae* INVSc1 strain cells using the lithium acetate method, with the empty vector pYES2-GFP as a control. *S. cerevisiae* INVSc1 strain cells transformed with the empty vector PYES2-GFP alone and with pYES2-*GmTIP2;3*:GFP were induced with galactose and spotted on the SC-Ura medium in 0, 10, 100, 1000, and 10,000-fold-dilutions, and the drought tolerance of yeast cells expressing *GmTIP2;3* was tested by 30% PEG6000 treatment for 40 h. The GFP in yeast was observed using a fluorescence microscope (Olympus BX61). All experiments were repeated three times.

Accession Number

The accession numbers of proteins used for Multiple Sequence Alignment (MSA) and phylogenetic tree analysis are as follows: SsTIP1;1 (AJ242805.1) from *Sporobolus stapfianus*, PsTIP1;1 (AJ243309.1) from *Pisum sativum*, SoTIP2;1 (AJ245953.1) from *Spinacia oleracea*, PtTIP3;2 (XM_006372585.1) from *Populus trichocarpa*, and GmTIP2;3 (XM_006582773.1) from *Glycine max* were used for MSA. MtTIP2.1 (XP_003626979.1) from *Medicago truncatula*; AtTIP4.1 (NP_180152.1), AtTIP1.3 (NP_192056.1), AtTIP5.1 (NP_190328.1), AtTIP2.3 (NP_199556.1), AtTIP2.2 (NP_193465.1), AtTIP2.1 (NP_188245.1), AtTIP1.1 (NP_181221.1), AtTIP1.2 (NP_189283.1), AtTIP3.1 (NP_177462.1), and AtTIP3.2 (NP_173223.1) from *Arabidopsis thaliana*; OsTIP1.1 (P50156.1), OsTIP1.2 (NP_001045562.1) OsTIP2.1 (NP_001047632.1), OsTIP2.2 (Q5Z6F0.1), OsTIP3.1 (NP_001064933.1), OsTIP3.2 (NP_001053371.1), OsTIP4.1 (NP_001054979.1), OsTIP4.2 (BAA92993.1), OsTIP4.3 (NP_001042500.1), OsTIP5.1 (NP_001053493.1) from *Oryza sativa*; and ZmTIP1.1 (NP_001104896.1), ZmTIP1.2 (NP_001105029.1), ZmTIP2.1 (NP_001105030.1), ZmTIP2.2 (NP_001105031.1), ZmTIP2.3 (NP_001104907.1), ZmTIP3.1 (NP_001105032.1), ZmTIP4.1 (NP_001105033.1), ZmTIP4.2 (NP_001105034.1), ZmTIP4.3 (NP_001105035.1), ZmTIP4.4 (NP_001105641.1), and ZmTIP5.1

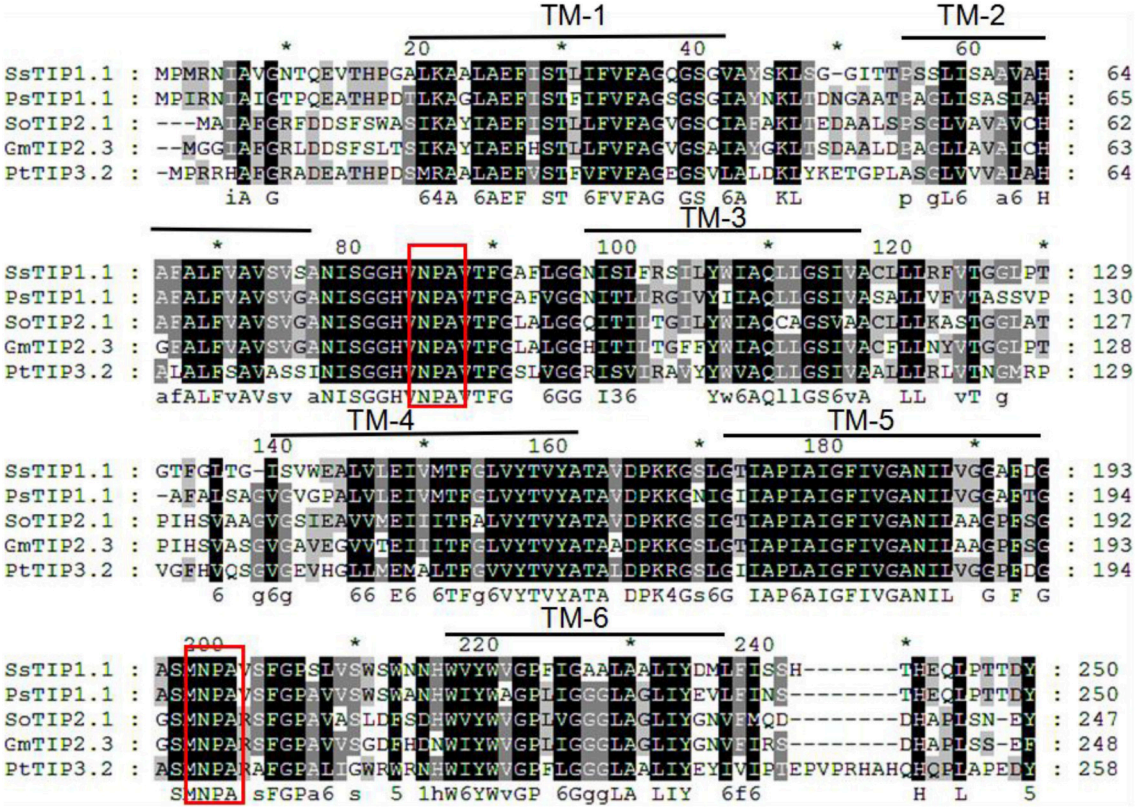


FIGURE 2 | Multiple sequence alignment of GmTIP2;3 protein amino acid sequence with other species. TM1-6 represents the six membrane-spanning helices of GmTIP2;3. The two red boxes represent two conserved "NPA" motifs of the MIP superfamily proteins. *Indicates the consensus sequence at this site.

(NP_001105036.1) from *Zea mays* were used for phylogenetic tree analysis.

Statistical Analysis

The data were analyzed by ANOVA testing using the EXCEL software. Significant differences among means were determined by the LSD at $P < 0.05$, and a–f represent the different significant levels.

RESULTS

The isolation and characterization of *GmTIP2;3*

The gene locus Glyma07 g02060 from the QTL region between the markers Satt590 and Satt567 on chromosome 7 in soybean (Specht et al., 2001) was selected as a candidate gene and further isolated by RT-PCR method. BLAST X showed that this locus encoded a protein with 89% identity to TIP2-1-like from *Cicer arietinum*. The phylogenetic trees were created using GmTIP and *Arabidopsis thaliana* TIPs (AtTIPs), *Oryza saliva* TIPs (OsTIPs), *Zea mays* TIPs (ZmTIPs), and *Medicago sativa* TIPs (MtTIPs). The phylogenetic tree showed that GmTIP had the highest similarity to TIP2-type proteins from other plants (**Figure 1**). Therefore, GmTIP was designated as *GmTIP2;3*. SMART software analysis showed that its protein sequence possessed two conserved NPA motifs and six transmembrane domains, indicating that it was a typical aquaporin protein (**Figure 2**).

Expression Analysis of *GmTIP2;3*

The temporal and spatial expression patterns of *GmTIP2;3* in various tissues/organs of soybean cv. Willimas 82 plants were examined using quantitative RT-PCR. *GmTIP2;3* appears to be expressed in most parts of the plant, with the highest expression in the root, stem, and pod, Moreover, the expression patterns of *GmTIP2;3* in different developmental stages of the same tissue, namely in different organs in the three-leaf, blooming, and podding stages, showed that the transcript abundance of *GmTIP2;3* in the stem exhibited a slight increase at the blooming stage, then significantly decreased in both the root and stem at the podding stage except in the pod tissue (**Figure 3A**).

To test whether *GmTIP2;3* responds to drought stress, soybean seedling roots were treated with PEG and ABA. Then, the expression of *GmTIP2;3* was analyzed by quantitative real-time RT-PCR. The results indicated that the expression of *GmTIP2;3* decreased within 2 h after PEG-6000 (20%) treatment, and then the mRNA level continuously increased from 4 to 12 h and reached a maximum at 12 h (**Figure 3B-1**) However, ABA treatment (100 μM) initially significantly suppressed *GmTIP2;3* expression after 10 min treatment, reached its minimum at 30 min ($p < 0.05$), then increased from 30 to 45 min and continuously decreased from 45 to 120 min, followed by a stable expression level (**Figure 3B-2**).

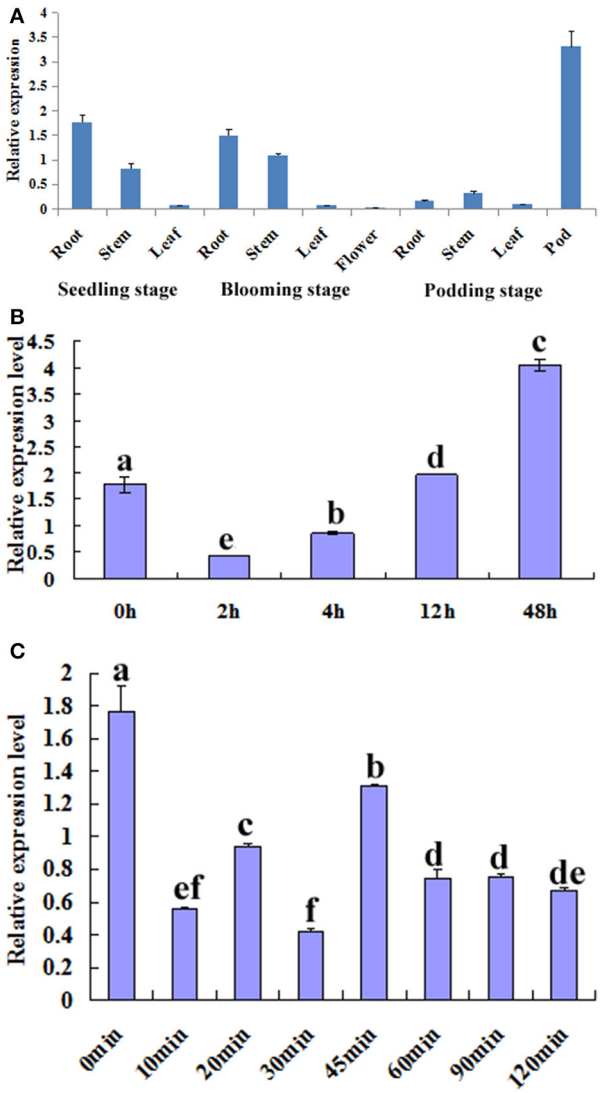


FIGURE 3 | Expression analysis of *GmTIP2;3*. (A) The temporal and spatial expression patterns of *GmTIP2;3* in different organs and at different stages. Root, stem, and leaf from three-leaf stage of young seedling; root, stem, leaf, and flower from blooming stage; and root, stem, leaf, and pod from podding stage. **(B)** The expression patterns of *GmTIP2;3* gene in soybean roots under PEG6000 and 100 μM ABA treatments; a–f indicate the significant difference level at $p < 0.05$. **(1)** Expression patterns of *GmTIP2;3* after PEG treatment for different time points. **(2)** Expression patterns of *GmTIP2;3* after ABA treatment for different time points.

The Promoter Activity Analysis of *GmTIP2;3*

To analyze the elements contained in the promoter region and the promoter activities of GmTIP2;3, a more than 2 kb (2081 bp)-long promoter region located upstream of the ATG start codon was amplified and inserted into the pGUSP vector by the T/A cloning method. The resulting construct was transformed into *Lotus japonicus*, and the transgenic plant was successfully obtained (**Figures 4A,B**). The non-transgenic plant was used

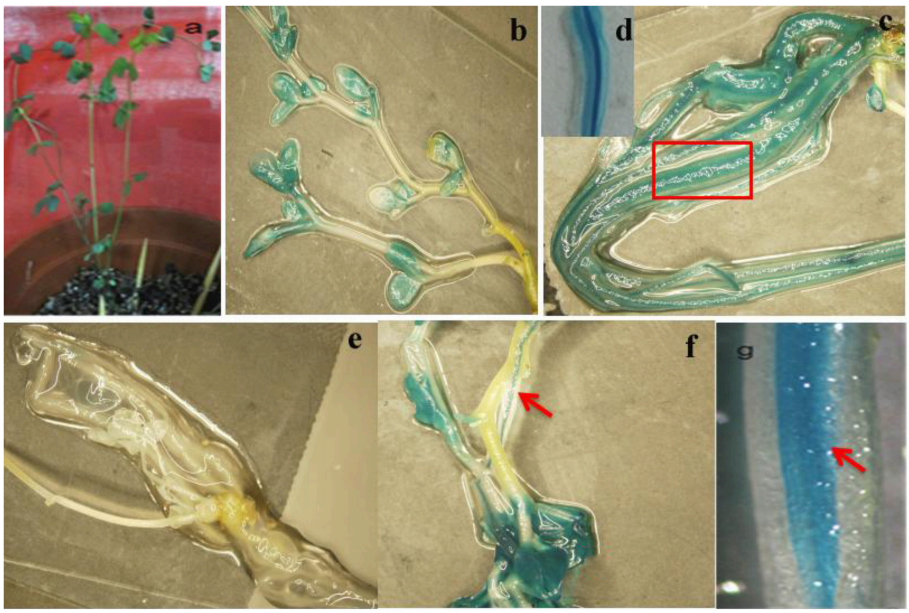


FIGURE 4 | Promoter activity analysis of *GmTIP2;3*. **(A)** The positive transgenic lotus plant transformed with pGUSGmTIP2;3 promoter vector. **(B)** GUS staining of transgenic positive plants. **(C)** High expression in root of positive plants. **(D)** Special expression in stele of root, magnified from root **(C)**. **(E)** GUS staining of negative plant. **(F)** High expression in leaf and stem stele of positive plant. **(G)** Special expression in stele of soybean hairy root. Red arrows indicate the stele of root and stem, and the red box indicates that this section was magnified into Panel **(D)**.

TABLE 2 | The cis-acting elements in *GmTIP2;3* promoter.

Site name	Organism	Position	Strand	Matrix score	Sequence	Function
Box 4	*Petroselinum crispum*	117	+	6	ATTAAT	Part of a conserved DNA module involved in light responsiveness
Box 4	*Petroselinum crispum*	275	+	6	ATTAAT	Part of a conserved DNA module involved in light responsiveness
Box I	*Pisum sativum*	322	–	7	TTTCAAA	Light-responsive element
Box I	*Pisum sativum*	757	–	7	TTTCAAA	Light-responsive element
G–box	*Solanum tuberosum*	475	+	7	CACATGG	Cis-acting regulatory element involved in light responsiveness
GA–motif	*Arabidopsis thaliana*	1008	–	8	ATAGATAA	Part of a light-responsive element
I–box	*Zea mays*	60	–	9	gGATAAGGTG	Part of a light-responsive element
I–box	*Triticum aestivum*	1006	–	8	AGATAAGG	Part of a light-responsive element
I–box	*Flaveria trinervia*	558	–	10	cCATATCCAAT	Part of a light-responsive element
Sp1	*Oryza sativa*	353	+	6	GGGCGG	Light-responsive element
TCT–motif	*Arabidopsis thaliana*	455	+	6	TCTTAC	Part of a light-responsive element

as a negative control (**Figure 4C**). GUS staining revealed that GmTIP2;3 was mainly expressed in the root (stele), stem (stele), and leaf (**Figures 4D–F**). Moreover, transgenic soybean hair roots using this construct also showed higher expression at the stele of the root (**Figure 4G**), which was consistent with its function as a water transporter. The GmTIP2;3 expression patterns in transgenic plants or hairy roots were identical to the patterns in different organs in the soybean plant. In addition, promoter sequence analysis using the PlantCARE online software indicated that it contained many light responsive elements, such as Box4, G-Box and I-Box, GATA-motif, MBS, and GARE-motif (**Table 2**). To further dissect the core region of the GmTIP2;3 promoter and explore the impact of external factors on its expression, a series of 8 truncated vectors were constructed, 2081, 1524, 1035, 935, 835, 735, 663, and 581 bp in length, named P1–P8, and transformed into *Agrobacterium rhizogenes* strain K599 to generate soybean hairy roots. The GUS staining and quantity assays demonstrated that only P1 and P3 exhibited GUS activities, and the activity of P3 was stronger than for P1 (**Figure 5A**). P5–P8 had no GUS signal, indicating that the core promoter region of GmTIP2;3 was 935 bp long from the ATG site. Interestingly, no GUS signal or GUS activity was detected for P2, implying that the inhibitor sequence occurred between P1 and P3, which also explained why the activity of P3 was stronger than for P1. Meanwhile, the expression of GmTIP2;3 was down-regulated under dark, drought (PEG and ABA), and salinity treatments for 2 h but showed no response to wounding treatment in transgenic lotus plants (**Figure 5B**). These results

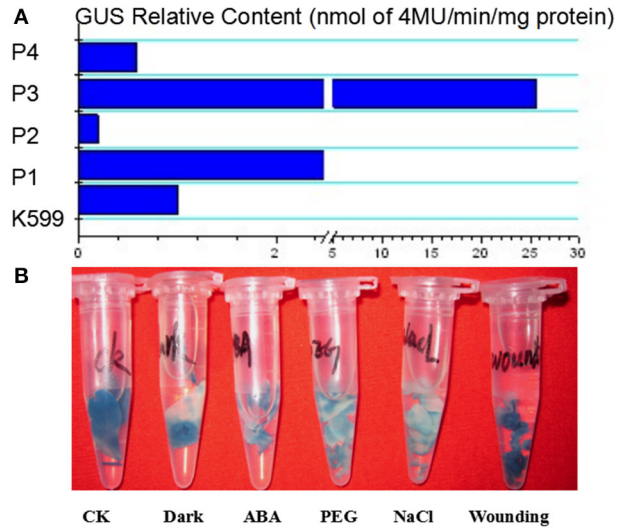


FIGURE 5 | The relative content of GUS protein harboring differently truncated promoter regions of *GmTIP2;3* and GUS expression patterns under different treatments using promoter 3.

were consistent with the results of the expression patterns after treatments with ABA and PEG in soybean roots.

Plasma Membrane Localization of *GmTIP2;3*

To examine the localization of the GmTIP2;3 protein, the coding sequences were fused in frame with the coding region of the N-terminal side of green fluorescent protein (GFP). The fusion genes were expressed under the control of the CaMV 35S promoter. GFP fluorescence was evident in the cell plasma membrane transformed with the *GmTIP2;3*::GFP fusion plasmid (**Figure 6A**), whereas GFP fluorescence (control) was detected throughout the cells transformed with GFP control plasmid (**Figure 6B**).

Heterologous Expression *GmTIP2;3* Improved Osmotic Stress Resistance in Yeast

Yeast cells carrying pYES2-*GmTIP2;3*:GFP or PYES2-GFP (control) were treated with PEG6000 for 40 h, and the survival state was detected. The results revealed that GmTIP2;3 was specifically expressed at the yeast cell membrane, and the heterologous expression of *GmTIP2;3* in yeast cells could improve the survival efficiency under osmotic stress (**Figure 7**), indicating that GmTIP2;3 played an important role in osmotic tolerance in eukaryotes.

DISCUSSION

In this study, we isolated and characterized GmTIP2;3, an MIP family protein showing the highest similarity to *Arabidopsis*, rice, and corn TIP5. SMART software showed that GmTIP2;3 contains six transmembrane domains, single "AEFH" and "NWIYWVGP" motifs, and two conserved NPA motifs. Fujiyoshi et al. (2002) reviewed the structure and function of water channels in mammalian aquaporins, reporting that the sequence alignment of aquaporins shows several highly conserved motifs including two "NPA" sequences and single "AEFL" and "HW[V/I][F/Y]WXGP" sequences. Here, we found that plant TIPs contain AEFI or AEFV/H, and TIPs from other plants do possess HW[V/I][F/Y]WXGP, but the soybean TIP5 had the motif NWIYWVGP, thereby implying the differences in function and localization between GmTIP2;3 and other plant TIPs.

Spatial and temporal expression analysis showed that *GmTIP2;3* was constitutively expressed in all tested organs, with higher expression in the root and stem, indicating that it can absorb water from the soil through the root and then transport water through the stem to other organs, such as the leaf, flower, and pod. Tungngoen et al. (2009) cloned and characterized two aquaporins, *HbPIP2;1* and *HbTIP1;1*, and induced expression analysis found that *HbTIP1;1* was down-regulated in liber tissues but up-regulated in laticifers in response to bark Ethrel treatment. Regon et al. (2014) also analyzed the expression patterns of 100 TIP aquaporin genes from dicots and monocots and indicated that the expression of *TIP* genes varies during different developmental stages and under stressed conditions. da Silva et al. (2013) identified and analyzed the expression patterns of sugarcane aquaporin genes under water deficit, thereby finding the aquaporin transcription in sugarcane to be potentially genotype specific. These findings demonstrated that *TIP* expression was organ specific or genotype specific and performed different regulator roles in different tissues. Recently, Lee et al. (2015) showed that the expressions of barley *HvTIP1;2* and *HvTIP3;1* were regulated by gibberellic acid (GA) and ABA and that these two hormones were involved in the fusion of protein storage vacuoles in aleurone cells, indicating that TIP plays another role in vacuole formation and transportation. When subjected to drought stress (ABA and PEG), the expression of *GmTIP2;3* showed a dynamic trend at different time points, with an increase after PEG and ABA treatments for 48 h and 45 min, respectively, indicating that the expression of *GmTIP2;3* exhibited a response to osmotic stress.

In fact, GmTIP2;3 should be a plasma membrane intrinsic protein (PIP). It was predicted to be localized at the plasma membrane by the online software http://www.predictprotein.org/, and this subcellular localization was proven using *Arabidopsis* protoplasts, yeast cells, and onion epidermal cells (data not shown) harboring GFP. However, BLAST result at NCBI showed GmTIP2;3 to be a tonoplast intrinsic protein (TIP). Analysis of the promoter activity of *GmTIP2;3* indicated that the activity of P3 (∼1000 bp in length) was stronger than the activity of P1 (∼2000 bp), implying that the inhibitor region occurred between these two regions, and P4 (∼550 bp) exhibited no GUS activity. To further determine the core or minimum region for the *GmTIP2;3* promoter, five truncated constructs at 100 bp intervals between P3–P4 were prepared, but no GUS signal was detected. Therefore, we concluded that the core promoter region for *GmTIP2;3* was located +1000 bp

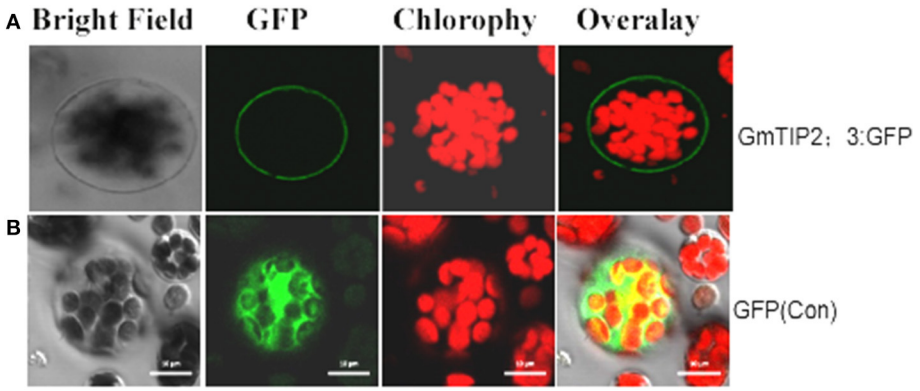


FIGURE 6 | Subcellular localization of GmTIP2;3 protein in *Arabidopsis* protoplasts. The fusion construct of the GmTIP2;3-green fluorescent protein (p35S::GmTIP2;3-GFP) in the pJIT166-GFP vector without a termination codon to create an in-frame fusion between the CDS and GFP, and the GFP control plasmid (p35S::GFP), was transformed into *Arabidopsis* protoplasts by PEG4000-mediated method. The transformed *Arabidopsis* protoplasts were incubated for 18–24 h at room temperature and observed under a confocal fluorescence microscope. GmTIP2;3 was mainly located at the cell membrane **(A)**. However, the GFP control was distributed throughout the whole cell **(B)**. Scale bars = 10 μm.

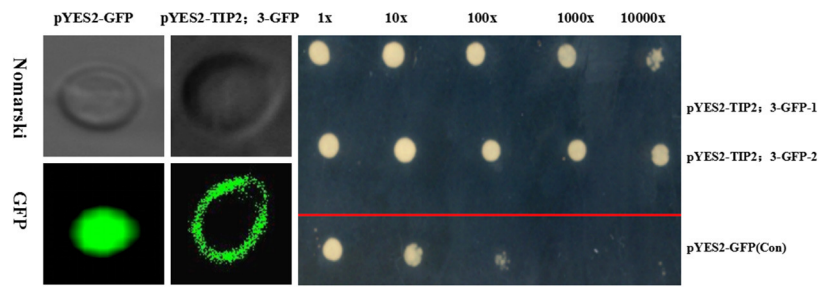


FIGURE 7 | Osmotic response of yeast cells in heterogeneously expressing *GmTIP2;3* S. *cerevisiae* INVSc1 strain cells transformed with the empty vector pYES2-GFP alone (Con) and pYES2-*GmTIP2;3*:GFP. GFP signal indicated that GmTIP2;3 was mainly located at the cell membrane, and the empty vector was distributed throughout the cells. S. *cerevisiae* INVSc1 strain cells transformed with the empty vector pYES2-GFP alone and pYES2-*GmTIP2;3*:GFP were spotted on SC-Ura medium in 0, 10, 100, 1000, and 10,000-fold dilutions, repeated twice for two different positive clones of pYES2-*GmTIP2;3*:GFP. The results showed the osmotic tolerance of yeast cells expressing *GmTIP2;3*. All experiments were repeated three times.

upstream of the ATG start codon containing the 5′UTR region of the *GmTIP2;3* gene. The promoter–GUS system was used to detect the GUS activity changes of transgenic *Lotus* leaf under different treatments, including ABA, Nacl, dark, wounding, and PEG for 2 h. The results showed that the expression of *GmTIP2;3* decreased under all treatments except wounding. The plant CARE software revealed that the promoter region contains many light-responsive elements, so the down-regulated expression under dark conditions was reasonable. Moreover, GUS activity under drought treatment for 2 h was consistent with the expression patterns after ABA and PEG treatments for 2 h. Lee et al. (2015) detected the promoter activity of *HvTIP3;1* in response to ABA and revealed that the ABA responsiveness of the *HvTIP3;1* promoter is likely to occur via a unique regulatory system distinct from the one involving the ABA-response promoter complexes. Therefore, the mechanism of the ABA responsiveness of *GmTIP2;3* should be further examined. Here, we can hypothesize that the plants first reduce the water hole number or close water channels to reduce the loss under stress by decreasing the transcription level of *GmTIP2;3*, and then when the plants have adapted to the stress environment, the expression of *GmTIP2;3* recovers to its original level and continues to increase its transcript abundance to respond to stressed conditions.

The plant response to drought is dependent on the SPAC (Soil-Plant-Air-Continuum). Root absorption and soil play important roles in plant adaption to drought stress (Shao et al., 2009). Higher expression of aquaporin proteins in plants can allow them to effectively absorb water from the soil using the roots and then transport water by the stem to other organs, such as the leaf, flower, and seed, especially under osmotic stress (Devi et al., 2015; Ding et al., 2015; Miniussi et al., 2015; Olaetxea et al., 2015). Here, the higher expression of *GmTIP2;3* in the steles of the root and stem might promote and speed up water transportation from the roots to other organs under osmotic stress, improving plant tolerance to osmotic stress.

Azad et al. (2009) analyzed water channels by yeast heterologous expression of tulip petal plasma membrane aquaporins from *Pichia pastoris* and monitored their water

channel activity (WCA) by *in vivo* spheroplast-bursting and hypo-osmotic shock assays, suggesting that *P. pastoris* can be employed as a heterologous expression system to assay the WCA and to monitor the AQP-mediating channel gating mechanism of aquaporins. The yeast heterologous expression assay in this study showed that *GmTIP2;3* could effectively improve the tolerance of yeast to drought stress. Previously, we performed this assay using salinity and drought treatments simultaneously, but the results indicated that yeast cells expressing *GmTIP2;3* did not show improved survival rates under salinity stress, implying that *GmTIP2;*3 had the ability to transport water but not ions.

