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THIOL-BASED REDOX HOMEOSTASIS AND SIGNALLING

Topic Editors
Francisco J. Cejudo, Andreas J. Meyer, Jean-Philippe Reichheld, Nicolas Rouhier and Jose A. Traverso

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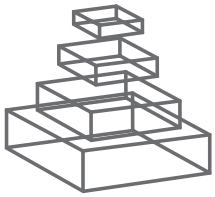

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THIOL-BASED REDOX HOMEOSTASIS AND SIGNALLING

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In contrast to the situation in heterotrophic organisms, plant genomes code for a significantly larger number of oxidoreductases such as thioredoxins (TRXs) and glutaredoxins (GRXs). These proteins provide a biochemical mechanism that allows the rapid and reversible activation or deactivation of protein functions in response to changing environmental conditions, as oxidative conditions caused by excessive photosynthesis. Indeed, owing to the fact that cysteines are sensitive to oxidation, TRXs and GRXs play an essential role in controlling the redox state of protein thiol groups. These redox-dependent post-translational modifications have proven to be critical for many cellular functions constituting regulatory, signalling or protective mechanisms.

The articles contained in this Research Topic provide timely overviews and new insights into thiol-dependent redox regulation mechanisms with a focus on TRX- and GRX-based reduction systems in plants. The different contexts discussed take into account physiological, developmental and environmental conditions.

Table of Contents

05 ***Thiol-Based Redox Homeostasis and Signaling***
Francisco J. Cejudo, Andreas J. Meyer, Jean-Philippe Reichheld, Nicolas Rouhier and Jose A. Traverso

08 ***Redox Regulation of the Calvin–Benson Cycle: Something Old, Something New***
Laure Michelet, Mirko Zaffagnini, Samuel Morisse, Francesca Sparla, María Esther Pérez-Pérez, Francesco Francia, Antoine Danon, Christophe H. Marchand, Simona Fermani, Paolo Trost and Stéphane D. Lemaire

29 ***Plastid Thioredoxins: A "One-For-All" Redox-Signaling System in Plants***
Antonio J. Serrato, Juan Fernández-Trijueque, Juan-de-Dios Barajas-López, Ana Chueca and Mariam Sahrawy

39 ***Overexpression of Plastidial Thioredoxins F and M Differentially Alters Photosynthetic Activity and Response to Oxidative Stress in Tobacco Plants***
Pascal Rey, Ruth Sanz-Barrio, Gilles Innocenti, Brigitte Ksas, Agathe Courteille, Dominique Rumeau, Emmanuelle Issakidis-Bourguet and Inmaculada Farran

52 ***The CP12 Protein Family: A Thioredoxin-Mediated Metabolic Switch?***
Patricia E. López-Calcagno, Thomas P. Howard and Christine A. Raines

61 ***Operation of Trans-Thylakoid Thiol-Metabolizing Pathways in Photosynthesis***
Mohamed Karamoko, Stéphane T. Gabilly and Patrice P. Hamel

67 ***Overoxidation of Chloroplast 2-Cys Peroxiredoxins: Balancing Toxic and Signaling Activities of Hydrogen Peroxide***
Leonor Puerto-Galán, Juan M. Pérez-Ruiz, Julia Ferrández, Beatriz Cano, Belén Naranjo, Victoria A. Nájera, Maricruz González, Anna M. Lindahl and Francisco J. Cejudo

73 ***Thiol-Based Redox Control of Enzymes Involved in the Tetrapyrrole Biosynthesis Pathway in Plants***
Andreas S. Richter and Bernhard Grimm

84 ***Redox-Dependent Functional Switching of Plant Proteins Accompanying With their Structural Changes***
Yong Hun Chi, Seol Ki Paeng, Min Ji Kim, Gwang Yong Hwang, Sarah Mae B. Melencion, Hun Taek Oh and Sang Yeol Lee

91 ***Overexpression of Chloroplast NADPH-Dependent Thioredoxin Reductase in Arabidopsis Enhances Leaf Growth and Elucidates in Vivo Function of Reductase and Thioredoxin Domains***
Jouni Toivola, Lauri Nikkanen, Käthe M. Dahlström, Tiina A. Salminen, Anna Lepistö, Florence Vignols and Eevi Rintamäki

109 ***Dissecting the Integrative Antioxidant and Redox Systems in Plant Mitochondria. Effect of Stress and S-Nitrosylation***
Juan J. Lázaro, Ana Jiménez, Daymi Camejo, Iván Iglesias-Baena, María Del Carmen Martí, Alfonso Lázaro-Payo, Sergio Barranco-Medina and Francisca Sevilla

129 ***Kinetic Analysis of the Interactions Between Plant Thioredoxin and Target Proteins***
Satoshi Hara and Toru Hisabori

137 ***Plant Cytoplasmic GAPDH: Redox Post-Translational Modifications and Moonlighting Properties***
Mirko Zaffagnini, Simona Fermani, Alex Costa, Stéphane D. Lemaire and Paolo Trost

155 ***Toward a Refined Classification of Class I Dithiol Glutaredoxins From Poplar: Biochemical Basis for the Definition of two Subclasses***
Jérémy Couturier, Jean-Pierre Jacquot and Nicolas Rouhier

169 ***Glutaredoxins are Essential for Stress Adaptation in the Cyanobacterium Synechocystis Sp. PCC 6803***
Ana M. Sánchez-Riego, Luis López-Maury and Francisco J. Florencio

180 ***Utility of Synechocystis Sp. PCC 6803 Glutaredoxin A as a Platform to Study High-Resolution Mutagenesis of Proteins***
David B. Knaff and Roger B. Sutton

190 ***Missing Links in Understanding Redox Signaling Via Thiol/Disulfide Modulation: How is Glutathione Oxidized in Plants?***
Marie-Sylviane Rahantaniaina, Andrée Tuzet, Amna Mhamdi and Graham Noctor

203 ***A Phenomics Approach to the Analysis of the Influence of Glutathione on Leaf Area and Abiotic Stress Tolerance in Arabidopsis Thaliana***
Daniel Schnaubelt, Philipp Schulz, Matthew A. Hannah, Rosita E. Yocgo and Christine H. Foyer

212 ***Thiol-Based Redox Signaling in the Nitrogen-Fixing Symbiosis***
Pierre Frendo, Manuel A. Matamoros, Geneviève Alloing and Manuel Becana

222 ***Thiol-Based Redox Regulation in Sexual Plant Reproduction: New Insights and Perspectives***
Jose A. Traverso, Amada Pulido, María I. Rodríguez-García and Juan D. Alché

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Thiol-based redox homeostasis and signaling

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Plants imperatively have to cope with adverse conditions owing to their lack of mobility and to the high amounts of reactive oxygen species (ROS) generated from both respiration and photosynthetic metabolism. Although thiol redox homeostasis in plants is mainly preserved by the cellular glutathione pool, specific strategies have been adopted by the plant kingdom during evolution to manage these "extra" pro-oxidative conditions. Unlike human or yeast, plants generally possess a higher number of genes coding for antioxidant proteins, including protein families responsible of dithiol/disulfide exchange reactions. During the last decades, redox-dependent post-translational modifications of proteins proved to be pivotal to many cellular functions. In particular, this is critically important under some situations of environmental constraints taking into account the alterations and fine adjustment of the cellular redox status occurring during and after any biotic or abiotic stresses.

Indeed, thiol groups of cysteinyl residues are highly sensitive to oxidation which might critically perturb cellular homeostasis. Members of the thioredoxin superfamily are key proteins involved in the regulation of cysteine/protein redox state. They share two common and well-known features: (i) the presence of an active center containing at least one catalytic cysteine residue, and (ii) a highly conserved 3D-structure, the so-called thioredoxin fold, which consists of a four-stranded anti-parallel β-sheet surrounded by three α-helices. Key members of this super family are thioredoxins (TRX) and glutaredoxins (GRX). Representatives of both subgroups are distributed in most cellular compartments and contain at least one TRX motif in their structures. While TRXs are generally reduced by thioredoxin reductases (TR), the reduction of GRXs depends on reduced glutathione (GSH).

The 19 reports of this Research Topic provide timely overviews and new insights into redox regulation, focusing on both TR/TRX and GSH/GRX reduction systems in plants. The biochemical characteristics of these systems as well as their target proteins and functions in metabolic and signaling pathways are discussed. Several contributions to this Research Topic deal with the role of TRX systems in plastid metabolism. Michelet et al. (2013) summarize the seminal contributions reported in the 70s and 80s indicating that several Calvin-Benson cycle enzymes are regulated by the ferredoxin/ferredoxin-thioredoxin-reductase/thioredoxin system. Besides this, based on the observation that multiple redox post-translational modifications including glutathionylation and nitrosylation may affect a single protein, Michelet and collaborators propose that these multiple layers of redox regulation could serve for the fine-tuning of the Calvin-Benson cycle enzyme activities in response to changing conditions. Serrato et al. (2013) reviewed the current knowledge on the functions of plastidial TRXs and discuss their emerging role in non-photosynthetic organs. By analyzing transplastomic tobacco plants overexpressing plastidial TRXs f or m, Rey et al. (2013) show that these types of TRXs fulfill distinct physiological functions and propose a role of TRXm in linking photosynthetic activity, redox homeostasis and antioxidant mechanisms in the chloroplast. In another report, López-Calcagno et al. (2014) discuss the possibility that CP12 proteins are part of a redox-mediated metabolic switch in response to rapid environmental changes, in addition to their classical regulatory role of the Calvin-Benson cycle.

Within the photosynthetic context, Karamoko et al. (2013) propose that the enzymatically assisted sulfhydryl oxidation in plant thylakoid lumen is required for the biogenesis of the energy-transducing membrane systems associated to photosynthesis. Their suggestions are based on both the existence of several luminal disulfide bond containing proteins in *Arabidopsis*, and on the discovery of trans-thylakoid redox pathways controlling disulfide bond formation and reduction.

In addition, the importance of NADPH thioredoxin reductase C (NTRC) in plastid redox regulation is also reported in four articles. The paper by Puerto-Galán et al. (2013) discusses the role of NTRC in the control of the overoxidation status of chloroplast 2-Cys peroxiredoxins (2-Cys-PRX), thus having a crucial function balancing the toxic and signaling activities of hydrogen peroxide. Moreover, Richter and Grimm (2013)

emphasize the relevant function of NTRC in conjunction with the FTR/TRX pathway on the redox regulation of tetrapyrrole biosynthesis. This pathway needs to be tightly regulated to adjust chlorophyll content of photosynthetic cells and to avoid the accumulation of chlorophyll biosynthesis intermediates, which may cause oxidative damage. In support of the overarching significance of redox regulation for chloroplast function and adaptation to environmental changes, Chi et al. (2013) discuss the molecular mechanisms and physiological significance of redox-dependent structural changes observed for some proteins including transcriptional factors and co-activators, which switch the chaperone activity of these proteins. Finally, Toivola et al. (2013) report an *in vivo* approach showing the positive effect of NTRC overexpression in plant performance.

The importance of redox regulation in plant mitochondria is also highlighted in this Research Topic by Lázaro et al. (2013). These authors update and discuss the complex antioxidant systems of this organelle, including the complex thiol-based TRX/PRX/Sulfiredoxin (SRX) system. Moreover, these authors focus on the function of this system in the response of plants to abiotic stresses and the regulation by redox post-translational modifications.

Some aspects of the cytosolic redox regulation pathways have also been developed in this Research Topic. Hara and Hisabori (2013) analyzed the kinetics of interactions of a cytosolic TRX *h* with selected target proteins using surface plasmon resonance. The presented data reveal a stronger preference of TRX *h* for an oxidized target, thus explaining the selective association of TRX with oxidized proteins. Zaffagnini et al. (2013) review how cysteine-based modifications of the plant cytosolic glyceraldehyde phosphate dehydrogenase (GAPDH) provoke the inactivation of classical enzymatic activity of this enzyme, but provide additional non-metabolic functions in stress signaling pathways, similar to those that have been identified in animal cells.

GRXs are oxidoreductases of the TRX family which display a particularly rich diversity in higher plants. Beyond classification in three main subgroups based on sequence and structural features, Couturier et al. (2013) indicated that class I GRXs can be subdivided into different groups which can be distinguished by different biochemical and catalytic properties. The importance of a GRX-dependent redox regulation is also evidenced here by Sánchez-Riego et al. (2013) who demonstrate, by performing a physiological and biochemical characterization of single, double and triple *grx* mutants, that GRXs are essential for stress adaptation in cyanobacteria. Finally, Knaff and Sutton (2013) describe an interesting work about the use of GRX to initiate a long-term educational project which will allow examining the structural and biochemical changes in GRXs according to single-point mutational replacements.

Glutathione is a key component in regulation and maintenance of cellular thiol redox homeostasis. It also plays key roles in different aspects of plant development. Here, Rahantaniaina et al. (2013) provide an overview of the main pathways influencing the glutathione redox status and their impact on signaling pathways through regulation of protein thiol status. Schnaubelt et al. (2013) examine the influence of glutathione in plant development and stress tolerance in *Arabidopsis*. The data demonstrate that cellular glutathione homeostasis influences the root architecture and the leaf area under optimal and stress conditions. Another aspect where glutathione and homoglutathione are crucial molecules is nodule development and in the context of legume-rhizobium mutualistic interactions. Indeed, these organs are peculiar due to the formation of a bacteroid in which the oxygen-sensitive nitrogenase reduces di-nitrogen to ammonia. However, the importance of other redox systems in this unique organ has been poorly documented. Frendo et al. (2013) review the current knowledge on the roles played by redox components in nitrogen-fixing symbioses. Finally, Traverso et al. (2013) summarize the insights of the precise involvement of thiol/disulfide-containing proteins at different stages of the sexual plant reproduction, suggesting specific and critical involvement of thiol-based redox modifications in different reproductive processes.

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Redox regulation of the Calvin–Benson cycle: something old, something new

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Reversible redox post-translational modifications such as oxido-reduction of disulfide bonds, S-nitrosylation, and S-glutathionylation, play a prominent role in the regulation of cell metabolism and signaling in all organisms. These modifications are mainly controlled by members of the thioredoxin and glutaredoxin families. Early studies in photosynthetic organisms have identified the Calvin–Benson cycle, the photosynthetic pathway responsible for carbon assimilation, as a redox regulated process. Indeed, 4 out of 11 enzymes of the cycle were shown to have a low activity in the dark and to be activated in the light through thioredoxin-dependent reduction of regulatory disulfide bonds. The underlying molecular mechanisms were extensively studied at the biochemical and structural level. Unexpectedly, recent biochemical and proteomic studies have suggested that all enzymes of the cycle and several associated regulatory proteins may undergo redox regulation through multiple redox post-translational modifications including glutathionylation and nitrosylation. The aim of this review is to detail the well-established mechanisms of redox regulation of Calvin–Benson cycle enzymes as well as the most recent reports indicating that this pathway is tightly controlled by multiple interconnected redox post-translational modifications. This redox control is likely allowing fine tuning of the Calvin–Benson cycle required for adaptation to varying environmental conditions, especially during responses to biotic and abiotic stresses.

Keywords: Calvin–Benson cycle, CO_2 fixation, thioredoxin, glutaredoxin, glutathionylation, nitrosylation, photosynthesis, redox regulation

INTRODUCTION

Redox post-translational modifications (PTM) of cysteine residues play a prominent role in the regulation of cell metabolism and signaling in all organisms. Indeed, cysteine residues can undergo different states of oxidation such as sulfenic (−SOH), sulfinic ($-SO_2H$) and sulfonic acids ($-SO_3H$) but also protein disulfide bonds (intra- or intermolecular, −SS−), S-thiolation (mainly glutathionylation, −SSG) or nitrosylation (−SNO). Most of these modifications are controlled by small disulfide oxidoreductases named thioredoxins (TRXs) and glutaredoxins (GRXs).

The importance of redox PTMs has been recognized very early in plants through studies aimed at understanding the mechanisms underlying the regulation of enzymes of the Calvin–Benson cycle (CBC). This pathway is responsible for CO_2 fixation by photosynthetic organisms and is therefore at the basis of our food chain. The CBC, or more generally photosynthesis, fuels all life on Earth with energy (Pfannschmidt and Yang, 2012). Without photosynthesis, no complex ecosystems and higher life forms including man would exist (Blankenship, 2002; Buchanan et al., 2002a).

After the initial discovery of the pathway by Bassham et al. (1950), the enzymes of the cycle were purified and characterized from diverse sources including C3 and C4 plants, algae and cyanobacteria (**Figure 1**). In the 60s, the activity of several enzymes of the CBC was found to be regulated by light. These enzymes were found to have a low activity in the dark and to be activated in the light. Investigation of the molecular mechanism of this light-dependent regulation led to the identification of the ferredoxin/thioredoxin (Fd/TRX) system that plays a crucial role in numerous redox- and light-dependent reactions in chloroplasts. Four enzymes of the CBC regulated by light were shown to contain a regulatory disulfide oxidized in the dark and reduced in the light by TRX. This reduction allows transition from a low active form to a fully active enzyme. Additional proteins were also recognized as TRX-regulated targets such as proteins involved indirectly in the regulation of the CBC, in light-dependent ATP production or in diverse carbon metabolism pathways (Lemaire et al., 2007; Schürmann and Buchanan, 2008). All these enzymes are regulated by light through TRX-dependent reduction of disulfide bonds. The mechanisms of this light-dependent regulation of carbon assimilation enzymes are considered as the best characterized mechanisms of redox signaling in photosynthetic organisms (Foyer and Noctor, 2005) since they were investigated in detail at the molecular and structural level in different model systems.

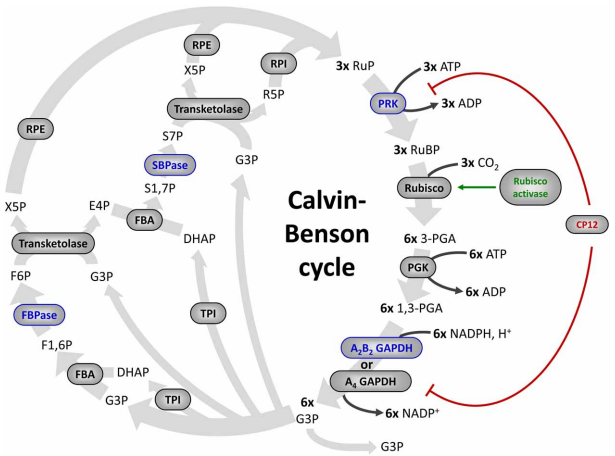

FIGURE 1 | The Calvin–Benson cycle. The eleven enzymes of the Calvin–Benson cycle are indicated in gray ellipses. Four enzymes (in blue) are activated directly by TRXs. Some proteins that control the activity of Calvin–Benson cycle enzymes are also regulated by TRXs: Rubisco activase (in green), and CP12 (in red), which forms a complex with PRK and A_4-GAPDH and inhibits both enzymes. Enzymes: Rubisco, ribulose-1,5-bisphosphate Carboxylase/Oxygenase; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triose phosphate isomerase; FBA, fructose-1,6-bisphosphate aldolase; FBPase, fructose-1,6-bisphosphatase; TK, transketolase; SBPase, sedoheptulose-1,7-bisphosphatase; RPE, ribulose-5-phosphate 3-epimerase; RPI, ribose-5-phosphate isomerase; PRK, phosphoribulokinase. Metabolites, RuBP, ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; 1,3-PGA, 1,3-bisphosphoglycerate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; F1,6P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; X5P, xylulose-5-phosphate; E4P, erythrose-4-phosphate; S1,7P, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; R5P, ribulose-5-phosphate; RuP, ribulose-5-phosphate.

However, recent proteomic studies are now challenging this rather simple view. Indeed, proteomic analyses aimed at identifying new TRX targets suggested that not only four but all CBC enzymes might be redox-regulated through mechanisms likely involving cysteine residues (Lemaire et al., 2007; Lindahl et al., 2011). Moreover, all enzymes of the cycle were also identified by proteomic approaches as potential targets of nitrosylation and glutathionylation, two redox PTMs whose importance in signaling and regulation has emerged recently (Zaffagnini et al., 2012a). This suggests that the CBC is likely regulated by a complex network of interconnected redox PTMs that remain to be characterized. In this review we will provide an overview of our current knowledge on the redox regulation of CBC enzymes and related proteins and discuss the potential importance of novel types of redox modifications on our understanding of the regulation of CO_2 fixation in photosynthetic organisms.

THE FERREDOXIN/THIOREDOXIN SYSTEM

During the 60s and 70s numerous studies have reported that the activity of four CBC enzymes was regulated by light (reviewed in Buchanan, 1980, 1991; Schürmann and Jacquot, 2000; Lemaire et al., 2007; Schürmann and Buchanan, 2008; Buchanan et al., 2012). These enzymes were phosphoribulokinase (PRK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase (FBPase), and sedoheptulose-1,7-bisphosphatase (SBPase) (**Figure 1**). These four enzymes were found to have a low activity in the dark and to be activated under illumination. During the same period, a similar regulation was reported for NADP-malate dehydrogenase (NADP-MDH) (Johnson and Hatch, 1970) and chloroplast ATP synthase (McKinney et al., 1978, 1979). NADP-MDH is involved in CO_2 fixation in C4 plants and in the export of excess reducing power from the chloroplast in C3 plants while chloroplast ATP synthase produces ATP equivalents required for CO_2 fixation by the CBC. The analysis of the molecular mechanisms underlying these light-dependent activations were mostly performed in the laboratory of Prof. Bob Buchanan in Berkeley and led to the identification of the so-called ferredoxin/thioredoxin system (Buchanan, 1991; Buchanan et al., 2002b). This system is composed of three types of chloroplast soluble proteins located in the stroma: ferredoxin (Fd), ferredoxin/thioredoxin reductase (FTR) and thioredoxin (TRX) (**Figure 2**). Upon illumination, ferredoxin is reduced by the photosynthetic electron transfer chain at the level of photosystem I (PSI). Chloroplastic Fd is located at a metabolic crossroad in the chloroplast and once reduced can transfer its electron(s) to enzymes involved in diverse metabolic pathways including photoreduction of NADP through ferredoxin/NADP reductase (FNR), reduction of sulfites and nitrites, lipid biosynthesis, hydrogen production (Winkler et al., 2010) and photosynthetic cyclic electron flow via ferredoxin-plastoquinone reductase (Hertle et al., 2013). Several isoforms of PSI-reduced Fd are present in photosynthetic organisms: 4 distinct Fd were described in the land plant *Arabidopsis thaliana* whereas 6 isoforms were reported in the green alga *Chlamydomonas reinhardtii*. These Fds are not equivalent as they exhibit some specificities toward their target enzymes and distinct expression profiles (Hanke et al., 2004; Terauchi et al., 2009). Under optimal growth conditions, most of the electron flux is likely directed to FNR to produce, in the form of NADPH, the reducing power required for CO_2 fixation by the CBC. Part of the reduced Fd pool also transfers electrons to FTR which can subsequently reduce the disulfide bond present in the active site of several types of chloroplastic TRXs. FTR is a thin and flat [4Fe-4S] enzyme interacting with Fd on one side and TRX on the other side (Dai et al., 2007). A first Fd molecule binds FTR and transfers one electron to the FTR [4Fe-4S] cluster. An intermediate is then formed in which one sulfur atom of FTR active site is free to attack the disulfide of TRX and the other sulfur atom forms a fifth ligand for an iron atom in the FTR [4Fe-4S] center. A second Fd molecule then delivers a second electron that cleaves the FTR-TRX mixed-disulfide. FTR is therefore unique in its use of a [4Fe-4S] cluster and a proximal disulfide bridge in the conversion of a light signal into a thiol signal (Dai et al., 2007). Once reduced, TRXs are able to reduce regulatory disulfides on their target enzymes, including PRK, GAPDH, FBPase, and SBPase, allowing their activation upon illumination through reduction of their regulatory disulfide by the Fd/TRX system (**Figure 3**).

Originally identified for their ability to activate enzymes of the CBC, TRXs were later found to contribute to the regulation of

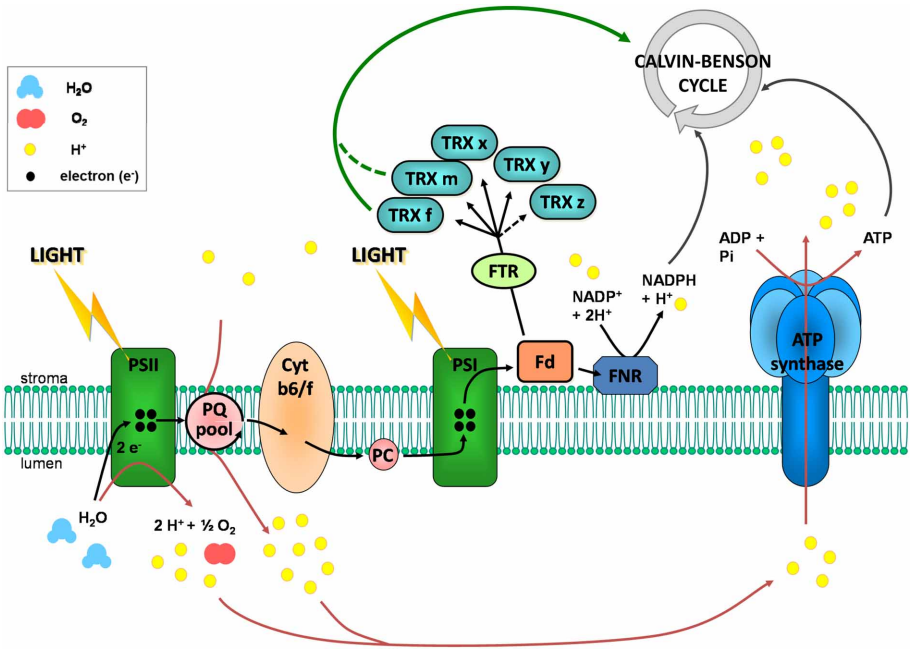

FIGURE 2 | The photosynthetic electron transfer chain and the reduction of chloroplastic TRXs. In the thylakoid membrane, when photosystem II (PSII) is excited by absorption of a photon light energy, the reaction center chlorophyll molecule transiently loses an electron. This electron is transmitted to the plastoquinone pool (PQ) which takes a proton from the stroma. Upon oxidation, the reaction center chlorophyll is a very strong oxidizing agent which is able to accept electrons from water, resulting in oxygen and protons production in the lumen. The chlorophyll can then be excited again. Reduced plastoquinone can move through the membrane from PSII to cytochrome b6/f (Cyt b6/f). There, plastoquinone is oxidized and its proton is released in the lumen, leading to a proton transport from stroma to lumen. Its electron is further transferred to photosystem I (PSI) via cytochrome b6/f and plastocyanin (PC). This electron transfer allows reduction of excited PSI reaction center chlorophyll. Upon excitation, this chlorophyll gives its electron to stromal ferredoxin (Fd) which can reduce chloroplastic thioredoxins (TRX) via the ferredoxin-thioredoxin reductase (FTR) and $NADP^+$ via the ferredoxin NADP reductase (FNR). Water photolysis and proton transport via plastoquinones contribute to the establishment of a proton gradient between stroma and lumen. This gradient is used as an energy source by the ATP synthase for ATP synthesis.

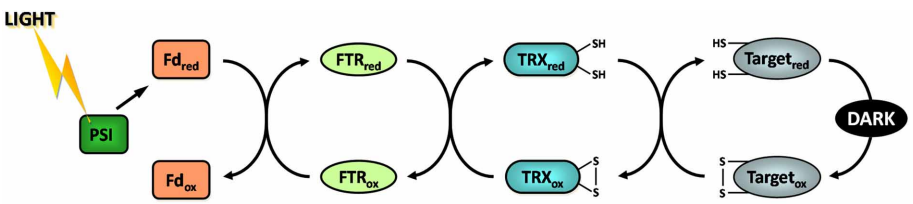

FIGURE 3 | The ferredoxin/thioredoxin system. Fd, ferredoxin; FTR, ferredoxin thioredoxin reductase; PSI, photosystem I; ox, oxidized; red, reduced.

diverse chloroplastic enzymes involved in other metabolic pathways such as ATP synthase which produces ATP in the light, Acetyl-CoA carboxylase which catalyzes the first committed step of fatty acid biosynthesis, ADP-glucose pyrophosphorylase, glucan:water dikinase and beta-amylase BAM1 involved in starch metabolism (Ballicora et al., 2000; Geigenberger et al., 2005; Mikkelsen et al., 2005; Sparla et al., 2006) or the oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (Wenderoth et al., 1997; Nee et al., 2009). In addition to their role in the control of metabolic enzymes through reduction of regulatory disulfides, TRXs also play a major role in the detoxification of reactive oxygen species (ROS) and the maintenance of redox homeostasis in the chloroplast. Indeed, TRXs serve as substrate and provide electrons for the regeneration of different types of antioxidant chloroplast enzymes including peroxiredoxins (PRXs) (Dietz, 2011), glutathione peroxidases (GPXs) (Navrot et al., 2006) and methionine sulfoxide reductases (Tarrago et al., 2009). Early studies on the regulation of FBPase and NADP-MDH led to the identification of two types of TRX named TRXf and TRXm due to their substrate specificity (Jacquot et al., 1978; Wolosiuk et al., 1979). Indeed, TRXf was initially found to activate FBPase while TRXm appeared to preferentially activate NADP-MDH. TRXf was also found to be more effective than TRXm for the reduction of all other CBC enzymes (Wolosiuk et al., 1979).

The availability of genome sequences revealed the existence of three other types of TRXs in the chloroplast. TRXx was identified in Arabidopsis (Mestres-Ortega and Meyer, 1999), TRXy

in Chlamydomonas (Lemaire et al., 2003), and TRXz in tomato and Arabidopsis (Rivas et al., 2004; Arsova et al., 2010; Meng et al., 2010; Schroter et al., 2010). In cyanobacteria, such as *Synechocystis* sp. PCC6803, four TRXs are present: 1 TRXm, 1 TRXx, 1 TRXy, and 1 TRX encoded by the *trxC* gene that has no ortholog in eukaryotes (Chibani et al., 2009; Pérez-Pérez et al., 2009). All five chloroplast TRX types (f, m, x, y, z) are conserved in land plants which usually contain several isoforms of each TRX type, such as Arabidopsis (2 TRXf, 4 TRXm, 1 TRXx, 2 TRXy, 1 TRXz) or poplar (1 TRXf, 8 TRXm, 1 TRXx, 2 TRXy, 1 TRXz) (Lemaire et al., 2007; Chibani et al., 2009). This multiplicity is more limited in unicellular eukaryotes such as the green algae *Chlamydomonas reinhardtii* (2 TRXf, 1 TRXm, 1 TRXx, 1 TRXy, 1 TRXz) or *Ostreococcus lucimarinus* (2 TRXf, 1 TRXm, 1 TRXx, no TRXy, 1 TRXz). Although TRXx and TRXy appear to be reduced by FTR (Bohrer et al., 2012), their biochemical properties do not allow them to activate CBC enzymes. These TRX types appear to be more specifically involved in the response to oxidative stress and the maintenance of ROS homeostasis. Indeed, TRXx and TRXy were found to be the most efficient TRXs for the reduction of PRXs, GPXs and MSRs (Collin et al., 2003, 2004; Chibani et al., 2011). TRXz has been recently characterized as a subunit of the plastid encoded RNA polymerase and plays an important role in chloroplast transcription and chloroplast development (Arsova et al., 2010; Schroter et al., 2010). In addition to this role, TRXz was suggested to be redox active although its mode of reduction remains controversial since TRXz was reported to be reduced by FTR in poplar (Chibani et al., 2011) or by other TRX types in Arabidopsis (Bohrer et al., 2012). Nonetheless, as TRXx and TRXy, TRXz appears to reduce some antioxidant enzymes but is most probably not able to reduce classical carbon metabolism targets including CBC enzymes, as demonstrated for NADP-MDH (Chibani et al., 2011; Bohrer et al., 2012). By contrast, TRXf and TRXm are clearly dedicated to the regulation of the CBC although TRXf appears to play a more prominent role. Indeed, all TRX-dependent enzymes of the CBC analyzed are exclusively or preferentially reduced by f-type TRXs. TRXm may thus play a more important role in the regulation of other chloroplastic processes and pathways such as transfer of reducing equivalents from the stroma to the thylakoid lumen (Motohashi and Hisabori, 2006, 2010) or regulation of chloroplastic proteins involved in electron transfer pathways (Courteille et al., 2013). Alternatively, TRXm isoforms may serve as alternative regulators of some CBC enzymes when TRXf activity is limiting, e.g., under oxidative stress conditions leading to protein glutathionylation in chloroplasts. Indeed, TRXf itself can be glutathionylated and consequently lose capability to regulate its targets (Michelet et al., 2005). In addition to canonical chloroplastic TRXs, numerous non chloroplastic TRX types and TRX-like isoforms are present in the genomes of photosynthetic eukaryotes (Chibani et al., 2009). Forty-one isoforms of TRXs and TRX-like proteins were reported in Arabidopsis and forty-five in poplar. Some of these TRX-related proteins are likely located in chloroplasts as shown for the peculiar CDSP32 protein composed of two TRX domains (Broin et al., 2002) and which participates in responses to oxidative stress (Rey et al., 2005; Tarrago et al., 2010).

MOLECULAR MECHANISMS OF LIGHT-DEPENDENT REGULATION OF THE CALVIN–BENSON CYCLE

After the discovery of the Fd/TRX system, numerous efforts have been put on the analysis at the biochemical and structural level of the molecular mechanism underlying TRX-dependent activation of chloroplast enzymes. The insights obtained from these studies are detailed in this section.

CALVIN–BENSON CYCLE ENZYMES

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH catalyzes the reversible interconversion of 1,3-bisphosphoglycerate (BPGA) into glyceraldehyde-3-phosphate (G3P) (**Figure 1**). At difference with NAD(H)-specific glycolytic GAPDH (GAPC) (Zaffagnini et al., 2013a,b, this series), photosynthetic GAPDH uses both NAD(H) and NADP(H) as coenzymes (Melandri et al., 1968). GAPDH was the first CBC enzyme reported to be activated by light: in leaves and chloroplast extracts subjected to short periods of illumination, the NADP(H)-dependent activity of GAPDH was found several-fold higher than in samples maintained in the dark, but the NAD(H)-GAPDH activity remained low and stable (Ziegler and Ziegler, 1965). GAPDH activation *in vivo* was strictly dependent on photosynthetic electron transport, i.e., was inhibited by the PSII inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), and could be mimicked *in vitro* by addition of NADPH and the strong chemical reductant dithiothreitol (DTT) (Ziegler and Ziegler, 1966). These results were confirmed in several species of land plants and green algae (Anderson and Lim, 1972; Huber, 1978; Austin et al., 1992; Scagliarini et al., 1993; Baalmann et al., 1994). Wolosiuk and Buchanan (1978a) first demonstrated the increase of the NADP(H)-dependent GAPDH activity by reduced TRX, but the complex mechanism of GAPDH regulation involves also the inter-conversion between active tetramers and low activity oligomers (Pupillo and Piccari, 1973) and this change of quaternary structure is controlled by different ligands, including NAD(P)(H), ATP and BPGA (Pupillo and Piccari, 1973; O'brien et al., 1976; Wolosiuk and Buchanan, 1976; Cerff, 1978; Trost et al., 1993). *In vivo*, oligomers can be formed by GAPDH only (Pupillo and Piccari, 1973; Scheibe et al., 2002; Howard et al., 2011b), or include PRK as suggested by early works (Wara-Aswapati et al., 1980; Nicholson et al., 1987; Avilan et al., 1997). Later, it was understood that GAPDH-PRK complexes were actually assembled by a small TRX-regulated protein named CP12 (Wedel et al., 1997; see below).

Photosynthetic GAPDH is coded by two types of genes (*gapA, gapB*) in Streptophytes [land plants and Charophytes (Petersen et al., 2006)], and Prasinophycean green algae [e.g., Ostreococcus, (Robbens et al., 2007)]. The *gapB* gene is absent in all other oxygenic phototrophs that usually contain a single *gapA* gene, except for cryptomonads, diatoms, and chromalveolates in general in which chloroplast GAPDH is encoded by *gapC*-type genes (Liaud et al., 1997). At the protein level, GAPA and GAPB are almost identical, but GAPB contains a specific C-terminal extension (CTE) of about 30 amino acids (Baalmann et al., 1996). The CTE contains the pair of Cys residues that are targeted by TRX and are responsible for the light/dark regulation of the enzyme (Sparla et al., 2002) (**Figure 4**). GAPA, or A-subunits, form stable

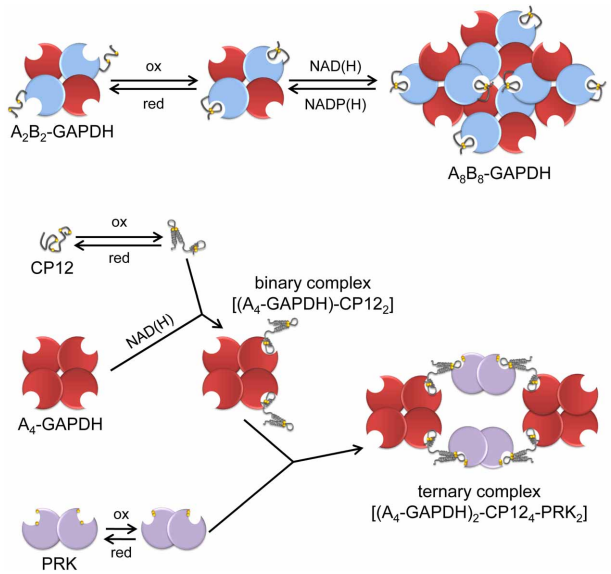

FIGURE 4 | A schematic view of the regulation and interactions of chloroplast A_4-GAPDH, CP12, PRK and AB-GAPDH. "Light conditions" (left side of the figure), consisting of increased levels of reduced TRXs and high NADP(H)/NAD(H) ratio, promote the reduction and dissociation of the supramolecular complexes. A_4-GAPDH, reduced PRK and reduced A_2B_2-GAPDH are fully active in the light while reduced CP12 does not interact with partner proteins. "Dark conditions" (right side of the figure), consisting of decreased level of reduced TRXs and low NADP(H)/NAD(H) ratio, promote disulfide formation in PRK, CP12 and A_2B_2-GAPDH. These conditions promote the formation of the binary complex between A_4-GAPDH and oxidized CP12 that further reacts with oxidized PRK forming the ternary complex A_4-GAPDH/CP12/PRK. Concomitantly, A_2B_2-GAPDH undergoes oxidation and shifts to oligomeric form (A_8B_8-GAPDH). Within these complexes, the activity of both GAPDHs and PRK is strongly suppressed.

homotetramers [A_4-GAPDH, (Fermani et al., 2001)] that resemble the structure of glycolytic GAPDHs (Zaffagnini et al., 2013a,b, this series), or alternatively bind B-subunits in stoichiometric ratio to form heterotetramers (A_2B_2) and higher order oligomers [mainly A_8B_8; (Pupillo and Piccari, 1973)]. A_4-GAPDH is a minor GAPDH isoform in higher plants (Scagliarini et al., 1993; Howard et al., 2011b), but is the only isoform of photosynthetic GAPDH in green algae, red algae and cyanobacteria (Petersen et al., 2006). Lacking the CTE, A_4-GAPDH is not TRX-regulated *per se* but acquires this regulation through the interaction with the TRX-regulated proteins CP12 and PRK (Wedel and Soll, 1998; Graciet et al., 2004; Trost et al., 2006). This is the only known mechanism of light/dark regulation of GAPDH in green algae and cyanobacteria, and it will be discussed further below in the section on CP12. In chloroplasts of C_3 plants, CTE- and CP12-based regulation of GAPDH co-exist (Scheibe et al., 2002), and CP12-assembled complexes may contain either A_4- or A_2B_2-GAPDH (Carmo-Silva et al., 2011; Howard et al., 2011b). In C_4 plants, the two systems appear instead to be separated: a proteomic study on maize revealed that CP12 is enriched in bundle sheath chloroplasts, together with GAPA and PRK, while GAPB is enriched in mesophyll chloroplasts (Majeran et al., 2005).

In land plants, the activity of AB-GAPDH isoforms primarily depends on the redox state of the two cysteines of the CTE [Cys349 and Cys358 in spinach (Sparla et al., 2002)]. The Cys349-Cys358 disulfide has a midpoint redox potential of −353 mV at pH 7.9 and is specifically reduced by TRXf (Marri et al., 2009). In the presence of reduced TRXf and at NADPH concentrations expected for illuminated chloroplasts, the NADPH-activity of AB-GAPDH is maximal and the enzyme is tetrameric (Scagliarini et al., 1993; Baalmann et al., 1994; Sparla et al., 2002). In this activated form, the 2′-phosphate group of NADPH interacts with Arg77 and Ser188, and these interactions are crucial for both coenzyme specificity [NADH contains an −OH in the 2′-position; (Falini et al., 2003)] and TRX-dependent regulation of GAPDH (Fermani et al., 2007). In fact, the disulfide bond between Cys349 and Cys358 shapes the CTE into a bulky hairpin structure that is harbored in proximity of the coenzyme-binding site. In this position, the negatively charged CTE distracts Arg77 from the 2′-phosphate of bound NADPH, with the consequence of depressing the NADPH-dependent activity, while leaving unaffected the NADH-dependent one. Both the crystal structure of oxidized A_2B_2-GAPDH (Fermani et al., 2007) and the kinetic characterization of site-specific mutants (Sparla et al., 2005) support this regulatory mechanism in which the CTE of GAPB acts as a redox-sensitive auto-inhibitory domain.

The CTE also controls the capability of A_2B_2-GAPDH to associate into the A_8B_8 isoform upon binding NAD(H) in place of NADP(H) (**Figure 4**). The effect is completely dependent on the CTE that must bear the Cys349-Cys358 disulfide (Baalmann et al., 1996; Sparla et al., 2002). Oligomeric AB-GAPDH has very low NADPH-activity and accumulates in chloroplasts in the dark (Scagliarini et al., 1993; Baalmann et al., 1994). Full recovery of GAPDH activity is obtained by reducing the CTE with reduced TRXf or by displacing oxidized CTE from the active site with ligands such as NADP(H), ATP, or BPGA (Trost et al., 2006). As recalled below, the CTE is homologous to the C-terminal half of CP12 and CP12 is engaged in protein-protein interactions with GAPA and PRK when containing a disulfide bond.

Phosphoribulokinase (PRK)

PRK catalyzes the phosphorylation of ribulose-5-phosphate to ribulose-1,5-bisphophate using ATP generated by thylakoid ATP synthase (**Figure 1**). The light-dependent activation of PRK was initially reported in the unicellular green alga Chlorella (Pedersen et al., 1966; Bassham, 1971) and later confirmed with isolated chloroplasts from spinach (Latzko et al., 1970; Avron and Gibbs, 1974). As in the case of GAPDH, the activation of PRK was found to be blocked by DCMU (Latzko et al., 1970; Avron and Gibbs, 1974; Champigny and Bismuth, 1976) and mimicked by DTT (Latzko et al., 1970; Anderson, 1973b; Anderson and Avron, 1976). Contrary to GAPDH, PRK has no cytosolic counterpart since this enzyme exclusively participates in the CBC. The enzyme is a homodimer in eukaryotes (Porter et al., 1986) and a homotetramer in cyanobacteria (Wadano et al., 1998). In anoxygenic photosynthetic prokaryotes PRK is octameric, but is not redox-regulated (Harrison et al., 1998). In plants, each PRK monomer contains 4 strictly conserved cysteines. PRK

has a low activity in the oxidized form and is activated by TRX (Wolosiuk and Buchanan, 1978b). The molecular mechanism of PRK redox regulation was investigated on the spinach enzyme by chemical modification and site-directed mutagenesis of Cys16 and Cys55 that are located on the N-terminal part of the monomer and form a disulfide reduced by TRX (Porter and Hartman, 1988; Milanez et al., 1991; Brandes et al., 1996). Since Cys55 appears to play an important role in the catalysis by binding the sugar phosphate substrate (Porter and Hartman, 1990; Milanez et al., 1991), formation of the Cys16-Cys55 disulfide efficiently blocks the activity. The midpoint redox potential of PRK was found somehow variable between species [at pH 7.9: −315 mV in tomato (Hutchison et al., 2000); −330 mV in Arabidopsis (Marri et al., 2005); −349 mV in spinach (Hirasawa et al., 1999)], but tends to be less negative compared to that measured for other CBC enzymes [between −350 and −385 mV (Hirasawa et al., 1999, 2000; Hutchison et al., 2000; Sparla et al., 2002; Marri et al., 2005)]. Recently, the TRX specificity for Arabidopsis PRK activation was investigated and TRXf was found to be the most efficient compared to m-type TRXs, while no PRK reactivation was observed with x- and y-types TRXs (Marri et al., 2009). The three dimensional structure of plant PRK remains unknown precluding full understanding of the molecular mechanisms involved in the redox regulation mediated by TRX.

Fructose-1,6-bisphosphatase (FBPase)

FBPase catalyzes the dephosphorylation of fructose-1,6-bisphosphate (FBP) into fructose-6-phosphate (F6P) with the concomitant release of inorganic phosphate (**Figure 1**). The enzyme is a homotetramer of *ca.* 160 kDa. The cytosolic isoform of FBPase, which participates in gluconeogenesis, is not redox regulated by TRX. The light-dependent activation of FBPase was also initially reported in Chlorella (Pedersen et al., 1966; Bassham, 1971) and later confirmed in isolated chloroplasts from higher plants where the activation was found to be DCMU sensitive (Champigny and Bismuth, 1976; Kelly et al., 1976) and could be mimicked by DTT (Baier and Latzko, 1975). Oxidized FBPase has a basal activity (20–30%) and becomes fully activated upon disulfide reduction which is strictly dependent on TRXf, all other TRX types being inefficient. The molecular mechanism of TRX dependent activation of FBPase was initially unraveled for pea FBPase. Compared to its cytosolic counterparts, pea stromal FBPase contains two insertions of 5 and 12 amino acids containing 1 (Cys153) and 2 cysteines (Cys173 and Cys178), respectively. The mutation C153S or the double mutation C173S/C178S yielded a permanently active FBPase while the single mutant C173S and C178S retained a partial redox dependent regulation (Jacquot et al., 1995, 1997). These results suggested that the regulatory disulfide is formed between Cys153 and either with Cys173 or Cys178. The structure of pea FBPase revealed the presence of a unique Cys153-Cys173 disulfide, suggesting that the Cys153-Cys178 disulfide was only formed upon mutation of Cys173 although a more complex regulation implicating isomerization of disulfide bonds could not be completely ruled out (Chiadmi et al., 1999). Strikingly similar results were obtained for the 3 cysteines present in the insertions found in rapeseed FBPase (Rodriguez-Suarez et al., 1997). The midpoint redox potential of the regulatory disulfide bond was found to be −369 mV and −384 mV at pH 7.9 for pea and spinach FBPases, respectively (Hirasawa et al., 1999). Comparison of the structure of oxidized and reduced FBPase allowed understanding the conformational changes linking the redox state of the regulatory disulfide and the level of activation of the enzyme (Villeret et al., 1995; Chiadmi et al., 1999; Dai et al., 2004). Although the regulatory disulfide is at a distance of more than 20 Å from the active site, its formation forces a loop connecting two antiparallel beta strands to slide in toward the active site, thereby disrupting the binding sites for the catalytic Mg^{2+} cations (Chiadmi et al., 1999). Therefore, in light-regulated FBPase, the regulatory insertions that form the disulfide do not interact directly with the active site (like in malate dehydrogenase as described below) or in its proximity (like in AB-GAPDH), but stabilizes a general conformation in which the active site is almost non-functional.

Sedoheptulose-1,7-bisphosphatase (SBPase).

SBPase catalyzes the dephosphorylation of sedoheptulose-1,7-bisphosphate (SBP) into sedoheptulose-7-phosphate (S7P) with the concomitant release of inorganic phosphate (**Figure 1**). SBPase is a homodimer of *ca.* 70 kDa that is unique to the CBC and has no cytosolic counterpart. SBPase is found in all photosynthetic eukaryotes but not in cyanobacteria which encode a bifunctional FBPase possessing also SBPase activity (Tamoi et al., 1996). As in the case of GAPDH and FBPase, the light-dependent activation of SBPase was also initially reported in Chlorella (Pedersen et al., 1966; Bassham, 1971), confirmed in isolated chloroplasts from land plants and found to be mimicked by DTT (Anderson, 1974; Schürmann and Buchanan, 1975; Anderson and Avron, 1976). By contrast with other CBC enzymes, the oxidized form of SBPase is completely inactive and its reactivation absolutely requires the TRX-mediated light activation. SBPase redox regulation has only been analyzed for the wheat enzyme that possesses 7 cysteines among which 4 are strictly conserved in all organisms. Site-directed mutagenesis of the wheat enzyme suggested the existence of a single regulatory disulfide between Cys52 and Cys57 (Dunford et al., 1998). However, in this study, the activity of recombinant wheat mutant SBPase was only measured in *E. coli* crude extracts using DTT as electron donor that may have mediated SBPase activation through *E. coli* TRXs. Therefore, the molecular mechanism underlying the redox regulation of SBPase remains to be clearly established with the purified enzyme. The enzyme from maize leaves was reported to be, like FBPase, specifically activated by TRXf (Nishizawa and Buchanan, 1981) but not all TRX types have yet been tested. SBPase sequences share a significant homology with FBPase, possibly due to a common origin (Raines et al., 1992; Martin et al., 1996). Therefore, although the structure of plant SBPase remains undetermined, a model has been proposed based on the structure of pig FBPase (Dunford et al., 1998). This model suggested that like in FBPase, the reduction of SBPase disulfide would trigger its activation through a general conformational change of the enzyme that shapes the active site.

OTHER TRX-DEPENDENT ENZYMES LINKED TO THE CALVIN–BENSON CYCLE

NADP-dependent malate dehydrogenase (NADP-MDH)

NADP-MDH catalyzes the reduction of oxaloacetate (OAA) into malate using NADPH as electron donor. The enzyme is a homodimer of *ca*. 70 kDa. NADP-MDH plays a key role for CO_2 fixation in C4 and CAM plants where photorespiration, linked to the oxygenase activity of Rubisco, is limited through an alternative CO_2 fixation pathway initiated by phosphoenolpyruvate carboxylase (PEPC) (Foyer et al., 2009) (**Figure 5**). CO_2 fixation by PEPC yields oxaloacetate that is converted to malate by NADP-MDH. The malic enzyme converts malate into phosphoenolpyruvate and CO_2 in the proximity of Rubisco. These carbon concentration mechanisms limit photorespiration and ensure optimal photosynthesis efficiency under specific growth conditions. In C3 plants, NADP-MDH is involved in the export of reducing power, in the form of malate, from the stroma to the cytosol through the malate valve. Light-dependent activation of NADP-MDH was initially reported in C4 plants (Johnson and Hatch, 1970) and later confirmed in C3 plants (Johnson, 1971). As in the case of CBC enzymes, the light-dependent activation was confirmed on isolated chloroplasts, found to be blocked by DCMU and mimicked by DTT (Anderson, 1973a; Anderson and Avron, 1976). NADP-MDH is probably one of the best studied light-regulated enzyme in the chloroplast both at the molecular and structural levels. By contrast with most CBC enzymes which exhibit a basal activity in the oxidized form, oxidized NADP-MDH is totally inactive and strictly dependent on light-driven TRX activation. The molecular mechanism of NADP-MDH activation has been initially unraveled on the enzyme from the C4 plant *Sorghum bicolor*. Compared to the redox independent NAD-dependent MDH isoforms, Sorghum NADP-MDH possesses N- and C-terminal extensions and contains 8 cysteine residues strictly conserved in land plants. Site-directed mutagenesis and chemical modifications allowed understanding the molecular mechanism of TRX-dependent activation of this enzyme (reviewed in Miginiac-Maslow and Lancelin, 2002). Two regulatory disulfides, located in each extension, are present in the oxidized form and have to be both reduced by TRX for full activation of the enzyme. Reduction of the Cys24-Cys29 N-terminal disulfide allows a slow conformational change of the enzyme while reduction of the Cys365-Cys377 C-terminal disulfide is required to give access to the active site (Issakidis et al., 1992, 1993, 1994, 1996; Lemaire et al., 1994). Indeed, in the oxidized form, the penultimate glutamate residue interacts with the active site Arg204 thereby blocking access of OAA (Ruelland et al., 1997; Hirasawa et al., 2000). In addition, the internal Cys207 can form a TRX-reducible disulfide with Cys24, suggesting that disulfide isomerization may be required during activation (Ruelland et al., 1997; Hirasawa et al., 2000). These results were later found to be consistent with the structures of Sorghum and Flaveria NADP-MDH (Carr et al., 1999; Johansson et al., 1999) and have allowed to propose a detailed model of the molecular mechanism of activation of NADP-MDH (**Figure 6**). The standard redox potentials of the two regulatory disulfides are not equivalent, the N-terminal disulfide ($E_m = -344$ mV at pH 7.9) being more positive, and therefore easier to reduce, than the C-terminal disulfide ($E_m = -384$ mV at pH 7.9) that will require an excess of reduced TRX for its reduction. This difference suggests the existence of a sequential activation: reduction of the N-terminal disulfide would occur first and allow "pre-reduction" of the enzyme in a form that can be rapidly activated when the reducing power of the chloroplast reaches a threshold level. The knowledge acquired on the redox regulation of Sorghum NADP-MDH allowed transforming the constitutively active NAD-MDH from the thermophilic bacteria *Thermus flavus* into a TRX-dependent enzyme by grafting of the Sorghum N- and C-terminal extensions (Issakidis-Bourguet et al., 2006).

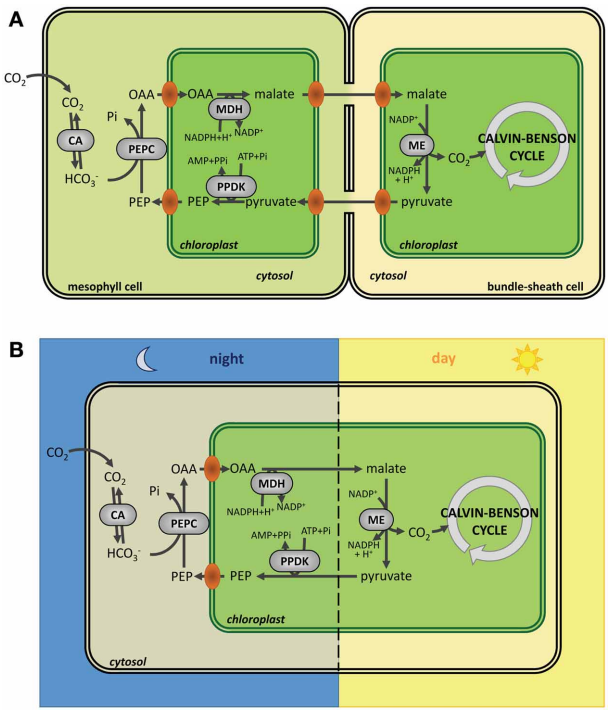

FIGURE 5 | Carbon fixation in C4 and CAM plants. (A) Carbon fixation in C4 plants. Compared to C3 plants, carbon fixation in C4 plants occurs in two steps: CO_2 is first fixed by PEPC (phosphoenolpyruvate carboxylase) in mesophyll cells to form oxaloacetate (OAA) that is converted to malate by NADP-MDH (NADP-malate dehydrogenase) before export to bundle sheath cells were CO_2 is released by ME (malic enzyme). Finally, CO_2 is fixed by Rubisco. **(B)** Carbon fixation in CAM plants. In CAM plants, carbon fixation is split in two steps, but, compared to C4 plants, this fixation is separated in time instead of being separated in space with cells fixing CO_2 by PEPC (phosphoenolpyruvate carboxylase) and accumulating malate during the night before release of CO_2 by ME (malic enzyme) and fixation by Rubisco during the day. CA, carbonic anhydrase; PEPC, phosphoenolpyruvate carboxylase; MDH, NADP malate dehydrogenase; ME, malic enzyme; PPDK, pyruvate, phosphate dikinase; OAA, oxaloacetate; PEP, phosphoenolpyruvate.

Interestingly, Chlamydomonas NADP-MDH was found to also possess N- and C-terminal extensions but the N-terminal extension does not contain any cysteine. The enzyme is also strictly dependent on TRX-mediated activation through reduction of a single regulatory disulfide located in its C-terminal extension (Lemaire et al., 2005). The redox potential of this disulfide ($E_m = -369$ mV at pH 7.9) is more positive than the

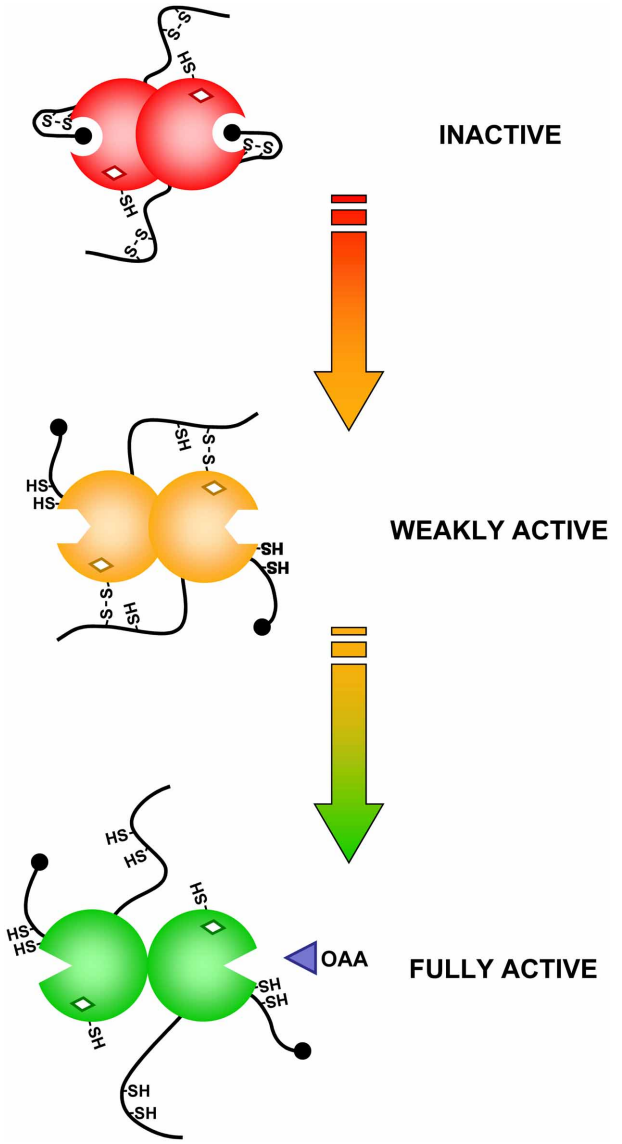

FIGURE 6 | Schematic representation of the activation mechanism of Sorghum NADP-MDH.

C-terminal disulfide of Sorghum NADP-MDH ($E_m = -384$ mV pH 7) and can be reduced by both TRXm and TRXf1 from Chlamydomonas. Conversely, Chlamydomonas TRXf1 is not able to activate Sorghum NADP-MDH, suggesting structural and thermodynamic differences between algal and land plants TRXf. Analysis of different Sorghum NADP-MDH mutants suggested that the redox potential of the algal TRXf is significantly lower than that of land plant TRXf (Lemaire et al., 2005). This surprising result suggests the existence of a coevolution of the redox properties of TRXs and NADP-MDH. From an evolutionary point of view, the redox regulation of Chlamydomonas NADP-MDH appears like a first step toward the complex regulation existing in land plants. The requirement for such a sophisticated control may be linked to the multicellular nature of land plants where malate is a circulating form of reducing power (Scheibe, 2004) while in Chlamydomonas malate is only exchanged between the unique chloroplast and the cytosol. These results also suggest that the redox regulatory sequences have been progressively added to non-regulated enzymes during the course of evolution.

Rubisco activase

Rubisco activase is a molecular chaperone of the AAA+ family that uses the energy from ATP hydrolysis to release tight binding inhibitors from the active site of Rubisco (reviewed in Portis, 2003; Portis et al., 2008). The ATPase activity of Rubisco activase is controlled by the ADP/ATP ratio and/or by the Fd/TRX system. In many species, such as Arabidopsis, two isoforms of activase are present: a short form (beta isoform) and a long form (alpha isoform). The two forms are either generated by alternative splicing or encoded by distinct genes (Werneke and Ogren, 1989; Salvucci et al., 2003; Yin et al., 2010). Compared to the beta isoform, the alpha isoform differs by the presence of a C-terminal extension containing two conserved cysteines. In Arabidopsis, site-directed mutagenesis revealed that these residues, Cys392 and Cys411, form a disulfide reduced by TRXf in the light (Zhang et al., 2001). The extension contains negative charges that interact with the ATP binding site (Wang and Portis, 2006; Portis et al., 2008). Indeed, the oxidized enzyme has a decreased affinity for ATP and is inhibited by ADP while reduction by TRXf alleviates this inhibition. This regulation controls the activity of both alpha and beta isoforms in the holoenzyme (Zhang et al., 2001). Some species such as tobacco, maize, or green algae only contain the short beta isoform but still exhibit light dependent regulation of Rubisco activase activity (Salvucci et al., 1987). A recent study revealed that tobacco beta isoform, by contrast with the beta isoform from Arabidopsis, has a unique sensitivity to ADP/ATP ratios that is responsible for the light regulation of the activity (Carmo-Silva and Salvucci, 2013). Several studies have concluded that Rubisco activase forms hexamers in solution, and that this may be the active form (Keown et al., 2013; Mueller-Cajar et al., 2013). The structure of tobacco Rubisco activase forms a helical arrangement in the crystal structure, with six subunits per turn (Stotz et al., 2011). However, Rubisco activase appears to form a wide range of structures in solution, ranging from monomers to oligomers, and an open spiraling structure rather than a closed hexameric structure has been recently proposed (Keown et al., 2013). Numerous features of Rubisco activase are reminiscent of GAPDH which also can assemble into higher oligomeric states and has both redox-regulated (GAPB) and non-redox-regulated (GAPA) subunits that differ by a C-terminal extension containing a TRX-reduced regulatory disulfide that exerts control on the activity of the mixed oligomer (A_nB_n-GAPDH).

CP12

CP12 was discovered by serendipity in higher plants as a novel protein of 78 amino acids with a C-terminal sequence homologous to the CTE of GAPB subunits of GAPDH (Pohlmeyer et al., 1996). CP12 was then found to be widespread in oxygenic photosynthetic organisms, including cyanobacteria, and GAPB

subunits of land plants are now believed to be the result of a gene fusion event between GAPA and CP12 that must have occurred at the origins of Streptophytes (Petersen et al., 2006) or before (Robbens et al., 2007).

Most CP12s contain four conserved cysteines able to form two disulfide bonds (Groben et al., 2010; Marri et al., 2010; Stanley et al., 2013). CP12 from both *Synechococcus* sp. PCC7942, Chlamydomonas and Arabidopsis were the most extensively studied. The protein is intrinsically disordered, particularly so when it is fully reduced, but still poorly structured when it bears both disulfide bonds (Graciet et al., 2003; Marri et al., 2008, 2010). In Arabidopsis, the C-terminal disulfide has redox properties similar to GAPB disulfide [$E_m = -352$ mV at pH 7.9, (Marri et al., 2008)] and is reduced by TRXs, though with no strict specificity (Marri et al., 2009). Oxidized CP12 binds to A_4-GAPDH more tightly than CTE through interactions with both the bound coenzyme and the catalytic site of the enzyme. (Fermani et al., 2007, 2012; Matsumura et al., 2011). In Arabidopsis (Marri et al., 2008; Fermani et al., 2012) and Chlamydomonas (Kaaki et al., 2013), A_4-GAPDH binds two CP12, while four CP12 are bound to A_4-GAPDH in Synechococcus (Matsumura et al., 2011). CP12 binding is very strong in Chlamydomonas (k_D 0.4 nM) and causes inhibition of GAPDH activity (Erales et al., 2011), whereas in Arabidopsis CP12 binding is much weaker [k_D 0.2 μM, (Marri et al., 2008)] and inhibition is negligible (Marri et al., 2005, 2008). However, in these and other organisms, the binary complex GAPDH/CP12 can then bind PRK forming the GAPDH/CP12/PRK ternary complex, in which both enzyme activities are strongly down-regulated (Marri et al., 2005). NAD(H) binding to GAPDH is an absolute requirement for complex formation because the 2′-phosphate of NADPH sterically hinders the attachment of CP12 (Matsumura et al., 2011; Fermani et al., 2012). Dissociation of the complex, and recovery of enzyme activity is rapidly obtained by reduced TRXs (Marri et al., 2009), but also by BPGA, NADPH or ATP that displace CP12 from its binding sites on GAPDH and PRK, respectively (Wedel et al., 1997; Scheibe et al., 2002; Graciet et al., 2004; Marri et al., 2005; Tamoi et al., 2005; Howard et al., 2008). CP12-assembled complexes of GAPDH and PRK accumulate in the dark, both in cyanobacteria and in chloroplasts, probably favored by the oxidation of the TRX pool and by low NADP(H)/NAD(H) ratios (Scheibe et al., 2002; Tamoi et al., 2005; Howard et al., 2008, 2011b). In Synechococcus, inactivation of *cp12* gene impaired cell growth in normal light-dark cycles, but not under continuous illumination, supporting the role of CP12 in light-dark regulation of the CBC in this organism (Tamoi et al., 2005). CP12 is also coded by the small genome of cyanophages that infect marine cyanobacteria including Synechococcus, apparently with the function of redirecting the carbon flux of the prokaryote from the CBC to the pentose phosphate pathway, thereby sustaining NADPH production for phage replication (Thompson et al., 2011). However, other functions of CP12 have been proposed, particularly in land plants like tobacco, where antisense suppression of CP12 severely restricts growth and alters carbon partitioning through mechanisms that are still poorly understood (Howard et al., 2011a).

EVOLUTION OF REGULATORY SEQUENCES

Comparison of the redox regulatory properties of enzymes from cyanobacteria, diatoms, algae and higher plants suggest that the light-dependent regulation mediated by TRX has been progressively introduced during evolution (Ruelland and Miginiac-Maslow, 1999; Lemaire et al., 2007) (**Figure 7**). Comparison with non-redox-regulated forms suggest that regulatory sequences have been grafted within N- or C-terminal extensions (GAPDH, NADP-MDH, Rubisco Activase) or inserted in the sequence (FBPase). For some enzymes, there is no obvious insertion or extension but this mainly applies to enzymes unique to the CBC (PRK, SBPase). Interestingly, in the diatom *O. sinensis* PRK contains the regulatory cysteines but the redox potential of the disulfide is less negative than in PRK from higher plants, suggesting that the enzyme might not be regulated by TRX *in vivo* (Michels et al., 2005). In a survey on different algal groups, redox-regulation of PRK was found to be greatest in chlorophytes, but low or absent in a red alga and most chromalveolates (including diatoms), and linked to the number of amino acids separating the two regulatory cysteine residues (Maberly et al., 2010). Several other enzymes in diatoms may also be TRX independent due to the absence of regulatory cysteines such as NADP-MDH (Ocheretina et al., 2000) or GAPDH (Liaud et al., 2000).

PROTEOMICS UNRAVEL NEW REDOX-DEPENDENT REGULATIONS

Recent advances in the field of proteomics and genomics associated with our increasing understanding of redox post-translational modifications have considerably challenged our current models of the redox dependent regulation of the CBC described in the above sections. These new data suggest that all enzymes of the CBC are regulated by a complex network of redox PTMs that is only starting to emerge. The following sections will describe these new developments and discuss their potential physiological and cellular importance.