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Proline accumulation and metabolism-related genes expression profiles in *Kosteletzkya virginica* seedlings under salt stress

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Proline accumulation is a common response to salt stress in many plants. Salt stress also increased proline concentration in roots, stems, and leaves of *Kosteletzkya virginica* seedling treated with 300 mM NaCl for 24 h and reached 3.75-, 4.76-, and 6.83-fold higher than controls. Further study on proline content in leaves under salt stress showed that proline content increased with increasing NaCl concentrations or time. The proline level peaked at 300 mM NaCl for 24 h and reached more than sixfold higher than control, but at 400 mM NaCl for 24 h proline content fell back slightly along with wilting symptom. To explore the cause behind proline accumulation, we first cloned full length genes related to proline metabolism including *KvP5CS1*, *KvOAT*, *KvPDH*, and *KvProT* from *K. virginica* and investigated their expression profiles. The results revealed that the expressions of *KvP5CS1* and *KvProT* were sharply up-regulated by salt stress and the expression of *KvOAT* showed a slight increase with increasing salt concentrations or time, while the expression of *KvPDH* was not changed much and slightly decreased before 12 h and then returned to the original level. As the key enzyme genes for proline biosynthesis, the up-regulated expression of *KvP5CS1* played a more important role than *KvOAT* for proline accumulation in leaves under salt stress. The low expression of *KvPDH* for proline catabolism also made a contribution to proline accumulation before 12 h.

Keywords: ***Kosteletzkya virginica*, salt stress, proline metabolism, proline accumulation, expression profiles**

Introduction

Soil salination has become one of the major abiotic tresses limiting crop growth and productivity all over the world. Saline soil is still rapidly expanding due to the deterioration of global climate and many human activities such as developing land unreasonably, improper irrigation and industrial pollution. The decreasing arable land cannot satisfy the demand of the increasing world population, which is expected to reach about 9.1 billion by 2050, about 34% higher than today's population. To cope with the serious challenge, there is an urgent need to identify and utilize salt-tolerant plants, which can be used to reclaim salt-affected soils and improve the salt tolerance of crops, or be directly domesticated into crops.

Salinity stress damages plant development by adversely affecting a series of biochemical and physiological processes such as photosynthesis, antioxidant metabolism, mineral nutrients homeostasis, osmolytes accumulation, and hormonal signaling (Misra and Gupta, 2005; Khan et al., 2012). Correspondingly, plants have evolved complex physiological and molecular mechanisms to endure and defend themselves from these adverse environments (Yamaguchi- Shinozaki and Shinoaki, 2006). As one of the most common responses to abiotic stresses, the accumulation of cellular osmolytes has been widely confirmed in many plants. Among many osmolytes, proline is the most widely accumulated compound in plants under stress conditions and it has attracted a lot of studies. The role of proline and its metabolism under stress conditions have received considerable attention in many plants, and now it is generally accepted that proline has multifunctional roles. In addition to functioning as a compatible osmolyte, it can also contribute to scavenging reactive oxygen species (ROS), stabilizing subcellular structures, modulating cell redox homeostasis, supplying energy, and functioning as a signaling molecule to interact with other metabolic pathways under stress conditions (Kavi-Kishor et al., 2005; Verbruggen and Hermans, 2008; Szabados and Savouré, 2010; Sharma et al., 2011). So it is of great importance to understand and utilize the regulatory mechanism of proline metabolism to improve the stress resistance of plants.

In higher plants, there are two pathways involved in proline biosynthesis: the glutamate (Glu) and ornithine (Orn) pathways (Hu et al., 1992; Roosens et al., 1998). The Glu pathway normally lies in the cytosol and chloroplasts. Glu is reduced to glutamate-semialdehyde (GSA) by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and spontaneously converted to Δ^1-pyrroline-5-carboxylate (P5C). P5C is then reduced to proline by Δ^1-pyrroline-5-carboxylate reductase (P5CR). The Orn pathway occurs in mitochondria. Orn is transaminated to P5C by ornithine-δ-aminotransferase (δ-OAT) and P5C is then transported to the cytosol and converted to proline by P5CR. The Glu pathway generally occurs under stress conditions while the Orn pathway is involved in seedling development (Armengaud et al., 2004). Proline degradation takes place in mitochondria through the sequential actions of proline dehydrogenase (PDH) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH), which produce P5C and Glu, respectively. Among the above-mentioned enzymes involved in proline metabolism, P5CS is generally considered to be the key enzyme for proline synthesis while PDH plays a key role in degradation. The enhanced synthesis and decreased degradation of proline are supposed to result in proline accumulation under stress (Chaitanya et al., 2009; Sharma et al., 2011). Thus genetic manipulation of the key enzyme genes by overexpressing *P5CS* gene or suppressing *PDH* gene expression has been widely studied in model plant *Arabidopsis thaliana*, tobacco as well as other plants (Ribarits et al., 2007; Parida et al., 2008; Miller et al., 2009; Sharma et al., 2011). These studies all reveal that there is a close correlation between proline accumulation and stress tolerance (Saradhi et al., 1995; Hare et al., 1999; Siripornadulsil et al., 2002; Kavi-Kishor et al., 2005; Mizoi and Yamaguchi-Shinozaki, 2013). In addition, because of the subcellular compartmentation of proline metabolism in plants, the dynamic transport process of proline is also vital for the protective role of proline, but it is not well understood until now. Although some specific proline transporters have been isolated and characterized, there are only few reports showing a direct role of ProTs in proline transport in plants (Kavi-Kishor et al., 2005; Lehmann et al., 2010; Kavi-Kishor and Sreenivasulu, 2014). A lot of efforts are needed to reveal the roles of proline transport and identify the proline transport systems in the future.

Kosteletzkya virginica (L.) is a perennial facultative halophytic species in Malvaceae family, natively distributing in coastal areas from Long Island along the Atlantic coast of the US west to eastern Texas, and is also found in coastal areas of Eurasia (Gallagher, 1985; Blanchard, 2008). It grows frequently in seashore soil containing 0.3–2.5% sodium salt (mainly NaCl; Zhou et al., 2010). Because of its economic values and the tolerance to saline soil, this species has been introduced in China and recommended as a potential cash crop for alternative saline agriculture (Gallagher, 1995). Many studies demonstrate that it is indeed a halophytic plant and is able to act as a model for the exploration of plant resistance. More importantly it is also a genetic resource to serve for our salt-tolerant crops breeding (Tang et al., 2015). Previously, we have studied its salt tolerance physiological characteristics and the results show that salt stress drastically increases proline accumulation, especially under severe salt stress (Wang et al., 2015). However, the specific mechanism of proline accumulation in *K. virginica* has not been reported so far. It is well known that proline level in plants is a combination result of biosynthesis, catabolism and transport processes and this prompts us to clone proline metabolism-related genes from *K. virginica* and further investigate their expression profiles under salt stress. Here we report the results of our experiment.

Materials and Methods

Plant Materials and Growth Conditions

The seeds of *K. virginica* were collected from Yellow River Delta, Shandong Province, China. The seeds were soaked in concentrated sulfuric acid for 20 min to remove the hard shell and then thoroughly rinsed with deionized water. The treated seeds were sown in plastic pots (with drain holes in bottom) containing washed sand and grown in the artificial climatic chambers (Huier, China) with temperature of 25°C/20°C(day/night), photoperiod of 16 h/8 h (light/dark) and relative air humidity of 60%. Seedlings were sufficiently watered with 1/2 Hoagland nutrient solution every 3 days. Six seedlings of uniform growth were kept in each pot.

Stress Treatments and Sampling

Two-week-old seedlings were treated with salt stress. To analyze organ-specific distribution of proline, roots, stems, and leaves were sampled in the unstressed condition and at 24 h after salt stress (300 mM NaCl), respectively. For salt concentration gradient treatments, seedlings were irrigated with 1/2 Hoagland

nutrient solution containing different concentrations of NaCl (100, 200, 300, or 400 mM) for 24 h. For the time-course treatments, seedlings were irrigated with 1/2 Hoagland nutrient solution containing 300 mM NaCl for 2, 6, 12, and 24 h treatments. Seedlings with only 1/2 Hoagland nutrient solution treatments were used as controls. Each treatment had three pot replications and the sample from each pot was mixed together as a replication. All samples were snap-frozen in liquid nitrogen and stored at −80°C until use.

RNA Extraction and First-strand cDNA Synthesis

Total RNA was extracted according to the manufacturer's instructions of RNAiso Plus (TaKaRa, Japan). The quality and quantity of total RNA were measured by using a NanoDrop-2000c spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized with TransScript All-in-One First-Strand cDNA Synthesis SuperMix for PCR (Transgen, China) according to the manufacturer's instructions.

Cloning of Proline Metabolism-related Genes

The first strand cDNA synthesized from mixed RNA samples treated by salt stress was used as the template for PCR amplification. According to the conserved region of each gene, a pair of primers (sequences given in Supplementary Table S1) were designed and used to amplify the core fragment of *P5CS*, *OAT*, *PDH*, and *ProT*, respectively. The amplified fragments were ligated into the *pEASY*-Blunt Zero Cloning Vector (TransGen, China) and sequenced. After the fragments were confirmed to be part of candidate genes by Blast analysis, the 5′ and 3′ ends of the full-length cDNA were further amplified according to the instruction of SMART^{TM} RACE cDNA Amplification Kit (Clotech, USA). Gene-specific primers and nested primers of each target gene were designed according to the obtained cDNA fragments (sequences given in Supplementary Table S2). The PCR products were separated by 1% agarose gel electrophoresis and the desired bands with predicted size were excised from the gels and purified, then ligated into the *pEASY*-Blunt Zero Cloning Vector (TransGen, China) and sequenced. Finally, the above obtained three sequences were spliced and assembled into the full length cDNA for each gene by DNAMAN software.

Bioinformatic Analysis

The nucleotide sequence and the deduced amino acid sequence were analyzed using the DNAMAN software and the BLAST software online[1]. ProtParam software[2] was used to analyze the basic characteristics of the encoded proteins. TMHMM software[3] was used to predict transmembrane domains in the encoded proteins. Conserved domains were analyzed using CDD of NCBI[4]. Multiple peptide alignments and phylogenetic analysis were carried out using Clustal X 2.1 and MEGA 5 programs, respectively.

[1]http://www.ncbi.nlm.gov/blast

[2]http://web.expasy.org/protparam/

[3]http://www.cbs.dtu.dk/services/TMHMM/

[4]http://www.ncbi.nlm.nih.gov/cdd

Determination of Proline Content

Fresh samples for the determination of proline content were the same as those for gene expression analysis. Each treatment had three pot replications and the sample from each pot was mixed together as a replication. Free proline content was determined by ninhydrin assay at A520 nm according to the method described by Bates et al. (1973).

Expression Analysis of Isolated Genes

Quantitative real-time PCR (qRT-PCR) was performed on an ABI fast 7500 Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's instructions. The gene EF-1αcloned in our previous study from *K. virginica* was used as the reference gene to normalize the amount of cDNA in each reaction. The qRT-PCR amplifications were carried out in triplicate in a total volume of 20 μL according to the manufacturer's instructions of SYBR® Green Realtime PCR Master Mix (Applied Biosystems). The qRT-PCR program was holding stage, 50°C for 20 s and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and melt curve stage, 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. All analyses were based on the C_T values of the PCR products. The amplification specificity was determined by analyzing the dissociation curves. Experiments were repeated three times and the C_T values of the triplicate PCRs were averaged and used for the quantification of transcript levels. The quantification of the relative expression levels was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer sequences for expression analysis are listed in Supplementary Table S3.

Statistical Analysis

Data was analyzed by Microsoft Excel 2007 and SPSS 16.0. Mean and standard error (SD) values of three replications were calculated. Data was compared with the control or among treatments by analysis of variance (ANOVA) to discriminate significant differences at $P \leq 0.05$ followed by least significant difference tests (LSD). Figures were created using Origin 7.5.

Results

Isolation and Characterization of Proline Metabolism-related Genes

The transcriptome information of *K. virginica* seedlings with or without salt stress has been established through high-throughout sequencing technology in our laboratory, which was deposited at GenBank with the accession number GCJL00000000 (Tang et al., 2015). Based on the transcriptome information, four cDNA fragments of proline metabolism-related genes including *P5CS*, *OAT*, *PDH*, and *ProT* were obtained. Blast analysis showed these fragments shared significant homologies with genes in the databases of NCBI. Then, by means of RT-PCR and RACE, the full-length cDNAs of *P5CS*, *OAT*, *PDH*, and *ProT* genes were isolated from *K. virginica* and designated as *KvP5CS1*, *KvOAT*, *KvPDH*, and *KvProT*, respectively. The

TABLE 1 | Isolated genes related to proline metabolism in *Kosteletzkya virginica*.

Gene name	GenBank accession	Full length of cDNA (bp)	5′-UTR (bp)	ORF length (bp)	3′-UTR (bp)
KvP5CS1	KR029088	2420	90	2139	191
KvOAT	KR029089	1738	137	1434	167
KvPDH	KR029090	1781	47	1560	174
KvProT	KR029091	1654	48	1326	280

UTR, untranslated regions; ORF, open reading frame.

GenBank Accession numbers and their basic characteristics were listed in **Table 1**. The deduced protein sequences are characterized by the basic features such as the number of amino acid residues, molecular weight and isoelectric point (pI), which were shown in **Table 2**.

Bioinformatics Analysis of Four Proline Metabolism-related Genes

Blast analysis and multiple sequence alignments revealed that these isolated genes had high homology with known genes in GenBank involved in proline metabolism. The deduced amino acid sequence of KvP5CS1 was more than 80% identical to those homologues in GenBank and shared the highest identity of 89% with P5CS in *Theobroma cacao*. As shown in Supplementary Figure S1, it had the same conserved domains as other species, such as ATP and NADPH binding sites, γ-GK and GSA-DH domains (Savouré et al., 1995; Su et al., 2011). The putative KvOAT protein also shared a very high identity with OATs from other plants and had the highest identity of 90% with OAT in *T. cacao* (Supplementary Figure S2). The putative KvPDH and KvProT proteins had lower identity with their homologues and shared 86 and 83% identity with *Gossypium hirsutum* (AFV28788.1) and *T. cacao* (XP_007009121.1), respectively. The prediction of transmembrane domains showed that KvP5CS1, KvOAT, and KvPDH proteins did not contain transmembrane domains (Supplementary Figures S3A–C), while 11 transmembrane domains were found in KvProT protein (Supplementary Figure S3D).

Phylogenetic analysis was performed based on multiple protein sequence alignments of different species using a Neighbor-Joining method in the MEGA 5 program. The phylogenetic tree of P5CS proteins from 10 species showed that KvP5CS1 was closely related to *T. cacao* (**Figure 1A**). Both KvOAT and KvProT were also the closest relatives of *T. cacao* in the phylogenetic tree of OAT and ProT, respectively (**Figures 1B,C**), while KvPDH was closely related to *G. hirsutum* (**Figure 1D**).

TABLE 2 | The basic features of protein sequences encoded by isolated genes.

Protein name	Number of amino acid residues	Molecular weight (kDa)	Isoelectric point
KvP5CS1	712	76.894	6.61
KvOAT	477	52.167	8.32
KvPDH	519	57.031	7.64
KvProT	441	48.256	9.35

Salt Stress Increased Proline Content

To understand whether proline accumulation is organ specific, we analyzed the proline content in roots, stems and leaves of *K. virginica* seedling treated with 300 mM NaCl for 24 h (**Figure 2A**). Under non-stress conditions, the proline content was the highest in leaves while in roots and stems it was lower. After NaCl stress, the proline content in these three organs all increased remarkably and reached 3.75-, 4.76-, and 6.83-fold higher than the corresponding control, respectively. Obviously, more proline was accumulated in leaves than roots and stems under salt stress. Therefore, the leaves were used in the following experiments.

To find out the correlation between the severity of salt stress and the level of proline accumulation, two-week-old seedlings were treated for 24 h with 0, 100, 200, 300, and 400 mM NaCl and the proline content in leaves was determined. As shown in **Figure 2B**, the proline level peaked at 300 mM NaCl for 24 h and reached more than sixfold higher than control, but at 400 mM NaCl for 24 h proline content fell back slightly along with wilting symptom. So we next chose 300 mM NaCl to treat seedlings for 0, 2, 6, 12, and 24 h in order to investigate the proline accumulation in leaves. The results showed that the proline content gradually increased over time and reached about sixfold higher after 24 h than control (**Figure 2C**).

Expression Analysis of Four Proline Metabolism-related Genes in Leaves

In the experiment of salt concentration gradient treatments (**Figure 3A**), the expression of *KvP5CS1* was slightly increased under low salt stress (100 mM NaCl), and then significantly increased by higher salt stress and peaked at 300 mM NaCl which was approximately 33-fold higher than control, but fell back again slightly at 400 mM NaCl. Expression pattern of *KvProT* showed a similar trend with *KvP5CS1*. The expression of *KvOAT* showed a slight increase with the increasing salt concentrations. The expression of *KvPDH* increased slightly only under 300 mM NaCl stress and under other treatments no significant difference was observed compared to the control.

In view of the obvious effect of 300 mM NaCl on gene expression, we further studied their expression profiles treated with 300 mM NaCl for different time (**Figure 3B**). The expression of *KvP5CS1* was not changed within 2 h and began to increase sharply from 6 to 12 h. The expression of *KvP5CS1* reached the highest level (52.75-fold higher than control) at 12 h and then fell back at 24 h. The expression of *KvOAT* showed a slight increase over time and then stayed at a stable level. The expression of *KvProT* gradually increased over time and reached the highest level at 24 h (27.11-fold higher than control). On the contrary,

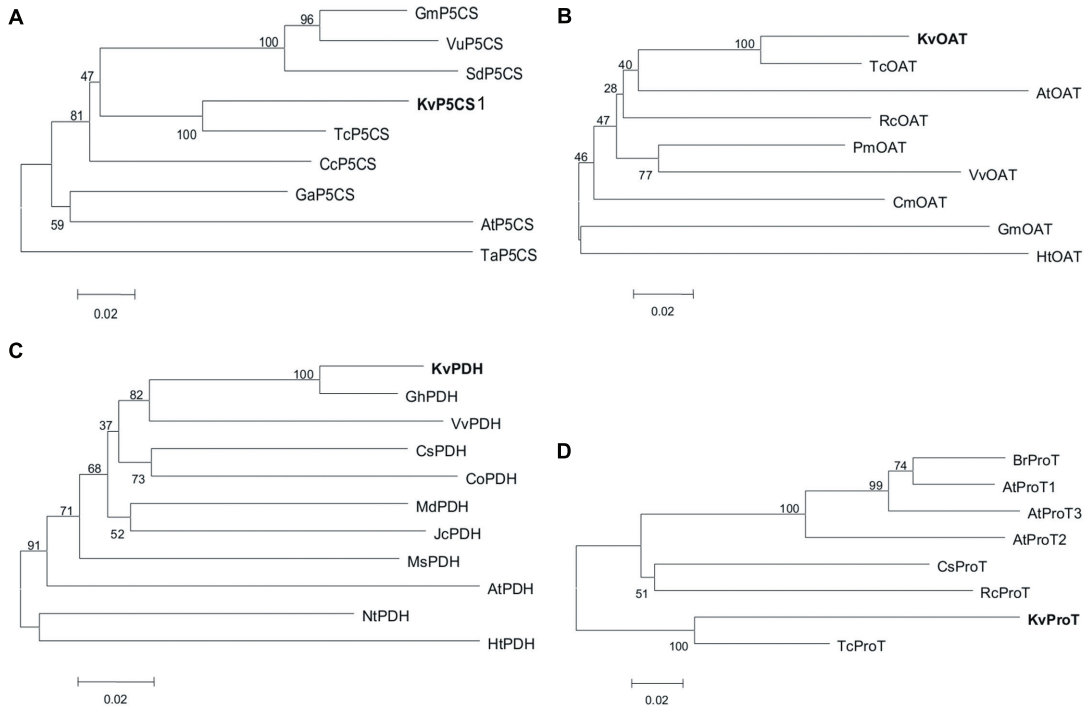


FIGURE 1 | The phylogenetic analysis for KvP5CS1 (A), KvOAT (B), KvPDH (C), and KvProT (D). Protein sequences used in phylogenetic analysis were listed in Supplementary Table S4.

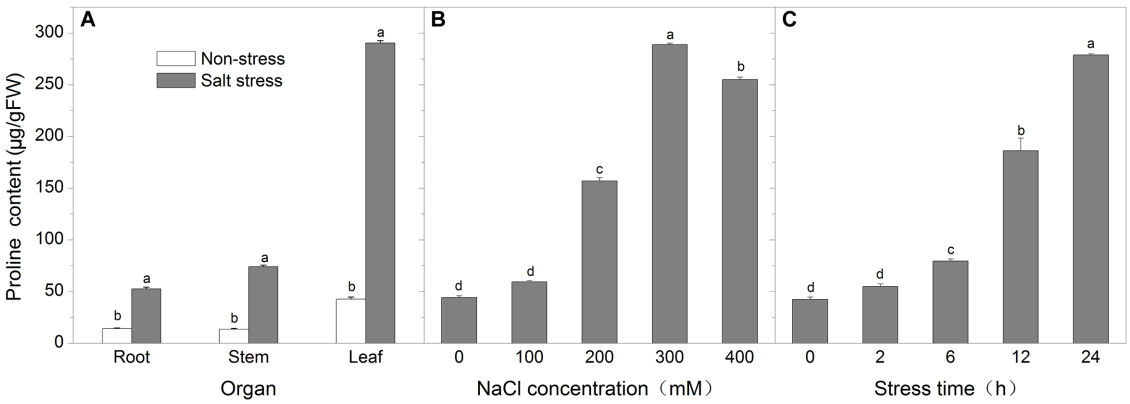


FIGURE 2 | The changes of free proline content. (A) The proline content in roots, stems, and leaves under non-stress and 300 mM NaCl for 24 h; **(B)** The proline content in leaves under salt concentration gradient treatments for 24 h; **(C)** The proline content in leaves under time-course treatments with 300 mM NaCl. Each data point represents the average of three replications and the error bars represents standard deviation based on three replications. Data was analyzed by analysis of variance (ANOVA), followed by least significant difference (LSD) tests.

the expression of *KvPDH* slightly decreased before 12 h and then returned to the original level.

Discussion

As proline level in plants is a combination result of biosynthesis, catabolism, and transport processes, it is necessary to analyze the expression profiles and functions of genes involved in these processes to understand the mechanism of proline metabolism. So far, there has been no any report about proline-metabolism genes in *K. virginica.* Therefore, we cloned four full-length of cDNAs from *K. virginica*-encoding proline synthetase (KvP5CS1 and KvOAT), proline dehydrogenase (KvPDH), and proline transporter (KvProT). Bioinformatics analysis revealed that the nucleotide and deduced amino sequences of these genes all shared high similarities with the known genes in other plants. For the putative KvP5CS1 protein, it shared the highest similarity

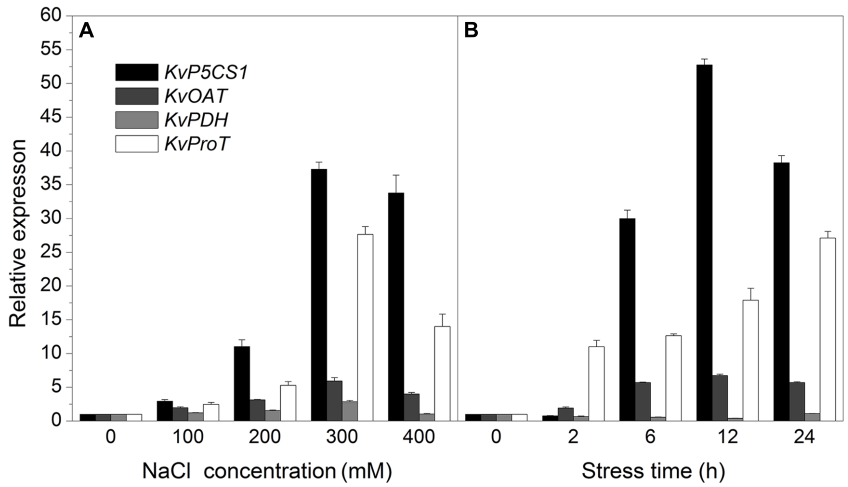


FIGURE 3 | The expression analysis of *KvP5CS1*, *KvOAT*, *KvPDH*, and *KvProT* in leaves of *Kosteletzkya virginica* under salt stress. (A) Salt concentration gradient treatments; **(B)** Time-course treatments. Each data point represents the average of three replications and the error bars represents standard deviation based on three replications.

(89%) with P5CS in *T. cacao*. Since there were at least two *P5CS* genes characterized in many other plants according to the available reports (Szabados and Savouré, 2010), in the next work we should try to clone another *P5CS* gene from *K. virginica*. KvOAT protein also shared the highest similarity with OAT (90%) in *T. cacao*, while KvPDH was closely related to PDH in *G. hirsutum*. Moreover, the putative KvPDH protein was predicted in the mitochondria by TargetP 1.1 server where proline catabolism regulated by PDH occurs. In contrast, proline synthesis regulated by P5CS and OAT takes place mainly in the cytosol and chloroplasts. As this subcellular compartmentation of proline synthesis and degradation in plants, the dynamic transport of proline is supposed to exist and be of great importance for proline metabolism, especially for stress-induced proline accumulation. In our study, one *KvProT* gene was obtained and its deduced protein shared high similarity with ProT (83%) in *T. cacao* and had 11 transmembrane domains, which indicated that KvProT protein might be located at the mitochondria membrane and involved in the transport of proline. Up to now, some proline transporters have been isolated and characterized in many plants such as *A. thaliana* (Grallath et al., 2005), *Lycopersicon esculentum* (Schwacke et al., 1999), *Oryza sativa* (Igarashi et al., 2000), *Hordeum vulgare* (Fujiwara et al., 2010), and *Chrysanthemum lavandulifolium* (Zhang et al., 2014), but research about their roles in proline transport is still scarce and superficial. It will need a great deal of work to identify the proline transport systems and their functions in plants.

Proline accumulation is a common response to abiotic stress in many plants, but the extent of proline accumulation varies in different plant species. In our study, we firstly analyzed the proline accumulation in different organs of *K. virginica* seedling under salt stress. Like many other plant species, salt stress induced a significant proline accumulation in roots, stems and leaves, especially in leaves where the proline content reached about sixfold higher than control. Furthermore, the proline content in leaves increased with increasing NaCl concentrations and prolonged stress time. The similar finding was reported in green gram (Misra and Gupta, 2005), mulberry (Surabhi et al., 2008), canola (Xue et al., 2009), and *Jerusalem artichoke* (Huang et al., 2013). It has been proposed that leaves accumulate more proline in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress (Silva-Ortega et al., 2008).

In previous studies, expression of *Arabidopsis P5CS1* was induced by various types of abiotic stress including salt stress (Savouré et al., 1995; Yoshiba et al., 1995, 1997, 1999; Peng et al., 1996). *P5CS1* overexpression plants increased proline accumulation, while p5cs1 mutants restricted proline accumulation (Kavi-Kishor et al., 1995; Zhang et al., 1995; Szekely et al., 2008). Conversely, reduced expression of *Arabidopsis PDH1* is also thought to be needed for stress-induced proline accumulation (Kiyosue et al., 1996; Yoshiba et al., 1997; Miller et al., 2005; Sharma et al., 2011). These studies on *P5CS1*and *PDH1* in *Arabidopsis* established a "standard model" which meant increased proline synthesis and reduced proline degradation could lead to proline accumulation (Miller et al., 2005, 2009; Ribarits et al., 2007; Parida et al., 2008; Chaitanya et al., 2009; Sharma et al., 2011). Identification of the key genes in proline metabolism from other plant species prompted a wave of studies that sought to overexpress P5CS1 or suppress PDH1expression to increase proline and enhance stress tolerance (Sawahel and Hassan, 2002; Su and Wu, 2004; Tateishi et al., 2005; Molinari et al., 2007). Our results also seemed to fit this model. The expression of *KvP5CS1* was rapidly up-regulated by salt stress, while the expression of *KvPDH* was inhibited before 12 h and only increase slightly by 300 mM NaCl for 24 h. We speculated that inhibited *KvPDH* expression also made a contributed to proline accumulation before 12 h salt stress, while under severe or prolonged salinity, the recovered

or increased *KvPDH* expression might speed up the degradation of proline to provide energy and electrons for the respiratory chain (Szabados and Savouré, 2010). As another key enzyme gene for proline synthesis through Orn pathway, *KvOAT* expression showed a slight increase with increasing salt concentrations or prolonged salt stress. Thus, for proline biosynthesis, the up-regulated expression of *KvP5CS1* played a more important role than *KvOAT* for proline accumulation in leaves under salt stress. It is consistent with the findings of previous research that proline biosynthesis from Glu is considered to be the predominant pathway, especially under stress conditions (Armengaud et al., 2004; Lehmann et al., 2010; Szabados and Savouré, 2010). In addition, one *KvProT* gene was isolated and its expression could be obviously up-regulated by salt stress. The increased proline transport might contribute to proline accumulation in leaves from other organs or keep the dynamic balance between proline synthesis and degradation and this still needs to be confirmed by further experiments. Previous studies showed that the expression patterns of different members of the ProT subfamily exhibited organ specificity and disparity under stresses (Rentsch et al., 1996; Igarashi et al., 2000; Fujiwara et al., 2010). In further study, much more attention should be paid to identify other proline transport members and reveal their location as well as their roles in proline transport.

Conclusion

We cloned four genes related to proline metabolism including *KvP5CS1*, *KvOAT*, *KvPDH*, and *KvProT* from *K. virginica* and investigated their expression profiles in leaves under salinity using quantitative RT-PCR method. The up-regulated expression of *KvP5CS1* and *KvOAT* resulted in proline accumulation in leaves under salt stress, and low expression of *KvPDH* also made a contribution to proline accumulation before 12 h.

Author Contributions

HW performed the experiments and wrote the manuscript. XT and HW helped with the experimental process. HS revised the paper. All authors reviewed the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00792

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ORIGINAL RESEARCH
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Identification and Validation of Selected Universal Stress Protein Domain Containing Drought-Responsive Genes in Pigeonpea (*Cajanus cajan* L.)

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Pigeonpea is a resilient crop, which is relatively more drought tolerant than many other legume crops. To understand the molecular mechanisms of this unique feature of pigeonpea, 51 genes were selected using the Hidden Markov Models (HMM) those codes for proteins having close similarity to universal stress protein domain. Validation of these genes was conducted on three pigeonpea genotypes (ICPL 151, ICPL 8755, and ICPL 227) having different levels of drought tolerance. Gene expression analysis using qRT-PCR revealed 6, 8, and 18 genes to be ≥ 2-fold differentially expressed in ICPL 151, ICPL 8755, and ICPL 227, respectively. A total of 10 differentially expressed genes showed ≥ 2-fold up-regulation in the more drought tolerant genotype, which encoded four different classes of proteins. These include plant U-box protein (four genes), universal stress protein A-like protein (four genes), cation/H(+) antiporter protein (one gene) and an uncharacterized protein (one gene). Genes *C.cajan_29830* and *C.cajan_33874* belonging to *uspA*, were found significantly expressed in all the three genotypes with ≥ 2-fold expression variations. Expression profiling of these two genes on the four other legume crops revealed their specific role in pigeonpea. Therefore, these genes seem to be promising candidates for conferring drought tolerance specifically to pigeonpea.

Keywords: *in-silico* analysis, drought responsive genes, expression profiling, pigeonpea, legumes

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INTRODUCTION

Abrupt climate changes and unavailability of sufficient water supply can severely affect the productivity of agriculturally important crops. Additionally, frequent exposure of environmental stresses such as drought is adversely affecting the plant growth and yield. Drought can occur at any stage of plant growth and the degree of yield loss depends on the onset time, intensity and duration of stress (Hu and Xiong, 2014). Pigeonpea is usually grown under marginal environments that are often subjected to water stress at different stages of growth and development. Even for short-duration varieties, yield gets affected due to water stress during late flowering and early pod development stages (Lopez et al., 1996). During seed hardening, the crop requires considerable amount of water and at this crucial stage unavailability of water often causes terminal drought. Despite having a deeper root system, drought is still one of the major yield-limiting factors,

especially at critical seedling and reproductive stages of pigeonpea (Saxena, 2008). There has been a rousing progress made in developing drought-tolerant pigeonpea genotypes, but still it is difficult to meet the conditions arisen due to climate change. It is feasible to develop drought tolerant varieties through genomics-assisted breeding that would facilitate yield stability under water-deficient conditions (Varshney et al., 2014).

Since drought is a complex trait and is controlled by multigenes, identification of candidate genes and understanding the molecular mechanism associated with drought tolerance in pigeonpea is critical. Many studies have been carried out in model plants to identify candidate genes associated with drought response (see Mir et al., 2012). In pigeonpea, ample amount of genomics resources has been developed which can be deployed to identify candidate drought tolerant genes specific to pigeonpea. Moreover, the pigeonpea genome sequence reported 111 homologous sequences corresponding to universal drought-responsive protein sequences from the Viridiplantae (Varshney et al., 2012). Similarly, the development of comprehensive transcriptome assembly (Kudapa et al., 2012) and the identification of genes involved in abiotic stresses tolerance have been reported (Priyanka et al., 2010; Sekhar et al., 2010; Saxena et al., 2011; Deeplanaik et al., 2013).

Functional characterization of genes involved in different stress-responsive pathways such as photosynthesis and carbohydrate metabolism (Basu et al., 1999), related to stress-responsive transcription factors (Nakashima et al., 2009), signal transduction and regulatory compounds (Ramanjulu and Bartels, 2002; Sreenivasulu et al., 2007) gives an insight into the mechanisms adopted by plants to cope with drought stress. In this context, using bioinformatics approach, a total of 32 drought-responsive ESTs were retrieved from seven plant genera namely, *Glycine*, *Hordeum*, *Manihot*, *Medicago*, *Oryza*, *Pinus,* and *Triticum* (Isokpehi et al., 2011). Similarly, in soybean, 32 drought responsive genes involved in 17 metabolic pathways were identified and were validated in pigeonpea to know their association with drought stress (Deeplanaik et al., 2013).

To identify differentially expressed genes, many technologies such as microarray, DNA chip-based array, genome-wide transcript profiling, and quantitative real-time PCR (qRT-PCR) have been deployed in a number of studies (Ozturk et al., 2002; Degenkolbe et al., 2009; Lenka et al., 2011). qRT-PCR is the most commonly used approach for expression analysis of genes in many crop species including pigeonpea (Borges et al., 2012; Qiao et al., 2012; Deeplanaik et al., 2013; Recchia et al., 2013; Turyagyenda et al., 2013; Da Silva et al., 2015; Sinha et al., 2015).

The present study involves *in-silico* identification of selected universal stress protein domain containing drought-responsive genes. The qRT-PCR validation of these genes was carried out on pigeonpea genotypes with different levels of drought tolerance. Drought stress was imposed on all the selected genotypes and compared with well-watered controls to validate the candidate genes involved with drought tolerance in pigeonpea. The genes were also validated using a tolerant and a susceptible genotype each from four legumes namely, chickpea, groundnut, common bean, and cowpea. The identified candidate genes in future, can be functionally validated using transgenic approaches. Additionally to utilize the identified drought tolerant genes, markers can be developed using haplotype analysis approach, which will accelerate crop yield even under drought stress conditions.

MATERIALS AND METHODS

Plant Materials

Three genotypes, ICPL 227, ICPL 8755, and ICPL 151, which are the parents of two mapping populations segregating for drought tolerance, were used. ICPL 151 and ICPL 8755 are known to have a low-level of drought tolerance as compared to ICPL 227 (Lopez et al., 1996; Saxena et al., 2011). To validate the putative pigeonpea drought-responsive candidate genes in other legumes, one tolerant and one susceptible genotype of each legume crop namely, chickpea (ICC 4958, tolerant and ICC 1882, susceptible), groundnut (CSMG 84-1, tolerant and ICGS 76, susceptible), common bean (BAT 477, tolerant and DOR 364, susceptible), and cowpea (IT93K503-1, tolerant and UC-C B46, susceptible) were selected, respectively (**Table 1**).

Drought Stress Treatment and Tissue Harvesting

Seeds were thoroughly washed with DEPC treated water, sown in 3 inches plastic pots (one seed per pot) filled with autoclaved black soil, sand, and vermicompost (10:10:1 v/v) mixture. All the plants were grown under controlled conditions in three replications. For imposing drought stress, slow drought (dry down) stress was imposed on the plants when they reached 22 days old seedling stage. A calculated amount of water was added to each pot, which was weighed at regular intervals. Control plants were maintained throughout at 80% relative water content (RWC) whereas stressed plants were dried down gradually to 20% RWC. The intensity of the drought stress was measured by recording the transpiration ratio (TR) on a daily basis. Stressed

TABLE 1 | Details of genotypes used for expression analysis.

Genotypes	Features	References
PIGEONPEA		
ICPL 151	Less drought tolerant	Saxena et al., 2011
ICPL 8755	Less drought tolerant	Saxena et al., 2011
ICPL 277	More drought tolerant	Saxena et al., 2011
CHICKPEA		
ICC 4958	Tolerant	Varshney et al., 2014
ICP 1882	Susceptible	Varshney et al., 2014
GROUNDNUT		
CSMG 84-1	Tolerant	Gautami et al., 2012
ICGS-76	Susceptible	Gautami et al., 2012
COMMON BEAN		
BAT 477	Tolerant	Galindo et al., 2007
DOR 364	Susceptible	Galindo et al., 2007
COWPEA		
IT93K503-1	Tolerant	Barrera-Figueroa et al., 2011
UC-C B46	Susceptible	Barrera-Figueroa et al., 2011

plants were allowed to dry through transpiration until the TR reached 0.1. Root tissues were harvested from the stressed plants from all three replicates. The root samples were gently wiped with 70% ethanol to remove soil particles. All tissues were immediately frozen in liquid nitrogen and stored at −80°C for RNA isolation.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from all the frozen root samples using TRIzol (Invitrogen, USA) and was purified using DNase (Qiagen, GmbH, Germany) through an RNeasy Plant Mini kit according to the manufacturer's instructions. The concentration of total RNA was checked using NanoDropND-1000 (NanoDrop Technologies, USA) and RNA integrity was assessed on 1% denaturing formaldehyde agarose gel. cDNA was prepared using one microgram of total RNA using the SuperScript® III RT enzyme (Invitrogen, USA).

In-silico Identification of Drought Responsive Genes

To predict drought responsive genes in pigeonpea, gene set annotated with pigeonpea genome assembly v5 (Varshney et al., 2012) was downloaded from International Initiative for Pigeonpea Genomics (IIPG: http://www.icrisat.org/gt-bt/iipg/Home.html). In addition, HMM profile of USP domain (PF00582) was retrieved from Pfam database (http://pfam.sanger.ac.uk/; Finn et al., 2010). The whole gene set was searched using "hmmsearch" program of HMMER 3.0 with HMM profile of the USP domain. Genes that detected sections encoding the USP domain, above the default inclusion threshold with statistically significant domain architecture were selected as USP domain-encoding genes.

Sequences were also subjected to protein homology search using BLASTP against Swiss-Prot and TrEMBL databases to further determine the identity of the selected genes containing USP domains. The identities obtained from the two databases were used in UniProtKB database to retrieve the protein names, location, biological pathways, and gene ontology identity with the help of an in-house Perl script. Gene ontology enrichment analysis was performed using the BiNGO tool with *p*-value cut-off of ≤ 0.05 (Maere et al., 2005).

Primer Designing and qRT-PCR

Gene specific primer pairs were designed from the exonic regions of the selected genes. Primer3 software (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) was used for primer designing using the following criteria: annealing temperature (Tm) in the range of 55–60°C with an average of 57°C, amplicon size of 150–200 bp, primer length of 20 ± 5 bp and GC% of 50 ± 5 (**Supplementary Table 1**). All the designed primer pairs were custom synthesized by MWG (MWG-Biotech AG, Bangalore, India).

qRT-PCR was carried out using ABI SYBR® GREEN PCR reaction on an ABI Fast7500 System (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. PCR conditions maintained for all qRT-PCR reactions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The melt curve analysis was conducted for all 51 primer pairs. Only after confirming the observed single peak with all the selected tissue samples, primers were used further for qRT-PCR analysis.

Each reaction was carried out in three biological and two technical replicates along with no template control. The differential expression values of drought responsive genes were normalized with *ACT1* as the reference gene (**Supplementary Table 2**). Statistical comparison between data obtained from three genotypes (ICPL 227, ICPL 8755 and ICPL 151) was performed using Tukey's *post-hoc* multiple comparison test using SPSS (version 16.0) whereas Student *t*-test was used to compare tolerant and susceptible genotypes of other four legume crops.

RESULTS

Identification of Drought Responsive Genes

The gene set consisting of 48,680 gene models (Varshney et al., 2012) was used to search the USP domain-encoding genes. As a result, 71 genes were found to encode USP domain, of which 51 were identified to be above the inclusion threshold with $E < 0.01$ (**Supplementary Table 3**). Of these genes, 49 also showed identity to the 111 drought responsive genes reported earlier in pigeonpea (Varshney et al., 2012).

Functional Classification of Drought Responsive Genes

To classify the 51 drought responsive genes based on their functional annotations, BLASTP search was performed against Swiss-Prot and TrEMBL databases. This analysis revealed that about 25.5% of the genes were classified as related to molecular functions such as catalytic activity (8%), transporter activity (5%), and binding (6%), whereas 27.4% of the genes were found to be related to cellular component such as ubiquitin ligase complex (5%), membrane (6%), organelle (5%), membrane part (5%), plastid part (1%), and cell part (11%). The genes involved in biological processes formed 47% and included response to stress (13%), metabolic process (8%), cellular process (12%), homeostatic process (3%), single-organism process (6%), localization (5%) and establishment of localization (5%). Gene ontology (GO) term enrichment performed using BiNGO for these 51 genes as visualized in Cytoscape has been presented in **Figure 1**. Detailed information of the corresponding protein name, GO term and ontology identities of these genes has also been provided in the **Supplementary Table 4** and **Supplementary Figure 1**.

On the basis of encoded proteins, the analyzed genes were further classified into six different groups namely, uncharacterized proteins (10 gene), universal stress protein A-(*uspA*) like protein (17 genes), plant U-box proteins (13 genes), cation/H(+) antiporter (*CHX*) proteins (6 genes), serine/threonine-protein kinase (4 genes), and probable nucleoredoxin (1 gene) (**Supplementary Table 4**).

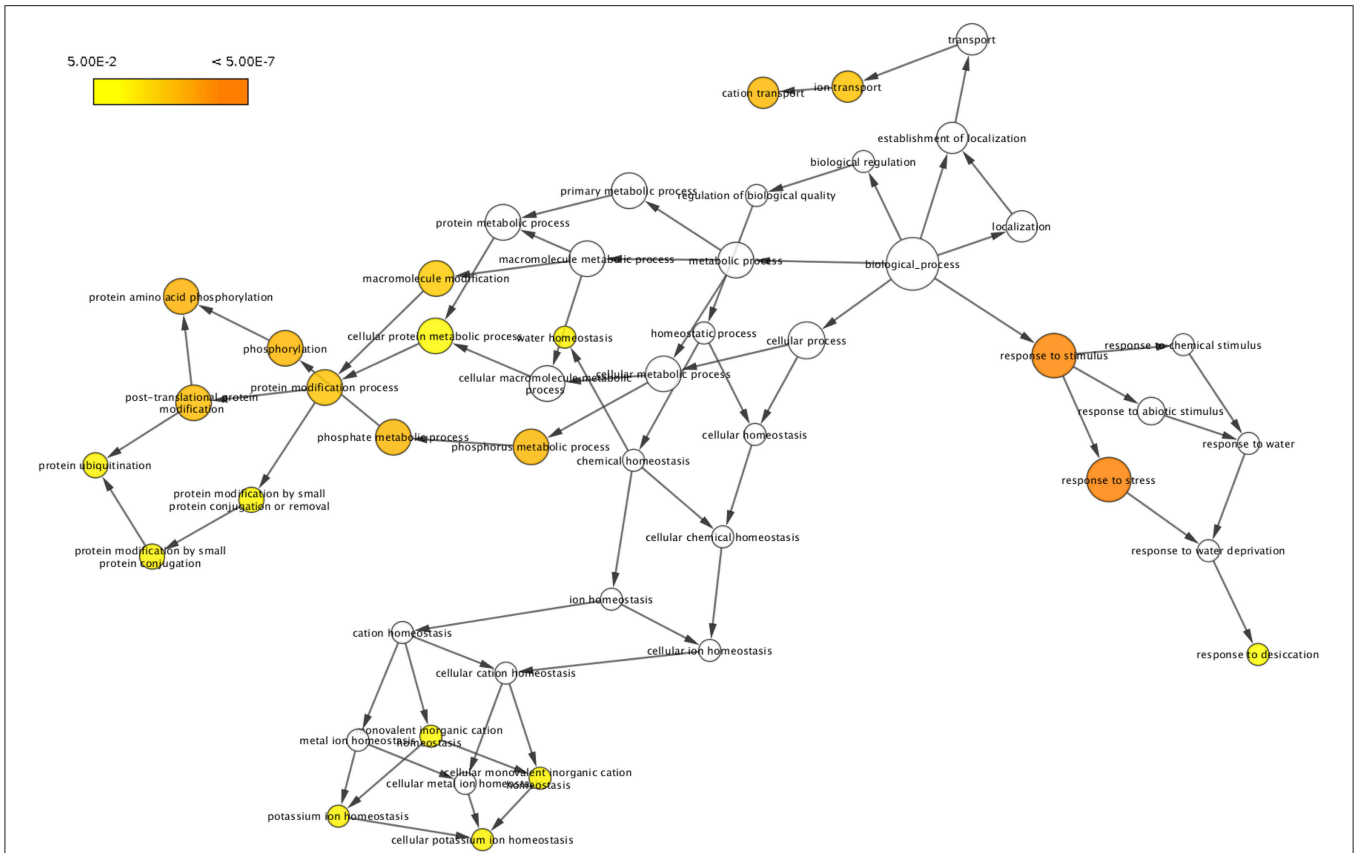


FIGURE 1 | Gene ontology (GO) enrichment of drought responsive genes. Genes showing differential regulation were analyzed using BiNGO and the biological process terms showing significant enrichment are presented. The colors shades represent the following significance level; white-no significance difference; yellow $P = 0.05$, orange $P < 0.0000005$.

Differentially Expressed Drought Responsive Genes

Gene specific primer pairs were designed from the exonic regions of 51 selected drought responsive genes for validation using qRT-PCR (**Supplementary Table 3**). Hierarchical cluster analysis of expression data of these genes showed a range of differential gene expression among three genotypes (ICPL 151, ICPL 8755, and ICPL 227) under stressed and controlled conditions. This analysis revealed that the less drought tolerant (LDT) genotypes, ICPL 151 and ICPL 8755 clustered separately from the more drought tolerant (MDT) genotype, ICPL 227 (**Figure 2**). Expression data was analyzed further in two different ways: (1) comparison between stressed and control samples for each genotype and (2) pair-wise comparison between genotypes. Expression analysis for each genotype with respect to stressed and control samples identified 18 genes in ICPL 227, six genes in ICPL 151 and eight genes in ICPL 8755 with significant expression variation. Genes with more than two-fold expression difference in each of these genotypes have been listed in **Table 2**. However, the expression analysis among the genotype pairs, ICPL 227 with ICPL 8755 (**Supplementary Table 5**) and ICPL 227 with ICPL 151 (**Supplementary Table 6**) has identified 11 genes in each case.

Furthermore, the relative transcript abundance was compared between the MDT genotype, ICPL 227 and the LDT genotypes, ICPL 8755 and ICPL 151 to identify the common genes. As a result, among these genotypes, 10 genes were found to be common and showed large differences in the relative transcript abundance (**Table 2** and **Figure 3**). These genes encodes four different classes of proteins, which include plant U-box proteins (four genes), cation/H(+) antiporter (*CHX*) proteins (one gene), uncharacterized proteins (one gene), and universal stress protein A-(*uspA*) like protein (four genes). Four genes encoding plant U-box proteins namely, *C.cajan_26230, C.cajan_39705, C.cajan_09181,* and *C.cajan_30211* showed significant up-regulation in ICPL 227 in comparison to ICPL 151 and ICPL 8755. The gene, *C.cajan_26230* showed 5.14-fold expression variation in ICPL 227 in comparison to ICPL 151 (–0.20-fold) and ICPL 8755 (–0.40-fold). Similarly, the gene *C.cajan_39705* showed higher level of expression (5.19-fold) in ICPL 227 as compared to ICPL 151 (–0.95-fold) and ICPL 8755 (–0.16-fold), whereas *C.cajan_09181* showed 13.5-fold gene expression variations in ICPL 227 compared to 3.16-fold in ICPL 151 and 0.80-fold in ICPL 8755. Likewise, for *C.cajan_30211,* the expression difference observed in ICPL 227 was high (7.13) as compared to that in ICPL 151 (0.86) and ICPL 8755 (1.00).

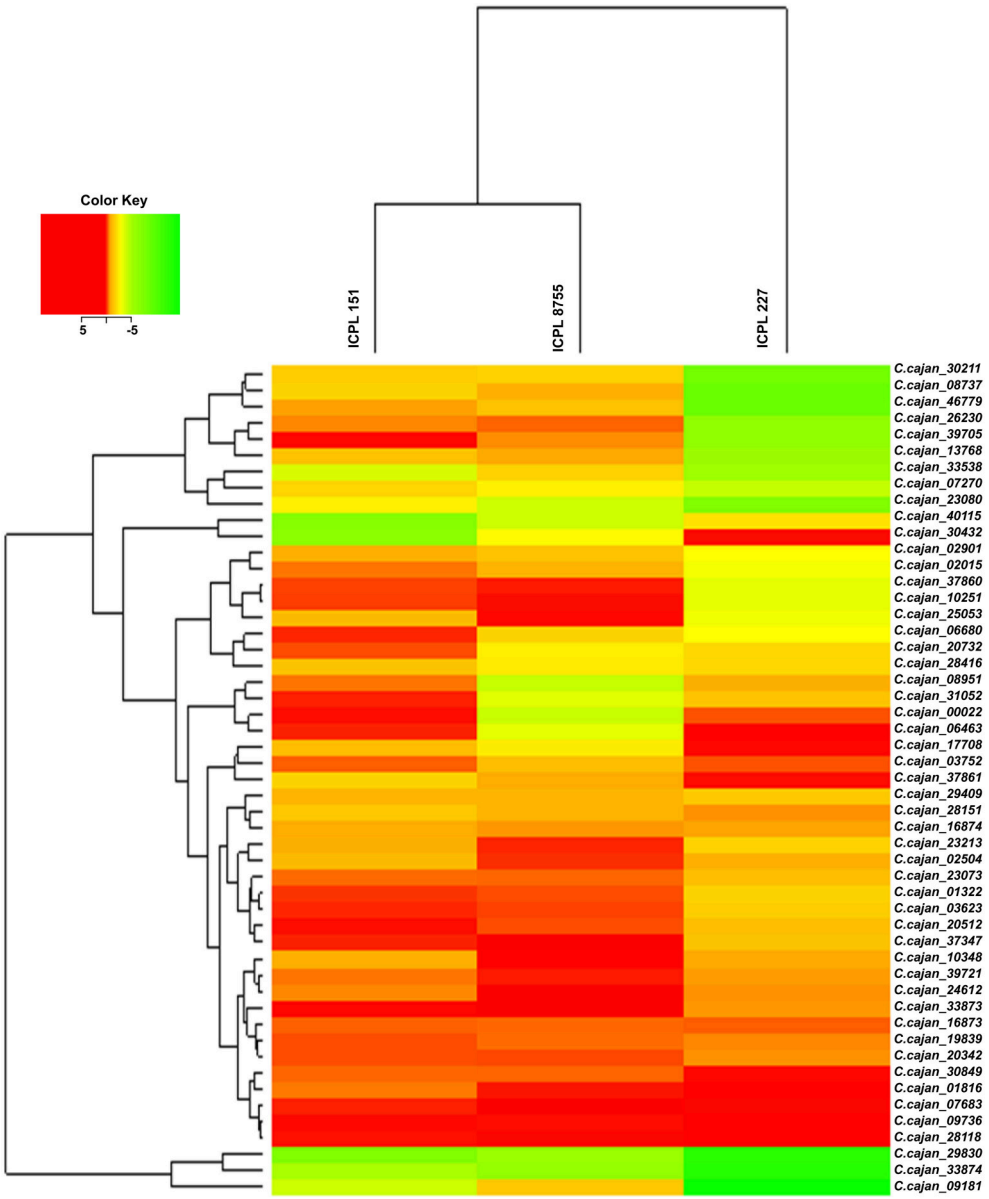


FIGURE 2 | Heat map of 51 drought responsive genes in MDT and LDT genotypes during drought stress condition. Heat map depicting clusters of differentially expressed 51 drought responsive genes. Induced genes are represented in red and suppressed genes are represented in green. The color scale at the top right represents the log-transformed RPKM-value.

In the case of *CHX* gene, *C.cajan_46779* showed significant up-regulation in ICPL 227 (7.47-fold) unlike ICPL 151 (−0.03-fold) and ICPL 8755 (0.6-fold). Also, expression profiling of the gene, *C.cajan_08737* encoding uncharacterized protein revealed 7.70-folds relative expression variation in ICPL 227 as compared to ICPL 151 (1.03) and ICPL 8755 (0.24). Four genes namely, *C.cajan_13768, C.cajan_23080, C.cajan_29830*, and *C.cajan_33874* encoding universal stress protein showed large relative transcript abundance differences among three genotypes. The gene, *C.cajan_13768* showed 4.56-fold relative expression variation in ICPL 227 as compared to ICPL 151 (0.58-fold) and ICPL 8755 (0.04-fold). Another gene *C.cajan_23080* was having 6.19-fold relative expression in ICPL 227 as compared to ICPL151 (1.70-fold) and ICPL 8755 (3.10-fold). Interestingly, two genes showed 11.40, 6.30, 4.78 (*C.cajan_29830*) and 11.68, 3.98, 5.03 (*C.cajan_33874*) folds up-regulation in ICPL 227, ICPL 151, and ICPL 8755, respectively (**Figure 3**).

Comparative Expression Profiling of Candidate Genes across Legumes

Among the 10 common differentially expressed genes among MDT and LDT genotypes, two genes namely, *C.cajan_29830*

TABLE 2 | List of common genes with more than two-fold difference between more and less drought tolerant genotypes.