THIOREDOXIN TARGETS

As described above, the availability of plant genome sequences revealed the existence of an unsuspected multiplicity of TRXs. At the beginning of the 2000s, the number of TRXs was even higher than the number of known TRX-regulated targets. This multiplicity raised questions about the specificity of the different TRX isoforms for their target enzymes. Moreover, systematic biochemical analysis of the ability of Arabidopsis TRX isoforms to activate different chloroplastic TRX targets revealed that TRXs are not equivalent and exhibit strong specificities (Collin et al., 2003, 2004). This suggested that additional TRX targets probably remained to be identified and prompted several groups to develop new proteomic-based strategies to identify these unrecognized targets (reviewed in Lemaire et al., 2007; Schürmann and Buchanan, 2008; Lindahl et al., 2011). Two main strategies have been employed. The most common is based on the ability of a monocysteinic TRX to form covalent heterodimers with its target enzymes. Indeed, studies on the reaction mechanism allowing the reduction of an oxidized target by a reduced TRX had established that the most N-terminal cysteine of TRX active site performs an initial nucleophilic attack leading to the formation

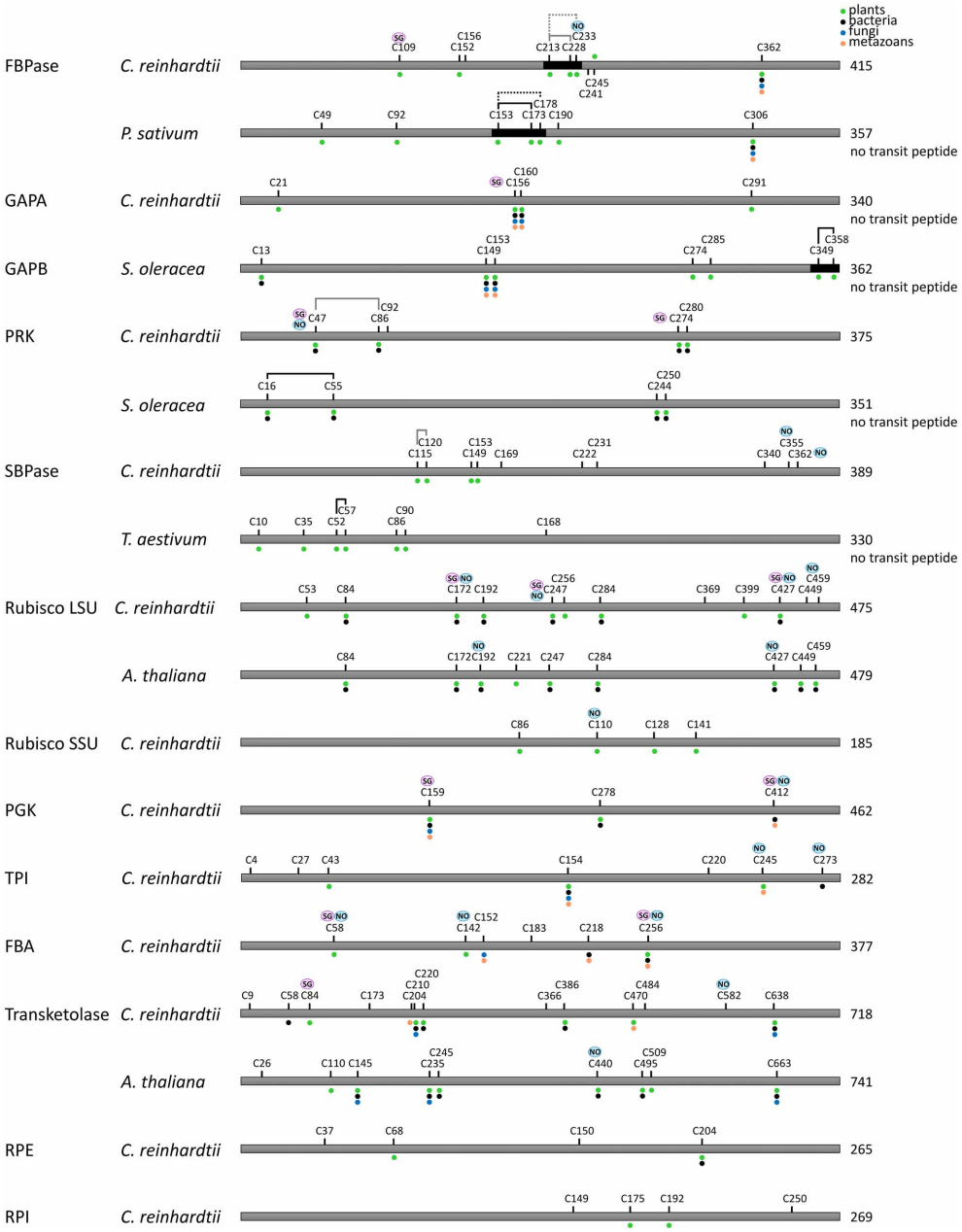

FIGURE 7 | Redox regulated cysteines in Calvin–Benson cycle enzymes. All proteins are schematically represented linearly. The numbering corresponds to the sequence with the transit peptide, except when "no transit peptide" is indicated on the right. Lines between cysteines indicate confirmed (black) or suggested (black and dashed) disulfide bonds, whereas gray lines indicate putative regulatory disulfides in *C. reinhardtii* by homology with the disulfide identified for other species. Cysteines identified as nitrosylated and glutathionylated are labeled with NO (blue) and SG (purple), respectively. The dots indicate cysteine conservation in photosynthetic organisms (green), bacteria (black), fungi (blue) and metazoans (orange). Dark boxes correspond to insertions/extension present in the TRX-regulated isoforms and absent in the isoforms not regulated by TRX.

of a transient mixed-disulfide between the TRX and the target. This mixed-disulfide is then immediately reattacked by the second cysteine of the active site to yield an oxidized TRX and a reduced target. Consequently, mutating the second cysteine of the active site allows stabilization of the mixed-disulfide. TRX-affinity columns, based on a resin-bound monocysteinic TRX, have been employed to trap TRX targets which can be eluted by DTT reduction and identified by proteomic analysis. Many targets have been identified using this type of affinity columns (Motohashi et al., 2001; Goyer et al., 2002; Balmer et al., 2003, 2004b, 2006b; Lindahl and Florencio, 2003; Lemaire et al., 2004; Wong et al., 2004; Yamazaki et al., 2004; Hosoya-Matsuda et al.,

2005; Marchand et al, 2006; Marchand et al., 2010; Pérez-Pérez et al., 2006, 2009). In addition, affinity columns based on wild-type TRX have also been used to detect proteins interacting electrostatically with TRX (Balmer et al., 2004a). The second most widely used approach is based on the visualization of proteins reduced by TRX *in vitro* by specific labeling of exposed thiols by fluorescent probes like monobromobimane (mBBr) (Yano et al., 2001; Marx et al., 2003; Wong et al., 2003, 2004; Balmer et al., 2006a,b; Yano and Kuroda, 2006; Hall et al., 2010) or Cy5 maleimide (Maeda et al., 2004) or by radioactive probes like ^{14}C-iodoacetamide (Marchand et al., 2004; Marchand et al, 2006). A comparison between both methods suggested that they are complementary since only a partial overlap is found between the different targets identified (Marchand et al, 2006).

All these proteomic studies have allowed identifying more than 300 putative TRX targets from the cyanobacteria *Synechocystis* sp. PCC6803, the unicellular green alga *Chlamydomonas reinhardtii* and numerous higher plant species (reviewed in Michelet et al., 2006; Lindahl et al., 2011). These proteomic methods are therefore powerful but they also suffer from a lack of specificity. Indeed, proteomic approaches with different types of TRX yielded roughly the same targets while a strong or exclusive specificity of most targets for a specific TRX type is generally observed *in vitro*. For example, columns based on monocysteinic TRXf and TRXm basically retain the same targets while FBPase and GAPDH are exclusively activated by TRXf and not by TRXm (Collin et al., 2003; Marri et al., 2009). This suggests that monocysteinic TRX have peculiar properties distinct from the WT enzyme and/or that the loss of specificity is due to the use of high concentrations of TRX. Indeed, while enzymes show a preference for some TRX types at physiological TRX concentration, many TRX types become able to significantly activate a number of TRX targets if used at high concentration (Collin et al., 2003, 2004). By contrast, the diversity of the targets appeared to be strongly dependent on the type of protein extracts employed (organism, tissue, growth conditions. . .). The problem of specificity is even larger since monocysteinic GRX columns also retain the same type of targets than those bound on TRX columns (Rouhier et al., 2005; Li et al., 2007). Classical GRXs belong to the TRX family and contain an active site disulfide (Cys-Pro-Trp/Phe-Cys) that is reduced by glutathione (Rouhier et al., 2008; Zaffagnini et al., 2012c). GRXs can reduce disulfide bonds on their target proteins, although much less efficiently than TRX, but they are thought to play a more prominent role in the control of protein (de)glutathionylation (Zaffagnini et al., 2012c).

Among all putative TRX targets, more than 130 are located in chloroplasts (Lemaire et al., 2007; Lindahl and Kieselbach, 2009). The well-established targets of TRX participating directly or indirectly in the CBC were recovered by proteomic approaches (FBPase, SBPase, GAPDH, PRK, CP12, Rubisco activase). More surprisingly, all other enzymes of the CBC were also identified among putative targets, suggesting that they might all be redox regulated (**Table 1**). All CBC enzymes were, however, not identified within the same study, possibly because of a low coverage rate due to the use of 2D-gels and MALDI-TOF mass spectrometry in most studies. This suggests that the number of TRX targets could be significantly higher than presently known and that the combination of TRX-affinity chromatography or thiol-labeling with modern gel-free proteomic methods may reveal a much greater diversity of new potential targets of TRXs.

Nevertheless, the identification of all CBC enzymes as potential targets of TRXs suggested the existence of a complex redox control of these enzymes that may be much more sophisticated than the light-dependent regulation initially uncovered for four enzymes of the cycle. These features may allow a fine tuning of the Calvin–Benson cycle in response to environmental changes that affect ROS production and the intracellular redox state. There are two non-mutually exclusive possibilities to explain this surprising result. First, all the CBC enzymes may contain a TRX-reducible disulfide bond, like the four established TRX-targets. These regulations may have been initially missed, for example, because of their low activation upon reduction (e.g., below 50%) or because the disulfide controls enzyme properties that have not been investigated (protein stability, cooperativity between subunits, protein-protein interactions, etc.). This may be the case for phosphoglycerate kinase (PGK) which was shown to be redox regulated, possibly by TRX, in *Phaeodactylum tricornutum* (Bosco et al., 2012) and *Synechocystis* sp. PCC6803 (Tsukamoto et al., 2013). To date, none of these putative TRX dependent redox regulations have been confirmed experimentally on any other CBC enzyme. The second possibility is that these cysteines are not attacked (or reduced) by TRX because they are engaged in a disulfide bond but because they harbor another type of cysteine oxidative modification. Indeed, although TRX are efficient protein disulfide reductases, they are also playing a role in the control of other post-translational modifications including sulfenylation, nitrosylation or glutathionylation. Therefore, putative TRX targets identified by proteomic approaches may represent proteins containing diverse types of redox PTM and should therefore be considered as putative redox regulated proteins rather than proteins containing a TRX-reducible disulfide. This was demonstrated for Chlamydomonas isocitrate lyase, an enzyme of the glyoxylate cycle participating in acetate assimilation which was retained on a monocysteinic TRX affinity column (Lemaire et al., 2004). Detailed biochemical analysis of this enzyme revealed that the enzyme is strongly and reversibly inhibited by glutathionylation but does not contain any TRX-reducible disulfide bond (Bedhomme et al., 2009). We recently obtained comparable results with the Calvin–Benson enzyme triosephosphate isomerase (TPI) which does not appear to contain a regulatory disulfide but was found to undergo glutathionylation and nitrosylation *in vitro* (Zaffagnini et al., 2013a). Although the number of studies is more limited, mounting evidence suggests that glutathionylation and nitrosylation also control enzymes of the CBC. These recent developments are detailed in the next sections.

MULTIPLE REDOX POST-TRANSLATIONAL MODIFICATIONS

During the last decade, glutathionylation and nitrosylation have emerged as crucial PTMs playing a major role in numerous fundamental cell processes, especially cell signaling pathways (Hess et al., 2005; Besson-Bard et al., 2008; Mieyal et al., 2008; Rouhier et al., 2008; Dalle-Donne et al., 2009; Foster et al., 2009; Astier et al., 2011; Hess and Stamler, 2012; Zaffagnini et al., 2012c).

Table 1 | Summary of redox proteomic analyses of Calvin–Benson cycle enzymes and related proteins.

	Putative nitrosylation targets (references)	Putative nitrosylated cysteine (Cys numbering)	Putative glutathionylation targets (references)	Putative glutathionylated cysteine (Cys numbering)	Putative thioredoxin targets (references)	Putative glutaredoxin targets (references)
Rubisco S	2, 3, 5, 6, 8, 11, 12, 26	C^{64}	1		10, 13, 14, 15, 16, 20	17
Rubisco L	2, 3, 4, 5, 6, 7, 8, 12, 26	$C^{172/247/427/459}$/$C^{192/427}$	1	$C^{172/247/427}$	10, 15, 16, 19, 20, 22, 23	17
PGK	2, 6, 8	C^{411}	1, 9	C^{411}/C^{158}	16, 21	17
GAPDH A	2, 3, 5, 7, 8, 11		1		13, 16, 18, 20, 22	17
GAPDH B	5, 8				14, 16	
TPI	2, 3, 5, 8, 11	$C^{245/273}$			14, 18, 20	17
FBA	2, 3, 5, 6, 8, 12, 26	$C^{58/142/256}$	1, 25	$C^{58/256}$	10, 15, 22, 24	17
FBPase	2	C^{233}	1	C^{109}	16, 18, 22	17
Transketolase	2, 4, 5, 8, 11, 12	C^{582}/C^{440}	1	C^{84}	14, 16, 18, 20, 21, 22	17
SBPase	2, 8, 12	$C^{355/362}$	1		10, 13, 14, 18, 20	
RPE	2		1		14, 18, 20	
RPI	2, 3		1, 9		10, 18, 20	
PRK	2, 3, 6, 7, 8, 11	C^{47}	1	$C^{47/274}$	10, 14, 18, 20, 22	17
CP12*	3				18, 20	
NADP-MDH	2	C^{74}	1		14, 16, 18, 20	
Rubisco Activase	2, 3, 4, 6, 8, 11	$C^{196/289}$/C^{175}	1		13, 14, 15, 16,18, 20	17

Calvin–Benson cycle enzymes are listed in the order of the reactions starting with Rubisco. The three last enzymes participate indirectly in the regulation of the cycle. The references correspond to proteomic studies that allowed identification of the enzyme as nitrosylated, as glutathionylated or as thioredoxin or glutaredoxin targets. Modification sites identified by proteomic methods are listed with the numbering of the cysteines identified as glutathionylated (Zaffagnini et al., 2012a) and nitrosylated (Morisse et al. manuscript in preparation) in Chlamydomonas reinhardtii in normal text and the cysteines found as nitrosylated in Arabidopsis thaliana (Fares et al., 2011) in italics. Numberings correspond to the full-length protein with its transit peptide. References: 1, (Zaffagnini et al., 2012a); 2, Morisse et al. manuscript in preparation; 3, (Lin et al., 2012); 4, (Fares et al., 2011); 5, (Lindermayr et al., 2005); 6, (Kato et al., 2013); 7, (Romero-Puertas et al., 2008); 8, (Tanou et al., 2012); 9, (Michelet et al., 2008); 10, (Lemaire et al., 2004); 11, (Tanou et al., 2009); 12, (Abat and Deswal, 2009); 13, (Motohashi et al., 2001); 14, (Balmer et al., 2003); 15, (Balmer et al., 2006b); 16, (Balmer et al., 2004a); 17, (Rouhier et al., 2005); 18, (Marchand et al., 2004); 19, (Lindahl and Florencio, 2003); 20, (Marchand et al, 2006); 21, (Balmer et al., 2006a); 22, (Pérez-Pérez et al., 2009); 23, (Hall et al., 2010); 24, (Marchand et al., 2010); 25, (Ito et al., 2003); 26, (Abat et al., 2008).

Glutathione is the major low molecular weight antioxidant in most species and exists in the reduced (GSH) and oxidized (GSSG) forms. GSH is the major form due to constant reduction of GSSG to GSH by glutathione reductase (GR) at the expense of NADPH. Glutathione is considered as a major cellular antioxidant and redox buffer but also plays an important role in a myriad of cellular and physiological functions including detoxification of heavy metals and xenobiotics, root growth or pathogen responses (Noctor et al., 2012). Glutathionylation is a post-translational modification triggered by oxidative stress conditions and consisting of the formation of a mixed-disulfide between a protein free thiol and the thiol of a molecule of glutathione. Although the precise mechanism leading to glutathionylation is still unclear *in vivo*, it is considered to occur mainly either through reactive oxygen species (ROS)-dependent sulfenic acid formation followed by reaction with reduced glutathione (GSH) or by thiol/disulfide exchange with oxidized glutathione (GSSG). The reverse reaction, named deglutathionylation, is mainly catalyzed by GRXs.

Nitrosylation consists in the formation of nitrosothiols by reaction of protein thiols with nitric oxide (NO). It can be triggered chemically by reactive nitrogen species (RNS) which includes NO and its related species (such as the nitrosonium cation, NO^+; nitroxyl anion, NO^-; dinitrogen trioxide, N_2O_3 or peroxynitrite, $ONOO^-$) but also by transnitrosylation reactions mediated by small nitrosothiols (e.g., nitrosoglutathione, GSNO) or by other nitrosylated proteins (Hogg, 2002; Hess et al., 2005; Benhar et al., 2009; Zaffagnini et al., 2013b). The reduction of nitrosothiols on proteins, i.e., denitrosylation, entails two possible mechanisms dependent on reduced glutathione (GSH) or reduced TRX (Benhar et al., 2009; Sengupta and Holmgren, 2013).

To date, several hundreds of targets of glutathionylation and nitrosylation have been identified in bacteria, yeast, animals and plants, suggesting a role for these redox modifications in many cellular processes (Mieyal et al., 2008; Astier et al., 2011; Hess and Stamler, 2012; Zaffagnini et al., 2012c; Maron et al., 2013). In our two recent studies, the use of biotin-based enrichment strategies using streptavidin affinity chromatography combined

with up-to date mass spectrometry instruments allowed identification of 225 glutathionylated proteins and 492 nitrosylated proteins in Chlamydomonas (Zaffagnini et al., 2012a; Morisse et al. manuscript in preparation). There is a striking overlap between potential TRX targets and proteins identified as nitrosylated or glutathionylated through proteomic studies. This suggests that these proteins are regulated by multiple redox PTMs or that the methods aimed at identifying TRX targets also identified nitrosylated and glutathionylated proteins. The latter possibility is consistent with the fact that TRX was proposed to catalyze both (de)nitrosylation and (de)glutathionylation reaction for some targets (Benhar et al., 2008; Greetham et al., 2010; Bedhomme et al., 2012; Zaffagnini et al., 2013b).

Notably, all CBC enzymes appear to be modified by glutathionylation (**Table 1**). Proteomic studies initially reported *in vivo* glutathionylation for fructose-1,6-bisphosphate aldolase (FBA) in Arabidopsis (Ito et al., 2003) and for phosphoglycerate kinase (PGK) and ribose-5-phosphate isomerase (RPI) in Chlamydomonas (Michelet et al., 2008). More recently, all CBC enzymes were found to undergo glutathionylation in Chlamydomonas (Zaffagnini et al., 2012a, 2013a). The modifications of FBA, PGK and A_4-GAPDH from Chlamydomonas were confirmed by demonstrating that the purified protein is glutathionylated after treatment with BioGSSG (biotinylated oxidized glutathione) *in vitro*. Chlamydomonas PRK was found to be strongly inhibited by GSSG and the activity could be fully recovered after DTT treatment (Zaffagnini et al., 2012a). The glutathionylation of different isoforms of GAPDH from Arabidopsis was investigated in detail (Zaffagnini et al., 2007). Arabidopsis A_4-GAPDH was shown to be glutathionylated *in vitro* on its catalytic cysteine with a concomitant loss of enzyme activity. The enzyme is very sensitive to oxidants and is rapidly and irreversibly inactivated by H_2O_2. However, incubation of the enzyme with H_2O_2 in the presence of GSH leads to glutathionylation, most likely through a mechanism involving a sulfenic acid intermediate. Therefore, glutathionylation efficiently protects A_4-GAPDH from irreversible oxidation and glutathionylated A_4-GAPDH was reported to be efficiently reactivated by GRXs (Zaffagnini et al., 2008; Couturier et al., 2009; Gao et al., 2010). Similar results were reported for the cytoplasmic GAPDH isoform (GAPC) (Holtgrefe et al., 2008; Bedhomme et al., 2012). By contrast, the A_2B_2-GAPDH isoform and its higher oligomeric state A_8B_8-GAPDH were not found to undergo glutathionylation *in vitro* (Zaffagnini et al., 2007). Chlamydomonas TPI was also found to be glutathionylated *in vitro* but with no apparent effect on the enzyme activity (Zaffagnini et al., 2013a). Finally, glutathionylation is also likely affecting the CBC indirectly through regulation of TRXf (Michelet et al., 2005). Among all chloroplastic TRXs, TRXf from diverse species specifically undergo glutathionylation on a strictly conserved extra cysteine that is distinct from the two-active site cysteines and located in the vicinity of the active site. The glutathionylation of TRXf strongly decreases its ability to activate A_2B_2-GAPDH and NADP-MDH likely by perturbing the interaction with FTR since glutathionylated TRXf is less efficiently reduced in the light. This suggests that glutathionylation could affect all TRXf targets which include many enzymes involved in carbon fixation and other chloroplast metabolic pathways. A_2B_2-GAPDH being specifically activated by TRXf, GAPDH activity is likely fully down-regulated under conditions leading to protein glutathionylation in chloroplasts, such as enhanced ROS production either by direct glutathionylation of A_4-GAPDH on its catalytic cysteine or indirectly through glutathionylation of TRXf that decrease the activation of A_2B_2-GAPDH.

All CBC enzymes were also identified as nitrosylated proteins by proteomic approaches (**Table 1**). None of these putative regulations has been confirmed biochemically with the exception of Chlamydomonas TPI that was shown to be partially inhibited by nitrosylation (Zaffagnini et al., 2013a) and land plant Rubisco which appears to be inhibited by nitrosylation (Abat et al., 2008; Abat and Deswal, 2009). Cytosolic GAPDH was shown to be completely inhibited by nitrosylation and fully reactivated by GSH or, less efficiently, by TRXs (Holtgrefe et al., 2008; Zaffagnini et al., 2013b). These properties may likely apply to chloroplastic GAPDH considering its strong structural and biochemical similarities with cytosolic GAPDH.

Recent proteomic studies also allowed identification of the cysteine residues undergoing nitrosylation and glutathionylation (**Table 1**). A schematic representation of the sites identified as nitrosylated, glutathionylated or forming a TRX-reducible disulfide bond is presented in **Figures 7**, **8**. Many of these cysteines are conserved, especially in photosynthetic organisms. Some sites are shared between different modifications while others are unique. For example, in Chlamydomonas PRK, Cys47 appears modified by the 3 types of redox PTMs while Cys274 was only found to undergo glutathionylation. By contrast, Chlamydomonas FBPase is modified on 4 distinct cysteines that are all conserved in photosynthetic organisms: the enzyme undergoes glutathionylation on Cys109, nitrosylation on Cys233 while the TRX-reducible disulfide bond is most likely located between Cys213 and Cys219 by homology with higher plant FBPase. The large subunit of Rubisco appears to undergo multiple modifications with Cys459 identified as nitrosylated and 3 cysteines (Cys172, Cys247, Cys427) undergoing both nitrosylation and glutathionylation (**Table 1**, **Figure 7**). These results are consistent with previous studies (reviewed in (Moreno et al., 2008)) that suggested a redox control of the activity and/or the stability of Rubisco involving Cys172 (Moreno and Spreitzer, 1999; Marcus et al., 2003), Cys427 (Muthuramalingam et al., 2013) or Cys 459 (Marin-Navarro and Moreno, 2006). The multiplicity of the modification sites suggest that CBC enzymes are indeed regulated by multiple redox PTMs although the different modifications may not occur at the same time, at the same site, to the same extent or under the same physiological/cellular conditions.

CBC enzymes appear to be regulated by an intricate network of redox PTMs whose dynamics remains to be explored. These regulations may allow a tight coupling between the activity of CBC enzymes and the intracellular redox state linked to environmental conditions. While dithiol/disulfide exchange reactions controlled by TRXs allow light-dependent activation of CBC enzymes, glutathionylation could constitute an alternative mechanism of regulation of the CBC pathway occurring under illumination and dependent on ROS production and glutathione.

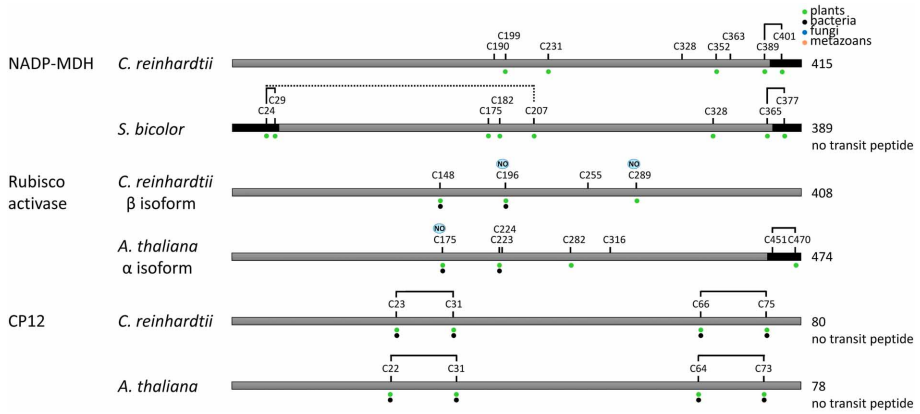

FIGURE 8 | Redox regulated cysteines in proteins controlling indirectly the Calvin–Benson cycle. All proteins are schematically represented linearly. The numbering corresponds to the sequence with the transit peptide, except when "no transit peptide" is indicated on the right. Lines between cysteines indicate confirmed (black) or suggested (black and dashed) disulfide bonds. Cysteines identified as nitrosylated and glutathionylated are labeled with NO (blue) and SG (purple), respectively. The dots indicate cysteine conservation in photosynthetic organisms (green), bacteria (black), fungi (blue) and metazoans (orange). In *A. thaliana* Rubisco activase, the regulatory cysteines Cys451 and Cys470 in the precursor correspond to Cys392 and Cys411 in the mature form. Dark boxes correspond to insertions/extension present in the TRX-regulated isoforms and absent in the isoforms not regulated by TRX.

Indeed, all available data suggest that glutathionylation down-regulates the activity of numerous CBC enzymes. Therefore, it has been proposed that glutathionylation could constitute a new mechanism of regulation of photosynthetic metabolism allowing a fine tuning of the CBC cycle in order to redistribute reducing power (in the form of NAPDH) and energy (in the form of ATP) within chloroplasts under oxidative stress, thereby favoring ROS scavenging (Michelet et al., 2005; Lemaire et al., 2007). This redistribution may be required transiently to cope with stress conditions. Glutathionylation also constitutes a mechanism of protection of CBC enzymes containing highly reactive cysteines from irreversible oxidation in the presence of ROS, as demonstrated for GAPDH (Zaffagnini et al., 2007). It has also been proposed that the glutathionylation/deglutathionylation cycle catalyzed by GRXs may contribute to ROS scavenging within chloroplasts (Zaffagnini et al., 2012b). In addition to regulation of CBC enzymes, redox signaling contributes to numerous short- and long-term acclimation responses that allow plants to adapt to fluctuating environmental conditions by enabling metabolic readjustments to maintain cellular homeostasis (Scheibe and Dietz, 2012).

It should be kept in mind that redox PTMs of CBC enzymes do not necessarily imply that the modification regulates the CBC pathway. First, it is possible that the modification does not affect the activity of the protein as shown for the glutathionylation of Chlamydomonas TPI (Zaffagnini et al., 2013a). However, even if it does, the extent of the modification *in vivo* being undetermined, it is possible that it only affects a minor pool of the total protein (especially for abundant proteins such as those involved in the CBC) and modification of this pool may not be limiting for the pathway. Finally, numerous identified proteins may represent moonlighting proteins that, upon redox PTMs, are diverted to new functions unrelated to their metabolic role in carbon metabolism as shown for cytosolic GAPDH in mammals (Hara et al., 2005) (see Zaffagnini et al. this series). Indeed, upon apoptotic stimulation, nitrosylation of mammalian GAPDH triggers its translocation to the nucleus where it regulates gene expression through several mechanisms including transnitrosylation of nuclear proteins (Kornberg et al., 2010). Plant cytosolic GAPDH was also shown to undergo nitrosylation and to relocalize to the nucleus under stress condition but the exact physiological function of the modification remains to be established (Holtgrefe et al., 2008; Vescovi et al., 2013; Zaffagnini et al., 2013b). Moreover, the glutathionylation of mammalian GAPDH has been shown to regulate endothelin-1 (ET-1) expression by altering the binding of GAPDH to the 3′ untranslated region of ET-1 mRNA thereby increasing its stability and resulting in increased ET-1 protein levels and endothelial vasoconstriction (Rodriguez-Pascual et al., 2008). The only CBC enzyme for which a moonlighting function has been reported is Rubisco. Indeed, under oxidative stress the Rubisco holoenzyme, composed of 8 small subunits (SSU) and 8 large subunits (LSU) disassembles into its constituents and LSU subsequently binds chloroplast mRNAs non-specifically and forms large particles (Yosef et al., 2004; Knopf and Shapira, 2005; Cohen et al., 2006). Recently, Chlamydomonas LSU but not SSU was shown to accumulate in chloroplast stress granules (cpSGs) under oxidative stress conditions (Uniacke and Zerges, 2008). cpSGs are RNA granules related to mammalian stress granules that form during oxidative stress and disassemble during recovery from stress. This study therefore suggested a novel function of Rubisco LSU as an mRNA-localizing and assembly factor of cpSGs (Uniacke and Zerges, 2008). This moonlighting function being triggered by oxidative stress conditions, it may likely be regulated by redox PTMs. Since cysteine modifications control several moonlighting functions of cytosolic GAPDH, the same may be true for chloroplastic GAPDH isoforms participating in the CBC which also undergo multiple redox PTMs. These redox PTMs are likely to play an important role in ROS sensing and to allow adaptation or alternatively

trigger programmed cell death under varying environmental conditions.

CONCLUDING REMARKS

Recent proteomic studies suggest that CBC enzymes undergo multiple types of redox PTMs including nitrosylation, glutathionylation and oxido-reduction of disulfide bonds. The possible regulations by additional redox PTMs whose importance starts to emerge in non-photosynthetic organisms, such as sulfenylation, sulfhydration or cysteinylation, will also have to be explored. Many data also suggest the existence of a strong interplay between the different types of redox PTMs as recently described for nitrosylation and glutathionylation (Zaffagnini et al., 2012c). All these data suggest that CBC enzymes are regulated by a complex and highly dynamic network of redox PTMs. Unraveling the importance and function of these redox modifications under diverse physiological and growth conditions and characterizing the underlying molecular and structural determinants will certainly constitute a major challenge for future studies. Acquiring this knowledge is highly desirable considering the central role of the CBC in the determination of crop yields, CO_2 fixation, biomass and biofuel production and plant adaptation to stress conditions.

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Plastid thioredoxins: a "one-for-all" redox-signaling system in plants

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The sessile nature of plants forces them to face an ever-changing environment instead of escape from hostile conditions as animals do. In order to overcome this survival challenge, a fine monitoring and controlling of the status of the photosynthetic electron transport chain and the general metabolism is vital for these organisms. Frequently, evolutionary plant adaptation has consisted in the appearance of multigenic families, comprising an array of enzymes, structural components, or sensing, and signaling elements, in numerous occasions with highly conserved primary sequences that sometimes make it difficult to discern between redundancy and specificity among the members of a same family. However, all this gene diversity is aimed to sort environment-derived plant signals to efficiently channel the external incoming information inducing a right physiological answer. Oxygenic photosynthesis is a powerful source of reactive oxygen species (ROS), molecules with a dual oxidative/signaling nature. In response to ROS, one of the most frequent post-translational modifications occurring in redox signaling proteins is the formation of disulfide bridges (from Cys oxidation). This review is focused on the role of plastid thioredoxins (pTRXs), proteins containing two Cys in their active site and largely known as part of the plant redox-signaling network. Several pTRXs types have been described so far, namely, TRX *f*, *m*, *x*, *y*, and *z*. In recent years, improvements in proteomic techniques and the study of loss-of-function mutants have enabled us to grasp the importance of TRXs for the plastid physiology. We will analyze the specific signaling function of each TRX type and discuss about the emerging role in non-photosynthetic plastids of these redox switchers.

Keywords: thioredoxin, redox signaling, photosynthesis, oxidative stress, carbon metabolism

INTRODUCTION

Plant H_2O photolysis provides electrons (and protons) to feed the photosynthetic electron transport chain (PETC) to allow NADPH and ATP synthesis for CO_2 fixation. During the process, O_2 and O_2-derived by-products, called reactive oxygen species (ROS), are also released. However, ROS molecules ($^1O_2$, O_2^-, H_2O_2, and $^\bullet OH$) are even more oxidant than O_2 itself (Noctor and Foyer, 1998). ROS-exposed cellular components (proteins, lipids, polysaccharides, and DNA) can be damaged, especially under environmental conditions leading to oxidative stress. Recent works also point to NO as an emerging oxidative compound (Lamotte et al., 2005; Grun et al., 2006; Neill et al., 2008a,b; Wilson et al., 2008) The NO-derived species are called reactive nitrogen species (RNS) and *S*-nitrosylation is the post-translational change they promote. ROS and RNS levels are controlled by thiol peroxidase-like plastid peroxiredoxins (PRX). Nevertheless, despite their toxicity at high concentrations, ROS and RNS play an important role as central signaling molecules (Laloi et al., 2004, 2007; Bellin et al., 2013).

Thiol groups (−SH) of cysteine residues are susceptible to ROS oxidation, provoking post-translational changes leading to enzyme inactivation. Upon oxidation, new chemical species can be generated from −SH, which can be gradually oxidized to sulphenic (−SOH), sulphinic (−SOOH), and sulphonic (−SOOOH) acids, the latter being an irreversible oxidation state. When a −SOH group is in the proximity of an −SH group, a disulphide bridge can form (-S-S-). This chemical reaction is of great biological relevance because thiol/disulphide inter-conversion operates as a molecular on/off switch for redox-regulated enzymes (Couturier et al., 2013). Plants, like other organisms, have developed sensing systems to monitor and maintain optimal redox conditions to avoid metabolic collapse. These sensing and signaling mechanisms (König et al., 2012) could be considered to be true "redox eyes".

Glutathione (GSH) and ascorbate (Asc), the most abundant antioxidant compounds in plant cells (Noctor and Foyer, 1998), are considered to be unspecific reducer molecules because of their small molecular masses. However, plants have complex enzymatic antioxidant systems composed of thioredoxins (TRX) and glutaredoxins (GRX), known under the name of redoxins (RX). On the contrary to GSH and Asc, surface topology of RX allows specific target interactions (Wangensteen et al., 2001; Barranco-Medina et al., 2009; Arsova et al., 2010). Among the first RX targets identified at the beginning of proteomic era were PRX (Baier and Dietz, 1999a,b, 2005; Dietz et al., 2006), which are involved in ROS/RNS signaling (König et al., 2012) and, as mentioned above, key ROS

detoxifying enzymes. RX are highly diversified in plants and display a conserved tertiary structure (TRX folding) holding one or two Cys at their active sites. Useful reviews are available on plant GRX and their cross-talk with TRX (Rouhier et al., 2006; Xing et al., 2006; Meyer et al., 2009, 2012; Zaffagnini et al., 2012). TRX are low-redox-potential proteins (<−270 mV) of approximately 10–12 kDa with the conserved active site WC(G/P)PC (classical TRX). The Cys residues of the TRX active site switch from a reduced (sulphydryl groups) to an oxidized form (disulphide bridge) as part of the enzymatic mechanism resulting in the reduction of a target protein.