Gene-Id	ICPL 227 (More tolerant)	ICPL 151 (Less tolerant)	ICPL 8755 (Less tolerant)	Uniprot-Id	Protein name
C.cajan_26230	5.14	−0.20	−0.40	Q9SW11	U-box domain-containing protein 35
C.cajan_39705	5.19	−0.95	−0.16	Q9SW11	U-box domain-containing protein 35
C.cajan_09181	13.50	3.16	0.80	Q8GZ84	U-box domain-containing protein 36
C.cajan_30211	7.13	0.86	1.00	Q9FKG6	U-box domain-containing protein 52
C.cajan_46779	7.47	−0.03	0.60	Q9SIT5	Cation/H(+) antiporter 15
C.cajan_08737	7.70	1.03	0.24	I1JEJ0	Uncharacterized protein
C.cajan_13768	4.56	0.58	0.04	Q8LGG8	Universal stress protein A-like protein
C.cajan_23080	6.19	1.70	3.10	Q57951	Universal stress protein
C.cajan_29830	11.40	6.30	4.78	Q8LGG8	Universal stress protein A-like protein
C.cajan_33874	11.68	3.98	5.03	Q8LGG8	Universal stress protein A-like protein

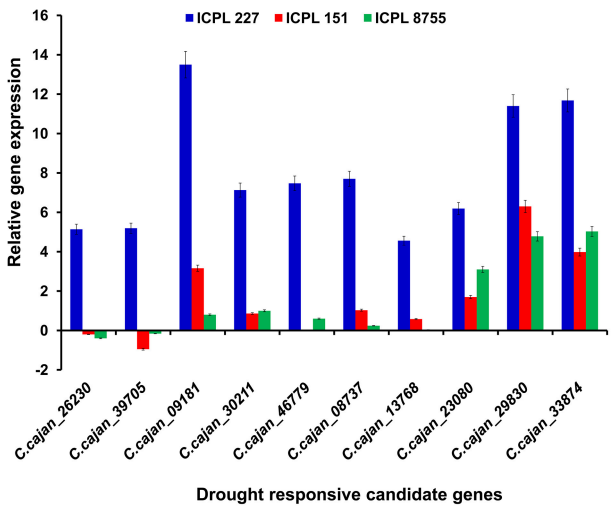


FIGURE 3 | Graph showing the relative transcript abundance for 10 genes in three genotypes, namely ICPL 227 (MDT genotype), ICPL 151, and ICPL 8755 (LDT genotypes). The y-axis represents the relative transcript abundance for the drought responsive genes depicted on the x-axis. The graph clearly shows ≥ 2-fold up-regulation of two genes namely, *C.cajan_29830* and *C.cajan_33874* in all the three genotypes with varying drought tolerance.

and *C.cajan_33874* showed marked up-regulation in all the three drought tolerant genotypes (**Supplementary Figure 2**). These two genes were further selected for validation using tolerant and susceptible genotypes in chickpea, groundnut, common bean, and cowpea. For gene normalization, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for chickpea (Garg et al., 2010), alcohol dehydrogenase (*ADH*) for groundnut (Reddy et al., 2013) while β-tubulin for common bean and cowpea (Eticha et al., 2010) were used as internal control (**Supplementary Table 2** and **Supplementary Figure 3**). To perform normalization, the reference genes which were reported to be stable for each of the legume crops were considered. In terms of expression profiling, in chickpea, ICC 4958 (tolerant genotype), showed 0.53, 0.14, and ICC 1882 (susceptible genotype), showed 0.23-, 1.01-fold differential gene expressions for *C.cajan_29830* (**Figure 4**) and *C.cajan_33874* (**Figure 5**), respectively. Similarly in groundnut, these two genes showed an expression variation of 0.58, 1.19 in CSMG 84-1 (tolerant genotype) for *C.cajan_29830* and 1.49, 0.56 in ICGS-76 (susceptible genotype) for *C.cajan_33874*. In the case of cowpea, the tolerant genotype (IT93K503-1) showed 0.55, 0.86 while the susceptible genotype (UC-C B46), showed 1.84-, 1.52-fold expression difference for the two genes, *C.cajan_29830* and *C.cajan_33874*, respectively. Similarly, in common bean, BAT 477 (tolerant genotype), showed 0.21, 0.03, and DOR 364 (susceptible genotype) showed 0.39, 0.48 differential gene expression for *C.cajan_29830* and *C.cajan_33874*, respectively. Overall, the gene expression variation between the tolerant and susceptible genotypes of the four legumes for the selected genes was less than 2-folds. Thus, the present study implies that these two genes might specifically be involved in contributing drought tolerance in pigeonpea.

DISCUSSION

This study has utilized genome sequence information for selecting genes encoding proteins containing USP domain and were validated for their role in drought tolerance in pigeonpea using qRT-PCR. Genes encoding protein with USP domain are known to be involved in a myriad of stress responses and any mutation in these genes may cause loss of efficacy in combating stresses (Drumm et al., 2009; Isokpehi et al., 2011; Shokry et al., 2014). USP domain has been found to be evolutionary conserved in a number of crop species such as cassava, soybean, finger millet, and peanut (Govind et al., 2009; Deeplanaik et al., 2013; Turyagyenda et al., 2013).

Earlier studies have provided evidences that stress-responsive genes encoding proteins with USP domain are useful in stress signal perception and subsequently lead to functionally efficient proteins. These proteins have been found to be involved in protecting cellular structures and cell molecules under stress conditions (Waditee et al., 2002; Majee et al., 2004; Dastidar et al., 2006; Govind et al., 2009). For instance, under water deficit conditions, out of 50 genes selected in peanut, only *HSP70* gene showed association with drought stress response (Govind et al., 2009). Similarly, 10 genes conferring drought tolerance were characterized in cassava (Turyagyenda et al., 2013). In the

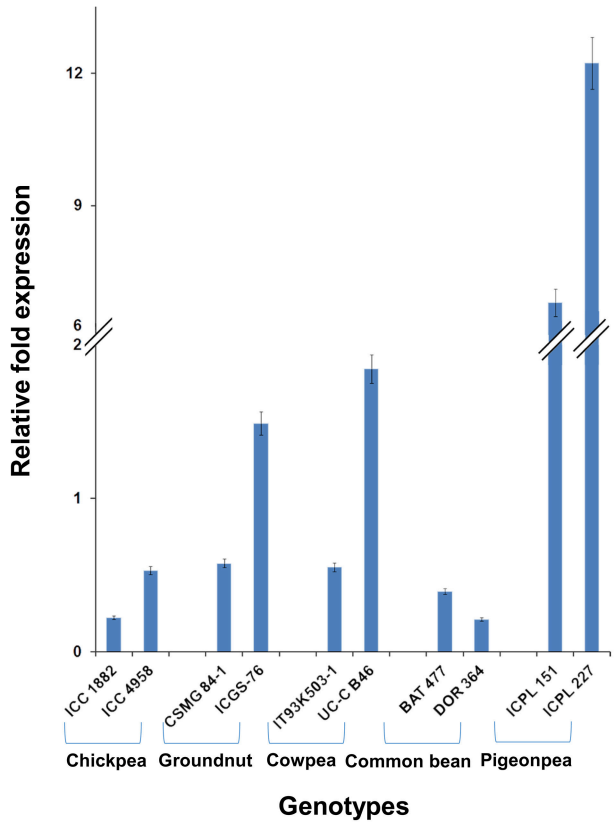


FIGURE 4 | Expression analysis of identified drought responsive gene, *C.cajan_29830* in the root tissues of tolerant and susceptible genotypes of the four legumes. The expression analysis was carried out using chickpea genotypes, ICC 4958 (tolerant) and ICC 1882 (susceptible), CSMG 84-1 (tolerant) and ICGS-76 (susceptible) for groundnut, IT93K503-1 (tolerant) and UC-C B46 (susceptible) for cowpea, and BAT 477 (tolerant) and DOR 364 (susceptible) for common bean, in comparison to the pigeonpea genotypes, ICPL 151 (LDT genotype) and ICPL 227 (MDT genotype).

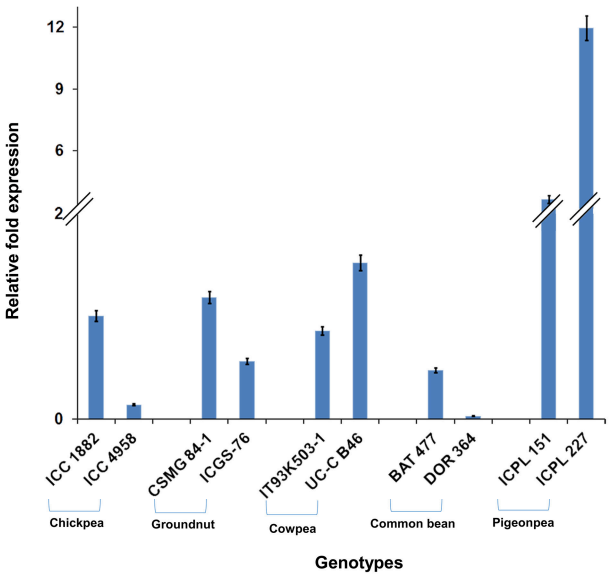


FIGURE 5 | Expression analysis of identified drought responsive gene, *C.cajan_33874* in the root tissues of tolerant and susceptible genotypes of the four legumes. The expression analysis was carried out using chickpea genotypes, ICC 4958 (tolerant) and ICC 1882 (susceptible), CSMG 84-1 (tolerant) and ICGS-76 (susceptible) for groundnut, IT93K503-1 (tolerant) and UC-C B46 (susceptible) for cowpea, and BAT 477 (tolerant) and DOR 364 (susceptible) for common bean, in comparison to the pigeonpea genotypes, ICPL 151 (LDT genotype) and ICPL 227 (MDT genotype).

case of pigeonpea, homology search provided 71 genes encoding USP domain, of which 51 genes with pure domain architecture were selected for further validation in pigeonpea. During water stress conditions, plant responds at both cellular as well as molecular level by accumulating osmolytes and proteins involved in stress response and/or tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006). Stress response at cellular level such as cell proliferation, differentiation, stomatal closure, repression of cell growth generally lead to induced expression of drought responsive genes (Yamaguchi-Shinozaki and Shinozaki, 2005). Among the selected 51 genes, majority of the genes were found to be related to response to stress followed by cellular processes.

The three pigeonpea genotypes selected for expression profiling in the present study exhibited varying degree of tolerance to drought stress (Lopez et al., 1996; Saxena et al., 2011). The expression variation of candidate genes in stressed tissues can be compared with well water controls at specific time frame (VanGuilder et al., 2008) using qRT-PCR. A set of 10 genes, which were identified in the MDT and LDT genotypes showed large difference in the relative transcript abundance. These genes were found to be related to plant U-box proteins, cation/H(+) antiporter (*CHX*) proteins, uncharacterized protein and universal stress protein A-(*uspA*) like protein.

Plant U-box E3 ligases have been found to be involved in enhanced drought, salinity, cold and heat tolerance in *Arabidopsis thaliana* (Lyzenga and Stone, 2011). Whereas, Ubiquitin-protein ligases (E3s) determine the substrate specificity of ubiquitylation and plays an important role in protein post-translational modification in higher plants (Liu and Walters, 2010). Based on the structure, Ubiquitin-protein ligases (E3s) has been classified into two families, the HECT and RING-finger (U-Box) families (Hatakeyama and Nakayama, 2003). Molecular and cellular characterization of U-Box protein-coding genes in hot pepper (Cho et al., 2006) and *Arabidopsis* (Cho et al., 2008) has clearly demonstrated the role of U-Box protein-coding genes in drought tolerance. The four plant U-Box protein-coding genes (*C.cajan_26230, C.cajan_39705, C.cajan_09181,* and *C.cajan_30211*) identified between the MDT and LDT genotypes showed significant differences in the relative transcript abundance.

One gene (*C.cajan_46779*) belonging to cation/H(+) exchanger (*CHX*) group was also found to be differentially expressed between the two genotypes studied. In *Arabidopsis, CHX* gene family was found to play an important role in osmotic adjustment and K+ homeostasis (Sze et al., 2004). Therefore, the finding suggests that the gene, *C.cajan_46779* belonging to *CHX* gene family may also be playing an important role during drought stress condition in pigeonpea. Another gene,

C.cajan_08737, annotated as uncharacterized protein, was also found to be differentially expressed among MDT and LDT genotypes suggesting its role in drought tolerance. Another class of genes (*C.cajan_13768, C.cajan_23080*, *C.cajan_29830*, and *C.cajan_33874*) having differences in the relative transcript abundance between MDT and LDT genotypes was found to have the *uspA* domain. The *uspA* domain is also known to play a vital role in survival during cellular growth arrest. Genes belonging to this domain help in oxidative stress resistance and initiates defense against superoxide-generating agents (Nachin et al., 2005).

Pigeonpea is one of the most drought tolerant legume crops (Varshney et al., 2009). Therefore, the expression variation of the two differentially expressed genes, *C.cajan_29830* and *C.cajan_33874* identified across all the three pigeonpea genotypes was studied across four other legumes. For these two genes (*C.cajan_29830* and *C.cajan_33874*), chickpea (78.5 and 82%), groundnut (77.6 and 79.5%) and common bean (85.9 and 86.4%) genes showed high sequence identity with pigeonpea, respectively. Based on this observation, two genes encoding Universal stress protein A-like protein seem to be highly conserved among the legumes studied. However, in the four legume crops, namely chickpea, groundnut, common bean, and cowpea, these two genes did not show any expression variation between the drought tolerant and susceptible genotypes. This may also be due to different strategies acquired by different legumes for drought adaptation mechanism (Mir et al., 2012). This study also showed the involvement of U-Box protein-coding in having some specific role in drought tolerance mechanisms in pigeonpea. Many such conserved U-Box protein-coding genes were found to be over-expressed in different plant species to enhance drought tolerance (Lyzenga and Stone, 2011).

Thus, expression analysis of the 51 drought responsive genes has provided a set of 10 genes belongs to plant U-Box proteins, cation/H(+) antiporter (*CHX*) proteins, uncharacterized proteins and universal stress protein A-(*uspA*) like protein. This candidate gene-based approach could provide useful insights into the molecular mechanisms involved in drought tolerance in pigeonpea. Moreover, the identified genes can also be validated at sequence level in different genetic backgrounds to detect the presence of sequence variations for the development of gene-based marker(s) for crop improvement and development of more tolerant breeding lines/hybrids through genomics-assisted breeding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01065

Supplementary Figure 1 | Functional classification of the 51 drought responsive pigeonpea genes having USP domains. These categories include (a) 25.5% in molecular function: catalytic activity (8%), transporter activity (5%) and binding (6%), (b) 27.4% in cellular component: ubiquitin ligase complex (5%), membrane (6%), organelle (5%), membrane part (5%), plastid part (1%) and cell part (11%), and (c) 47% in biological process: response to stress (13%), metabolic process (8%), cellular process (12%), homeostatic process (3%), single-organism process (6%), localization (5%), and establishment of localization (5%).

Supplementary Figure 2 | Expression variation of two candidate genes (*C.cajan_29830* and *C.cajan_33874*) between MDT and LDT genotypes. Differentially expressed genes were identified with ≥ 2-fold expression variation across the three pigeonpea genotypes, namely ICPL 227 (MDT genotype), ICPL 151, and ICPL 8755 (LDT genotypes). The different letters above the bars were considered as statistically significant between each other.

Supplementary Figure 3 | Cross generic amplification check. (A) The melt curve obtained for the two qRT-PCR primer sets, namely *C.cajan_29830* (Top) and *C.cajan_33874* (Bottom) in the resistant and the susceptible genotypes of the four legumes studied. A: ICC 4958; B: ICC 1882; C: IT93K503-1; D: CSMG 84-1, E: BAT 477; F: UC-C B46; G: DOR 364 and H: ICGS-76. **(B)** 2% agarose gel showing the amplification of corresponding genes in the resistant and the susceptible genotypes of the four legumes studied to the pigeonpea genes (*C.cajan_29830*-Top and *C.cajan_33874*-Bottom).

Supplementary Table 1 | List of primer pairs used for qRT-PCR analysis.

Supplementary Table 2 | List of housekeeping genes used for qRT-PCR analysis.

Supplementary Table 3 | HMM Search output for USP domain in pigeonpea genes set.

Supplementary Table 4 | Protein names, gene ontology terms (GO_term) and ontology identities (GO_ID) of 51 drought responsive genes.

Supplementary Table 5 | List of genes showing more than two-fold difference between ICPL 227 and ICPL 8755.

Supplementary Table 6 | List of genes showing more than two-fold difference between ICPL 227 and ICPL 151.

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Conservation of *AtTZF1, AtTZF2,* and *AtTZF3* homolog gene regulation by salt stress in evolutionarily distant plant species

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Arginine-rich tandem zinc-finger proteins (RR-TZF) participate in a wide range of plant developmental processes and adaptive responses to abiotic stress, such as cold, salt, and drought. This study investigates the conservation of the genes *AtTZF1-5* at the level of their sequences and expression across plant species. The genomic sequences of the two *RR-TZF* genes *TdTZF1-A* and *TdTZF1-B* were isolated in durum wheat and assigned to chromosomes 3A and 3B, respectively. Sequence comparisons revealed that they encode proteins that are highly homologous to AtTZF1, AtTZF2, and AtTZF3. The expression profiles of these RR-TZF durum wheat and *Arabidopsis* proteins support a common function in the regulation of seed germination and responses to abiotic stress. In particular, analysis of plants with attenuated and overexpressed AtTZF3 indicate that AtTZF3 is a negative regulator of seed germination under conditions of salt stress. Finally, comparative sequence analyses establish that the *RR-TZF* genes are encoded by lower plants, including the bryophyte *Physcomitrella patens* and the alga *Chlamydomonas reinhardtii*. The regulation of the *Physcomitrella AtTZF1-2-3-like* genes by salt stress strongly suggests that a subgroup of the RR-TZF proteins has a function that has been conserved throughout evolution.

Keywords: RR-TZF, CCCH zinc finger proteins, abiotic stress, germination, phylogenetic analysis, durum wheat, *Arabidopsis*, *Physcomitrella patens*

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Introduction

Zinc-finger proteins constitute one of the largest and most diverse families of plant regulatory proteins, and increasing evidence indicates that they act as key factors in several biological processes, such as morphogenesis and responses to environmental stress (Hall, 2005; Lin et al., 2011). These proteins are characterized by zinc-finger motifs that consist of cysteine and/or histidine residues that coordinate a zinc ion to form local peptide structures that are generally associated with specific molecular functions (Guo et al., 2009). The CCCH zinc-finger motif, in particular, identifies a specific zinc-finger family, members of which have been found in many species from yeast to human (Blackshear et al., 2005; Wang et al., 2008; Kramer et al., 2010). The gene family for these CCCH zinc-finger proteins has also been studied in plants, and various

members have been identified by genome-wide analysis and have been implicated in a broad range of developmental and adaptive processes (Wang et al., 2008; Chai et al., 2012; Peng et al., 2012; Zhang et al., 2013; Liu et al., 2014; Xu, 2014).

A particular group of the plant CCCH protein family is characterized by an arginine-rich region that contains a CHCH motif (known as the RR region) that is linked to a tandem zinc-finger (TZF) domain, which is unique to plants (Wang et al., 2008; Pomeranz et al., 2010b). Members of the RR-TZF subfamily have been identified in several higher plants, including *Arabidopsis* (AtTZF1-11) (Wang et al., 2008; Chai et al., 2012; Peng et al., 2012; Zhang et al., 2013; Liu et al., 2014; Xu, 2014). The functions of some of the RR-TZF *Arabidopsis* proteins have been defined in relation to responses to a number of stress stimuli and developmental processes. PEI1 (AtTZF6) is involved in embryogenesis (Li and Thomas, 1998), and AtTZF10 and AtTZF11 (which are also known as AtSZF2 and AtSZF1, respectively) have been described as positive regulators of salt tolerance (Sun et al., 2007). The overexpression of AtTZF2 and AtTZF3, which are two RR-TZF genes that are regulated by abscisic acid (ABA), conferred ABA hypersensitivity, reduced transpiration, enhanced drought tolerance, and delayed jasmonic-acid-induced senescence, whereas their attenuation showed reduced tolerance to salt stress (Lee et al., 2012). Overexpression of another member of the RR-TZF subfamily, AtTZF1, resulted in late flowering and enhanced tolerance to cold and drought stress, while concurrent down-regulation of the AtTZF1-3 genes by RNA interference (RNAi) caused early germination and phenotypes that were relatively stress-sensitive (Lin et al., 2011). These studies have suggested a connection between AtTZF1 and ABA- and gibberellic-acid-mediated responses (Lin et al., 2011). The rice OsTZF1 gene is positively regulated by polyethylene glycol and ABA, and its overexpression in transgenic rice seedlings conferred hypersensitivity to ABA (Zhang et al., 2012). OsTZF1 has been reported to be involved in seed germination, seedling growth, leaf senescence, and oxidative-stress tolerance (Kong et al., 2006; Jan et al., 2013). In transgenic *Arabidopsis* plants, overexpression of TaZnFP, a *T. aestivum* gene that is induced by cold, salt, drought, and ABA, enhanced the *Arabidopsis* drought and salt tolerance (Min et al., 2013). Taken together, these data indicate a role for some of the RR-TZF proteins in ABA and gibberellic-acid pathway(s). Indeed, it has been established that the *Arabidopsis* SOMNUS (AtTZF4/SOM) protein acts as a negative regulator of light-dependent seed germination, and *som*-mutants are characterized by reduced levels of ABA and elevated levels of gibberellic acid, which appears to be due to changes in the expression of the ABA and gibberellic-acid metabolic genes (Kim et al., 2008). AtTZF4/SOM is positively regulated by the phytochrome-interacting factor-3-like 5 (PIL5) and ABA insensitive 3 (ABI3) transcription factors, which together bind to the AtTZF4/SOM promoter (Kim et al., 2008; Park et al., 2011).

A putative *RR-TZF* gene was previously identified in durum wheat as a cold-responsive EST (EST002H8). Interestingly, the expression of this *RR-TZF* gene was also modulated by drought in a developmental- and genotype-dependent manner (Mastrangelo et al., 2005; De Leonardis et al., 2007). To gain further insight into this putative *RR-TZF* gene and its regulation, we identified and characterized two homeologous genes, named *TdTZF1-A* and *TdTZF1-B* that correspond to EST002H8. Based on the sequence similarity, gene structure, and expression analysis, we established that *AtTZF1, AtTZF2,* and *AtTZF3* (indicated as *AtTZF1-2-3*) are putative orthologs of the durum wheat *TdTZF1-A* and *TdTZF1-B* genes. In addition, we showed that AtTZF3 is a negative factor in the control of *Arabidopsis* seed germination in the presence of salt. Finally, an evolutionary computational analysis indicated the presence of highly conserved AtTZF1-2-3-like proteins in phylogenetically distant species, such as the bryophyte *Physcomitrella patens.* Similar to the *Arabidopsis AtTZF1-5* and durum wheat *TdTZF1-A* and *TdTZF1-B* genes, the *Physcomitrella TZF* genes were shown to be regulated by salt stress. Taken together, our data suggest that the function of the AtTZF1-2-3-like proteins in the regulation of seed germination has emerged from their roles in pre-existing NaCl-stress signaling pathways that control growth and development in lower plants.

Materials and Methods

Cloning of Full-Length *TdTZF1-A* and *TdTZF1-B* Genomic and cDNA Sequences

Genomic DNA was extracted from durum wheat leaves of cv. 'Creso' using the cetyl trimethyl ammonium bromide (CTAB)-based method (Hoisington et al., 1994). Total RNA was isolated with Trizol reagent (Invitrogen), according to the manufacturer instructions. The single-stranded cDNA was synthesized from total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen) and an oligod$(T)_{18}$ primer, following the manufacturer recommendations.

A partial DNA sequence that codes for the RR-TZF protein (*EST002H8*) was used as a query in a BLASTN search with the database of the wheat separate chromosome arms promoted by the International Wheat Genome Sequencing Consortium[1] (IWGSC), to gain the corresponding full-length genomic sequence. Two very similar hits were identified on chromosomes 3A and 3B (IWGSC_chr_3AS_ab_k71_contings_longerthan_200_3415369, and IWGSC_chr_3B_ab_k71 contings_longerthan_200_10407484). However, the genomic clones identified contained only partial sequences that corresponded to the 3′ part of *EST002H8*. A BLASTN search carried out against the Triticeae full-length CDS database (TriFLDB) using the *EST002H8* sequence as the query allowed the identification of a full-length cDNA sequence (AK330326) that was 100% identical to the partial sequence on chromosome 3A (IWGSC_chr_3AS_ab_k71_contings_longerthan 200_3415369). Specific primers were designed on the AK330326 sequence and used to amplify this gene in the durum wheat cultivar 'Creso', which we named as *TdTZF1-A*.

[1]https://urgi.versailles.inra.fr/blast/blast.php

The full-length *TdTZF1-A* sequence was used as a query in a BLASTN search with the database of the wheat 3B chromosome promoted by the IWGSC. A new clone that showed 95% identity with respect to the query sequence was obtained (IWGSC_chr_3B_ab k71_contings_longerthan_200_10722038). However, this clone corresponded to a partial sequence; in particular, it contained the 5′ portion of the putatively homeologous gene of *TdTZF1-A*. Therefore, it was used together with the *EST002H8* sequence corresponding to the 3′ region of the gene to design specific primers to isolate *TdTZF1* on durum wheat genome B (*TdTZF1-B*). The forward primer was designed on the clone IWGSC_chr_3B_ab_k71_contings_longerthan_200_10722038 within the 5′-UTR region of the gene, and the reverse primer was designed on the *EST002H8* durum sequence. The bread-wheat cv. 'Chinese Spring' and the durum wheat cv. 'Creso' DNA and genomic sequences described in this study have been submitted to GenBank, with the accession numbers: KP717034-39.

Physical Mapping of the *TdTZF1-A* and *TdTZF1-B* Genes in Heat

Nulli-tetrasomic lines of 'Chinese Spring' wheat for chromosomes 3A, 3B, and 3D (N3AT3D, N3BT3D, and N3D3B, respectively; Endo and Gill, 1996) were used to assign the *TdTZF1-A* and *TdTZF1-B* genes to specific chromosomes. Ditelosomic and deletion lines of chromosomes 3A and 3B were also used to further restrict the position of the *TdTZF1-A* and *TdTZF1-B* genes to specific bins. The primer pairs and PCR conditions were the same as those described for the cloning of the full-length sequences. The amplification products were separated on agarose gels and sequenced.

Identification of Arginine-Rich Tandem Zinc-Finger Proteins in Plant Species

To identify the RR-TZF proteins in the plant kingdom, the genomes of 54 plant species that ranged from green algae to angiosperms were investigated. The AtTZF3 protein sequence IDAYSCDHFRMYDFKVRRCARGRSHDWTECPYAH was used as the query to search the phytozome database using the BLASTP program[2]. As gymnosperm genomes were not available in the phytozome database, the *Picea abies, Picea glauca, Picea sitchensis*, and *Pinus taeda* RR-TZF proteins were obtained from the Plant Transcription Factor Database[3]. The wheat RR-TZF proteins were obtained from the TriFLDB[4], The Institute for Genomic Research (TIGR) Plant Transcript Assemblies[5], the IWGSC[6], and the National Center for Biotechnology Information[7] (NCBI). The most similar proteins in each plant species were selected, and these were subsequently filtered based on the simultaneous presence of a CHCH motif, which is a distinctive feature of this subfamily (Wang et al., 2008), and TZF CCCH domains. The RR-TZF proteins from each species have been named according to the TZF nomenclature previously used by (Pomeranz et al., 2010a).

Identification of the AtTZF1-2-3-like and AtTZF4-5-like Proteins

The AtTZF1-5 proteins are characterized by the absence of ANK repeats and by specific spacings between the Cys residues in the CCCH domains, which are typically $C-X_{7-8}-C-X_5-C-X_3-H$ for the first CCCH domain, and $C-X_5-C-X_4-C-X_3-H$ for the second CCCH domain. To search for putative orthologs of these proteins, a structure-based and homology-based approach was used: first, sequences structurally different from the AtTZF1-5 proteins were excluded (i.e., with ANK domains or with altered spacings between the Cys residues in the CCCH domains); then, for each sequence the most similar sequences in *Arabidopsis* were identified, based on a minimum similarity of 23%; and finally, these AtTZF1-2-3-like and AtTZF4-5-like proteins were clustered into two separate groups. Sequence similarities were obtained from the distance matrix of multi-alignments of all of the RR-TZF proteins.

Alignments and Phylogenetic Analysis

All of the multiple alignments of amino-acid sequences were performed using the Clustal W algorithm in the Geneious software (version 5.5.3; Biomatters, New Zealand). The phylogenetic tree was constructed with the MEGA 5.0 software using the neighbor-joining method, after alignment of the RR-TZF full-length proteins from selected species through the Clustal W algorithm. For statistical reliability, bootstrap analysis with 1,000 replicates was used to evaluate the significance of each node.

Plant Materials and Stress Conditions

Durum Wheat

Triticum durum cv. 'Creso' was used in this study. For all of the analyses, dry seeds were sterilized with 70% ethanol for 1 min, then with 3% sodium hypochlorite solution for 20 min, and finally washed five times with sterile water.

For the salt-stress gene expression analyses in germinating seeds, 15 seeds per dish were incubated on two layers of filter paper that had been moistened with distilled water, at 4°C in the dark for 72 h, to synchronize the germination. The seeds were moved to 21°C in the presence of light, with or without 150 mM NaCl. The germinating seeds were harvested for the expression analysis after 6 h and 12 h.