Plants are sessile eukaryotic photosynthetic organisms that have colonized multitude of terrestrial environments with fluctuating light intensities, water availability, temperature variations, and other environmental factors continuously challenging plant life. Success of this adaptation lies partially in the versatile redox signaling and regulation exerted by TRX (König et al., 2012). With the arrival of the genomics era and massive sequencing projects, many plant species have been already sequenced (e.g., *Arabidopsis thaliana* and *Oryza sativa*). The knowledge of full genomes offered the possibility of discovering tissue-specific or faintly expressed TRX, elusive before genomics. At present, the number of TRX, TRX-like proteins, or proteins with TRX-domains in *Arabidopsis* have risen to 44 members (Meyer et al., 2012), many of them without any assigned function. TRX are classified, according to their subcellular location and sequence similarity, into 15 subgroups (Meyer et al., 2012). While classical TRX *h* and *o* are located in cytosol/nucleus and mitochondria, respectively, five typical TRX exist in plastids, namely, TRX *f*, *m*, *x*, *y*, and *z*. TRX receive electrons from two compartment-specific and well-defined systems: the ferredoxin-thioredoxin system (FTS), which reduces plastid TRX with electrons coming from ferredoxin through the action of ferredoxin-thioredoxin reductase (FTR); and the NADP-thioredoxin system (NTS), involving the NADPH-thioredoxin reductase (NTR) to furnish electrons from NADPH to TRX *h* and *o*. Apart from FTS and NTS, NTRC is a bi-modular protein with NTR and TRX domains reported in 2004 (Serrato et al., 2004). Although NTRC is located in plastids, it is nevertheless reduced by NADPH (Spínola et al., 2008; Pérez-Ruiz and Cejudo, 2009) and behaves as a condensed NTS system important for the response to abiotic and oxidative stress (Serrato et al., 2004; Pérez-Ruiz et al., 2006). Besides the antioxidant role, NTRC functions related to carbon metabolism have been recently proposed (Michalska et al., 2009).

Phylogenetic studies on plastid TRX and sequence comparisons have demonstrated that while TRX *m*, *x*, *y*, and *z* are of prokaryotic origin (Sahrawy et al., 1996; Arsova et al., 2010), TRX *f* is closely related to eukaryotic TRX (Sahrawy et al., 1996; Issakidis-Bourguet et al., 2001). It seems reasonable that TRX diversification reflects the complexity of the plastid redox network and the extent of their role played in plant physiology. In recent years, due mainly to the availability of collections of mutant lines, many studies on plastid TRX have focused on the model plant *A. thaliana*. In this species, two *f*, four *m*, two *y*, and one *x* and *z* TRX isoforms have been described. This multiplicity has raised the question of functional redundancy or a specific role for each isoform. In this sense, Issakidis-Bourguet et al. (2001) showed that chloroplast TRX *f*, *m*, and *x* are differentially able to compensate for TRX deficiency in yeast. Since the discovery some decades ago of the preferential activation of chloroplast fructose-1,6-bisphosphatase (FBPase) by TRX *f* and NADP-malate dehydrogenase (MDH) by TRX *m* (Schürmann et al., 1981), linking carbon fixation and TRX-mediated activation, many other essential plastid processes such as PETC, oxidative-stress response, starch metabolism, nitrogen metabolism, lipid biosynthesis, protein folding, protein import, translation, or chaperone activity (Balmer et al., 2004; Buchanan and Balmer, 2005; Balsera et al., 2010; Chibani et al., 2010; Sanz-Barrio et al., 2012) have been reported to be under the redox regulation exerted by TRXs. Moreover, initially confined to chloroplasts, growing evidence points to new physiological functions in roots and other heterotrophic organs (Barajas-López et al., 2007; Traverso et al., 2008; Benitez-Alfonso et al., 2009; Fernández-Trijueque et al., 2012).

TRX *f* AND *m* ARE PHOTOSYNTHESIS-RELATED ENZYMES, BUT NOT EXCLUSIVELY SO

Discerning between functional specificity and redundancy among components of multigenic families proves difficult. Single loss-of-function lines are frequently phenotypically undistinguishable from wild-type plants. To address this question, one possibility would entail obtaining double, triple, or even quadruple loss-of-function mutants. Nevertheless, this approach is time consuming and, in the case of TRX, can be complex because of cross-talks with the GRX family. Some authors have evidenced this cross-talk by inhibiting GSH synthesis (Reichheld et al., 2007). Expression patterns (abundance and tissue location), protein topologies (determining electrostatic and/or hydrophobic interactions), redox potentials, and post-translational modifications are distinctive features that would address the specificity for each pTRX toward a particular target in a specific cell type. Described long before other pTRXs, greater information has been compiled on TRX *f* and *m* than on the *x*, *y*, or *z* isoforms. Most recent works have offered further insight into the specific role of TRX *f* and *m* in photosynthesis, carbohydrate metabolism, NADPH synthesis, response to abiotic stress and, notably, putative new functions in heterotrophic organs.

REDOX SIGNALING IN PHOTOSYNTHESIS REGULATION

A large cluster of genes involved in light-harvesting reactions of photosynthesis genes coding for LHCA and LHCB (Light Harvesting Complex) proteins, protoporphyrin IX Mg chelatase, and several proteins of the photosystem I and II reaction centers (PSI and PSII, respectively) are found to be under clock control (Harmer et al., 2000). ATPase activity of Mg chelatase CHLI subunit, from the tetrapyrrole biosynthesis pathway, is activated *in vitro* by *Pissum sativum* TRX *f* (Luo et al., 2012). *In vivo* experiments with TRX *f* virus-induced gene-silenced plants produced no phenotype changes in the treated plants, suggesting that low levels of TRX *f* could be compensated for by the *m*-type isoform (Balmer et al., 2003; Luo et al., 2012). Nevertheless, in the same work, the silencing of pea TRX *f*/*m* induced a pale-green phenotype and ROS accumulation. The authors suggest two possible types of TRX-mediated regulations for the tetrapyrrole biosynthesis pathway:

one being transcriptional regulation through plastid-mediated retrograde signaling; and another being an indirect result of the lower Mg chelatase (interacting *in vitro* with TRX *f*) activity due to lower TRX *f*/*m* activity (**Figure 1A**).

It is known that carbon metabolism is concomitantly under this circadian rhythm. Therefore, it seems reasonable, given the tight relationship with photosynthesis and carbon fixation, that pTRX genes should have a similar transcriptional regulation in order to optimize these physiological processes. Surprisingly, only two (TRX *f2* and *m2*) out of the six TRX *f* and *m* of *A. thaliana* and the pea TRX *f* and *m1* follow a circadian rhythm (Barajas-López et al., 2011). The rest of TRXs *f* and *m*, with the exception of TRX *m3*, are directly induced by light. This light-independent gene expression could correspond to a more functional specificity of TRX *m3* (Benitez-Alfonso et al., 2009), clustered away in phylogenetic trees based on protein-sequence comparisons (Arsova et al., 2010).

Regarding photosynthesis regulation (**Figure 1A**), TRX is involved in state transitions (Rintamäki et al., 2000). Both TRX *f* and *m* are able to *in vivo* inactivate LHC kinase in response to high light intensities, controlling the relocation of LHC between PSI and PSII under this condition. More recently, in 2006, TRX *m* and *f* were found to transfer reducing equivalents (the *m*-type isoform being more efficient) to HCF164, a thylakoid-membrane-spanning protein with two thioredoxin-like domains participating in the assembly of cytochrome b_6f (Lennartz et al., 2001), mediating electron transport between PSII and PSI, and the reduction of the photosynthetic protein PSI-N both *in vitro* and in isolated thylakoids (Motohashi and Hisabori, 2006). HCF164 reduction might involve CcdA, a thylakoidal protein from bacterial origin targeted by TRX *m* (Motohashi and Hisabori, 2010). However, this hypothesis is only based on indirect results obtained from *in vitro* subcellular localization experiments and phenotype similarities between the *ccda* and *hcf164 Arabidopsis* mutants. Further *in vitro* and/or *in vivo* experimental approaches will be necessary to corroborate this putative interaction and its regulation. Very recently, the analysis of the photosynthetic parameters of an *A. thaliana* loss-of-function mutant has allowed to uncover a direct photosynthesis control carried out by TRX *m4* (Courteille et al., 2013). According to the authors, this TRX *m* isoform would play an important role in regulating photosynthetic alternative electron pathways in *A. thaliana* and *Nicotiana tabacum* chloroplasts, acting as a repressor of the cyclic electron flow (CEF) involved in preserving a proper ATP/NADPH balance. Although this was the first specific function assigned to TRX *m4*, the physiological significance of this regulation still remains to be clarified. In this sense, the photosynthesis regulation of TRX *m* has been shown in rice, when the repression of OsTRX *m* level is responsible for a greater reduction, compared to wild-type plants, in the photosynthetic efficiency under high-irradiance treatments together with other impairments such as thylakoidal ultrastructural changes and a reduced chlorophyll and pigment content (pale-green leaves; Chi et al., 2008).

Until now, pTRX regulation of photosynthesis has been *in vivo* studied in C3 plants. In C4 photosynthesis, the redox state of the bundle sheat cells and mesophyll cells are expected to have very different redox status due to the spatial separation of the photosynthetic process. This C4-photosynthesis peculiarity could be influencing the relative expression pattern of the different pTRX isoforms in bundle sheat and mesophyll cells and how the photosynthesis is redox controlled in C4 plants. We hope that this intriguing topic could be addressed in a near future.

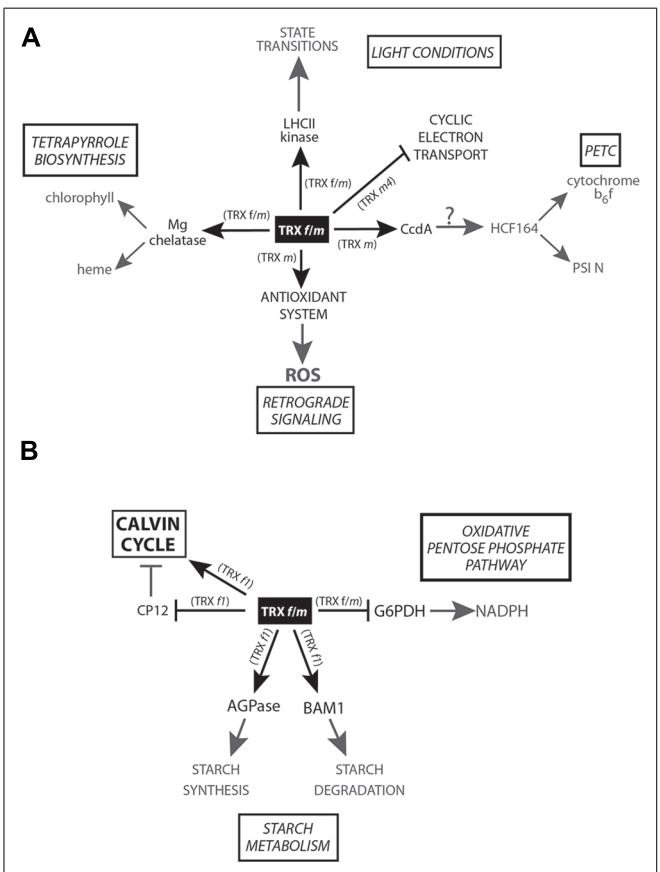

FIGURE 1 | Scheme of the TRX *f* and *m*-mediated redox signaling in plastids. (A) Main physiological processes related to photosynthesis and retrograde signaling controlled by TRX *f* and/or *m* are shown in the figure. **(B)** Regulation of carbon metabolism carried out by TRX *f* and/or *m*.

CONTROL OF CARBOHYDRATE METABOLISM IN PLASTIDS

Sugars are photosynthetic products and, recently, it has been reported that these molecules (together with thiol status in leaves) are regulating the expression of *PsTRXf* and *m1* genes in pea plants (Barajas-López et al., 2012). This regulation is mainly exerted by glucose and sucrose and might involve the transcription factor *PsDOF7*, able to bind to a pea DOF motif present in *PsTRX f* and *m1* promoters (Barajas-López et al., 2012).

Today, the redox regulation of all Calvin cycle (CC) enzymes exerted by pTRXs is widely acknowledged (Lindahl and Kieselbach, 2009; Meyer et al., 2012). Concerning redox regulation, one of the most intensely studied CC enzymes has been the chloroplast fructose-1,6-bisphosphatase (cFBPase), whose redox activation mechanism is a classical model in enzyme post-translational regulation (Jacquot et al., 1995; Chen and Xu, 1996; Hermoso et al., 1996; Jacquot et al., 1997; López Jaramillo et al., 1997; Chiadmi et al., 1999; Wangensteen et al., 2001; Cazalis et al.,

2004). Nevertheless, most of the interaction evidence comes from *in vitro* studies. In immunocytolocalization experiments with pea chloroplasts, TRX *f* and *m* have been found to be non-randomly distributed with respect to some CC-analyzed enzymes, NADP-dependent malate dehydrogenase (NADP-MDH), heat-shock protein 70 (Hsp70), and ATP synthase (Anderson et al., 2008). Based on pTRXs co-localization with non-light activated enzymes, the authors proposed a secondary function for pTRXs as protein linkers facilitating enzyme interactions and/or substrate channeling. Additionally, it has been suggested that mechanisms by means pTRXs would exert a fine-tuned modulation of enzyme activities based on short-lived interactions, not only involving a simple binary on/off mechanism (König et al., 2012).

Apart from the pTRX-mediated activation of CC target enzymes, an additional and indirect activation mode involving CP12, a small chloroplast protein containing four redox-active Cys, was described in the 1990s (Wedel et al., 1997). In its oxidized state, CP12 forms an inhibited complex (**Figure 1B**) with the CC enzymes NADPH-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK), fully restored upon pTRXs reduction (Marri et al., 2009). In plant cells, pTRXs/CP12 system broadens the redox regulatory complexity, providing a faster activation of CC oxidized enzymes and pointing to TRX *f1* as the physiological reducer of GAPDH/CP12/PRK complexes in *A. thaliana*. Further *in vivo* studies have highlighted the importance of CP12 in carbon partitioning and growth in tobacco plants, leading to proposal of functions other than the single formation of PRK and GAPDH complexes (Howard et al., 2011).

Plastid synchronization of the starch synthesis/degradation processes is crucial for plant growth and development. ADP-glucose pyrophosphorylase (AGPase) is a key redox-activated enzyme for starch biosynthesis in plastids (Fu et al., 1998; Ballicora et al., 2000). Very recently, in *Arabidopsis* leaves, the role of TRX *f1* as AGPase activator (**Figure 1B**) during light period has been evidenced (Thormählen et al., 2013). In a loss-of-function *trx f1* mutant, a lower redox activation of AGPase and a decrease in the starch:sucrose ratio have been detected during the day. AmongTRX *f1*, *m1*, *x*, and *y1*, AGPase is more efficiently *in vitro* activated by TRX *f1* (followed by TRX *m1*). These authors hypothesized that AGPase could be activated by TRX *f1* during the light period while NTRC would be the activating enzyme in the dark. However, other authors have reported contradictory experimental results and contend that redox modulation is of minor importance for AGPase activity in response to light (Li et al., 2012). Nevertheless, further experiments will be necessary to clarify this important point.

Surprisingly, not only starch synthesis is TRX *f1* controlled but also degradation through the redox-activated enzyme BAM1 (**Figure 1B**), an *A. thaliana* β-amylase controlling stomata opening (Valerio et al., 2011). Although TRX *f1* activates both starch synthesis and degradation during the day, these processes take place in different cell types (mesophyll cells and guard cells, respectively). However, light degradation in mesophyll tissue is activated during osmotic-stress situations that trigger BAM1 induction. *BAM1* is also expressed in *Arabidopsis* roots, where NTRC (proved to be less efficient than TRX *f* in *in vitro* assays) is the putative activating enzyme (Valerio et al., 2011). Besides the presumed role of NTRC as a redox alternative activator under dark conditions and/or in non-photosynthetic organs, it is feasible that classical pTRXs (the specifically expressed in non-photosynthetic organs) could be activated by the heterotrophic ferredoxin NADP reductases (FNR) isoforms and NADPH (Hanke et al., 2004; Balmer et al., 2006; Barajas-López et al., 2007; Bohrer et al., 2012; Fernández-Trijueque et al., 2012). Despite that some works have focused on the study of pTRX-mediated redox regulation in non-photosynthetic organs, much effort still needs to be done in order to ascertain the true extent of pTRXs in heterotrophic tissues.

OXIDATIVE ACTIVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the first committed step of the oxidative pentose phosphate pathway (OPPP), a major source of NADPH for plant heterotrophic cells as well as for photosynthetic tissues during the night period. Six genes coding for G6PDH have been identified in *Arabidopsis*, four predicted to code for plastid isoforms (Wakao and Benning, 2005). The higher number of G6PDH identified in plastids points to the significance of OPPP taking place in this subcellular compartment. *In vitro* assays have shown the reductive inactivation by DTT, a common feature of at least three out of the four plastidial enzymes (AtG6PDH1, AtG6PDH2, and AtG6PDH3) and not shared with the cytosolic isoforms. *AtG6PDH1* is expressed mostly in photosynthetic tissues while *AtG6PDH2* and *AtG6PDH3* transcripts are accumulated predominantly in roots (Wakao and Benning, 2005). Although a specific TRX *m*-mediated G6PDH inactivation (**Figure 1B**) has been previously reported (Wenderoth et al., 1997), Née et al. (2009) have demonstrated that *A. thaliana* TRX *f1* regulate AtG6PDH1 activity *in vitro* as efficiently as TRX *m1* or *m4*. Nevertheless, these *in vitro* results must be carefully interpreted and need to be supported by complementary *in vivo* interaction approaches or by determining the *in vivo* redox state of G6PDH1 in a TRX *f1* loss-of-function mutant. In addition, it would be helpful to know whether pTRXs also regulate root-expressed AtG6PDH2 and AtG6PDH3 in order to elucidate the role of pTRXs in OPPP control and NADPH synthesis. It is tempting to conclude that some of the pTRXs could control their own redox status through the activation/inactivation of OPPP in heterotrophic organs.

ROS HOMEOSTASIS IN ROOTS

TRX m3 transcripts are one of the least abundant pTRXs mRNA in leaves, while higher root-transcript levels are comparable to those of *TRX m2*, *m4*, and *x* (Bohrer et al., 2012). Nevertheless, until now, the published results highlight the importance of TRX *m3* for root ROS homeostasis (Benitez-Alfonso et al., 2009; Benitez-Alfonso and Jackson, 2009). The results shown in this work localize TRX *m3* in root and shoot meristem plastids, this isoform being important for callose deposition and plasmodesmal transport, as well as for arresting the growth of TRX *m3* loss-of-function seedlings (*gat1* mutant). It is quite surprising, however, that, given the redundancy of ROS detoxifying mechanisms in plant cells and the existing interplay between TRX and GRX signaling pathways, in vital plant meristems, the ROS content was not buffered by other TRX isoforms or GRX members

also expressed in roots (Bohrer et al., 2012; Meyer et al., 2012). In fact, other authors hold that the lethality due to TRX *m3* inactivation needs to be firmly established (Reichheld et al., 2010).

Although some studies have experimentally proved the presence of *Pisum sativum* TRX *f* and *m* isoforms in roots (Barajas-López et al., 2007) and the response of TRX *m* to NaCl-induced stress in root pea seedlings (Fernández-Trijueque et al., 2012) there is no information about the precise tissue expression in this organ (excepting *TRX m3*). According to the online available microarray data from "*Arabidopsis* eFP Browser" (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), *TRX m2* would be the most abundantly expressed gene in roots, principally in procambium cells. In contrast, *TRX m1* would be pericycle and phloem specific while *TRX m4* could be mostly expressed in cortex and procambium tissues. The analysis of the data suggests a poor expression in *A. thaliana* of both *TRX f* genes in roots compared to the other *TRX m* genes. Nonetheless, although these data may help to have an approximate idea about the pTRX accumulation in root tissues, we must taking into account that putative further post-transcriptional control could alter the final protein-expression pattern.

TRX *x* AND *y*: THE SUBSET OF pTRXs SPECIALIZED IN RESPONDING TO OXIDATIVE STRESS

The first reports describing TRX *x* and *y* as members of the plant pTRXs appeared in 2003 and 2004, respectively (Collin et al., 2003; Collin et al., 2004). It bears noting that TRX *x*, *y*, and *z* have higher redox potentials (>−340 mV) than do TRX *f* and *m* (<−350 mV; Collin et al., 2003; Collin et al., 2004; Chibani et al., 2011). As mentioned above, one of the key TRX features determining their functional specificity is redox potential. This biochemical characteristic confers these proteins a poor capacity to activate carbon-metabolism enzymes such as FBPase and MDH. On the contrary, *x*- and *y*-type TRX can efficiently activate plastid 2-Cys PRX and PRX Q, respectively (**Figure 2**). The absence of TRX *x* in the *A. thaliana* mutant *trxx* triggers protein carbonylation (stress marker) but does not affect photosynthesis or carbon fixation under long-day conditions (Pulido et al., 2010). Nevertheless, under continuous-light conditions, CO_2 fixation is affected in *trxx*, suggesting that TRX *x* can undertake a more important role under non-optimal environmental conditions (Pulido et al., 2010).

Two *y*-type isoforms, TRX *y1* and *y2*, are present in *Arabidopsis*. These oxidoreductases do not activate FBPase and only partially MDH, being efficient reducers of PRX Q (Collin et al., 2004). In *Arabidopsis* plants, TRX *y1* is preferentially expressed in heterotrophic organs (e.g., roots and seeds), while TRX *y2* is a more photosynthesis-associated protein. Expression in heterotrophic organs of the *y1*-type implies the existence of a functional redox system, furnishing reducing power to these TRX. In this sense, Marchand and colleagues reported a list of TRX *y* targets in *Arabidopsis* roots, finding numerous proteins involved in detoxification and defense (**Figure 2**) like monodehydroascorbatereductase (MDAR; Marchand et al., 2010). Notably, although *y*-type TRX cannot activate FBPase, another of the root-found targets is a putative fructose bisphosphate aldolase, whose reaction product that serves as a substrate for cFBPase and that participates in plant responses to abiotic stress (Lu et al., 2012).

Recently, from the analysis of methionine sulfoxidereductase (MSR) capacity in different TRX loss-of-function mutants, TRX *y2* has been proposed as the physiological electron donor of MSR (**Figure 2**; Laugier et al., 2013). However, according to the results shown in this work, overlapping functions of TRX *m1* and *m4* as MSR activators cannot be ruled out.

TRX *z*, A REDOX REGULATOR OF THE PLASTID TRANSCRIPTION

Although a *Solanum lycopersicum* TRX *z* ortholog (CITRX) has been reported as an interacting cytosolic partner of the resistance protein Cf-9 (Rivas et al., 2004), in 2006 this protein was

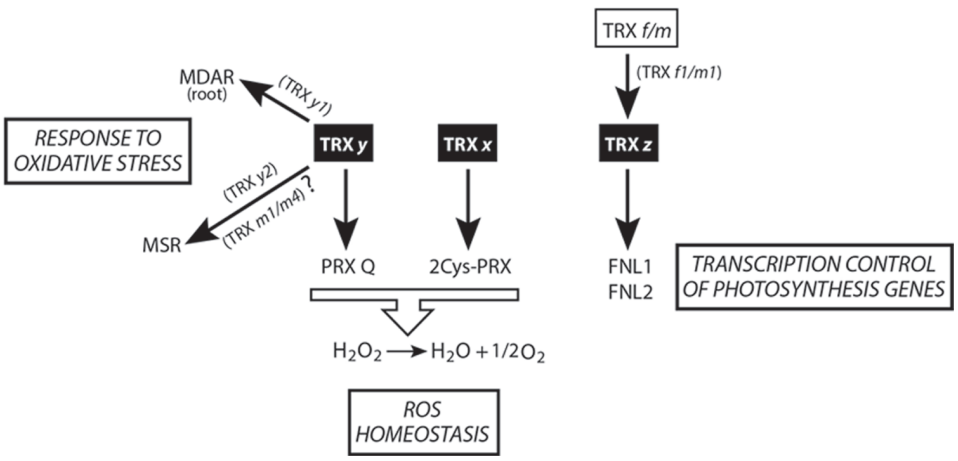

FIGURE 2 | Scheme of TRX *x*, *y*, and *z*-mediated redox signaling in plastids. The central physiological processes regulated by these isoforms are shown.

identified for the first time as a component of plastid transcriptionally active chromosomes (TACs) from mustard (*Sinapis alba*) and *Arabidopsis* (Pfalz et al., 2006) and, 4 years later, named as TRX *z* (Arsova et al., 2010) or TRX *p* (Meng et al., 2010) and designated as a new member of pTRXs. The lack of this protein affects transcription (**Figure 2**) of genes dependent on plastid-encoded RNA polymerase (PEP), essentially photosynthetic-related genes (class I; Arsova et al., 2010). Consequently, *A. thaliana trxz* has yellow leaves and lacks the ability of autotrophic growth while in *Nicotiana benthamiana* low TRX *z* protein levels induce a chlorotic phenotype (Arsova et al., 2010; Meng et al., 2010). Two fructokinase-like proteins (FLN1 and FNL2) reportedly interact *in planta* with TRX *z* in a thiol-dependent way. Recombinant FLN1 and FLN2 lack any sugar-phosphorylating activity, suggesting a regulatory rather than a metabolic function (Arsova et al., 2010). Several pieces of evidence, such as *in planta* interaction between FLNs and TRX *z*, a similar *Arabidopsis* leaf phenotype of the *trx z* and the FLN1 and FNL2 silenced mutants, and a reduced expression of PEP-dependent class I genes in both mutants suggest that TRX *z* and FLN1 and FLN2 might take part in a signaling pathway, regulating PEP activity in chloroplasts (Arsova et al., 2010). It is noteworthy that, in a parallel work, both TRX *z* and FNL1, together with other redox proteins, have been reported to take part of PEP complexes in mustard (Schröter et al., 2010). Curiously, the TRX *f*-target FBPase (Schürmann et al., 1981) is among the proteins found in mustard transcriptional complexes.

Biochemical assays with poplar TRX *z* have shown that this protein can be reduced by NTRB (Chibani et al., 2010), physiologically important in the case that TRX *z* is dually targeted to plastids and cytosol (Rivas et al., 2004). The activation of some peroxidases and MSR has led Chibani et al. (2011) to propose TRX *z* as an alternative electron donor to ROS-detoxifying enzymes. *Arabidopsis* TRX *z* is able to form dimers in its oxidized state, being monomerized upon reduction by DTT and, unlike TRX *x*, *y*, and *f1*, is the first pTRX not reduced by FTR (Bohrer et al., 2012). The higher redox potential of TRX *z* with respect to other pTRXs prompted to Bohrer and colleagues to conduct *in vitro* reduction assays by using other pTRXs. TRX *f1* and *m1* behaved as good TRX *z* reducers, being the first available case of TRX reduced by other TRX (**Figure 2**). However, additional *in vivo* experiments (as the determination of the reduction/oxidation TRX *z* state in loss-of-function pTRX mutants) need to be performed in order to corroborate these intriguing results.

PLASTID CYSTATHIONINE β-SYNTHASE DOMAIN-CONTAINING PROTEINS REGULATE pTRXs ACTIVITY

In the literature, no activating-TRX protein has been reported prior to the work of Yoo et al. (2011), which demonstrated the activating role of plastid cystathionine β-synthase (CBS) domain-containing proteins (CDCPs) over FTS and NTS. CDCPs are members of a large superfamily of ubiquitous proteins able to bind to adenosine-containing ligands such as AMP, ATP, or *S*-adenosyl methionine (Yoo et al., 2011). In *A. thaliana* and rice, 34 and 59 CDCPs have been reported, respectively (Kushwaha et al., 2009). CDCPs are located in different subcellular compartments. Two of these proteins, CBSX1 and CBSX2, are located in plastids and are able to activate TRX *f*, *m*, *x*, and *y* (Yoo et al., 2011). The loss-of-function mutant *cbsx1* shows severe growth retardation while CBSX1 overexpressing plants are able to grow faster in free-sucrose medium and display a delayed senescence compared to wild-type plants, resembling transgenic plants overexpressing TRX *m* (Benitez-Alfonso et al., 2009). Notably, the authors have suggested that CBSX1 would regulate physiological processes in non-green tissues while CBSX2 would be a green-tissue specific protein, reinforcing the above-mentioned idea of the presence of a fully active FTS in heterotrophic organs.

CONCLUDING REMARKS

Sometimes, phenotypic differences between TRX mutant lines and wild-type plants are subtle or even missing. However, in order to see whether novel isoforms have conferred adaptive advantages during evolution it would be necessary to perform population-dynamics studies of loss-of-function mutants grown under natural conditions. Although we know that this approach would be time-consuming and difficult to develop, it would give a definite answer to the perennial question of the functional specificity or redundancy of the members of the family of TRX. Additionally, to find a putative relationship between environment adaptation and diversification of plant pTRXs, it would be interesting to analyze whether there would be differences between the number of pTRX isoforms found in plant species living in extreme environments (e.g., deserts) compared with other species living in more stable environments (e.g., rain forests). It is quite probable that the diversification of the pTRXs also responds, at least in part, to the demand of a more complex redox signaling due to the appearance of new specialized organs (and plastid types), as roots and flowers, necessary for the successful land colonization. In roots and some flower tissues, and instead of chloroplasts, specialized non-green plastids are present. It would be logical to think that plants have reprogrammed or adapted the redox-signaling machinery already present in green plastids to redox regulate the light-independent processes occurring in non-green plastids. However, with the exception of a few works already mentioned in this review, there is very little information about the pTRX targets in heterotrophic organs and the light-independent processes they are redox regulating. Further comprehensive studies are still necessary to realize the extent of the redox-signaling role mediated by pTRX in the whole plant.

In our opinion, when we study multigenic families, we almost exclusively focus our attention on the gene coding sequences and on comparative analyses of the primary structures of the peptides they are coding for. However, regulatory sequences (DNA motifs) present in promoters are also a basic part of genes (and sometimes neglected). When revising TRX literature it is quite usual to find *in vitro* interaction experiments in which two or more pTRX isoforms are reported to have the same or similar affinity for a given target. For instance, *Arabidopsis* TRX *f2* isoform has been omitted from the *in vitro* interaction experiments of some works reasoning a high sequence similarity with TRX *f1*. We think that the diversification of the TRX family and other

multigenic families could also respond to a plant strategy leading to a more efficient transcriptional regulation. The increase in the number of plant transcription factors and the complexity of the transcriptional machinery could have compromised gene regulation and, consequently, plant survival. One solution could have consisted in organizing regulatory DNA motifs in several promoters. These regulatory sequences could have co-evolved together with the coding regions following a gene duplication event. During evolution, both the promoter changes as the amino acid substitutions of the pTRXs could allow a precise and specific redox signaling in non-green plastid of the heterotrophic organs.

As we have pointed out, pTRXs redox proteins may regulate a large number of plant physiological processes, and compelling evidence points toward the existence of fully active pTRXs in heterotrophic tissues. According to the works cited in this review, three pTRX functional subsets can be inferred (**Figure 3**). The first subgroup, related to photosynthesis and carbon metabolism, would be composed of the TRXs *f* and *m* isoforms, coupling light and redox-signaling pathways (excluding the *Arabidopsis* TRX *m3* isoform, possibly developing a specific physiological role). In the second subcategory, we could find the *x*- and *y*-type TRXs, involved mostly in ROS detoxification and taking part of the complex redox-signaling network regulating plant development. The last subset, related to redox signaling and regulation of photosynthesis-related transcription in chloroplasts, would be composed of only one member, i.e., TRX *z*. Intriguingly, TRXs *f* and *m* are efficient TRX *z* reducers. Subsequently, it is tempting to conclude that *f*- and *m*-type isoforms, in addition to regulate the photosynthesis-related processes mentioned in this review, could also act as redox-signaling molecules linking photosynthesis and plastid transcription. In our opinion, the discovery of CDCPs as pTRX activity regulators should be taken into account in order to improve our knowledge of the external elements modulating the multilevel redox signaling mediated by pTRXs.

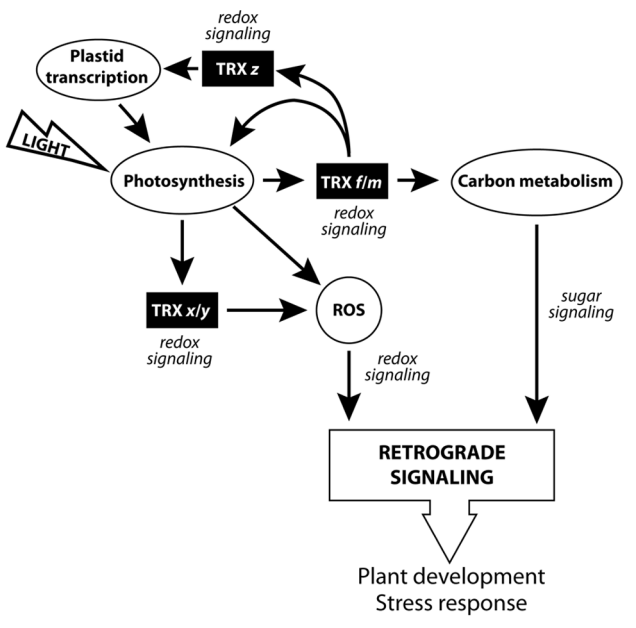

FIGURE 3 | Scheme of the pTRXs implication in the chloroplast signaling network.

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Overexpression of plastidial thioredoxins f and m differentially alters photosynthetic activity and response to oxidative stress in tobacco plants

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Plants display a remarkable diversity of thioredoxins (Trxs), reductases controlling the thiol redox status of proteins. The physiological function of many of them remains elusive, particularly for plastidial Trxs f and m, which are presumed based on biochemical data to regulate photosynthetic reactions and carbon metabolism. Recent reports revealed that Trxs f and m participate *in vivo* in the control of starch metabolism and cyclic photosynthetic electron transfer around photosystem I, respectively. To further delineate their *in planta* function, we compared the photosynthetic characteristics, the level and/or activity of various Trx targets and the responses to oxidative stress in transplastomic tobacco plants overexpressing either Trx f or Trx m. We found that plants overexpressing Trx m specifically exhibit altered growth, reduced chlorophyll content, impaired photosynthetic linear electron transfer and decreased pools of glutathione and ascorbate. In both transplastomic lines, activities of two enzymes involved in carbon metabolism, NADP-malate dehydrogenase and NADP-glyceraldehyde-3-phosphate dehydrogenase are markedly and similarly altered. In contrast, plants overexpressing Trx m specifically display increased capacity for methionine sulfoxide reductases, enzymes repairing damaged proteins by regenerating methionine from oxidized methionine. Finally, we also observed that transplastomic plants exhibit distinct responses when exposed to oxidative stress conditions generated by methyl viologen or exposure to high light combined with low temperature, the plants overexpressing Trx m being notably more tolerant than Wt and those overexpressing Trx f. Altogether, these data indicate that Trxs f and m fulfill distinct physiological functions. They prompt us to propose that the m type is involved in key processes linking photosynthetic activity, redox homeostasis and antioxidant mechanisms in the chloroplast.

Keywords: antioxidant mechanisms, oxidative stress, photosynthesis, redox homeostasis, thioredoxin, tobacco

INTRODUCTION

Thioredoxins (Trxs) are ubiquitous and evolutionarily conserved enzymes of *ca.* 12 kDa catalyzing the reduction of disulfide bonds through a redox-active dithiol CxxC motif (Arnér and Holmgren, 2000). Trxs, discovered 50 years ago in bacteria, cover functions as redox carriers in numerous physiological processes such as DNA synthesis, sulfur assimilation or regulation of transcription factors (Arnér and Holmgren, 2000). In plants, two plastidial Trx types, named Trx f and Trx m, were primarily identified as light-dependent regulators of enzymes related to photosynthetic processes and carbon metabolism (Jacquot et al., 1978; Wolosiuk et al., 1979). Their denomination was based on *in vitro* ability to activate by reduction fructose-1,6-bisphosphatase (FBPase) and NADP-dependent malate dehydrogenase (NADP-MDH), respectively. These Trxs are reduced by the ferredoxin/thioredoxin system (Wolosiuk and Buchanan, 1977). A third plant Trx type located in cytosol was identified later (Wolosiuk et al., 1979). This type named Trx h is reduced by cytosolic NADPH thioredoxin reductase (Jacquot et al., 1994) and participates in various processes such as mobilization of seed reserves (Kobrehel et al., 1992) and responses to oxidative stress (Verdoucq et al., 1999).

Whereas most organisms possess a low number (two or three) of Trxs achieving multiple functions, plants display a remarkable diversity of these reductases. A survey of genomic and EST sequences from *Arabidopsis* and other species revealed the presence of almost 50 genes encoding Trx or Trx-like proteins in higher plants (Meyer et al., 2005). On the basis of gene and peptide sequences, other Trx types, o, x, y, and z, have been defined and in *Arabidopsis*, Trxs f, h, m and y include 2, 9, 4, and 2 isoforms, respectively (Meyer et al., 2005; Lemaire et al., 2007). This outstanding diversity raised the question of functional specialization or redundancy. Genetic studies revealed

that several plant Trxs possess specific and unique physiological functions in development, metabolism and stress responses. For instance, *Arabidopsis* mutants deficient in Trx h9 display impaired root and leaf development. This cytosolic Trx is associated with plasma membrane and presumed to participate in cell-to-cell communication processes (Meng et al., 2010). In *Arabidopsis* plants lacking plastidial Trx z, chloroplast biogenesis is inhibited revealing a critical role of this Trx (Arsova et al., 2010). Trx z has been proposed to regulate transcription via a redox control of plastid-encoded plastid RNA polymerase. In other respects, Trx h5 is required for the response to victorin, a fungal toxin inducing programmed cell death in sensitive plants (Sweat and Wolpert, 2007).

Other Trxs participate in plant responses to the oxidative stress conditions resulting from environmental constraints (Vieira Dos Santos and Rey, 2006), mainly due to their ability to provide reducing power to peroxiredoxins (Prxs) and methionine sulfoxide reductases (MSRs), enzymes reducing organic peroxides and repairing oxidized proteins, respectively. Thus, CDSP32 (chloroplastic drought-induced stress protein of 32 kDa), a double module Trx initially isolated in potato plants subjected to water deficit (Rey et al., 1998) supplies Prxs and MSRs with electrons (Broin et al., 2002; Rey et al., 2005; Tarrago et al., 2010). Another plastidial Trx-like protein, NADPH thioredoxin reductase C (NTRC), uses NADPH to reduce 2-Cys Prx and has been proposed as a protection system against oxidative damage (Pérez-Ruiz et al., 2006). Trxs x and y are also presumed to participate in responses to oxidative stress based on their ability to reduce Prxs and MSRs *in vitro* (Collin et al., 2003, 2004; Navrot et al., 2006; Vieira Dos Santos et al., 2007), but evidence for such a function *in planta* is still scarce. Very recently, we showed that Trx y2 maintains growth under high light and long day in *Arabidopsis*, likely through electron supply to plastidial MSRs (Laugier et al., 2013). Note that the other Trx y isoform, y1, which is specifically expressed in non-photosynthetic organs (Collin et al., 2004) could also fulfill a protective function in seeds, in which MSRs likely play a key role in preserving longevity (Châtelain et al., 2013).

Although Trxs f and m have been the first Trxs discovered in plants, the knowledge concerning their physiological functions is only emerging. Based on biochemical studies, Trxs f and m are presumed to regulate photosynthesis and carbon metabolism although Trx f seems more efficient than Trx m to redox regulate most enzymes involved in these processes. Trx f specifically activates glyceraldehyde-3-phosphate dehydrogenase (B-containing GAPDH isoforms) and FBPase, and controls the activity of other redox-sensitive enzymes like NADP-MDH and glucose-6-phosphate dehydrogenase (G6PDH; Collin et al., 2003; Lemaire et al., 2007; Marri et al., 2009; Née et al., 2009). Similarly, Trx m reduces enzymes involved in carbon metabolism and catabolism such as NADP-MDH and G6PDH, but also regenerates the activity of enzymes involved in antioxidant mechanisms like Prxs and MSRs (Collin et al., 2003; Vieira Dos Santos et al., 2007). Among Trx m isoforms, Trx m3 displays highly distinct properties, since it cannot reduce known Trx targets (Collin et al., 2003; Vieira Dos Santos et al., 2007). This isoform, expressed in non-green plastids of meristems and organ primordia, could be involved in redox regulation of symplastic permeability (Benitez-Alfonso et al., 2009). Using RNA-interference, Chi et al. (2008) showed that rice plants knockdown for Trx m expression display abnormal chloroplast development and impaired growth. In contrast, no obvious phenotype was observed in *Arabidopsis* plants lacking either Trx m1 or Trx m4 (Laugier et al., 2013). But, most interestingly, Trx m4-deficient mutant plants specifically display strongly increased cyclic photosynthetic electron transfer around PSI (Courteille et al., 2013). Only very recent papers brought information regarding the physiological function of Trx f. In pea plants displaying a dramatically reduced Trx f transcript level due to silencing, no phenotype was noticed (Luo et al., 2012). Similarly, no change was found in growth and photosynthesis in *Arabidopsis* knockout lines for Trx f1, but reduced light-activation of ADP-glucose pyrophosphorylase (AGPase) in leaves accompanied by a decrease in starch accumulation was observed in these mutants (Thormählen et al., 2013). Consistently, we observed that transplastomic tobacco plants overexpressing Trx f show a strong increase in starch content (Sanz-Barrio et al., 2013).

The data gained in various species indicate that Trx f is involved in the regulation of starch metabolism, whereas the role of Trx m seems more complex. In this work, we compare the phenotypes of tobacco plants overexpressing either Trx f or Trx m with regards to growth, photosynthetic metabolism, activation and content of Trx targets and response to oxidative stress. We show that overexpression of Trx m leads to delayed growth, reduced pigment content and impaired photosynthetic activity. Further, we found a differential behavior of plants overexpressing Trx f or Trx m exposed to oxidative stress conditions, revealing that Trx m very likely displays, compared to Trx f, a broader range of physiological functions.

MATERIALS AND METHODS

PLANT MATERIAL AND STRESS TREATMENTS

Nicotiana tabacum L. plants, cv Petit Havana (Wt and transplastomic lines), were sown and grown on compost in phytotron under a 12-h photoperiod (300 μmol photons m^{-2} s^{-1}) and a 25°C/19°C (day/night) temperature regime for standard conditions. Transplastomic plants overexpressing either Trx f or Trx m were generated and characterized as reported in Sanz-Barrio et al. (2013). Photosynthetic and biochemical analyses were carried out on young well-expanded leaves from 35- to 40-day old plants.

Photooxidative treatment was carried out by exposing 30-day-old tobacco plants grown under standard conditions to high light intensity (950 μmol photons m^{-2} s^{-1}) and low temperature (8°C) for 5 to 8 days under a 12-h photoperiod. Methyl viologen (MV) treatment was performed both on whole plants and leaf disks. For whole plant experiments, 40-day-old tobacco plants were sprayed with 30 μM MV in 0.05% (v/v) Tween 20 and placed in phytotron under 200 μmol photons m^{-2} s^{-1} and a 16-h light (28°C)/8-h dark (25°C) photoperiod. Leaf damage caused by MV was photographed 2 days after treatment. For leaf disk experiments, 15 disks (12 mm diameter) were punched from young fully expanded leaves from 40-day-old plants, floated topside up on 15 mL of water or 1 μM MV, and illuminated at 600 μmol photons m^{-2} s^{-1} and 28°C during 14 h. Electrolyte content in solution was measured

after treatment using a HI9813-5 conductivity meter (Hanna Instruments, Woonsocket, RI, USA). Total electrolyte content was determined in the same way after autoclaving samples. Results were expressed as the percentage of total electrolytes released after treatment.

PHOTOSYNTHETIC MEASUREMENTS

Chlorophyll fluorescence parameters were measured using a PAM-2000 modulated fluorometer (Waltz Effeltrich, Germany) as previously described (Havaux et al., 2000). A saturating pulse of white light was applied on leaf and measurements were recorded during actinic light illumination (from 25 to 2,500 μmol photons m^{-2} s^{-1}). The PSII photochemical efficiency (Φ_{PSII}) during actinic illumination was estimated by calculating $\Delta F/F_{m'}$, where ΔF is the steady-state chlorophyll fluorescence level and $F_{m'}$ is the maximal level. NPQ (non-photochemical quenching) reflects the dissipation of absorbed light energy from PSII as heat. NPQ was calculated as $(F_m/F_{m'})-1$ where F_m is the maximal fluorescence level in the dark.

ANALYSIS OF CHLOROPHYLL CONTENT

One-cm diameter leaf disks were collected from fully expanded mature leaves and immediately frozen in liquid nitrogen and stored at −80°C until use. Leaf disks were crushed in 1 mL 80% acetone. After storing overnight in the dark at 4°C and centrifugation (14,000 *g*, 10 min), the content in chlorophylls *a* and *b* was measured spectrophotometrically and calculated according to Lichtenthaler (1987).

GLUTATHIONE CONTENT

Three tobacco leaf disks of 1 cm diameter (about 100 mg) were ground to a fine powder in liquid nitrogen and extracted in 1 mL 6.3 mM diethylene triamine-pentaacetic acid (DTPA), 40 μM *N*-acetyl-L-cysteine and 0.15% trifluoroacetic acid (TFA). After centrifugation (15,000 *g*, 10 min), the supernatant containing non-protein thiols was filtered on 0.2 mm nylon membrane. 125 μl were added to 225 μL buffer A (6.3 mM DTPA, 0.2 M 4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid, pH 8.2) or buffer B (buffer A + 0.5 mM Tris(2-carboxy-ethyl)phosphine hydrochloride, TCEP). For measurements of GSH content, samples in buffer A were immediately alkylated with monobromobimane in acetonitrile at a final concentration of 500 μM and stabilized by adding 150 μL cold 1 M methane sulfonic acid following 20 min incubation in the dark. For measurements of total glutathione (GSH), samples in buffer B were alkylated for 45 min at room temperature. Reactions were stopped by adding 150 μL of cold 1 M methane sulfonic acid. 20 μl were analyzed by HPLC and measurements of the fluorescence of bimane derivates were carried out as in Collin et al. (2008). Quantification of GSH amount was based on peak area and calibration was performed using GSH (Sigma). The concentration of oxidized glutathione (GSSG) was calculated as the difference between total GSH and reduced GSH values.

ASCORBIC ACID CONTENT

Ascorbate (AsA) was analyzed by HPLC as described by Havaux et al. (2005). Three leaf disks of 1 cm in diameter were ground in 750 μL 0.1 M metaphosphoric acid. Samples were filtered on nylon 0.2 μM membrane (Spin-X Costar). 6 μL were immediately injected for assaying reduced ascorbate. Total ascorbate was measured in the same volume following reduction of dehydroascorbic acid into ascorbic acid using 10 mM TCEP for 2 h in the dark at 25°C. AsA was detected at 245 nm in sulfuric acid-acidified water (pH 2.5) at a retention time of 1 min under a 0.65 mL min^{-1} flow. Quantification of AsA amount was based on peak area and calibration was performed using AsA from Sigma.

PROTEIN EXTRACTION, SDS-PAGE AND WESTERN ANALYSIS

Leaf pieces were blended in liquid nitrogen, and the powder was used to prepare soluble proteins as described in Rey et al. (2005). The protein content was determined using the BC Assay Reagent (Interchim, Montluçon, France). Proteins were separated using SDS-PAGE and Coomassie Brilliant Blue staining of gels was carried out to check quality of protein extracts. For immunoblot analysis following electrophoresis, proteins were electro-transferred onto nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA) and Red Ponceau staining was achieved to ensure equal loading in all lanes. Western analysis was carried out using primary antibodies raised in rabbit against NtTrx m or NtTrx f (1:5,000; Sanz-Barrio et al., 2011), AtMSRB1 and AtMSRB2 (1:1,000; Laugier et al., 2010), poplar PrxQ (1:2,000; Rouhier et al., 2004) and catalase (1:1,000; Agrisera, Vännas, Sweden). Western analysis of 2-Cys Prx abundance and redox status was performed as in Rey et al. (2007). Bound antibodies were detected using either an anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) or a goat anti-rabbit "Alexa Fluor® 680" IgG from Invitrogen diluted 1:10,000. When using the latter, antibodies were revealed at 680 nm using the "Odyssey Infrared Imager" from Licor.

NADP-MDH AND NADP-GAPDH ACTIVITY ASSAYS

NADP-dependent malate and glyceraldehyde-3-phosphate dehydrogenases activities in tobacco leaf crude extracts were assayed spectrophotometrically as previously described in Keryer et al. (2004) and Marri et al. (2009), respectively. Extractable enzymatic activities were measured on aliquots of freshly prepared extracts and maximal activities were measured after pre-treatment (reductive activation) of extracts with 25 mM DTT for 20 min at room temperature.

MSR ACTIVITY ASSAY

Maximal MSR activity in tobacco leaf extracts was assayed by monitoring the reduction of the synthetic substrate, dabsyl-MetO, in the presence of DTE (Vieira Dos Santos et al., 2005; Laugier et al., 2010). After blending leaves and suspension in extraction buffer, the content in soluble proteins was determined as above. The reaction mixture contained 15 mM HEPES pH 8, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTE, 0.25 mM dabsyl-MetO and 30 or 300 μg soluble proteins. After incubation for 3 h at 37°C, stopping using an ethanol:acetate buffer (50:50) and centrifugation, a supernatant aliquot was loaded on a C18 reverse phase 3.5 μm, 3 mm × 50 mm column SunFire™ (Waters, Milford, MA, USA) to separate dabsyl-MetO and dabsyl-Met.

RESULTS

GROWTH CHARACTERISTICS OF TOBACCO PLANTS OVEREXPRESSING Trx f OR Trx m

The transplastomic tobacco plants studied in this work were generated as described previously (Sanz-Barrio et al., 2013) by inserting tobacco Trx f or Trx m sequences (GenBank Acc. N° HQ338526 and HQ338525, respectively) without predicted transit peptides in the chloroplast genome under the control of the *psbA* regulatory sequence. Compared to Wt, the tobacco transformant lines termed Trx f^+ and Trx m^+ were shown to contain strongly increased Trx protein levels (at least 20 times more for Trx f^+ and 15 times for Trx m^+; Sanz-Barrio et al., 2013 and data not shown). The first phenotype analysis revealed that compared to Wt and Trx f^+ plants, Trx m^+ plants display some delay (2–3 days) in germination in *in vitro* conditions, a pale-green phenotype and a delay of few days in flowering time when grown in greenhouse conditions (Sanz-Barrio et al., 2013). In the present work, we further investigated the growth parameters of the transplastomic lines grown on compost in phytotron conditions. We noticed that plants overexpressing Trx m do not exhibit any delay in germination in these conditions, but show a slower growth compared to Wt and those overexpressing Trx f (**Figures 1A–C**). Thus at 40 days, the height stem of Trx m^+ plants (15.3 ± 2.1 cm) is significantly lower than that of Wt plants (22.9 ± 6.5 cm), whereas the growth of Trx f^+ plants is slightly altered, their height stem being 19.3 ± 5 cm (**Figure 1C**). The slower growth of Trx m^+ plants is associated with some delay (*ca.* 3 days) in flowering, but the plant size at this developmental stage is very similar to that of Wt and Trx f^+ plants (data not shown), as previously observed (Sanz-Barrio et al., 2013). We determined the leaf specific weight and did not notice any difference for the three lines (**Table 1**). Regarding the dry matter percentage, we observed a slightly, but significantly higher ratio in Trx f^+ plants (**Table 1**).

Table 1 | Leaf specific weight, dry matter percentage and chlorophyll content in 40-day old Wt and transplastomic tobacco plants.

Genotype	Wt	Trx f^+	Trx m^+
Leaf specific weight (mg FW cm^{-2})	21.4 ± 0.9	20.8 ± 1.6	21.0 ± 1.7
% Dry matter	10.3 ± 0.8	11.4 ± 1.1*	10.2 ± 1.3
Chl (μg cm^{-2})	44.6 ± 1.4	41.1 ± 1.0**	30.9 ± 1.3***
Chl *a* (μg cm^{-2})	31.6 ± 1.7	28.9 ± 1.0*	23.4 ± 1.0***
Chl *b* (μg cm^{-2})	13.0 ± 0.6	12.2 ± 1.1	7.6 ± 0.3***
Chl *a*/Chl *b*	2.4 ± 0.2	2.4 ± 0.2	3.1 ± 0.1***

*Chlorophyll content was measured in young well-expanded leaves of 40-day old Wt and transplastomic tobacco plants. Chlorophyll data are mean values ± SD from five independent measurements per plant and genotype. Leaf specific weight and dry matter data are mean values ± SD from eight independent measurements per plant and genotype. Each measurement was carried out using three 1-cm leaf disks. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. Values significantly different from Wt values with *p < 0.05, **p < 0.01, and ***p < 0.001 (t-test).*

This characteristic could originate from the much higher starch content measured in this line (Sanz-Barrio et al., 2013).

We then measured chlorophyll content in fully expanded leaves (**Table 1**) and observed that Trx f^+ plants display a pigment content slightly, but significantly, lower than that of Wt (41.1 ± 1.0 against 44.6 ± 1.4 μg cm^{-2}). This difference has not been observed when plants were grown in greenhouse under longer photoperiod and higher temperature (Sanz-Barrio et al., 2013). Thus, we presume that this phenotype feature originates from the different environmental conditions used in the present study. Consistent with visual observations, a chlorophyll content reduced by more than 30%

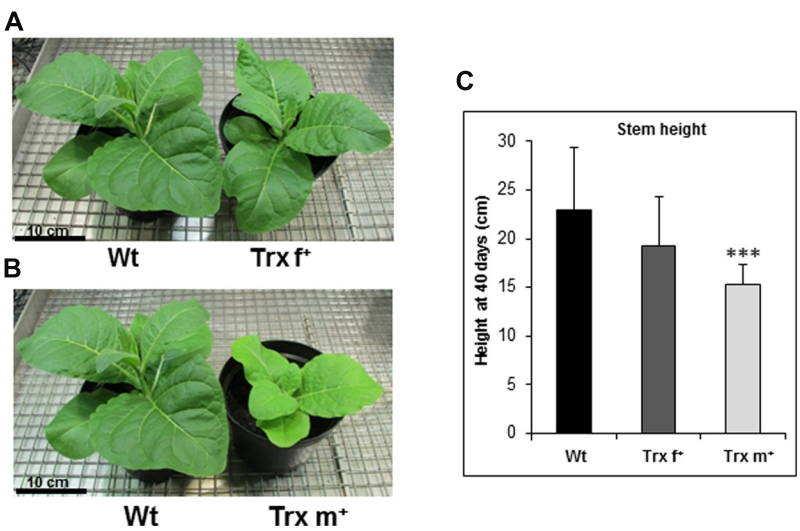

FIGURE 1 | Growth of transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Wt and Trx f^+ plants grown in phytotron for 35 days in standard conditions (300 μmol photons m^{-2} s^{-1}, 12-h photoperiod and 25°C/19°C, day/night). **(B)** Wt and Trx m^+ plants grown in phytotron for 35 days under standard conditions. The same Wt plant is shown in panels **(A)** and **(B)**. **(C)** Stem height of Wt and transplastomic plants grown for 40 days in standard conditions. Mean stem height values ±SD were gained from 15 plants per genotype. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. ***Significantly different from Wt with $p < 0.001$ (*t*-test).

(30.9 ± 1.3 μg cm^{-2}) compared to Wt was recorded in Trx m^+ plants. Note that when plants were grown at higher temperature, the pigment content was reduced in a less pronounced manner (−25%; Sanz-Barrio et al., 2013). The chlorophyll *a*/chlorophyll *b* ratio was similar (2.4) in both Wt and Trx f^+ plants, but higher (3.1) in Trx m^+ plants (**Table 1**), due to a chlorophyll *b* content reduced to a larger extent (−42% compared to Wt) than that of chlorophyll *a* (−26%; **Table 1**).

PHOTOSYNTHETIC PROPERTIES OF TOBACCO PLANTS OVEREXPRESSING Trx f OR Trx m

We then investigated the photosynthetic properties of transplastomic tobacco plants and first measured maximal PSII photochemical efficiency (**Figure 2A**) by recording the chlorophyll fluorescence parameter, F_v/F_m, which reflects the PSII capacity to reduce the primary Q_A electron acceptor. We observed a typical value close to 0.8 in Wt plants (0.793 ± 0.007). A slightly altered F_v/F_m value (0.766 ± 0.017) was recorded in the Trx f^+ line, whereas this fluorescence parameter was noticeably reduced in plants overexpressing Trx m (0.692 ± 0.028). These data reveal impairment in PSII functioning in plants accumulating Trx m. Photosynthetic electron transport activity was also estimated by measuring Φ_{PSII}, a chlorophyll fluorescence parameter indicative of the efficiency of PSII photochemistry as a function of light intensity. While only slightly decreased in Trx f^+ plants, PSII photochemical performance appeared more impaired in plants overexpressing Trx m (**Figure 2B**). Thermal dissipation of absorbed light energy, expressed by the NPQ of chlorophyll fluorescence coefficient, was also recorded in the different lines (**Figure 2C**). No noticeable difference was detected between Wt and Trx^+ transplastomic lines since they displayed a similar ability to recover photosynthesis and PSII photochemical efficiency following light irradiation.

ACTIVITY OF Trx TARGETS INVOLVED IN CARBON METABOLISM

To get evidence that overexpression of plastidial Trxs has a functional impact *in planta* on Trx target proteins, we first measured in leaves of transplastomic lines the activities of two well-known Trx-regulated enzymes, NADP-dependent MDH and GAPDH (Lemaire et al., 2007). Both Trx f^+ and Trx m^+ plants show similar marked changes in enzymatic activities. Extractable leaf activities were very strongly lowered to *ca.* 40 and 30% of the Wt value for MDH and GAPDH, respectively (**Figures 3A,B**, left panels). Conversely, leaf MDH and GAPDH capacities corresponding to the maximal activities measured in extracts chemically reduced with DTT, which allows Trx reduction but not direct Trx-target activation, were substantially increased in both transplastomic lines (**Figures 3A,B**, right panels). These findings show that overexpressed Trxs modulate the activity of known targets *in planta*. Further, they indicate that both Trx f and m, when they are in large excess, regulate NADP-dependent MDH and GAPDH enzymes in a similar manner.

Prx AND MSR ABUNDANCE

Peroxiredoxins are ubiquitous thiol-based peroxidases detoxifying hydrogen and organic peroxides. In plants, several Trx types such as NTRC, Trx x, CDSP32 and Trx y supply with electrons the main Prx plastidial type, 2-Cys Prx, and another isoform, PrxQ (Dietz, 2011). We thus investigated the abundance of these Prxs in tobacco plants by performing Western analysis. First, we confirmed the high abundance of Trxs f and m in transplastomic lines (**Figure 4A**). The antibodies raised against *Arabidopsis* 2-Cys Prx

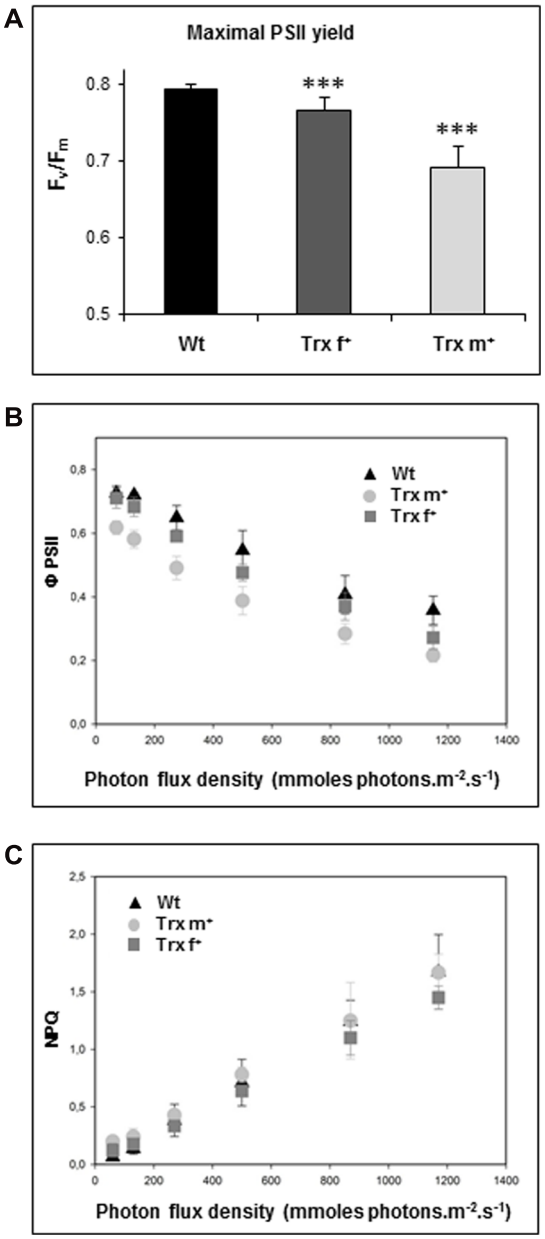

FIGURE 2 | Photosynthetic fluorescence parameters of transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Maximal photosystem II photochemical efficiency in tobacco plants (F_v/F_m). **(B)** Effective photosystem II yield (ΦPSII). **(C)** Non-photochemical quenching (NPQ). Light response curves are shown for ΦPSII **(B)** and NPQ **(C)**. Measurements were achieved in leaves from 35-day-old Wt and transplastomic tobacco plants grown in standard conditions. Data are mean values ± SD from at least five independent measurements per genotype. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. ***Significantly different from Wt with $p < 0.001$ (*t*-test).

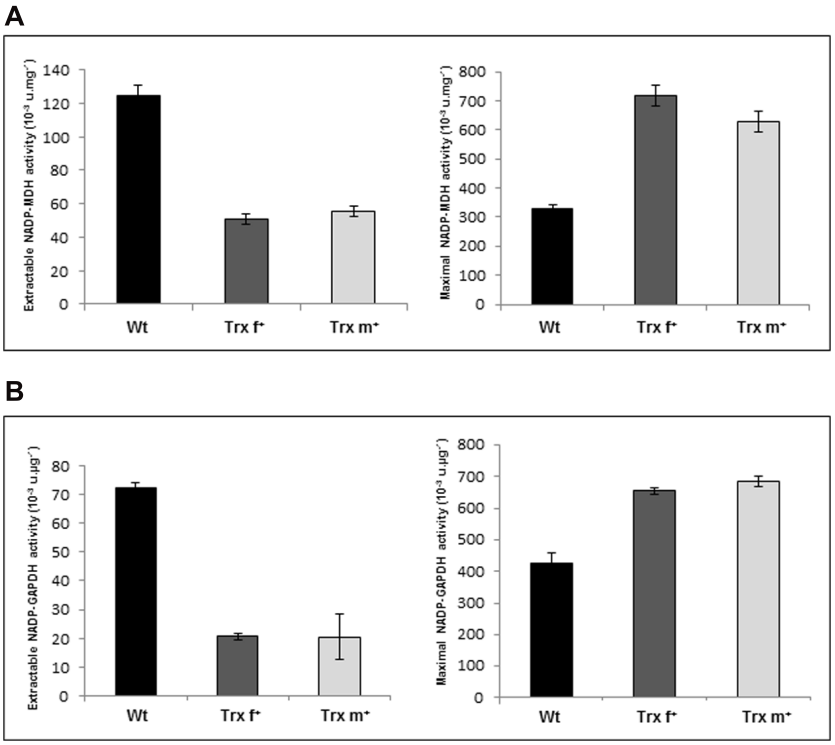

FIGURE 3 | NADP-dependent malate and glyceraldehyde-3-phosphate dehydrogenases enzymatic activities in leaf extracts from transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Extractable and maximal NADP-MDH activities. **(B)** Extractable and maximal NADP-GAPDH activities. Maximal activities (capacities) were measured after a reducing treatment of protein leaf extracts (25 mM DTT, 20 min at room temperature). Data are mean values ± SD from at least four independent measurements per genotype. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively.

and poplar PrxQ homologues specifically revealed bands with the expected molecular masses in tobacco extracts (**Figure 4B**). No change was detected in the amount of the two plastidial Prxs in Trx^+ plants (**Figure 4B**). We also used antibodies raised against overoxidized 2-Cys Prx, an inactivated form possibly involved in signaling processes related to redox homeostasis (Rey et al., 2007). Whereas there was no change in 2-Cys Prx abundance in plants overexpressing Trx f or Trx m compared to Wt, we observed a more variable amount of overoxidized 2-Cys Prx in the same leaf extracts. We noticed that such a variation occurred within the same genetic background (**Figure 4B**). A quantitative analysis, performed on data gained from six independent plants per line (data not shown), lead us to conclude that there was no significant variation in the 2-Cys Prx redox status in transplastomic tobacco plants.

Methionine sulfoxide reductase enzymes, which repair oxidized methionine in proteins, are also well-known targets of Trxs involved in plant responses to environmental constraints (Laugier et al., 2010). The abundance of plastidial MSRs was investigated in tobacco plants using sera raised against *Arabidopsis* MSRB1, MSRB2 and poplar MSRA4. MSRA4 antiserum recognized two bands at *ca.* 25 kDa in tobacco extracts (**Figure 4C**), corresponding to the reduced and oxidized forms of plastidial MSRA as observed in other species (Vieira Dos Santos et al., 2005; Bouchenak et al., 2012; Marok et al., 2013). No substantial change was observed regarding the abundance of MSRA4 forms in Trx-overexpressing plants compared to Wt. For MSRB1, no unambiguous signal could be detected in tobacco extracts due to poor cross reactivity of the serum generated against *Arabidopsis* homologue (data not shown). Like in *Arabidopsis* extracts, a faint band at *ca.* 15 kDa was revealed for MSRB2, next to a lower non-specific and intense band (Laugier et al., 2010). Again, overexpression of either Trx f or Trx m was not found to result in any substantial change in the amount of this plastidial MSR isoform. Altogether, Western data reveal that the levels of some major Trx targets involved in antioxidant mechanisms are not modified when Trxs f and m are overproduced.

LEAF MSR CAPACITY

We then measured the MSR enzymatic capacity in leaf extracts from tobacco plants using dabsyl-MetO, a substrate mimicking peptide-bound MetO (**Figure 4D**). In Wt, a maximal activity in the range of 22 pmol Met. mg $prot^{-1}$ min^{-1} was measured in the presence of reductant. Note that this value is noticeably lower than that measured in *Arabidopsis*, *ca.* 50 pmol Met. mg $prot^{-1}$ min^{-1} (Laugier et al., 2010), but higher than that recorded in barley, *ca.* 10 pmol Met. mg $prot^{-1}$ min^{-1} (Marok et al., 2013). In Trx f^+ plants, a rather similar value was found, *ca.* 25 pmol Met. mg $prot^{-1}$ min^{-1}. In sharp contrast, a twice higher value (46 pmol Met. mg $prot^{-1}$ min^{-1}) was measured in plants overexpressing Trx m. These data, showing a more elevated MSR capacity

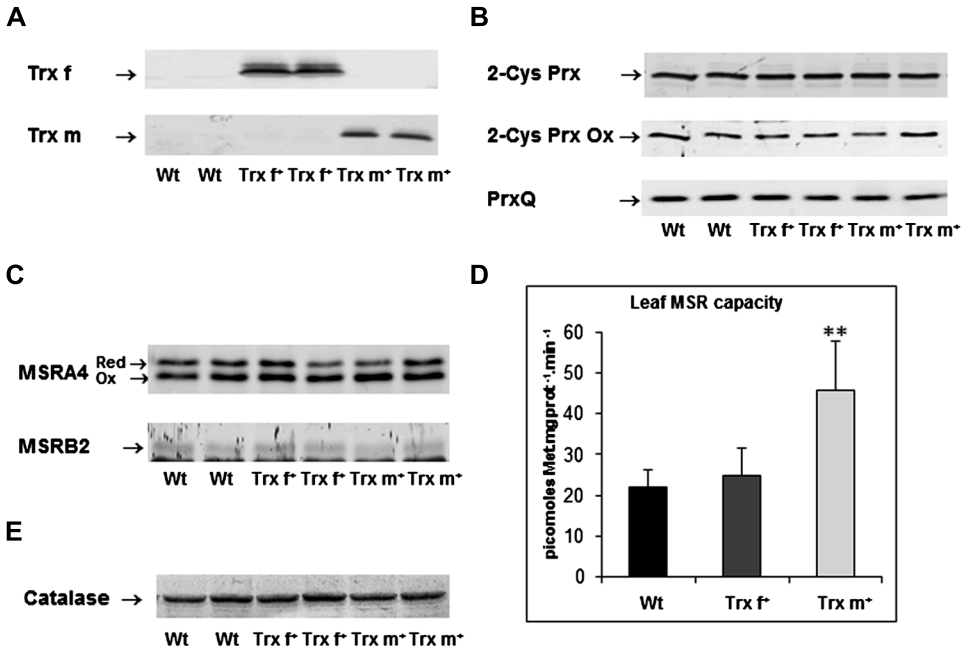

FIGURE 4 | Abundance of proteins involved in antioxidant mechanisms and leaf peptide methionine sulfoxide reductase enzymatic capacity in leaf extracts from transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Western analysis of Trx f and Trx m abundance. **(B)** Western analysis of the abundance and redox status of plastidial 2-Cys peroxiredoxin (2-Cys Prx) and of the abundance of peroxiredoxin Q (PrxQ). **(C)** Western analysis of the abundance of plastidial methionine sulfoxide reductases A4 and B2, MSRA4 and MSRB2, respectively. **(D)** Peptide methionine sulfoxide reductase enzymatic capacity measured using HPLC in crude leaf extracts using dabsyl-MetO, a synthetic substrate mimicking peptide-bound MetO. Data are mean values ± SD from at least five independent measurements per genotype. **Significantly different from Wt with $p < 0.01$ (*t*-test). **(E)** Western analysis of catalase abundance. Similar Western data were obtained using leaf extracts from two independent plants originating from at least two distinct growth experiments. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively.

in Trx m^+ plant extracts, reveal that *in planta* Trx m, but not Trx f, very likely regenerates and sustains the activity of plastidial MSRs.