For the salt-stress gene expression analyses in seedlings, the sterilized wheat seeds were germinated and grown under controlled conditions (16 h photoperiod, at 100 μmol m^{-2} s^{-1} photon flux density) on two layers of filter paper that were moistened with distilled water. Then, the 4-day-old plantlets were transferred during light exposure to paper soaked with water or NaCl (at 150, 250, or 400 mM). The seedlings were sampled at the time of the transfer and after 1, 3, and 6 h.

For the cold-stress gene expression analyses, the sterilized wheat seeds were germinated and cultured on a mixture of

[2]http://www.phytozome.net/

[3]http://planttfdb.cbi.pku.edu.cn/

[4]http://trifldb.psc.riken.jp/ver.2.0/blast.pl

[5]http://plantta.jcvi.org/search.shtml

[6]http://www.wheatgenome.org/content/view/full/625

[7]http://blast.ncbi.nlm.nih.gov/Blast.cgi

soil, sand, and peat (6:3:1) in a growth chamber at 20°C (16 h light, at 500 μmol m^{-2} s^{-1} photon flux density)/ 18°C (8 h darkness), and 60% relative humidity, up to the full expansion of the third leaf. Then, the plants to be cold-stressed were moved to a chamber at 4°C (continuous light, at 500 μmol m^{-2} s^{-1} photon flux density), while the control plants were left under the initial conditions. The leaves were sampled at the time of the transfer and after 1, 3, and 6 h.

Arabidopsis thaliana

The *Arabidopsis thaliana* ecotype 'Columbia' (Col-0) was used in all of the experiments. The seeds were sterilized with 5% sodium hypochlorite solution that contained 0.02% Triton X-100, and then they were rinsed with water.

For the salt-stress gene expression analyses in germinating seeds, the sterilized seeds were spread on two layers of filter paper soaked with water or 150 mM NaCl and exposed to continuous light for 6 and 12 h. The material was collected from dry seeds and water-treated and salt-treated seeds.

For the salt-stress gene expression analyses in seedlings, the Col-0 seeds were spread on nylon sheets (Sefar Nitex 03-100/44) on germination medium (1x Murashige and Skoog salts, vitamins and 1% sucrose) solidified with 0.8% agar, and the seedlings were grown for 7 days at 21°C with a long photoperiod (16 h light, at 100 μmol m^{-2} s^{-1} photon flux density). Then, the nylon sheets were transferred onto the same substrate or onto germination media containing NaCl (150, 250, or 400 mM). Whole seedlings were collected at the time of the transfer and after 1, 3, and 6 h.

For the cold-stress gene expression analyses in seedlings, 7-day-old seedlings were grown on solid germination medium (0.8% agar) at 21°C with a long photoperiod (16 h light, at 100 μmol m^{-2} s^{-1} photon flux density) and were subjected to 10°C for 1, 3, and 6 h. The control seedlings were left at 21°C.

Physcomitrella patens

Physcomitrella patens ecotype 'Gransden 2004' was used in the experiments described in this study. The plants were grown at 21°C under a 16-h-light (40 μmol m^{-2} s^{-1})/8-h-dark photoperiod. Protonemata were propagated on a cellophane overlay on rich medium (BCD medium supplemented with 5% [w/v] glucose and 0.5 g/l ammonium tartrate; Roberts et al., 2011) solidified with 0.7% agar. After propagation, the protonemata were blended in water with a homogenizer. The homogenate was spread on a new cellophane disk on solid minimum BCD medium. After 6 days, the cellophane overlay with the protonemal tissue was transferred either onto the same substrate or onto media containing NaCl (150, 250, or 400 mM). Samples were collected at the time of the transfer and after 1, 3, and 6 h.

Germination Tests

Arabidopsis wild-type and mutant (attenuated and over-expressing) AtTZF3 lines were propagated under controlled conditions in a growth room with a long photoperiod (16 h light, at 100 μmol m^{-2} s^{-1} photon flux density) at 21°C. Following seed harvesting, the seeds were dried at 21°C for at least 1 month prior to the germination assays. After sterilization, seeds were germinated on filter paper soaked with either water, 150 mM NaCl or 1 μM ABA solutions, at 21°C under continuous light (at 100 μmol m^{-2} s^{-1} photon flux density). The germination rate was scored after 72 h of incubation, with the emergence of a visible root used as the morphological marker for germination. Two experiments were carried out with two independent seed stocks, and each of these was performed using triplicate samples (each containing 50−100 seeds).

Expression Analysis of *RR-TZF* Genes in Durum Wheat, *Arabidopsis*, and *Physcomitrella patens*

Total RNA was extracted from *T. durum*, *Arabidopsis* seedlings, and *P. patens* protonemata using RNeasy Plant Mini kits (Qiagen) with RNase-Free DNase (Qiagen) treatment, and from *Arabidopsis* and *T. durum* seeds using Spectrum plant total RNA kits (Sigma−Aldrich) with On-Coloumn DNase Digestion (Sigma−Aldrich). The RNA concentration and quality were determined spectrophotometrically (NanoDrop ND-1000 spectrophotometer, NanoDrop Technologies Inc., Montchanin, Germany).

The removal of any genomic DNA contamination from the total RNA and the first-strand cDNA synthesis were performed using QuantiTect Reverse Transcription kits (Qiagen), according to the manufacturer instructions, except for step 6, for which the incubation time was extended to 1 h.

Quantitative PCR was performed on an ABI7900HT PCR machine (Applied Biosystems), according to the manufacturer instructions, using TaqMan Universal PCR Master Mix (Applied Biosystems) and Universal ProbeLibrary Probes (Roche). The amplification reactions were carried out using a final Universal ProbeLibrary probe concentration of 100 nM, and a final primer concentration of 200 nM. The 384-well plates were set up using the Tecan Freedom Evo 75® platform (Tecan). Each gene-specific expression quantification assay was designed using the free online ProbeFinder Roche software (www.universalprobelibrary.com), which allows an automated search for available probes, with annexed primers specific for the targets to be amplified. In some cases, the primer sequences were manually designed or just optimized. The primers and probes for each of the assays are listed in Supplementary Table S1. The expression levels of the reference gene (as indicated in each experiment) were used as the internal control. The relative expression level was calculated using the $2(-\Delta\Delta CT)$ method (Livak and Schmittgen, 2001). The CT (cycle threshold) values used for both the target and the internal control genes were the means of three technical replicates. The experiments were performed at least twice, and the relative transcript levels were calculated and normalized as described previously (Willems et al., 2008).

Construction of Attenuated and Overexpressing *Arabidopsis* Lines for the *AtTZF3* Gene

For the overexpression of *AtTZF3*, the *AtTZF3* open-reading frame was PCR amplified from genomic DNA and cloned into

the 2x35S expression vector pMDC32 (Curtis and Grossniklaus, 2003).

Due to a lack of *AtTZF3* knock-out T-DNA insertions in mutant collections, RNAi, and artificial microRNAs (amiRNAs) were used to knock-down the expression of the gene. The amiRNA were generated by site-directed mutagenesis of the endogenous microRNA miR319a of *A. thaliana*, following the protocol described by Schwab et al. (2006), using modified primers (Supplementary Table S1). The RNAi was performed using an ihpRNA (intron hairpin RNA) constructs (Wesley et al., 2001), using the primers indicated in Supplementary Table S1.

The transformation of *Arabidopsis* was conducted according to the floral dip method (Clough and Bent, 1998), and single insertion T_3 homozygous generation was used to determine the expression level of the *AtTZF3* transcript by RT-PCR.

Statistical Analysis

Statistical analysis was performed after angular transformation of the germination ratios (Little and Hills, 1978), and after log2 transformation of the relative expression ratios. Statistical significance was evaluated by means of one-way ANOVA, followed by Bonferroni or Dunnett *post hoc* tests (Prism 6, GraphPad Software, CA, USA), as indicated in the Figure legends. $P < 0.05$ or < 0.01 were considered as statistically significant.

Results

Identification and Characterization of the *TdTZF1-A* and *TdTZF1-B* Genes in Durum Wheat

A partial DNA sequence coding for a RR-TZF protein named EST002H8 was previously identified as an abiotic stress-responsive gene in durum wheat (Mastrangelo et al., 2005; De Leonardis et al., 2007). To better characterize the genomic regions encoding EST002H8, the full-length sequences of two genes were isolated in the durum wheat cv. 'Creso,' which we named *TdTZF1-A* and *TdTZF1-B*. The genomic and cDNA sequences are identical, which indicated that no introns are present in these genes. *TdTZF1-A* and *TdTZF1-B* are 1,630-bp and 1,633-bp long, and they contain open-reading frames of 1,155 and 1,164 bp, respectively. The coded proteins are 384 and 387 amino-acids long, with calculated molecular weights of 41.17 kDa and 41.45 kDa, and isoelectric points of 7.53 and 7.51. TdTZF1-A and TdTZF-1B sequences show 89.5 and 96.4% identity at the nucleotide and amino-acid levels, respectively. The region containing the two CCCH domains is highly conserved, but the Ser-315 (polar) coded for by *TdTZF1-A* is replaced by the apolar Gly-319 coded for by *TdTZF1-B*. Moreover, the *TdTZF1-B* gene is characterized by a nucleotide insertion coding for three amino acids (i.e., serine, cysteine, glycine) at position 254, with respect to *TdTZF1-A* (Supplementary Table S2). *TdTZF1-A* and *TdTZF1-B* are putative homeologous genes, as a set of nullitetrasomic (i.e., N3AT3D, N3BT3D, N3DT3B) and deletion lines of bread wheat cv. 'Chinese Spring' and gene-specific primers allowed us to map the *TdTZF1-A* and *TdTZF1-B* sequences to the bins 3AS2-0.23-0.46 and C-3BS1-0.33 on chromosomes 3A and 3B, respectively (Supplementary Materials; Supplementary Figure S1).

Sequence comparisons of TdTZF1-A and TdTZF1-B with the *Arabidopsis* AtTZF1-5 revealed that the wheat proteins are more similar to AtTZF1-2-3 than to AtTZF4-5 (**Figure 1**). Indeed, AtTZF1-2-3, TdTZF1-A, and TdTZF1-B have some amino-acid positions in common within or near the conserved RR-TZF region (e.g., Cys-95, Ala-109, Gly-111, Thr-141, Ala-142, Lys/Arg-153, Ala/Ser-157, Gln-177, Pro-178, Asp/Glu-197) that might discriminate the AtTZF1-2-3 group and TdTZF1-A and TdTZF1-B from the AtTZF4-5 group (**Figures 1C,D**). The gene structures (absence of introns) and the very high similarities of the TZF domains suggest that *AtTZF1-2-3*, *TdTZF1-A*, and *TdTZF1-B* are indeed orthologous genes. Moreover, there are some other motifs that are conserved in the grouping of AtTZF1-2-3, TdTZF1-A, and TdTZF1-B that are not in the AtTZF4-5 group (e.g., 60-LX[R/Q]YLP-65, **Figure 1B**; 381-[L/I]EEXPPMERVESGR-397, **Figure 1G**). Other motifs are common to both of these groups of proteins (e.g., 16-VXIPP-20, **Figure 1A**; 255-SPPSESPPLSP-265, **Figure 1E**; 299-NDVVASL-305, **Figure 1F**; 429-DVGWVSDLL-437, **Figure 1H**).

In Silico Analysis of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* Genes during Development and Under Abiotic-Stress Conditions

As a first step to investigate whether there are any evolutionarily conserved features in the regulation of these genes, an *in silico* analysis of their expression profiles during plant development and in response to abiotic stress was performed (Supplementary Figure S2). The *AtTZF1-5* genes have well-defined counterparts in species in which the RR-TZF family has been described (Wang et al., 2008, 2014; Chai et al., 2012; Peng et al., 2012; Bogamuwa and Jang, 2014; Liu et al., 2014; Wang et al., 2014; Xu, 2014), and these were selected for this analysis. Furthermore, we identified other genes that belong to the *RR-TZF* family in wheat using an *in silico* search in which the *Arabidopsis* genes and the durum wheat *TdTZF1-A* and *TdTZF1-B* genes were used as queries against the TIGR, TriFLDB, NCBI, and IWGSC databases. As well as the bread-wheat AK330326 sequence, which is named here as *TaTZF1*, and a recently described bread-wheat gene, *TaZnFP* (*TaTZF2*) (Min et al., 2013), we retrieved six more CCCH sequences, as: AK335344 (*TaTZF3*), AK335750 (*TaTZF4*), and Tplb0012e12 (*TaTZF7*) in the TriFLDB database; EMS63094 (representing a *T. urartu* gene) in the NCBI; TC435797 (*TaTZF5*), as the only tentative consensus that corresponded to a full-length *TZF* gene in the wheat gene index database; and Ta377176 (*TaTZF6*) of 9456 bp, which was contained within IWGSC_chr2BS_ab_k71_contigs_longerthan_200_5175761.

All of these sequences were blasted against the IWGCS database to predict their putative chromosome locations. This information was obtained for *TaTZF4* (chromosome 2AS), *TaTZF6* (chromosome 2BS), *TaTZF3* (chromosome 1DS), *TaZnFP* (chromosome 3B), and *TaTZF5* (chromosome 1AL).The *in silico* expression analysis was conducted using the following

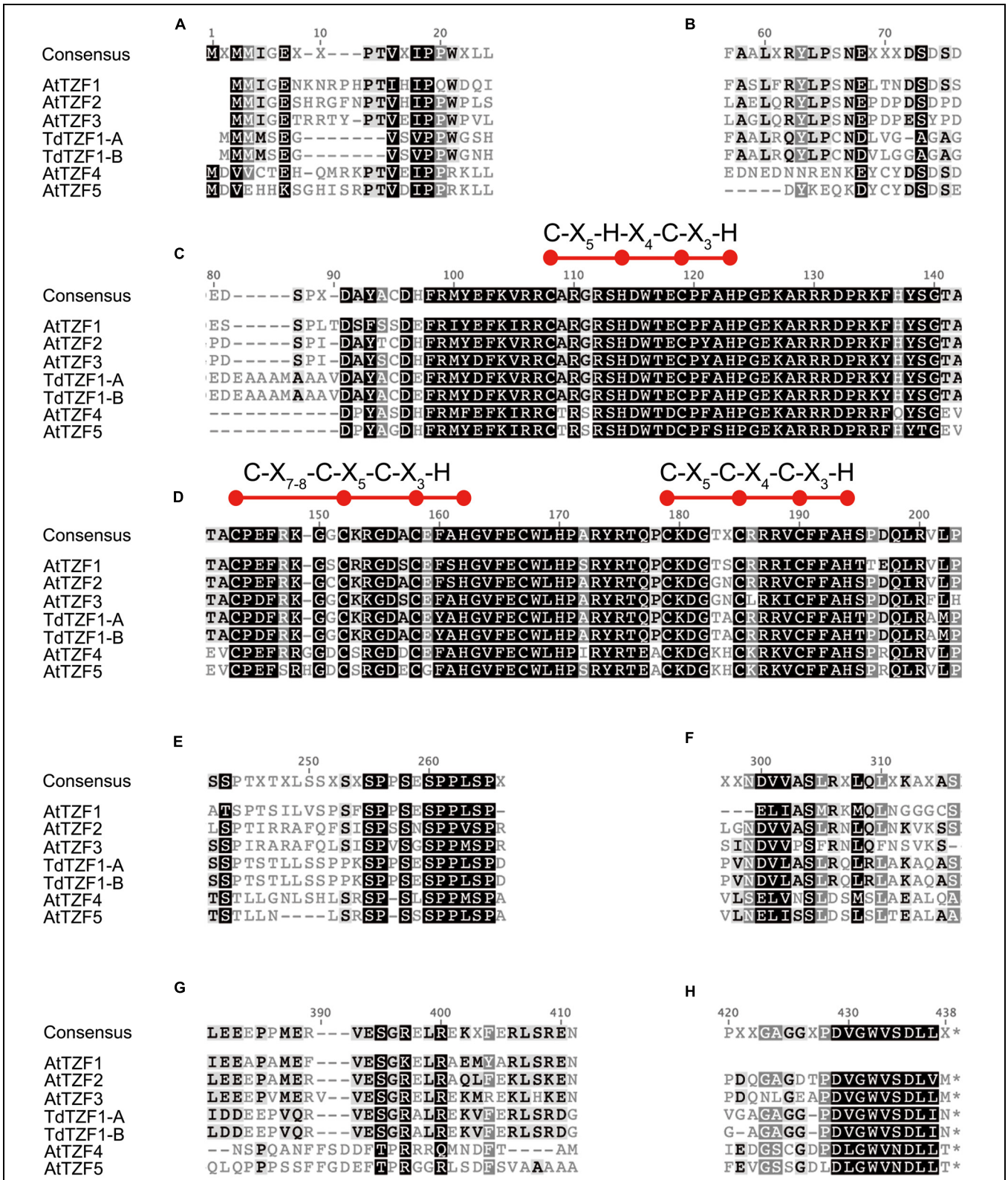


FIGURE 1 | Multi-alignment of the full-length amino-acid sequences of AtTZF1-5, TdTZF1-A, and TdTZF1-B. Selected portions of the alignment of the AtTZF1-5, TdTZF1-A, and TdTZF1-B amino-acid sequences, constructed using the Clustal W algorithm with the Geneious software (version 5.5.3) **(A–H).** Black highlights amino acids that are identical (or analogous) in all of the sequences. Red balls and horizontal bars represent the CHCH motif or CCCH domains.

public databases: the *Arabidopsis* eFP Browser[8] and PLEXdb[9] for *Arabidopsis* and wheat, respectively. The wheat *RR-TZF* sequences were blasted against the database of PLEXdb to identify the probe set that corresponded to each gene. The same probe set was found for the *TaTZF1*, *TdTZF1-A*, and *TdTZF1-B* genes.

The *AtTZF2-3* genes showed the highest expression levels in seeds and senescent leaves, whereas a more specific expression in seeds was observed for *AtTZF1-4-5* (Supplementary Figure S2A). Similar behavior was seen for the wheat *TdTZF1-A* and *TdTZF1-B* genes (Supplementary Figure S2C). Then, we revealed a common feature of these genes: that they are regulated by abiotic stress. Clear overexpression was observed for *AtTZF1-3* in response to cold, salt, and osmotic stress, in both leaves and roots (Supplementary Figure S2B), whereas *TaTZF1*, *TdTZF1-A*, *TdTZF1-B*, and *TaTZF5* were all up-regulated by drought and cold stress (Supplementary Figure S2D,E).

To compare the expression profiles of these putative orthologous genes more closely, we analyzed the accumulation of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* transcripts in response to cold and salt stress, and during seed germination.

Expression Profiles of the *TdTZF1-A* and *TdTZF1-B* Genes and *Arabidopsis* Homologs under Cold-Stress Conditions

The expression profiles of *Arabidopsis AtTZF1-5* and the durum wheat genes *TdTZF1-A* and *TdTZF1-B* were investigated using RT-qPCR in 7-day-old seedlings exposed to low temperature for 1, 3, and 6 h (**Figure 2**). At 21°C, excluding *AtTZF4/SOM*, the *Arabidopsis* and durum wheat genes showed marked variations in transcript accumulation (up to 1.9-fold at 6 h) during the experimental time course, which suggested that their expression changed in response to light or the circadian cycle. For this reason, all of the experiments were initiated at the same time of day. The shift to lower temperature affected the expression profiles of all of the genes compared to controls, again except for *AtTZF4/SOM*. In particular, *AtTZF1* showed clear repression by low temperature (about 2.4-fold repression at 3 h); *AtTZF3* showed up-regulation in response to low temperatures at each time point, with a maximum of induction of 3.3-fold at 3 h; *AtTZF2* was up-regulated transiently (about 1.7-fold) 3 h from the beginning of the cold period. In contrast, *AtTZF5* showed the opposite behavior, as it was transiently down-regulated about twofold by the cold after 3 h of the low-temperature exposure.

Slight up-regulation during the cold treatment was observed for the durum wheat genes during the experimental time course. In particular, with the experimental set-up that was used here, both the *TdTZF1-A* and *TdTZF1-B* genes were transiently up-regulated about twofold by the cold treatment and then clearly down-regulated about 2.2-fold after 6 h of low-temperature exposure. However, neither of these two durum wheat genes showed clear overlapping expression profiles with any of the putative orthologous *Arabidopsis* genes.

Expression Profiles of the *Arabidopsis* Homologs and the *TdTZF1-A* and *TdTZF1-B* Genes under Salt-Stress Conditions

Based on the data from the *in silico* expression analysis, we then determined the expression profiles of *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* in response to salt stress. The analysis was performed with the *Arabidopsis* and durum wheat seedlings exposed to

[8]http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi
[9]http://www.plexdb.org/index.php

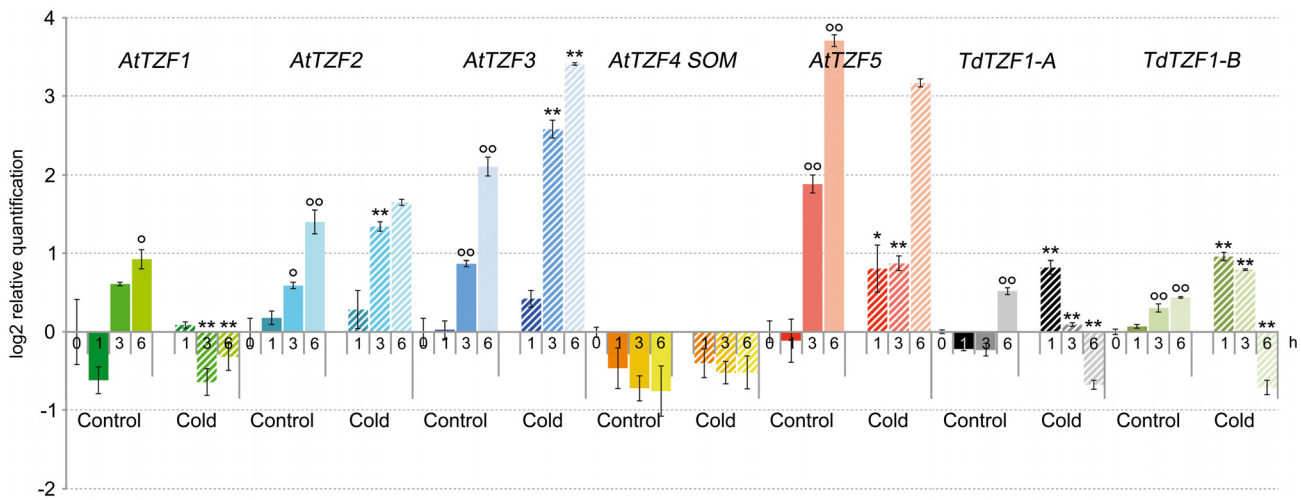


FIGURE 2 | Expression of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* genes under cold-stress conditions. *Arabidopsis thaliana* and *T. durum* seedlings were grown at 21°C and then maintained at the same temperature or subjected to cold stress for different times. The relative expression levels are shown for *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* under control conditions and upon cold stress. Relative mRNA expression levels were referred to the untreated controls (0 h). Data are means of relative quantification (Log2) of three biological replicates normalized to *AtACT2* or *Td_Polyubiquitin* for the *A. thaliana* and *T. durum* target genes, respectively (±SE). Statistical significance was assessed by one-way ANOVA analysis followed by Bonferroni's tests. °$P < 0.05$, °°$P < 0.01$ 1 h control, 3 h control, 6 h control vs 0 h control; *$P < 0.05$, **$P < 0.01$ 1 h cold vs 1 h control, 3 h cold vs 3 h cold, 6 h cold vs 6 h control.

different concentrations of NaCl (i.e., 0, 150, 250, 400 mM) for 1, 3, and 6 h (**Figure 3**). All of the analyzed genes showed rapid increases in their transcript levels in response to the salt stress, which were evident already at 1 h after the onset of the treatment. Clear dose-dependent regulation was seen in the responses to the increasing salt concentrations for all of these genes. However, *AtTZF2-4* showed a greater than twofold increases over the controls, which demonstrated stronger regulation with respect to *TdTZF1-A* and *TdTZF1-B*.

Expression Profiles of the *Arabidopsis* Homologs and the *TdTZF1-A* and *TdTZF1-B* Genes in Dry Seeds and during Germination

The analysis of gene expression conducted *in silico* (Supplementary Figure S2) suggested that the *RR-TZF* genes are actively transcribed in seeds. On the basis of these data, the expression profiles of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* genes were also investigated during the germination process (**Figure 4**). Interestingly, all of these genes showed very similar expression profiles. The steady-state levels of the transcripts in germinating seeds was always lower than in dry seeds, with the lowest levels reached at 6 h from the beginning of the germination process for *AtTZF2* (2.6-fold repression), *AtTZF1* (14-fold repression), and *AtTZF5* (1.5-fold repression), and at 12 h from the beginning of the germination process for *AtTZF3* (17.3-fold repression), *AtTZF4* (1.9-fold repression), *TdTZF1-A*, and *TdTZF1-B* (1.7- and 5.5-fold repression, respectively).

The effect of salt in the delaying of the germination process in both *Arabidopsis* and wheat is well known. Considering that in these seedlings the *RR-TZF* genes are regulated by salt (see **Figure 3**), we also analyzed their expression during the germination process in the presence of 150 mM NaCl. Interestingly, the decrease in the transcript levels of these *RR-TZF* genes during germination was lower in the presence of 150 mM NaCl than in water (**Figure 4**). In particular, *TdTZF1-A* and *TdTZF1-B* showed strong differences in their transcript accumulation after 12 h of salt treatment, with respect to the controls (about 1.8-fold), while all of the *Arabidopsis* genes showed differences between the NaCl-treated and untreated seeds even at 6 h after the beginning of the germination

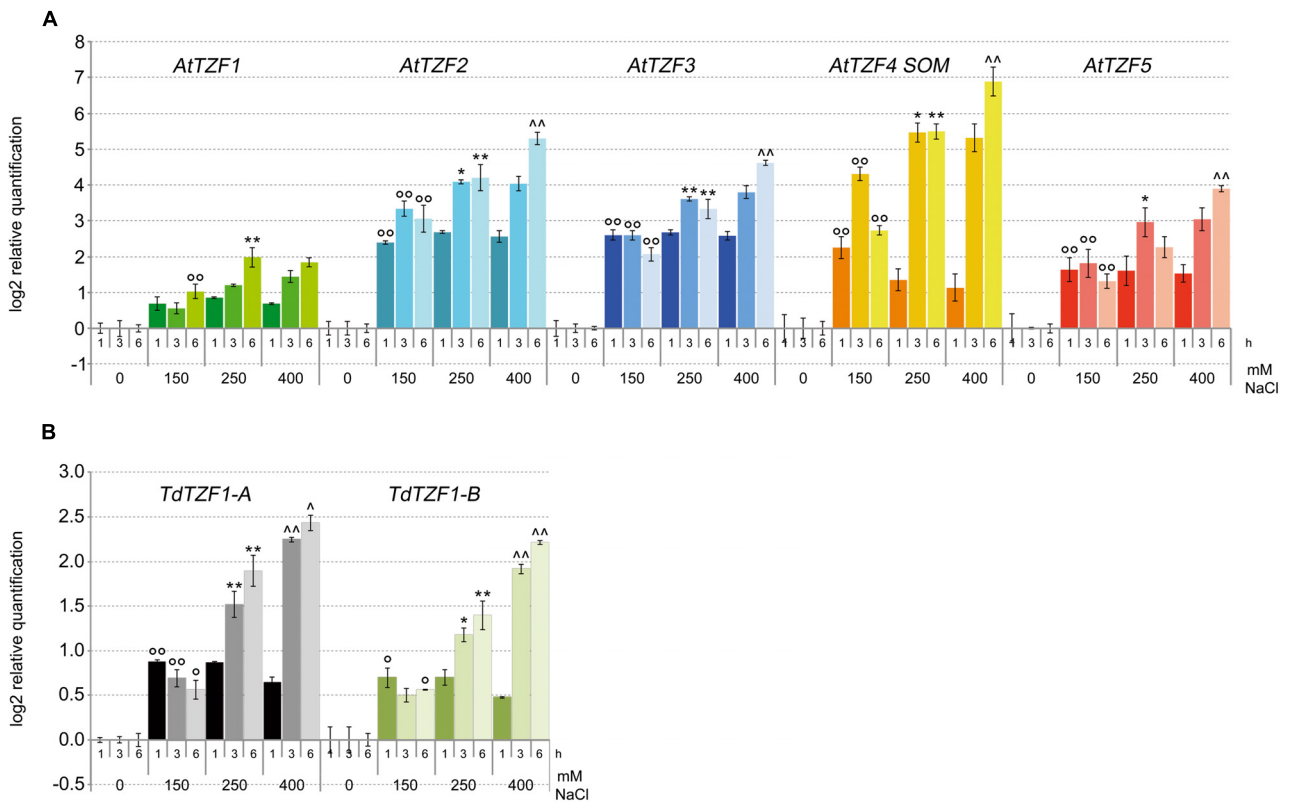


FIGURE 3 | Expression of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* genes under salt-stress conditions. *Arabidopsis* **(A)** and *T. durum* **(B)** seedlings were grown in the absence of salt, and then treated with no salt or 150, 250, or 400 mM NaCl for different times. The relative expression levels are shown for *AtTZF1-5* **(A)**, *TdTZF1-A*, and *TdTZF1-B* **(B)** under control conditions and upon exposure to salt stress for different times. The relative mRNA expression levels of each salt-treated sample were referred to the corresponding time point of the control experiment (no NaCl). Data are means of relative quantification (Log2) of two (*T. durum*) or three (*A. thaliana*) biological replicates normalized to *AtACT2* or *Td_Polyubiquitin* for the *A. thaliana* and *T. durum* target genes, respectively (±SE). Statistical significance was assessed by one-way ANOVA analysis followed by Bonferroni's tests. °$P < 0.05$, °°$P < 0.01$ 1 h 150 mM vs 1 h 0 mM, 3 h 150 mM vs 3 h 0 mM, 6 h 150 mM vs 6 h 0 mM; *$P < 0.05$, **$P < 0.01$ 1 h 250 mM vs 1 h 150 mM, 3 h 250 mM vs 3 h 150 mM, 6 h 250 mM vs 6 h 150 mM; ^$P < 0.05$, ^^$P < 0.01$ 1 h 400 mM vs 1 h 250 mM, 3 h 400 mM vs 3 h 250 mM, 6 h 400 mM vs 6 h 250 mM.