CATALASE ABUNDANCE

The previous data revealed that Trx m likely provides reducing power to MSRs. MSRs are enzymes repairing oxidized proteins, but could also play a more general antioxidant function since their action results in ROS scavenging at the expense of NADPH (Moskovitz et al., 1997). To further analyze a putative function of plastidial Trxs in the control of redox homeostasis in plant cells, we analyzed leaf catalase abundance using Western blot analysis. Catalase is one major enzymatic system responsible for H_2O_2 scavenging in plant cells. We did not notice any substantial difference in catalase amount in plants overexpressing either Trx f or Trx m compared to Wt (**Figure 4E**), likely indicating no important change in the catalase-based capacity for detoxifying H_2O_2 in these lines.

CONTENT AND REDOX STATUS OF GLUTATHIONE AND ASCORBATE

We then investigated whether non-enzymatic antioxidant systems could be altered in transplastomic tobacco lines and measured the leaf content in GSH and ascorbate, which are abundant soluble antioxidants fulfilling key roles in redox homeostasis (Noctor and Foyer, 1998). In young well-expanded leaves of 40-day-old Wt tobacco plants, a GSH content of *ca.* 0.90 μmol g FW^{-1} was measured, the proportion of reduced form being 91% (**Figures 5A,B**). In Trx f^+ plants, the content and percentage values were very similar: 0.95 μmol g FW^{-1} and 94%, respectively. Interestingly, in plants overexpressing Trx m, whereas the proportion of reduced GSH was not modified (93%), the content, 0.75 μmol g FW^{-1}, was significantly lower (*ca.* −20%) than in Wt and Trx f^+ plants (**Figures 5A,B**). Regarding ascorbate, both Wt and Trx f^+ plants display very similar total contents (0.82 and 0.85 μnmol g FW^{-1}, respectively) and proportions of reduced form (83 and 86%, respectively) in young well-expanded leaves (**Figures 5C,D**). In contrast, the total ascorbate content, 0.65 μnmol g FW^{-1}, was significantly lower in Trx m^+ plants, but with an unchanged redox status (82%) compared to the two other lines (**Figures 5C,D**). Taken together, these data reveal that GSH and ascorbate pools are not altered when Trx f is overexpressed, but significantly and similarly modified in Trx m^+ plants. Indeed, these plants exhibit a decrease of *ca.* 20% in the total content of ascorbate and GSH without any noticeable change in redox status.

TOLERANCE TO PHOTOOXIDATIVE TREATMENT OF TOBACCO PLANTS OVEREXPRESSING Trx f OR Trx m

Oxidative stress tolerance of transplastomic tobacco plants was monitored by exposing them to a treatment combining high light and low temperature, conditions known to generate loss of photosynthetic membranes within chloroplasts due to photooxidative damage. Thirty-day old plants grown in standard conditions were transferred to a light intensity of 950 μmol photons m^{-2} s^{-1} at

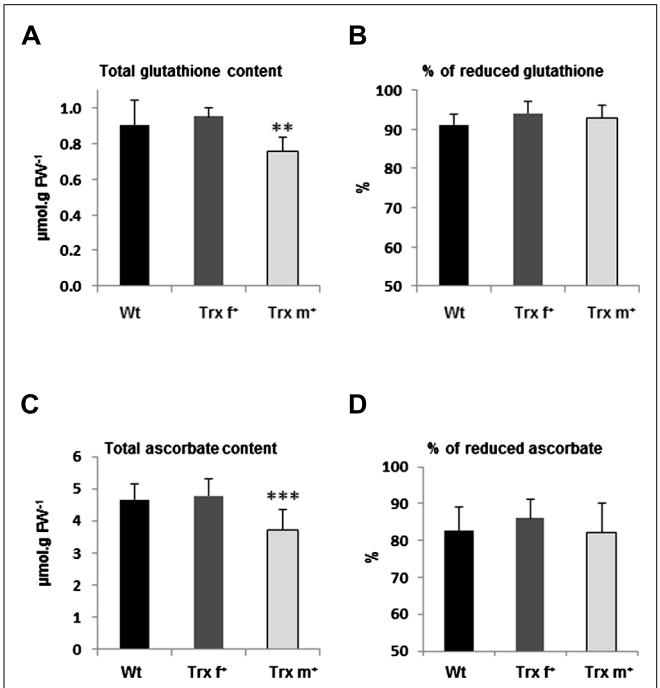

FIGURE 5 | Glutathione and ascorbate content in transplastomic tobacco plants overexpressing Trx f or Trx m. Total content **(A, C)** and redox status **(B, D)** of glutathione **(A, B)** and ascorbate **(C, D)** were determined in leaves of tobacco plants grown in standard conditions for 40 days. Measurements were performed using HPLC as described in Section "Materials and Methods" using three 1-cm leaf disks from young well-expanded leaves. Data are mean values ± SD from at least 10 independent measurements per genotype. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. Values significantly different from Wt values with $^{**}p < 0.01$ and $^{***}p < 0.001$ (*t*-test).

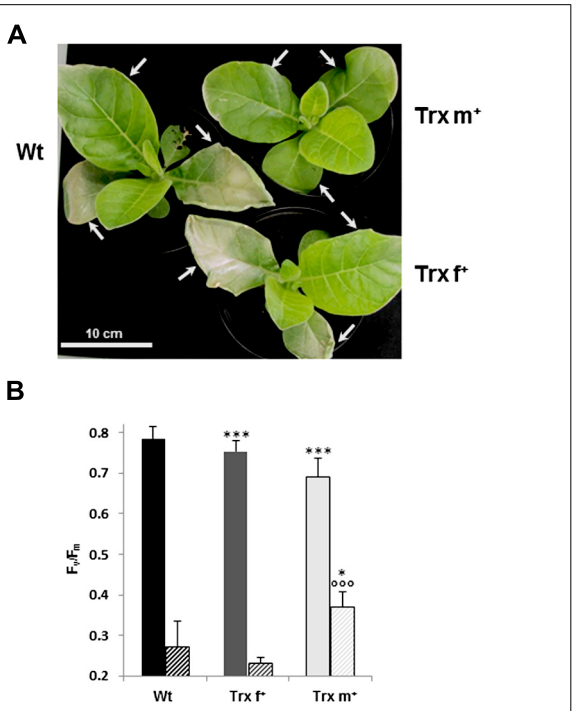

FIGURE 6 | Tolerance to photooxidative treatment of transplastomic tobacco plants overexpressing Trx f or Trx m. Photooxidative treatment was carried out by exposing 30-old tobacco plants grown in standard conditions to high light intensity (950 µmol photons m^{-2} s^{-1}) and low temperature (8°C) under a 12-h photoperiod in a controlled growth chamber. **(A)** Photograph showing representative plants subjected to the treatment for 5 days. Three independent experiments were carried out with three plants per genotype in each experiment. **(B)** Maximal photosystem II photochemical efficiency in tobacco plants. F_v/F_m values were measured in plants grown in control conditions for 32 days (full bars) and in plants further subjected to photooxidative conditions for 5 days (dashed bars). Data are mean values ± SD of the average values from eight measurements performed on five plants per genotype. Measurements were performed on representative areas from three leaves (indicated by arrows in panel **A**) per plant. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. Values significantly different from Wt values with $^{*}p < 0.05$ and $^{***}p < 0.001$ (*t*-test); °°°Value significantly different from the Trx f^+ value with $p < 0.001$ (*t*-test).

8°C. After 1 day, some bleaching appeared particularly in well-expanded and old leaves and damage intensity increased until the 4th day of treatment. Interestingly, a differential behavior was observed: Wt and Trx f^+ exhibited much larger bleached leaf areas compared to Trx m^+ plants, which displayed damage limited to leaf edges (**Figure 6A**). To further investigate the responses of tobacco plants to photooxidative stress conditions, we measured maximal photosystem II efficiency, using the chlorophyll fluorescence parameter, F_v/F_m, which constitutes a sensitive indicator of photosynthetic performance and reveals whether photosynthetic structures are damaged (Maxwell and Johnson, 2000). As previously observed in control conditions (**Figure 2**), Wt and Trx f^+ plants displayed close F_v/F_m values, 0.785 and 0.754, respectively, whereas this value was already substantially decreased in Trx m^+ plants (0.691; **Figure 6B**). F_v/F_m measurements in plants exposed to photooxidative treatment for a period of 5 days revealed a strong decrease in PSII photosynthetic efficiency in Wt and Trx f^+ plants since the recorded values were reduced by *ca.* 65 and 70% (0.272 and 0.233, respectively). In contrast, the decrease observed in Trx m^+ plants was much less pronounced, the mean F_v/F_m value measured in this line being reduced by *ca.* 45% (0.371). These data reveal that photosynthetic efficiency is less impaired in Trx m^+ plants exposed to photooxidative treatment and are consistent with the visual observations of limited leaf bleaching in this line.

TOLERANCE TO METHYL VIOLOGEN OF TOBACCO PLANTS OVEREXPRESSING Trx f OR Trx m

Trx overexpressing plants were also evaluated for protection against damage induced by MV, a redox-cycling herbicide that generates superoxide radicals by accepting electrons from PSI and transferring them to oxygen (Babbs et al., 1989). MV-mediated oxidative damage was assessed in whole plants sprayed with MV, and visual symptoms were registered 2 days following treatment. Wt and Trx f^+ plants were severely affected by MV treatment, whereas necrotic lesions were more limited in Trx m^+ plants (**Figure 7A** and data not shown). Maximal PSII efficiency (F_v/F_m) was measured 5 h after MV treatment. Similarly to the results reported above for photooxidative damage, PSII photosynthetic efficiency was significantly impaired in Wt and Trx f^+ plants (F_v/F_m values reduced by *ca.* 20 and 40%, respectively), whereas almost no change was observed in Trx m^+ plants (**Figure 7B**). To further evaluate the tolerance of transplastomic tobacco plants to

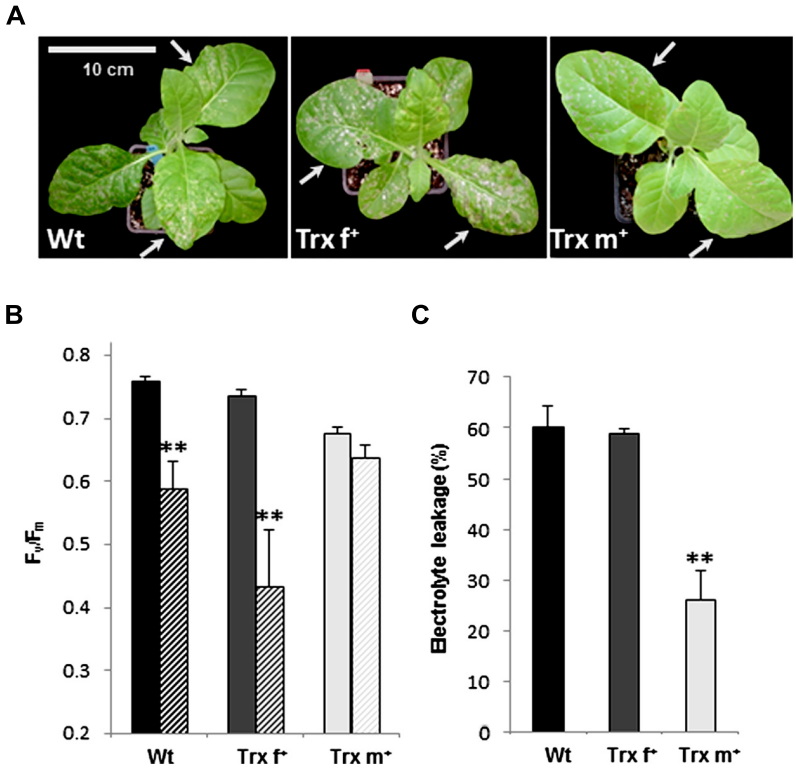

FIGURE 7 | Tolerance to methyl viologen of transplastomic tobacco plants overexpressing Trx f or Trx m. 40-day old tobacco plants were sprayed with 30 μM MV and grown in a controlled growth chamber as described in Section "Materials and Methods." **(A)** Visual symptoms 2 days after treatment. Photographs show representative plants subjected to the treatment. **(B)** Maximal photosystem II photochemical efficiency in tobacco plants. F_v/F_m values were measured in plants grown in control conditions (full bars) and in plants subjected to MV treatment for 5 h (dashed bars). Data are mean values ± SD from six plants per genotype. Measurements were performed on two leaves (indicated by arrows in panel **A**) per plant. **Value significantly different from the value measured in relative non-treated plants with $p < 0.01$ (*t*-test). **(C)** Electrolyte leakage measured on tobacco leaf disks after incubation on 1 μM MV for 14 h at 600 μmol photons m^{-2} s^{-1}. Results were expressed as the percentage of total electrolytes released after treatment relative to that of disks incubated on water under the same conditions. Data are mean values ± SD from three assays. Wt, wild-type; Trx f^+ and Trx m^+, plants overexpressing Trx f or Trx m, respectively. **Significantly different from Wt with $p < 0.01$ (*t*-test).

MV, membrane damage was estimated by measuring ion release from control and treated leaf disks. Following incubation on MV, the level of electrolyte leakage from Trx f^+ disks was relatively high and comparable to that of Wt (*ca.* 60% of the total electrolyte content, **Figure 7C**). In contrast, plants overexpressing Trx m displayed a much lower level of ion release (25%). Altogether, these results signify that overexpression of Trx m, but not of Trx f, increases tolerance to MV.

DISCUSSION

SPECIFICITY OF Trx m FUNCTION

In contrast to other organisms, where only two or three multifunctional Trx isoforms are present (Ritz et al., 2000; Garrido and Grant, 2002), plants display a great diversity of Trxs with physiological functions remaining elusive for many of them. Our work based on a genetic strategy contributes to decipher the role of Trx m *in planta*. Clear differences have been observed between plants overexpressing either Trx m or Trx f. Trx m^+ plants exhibit reduced growth and impaired photosynthesis (**Figures 1,2**), but increased tolerance to oxidative treatments (**Figures 6,7**). In comparison, Trx f^+ plants are almost undistinguishable from Wt, in full agreement with previous data (Sanz-Barrio et al., 2013) and others gained in *Arabidopsis* mutants knockout for Trx f (Thormählen et al., 2013) and in Trx f RNAi pea plants (Luo et al., 2012). Consistently, in heterologous complementation assays in yeast, *Arabidopsis* Trx m, but not Trx f, confers tolerance to oxidative stress (Issakidis-Bourguet et al., 2001). Taken together, these reports indicate that Trxs f and m fulfill distinct and non-overlapping physiological functions very likely through a marked specificity towards their targets *in planta*.

In the last years, knowledge has been acquired about the essential roles of some plant Trxs, particularly plastidial isoforms. Some are critical such as Trx z which is needed for plastid development and seedling viability (Arsova et al., 2010). NTRC-KO *Arabidopsis* plants display pale phenotype, impaired photosynthesis and sensitivity to oxidative stress, high temperature and prolonged darkness (Pérez-Ruiz et al., 2006; Lepistö et al., 2009; Chae et al., 2013). Other plastidial Trxs play more specialized functions such as CDSP32, which prevents oxidative damage during environmental constraints (Broin et al., 2002; Rey et al., 2005) and Trx y2, which maintains growth under high light conditions (Laugier et al., 2013). In other respects, cytosolic Trx h1 participates in responses to salt treatment (Zhang et al., 2011) and Trx h5 in sensitivity

to a fungal toxin (Sweat and Wolpert, 2007). Our present work reveals that Trx m fulfills essential functions in photosynthetic processes and in stress tolerance. Noteworthy, down-regulation of Trx m gene expression in rice also leads to impaired growth and reduced chlorophyll content in control conditions (Chi et al., 2008). This further argues for a function of Trx m as a central actor controlling photosynthesis. However, no obvious phenotype was recorded in *Arabidopsis* mutants deficient for either Trx m1 or Trx m4 (Courteille et al., 2013; Laugier et al., 2013). Since these Trxs share, with Trx m2, very similar sequence and biochemical properties (Collin et al., 2003), we hypothesize that the three isoforms could play overlapping functions in *Arabidopsis*.

PHENOTYPE OF Trx m$^+$ TOBACCO UNDER CONTROL CONDITIONS

Compared to Wt and Trx f$^+$ plants, Trx m$^+$ plants grown in control conditions display modified chlorophyll composition and impaired PSII activity (**Figure 2**; **Table 1**). Trx m overexpression in tobacco (**Table 1**), like Trx m deficiency in rice (Chi et al., 2008), leads to lower chlorophyll content and increased Chl *a*/Chl *b* ratio. Luo et al. (2012) showed that pea plants silenced for both genes encoding Trxs f and m exhibit reduced chlorophyll content and a much higher level of the oxidized form of CHLI, a subunit of magnesium chelatase, an enzyme essential for chlorophyll biosynthesis and regulated by Trxs (Ikegami et al., 2007). Moreover, Luo et al. (2012) reported that the expression of numerous genes involved in tetrapyrole biosynthesis was also strongly altered in these pea plants. Therefore, we can hypothesize that expression and redox activation of enzymes participating in chlorophyll synthesis are modified in Trx m$^+$ tobacco due to the large Trx excess, leading to change in pigment content. In other respects, one LHCII protein has been identified as a Trx target in spinach thylakoid membranes (Balmer et al., 2006). Interestingly in Trx m$^+$ plants, we recorded a much lower content in Chl *b* (**Table 1**), which is more specifically associated with LHC proteins, and preliminary proteomic analyses on transplastomic lines indicate that LHCII proteins are less abundant compared to Wt and Trx f$^+$ (unpublished data). All these data prompt us to propose that Trx m might be involved in the regulation of the light capture process via the control of the abundance and/or redox status of LHC proteins. We previously reported that Trx m$^+$ plants are characterized by the absence of cyclic electron transfer via the NDH pathway (Courteille et al., 2013). Thus, the suppression of this pathway might modify the NADPH/ATP ratio within plastids and finally impair the whole photosynthetic process. This hypothesis is not supported by the fact that tobacco plants knockout for the whole NDH complex do not exhibit any noticeable phenotype in control conditions (Horváth et al., 2000). Nonetheless, as the other cyclic electron pathway via the proton gradient regulation (PGR) complex is also negatively regulated by Trx m4 in *in vitro* experiments (Courteille et al., 2013), we cannot exclude that inhibition of both cyclic NDH and PGR pathways due to high Trx m abundance results in substantial impairment of photosynthetic processes. In other respects, in control conditions no obvious difference between the two transplastomic lines has been noticed regarding the activities in leaf extracts of two enzymes involved in carbon metabolism, NADP-MDH and NADP-GAPDH (**Figure 3**). Based on all these data, we conclude that the phenotype of Trx m$^+$ plants is probably not linked to changes in carbon metabolism, but more likely to modifications in photochemical processes from light capture to electron transfer.

Interestingly, plants overexpressing Trx m display reduced contents in ascorbate and GSH (*ca.* −20% compared to Wt and Trx f$^+$ plants), with no modification in redox status (**Figure 5**). This reveals that Trx m exerts a specific control on the amount of these compounds through mechanisms remaining to be delineated. Regarding AsA, redox regulation of enzymes involved in biosynthesis and regeneration pathways could account for the modified content. Indeed, dehydroascorbate reductase (DHAR), the enzyme regenerating AsA from its oxidized form, has been identified as a Trx target in several reports (Marchand et al., 2004; Hägglund et al., 2008; Montrichard et al., 2009) and reduction of DHAR by Trx is known to activate the enzyme (Dixon et al., 2002). Consequently, overexpression of Trx m in tobacco might trigger activation of DHAR and lead to increased AsA recycling, thus explaining the need for a reduced pool of AsA in Trx m$^+$ tobacco. But note that in tobacco plants overexpressing DHAR, increased AsA recycling is associated with increased AsA content (Chen et al., 2003). Currently, there is no evidence for a possible redox regulation of enzymes involved in GSH metabolism (Montrichard et al., 2009). It is worth mentioning that the increase in AsA content due to DHAR overexpression in tobacco plants is accompanied by a GSH pool increased in the same range (Chen et al., 2003). Further studies are needed to investigate whether the decreased GSH content in Trx m$^+$ plants is linked to modified AsA level or is a direct consequence of Trx overexpression. Whether the modified GSH and AsA pools in Trx m$^+$ plants lead to the observed growth and photosynthesis phenotype remains also unclear. Nonetheless, these data unveil a new role for Trx m in plant cell redox homeostasis through regulation of the content in major soluble antioxidants.

PHENOTYPE OF Trx m$^+$ TOBACCO UNDER OXIDATIVE TREATMENTS

Compared to Wt and to Trx f$^+$, Trx m$^+$ plants are more tolerant to conditions generating oxidative damage, induced by either MV or high light combined to low temperature (**Figures 6,7**), revealing a role of Trx m in the protection of plastidial structures. Accordingly, the abundance of Trx m1 is strongly up-regulated in cold-stressed *Arabidopsis* plants and this Trx has been proposed to preserve photosynthetic apparatus (Goulas et al., 2006). The tolerance of Trx m$^+$ plants is nonetheless rather puzzling when taking into consideration their reduced chlorophyll content (by more than 40% for Chl *b*) since an *Arabidopsis* mutant lacking Chl *b* is much more sensitive to photooxidative treatments, partly due to increased single oxygen production (Dall'Osto et al., 2010). Thus, the tolerance of Trx m$^+$ tobacco plants is not directly linked to chlorophyll content, but to other mechanisms also counterbalancing the deleterious effects due to Chl *b* shortage. Based on our data, we presume that neither NPQ, nor soluble antioxidants constitute primary determinants in this stress tolerance. Indeed, in Trx m$^+$ plants, NPQ is not modified (**Figure 2**) and both AsA and GSH pools are reduced by *ca.* 20% (**Figure 5**), with no change in redox status. In numerous cases, there is a positive correlation between stress tolerance level and AsA content. For instance, an *Arabidopsis* mutant deficient in AsA is highly sensitive to environmental constraints (Conklin et al., 1996) and tobacco plants with increased

AsA content are more tolerant to high light (Chen and Gallie, 2008). Regarding GSH, as reviewed very recently (Zagorchev et al., 2013), complex and contradictory data have been reported since tobacco plants with decreased GSH reductase activity are more sensitive to oxidative stress (Ding et al., 2009), but plants displaying elevated GSH biosynthesis capacity show high sensitivity to light (Creissen et al., 1999).

Trx m^+ plants display reduced PSII activity in control conditions (**Figure 2**), and surprisingly this activity is much less affected under oxidative conditions compared to Wt (**Figures 6,7**). Exposure to environmental constraints generally impairs PSII at the D1 protein level due to increased production of reactive oxygen species and inhibits PSII repair via the suppression of the synthesis of this subunit (Takahashi and Murata, 2008). Thus we can speculate that the excess of Trx m is associated with preservation of PSII structures in stress conditions. In connection with this hypothesis, it is worth mentioning that Trx has been proposed to regulate D1 synthesis as a function of light level (Danon and Mayfield, 1994).

Finally, we can hypothesize that the tolerance of Trx m^+ plants results from direct prevention by Trx m of damage in the photosynthetic apparatus. We recently reported that both plastidial Trxs f and m are able to form oligomers possessing chaperone-like properties (Sanz-Barrio et al., 2012). However, since there is a differential behavior of Trx m^+ and f^+ plants exposed to oxidative treatments, it appears unlikely that such a function could specifically account for the phenotype of Trx m^+ plants. In other respects, their stress tolerance could originate also from increased electron supply to Trx targets participating in antioxidant mechanisms, thus improving the plant capacity to adapt to challenging environmental conditions. Prxs are not likely involved in such a process since no noticeable difference was observed in abundance and/or redox status of the main plastidial Prxs in Trx m^+ plants (**Figure 4**). In contrast, we observed a twice higher MSR enzymatic capacity in Trx m^+ plants than in Trx f^+ and Wt plants with no change in protein abundance (**Figure 4**). This signifies that Trx m very likely constitutes a physiological electron donor to MSRs. This is consistent with our previous work showing that while MSR capacity is slightly lowered in *Arabidopsis* plants deficient for Trx f, it is significantly reduced in mutants lacking various Trx m types (Laugier et al., 2013). MSRs are key enzymes repairing oxidized proteins and possibly scavenging ROS via MetO (Moskovitz et al., 1997). In plants, they are involved in the protection against environmental constraints (Romero et al., 2004; Laugier et al., 2010) and in seed longevity (Châtelain et al., 2013). Altogether, these data give high credence for a role of the Trx m/MSR system in the tolerance of Trx m^+ plants to oxidative treatments.

To conclude, the complex phenotype of transplastomic tobacco Trx m^+ plants indicates that this Trx is very likely a central actor in plant cell redox homeostasis. In contrast to other types like f, the m type could regulate *in planta* numerous redox-based processes in photosynthesis and antioxidant responses. Further investigations, based for instance on co-immunoprecipitation methods to isolate Trx m partners in plant extracts (Rey et al., 2005), are needed to unveil the target proteins and the mechanisms underlying the physiological function(s) of this Trx type.

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The CP12 protein family: a thioredoxin-mediated metabolic switch?

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CP12 is a small, redox-sensitive protein, representatives of which are found in most photosynthetic organisms, including cyanobacteria, diatoms, red and green algae, and higher plants. The only clearly defined function for CP12 in any organism is in the thioredoxin-mediated regulation of the Calvin–Benson cycle. CP12 mediates the formation of a complex between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) in response to changes in light intensity. Under low light, the formation of the GAPDH/PRK/CP12 complex results in a reduction in the activity of both PRK and GAPDH and, under high light conditions, thioredoxin mediates the disassociation of the complex resulting in an increase in both GAPDH and PRK activity. Although the role of CP12 in the redox-mediated formation of the GAPDH/PRK/CP12 multiprotein complex has been clearly demonstrated, a number of studies now provide evidence that the CP12 proteins may play a wider role. In *Arabidopsis thaliana* CP12 is expressed in a range of tissue including roots, flowers, and seeds and antisense suppression of tobacco CP12 disrupts metabolism and impacts on growth and development. Furthermore, in addition to the higher plant genomes which encode up to three forms of CP12, analysis of cyanobacterial genomes has revealed that, not only are there multiple forms of the CP12 protein, but that in these organisms CP12 is also found fused to cystathionine-β-synthase domain containing proteins. In this review we present the latest information on the CP12 protein family and explore the possibility that CP12 proteins form part of a redox-mediated metabolic switch, allowing organisms to respond to rapid changes in the external environment.

Keywords: protein–protein interactions, redox, cystathionine-β-synthase (CBS)-domains, thioredoxin, intrinsically unstructured (disordered) protein

INTRODUCTION

Redox-mediated modulation of enzyme activity is an important post-translational mechanism involved in the regulation of cellular processes, enabling organisms to respond to changes in metabolic demands and environmental conditions. A group of well known redox-sensitive proteins, thioredoxins, play a major role in the regulation of cellular processes in plants, algae and cyanobacteria (Meyer et al., 2009; Buchanan et al., 2012). The mode of action of thioredoxin-mediated regulation is through the post-translational modification of cysteine residues on target proteins, bringing about the conversion of a disulphide bridge in the oxidized state, to a thiol group when reduced. In higher plants two thioredoxins (Trx f and Trx m) were first identified in the 1970s as activators of enzymes involved in photosynthetic carbon assimilation in the chloroplast (Buchanan, 1980; Buchanan and Balmer, 2005). The Calvin–Benson cycle is directly dependent on the energy adenosine triphosphate (ATP) and reducing power nicotinamide adenine dinucleotide phosphate (NADPH) derived from photosynthetic electron transport to drive the enzymatic reactions. In addition, reducing equivalents from electron transport are used to reduce thioredoxin via ferredoxin thioredoxin reductase. Trx f activates the Calvin–Benson cycle enzymes phosphoribulokinase (PRK), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose 1, 6-bisphosphatase (FBPase) and sedoheptulose 1, 7-bisphosphatase (SBPase) (**Figure 4**). Light intensity in the natural environment is variable and Trx redox state links the activity of these enzymes to the supply of ATP and NADPH in response to variations in light intensity. However, when temperature or light levels fall, the responses of thioredoxin-modulated enzymes in leaf tissues are not uniform. Under such conditions the activities of FBPase and SBPase can temporarily limit photosynthesis (Sassenrath-Cole et al., 1994; Hutchison et al., 2000) however, for PRK and GAPDH no such limitation has been reported and activation is rapid.

A second redox-mediated mechanism that regulates the activity of the Calvin–Benson cycle involves the aggregation of the enzymes PRK and GAPDH into a multiprotein complex which has been shown to be mediated by a small, nuclear-encoded chloroplast protein, CP12. This PRK/GAPDH/CP12 protein complex has been shown to be present in several higher plant (Wedel et al., 1997; Wedel and Soll, 1998; Scheibe et al., 2002; Howard et al., 2011a) and algal species (Avilan et al., 1997; Boggetto et al., 2007; Oesterhelt et al., 2007). The existence of this PRK/GAPDH/CP12 regulatory complex is well established (Avilan et al., 1997; Wedel et al., 1997; Scheibe et al., 2002; Marri et al., 2005b, 2009; Howard et al., 2011a) and when bound in this complex, the activity of the enzymes PRK

and GAPDH are decreased. Initially, evidence suggested that the association and dissociation of the PRK/GAPDH/CP12 complex is mediated via changes in NADP(H)/NAD(H) ratios within the chloroplast (Wedel et al., 1997; Wedel and Soll, 1998; Tamoi et al., 2005; Trost et al., 2006). However, more recently it has been shown that the status of the PRK/GAPDH/CP12 complex is regulated by changes in the redox state of Trx (Howard et al., 2008; Marri et al., 2009). *In vitro*, both chloroplastic Trx f and m have been shown to mediate the breakdown of the PRK/GAPDH/CP12 complex, via reduction of the two cysteine pairs on the CP12 protein (Marri et al., 2009). These studies provide evidence of a link between the redox state of Trx and that of CP12 in the formation and breakdown of the PRK/GAPDH/CP12 complex. When high levels of reduced Trx are available, CP12 will be maintained in a reduced state and little or no formation of the PRK/GAPDH/CP12 complex will occur. Conversely when levels of reduced Trx declines, levels of oxidized CP12 will increase resulting in the formation of the PRK/GAPDH/CP12 complex (**Figure 4**).

An important feature of the PRK/GAPDH/CP12 complex *in vivo* is the observation that dissociation and formation of the complex in pea leaves is rapid and that it responds to light intensity (Howard et al., 2008). In high light, dissociation occurred in under 1 min and on transfer to low light re-association was evident within 1 min; furthermore following 5 min in total darkness all of the PRK was found to be associated in the complex. A further important physiological observation is that although PRK contained within the complex is inactive it is found in both the reduced or oxidized state (Lebreton et al., 2003; Howard et al., 2008). The implication of this is that the PRK/GAPDH/CP12 complex provides a mechanism for sequestering and rapidly deactivating PRK and GAPDH in response to reduced light intensity. Conversely when light levels increase PRK and GAPDH are released and the reduced forms do not require Trx-activation and are instantly functional. This may provide an explanation for the rapid increase in PRK activity in response to an increase in light intensity, which is at least one order of magnitude quicker than the rate of Trx-mediated reductive activation of the oxidized form of this enzyme (Avilan et al., 2000). The physiological significance of the PRK/GAPDH/CP12 complex is that it provides a rapid response mechanism to regulate the rate of carbon fixation in the Calvin–Benson cycle, in response to changes in the availability of light to produce NADPH and ATP.

Despite a considerable body of data on the role of CP12 in the context of the regulation of PRK and GAPDH, some questions remain to be addressed in terms of the relative importance of this complex in regulating carbon metabolism and the possibility of a wider role for CP12 in redox regulation of metabolism. Firstly, it is still debatable whether the PRK/GAPDH/CP12 complex is a universal regulatory mechanism. Recent evidence has shown that the CP12 mediated regulation of PRK and GAPDH varies between different algal species (Maberly et al., 2010) and that there is heterogeneity in the PRK and GAPDH protein complex in higher plant species (Howard et al., 2011a). Furthermore, *in vitro* studies indicated that only fully oxidized *Arabidopsis* PRK is incorporated into the complex (Marri et al., 2005b) unlike the findings for the complex isolated from pea and *Chlamydomonas* (Lebreton et al., 2003; Howard et al., 2008). The importance of both CP12 and protein aggregation in the regulation of the Calvin–Benson cycle may therefore vary between species.

Four additional pieces of information raise further questions about the role of CP12. (1) In higher plants the CP12 proteins are encoded by a small gene family with different patterns of expression (Marri et al., 2005a; Singh et al., 2008). (2) Antisense suppression of CP12 in tobacco plants which resulted in a complex phenotype is not consistent with a loss of regulation of the Calvin–Benson cycle (Howard et al., 2011b,c). (3) Recent analysis of genome data from 126 species of cyanobacteria has revealed a wide diversity of CP12 protein sequences raising questions about the role of these different CP12 and CP12-like proteins (Stanley et al., 2013). (4) CP12 is a member of a class of proteins known as intrinsically unstructured proteins (IUPs; Gardebien et al., 2006; Erales et al., 2009b; Mileo et al., 2013). This article presents brings together information from a number of recent studies that suggest that CP12 may have a broader role in the regulation of metabolism, over and above the well established role of CP12 in the regulation of the Calvin–Benson cycle.

CP12 DISTRIBUTION AND STRUCTURE

Until recently the distribution of the CP12 proteins had been found exclusively within photosynthetic organisms and at least one CP12-like protein has been identified in all photosynthetic autotrophs including cyanobacteria, with the exception of the prasinophyte *Osterococcus* (Wedel et al., 1997; Wedel and Soll, 1998; Graciet et al., 2003; Marri et al., 2005a; Tamoi et al., 2005; Oesterhelt et al., 2007; Robbens et al., 2007; Groben et al., 2010; Stanley et al., 2013). More recently, it has been shown that proteins containing sequences with a high degree of similarity to the carboxy terminal region of CP12 have been identified in cyanophages (**Figure 1**).

In the *Arabidopsis* genome three genes have been identified and named *CP12-1* (At2g47400), *CP12-2* (At3g62410), and *CP12-3* (At1g76560). CP12-1 and CP12-2 are highly homologous and share 86% identity rising to 98% following cleavage of the transit peptide (**Figure 1**). Comparisons between these proteins in a variety of species have been unable to differentiate CP12-1 and CP12-2 into two separate *sub*-groups on the basis of their amino acid sequence (Singh et al., 2008; Groben et al., 2010). CP12-3 shares 41% and 48% identity with CP12-1 and CP12-2 respectively and phylogenetic analysis places *CP12-3* in a distinct clade. In all angiosperm species for which a full genome sequence is available, e.g., rice, maize and poplar, three CP12 encoding genes have been found with two being highly similar and a third being distinct, similar to the pattern in *Arabidopsis*. In contrast, in the green algae *Chlamydomonas reinhardtii* only one "canonical CP12" coding gene has been identified (Groben et al., 2010; Gontero and Maberly, 2012). In Gymnosperms, similar to *C. reinhardtii*, only one type of CP12 protein has been identified and this has more similarity to the CP12-3-like type. Furthermore, evidence to date suggests that the CP12-1/CP12-2-like forms are not present in this group of plants (Groben et al., 2010).

CP12 proteins in the green lineage have a highly conserved primary structure with three key features: an N-terminal cysteine pair, a C-terminal cysteine pair and a core "AWD_VEE" sequence (**Figures 1** and **2**). The N- and C-terminal cysteine pairs

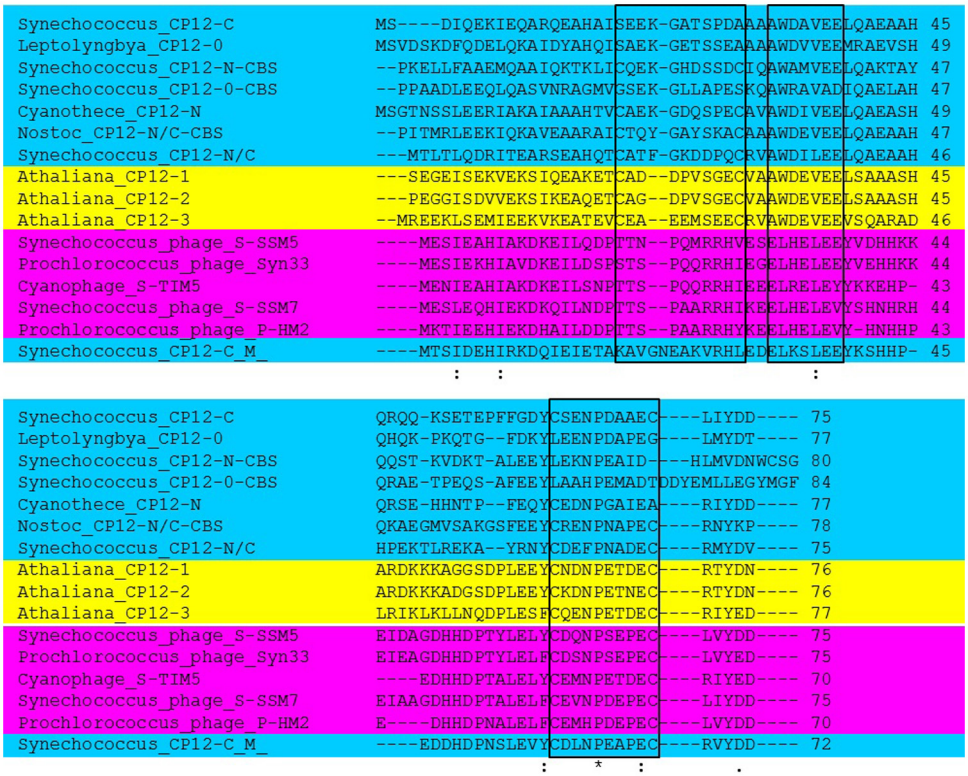

FIGURE 1 | Sequence analysis comparison of representative CP12 proteins. CP12 proteins from *Arabidopsis thaliana* (yellow), cyanobacterial CP12 sequences representing the eight different forms (blue) and representative CP12 sequences for cyanophages (pink). Conserved amino acid residues are denoted by (*); conservative changes (:) and semi-conservative (.); black boxes indicate the N- and C-terminal cysteine pairs and the AWD_VEE region. For the *A. thaliana* sequences only the mature proteins are shown and for the CP12-CBS proteins only the CP12-like region is presented. *Arabidopsis* sequences are: AT2G47400 (CP12-1), AT3G62410 (CP12-2), and AT1G76560 (CP12-3). NCBI Reference Sequence for Cyanophages: YP_004324623.1 (*Synechococcus* phage S-SSM5), YP_004323628.1 (*Prochlorococcus* phage Syn33), YP_007006052.1 (Cyanophage S-TIM5), YP_004324068.1 (*Synechococcus* phage S-SSM7), and YP_004323596.1 (*Prochlorococcus* phage P-HM2). Cyanobacterial CP12 sequence's IMG Gene ID (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=geneSearch) are: 637616925 (*Synechococcus*_CP12-N/C), 638958550 (*Synechococcus*_CP12-C(M)), 637616815 (*Synechococcus*_CP12-C), 643587017 (Cyanothece_CP12-N), 2509777734 (Leptolyngbya_CP12-0), 2503740304 (Nostoc_CP12-N/C-CBS), 641610209 (*Synechococcus*_CP12-N-CBS), 2506746062 (*Synechococcus*_CP12-0-CBS).

have been shown to form two intramolecular disulfide bridges when oxidized which are converted to thiol groups when reduced by Trx. Early studies provided evidence that both the N- and C-terminal disulfide bridges are necessary for the formation of the GAPDH/CP12/PRK complex (Wedel and Soll, 1998; Graciet et al., 2003). Although in the higher plants all CP12 proteins studied so far share all three of these features, there are exceptions to this in the red algae, haptophytes, cyanobacteria, and cyanophages (**Figure 1**; Groben et al., 2010; Thompson et al., 2011; Stanley et al., 2013). An unexpected diversity in the primary structure of CP12-like proteins was found to be present in cyanobacterial species. These have been classified into eight different groups based on the presence or absence of the three conserved features of classical CP12 proteins, i.e., the N- and C- terminal cysteine pairs and the central highly conserved "AWD_VEE" motif (Stanley et al., 2013). In addition, three of the cyanobacterial CP12-like protein classes described have a N-terminal cystathionine-β-synthase (CBS) domain (Stanley et al., 2013). No individual species of cyanobacteria has all eight of the different classes of CP12-like proteins, but with the exception of the marine picoplanktonic group, all other groups have at least one copy of the classical CP12 (CP12 C–N) form.

Structural and *in silico* studies have demonstrated that CP12 has physicochemical properties similar to those of IUPs (Graciet et al., 2003; Gardebien et al., 2006; Erales et al., 2009b; Marri et al., 2010; Mileo et al., 2013). IUPs possess relatively little structure *in vivo*. Instead, they adopt more structured conformations upon binding their target ligand. IUPs (and IUP regions within proteins) typically facilitate protein–protein interactions (Uversky, 2002; Tompa, 2005; Tompa et al., 2005; Uversky et al., 2005). It has therefore been proposed that CP12 is a conditionally unstructured protein and in the reduced state CP12 is disordered and inactive. But under oxidizing conditions, the formation of disulphide bridges and a α-helice results in a more structured active protein. *In silico* modeling of *C. reinhardtii* CP12 predicts the presence of two α-helices located in the N-terminal and central regions of the protein (Gardebien et al., 2006). In contrast, structural studies of cyanobacterial and higher plant GAPDH/CP12 binary complexes reveal only one alpha helix in the C-terminal region (**Figure 2**) while no structure in the N-terminal region

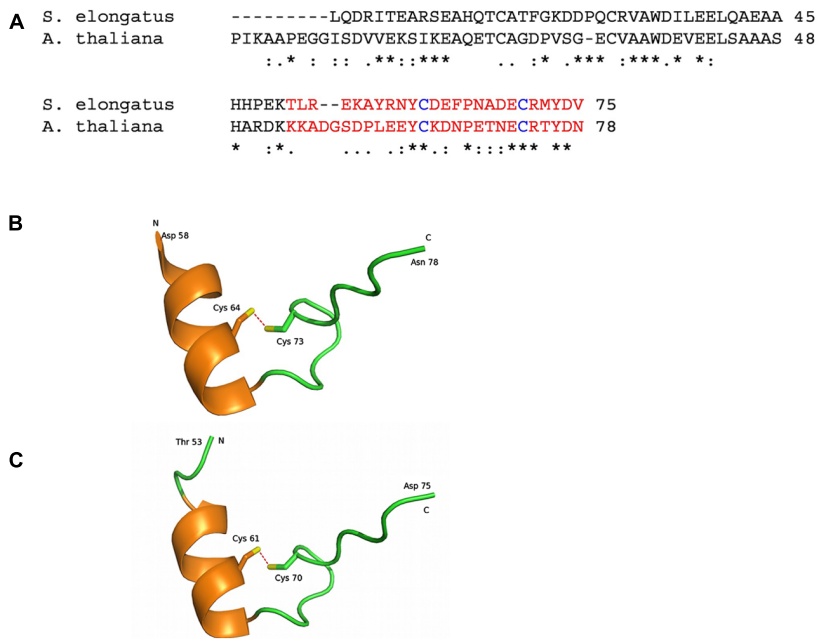

FIGURE 2 | Representations of crystal structures of the *Arabidopsis thaliana and Synechococcus elongatus* proteins. (A) The amino acid sequence alignment of Q9LZP9 *Arabidopsis thaliana* CP12-1 (mature protein) and Q31RN5 *S. elongatus* (strain PCC 7942). The regions in red is represented in the model structures in (**B**,**C**). **(B)** 2.0 Å resolution crystal structure of the C-terminal region of CP12 from *Arabidopsis thaliana* (Fermani et al., 2012; PDB accession code 3qv1). **(C).** 2.2 Å resolution crystal structure of the C-terminal region of CP12 from *S. elongatus* (Matsumura et al., 2011; PDB accession code 3b1j). Each structure contains a single alpha helix and a disulphide bond (red dashed lines). N and C represent the ends of the structured region, with the remaining amino acids being disordered and not modeled. Conserved amino acid residues are denoted by (*); conservative changes (:) and semi-conservative (.).

of either of these forms CP12 was evident, indicating that this region is highly unstructured (Matsumura et al., 2011; Fermani et al., 2012). On conversion to the reduced form CP12 loses the conserved α-helices present and becomes completely unstructured and this fully unfolded form is more flexible and mobile than oxidized CP12 (Gardebien et al., 2006; Gontero and Avilan, 2011; Matsumura et al., 2011; Fermani et al., 2012). The degree of disorder of CP12 has been shown to increase in higher plants compared to eukaryotic algae and cyanobacteria (apart from the green algal class Mesostigmatophyceae, which is ancestral to the streptophytes) and this has led to the suggestion that CP12 has evolved to become more flexible. This increasing disorder is likely to affect the functionality of CP12 and, given that higher flexibility has been found in other protein to be associated with a wider range of targets, CP12 proteins may have evolved additional roles in higher plants (Groben et al., 2010; Marri et al., 2010).

Bioinformatic analysis of CP12 proteins sequences has revealed some structural similarity with copper chaperones from *Arabidopsis* which have been shown to play different roles in copper homoeostasis (Himelblau et al., 1998; Mira et al., 2001a,b; Delobel et al., 2005). Furthermore, metal binding studies *in vitro* have shown that *Chlamydomonas* CP12 is able to bind both nickel (Ni^{2+}) and copper (Cu^{2+}) ions. Although the affinity for nickel is low (Kd 11 μM), the affinity for copper (Kd 26 μM) is within the same range of those reported for the prion protein (Kd of about 14 μM) and for copper chaperone proteins (Multhaup et al., 2001; Cobine et al., 2002; Delobel et al., 2005; Erales et al., 2009a). There is evidence showing that copper ions aid the formation of disulfide bonds in reduced CP12 leading to the recovery of fully oxidized CP12 which led to the hypothesis that the role of CP12 may be linked to copper metabolism (Delobel et al., 2005; Gontero and Maberly, 2012). However, structural studies have shown that GAPDH and PRK can interact with CP12 in the presence or absence of copper ions. In addition, the backbone structures of the GAPDH-CP12 binary complex of *Synechococcus elongatus* in copper-free and copper-bound forms are basically the same suggesting that copper is not essential for CP12 function in relation to the GAPDH/PRK/CP12 complex (Erales et al., 2009b; Matsumura et al., 2011).

CP12 GENE EXPRESSION

The three CP12 genes present in *Arabidopsis* are differentially expressed (Marri et al., 2005b; Singh et al., 2008). The expression of *CP12-2*, like *GAPDH* and *PRK*, is light dependent and is highest in photosynthetic tissues such as cotyledons, vegetative leaves and stalks. *CP12-1* transcripts are evident in dark-grown tissue and whilst it is abundantly expressed in photosynthetic tissues, it is also expressed in a range of tissues including flowers (siliques, styles, and sepals), seeds and root tips. In contrast *CP12-3* has very low expression in leaf tissue but accumulates in roots, stigma and anthers (Singh et al., 2008). Hypoxic conditions increase expression of *CP12-3* in the leaves while inhibiting the expression of *CP12-2*. Other environmental signals that affect the expression of the CP12 genes include low temperature, which decreases

expression of *CP12-2* (*CP12-1* and *CP12-3* are insensitive to this treatment).

In addition to the interesting results shown by the *in-vivo* expression studies, *in silico* co-expression analysis of the *Arabidopsis* gene family using the *Arabidopsis thaliana* trans-factor and cis-element prediction database, ATTED-II (http://atted.jp, Obayashi et al., 2011) has shown that the three CP12 genes have very distinct co-expression patterns and also correlate with expression of genes outside the Calvin–Benson cycle (**Figure 3**). Although this database is subject to updates which result in differences in the networks produced, the results from this bioinformatics package have consistently shown these tendencies. The co-expression network of *CP12-1* includes *GAPA-1* (a gene encoding the A subunit of GAPDH) and genes encoding the photosynthetic electron transport proteins. Interestingly, expression of *CP12-2* did not correlate with any of the genes encoding Calvin–Benson cycle enzymes, including GAPDH and PRK, but instead the co-expression network included the genes encoding subunits of the thylakoid membrane located NADH-dehydrogenase complex. The *CP12-3* gene shared expression patterns with genes encoding enzymes in phenylpropanoid biosynthesis, carbohydrate metabolism, regulatory kinases and transcription factors; but has failed to show co-expression with genes encoding the photosynthetic electron transport proteins or Calvin–Benson cycle components. The meaning and importance of these co-expression patterns is not yet clear and experimental approaches will be needed to understand the specific implications of these connections. Nevertheless, the differences in the co-expression patterns of the three plant CP12 genes raise questions about the role of the different CP12 isoforms and about the influence of CP12 regulation in wider metabolism.

CP12 *IN VIVO* FUNCTION

To date two studies have reported on the effects of reduced levels of CP12 protein *in vivo*. One using the cyanobacterial knock out mutant of *Synechococcus* PCC7942 (Tamoi et al., 2005) and the other tobacco antisense CP12 plants (Howard et al., 2011b,c). The resulting phenotypes indicate that CP12 has an important role in the regulation of metabolism. In *Synechococcus* PCC7942 the results were consistent with the proposal that CP12 was necessary for the separation of the activity of the Calvin–Benson cycle from the oxidative pentose phosphate pathway (OPPP) during day-night cycles. As a corollary to this study it was recently reported that cyanobacterial phage exploit this regulatory mechanism by introducing a copy of a CP12-like protein into the cyanobacterial host, which results in a down regulation of the Calvin cycle and an increase in flux through the OPPP. In addition to expression of a CP12 gene, phage genes involved in the light reactions, deoxynucleotide biosynthesis, and the OPPP, including a transaldolase gene, are all expressed in the host cyanobacteria. It is proposed that the cyanophage uses this metabolic switching strategy to boost production of NADPH to help fuel the biosynthesis of deoxynucleotides for phage replication (Thompson et al., 2011).

The situation in higher plants is likely to be different to that found in cyanobacteria. In higher plants not only are some Calvin–Benson cycle enzymes reductively activated in the light, but Trx f also reduces plastidic glucose 6-phosphate dehydrogenase (G6PDH), the first enzyme of the OPPP, which results in the inactivation of G6PDH (Wenderoth et al., 1997; Kruger and von Schaewen, 2003; Nee et al., 2009). Such a mechanism could be sufficient to prevent futile cycling between the Calvin–Benson cycle and the OPPP. Analysis of flux into the OPPP, measured by following the decarboxylation of 6-phosphogluconate to ribulose 5-phosphate catalyzed by 6-phosphogluconate dehydrogenase, together with measurements of G6PDH activity in antisense CP12 plants suggested that the cyanobacterial model cannot be applied to tobacco (Howard et al., 2011b). Furthermore, antisense suppression of CP12 had a limited effect on the ability of the PRK/GAPDH/CP12 complex to form in the presence of NAD and no significant impact on PRK or GAPDH enzyme activity or photosynthetic carbon fixation was detected. In contrast to this, significant changes in the growth rate and very dramatic alterations to morphology were observed in the CP12 antisense plants including a loss of apical dominance, fused cotyledons, altered leaf morphology and reduced fertility (Raines and Paul, 2006; Howard et al., 2011b,c). Furthermore, carbon allocation to the cell wall increase with a concomitant decrease in allocation of carbon to starch and soluble carbohydrates. Interestingly, in the CP12 antisense plants the activity of the Trx–activated enzyme NADP-malate dehydrogenase (NADP-MDH) was lower than in wild type plants and changes in pyridine nucleotide content were evident, suggesting a reduction in the activity of the malate valve. This observation is made more interesting because this decrease in NADP-MDH activity corresponded to changes in mobility of this enzyme analyzed using Blue Native PAGE (Howard et al., 2011b). These results indicated a structural change in NADP-MDH which could be due either to a change in conformation or aggregation state of this enzyme. Activation of NADP-MDH involves conformational changes and is subject to a two-stage process mediated by reduced Trx m. The change in mobility of this enzyme together with reduced activity in the antisense CP12 plants suggests that either CP12 is required for activation of NADP-MDH, or that the consequences of loss of CP12 in the plants impacts indirectly on NADP-MDH (Scheibe, 2004; Scheibe et al., 2005; Schneidereit et al., 2006; Howard et al., 2011b). Any reduction in NADP-MDH activity would be expected to impact on the ability to dissipate excess NADPH through the conversion of oxaloacetate to malate, via the malate valve (Scheibe, 2004; Scheibe et al., 2005). Interestingly, in the CP12 antisense plants significant reductions in levels of both 2-OG and malate were observed, indicating that there may have been impairment of the malate valve which in turn negatively affected 2OG cycling (Howard et al., 2011b). This result is consistent with the proposal that the AtpOMT1 transporter has a dual function in OAA/malate exchange in the malate valve and in 2-OG/malate exchange for carbon/nitrogen metabolism (Riebeseel et al., 2010).

CP12-LIKE FUSION PROTEINS

In *C. reinhardtii* the GAPDH holoenzyme is a homotetramer made up of A-type subunits and it has been shown that CP12 binding to this form of GAPDH confers redox regulation, mediated directly by Trx (Graciet et al., 2003). In higher plants there

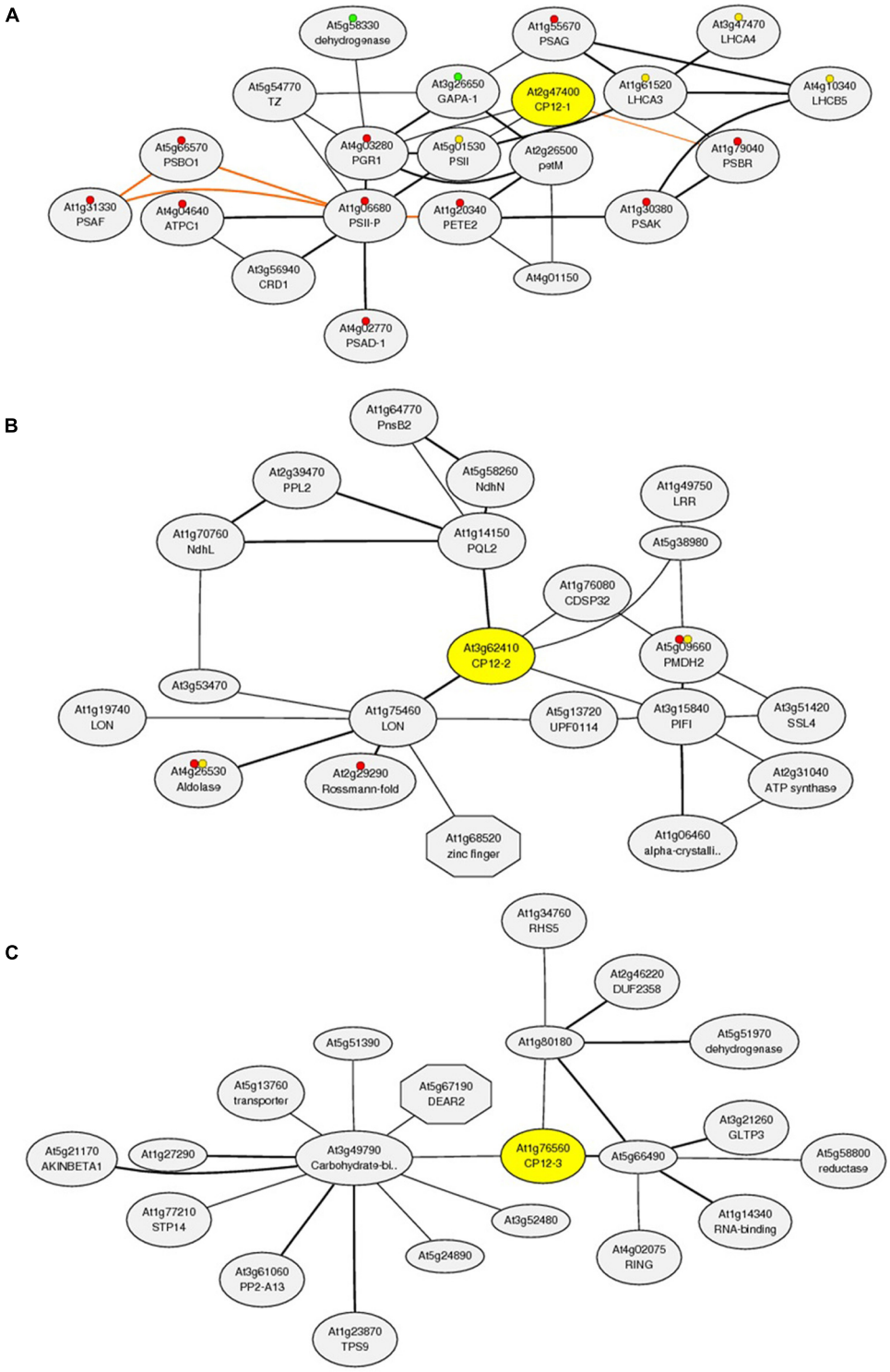

FIGURE 3 | Co-expression analysis of the CP12 gene family using the *Arabidopsis thaliana* trans-factor and cis-element prediction database, ATTED-II (produced 15/11/2013). Co-expressed gene network around **(A)** CP12-1 (http://atted.jp/data/locus/819353.shtml). **(B)** CP12-2 (http://atted.jp/data/locus/825414.shtml) and **(C)** CP12-3 (http://atted.jp/data/locus/843989.shtml). Colored marks accompanying some genes represent their involvement in: **(A) Red**: Photosynthesis, **Yellow**: Antenna proteins and **Green**: Carbon fixation in photosynthetic organisms. **(B) Red**: Biosynthesis of secondary metabolites and **Yellow**: Carbon fixation in photosynthetic organisms.

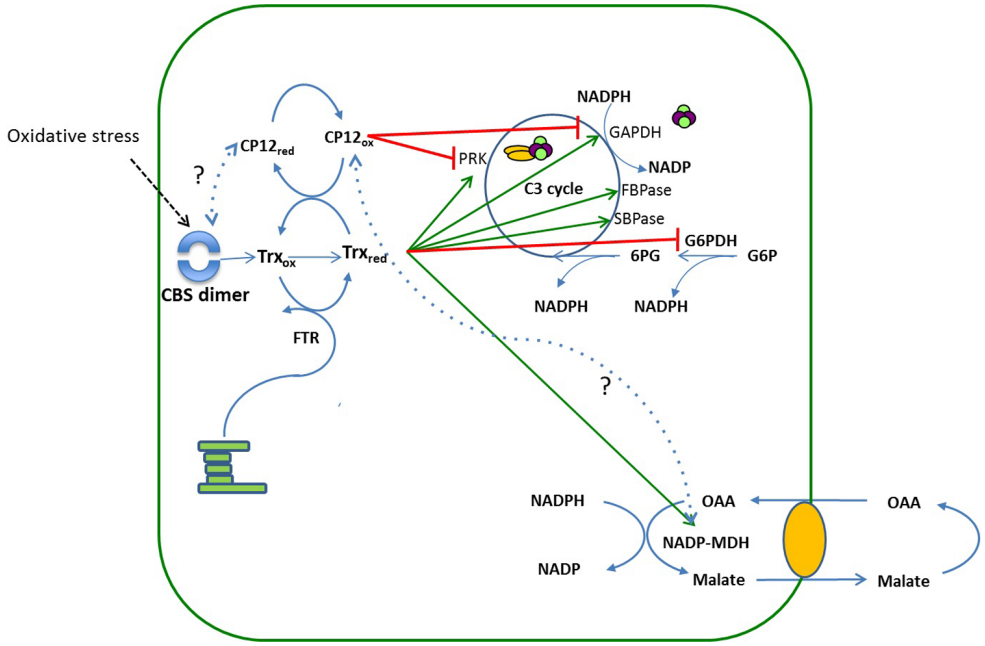

FIGURE 4 | A schematic showing the relationship between CBS domain containing proteins, thioredoxin and CP12 regulation of the Calvin cycle. The reducing equivalents from the electron transport chain reduce the disulphide bridges on thioredoxin to thiol groups. Reduced Trx then reductively activates enzymes of the Calvin–Benson cycle, PRK, GAPDH, SBPase, FBPase and the enzyme MDH. The redox state of CP12 is also determined by Trx, when light levels are high Trx is maintained in a reduced state and under these conditions CP12 is reduced. When light levels drop, Trx becomes oxidized and the levels of CP12 in the oxidized state also increase resulting in the formation of the PRK/GAPDH/CP12 complex and inactivation of PRK and GAPDH. Under oxidative stress dimerization of the plastid CBSX1 protein occurs, which increases the levels of reduced Trx, thereby maintaining CP12 in the reduced state. The green arrows indicate the Trx mediated activation of the enzymes GAPDH, PRK, SBPase, FBPase, and MDH and the red bars indicate deactivation of PRK and GAPDH by CP12 mediated formation of the complex or of G6PDH by reduced Trx. Dotted lines indicate hypothetical interactions between CBSX1 and CP12red and CP12ox and MDH.

is also a B form of the GAPDH subunit which forms a functional heterotetramer (A2B2) with the A subunit. The B subunit of GAPDH found in higher plants is believed to have arisen due to a gene duplication of the GapA gene and a subsequent fusion with the C-terminus of CP12 (Pohlmeyer et al., 1996; Petersen et al., 2006). This C-terminal extension contains two cysteine residues and has been shown to confer Trx-mediated redox regulation on the GAPDH A2B2 enzyme (Sparla et al., 2002). It had been suggested that CP12 provides redox regulation to the A4 homotetramer but that the A2B2 form is autoregulated (Trost et al., 2006). However, the picture is now less clear as CP12 has been identified as a component of a complex containing the A2B2 heterotetramer (Carmo-Silva et al., 2011). Furthermore higher plants species with no detectable A4 homotetramer have been shown to form a PRK/GAPDH/CP12 complex *in vitro* (Wedel et al., 1997; Scheibe et al., 2002; Howard et al., 2008; Carmo-Silva et al., 2011). More recently a second group of CP12 fusion proteins has been identified from analysis of cyanobacterial genomes. In addition to the diversity of classes of CP12 protein it has been shown that some CP12-like proteins exist as fusions with proteins containing a CBS domain (Stanley et al., 2013). The function of these CBS domain-containing proteins has not been defined and the role of the CP12 fusion in relation to the activity of this domain also remains to be resolved. Having said this, it is interesting to speculate that the fusion of the CP12-like motifs to the CBS domain containing proteins in cyanobacteria confers a redox regulation to the activity of the CBS protein, similar to that for GAPDH in higher plants.

A CBS-TRX-CP12 REDOX NETWORK?

The discovery of eight different classes of CP12 in cyanobacteria, some of which are fused to a CBS domain containing protein, raises interesting questions about the role of these fusion proteins in the regulation of metabolism. Evidence from *in silico* modeling studies indicates that the CBS-CP12 fusion proteins are unable to interact with GAPDH and therefore an alternative role for these proteins has to be considered (Stanley et al., 2013). In higher plants, although a large family of CBS domain type proteins has been identified, none have been found fused to a CP12-like domain (Kushwaha et al., 2009). However, two CBS domain-containing proteins in *Arabidopsis*, CBSX1 and 2 have been shown to be located in the chloroplast. Analysis of the CBSX1 and 2 insertion mutants revealed that theses proteins form a dimer under oxidative stress conditions and that in these mutants the level of reduced Trx f and m was increased (Yoo et al., 2011). This in turn will maintain a higher level of the reduced form of CP12, thereby modulating the activity of the Calvin–Benson cycle (**Figure 4**). CP12 may also influence directly the ability of the CBSX proteins to dimerize in response to changes in redox state in the chloroplast through Trx action on CP12 (**Figure 4**). Although there is no direct evidence to support this, it is worthy of consideration

given the presence of CP12 fusions with CBS domain containing proteins in cyanobacteria.

CONCLUSION

As yet no experimental evidence for a role for the CP12 proteins outside of the Calvin–Benson cycle has been shown nor has a unique function been assigned to the different forms of CP12 in any organism. However, evidence from a number of different sources is accumulating to suggest that the CP12-like proteins may act in combination with other regulatory proteins, e.g., Trx's and CBS domain containing proteins to modulate metabolism in response to changes in metabolic demand and environment, mediated by changes in the redox state. This raises the possibility that CP12 acts to switch on and off metabolic pathways in response to changes in redox status in the chloroplast network.

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Operation of trans-thylakoid thiol-metabolizing pathways in photosynthesis

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Thiol oxidation to disulfides and the reverse reaction, i.e., disulfide reduction to free thiols, are under the control of catalysts *in vivo*. Enzymatically assisted thiol-disulfide chemistry is required for the biogenesis of all energy-transducing membrane systems. However, until recently, this had only been demonstrated for the bacterial plasma membrane. Long considered to be vacant, the thylakoid lumen has now moved to the forefront of photosynthesis research with the realization that its proteome is far more complicated than initially anticipated. Several lumenal proteins are known to be disulfide bonded in *Arabidopsis*, highlighting the importance of sulfhydryl oxidation in the thylakoid lumen. While disulfide reduction in the plastid stroma is known to activate several enzymatic activities, it appears that it is the reverse reaction, i.e., thiol oxidation that is required for the activity of several lumen-resident proteins. This paradigm for redox regulation in the thylakoid lumen has opened a new frontier for research in the field of photosynthesis. Of particular significance in this context is the discovery of trans-thylakoid redox pathways controlling disulfide bond formation and reduction, which are required for photosynthesis.

Keywords: thylakoid lumen, photosynthesis, disulfide, thioredoxin

Thiol-disulfide chemistry refers to the oxidation of thiols (sulfhydryls) into disulfides and also the reversible reaction, i.e., the reduction of the disulfides to free thiols. *In vivo*, both chemical reactions are catalyzed by dedicated enzymes in cellular compartments where disulfide bonds and reduced thiols in protein targets need to be maintained at all times. Thiol-disulfide chemistry has been best studied in bacteria where it is required for the biogenesis of the periplasmic compartment (Denoncin and Collet, 2013). It has now become apparent that catalyzed thiol-disulfide reactions also operate in the mitochondrial intermembrane space (IMS) and the thylakoid lumen, which are topologically equivalent to the bacterial periplasm. In mitochondria, the Mia40/Erv1 proteins were discovered to be key enzymes of a disulfide relay system driving the import of cysteine-rich proteins into the IMS by an oxidative folding mechanism (Herrmann and Riemer, 2012). The thylakoid lumen has long been viewed as a vacant compartment, with the exception of the oxygen evolving complex (OEC) and some of the redox carriers (e.g., cytochrome *f*, Rieske, and plastocyanin-or its substitute cytochrome c_6) involved in electron transfer during the light reactions of photosynthesis. This dogma has now been revised with the realization that numerous molecules reside in the thylakoid lumen in addition to the previously known photosynthetic proteins. Proteomics studies revealed that the lumenal proteome (~80–200 proteins) is far more complicated than initially anticipated and includes proteases, chaperones, isomerases, redox enzymes, and other proteins of unknown activities (Kieselbach and Schröder, 2003). It is possible that the proteins revealed by proteomics regulate photosynthesis or other yet-to-be discovered processes unrelated to photosynthesis. A recent study demonstrated that lumenal PPD5 is not involved in photosynthetic electron transfer reactions, but rather controls the synthesis of strigonolactone, a plant hormone regulating axillary bud formation (Roose et al., 2011). There is mounting evidence that several lumenal proteins, including components of the photosynthetic chain, contain one or several disulfide bonds (Kieselbach, 2013; **Table 1**). However the functional importance of the disulfide(s) has only been examined for a small number of these proteins. The molecular mass of disulfide bonded proteins in the thylakoid lumen range from 10 to 55 kDa and the disulfide bond forming cysteines do not appear to occur in a motif. This is at contrast with most of the disulfide bonded molecules in the mitochondrial IMS which are small proteins (6–18 kDa) containing two disulfide bonds in either a $C(X)_3C$ or a $C(X)_9C$ motif (Sideris and Tokatlidis, 2010). Interestingly, while disulfide bond reduction in stroma-localized targets serves to activate several enzymes (Meyer et al., 2009), it appears that it is the reverse reaction, i.e., disulfide bond formation, that is required for the activities of several lumen-resident proteins (Buchanan and Luan, 2005).