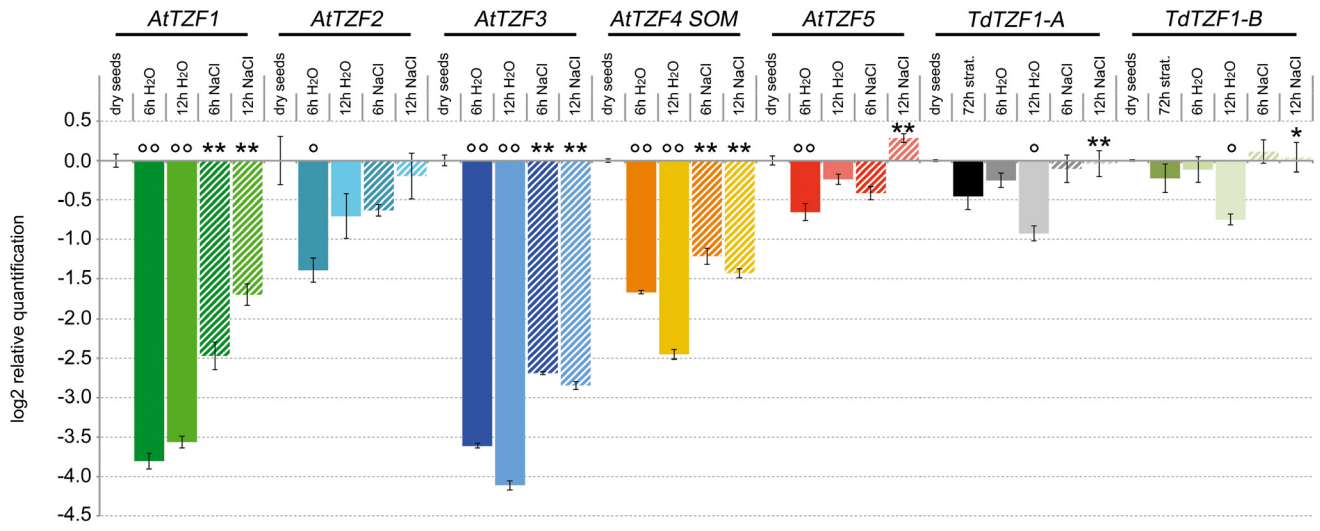


FIGURE 4 | Expression of the *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* genes during the germination process. *Arabidopsis* and *T. durum* seeds were germinated in water or in the presence of 150 mM NaCl. The relative expression levels are shown for *AtTZF1-5*, *TdTZF1-A*, and *TdTZF1-B* in germinating seeds in water and in NaCl solution after 6 h and 12 h from the onset of germination. The relative expression levels are also shown for *TdTZF1-A* and *TdTZF1-B* in stratified seeds. Relative mRNA expression levels were referred to the dry seeds. Data are means of relative quantification (Log2) of three biological replicates normalized to *AtTIP41-like* and *Td_17S1* for the *A. thaliana* and *T. durum* target genes, respectively (±SE). Statistical significance was assessed by one-way ANOVA analysis followed by Bonferroni's tests. °$P < 0.05$, °°$P < 0.01$ 72 h strat., 6 h H_2O, 12 h H_2O *vs* dry seeds; *$P < 0.05$, **$P < 0.01$ 6 h NaCl vs 6 h H_2O, 12 h NaCl vs 12 h H_2O.

process (**Figure 4**). In analogy with the role of light, which promotes germination by repressing the negative regulation of *AtTZF4*/*SOM* on this process, these data suggested that salt increases the expression of *AtTZF1-5*, which results in a delay of seed germination.

AtTZF3 is a Negative Regulator of Seed Germination in the Presence of NaCl and Abscisic Acid

The results of the expression analysis shown in **Figure 4** suggested that the *Arabidopsis* AtTZF1-5 proteins and their homologs TdTZF1-A and TdTZF1-B have redundant actions as negative regulators of seed germination in the presence of salt. To test this hypothesis, we generated *Arabidopsis* lines that expressed either lower or higher levels of the *AtTZF3* gene.

Two *AtTZF3* knocked-down lines showed relevant reduction in the *AtTZF3* transcript (by up to 90%; data not shown): pK35.4 (ihpRNA), and pB23.3 (amiRNA). Two *AtTZF3* overexpressing lines showed high levels of *AtTZF3* mRNA (about 200-fold and 400-fold higher, respectively, compared with wild-type; data not shown): FD7.9.2 and FD7.14.4. These were assessed for their germination under normal conditions and upon treatment with NaCl and ABA. When these seeds were germinated with water, there were no differences in the germination rates between the Col-0 seeds and the transgenic lines with altered levels of *AtTZF3* expression (data not shown). Interestingly, completely different behavior was shown by these transgenic lines when NaCl was present in the growth medium. Indeed, the *AtTZF3* attenuated (i.e., pK35.4, pB23.3) and the *AtTZF3* overexpressing (i.e., FD7.9.2, FD7.14.4) lines showed opposite responses to salt, as more tolerant and more sensitive, respectively (**Figure 5A**).

Based on the consideration of the negative role of ABA in the germination process, we next tested the sensitivity of the transgenic lines to ABA. In analogy with NaCl, in terms of the germination rates, the attenuated lines were less sensitive and the overexpressing lines were more sensitive to 1 mM ABA treatment (**Figure 5B**). These germination trials in the presence of NaCl and ABA were replicated several times using different batches of seeds, and similar data were obtained (data not shown).

Taken together, these data suggested that *AtTZF3* is involved in NaCl-mediated and ABA-mediated regulation of seed germination.

Identification of RR-TZF Proteins in Other Plant Species

Although many *RR-TZF* genes have been described, a complete survey and classification of all of the *RR-TZF* genes in plant species from disparate evolutionary groups is lacking. The completion of several high-quality plant-genome sequencing projects provided us with the unique opportunity to carry out a complete assessment and thorough comparative analysis of the plant RR-TZF proteins. As shown in **Figure 1**, the entire RR-TZF region is very well conserved between the *Arabidopsis* and wheat RR-TZF proteins. However, the conservation is particularly high both in the region upstream of the CCCH domains and in the CCCH domains themselves. Therefore, to establish the evolutionary conservation of this gene family across the plant kingdom, we used the AtTZF3 sequence IDAYSCDHFRMYDFKVRRCARGRSHDWTECPYAH, which

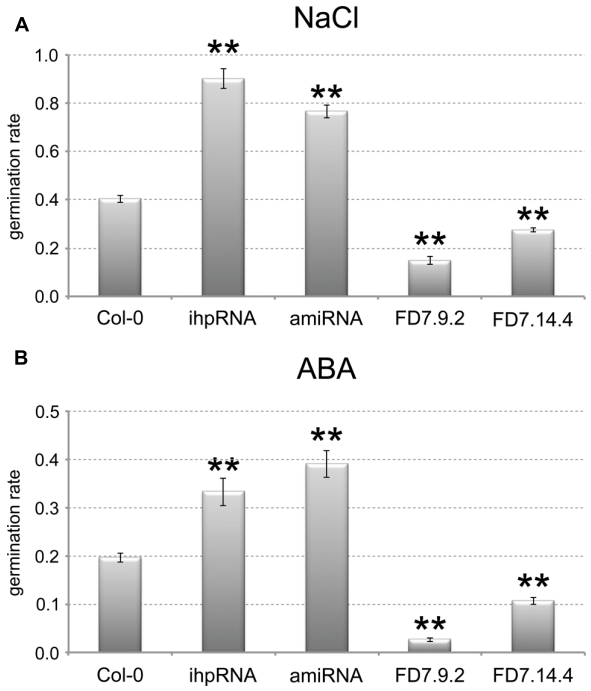


FIGURE 5 | AtTZF3 is a negative regulator of seed germination. The germination rates are shown for Col-0, for two attenuated lines for AtTZF3 (ihpRNA, amiRNA), and for two independent lines overexpressing AtTZF3 (FD7.9.2, FD7.14.4) in the presence of 150 mM NaCl **(A)** or 1 μM ABA **(B)**. Data are means of three biological replicates (±SD), each one conducted with 50–100 seeds per genotype. Statistical significance was determined by one-way ANOVA analysis followed by Dunnett's tests. $^{*}P < 0.05$, $^{**}P < 0.01$ ihpRNA, amiRNA, FD7.9.2 and FD7.14.4 vs Col-0.

includes the CHCH motif and some upstream amino acids, as the query in BLASTP searches to find the most similar sequences from several plant species. The sequences obtained were filtered using the presence of both the CHCH motif and the TZF CCCH domains. Through this, we defined 461 RR-TZF sequences, including several in lower plants and algae. The few proteins that showed high similarity with the query sequence but that lacked one or more of the requirements (i.e., the CHCH motif or the CCCH domain) were collected separately (Supplementary Table S3). These proteins are likely to have diverse or compromised functionality and/or to be encoded by pseudogenes, and they were found in *Coccomyxa subellipsoidea, P. patens, Capsella grandiflora, Amborella trichopoda, Glycine max, Zea mays, Malus domestica, Medicago truncatula*, and *Solanum lycopersicum*. The species analyzed and all of the sequences found are listed in Supplementary Tables S2 and S4, where the proteins are named according to the TZF nomenclature, with the indication of the structure and sequence of the CCCH domains and CHCH motifs, and the presence and type of ANK repeats.

For the first CCCH domain, in addition to the proteins with the conventional $C-X_{7-8}-C-X_5-C-X_3-H$ structure, several proteins that showed a more variable structure were found (i.e., altered spacing between the first and second Cys) (Supplementary Table S2). Several new sequences with a diverging CCCH domain were also identified in species in which the RR-TZF proteins have already been described, such as for *Z. mays*, *M. truncatula*, and *S. lycopersicum* (e.g., ZmTZF13, MtTZF6-14, SlyTZF9-12). In particular, ZmTZF13 contains 20 amino-acid residues between the first and the second Cys of the first CCCH domain. Although the amino-acid sequence $C-X_{20}-C-X_5-C-X_3-H$ would not be classified as a CCCH domain based on the definition by Wang et al. (2008), the high homology with the other RR-TZF proteins suggests that ZmTZF13 is indeed a member of the RR-TZF family. Therefore, the first CCCH domain might be better defined as the following more general structure: $C-X_{5-20}-C-X_5-C-X_3-H$. The $C-X_9-C-X_5-C-X_3-H$ domains were found across proteins of several lineages, such as for monocotyledons (e.g., *T. aestivum*), dicotyledons (e.g., *A. lyrata, Capsella rubella, Linum usitatissimum*) and gymnosperms (e.g., *P. abies*, *P. glauca*) (Supplementary Table S2). The $C-X_5-C-X_5-C-X_3-H$ domains were found only in algal proteins (e.g., *Chlamydomonas reinhardtii*, *Volvox carteri*), whereas the $C-X_{10-20}-C-X_5-C-X_3-H$ domains were exclusively found in monocotyledon proteins (e.g., *Brachypodium distachyon, Oryza sativa, Panicum hallii, Panicum virgatum, Sorghum bicolor, T. aestivum*, *Z. mays*). The second CCCH domain, which was typically $C-X_5-C-X_4-C-X_3-H$, is highly conserved in structure among all of the proteins, with the exception of two soybean (i.e., GmTZF17, GmTZF18) and two apple (i.e., MdTZF4, MdTZF16) proteins that are characterized by variant motifs (i.e., $C-X_4-C-X_4-C-X_3-H$, $C-X_7-C-X_4-C-X_3-H$, respectively).

Based on the sequence homology, we constructed a dendrogram that defined five distinct clusters of RR-TZF proteins (Supplementary Figure S3A). The largest group (i.e., RR-TZF I) corresponds to the proteins that are homologous to AtTZF7-11, and these are characterized by the presence of ANK repeats. The second group (i.e., RR-TZF II) contains the RR-TZF proteins that are highly homologous to AtTZF1-5; these proteins do not have ANK domains and they are relatively small (250 amino acids, on average). The third group (i.e., RR-TZF III) consists of proteins that are homologous to AtTZF6. Although these proteins are structurally similar to those of the AtTZF1-5 group, their amino-acid sequences are sufficiently different to form a separate group. Then the fourth group (i.e., RR-TZF IV) consists of proteins that are encoded by the gymnosperms and Solanum, and finally the fifth group (i.e., RR-TZF V) consists of proteins from monocotyledons (characterized by a larger spacing between the first and second Cys residues of the first CCCH domain) and algae. Interestingly, the multiple sequence alignments of the full-length RR-TZF proteins belonging to these five groups highlighted the presence of distinctive invariant residues in the RR-TZF region of all of the plant proteins (i.e., for monocotyledons, eudicotyledons, *Selaginella*, and *Physcomitrella*, and even among the unicellular green algae). Supplementary Figure S3B shows a schematic representation of the sequence conservation of the amino-acid residues, and the consensus sequence of the RR-TZF region. In addition, it is worth noting that the presence of several invariant amino-acid residues resulted in a signature that

uniquely identifies the plant RR-TZF proteins (Supplementary Figure S3B).

Identification of Genes Orthologous to *AtTZF1-3*, *TdTZF1-A*, and *TdTZF1-B*

To search for the *AtTZF1-3*, *TdTZF1-A*, and *TdTZF1-B* orthologs, we performed a comparative analysis according to a sequence-homology-based approach (see Materials and Methods). Here, we identified only two subgroups: the first subgroup (i.e., RR-TZF IIa) includes 117 AtTZF1-2-3-like sequences, and the second subgroup (i.e., RR-TZF IIb) consists of 49 AtTZF4-5-like proteins (Supplementary Table S5).

The AtTZF1-2-3-like proteins were found at all levels of the evolutionary scale, which included the green algae *C. subellipsoidea C-169* (CsTZF1), which thus suggested that their origin is very ancient. Moreover, even if their sequences show low similarity to the AtTZF1-2-3 proteins, some other RR-TZF proteins in green algae (e.g., CreTZF6, VcTZF6) might be orthologous to proteins of the RR-TZF IIa subgroup, as they share some specific amino-acid residues with them in the RR-TZF region (data not shown). The AtTZF4-5-like proteins were found only in angiosperm dicotyledon plants and in *A. trichopoda*, which is considered as the most primitive angiosperm, but not in monocotyledons. This indicates that their origin is more recent and that they are lineage-specific proteins.

To investigate the conservation and divergence of the RR-TZF IIa and RR-TZF IIb protein subgroups, we constructed relative multiple sequence alignments and carried out comparisons. Within the RR-TZF region, the same conserved amino-acid positions seen in **Figure 1** were observed, which confirmed that these amino acids are specific for the respective groups and are evolutionarily conserved; the frequencies of the amino acids at each position are detailed in Supplementary Table S6. Among the conserved amino-acid residues, we focused our attention on the Cys (**Figure 6**, blue diamond) at position -12 from the first Cys of the CHCH motif. Most of the RR-TZF IIa proteins (84%) have this additional Cys. With the exception of a few cases, i.e., some angiosperms (e.g., *T. urartu*, *A. halleri*, *Carica papaya*, *Ricinus communis*) and all of the algal proteins, the proteins with this additional Cys were all in species that are further along the evolutionary scale.

These protein alignments also showed that some small regions that are up-stream and down-stream of the RR-TZF motif are conserved (Supplementary Figures S4 and S5). In the N-terminal region, there was the conservation of an IPP motif, which in the RR-TZF IIb proteins was followed by RKLL, and in the RR-TZF IIa proteins, by W. This W residue is present only in angiosperms, monocotyledons and dicotyledons, and not in other species (data not shown). In addition, the AtTZF1-2-3-like proteins contained another conserved short motif, RYLP, which is not found in the AtTZF4-5-like proteins. In the C-terminal region, the AtTZF1-2-3-like and AtTZF4-5-like proteins share some common motifs, such as SP-rich regions, and the sequence PDVGWVSELV/DPDLGWVNDLL, which is highly conserved. Finally, an EE—PAMER-VESGRDLR motif is evolutionarily

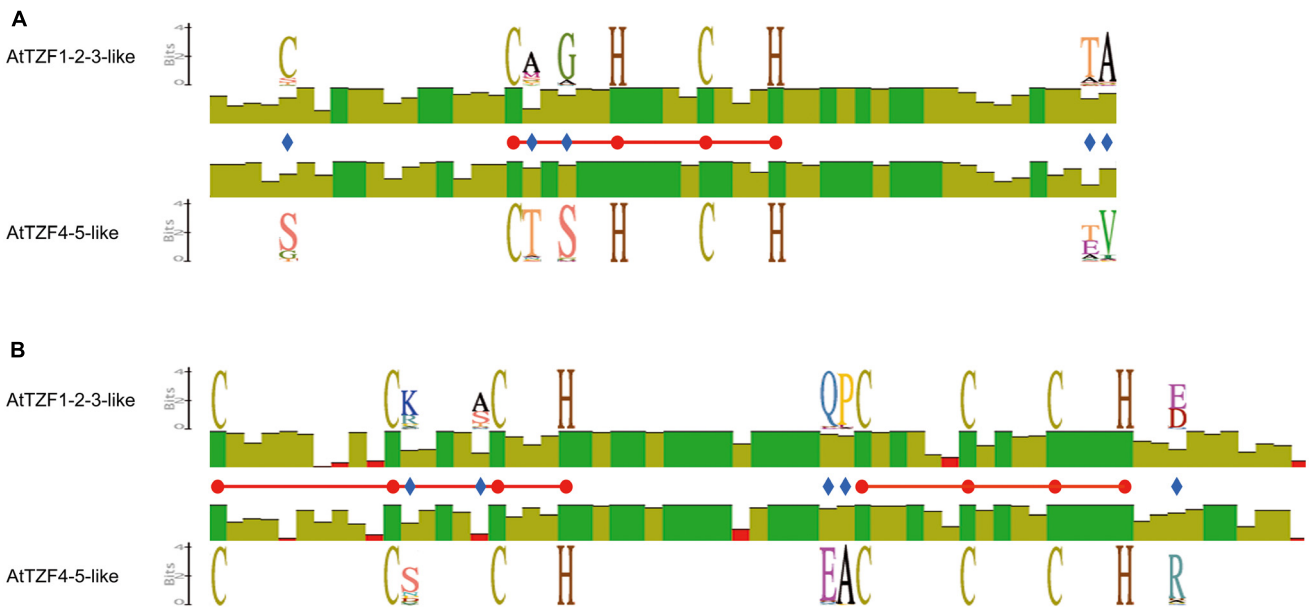


FIGURE 6 | Schematic representation of the conserved amino acids in the AtTZF1-2-3-like and AtTZF4-5-like proteins. The scheme shows identity levels (histograms) at each amino-acid position, the sequence logo of invariant but specific positions (blue diamonds) in the AtTZF1-2-3-like and the AtTZF4-5-like proteins and the CHCH motif **(A)** and CCCH domain **(B)** amino acids (red balls, horizontal bars). Data were extracted from multi-alignments of the AtTZF1-2-3-like or AtTZF4-5-like full-length amino-acid sequences, constructed using the Clustal W algorithm with the Geneious software (version 5.5.3).

conserved in the RR-TZF IIa proteins (Supplementary Figure S4), while there is a CCLFC motif in the RR-TZF IIb proteins (Supplementary Figure S5).

Phylogenetic Analysis of the RR-TZF Group II Proteins

To reveal the evolutionary relationships of the RR-TZF group II proteins, a neighbor-joining tree was constructed based on the alignment of the full-length amino-acid sequences of some selected plant species that are representative of each level of the evolutionary scale: *A. thaliana, O. sativa, T. durum, Spirodela polyrhiza, A. trichopoda, P. abies, Selaginella moellendorffii, P. patens*, and *C. reinhardtii* (**Figure 7**).

The CreTZF6 protein is located on the outer branch of the tree, which highlights the great phylogenetic distance between the algae and the land species. The other sequences can be divided into two groups, one that includes AtTZF4-5 and their *A. trichopoda* ortholog (AtrTZF4). As already indicated in the previous analysis, the AtTZF4-5 proteins appear not to have any orthologs in either monocotyledons or in non-angiosperm species, which indicates their recent origin and lineage specificity in dicotyledons. The presence of AtTZF4-5 in *A. trichopoda* suggests that this gene already existed in the ancestor of the angiosperms, but only in the dicotyledons has it been maintained, while in the monocotyledons it has been lost. The other group consists of 19 proteins, and it includes the AtTZF1-2-3 proteins and their relative orthologs that are present in all of the species analyzed, with the exception of algae. Moreover, clear separation is evident (which is statistically well supported) between the angiosperm and non-angiosperm (i.e., gymnosperm, bryophyte) proteins. Within the angiosperm group, the *T. durum* sequences are more similar to the *O. sativa* proteins, which is consistent with the evolutionary relationship between these species. Moreover, it is of note that despite it being a monocotyledon, the sequences of *S. polyrhiza* are closer to those of *Arabidopsis*, as compared to other monocotyledons. Within the non-angiosperm group, all of the *P. patens* sequences are highly related, so as to form a specific and well-supported group. The other sequences (e.g., *S. moellendorffii*, *P. abies*) are not distributed in a manner consistent with their phylogenetic relationships, which suggests that lycophytes and gymnosperms have undergone distinct and separate evolution.

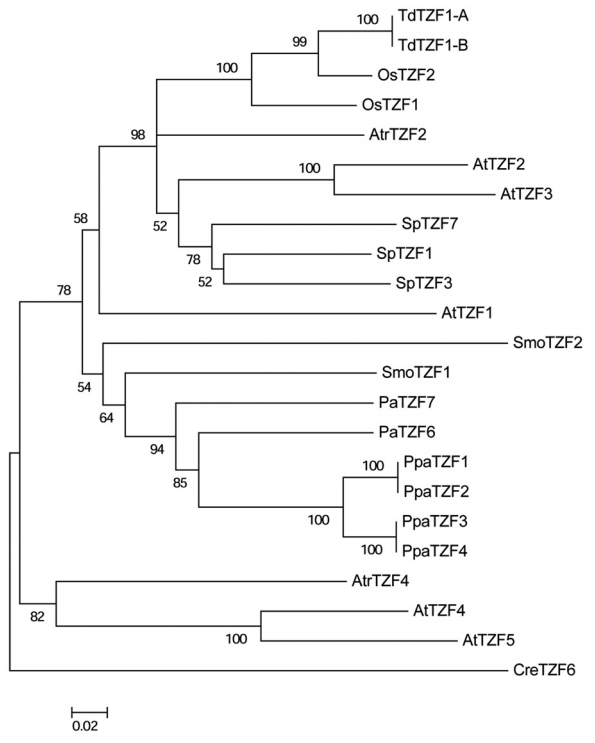


FIGURE 7 | Phylogenetic tree of the AtTZF1-2-3-4-5-like proteins from selected species. The unrooted tree was constructed using the neighbor-joining method after alignment of the full-length amino-acid sequences using the Clustal W algorithm. Bootstrap values from 1,000 replicates are indicated at each node, and only bootstrap values higher than 50% from 1,000 replicates are shown. Scale bar: estimated 0.2 amino-acid substitutions per site.

Expression Profiles of *RR-TZF Physcomitrella* Genes under Salt-Stress Conditions

Previous studies have shown that *P. patens* can survive severe dehydration, high salinity, low temperature, and high osmotic stress (Frank et al., 2005; Saavedra et al., 2006; Charron and Quatrano, 2009). To investigate whether the *RR-TZF Physcomitrella* genes have conserved responses to salt stress, we analyzed their expression profiles in response to NaCl. The *PpaTZF1* and *PpaTZF2* genes are identical, and the *PpaTZF3* and *PpaTZF4* genes are nearly identical. Therefore, only two distinct assays for the qPCR analysis were developed here, one that amplified the *PpaTZF1-2* pair, and the other that amplified the *PpaTZF3-4* pair. In analogy with the experiments performed in *Arabidopsis* and durum wheat (see **Figure 3**), the *Physcomitrella* protonemata were treated with different concentrations of NaCl (i.e., 0, 150, 250, 400 mM) for 1, 3, and 6 h (**Figure 8**). Very interestingly, both pairs of *Physcomitrella* genes showed clear dose-dependent up-regulation in response to the salt. At the lowest NaCl concentration (150 mM), *PpaTZF1-2* was slightly induced 6 h after the salt exposure. At 250 mM NaCl, the *PpaTZF1-2* transcript levels were about 1.4-fold higher than those observed under control conditions 3 h after this salt exposure. At the highest salt concentration (400 mM NaCl), induction of the *PpaTZF1-2* genes was transient, with the maximum transcript levels reached after 3 h of salt treatment (about 1.7-fold induction). Similar behavior was shown by the *PpaTZF3-4* pair, which overall appeared to be slightly more inducible than the *PpaTZF1-2* pair.

In conclusion, the expression profiles here indicated clear responses to salt for the *RR-TZF Physcomitrella* genes, which were similar to those of the durum wheat *TdTZF1-A* and *TdTZF1-B* genes, and of the *Arabidopsis* group II *RR-TZF* genes. This thus suggests a conserved function of these proteins across these evolutionarily distant plant organisms.

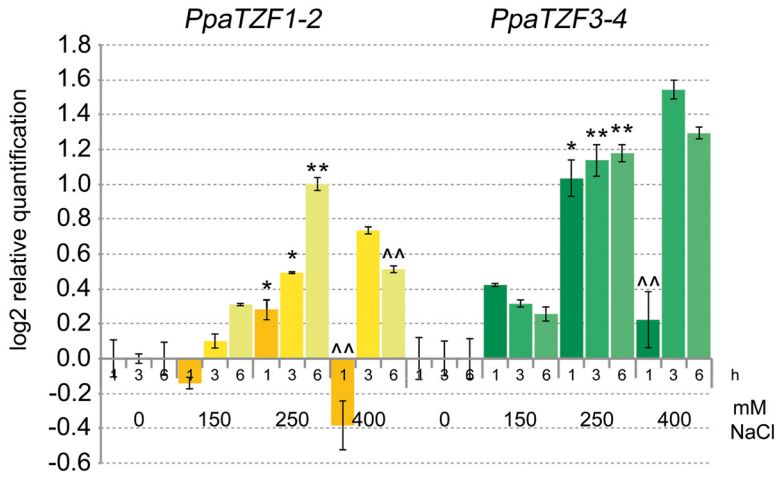


FIGURE 8 | Expression analysis of the *PpaTZF* genes under salt-stress conditions. Protonemata were grown for 6 days on control medium, and then either transferred onto the same medium or onto medium containing 150, 250, or 400 mM NaCl, for different times. The relative expression levels are shown for *PpaTZF1-2* and *PpaTZF3-4* under control conditions and upon exposure to salt stress for 1, 3, and 6 h. The relative mRNA expression levels of each salt-treated sample were referred to the corresponding time point of the control experiment (no NaCl). Data are means of relative quantification (Log2) of two biological replicates normalized to *PpaEF1a* (±SE). Statistical significance was assessed by one-way ANOVA analysis followed by Bonferroni's tests. °$P < 0.05$, °°$P < 0.01$ 1 h 150 mM vs 1 h 0 mM, 3 h 150 mM vs 3 h 0 mM, 6 h 150 mM vs 6 h 0 mM; *$P < 0.05$, **$P < 0.01$ 1 h 250 mM vs 1 h 150 mM, 3 h 250 mM vs 3 h 150 mM, 6 h 250 mM vs 6 h 150 mM; ^$P < 0.05$, ^^$P < 0.01$ 1 h 400 mM vs 1 h 250 mM, 3 h 400 mM vs 3 h 250 mM, 6 h 400 mM vs 6 h 250 mM.

Discussion

A number of *RR-TZF* genes have been identified previously in higher plants, and their products have been shown to have important roles in the regulation of some developmental processes and adaptive responses to abiotic stress, such as to cold, salt and drought (Wang et al., 2008; Chai et al., 2012; Lee et al., 2012). In the present study, we characterized two durum wheat *RR-TZF* genes, *TdTZF1-A*, and *TdTZF1-B*, which are highly homologous to *AtTZF2* and *AtTZF3*. Using a short conserved peptide sequence derived from the RR-region, we identified 461 putative RR-TZF plant proteins that share the unique signature of $KX_3CX_5HX_4CX_3HX_6RRX_6YX_4CX_{7-8}CX_5CX_3HX_2FEX_3HPX_7CX_5CX_4CFFAH$, which includes a highly conserved zinc-finger CCCH domain. Remarkably, *RR-TZF* genes were found in all levels of the evolutionary scale, including the green algae *Coccomyxa subellipsoidea C-169*, indicating the evolutionary origin of these genes in a common ancestor of green algae and land plants. Based on our homology analysis, we also divided the RR-TFZ family of proteins into five different groups, as RR-TZF I-V, and TdTZF1-A and TdTZF1-B belong to the second of these groups, RR-TZF II.

The RR-TZF II Group

The RR-TZF II group is formed by 168 proteins that are encoded by all of the plant genomes sequenced to date, with the only exception of some green algae. This suggests that the evolutionary origin of the RR-TZF II group is also very ancient, and is likely to have been before the divergence of the algae and the land plants. However, the homology analysis indicated that this group can be further divided in two subgroups: RR-TZF IIa and RR-TZF IIb. The first of these subgroups includes the AtTZF1-3-like proteins, and the second subgroup includes proteins that are homologous to AtTZF4/SOM and AtTZF5. Most of the RR-TZF IIa proteins (84%), including the TdTZF1-A, TdTZF1-B, AtTZF2-3, and *P. patens* proteins, have an additional Cys amino acid that is nine residues upstream of the invariant Lys of the RR-TZF signature (see **Figure 6**). The conservation of this amino-acid residue suggests that it might have a specific role in protein folding and/or protein-protein interactions; e.g., through disulfide bonding. As an alternative, this additional Cys might be involved in the formation of an atypical CCCH domain, as $C-X_{12}-C-X_{10}-C-X_3-H$, which partially overlaps with the CHCH motif, as suggested by Huang et al. (2011).

Evolutionarily Conserved Regulation for the RR-TZF IIa Proteins

We and others have shown that several genes belonging to the RR-TZF II group, which include the *P. patens* genes described here, respond to salt stress (Sun et al., 2007; Jan et al., 2013; Han et al., 2014; Wang et al., 2014; Zhou et al., 2014). The conservation of the gene expression response to salt stress suggests a function for the RR-TZF II proteins in assisting plants to cope with environmental stress, a common challenge to phylogenetically distant plant species. Interestingly, we observed very similar expression patterns for the *Arabidopsis* (i.e., *AtTZF2*, *AtTZF3*) and durum wheat (i.e., *TdTZF1-A*, *TdTZF1-B*) genes during the germination process in the presence of NaCl. The conservation of salt-stress regulation during germination is intriguing considering the physiological diversity of the process across these two species, due to the different morpho-physiological features of *Arabidopsis* and durum wheat seeds. Remarkably, the attenuation and overexpression of *AtTZF3* in plants showed altered germination ability exclusively in the

presence of NaCl and ABA, which thus suggests that the activity of the AtTZF3 protein is dependent on salt-induced stress and/or on the ABA levels. Previous studies have provided evidence for roles of the other *Arabidopsis RR-TZF II* genes in the germination process; however, in those cases, the germination appeared to correlate inversely to the level of expression of the selected genes. For instance, the concurrent suppression of *AtTZF1-3* by RNAi was characterized by early germination and relatively stress-sensitive phenotypes. Also, rice plants overexpressing *OsTZF1* showed delayed seed germination and growth delay at the seedling stage, whereas RNAi knock-down *OsTZF1* lines showed early seed germination and enhanced seedling growth (Jan et al., 2013). Similarly, knock-out mutants of *AtTZF4/SOM*, *AtTZF5*, or *AtTZF6* showed early germination, whereas the plants overexpressing these genes showed late germination, compared to the wild-type (Bogamuwa and Jang, 2013).

A role for the RR-TZF II proteins in salt-stress responses might have arisen very early during plant evolution. Later, in higher plants, the RR-TZF II proteins might have acquired new regulatory functions that are connected to the germination process. However, ABA represents a common factor that links together salt stress and germination. ABA has an ancient origin and conserved functions in the life of plants, as it affects growth and differentiation, increases dehydration stress tolerance in bryophytes, and induces accumulation of soluble sugars (such as compatible solutes) in association with enhancement of freezing tolerance in *P. patens* protonemata (Nagao et al., 2005, 2006). The core network that mediates ABA signaling is evolutionarily conserved between *P. patens* and angiosperms (Takezawa et al., 2011), and the mechanisms of regulation of ABA-mediated salt-stress responses are very similar in *P. patens* and in seed plants (Richardt et al., 2010). In particular, Richardt et al. (2010) demonstrated that the *P. patens* homologs of *Arabidopsis* genes have key roles in the regulation of processes like flowering and seed development. In light of these data, our results suggest that the regulation of *RR-TZF II* gene expression during germination might have evolved from the pre-existing pathway(s) that regulate ABA-mediated responses to salt stress. This hypothesis is also supported by the conservation of certain regulatory elements that are linked to the responses to ABA and to abiotic stress, and that are located within the promoters of the *AtTZF1-5* genes and their homologs in durum wheat and *P. patens* (data not shown). As an example, the ABRERATCAL element, which is an ABA-responsive element (Finkler et al., 2007), is present in the promoters of the *AtTZF1-4*, *TdTZF1-B*, and *P. patens RR-TZF* genes.

Expansion the RR-TZF II Genes in Dicotyledons

The level of sequence similarity and the physical position of the *TdTZF1-A* and *TdTZF1-B* genes on chromosomes 3A and 3B, respectively, suggest that these two genes are homologous in the durum wheat genome. As *TdTZF1-A* and *TdTZF1-B* are characterized by similar expression profiles, this strongly suggests a conserved function for these two genes in the diploid progenitors of durum wheat that was retained after the polyploidization event, probably because of their importance in stress responses and seed germination. A similar explanation can be proposed for the *RR-TZF-IIa* genes in *Arabidopsis*, which have highly conserved expression profiles across duplicated genes. This is consistent with the 'functional buffering' hypothesis that stipulates that genes with crucial functions tend to be retained to ensure that the essential functions can be carried out in the event of inactivation of one of the duplicates (Chapman et al., 2006). In contrast, the *RR-TZF-IIb* group is lacking in monocots and non-angiosperm species, which indicates a recent origin and lineage specificity in dicot plants. However, the occurrence of one *RR-TZF-IIb* gene in *A. trichopoda*, which is considered to be the most primitive angiosperm plant, suggests that genes related to the *RR-TZF-IIb* group might have been lost during the evolution of monocot plants.

Among these *RR-TZF-IIb* genes there is *AtTZF4*/*SOM*, for which a role as a negative regulator of phytochrome-dependent seed germination has been established. Although *Arabidopsis* shares some dormancy pathway components with cereals (e.g., ABI3, VP1), there are also major differences in terms of the responses to light, as well as to other environmental cues (Simpson, 1990; Goggin et al., 2008; Gubler et al., 2008). Interestingly, however, a model for temperate cereals, *Brachipodium distachyon*, shows phytochrome regulation of seed dormancy and germination (Barrero et al., 2011), even though it lacks any of the *RR-TZF-IIb* genes.

Altogether, these data suggest that the expansion of the *RR-TZF-II* genes might have been related to the divergence between the monocot and dicot lineages, rather than to a specialized functional response to light stimuli. Among other possibilities, it appears reasonable to hypothesize that once the *AtTZF4* gene acquired phytochrome-mediated control, its product became a crucial factor in light-regulated germination, because of its intrinsic function as a negative regulator of this process, which is a feature that it shares with other members of the *RR-TZF-II* family.

Conclusion

The present study provides a complete survey and classification of the *RR-TZF* genes in plants for which the genomic information is available. This analysis has allowed us to compile a detailed description of the structural features of the RR-TZF motifs in relation to the evolution of this gene family. The strong conservation in terms of their sequence and structural features that is shown here across phylogenetically distant species, together with the similar expression profiles of the *RR-TZF* genes in response to salt stress in *Arabidopsis*, durum wheat, and the moss *P. patens*, have allowed us to hypothesize that these genes emerged as key regulators of stress responses very early during plant evolution.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00394/abstract

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Selection and Validation of Housekeeping Genes as Reference for Gene Expression Studies in Pigeonpea (*Cajanus cajan*) under Heat and Salt Stress Conditions

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To identify stable housekeeping genes as a reference for expression analysis under heat and salt stress conditions in pigeonpea, the relative expression variation for 10 commonly used housekeeping genes (*EF1α, UBQ10, GAPDH, 18Sr RNA, 25Sr RNA, TUB6, ACT1, IF4α, UBC, and HSP90*) was studied in root, stem, and leaves tissues of Asha (ICPL 87119), a leading pigeonpea variety. Three statistical algorithms geNorm, NormFinder, and BestKeeper were used to define the stability of candidate genes. Under heat stress, *UBC, HSP90,* and *GAPDH* were found to be the most stable reference genes. In the case of salinity stress, *GAPDH* followed by *UBC* and *HSP90* were identified to be the most stable reference genes. Subsequently, the above identified genes were validated using qRT-PCR based gene expression analysis of two universal stress-resposive genes namely *uspA* and *uspB*. The relative quantification of these two genes varied according to the internal controls (most stable, least stable, and combination of most stable and least stable housekeeping genes) and thus confirmed the choice as well as validation of internal controls in such experiments. The identified and validated housekeeping genes will facilitate gene expression studies under heat and salt stress conditions in pigeonpea.

Keywords: heat stress, salt stress, quantitative real-time PCR, housekeeping genes

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INTRODUCTION

Pigeonpea, one of the major food legume of tropic and sub-tropic, encounters various abiotic stresses during its life cycle (Varshney et al., 2012). As a rain-fed growing crop, among different abiotic stresses, moisture stress is more prevalent during various stages of the life cycle in pigeonpea (Choudhary et al., 2011). For instance, in the north-western part of India, extreme high temperature (heat stress) during reproductive stage had hampered the crop, leading to severe yield loss (Choudhary et al., 2011). Similarly, accumulation of the excess amount of salt in the soil surface is very harmful and could result in the damage in plant growth by interfering with the mineral nutrient uptake (Chikelu et al., 2007). It has also been shown that higher salt concentration

reduced important agronomic traits like, plant height, leaf area, crop growth rate, total dry matter, net assimilation rate, and seed yield, etc. (Joshi and Nimbalkar, 1983).

In view of above, there is a demand of development of high yielding and multiple stresses resistant varieties in pigoenpea (Pazhamala et al., 2015). Availability of draft genome sequence has opened an unprecedented opportunity to investigate the genetic basis of abiotic stress resistance in pigeonpea (Varshney et al., 2012). For instance, it has become possible now to identify candidate genes either by mining directly from the pigeonpea genome or identifying the homo-/ortho-logous genes for the candidate genes identified in other crop species. qRT-PCR is one of the most robust and reliable techniques of gene expression studues. For an accurate measurement and reproducible expression profiling of target genes in qRT-PCR analysis, use of stable housekeeping genes, also called as 'internal control' is essential to normalize the expression level. Housekeeping genes work for the basic cellular and metabolic functions and maintains the stable and constitutive expression throughout, irrespective of any external physiological conditions (Yang et al., 2014). However, several reports available across species stated that the expressions of housekeeping genes may vary depending on different external factors (Greer et al., 2010; Liu et al., 2012; Duhoux and Délye, 2013). The selection of a suitable housekeeping genes to normalize the expression level is a challenging task and requires extensive study to get an accurate result (Wang et al., 2015). For instance, expression of commonly used reference genes, i.e., *ACT1* and *GAPDH* has been found varying across different tissues, developmental stages, and different experimental conditions (Fischer et al., 2005; Goossens et al., 2005; Brinkhof et al., 2006; Sinha et al., 2015). Therefore, for consistent and reliable results, housekeeping genes should be chosen and validated prudently (Wang et al., 2015). Additionally, a combination of multiple numbers of reference genes will give more precision, where the geometrical mean of multiple internal controls will minimize the expressional variation (Vandesompele et al., 2002). In the case of pigeonpea, *IF4α* and *TUB6* genes had recently been identified as stable housekeeping genes for undertaking gene expression studies under drought stress conditions in pigeonpea (Sinha et al., 2015).

Keeping in view of above, the present study reports identification of the most stable gene(s) for gene expression studies under heat and salt stress conditions. These genes are expected to accelerate gene expression studies especially for heat and salt stresses in pigeonpea.

MATERIALS AND METHODS

Plant Material and Growth Conditions

For the gene expression analysis, ICPL 87119 (Asha), a medium duration, high yielding variety was selected. Genetically pure seeds, developed by crossing C11 × ICP1-6-W3/W, were collected from Pigeonpea Breeding Division, ICRISAT, Patancheru. Seeds were surface sterilized with sodium hypochlorite, thoroughly washed with DEPC treated water and pre-soaked overnight. Germinated seedlings were sown in a three inch plastic pots (one per pot) filled with autoclaved black soil, sand, and vermicompost (10:10:1 v/v) mixture. Fresh root, shoot and leaf tissues were harvested from all the pots, immediately frozen in liquid nitrogen and stored in -80 deep freezer till RNA isolation.

Heat and Salt Stress Treatments

For heat stress, 45-days-old (vegetative stage) and 75-days-old-plants (reproductive stage) were transferred from glass-house to growth chamber (12 h/12 h light/dark), 32°C/20°C day/night and 50% relative humidity (RH) whereas control plants were maintained at normal glass-house conditions. The saline solution was added on 7-days-old seedlings (vegetative stage) and 75-days-old-plants (reproductive stage) for salt stress. Total of 120 mM NaCl solution was added to stress plants and tissues were harvested after 5 days of stress treatment.

RNA Isolation

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) and purified using DNase (Qiagen, GmbH, Germany) through an RNeasy Plant Mini kit according to the manufacturer's instruction. The integrity of isolated RNA was checked on 0.8% agarose/formaldehyde (FA) gel electrophoresis. The concentration of each sample was checked on the Qubit fluorometer (Invitrogen) and three micrograms of RNA was used for first-strand cDNA synthesis using the SuperScript® III RT enzyme (Invitrogen, USA) following the manufacturer's guidelines.

Selection of Housekeeping Genes

Based on various gene expression studies in different crops, a set of 10 genes namely *EF1α, UBQ10, GAPDH, 18Sr RNA, 25Sr RNA, TUB6, ACT1, IF4α, UBC,* and *HSP90* were selected. Details of these genes have been provided in **Table 1**. These genes were subjected to homology search in pigeonpea genome, and their homologs were used for primer designing. The amplicon size ranged from 95 bp for *GAPDH* and *IF4α* genes to 107 bp for *25Sr RNA*.

Primer Designing and Quantitative Real-time PCR

Ten commonly known housekeeping genes, listed in **Table 1** were subjected to get pigeonpea orthologous sequences and used for primer designing. The functional integrity of the obtained sequences were checked using BLASTN search against GenBank EST database[1] (IIPG). Primer pairs were designed from exonic regions using Primer3Plus software[2].

The qRT-PCR was carried out using ABI SYBR® GREEN PCR reaction on an ABI Fast7500 System [Applied Biosystems (ABI), Foster City, CA, USA] according to the manufacturer's instructions. The amplification efficiency of primers was estimated by SYBR Green chemistry RT-qPCR (Sinha et al., 2015). PCR conditions for all the qRT-PCR reactions were used

[1]http://www.icrisat.org/gt-bt/iipg/Home.html

[2]http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/

TABLE 1 | Details on primers used for qRT-PCR analysis.

Gene name	Gene ID	Primer sequence (5′–3′)	Amplicon size (bp)	qRT- PCR efficiency (%)	Correlation coefficient (R^2)
EF1α	B9SPV9	F-GAGAGGTCCACCAACCTTGA	103	103.09	0.9995
		R-TTGTAGACGTCCTGCAATGG			
UBQ10	Q8H159	F-CCAGACCAGCAGAGGTTGAT	102	104.43	0.9991
		R-GATCTGCATACCTCCCCTCA			
GAPDH	Q2I0H4	F-ATGGCATTCCGTGTTCCTAC	95	99.66	0.9996
		R-CCTTCAACTTGCCCTCTGAC			
18Sr RNA	A5COJ4	F-CCACTTATCCTACACCTCTC	102	104.43	0.9951
		R-ACTGTCCCTGTCTACTATCC			
25Sr RNA	B7FKH8	F-ACCCTTTTGTTCCACACGAG	107	97.23	0.9965
		R-GACATTGTCAGGTGGGGAGT			
TUB6	B9R897	F-GCCCTGACAACTTCGTCTTC	100	103.98	0.9878
		R-GCAGTTTTCAGCCTCTTTGC			
ACT1	C6TJ78	F-GGCATACATTGCCCTTGACT	97	100.92	0.9984
		R-GAACCTCGGGACATCTGAAA			
IF4α	C6T8X3	F-GCCGAGATCACACAGTCTCA	95	90.94	0.9985
		R-ACCACGAGCCAAAAGATCAG			
UBC	Q2V732	F-CGAGAAAAGGCAGTTGATCC	105	104.43	0.9988
		R-CAGAAAAGGCAAGCTGGAAC			
HSP90	A5AHA8	F-TGTCGAGCAAGAAGACGATG	103	100.50	0.9975
		R-GGGCAGTTTCAAAGAGCAAG			

as followings: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Each reaction was performed in three biological and two technical replicates along with no template control. Melting curve analysis and agarose gel electrophoresis were carried out to check the amplicon specificity.

Gene Expression Analysis

Gene expression stability of 10 selected housekeeping genes in the root, shoot, and leaf tissues under heat and salt stress conditions was determined by BestKeeper descriptive statistical tool (Pfaffl et al., 2004). The tool is a Microsoft Excel based, freely downloadable software[3] that identifies the most suitable reference gene by repeated pairwise correlation and regression analysis of each gene with the other remaining candidate reference genes.

For ranking and identification of the most stable housekeeping genes for given conditions, statistical algorithms geNorm and NormFinder were used. The geNorm[4] algorithm measures the average expression stability value (*M*-value) and identifies two most stable genes from the analysis (Vandesompele et al., 2002). NormFinder is a Microsoft Excel based program[5] that works on the linear mixed-effects modeling to calculate stability values. The programe identifies the optimum number of housekeeping genes to be used in normalization studies for qRT-PCR analysis (Andersen et al., 2004).

Validation of Identified Reference Genes

The most stable housekeeping genes, identified during the present study were validated in the root, shoot and leaves tissues from heat and salt-stressed conditions. Two previously identified drought responsive universal stress protein coding genes, *uspA* and *uspB* (data unpublished) were used to validate the most stable, combination of most stable, least stable, and commonly used housekeeping genes. The differential gene expression of heat and salt stressed samples were compared to their respective unstressed controls with respect to different reference genes using a Relative Expression Software Tool (REST©) (Pfaffl et al., 2002).

RESULTS

Expression Profiling of Housekeeping Genes

To identify the most stable housekeeping genes, mRNA levels in all 24 tissues (stress imposed and control) were quantified based on their cDNA concentration. Detailed information on these 24 tissue samples has been given in **Supplementary Table S1**. The PCR efficiencies of each of the primers used in the present study were calculated based on 10-fold serial dilutions of pooled cDNA as reported previously (Sinha et al., 2015). The qRT-PCR efficiency (%) ranged from 90.94 (I*F4α*) to 104.43 (*UBQ10, 18Sr RNA,* and *UBC*) (**Table 1**). The obtained results were in accordance to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, the ideal PCR efficiency is 100%, while the acceptable range is from 80 to 120% (Bustin et al., 2009). The mean cycle threshold (Ct) values of all the 10 candidate genes for 12 different samples of heat ranged from *7.8 (18Sr RNA* in LHRS*)* to *28.8 (TUB6* in EHSC*)*. Similarly for salt stress conditions, the mean Ct values ranged from 5.4 (*25Sr RNA* in LHRC) to 29.3 (*TUB6* in ESRC) (**Figure 1** and **Supplementary Figure S1**). Further, to define the

[3]http://download.gene-quantification.info/

[4]http://medgen.ugent.be/~jvdesomp/genorm/

[5]http://moma.dk/normfinder-software

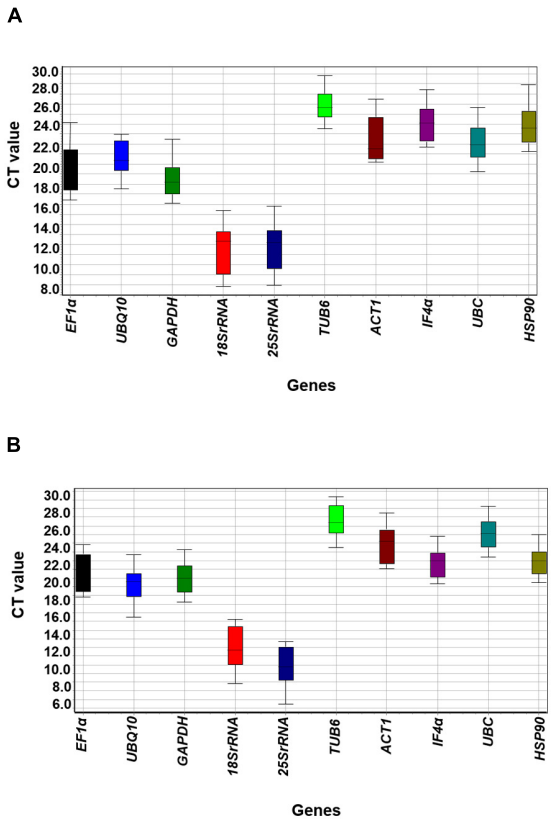


FIGURE 1 | Ct variation of tested housekeeping genes among different tissue samples under heat and salt stresses. (A) Boxplot depicting absolute Ct values of heat imposed/control samples and **(B)** Boxplot depicting absolute Ct values of salt imposed/control samples. Lower and upper boxes indicate the 25th and 75th percentile, respectively. The median is depicted by the horizontal line in the box.

ranking of targeted housekeeping genes for heat as well as salt stress conditions, three different algorithms namely BestKeeper, geNorm, and NormFinder were used as given in section below.

Identification of Suitable Reference Genes for Heat Stress Conditions

Descriptive statistics for each gene across tissues was estimated using BestKeeper algorithm. The analysis determined standard deviation (SD) value with ≤1 for all targeted housekeeping genes, indicating their consistent and stable performance (**Supplementary Table S2**). This analysis revealed that *TUB6* (SD, 1.13) showed the least SD followed by *UBQ10* (SD, 1.45) and *GAPDH* (SD, 1.49). Two genes namely *25Sr RNA* (SD, 2.08) and *18Sr RNA* (SD, 2.08), followed by *EF1α* (SD, 2.04) showed higher SD during the analysis reflecting their unstable nature under heat stress conditions. The coefficient of variations (CVs) of all the tested housekeeping genes ranged from 4.39 for *TUB6* to 17.83 for *18Sr RNA*.

To determine the ranking of selected housekeeping genes based on average expression stability value (*M*-value), geNorm algorithm was used. geNorm analysis of datasets revealed *UBC* and *HSP90* (*M*-value of 0.666) were the best pair of stable genes on the basis of their average expression stability value followed by *GAPDH* (*M*-value of 0.728) and *EF1α* (*M*-value of 0.780) (**Table 2**). Based on *M*-value, *18Sr RNA* (*M*-value of 1.154), *25Sr RNA* (*M*-value of 1.244) and *UBQ10* (*M*-value of 1.532) were found to be the least stable genes for expression studies. All 10 tested genes showed relatively high stability with *M*-value of less than 1.5 except *UBQ10*, indicating that genes used in the present study performed stable under heat stress conditions. Graphical representation of all the selected housekeeping genes are illustrated in **Figure 2A**.

In addition to above mentioned algorithms, NormFinder analysis was also used to identify the most stable genes, on the basis of stability value. Based on this analysis, *GAPDH* (stability value, 0.362), *UBC* (stability value, 0.496) and *HSP90* (stability value, 0.558) were identified as the most stable reference gene. Similar to the results obtained from geNorm analysis, *18Sr RNA* (stability value, 1.364), *25Sr RNA* (stability value, 1.481), and *UBQ10* (stability value, 2.755) were found as the least stable genes for heat stress conditions (**Figure 2B**) in the NormFinder analysis.

Although marginal differences were observed in the overall ranking of all the candidate genes tested in the present study, comparative analysis based on the geNorm and NormFinder output results showed that *UBC, HSP90* followed by *GAPDH* are the three most stable genes (**Table 2** and **Figures 2A,B**). The present findings were further supported by the heat map of the individual genes based on the Ct values, which correlates the stability ranking of the identified genes (**Supplementary Figure S2**).

Identification of Suitable Reference Genes for Salt Stress Conditions

For identification of the most appropriate reference gene under salinity stress, 10 housekeeping genes were analyzed in 12 different tissues (salinity imposed and control tissues). The descriptive analysis of datasets of all tested housekeeping genes under salinity stress conditions were determined by BestKeeper (**Supplementary Table S2**). Based on the SD value, *IF4α* (SD,

TABLE 2 | Ranking of tested housekeeping genes for heat stress conditions using geNorm and NormFinder algorithms.

Factor	geNorm		NormFinder	
	M-value	Ranking	Stability value	Ranking
EF1α	0.780	3	0.858	4
UBQ10	1.532	9	2.755	10
GAPDH	0.728	2	0.362	1
18Sr RNA	1.154	7	1.364	8
25Sr RNA	1.244	8	1.481	9
TUB6	1.007	6	0.951	7
ACT1	0.831	4	0.896	6
IF4α	0.938	5	0.879	5
UBC	0.666	1	0.496	2
HSP90	0.666	1	0.558	3

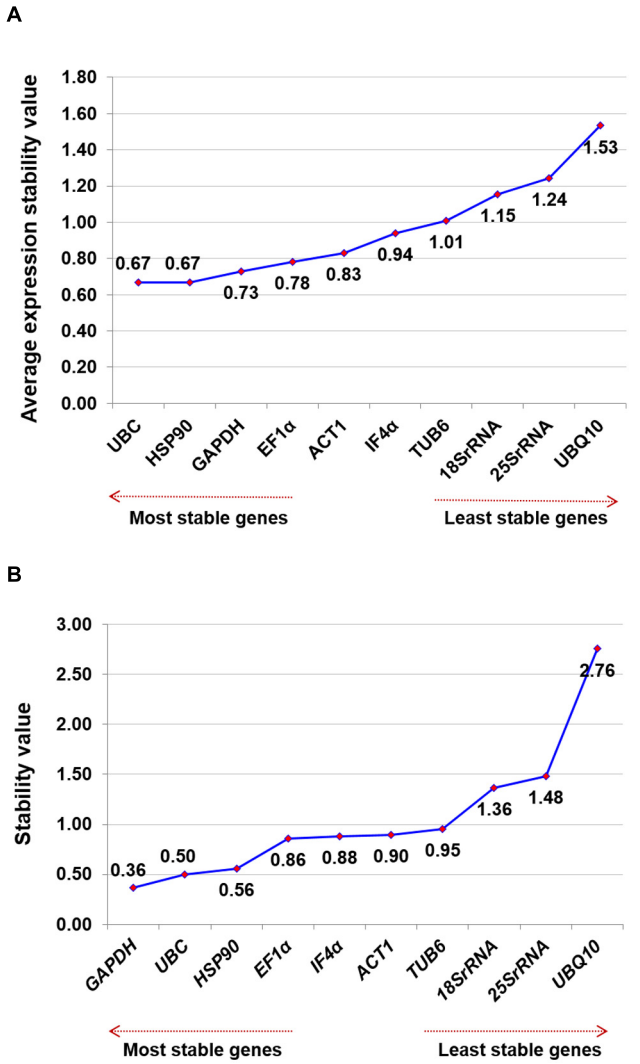


FIGURE 2 | Ranking of housekeeping genes for heat stress conditions. Gene expression studies for identification of most stable housekeeping genes under heat stress condition using two software programs. The direction of arrow indicates the most and least stable housekeeping genes in graphs **(A)** Gene expression stability graph of housekeeping gene using geNorm program based on average expression stability value (*M*), which is based on stepwise exclusion process. *M*-value is inversely related to gene stability **(B)** Gene expression stability graph using NormFinder program based on stability value and lower the stability value indicates higher stability of the housekeeping genes.

1.44) was identified as the most stable gene followed by *HSP90* (SD, 1.46) and *TUB6* (SD, 1.47). However, *EF1α* (SD, 1.85), *25Sr RNA* (SD, 1.86), and *18Sr RNA* (SD, 2.06) were found as the least stable genes under salt stress condition. The CV of all the tested genes were found higher and ranged from 5.56 for *TUB6* and 19.27 for *25Sr RNA*.

The two most stable housekeeping genes, namely *GAPDH* and *UBC* (*M*-value, 0.384) were identified using geNorm analysis for salinity stress conditions followed by *HSP90* (*M*-value, 0.422) and *IF4α* (*M*-value, 0.474). However, *TUB6* (*M*-value, 1.046) was found as the least stable gene compared to all other tested genes (**Figure 3A**). Even though on the basis of *M*-value, all of the tested genes showed relatively high stability with *M*-value of less than 1.5. Overall ranking of all tested genes using both the software for the salt stress condition is presented in **Table 3**.

NormFinder analysis of the datasets identified *GAPDH* (stability value, 0.192) as the most stable gene followed by *UBC* (stability value, 0.210) and *ACT1* (stability value, 0.491) (**Figure 3B**). Based on the geNorm and NormFinder analysis,

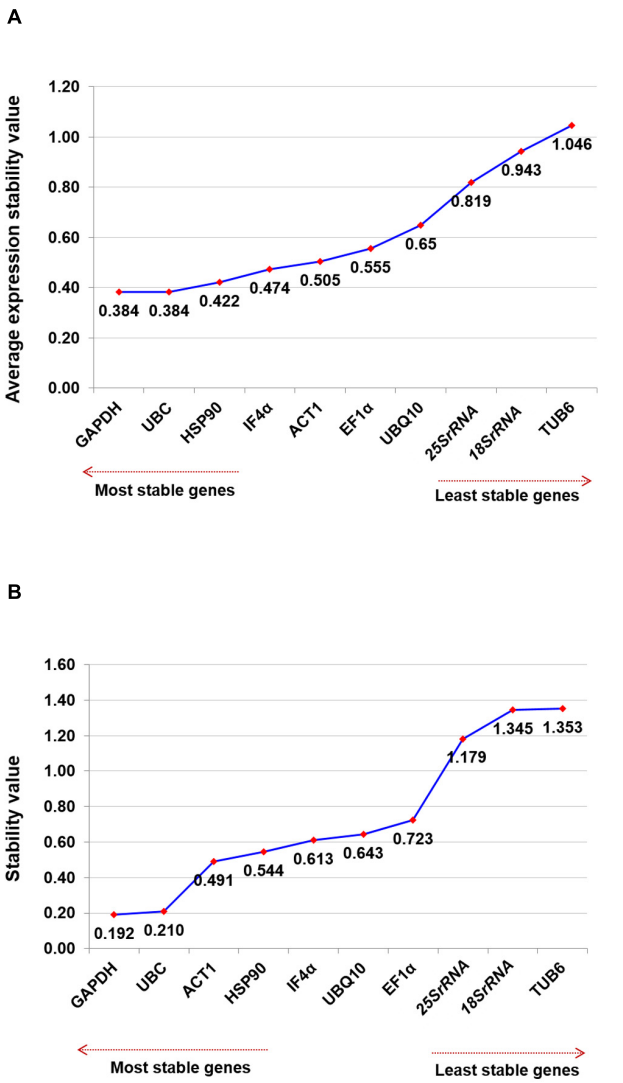


FIGURE 3 | Ranking of housekeeping genes for salinity stress conditions. Gene expression studies for identification of most stable housekeeping genes under salt stress condition using two software programs. The direction of arrow indicates the most and least stable housekeeping genes in graphs **(A)** Gene expression stability graph of housekeeping gene using geNorm program based on an average expression stability value (*M*), which is based on stepwise exclusion process. *M*-value is inversely related to gene stability **(B)** Gene expression stability graph using NormFinder program based on stability value and lower the stability value indicates higher stability of the housekeeping genes.

TABLE 3 | Ranking of tested housekeeping genes for salt stress conditions using geNorm and NormFinder algorithms.

Factor	geNorm		NormFinder	
	M-value	Ranking	Stability value	Ranking
EF1α	0.555	5	0.723	7
UBQ10	0.650	6	0.643	6
GAPDH	0.384	1	0.192	1
18Sr RNA	0.943	8	1.345	9
25Sr RNA	0.819	7	1.179	8
TUB6	1.046	9	1.353	10
ACT1	0.505	4	0.491	3
IF4α	0.474	3	0.613	5
UBC	0.384	1	0.210	2
HSP90	0.422	2	0.544	4

GAPDH was ranked as the most stable gene followed by *UBC* and *HSP90*. Similar to other stress conditions, *25Sr RNA* (stability value, 1.179) and *18Sr RNA* (stability value, 1.345) genes were found as the two least stable genes (**Table 3**). Additionally, using the Ct values, heat map was generated for all the candidate genes tested across the tissues. The heat map analysis revealed the stable level of expression of *GAPDH*, across the tissues and stages (**Supplementary Figure S2**).

Validation of Identified Stable Reference Genes for Heat Stress Conditions

To test the performance of identified most stable housekeeping genes, two earlier identified universal stress protein genes namely, *uspA* and *uspB* were used as target genes. Three most stable housekeeping genes identified in the present study (*UBC, HSP90,* and *GAPDH*), their combinations (*UBC* + *HSP90*, *UBC* + *GAPDH,* and *UBC* + *HSP90* + *GAPDH*), most commonly used housekeeping gene (*ACT1*) and least stable (*UBQ10*) genes were used as internal controls. The expression analysis was performed in three different tissues (root, stem, and leaf) at early and late heat stress conditions. As a result, varied level of expression differences was observed for both the target genes while normalized with different internal controls (**Figures 4A,B**).

For *uspA* gene, late heat root (LHR) tissues showed higher level of expression with *UBQ10* (5.20 fold) as compared to the stable *UBC* (0.20 fold), *HSP90* (0.13 fold), *GAPDH* (0.49 fold), and combination of stable genes, *UBC* + *HSP90* (0.16 fold),

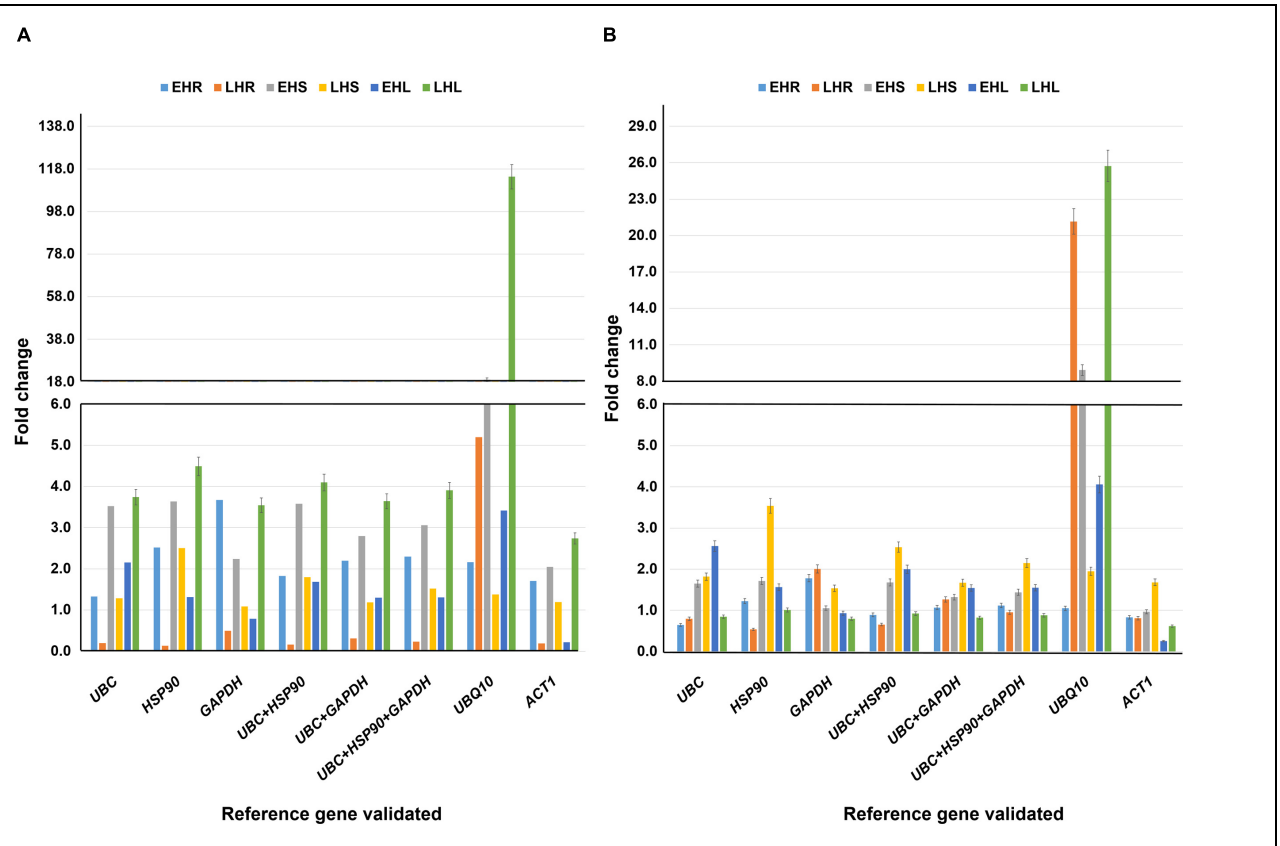


FIGURE 4 | Validation of reference genes under heat stress conditions. Expression profiling of candidate gene **(A)** *uspA* and **(B)** *uspB* in heat imposed tissues (root, stem, and leaves) and normalized with (i) *UBC* (ii) *HSP90* (iii) *GAPDH* (iv) *UBC* + *HSP90* (v) *UBC* + *GAPDH* (vi) *UBC* + *HSP90* + *GAPDH* (vii) *UBQ10* and (viii) *ACT1*. The analysis was completed in two different stages with six different tissues. The relative quantification values of selected drought responsive candidate gene were obtained after scaling to control samples. EHR, vegetative root stressed; LHR, reproductive root stressed; EHS, vegetative stem stressed; LHS, reproductive stem stressed; EHL, vegetative leaves stressed; LHL, reproductive leaves stressed.

UBC + *GAPDH* (0.31 fold) and *UBC* + *HSP90* + *GAPDH* (0.23 fold). Simillary, *uspA* gene in early heat stem (EHS) and late heat leaf (LHL) tissues showed very high level of gene expression with *UBQ10* as reference gene (18.94 fold for EHS and 114.30 fold for LHL). The expression *of uspA* in EHS and LHL varied when we used different stable reference genes, e.g., *UBC* (3.52 fold for EHS and 3.74 fold for LHL), *HSP90* (3.64 fold for EHS and 4.49 fold for LHL), *GAPDH* (2.24 fold for EHS and 3.55 fold for LHL) and combinations of different stable reference genes such as *UBC* + *HSP90* (3.58 fold for EHS and 4.10 fold for LHL), *UBC* + *GAPDH* (2.80 fold for EHS and 3.64 fold for LHL) and *UBC* + *HSP90* + *GAPDH* (3.06 fold for EHS and 3.91 fold for LHL) (**Figure 4A**).

In the case of *uspB* gene, *UBQ10* in comparison to stable and combination of stable genes showed higher gene expression in LHR and LHL tissues. The gene expression of *UBQ10* was 21.16 fold for LHR and 25.73 fold for LHL. For stable genes, *UBC* showed 0.80 fold in LHR and 0.84 fold gene expression in LHL tissues, *HSP90* showed 0.54 fold in LHR and 1.01 fold in LHL, *GAPDH* showed 2.01 fold in LHL and 0.80 fold in LHL tissues. Similarly, the combinations of stable genes, *UBC* + *HSP90* (0.65 fold in LHR and 0.92 fold in LHL tissues), *UBC* + *GAPDH* (1.26 fold in LHR and 0.82 fold in LHL tissues) and *UBC* + *HSP90* + *GAPDH* (0.95 fold in LHR and 0.88 fold in LHL tissues) showed similar level of expression as of stable genes (**Figure 4B**).

Validation of Identified Stable Reference Genes for Salt Stress Conditions

The identified most stable housekeeping genes for salt stress conditions were also validated with previously identified two universal stress protein genes namely, *uspA* and *uspB*. Three most stable housekeeping genes (*GAPDH, UBC,* and *HSP90*), combination of stable genes (*GAPDH* + *UBC*, *GAPDH* + *HSP90* and *GAPDH* + *UBC* + *HSP90*) along with the most commonly used (*ACT1*) and the least stable housekeeping gene (*TUB6*) identified during the present study were used as internal control (**Figures 5A,B**).

The relative expression of the target gene *uspA* under salt stress conditions were almost similar with all the tested reference genes or combinations. However, the least stable housekeeping gene, *TUB6* showed a different expression pattern with a very high gene expression value. Briefly, *TUB6* gene showed showed 8.41 fold gene expression in late salt shoot (LSS) tissues. However, with stable genes the expression was 2.41 fold (*GAPDH*), 2.39 fold (*UBC*), 3.45 fold (*HSP90*), 2.40 fold (*GAPDH* + *UBC*), 2.89 fold (*GAPDH* + *HSP90*), and 2.71 fold (*GAPDH* + *UBC* + *HSP90*)

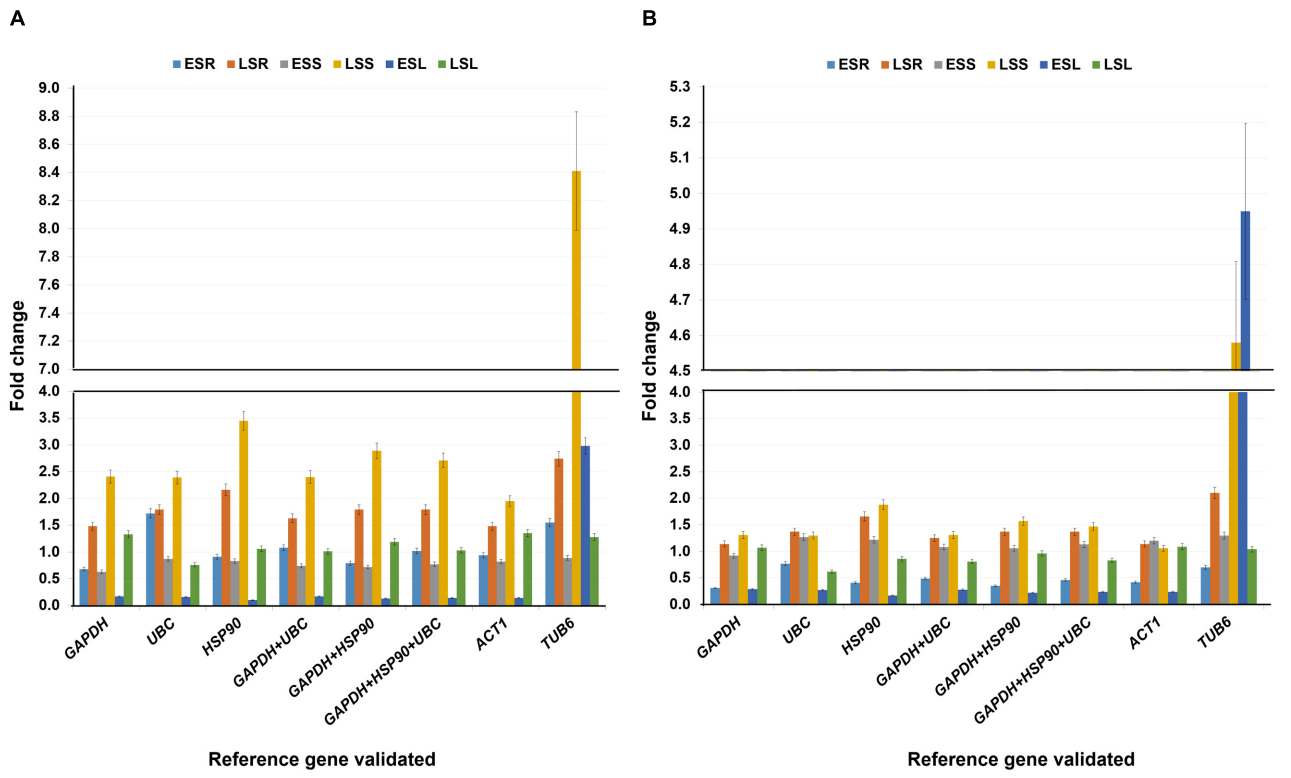


FIGURE 5 | Validation of reference genes under salt stress conditions. Expression profiling of candidate gene **(A)** *uspA* and **(B)** *uspB* in salt imposed tissues (root, stem, and leaves) and normalized with (1) *GAPDH* (ii) *UBC* (iii) *HSP90* (iv) *GAPDH* + *UBC* (v) *GAPDH* + *HSP90* (vi) *GAPDH* + *HSP90* + *UBC* (vii) *ACT1* and (viii) *TUB6*. The analysis was completed in two different stages with six different tissues. The relative quantification values of selected drought responsive candidate gene were obtained after scaling to control samples. ESR, vegetative root stressed; LSR, reproductive root stressed; ESS, vegetative stem stressed; LSS, reproductive stem stressed; ESL, vegetative leaves stressed; LSL, reproductive leaves stressed.

in the same tissue. Similarly, in the early salt leaf (ESL) tissues, *GAPDH* showed 0.17 fold, UBC 0.16 fold, HSP90 0.10 fold, *GAPDH + UBC 0.17* fold, *GAPDH + HSP90 0.13* fold, and *GAPDH + UBC + HSP90* 0.14 fold gene expression, which was very low as compared to *TUB6* with 2.98 fold expression (**Figure 5A**).

The *uspB* gene expression in the LSS and ESL also showed a similar pattern found with the *uspA* gene, during the validation studies. The expression level of *uspB* gene in LSS tissue was checked in presence of reference genes, e.g., *GAPDH* (1.31 fold), *UBC* (1.30 fold), *HSP90* (1.88 fold), and *TUB6* (4.58 folds) as well as combinations of different reference genes, such as *GAPDH + UBC* (1.31 fold), *GAPDH + HSP90* (1.57 fold), *GAPDH + UBC + HSP90* (1.47 fold). Following the same pattern, ESL tissue showed expression of 0.29 fold (*GAPDH*), 0.27 fold (*UBC*), 0.17 fold (*HSP90*), 0.28 fold (*GAPDH + UBC*), 0.22 fold (*GAPDH + HSP90*), and 0.24 fold (*GAPDH + UBC + HSP90*) in comparison to *TUB6* with a higher gene expression (4.95 fold) (**Figure 5B**).

DISCUSSION

For better understanding of regulation and function of genes involved in different stresses, it is pre-requisite to perform quantitative measurements and determine gene regulation patterns between samples (Van Hiel et al., 2009). To determine accurate measurement of target candidate gene(s), selection of a suitable reference gene, is pre-requisite during expression studies. An inappropriate reference gene can entirely change the base reference leading to an incorrect result interpretation (Dheda et al., 2005). Despite the fact that housekeeping genes exhibits no or minimum expression variations, many studies have proven the fact that such a perfect housekeeping gene has not yet reported which can be used as reference across different stress conditions (Zhu et al., 2013; Lopez-Pardo et al., 2013; Yang et al., 2014). Therefore, reference genes must be validated for each experimental condition in different species (Schmittgen and Zakrajsek, 2000).

To select appropriate reference genes for heat and salinity stress conditions, we have analyzed 10 commonly used housekeeping genes in a set of 24 diverse tissues (12 for each stress conditions) in pigeonpea. Three programs namely, BestKeeper, geNorm, and NormFinder were used to find out the stable housekeeping gene(s) in the given sample set and experimental design for different stress conditions. BestKeeper determines the optimal housekeeping gene employing the *pair-wise correlation analysis* of all pair of candidate genes (Pfaffl et al., 2004). Another program, geNorm works upon stepwise exclusion of the least stable genes, based on the average expression stability (*M*) value and which is indirectly proportional to stability of genes, i.e., lower the *M*-value higher the stability of genes (Vandesompele et al., 2002). The geNorm algorithm provides a pair of ideal housekeeping gene with identical expression ratios. NormFinder is an Excel based algorithm for identification of most stable gene based on the expression stability value (Andersen et al., 2004). As the three programs work on three different algorithms, they may provide different results (Mallona et al., 2010; Mafra et al., 2012; Zhu et al., 2013). Based on previous studies and algorithm they work upon, we have utilized BestKeeper for analyzing descriptive studies of different housekeeping genes and geNorm and NormFinder were used to determine the ranking of genes used in the present study.

The BestKeeper software provides two measures that can be used for assessing the stability of the reference genes. (i) raw SD of the Cq values and (ii) geometric mean of the reference genes and performs Pearson correlation of each of the reference genes to the BestKeeper Index. In the case of geNorm algorithms pairwise correlation known to be a strong algorithm for small sample sizes, but is biased toward selecting genes that are mutually correlated. Similarly, NormFinder has the strength that it can differentiate intragroup variation from intergroup variations. This software is useful for identifying candidate genes when different sample groups are to be compared. Therefore, differences among the underlying algorithms of three software packages are difficult for direct comparison among them. Recently, De Spiegelaere et al. (2015) analyzed all the three different softwares and revealed despite the differences among the algorithms between different softwares, the outcome of most stable and least stable reference genes was largely comparable for each sample set.

During analysis of different datasets, we observed that *UBC*, *HSP90* and *GAPDH* exhibited most stable gene expression across heat and salt stress conditions and can be used as a common stable internal control for expression studies under the given abiotic stresses. In contrast, several studies identified stress specific stable housekeeping genes, used as an internal control (Barsalobres-Cavallari et al., 2009; Garg et al., 2010; Sinha et al., 2015). However, not only for experimental conditions, in some cases different algorithms identified different stable housekeeping genes during analysis of the same datasets with different programs (Van Hiel et al., 2009; Reddy et al., 2013; Lopez-Pardo et al., 2013).

Considering the results examined by different programs for heat stressed tissue samples (root, stem, and leaves), *UBC* (Ubiquitin C), *HSP90* (Heat Shock Protein 90), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes are the most stable genes across the tissues used in the study using geNorm and NormFinder. *UBC* and *HSP90* were identified as the two most stable genes for heat stress conditions using geNorm with *M*-value of 0.666 were similar to earlier identified housekeeping genes for chickpea datasets with *M*-value of 0.28 (Jain et al., 2006).

Identified housekeeping gene *UBC*, (*Ubiquitin C*) has been associated with DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell signaling pathways. The ubiquitin–proteasome system is a major non-lysosomal proteolytic pathway that functions constitutively to degrade abnormal or damaged proteins (Hegde et al., 1997). After analyzing the datasets for salinity stress conditions, the gene *GAPDH*, an enzyme of glycolysis (Giulietti et al., 2001) outperformed in comparison to all other genes, and can be used as internal control for qRT-PCR analysis. *GAPDH* was also found stable housekeeping gene during expression analysis across tissues and genotypes in sugarcane (Iskandar et al., 2004). *GAPDH* been identified as a central metabolism enzyme is an

important energy-yielding step in carbohydrate metabolism and its ability to perform mechanistically different functions (Zimmer and Wen, 2013). Another identified gene, *HSP90* is known to play an important role in protein refolding in cells exposed to environmental stress and is required for the conformational maturation of several important signaling proteins (Jakob and Buchner, 1994). Additionally, *HSP90* has been shown role in the proteasome-dependent degradation of a selected group of cellular proteins (Whitesell et al., 1994). *Actin* is reported as one of the most commonly used housekeeping gene which is found to be essential for a range of cellular functions. Some of the major roles include cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking, and cell shape regulation (Perrin and Ervasti, 2010).

Validation of identified most stable and the combination of stable genes in comparison to the most unstable and widely used genes revealed significant differences in the fold change expression when normalized with the targeted candidate genes. Gene expression showed enhanced expression level with *18Sr RNA* in the case of heat stress and *TUB6* for salt stress conditions. These results indicated their low stability in the gene expression under targeted stress conditions. The validation result revealed that normalization with the most stable housekeeping genes based on the ranking had a similar level of gene expression for targeted genes, *uspA*, and *uspB*. Therefore, for better accuracy during gene normalization studies, a combination of identified stable housekeeping genes should be used. Our findings signify the importance of identification of specific housekeeping genes for specific stress conditions. In the case of pigeonpea, now together with this study, we got reference genes as *IF4α* and *TUB6* for drought stress (Sinha et al., 2015), *UBC, HSP90,* and *GAPDH* for heat stress and *GAPDH*, *UBC,* and *HSP90* for salt stress.

CONCLUSION

Our study identified *UBC, HSP90, GAPDH,* and *GAPDH, UBC, HSP90* as the most stable housekeeping genes under heat and salt stress conditions, respectively, for gene expression studies. Our data suggests that the expression of *18Sr RNA* is not very stable for heat stress condition, and for salt stress condition *TUB6* is the least stable gene. Validation of the identified stable housekeeping genes suggested that although, single reference gene gave reliable results, a combination of stable genes produces even better results for heat as well as salt stress conditions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01071

FIGURE S1| Gene expression analysis of candidate housekeeping genes across the tissues of heat and salt samples. This figure shows Ct distribution of each candidate reference genes among different samples of **(a)** heat and **(b)** salt tissues.

FIGURE S2| Heat map of candidate genes for heat and salt stress samples. This figure **(a)** heat and **(b)** salt, shows a heat map of candidate genes plotted based on normalized Ct mean values. Clustering of genes was based upon the Ct mean values of individual candidate genes across tissues. The detailed description of samples is provide in supplementary table S1.

TABLE S1| List of different tissue samples used for qRT-PCR analysis.

TABLE S2| Descriptive statistics of candidate genes under heat and salt stress conditions using BestKeeper software.

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