In bacteria, the requirement for disulfide bond reduction in the periplasmic space was established mainly through studies of cytochrome *c* maturation. Cytochromes *c* are metalloproteins with one or several hemes covalently linked to a C*XX*CH motif on the protein (Thony-Meyer, 1997; Hamel et al., 2009). The need for a disulfide-reducing activity in the context of bacterial cytochrome *c* assembly seemed obvious because the periplasmic space is also the compartment where cysteine-containing proteins are oxidized by dedicated catalysts (Denoncin and Collet, 2013). The current

Table 1 | Lumenal disulfide bonded proteins in *Arabidopsis*.

protein	Gene locus	Classification	Function
PsbO1 (2 cys, **1 SS**)	AT5G66570	PSII subunit	Oxygen evolution
PsbO2 (2 cys, **1 SS**)	AT3G50820	PSII subunit	Oxygen evolution
PSI-N (4 cys, **2 SS**)	AT5G64040	PSI subunit	Regulation of PSI activity?
Rieske (2 cys, **1 SS**)	AT4G03280	Cytochrome b_6f subunit	Electron transfer in b_6f complex
Cyt c_{6A} (2 cys, **1 SS**)	AT5G45040	*c*-type cytochrome	Unknown
STT7/STN7 (2 cys, 1 SS)	AT1G68830	Kinase	State transition
FKBP13 (4 cys, **2 SS**)	AT5G45680	Peptidyl-prolyl *cis-trans* isomerase	Unknown
FKBP20-2 (2 cys, **1 SS**)	AT3G60370	Peptidyl-prolyl *cis-trans* isomerase	PSII supercomplex assembly
PrxQ (2 cys, **1 SS**)	AT3G26060	Peroxiredoxin	Unknown
VDE (13 cys, ≥**4 SS**)	AT1G08550	Violaxanthin de-epoxidase	Photoprotection
PPD6 (2 cys, **1SS**)	AT3G56650	PsbP domain protein	Unknown
TL15 (2 cys, 1 SS)	AT2G44920	Pentapeptide repeat	Unknown
TL17 (4 cys, **2 SS**)	AT5G53490	Pentapeptide repeat	Unknown
TL20.3 (4 cys, ≥1 SS)	AT1G12250	Pentapeptide repeat	Unknown
TL29 (2 cys, 1 SS)	AT4G09010	Ascorbate peroxidase	Unknown
CtD1 (5 cys, ≥1 SS)	AT4G17740	Protease	Processing of D1 subunit
CtD1-like (4 cys, ≥1 SS)	AT5G46390	Protease	Unknown
Deg5 (2 cys, 1 SS)	AT4G18370	Protease	Degradation of lumenal proteins?

The number of lumenal cysteines (cys) that can form an intramolecular disulfide and the number of disulfides (SS) are indicated in parenthesis. The number of disulfide is experimentally determined. Bolded font indicates that the presence and the number of the disulfide has been confirmed. "≥n SS" indicates that the protein contain at least n disulfide(s). Experimental evidence for the presence of disulfides comes from a combination of thiol trapping/labeling experiments, structure determination or the finding that the enzymatic activity is sensitive to disulfide reducing agents such as DTT or abolished by site-directed mutagenesis of conserved cysteine residues. Several single cysteine containing lumenal proteins (not indicated here) were recovered in thiol trapping experiments, suggesting that intermolecular disulfides can be formed. See references (Hall et al., 2010; Hall, 2012; Kieselbach, 2013).

view is that the C*XX*CH motif is oxidized after translocation of apocytochromes *c* to the periplasmic space and then reduced by a reducing pathway to provide free sulfhydryls for ligation with heme (Bonnard et al., 2010; Sanders et al., 2010). This question had received little attention in the context of plastid cytochromes *c* because thiol-disulfide chemistry as a catalyzed process was not believed to take place in the thylakoid lumen, the compartment where heme attachment to apocytochromes *c* occurs. The discovery of trans-thylakoid redox pathways controlling disulfide bond formation and reduction in *Chlamydomonas* and *Arabidopsis* has now changed this perception.

TWO DISULFIDE-REDUCING PATHWAYS OPERATE IN THE LUMEN

A central component of the bacterial disulfide-reducing pathways is the thiol-disulfide oxido-reductase of the DsbD family (Cho and Collet, 2013). In bacteria, members of this family (CcdA, DsbD, ScsB) are cytoplasmic membrane proteins conveying reducing power from the cytosol to the active sites of several target molecules in the periplasm. Reducing power is transferred across the cytoplasmic membrane through sequential thiol-disulfide exchanges (Cho and Collet, 2013). One target required for cytochrome *c* assembly is the membrane-bound, periplasm-facing, thioredoxin-like protein (CcmG/ResA/CcsX). CcmG/ResA/CcsX is postulated to reduce a disulfide in the C*XX*CH heme-binding site of apocytochrome *c* prior to heme ligation to the cysteines (Bonnard et al., 2010; Sanders et al., 2010). In the plastid lumen, the involvement of a disulfide-reducing pathway was first suspected based on the presence of CCDA, an ortholog of bacterial CcdA from the DsbD family, at the thylakoid membrane (Page et al., 2004; Motohashi and Hisabori, 2010; **Figure 1**). Another component in the plastid is HCF164 (High Chlorophyll Fluorescence), a membrane-anchored, lumen-facing, thioredoxin-like protein with similarity to bacterial CcmG/ResA/CcsX (Lennartz et al., 2001; **Figure 1**). The disulfide-reducing activity of HCF164 was inferred from the fact that a recombinant form of its lumenal domain displays disulfide reductase activity *in vitro* (Lennartz et al., 2001; Motohashi and Hisabori, 2006). Loss of function of CCDA or HCF164 in *Arabidopsis* produces a photosynthetic-deficient phenotype due to a defect in cytochrome b_6f complex assembly (Lennartz et al., 2001; Page et al., 2004). However, the biochemical activity and site of action of CCDA and HCF164 in the assembly process remained unknown until the characterization of the *Chlamydomonas ccs4* and *ccs5* mutants (Xie et al., 1998). The *ccs4* and *ccs5* mutants (*ccs* for *c*ytochrome *c* synthesis), which are partially photosynthetic deficient, display a block in the conversion of apo to holoform of lumen resident cytochromes *c*, namely membrane-bound cytochrome

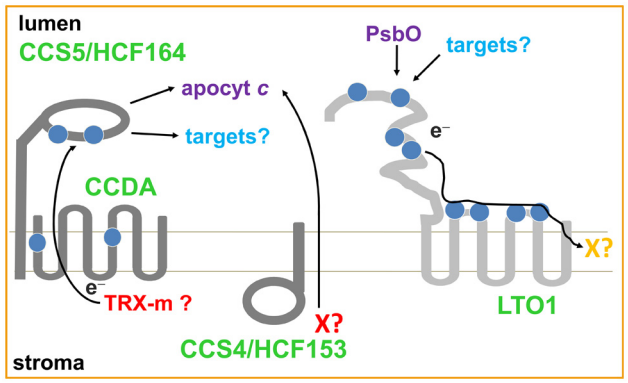

FIGURE 1 | *Trans*-thylakoid thiol metabolizing pathways. The disulfide-reducing (CCDA, CCS5/HCF164 and CCS4/HCF153) and thiol-oxidizing pathways (LTO1) are represented in dark and light grey, respectively. Cysteines are represented as blue balls. Electron (e^-) routes are indicated by arrows. The topology of CCS4/HCF164 is hypothetical but the positive-inside rule predicts a stromal localization for the C-terminal domain of the protein. CCS5/HCF164 is a membrane anchored lumen-facing thioredoxin-like protein. LTO1 contains a VKOR-like membrane domain and a lumen-facing thioredoxin-like domain. Stromal reductants for the disulfide reducing pathways are indicated in red. The electron acceptor for the thiol-oxidizing pathway is indicated in orange. Thioredoxin-*m* (Trx-m) is the possible reductant for the CCDA-CCS5/HCF164 pathway. The CCS4/HCF153 pathway might be involved in the transport of a reductant such as glutathione or ascorbate (X?). The final electron acceptor of the LTO1-dependent pathway is not known and is postulated to be a phylloquinone (X?). CCDA and LTO1 topology was deduced using PhoA/LacZ topological reporters (Page et al., 2004; Feng et al., 2011; Karamoko et al., 2011). The thylakoid membrane is shown in light grey.

f and soluble cytochrome c_6. The *ccs* mutants are deficient for cytochrome b_6f assembly, which is dependent on cytochrome *f*, the thylakoid membrane-bound cytochrome *c*. This assembly defect is at the step of heme attachment to apocytochromes *c*, a chemical reaction taking place in the thylakoid lumen (Howe and Merchant, 1992, 1993, 1994; Xie et al., 1998). The *Chlamydomonas CCS5* gene was cloned and shown to encode a thioredoxin-like protein with similarity to *Arabidopsis* HCF164 (Gabilly et al., 2010; **Figure 1**). *Arabidopsis* HCF164 can complement the cytochrome *c* assembly defect when expressed from the plastid genome of the *Chlamydomonas ccs5* mutant, demonstrating that HCF164 and CCS5 are functionally equivalent. This also suggests that the defect in cytochrome b_6f assembly in the *Arabidopsis hcf164* mutant is caused by a block in the conversion of apo to holocytochrome *f*. The role for CCS5 as an apocytochrome *c* disulfide reductase in cytochrome *c* assembly was formulated based on the following findings: (a) the *ccs5*-null mutant can be chemically rescued *in vivo* by application of exogenous thiols (such as DTT); (b) CCS5 interacts with plastid apocytochromes *c* in yeast two-hybrid assays; and c) a recombinant form of CCS5 is able to reduce a disulfide bonded C*XX*CH in apocytochrome *c in vitro* (Gabilly et al., 2010). The proposed model is that the thiol-disulfide oxido-reductase CCDA and the thioredoxin-like protein CCS5/HCF164 define a trans-thylakoid pathway for the delivery of reductants from the stroma to the lumen (Lennartz et al., 2001; Page et al., 2004; Motohashi and Hisabori, 2006; Gabilly et al., 2010; Motohashi and Hisabori, 2010; Gabilly et al., 2011). This pathway is required for cytochrome b_6f assembly but it is conceivable that it also acts as the transducer of reducing power to regulate the thiol-dependent activity of other lumenal targets (Motohashi and Hisabori, 2006). For instance, *in vitro* and *in organello* experiments showed that the reduction of the two disulfides in PSI-N (**Table 1**), a subunit of photosystem I (PSI), is dependent upon HCF164. The source of reducing power on the stromal side is not known but *in organello* experiments support the role of the thioredoxin Trx-*m* as a possible electron donor to CCDA and HCF164 (Motohashi and Hisabori, 2010).

The *Chlamydomonas CCS4* gene encodes a small protein with limited similarity to *Arabidopsis* HCF153, a thylakoid membrane anchored protein required for the assembly of the cytochrome b_6f complex (Lennartz et al., 2006; Gabilly et al., 2011; **Figure 1**). The CCS4 protein contains an N-terminal hydrophobic stretch that could serve as a membrane anchor and a C-terminal domain rich in charged residues. On the basis of the positive-inside rule that governs the topology of bacterial and thylakoid membrane proteins (von Heijne, 1989; Gavel et al., 1991), the C-terminal domain of CCS4 is predicted to be exposed to the stromal side of the thylakoid membrane. Surprisingly, CCS4 does not display any motif or residue (such as cysteines) suggesting a role in thiol-based redox chemistry. Yet, the thiol-dependent photosynthetic rescue of the *ccs4* mutant and the suppression of the *ccs4* phenotype by ectopic expression of CCDA, the thiol/disulfide oxido-reductase of the DsbD family, at the thylakoid membrane, confirms the activity of CCS4 in a disulfide-reducing pathway for cytochrome *c* assembly. Moreover, the CCDA-dependent suppression of the *ccs4* mutant confirms the placement role of CCDA in plastid cytochrome *c* maturation. Indeed, earlier studies in *Arabidopsis* supported, but did not establish, the requirement of plastid CCDA in the conversion of apo- to holocytochromes *c* (Page et al., 1997; Motohashi and Hisabori, 2010). Interestingly, the *ccs4*-null *ccs5*-null double mutant displays a synthetic phenotype characterized by a complete loss of b_6f assembly, an indication that CCS4 and CCS5 are redundant. This functional redundancy suggests that CCS4 might control a different disulfide-reducing pathway than the CCS5/HCF164 dependent one (**Figure 1**). The CCDA-dependent suppression of the *ccs4* mutant can be explained by a compensatory effect due to enhanced expression of the thiol-disulfide oxido-reductase CCDA. At the present time, the activity of CCS4 in such a pathway remains elusive. While the CCDA dependent pathway relies on the transfer of reductants through sequential thiol-disulfide exchanges, it is conceivable that CCS4 controls the transport across the thylakoid membrane of a molecule acting as a reducing agent (**Figure 1**). In bacteria, operation of such redundant routes for export of reductant to the periplasm have been postulated (Pittman et al., 2005). The nature of the exported reductant can only be speculated upon but glutathione or ascorbate is an obvious candidate. While the presence of glutathione in the thylakoid lumen remains to be established, ascorbate is known to function in this compartment as an alternative electron donor to PSII and a co-factor for violaxanthin de-epoxidase (VDE) dependent photoprotection (Tóth et al., 2013).

DISCOVERY OF A DISULFIDE-FORMING ENZYME IN THE THYLAKOID LUMEN

In the periplasmic space of most proteobacteria, the thiol-oxidizing pathway consists of a disulfide bond catalyzing system defined by soluble DsbA and membrane-bound DsbB (Denoncin and Collet, 2013). DsbA catalyzes disulfide bridge formation in cysteine-containing substrates that are translocated across the membrane into the periplasmic space. DsbB operates by recycling reduced DsbA to its oxidized form with transfer of electrons to quinones, which are membrane-soluble redox carriers in the respiratory chain. The fact that no DsbA or DsbB-like enzymes can be detected in the genomes of photosynthetic eukaryotes or Cyanobacteria, the presumed ancestors of the chloroplast, reinforced the view that disulfide bond formation did not take place in the thylakoid lumen. However, the operation of catalyzed disulfide bond formation in the lumen is supported by the finding that bacterial alkaline phosphatase (PhoA), an enzyme requiring two disulfide bonds for activity, and basic pancreatic trypsin inhibitor BPTI (aprotinin), a molecule containing three disulfide bonds, are active when targeted to this compartment in tobacco (Sone et al., 1997; Bally et al., 2008; Tissot et al., 2008). A novel class of disulfide-forming enzymes with similarity to VKOR (Vitamin K epoxide Oxidoreductase) was recently recognized in some bacterial phyla lacking the typical DsbAB components (including Cyanobacteria) and in all photosynthetic eukaryotes (Dutton et al., 2008; Singh et al., 2008; Grossman et al., 2010). VKOR is well studied for its involvement in the reduction of vitamin K, a phylloquinone required as a co-factor for the γ-carboxylation of clotting factors in blood (Tie and Stafford, 2008). Recent work shows that the enzymatic activity of VKOR is also linked to oxidative folding of proteins in the ER lumen (Rishavy et al., 2011; Schulman et al., 2011). A first indication that the thylakoid lumen houses a thiol-oxidizing pathway came from the identification of a VKOR-like protein in Cyanobacteria (Singh et al., 2008). *In vitro* reconstitution of disulfide bond formation with the purified cyanobacterial enzyme demonstrated the sulfhydryl oxidase activity of the protein (Li et al., 2010). However, the localization of the protein at the thylakoid membrane and its relevant targets of action in the lumen were not documented (Singh et al., 2008). The identity of the thiol-oxidizing catalyst in the thylakoid lumen of plastids has now emerged through the discovery of LTO1 (Lumen Thiol Oxidoreductase 1), a thylakoid membrane protein containing a VKOR domain fused to a thioredoxin-like moiety (**Figure 1**; Furt et al., 2010; Feng et al., 2011; Karamoko et al., 2011). Topological studies using bacterial topological reporters established a lumenal location for the LTO1 domains carrying the redox motifs and conserved cysteines (**Figure 1**). Previous studies with bacterial VKOR-like proteins have demonstrated that the thioredoxin-like domain carries a DsbA-like activity while the VKOR-like central domain is functionally equivalent to DsbB (Singh et al., 2008; Dutton et al., 2010; Wang et al., 2011). In *Arabidopsis*, loss of LTO1 function is associated with a severe phototrophic growth defect (Karamoko et al., 2011; Lu et al., 2013). Measurements of the photosynthetic activity indicate that *lto1* mutants display a limitation in the electron flow from Photosystem II (PSII). In accord with these measurements, *lto1* mutants show a severe depletion of several of the structural subunits of PSII (including subunits of the OEC) but no change in the accumulation of the cytochrome b_6f complex or PSI and no defect in the activity of ATP synthase. In a yeast two-hybrid assay, the lumen-facing thioredoxin-like domain of LTO1 was shown to interact with PsbO, a lumenal PSII subunit in the OEC known to be disulfide bonded (**Table 1**). *In vitro*, the thioredoxin-like domain of LTO1 is able to introduce a disulfide bond in the PsbO target when recombinant forms of the molecules are used. Because the redox state of the sulfhydryls in PsbO was shown to be a determinant for the stability of this subunit and also for PSII accumulation (Burnap et al., 1994; Nikitina et al., 2008; Hall et al., 2010), it is likely that loss of disulfide bond formation in PsbO in the *lto1* mutants accounts for the PSII assembly defect. It is not known if the ability of LTO1 to form a disulfide bond in PsbO is linked to the import of this protein into the lumen, similarly to the Mia40-dependent pathway in mitochondria (Herrmann and Riemer, 2012). *In organello* import experiments showed that PsbO translocates at a different site than PsbP, another subunit of the PSII OEC (Hashimoto et al., 1997). It is plausible that this translocation step is assisted by LTO1 but this awaits experimental testing.

The final electron acceptor of the LTO1-dependent disulfide bond forming pathway is currently unknown (**Figure 1**). It is likely to be a phylloquinone based on the fact that the *Arabidopsis* protein reduces phylloquinone in an *in vitro* enzymatic assay (Furt et al., 2010). The role of phylloquinone as a structural cofactor tightly bound to PSI is well documented (Brettel, 1997). However, the occurrence of a pool of phylloquinone that is not bound to PSI suggests phylloquinone might participate in redox processes in addition to the known electron transfer reactions through PSI (Gross et al., 2006; Lohmann et al., 2006). This pool may act as an electron acceptor for the LTO1-dependent disulfide bond forming pathway *in vivo*.

OUTLOOK

It is conceivable that catalyzed thiol-oxidation in the lumen extends to other disulfide-bond containing targets in addition to PsbO (**Table 1**). *In vitro* experiments suggest that the thioredoxin-like domain of LTO1 is also able to catalyze the formation of the two disulfide bonds in FKBP13 (Lu et al., 2013), a peptidyl-prolyl *cis-trans* isomerase whose activity is dependent upon sulfhydryl oxidation (Gopalan et al., 2004; **Table 1**). It is not known if additional enzymes with sulfhydryl oxidase activity besides LTO1 also operate in the thylakoid lumen. In bacteria, the disulfide-reducing pathway is also required to maintain the reduction state of periplasmic oxido-reductases that shuffle disulfide bonds that are incorrectly formed (Kadokura and Beckwith, 2010; Depuydt et al., 2011). The need for disulfide bond isomerization is critical for proteins containing more than two cysteines such as VDE, an enzyme involved in photoprotection whose activity depends on sulfhydryl oxidation (Kanervo et al., 2005; **Table 1**). Interestingly, the activity of recombinant VDE was initially reported to be low, presumably because of improper protein folding due to incorrect disulfide linkages (Bugos and Yamamoto, 1996; Hieber et al., 2002). Expression in the cytosol of a bacterial strain engineered for disulfide bond formation and isomerization resulted in a high level of VDE activity for the purified enzyme, an indication

that isomerization of disulfide bond in the lumen is also likely to be required for yielding an active enzyme (Saga et al., 2010). It is possible that LTO1 exhibits this activity based on the finding that its thioredoxin-like domain is active as a disulfide-bond isomerase *in vitro* (Lu et al., 2013). Another thylakoid membrane protein displaying disulfide-bond isomerase activity *in vitro* is LQY1, an enzyme required for the repair and re-assembly of photodamaged PSII (Lu, 2011). However, it is not known if the active site of LQY1 faces the lumenal or stromal side of the thylakoid membrane. Further experimental work is required to establish the molecular identity of the missing redox components defining the thiol-metabolizing pathways, identify their relevant targets of action and understand how they control photosynthesis or other processes in the thylakoid lumen.

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Overoxidation of chloroplast 2-Cys peroxiredoxins: balancing toxic and signaling activities of hydrogen peroxide

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Photosynthesis, the primary source of biomass and oxygen into the biosphere, involves the transport of electrons in the presence of oxygen and, therefore, chloroplasts constitute an important source of reactive oxygen species, including hydrogen peroxide. If accumulated at high level, hydrogen peroxide may exert a toxic effect; however, it is as well an important second messenger. In order to balance the toxic and signaling activities of hydrogen peroxide its level has to be tightly controlled. To this end, chloroplasts are equipped with different antioxidant systems such as 2-Cys peroxiredoxins (2-Cys Prxs), thiol-based peroxidases able to reduce hydrogen and organic peroxides. At high peroxide concentrations the peroxidase function of 2-Cys Prxs may become inactivated through a process of overoxidation. This inactivation has been proposed to explain the signaling function of hydrogen peroxide in eukaryotes, whereas in prokaryotes, the 2-Cys Prxs of which were considered to be insensitive to overoxidation, the signaling activity of hydrogen peroxide is less relevant. Here we discuss the current knowledge about the mechanisms controlling 2-Cys Prx overoxidation in chloroplasts, organelles with an important signaling function in plants. Given the prokaryotic origin of chloroplasts, we discuss the occurrence of 2-Cys Prx overoxidation in cyanobacteria with the aim of identifying similarities between chloroplasts and their ancestors regarding their response to hydrogen peroxide.

Keywords: chloroplast, hydrogen peroxide, peroxiredoxin, redox regulation, thioredoxin, oxidative stress

INTRODUCTION

Oxygenic photosynthesis is an essential process for life on Earth because it allows the use of light and water to produce biomass and oxygen. However, it is also a process potentially harmful due to the transport of electrons in the presence of oxygen, which inevitably produces reactive oxygen species (ROS). Several environmental challenges such as drought, low or high temperature, high light intensity, or salinity, alter chloroplast ROS homeostasis producing oxidative stress (Miller et al., 2010). To adequately respond to these stressful conditions chloroplasts are equipped with different antioxidant systems both enzymatic and non-enzymatic. It should be taken into account that besides their harmful effect, ROS have also signaling function (Laloi et al., 2007). This is the case of hydrogen peroxide, which is produced at high rate in chloroplasts of photosynthetic cells and has an important signaling activity (Mubarakshina et al., 2010), as confirmed by genome-wide expression analyses in tobacco and *Arabidopsis* (Vandenabeele et al., 2003; Vanderauwera et al., 2005).

Peroxiredoxins (Prxs), thiol-based peroxidases able to reduce hydrogen peroxide, peroxynitrite and organic peroxides, are among the most abundant chloroplast enzymatic antioxidant systems. Prxs are universally present in any type of organisms from bacteria to animals and plants (Dietz, 2003, 2011; Wood et al., 2003a; Rhee et al., 2005; Hall et al., 2009). These enzymes are classified into different classes including typical 2-Cys Prxs, which are homodimeric, atypical 2-Cys Prxs, which are monomeric, and 1-Cys Prxs. Both typical and atypical 2-Cys Prxs share a similar reaction mechanism involving two conserved Cys residues, termed peroxidatic and resolving, respectively (Wood et al., 2003a; Hall et al., 2009). During the catalytic cycle the peroxidatic Cys becomes transiently oxidized as sulfenic acid (-SOH) and then condenses with the resolving Cys to form a disulfide bridge. In the case of the typical 2-Cys Prxs, which are dimeric, the enzyme is fully oxidized when both pairs of catalytic Cys residues (peroxidatic and resolving) form disulfides, which have to be reduced to initiate a new catalytic cycle. This reduction is performed by a thiol-oxidoreductase, which usually is thioredoxin (Trx), though glutaredoxin and cyclophilins are also able to participate in this step. At high peroxide concentrations, the sulfenic acid intermediate of the peroxidatic Cys may become overoxidized to sulfinic ($-SO_2H$) or even sulfonic ($-SO_3H$) acids, which causes the inactivation of the enzyme. Based on the different sensitivities of 2-Cys Prxs to overoxidation, which is higher in enzymes from eukaryotes than from prokaryotes, Wood et al. (2003b) proposed the floodgate hypothesis. According to this hypothesis, oxidizing conditions promote the inactivation by overoxidation of sensitive 2-Cys Prxs in eukaryotic organisms, provoking a transient further increase of hydrogen peroxide, which may then be used as second messenger

(Wood et al., 2003b). In contrast, in prokaryotic organisms, the 2-Cys Prxs of which were considered to be insensitive, hydrogen peroxide is efficiently reduced and does not accumulate, thus having a less important function in signaling. Different reports confirm the relevant role of the hydrogen peroxide-dependent inactivation of 2-Cys Prxs in signaling processes in eukaryotic organisms (Karplus and Poole, 2012; Rhee et al., 2012). A notion reinforced by the recent finding that 2-Cys Prx overoxidation is a conserved marker of circadian rhythmicity (Edgar et al., 2012).

In plants, Prxs are encoded by a gene family, which in *Arabidopsis* is composed of ten members (Dietz, 2003). The first plant Prx identified was a 1-Cys Prx highly expressed in barley grains (Stacy et al., 1996). Later it was shown that this 1-Cys Prx accumulates in the nucleus of cereal seed tissues that undergo intense oxidative stress (Stacy et al., 1999; Pulido et al., 2009), suggesting a function in the antioxidant protection of nuclear structures. Chloroplasts are the organelles with the highest content of Prxs. The *Arabidopsis* chloroplast contains two almost identical typical 2-Cys Prxs, termed A and B, and atypical monomeric Prxs Q and IIE (Dietz, 2003, 2011). Although 2-Cys Prxs are among the most abundant plastidial proteins, a double mutant of *Arabidopsis*, which is a severe knock down for 2-Cys Prxs, shows a surprisingly mild phenotype (Pulido et al., 2010) suggesting that other antioxidant systems, such as the ascorbate-glutathione cycle in combination with superoxide dismutase, are able to compensate for 2-Cys Prx deficiency. Moreover, it was shown that chloroplast 2-Cys Prxs are sensitive to overoxidation, hence behaving as eukaryotic-type enzymes despite the endosymbiont origin of this organelle (Kirchsteiger et al., 2009). A more in-depth analysis of 2-Cys Prx in cyanobacteria showed that the enzyme from *Anabaena* is more sensitive to overoxidation than the *Synechocystis* enzyme (Pascual et al., 2010).

In this review we will discuss our present knowledge of the mechanisms controlling 2-Cys Prxs reduction and overoxidation in chloroplasts with emphasis in the effect of the redox status of 2-Cys Prxs on the activity of these enzymes. Moreover, we will discuss how the redox status of the chloroplast influences the signaling function of this organelle, which is essential to harmonize the growth and development of the different plant organs.

THE PATHWAYS OF 2-Cys Prxs REDUCTION IN CHLOROPLASTS

The *Arabidopsis* chloroplast is equipped with two almost identical typical 2-Cys Prxs, A and B, and atypical Prxs Q and IIE (Dietz et al., 2006). Although all these enzymes are relatively abundant, 2-Cys Prxs are among the most abundant proteins of the chloroplast (Dietz, 2011). Concerning their suborganellar localization, the presence of Prx IIE in the chloroplast stroma has been established, though its interaction with internal membranes was not analyzed (Bréhélin et al., 2003). Prx Q, which was described initially as associated to thylakoids (Lamkemeyer et al., 2006), was later localized in the thylakoid lumen (Petersson et al., 2006). Therefore, the exact localization of Prx Q still awaits confirmation (Dietz, 2011). 2-Cys Prxs A and B are localized in the chloroplast stroma in dimeric form, but become associated to the thylakoid membrane in their oligomeric form (Konig et al., 2002).

The function of the chloroplast-localized Prxs was first addressed by the generation of transgenic plants with antisense suppression of 2-Cys Prx expression (Baier et al., 2000), and then by the analysis of *Arabidopsis* mutants (Pulido et al., 2010). Single mutants with reduced levels of 2-Cys Prx A or lacking 2-Cys Prx B showed no phenotypic differences as compared with wild type plants, suggesting redundant functions of these enzymes (Pulido et al., 2010). Surprisingly, the double mutant Δ*2cp*, which is knock out for 2-Cys Prx B and a severe knock down for 2-Cys Prx A, shows almost wild type phenotype (Pulido et al., 2010). However, it was not possible to obtain a double knock out mutant, which suggests that plants cannot survive without at least a small amount of chloroplast 2-Cys Prxs. Most probably this is not exclusively due to their peroxidase activity, which can be compensated for by the other antioxidant systems of the chloroplast, such as the ascorbate-glutathione cycle in combination with superoxide dismutase. 2-Cys Prxs are complex enzymes showing different activities associated with different quaternary structures. As initially shown for the enzyme from yeast (Jang et al., 2004), the low-molecular-weight (LMW) form of 2-Cys Prxs shows predominantly peroxidase activity, whereas higher molecular weight (HMW) forms lack peroxidase activity while gaining chaperone activity. Interestingly, the switch from LMW to HMW is triggered under conditions of oxidative stress.

Chloroplast 2-Cys Prxs have a reaction mechanism similar to the enzyme from other eukaryotic organisms. The LMW form of the enzyme is arranged as a head-to-tail homodimer, which in its reduced form displays peroxidase activity (**Figure 1**). Therefore, 2-Cys Prxs can be considered as symmetric enzymes having two identical active sites. The catalytic cycle is initiated by the attack of the peroxidatic Cys to the peroxide rendering the corresponding alcohol, or water in the case of hydrogen peroxide, and the Cys residue oxidized to sulfenic acid (**Figure 1**). In a second step, the sulfenic acid intermediate is condensed with the resolving Cys producing a molecule of water and both Cys linked by a disulfide bridge (**Figure 1**). For a new catalytic cycle this disulfide has to be reduced. In chloroplasts Broin et al. (2002) proposed that a previously identified protein, termed CDSP32, which is formed by two Trx folds, with only one of them harboring a Trx active site, acted as reductant of 2-Cys Prxs. The *in vitro* analysis of several plastidial Trxs led Collin et al. (2003) to propose Trx *x* as the most efficient reductant of these enzymes. Finally, the chloroplast localized NADPH-dependent thioredoxin reductase C (NTRC), a peculiar NTR with a joint Trx domain at the C-terminus (Serrato et al., 2002, 2004) was shown to combine both NTR and Trx activity to efficiently reduce plastidial 2-Cys Prx (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). The notion that NTRC is the most efficient reductant of chloroplast 2-Cys Prxs was subsequently confirmed in further studies by *in vivo* analysis, based on fluorescence resonance energy transfer (FRET) assays, which showed interaction of 2-Cys Prx with NTRC but not with Trx *x* (Muthuramalingam et al., 2009). In addition, the redox status of 2-Cys Prxs was similar in wild type and Trx *x* knock out mutant plants, whereas the *ntrc* mutant showed a severely impaired redox status (Pulido et al., 2010). In summary, as depicted in **Figure 1**, chloroplast 2-Cys Prxs have a mode of action similar to the

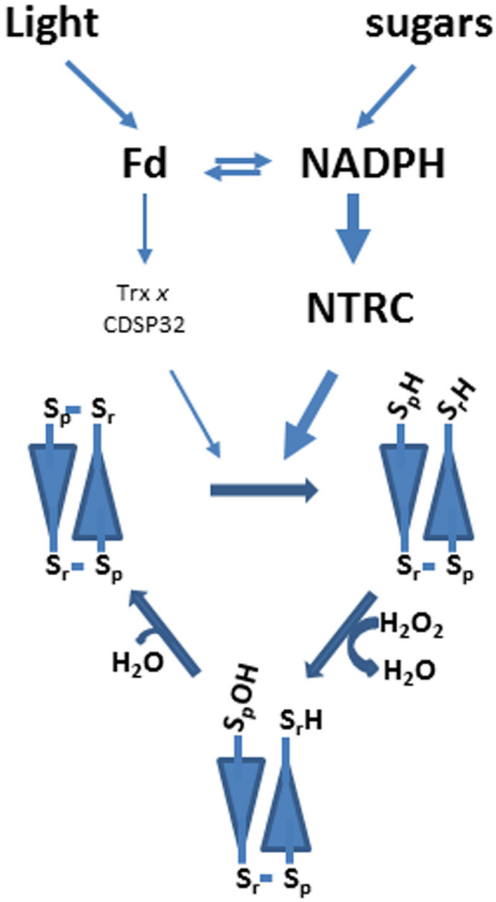

FIGURE 1 | Reaction mechanism and pathways for 2-Cys Prx reduction in chloroplasts. Typical 2-Cys Prxs are homodimeric enzymes arranged in head-to-tail conformation. Catalysis is performed by two cysteine residues, peroxidatic (S_p) and resolving (S_r). The peroxidatic cysteine reacts with hydrogen peroxide producing a molecule of water and becoming oxidized as sulfenic acid. In a second step the resolving cysteine condenses with the sulfenic acid intermediate so that a molecule of water is produced and both cysteines are linked by a disulfide bridge. For a new catalytic cycle the disulfide has to be reduced. In plant chloroplasts both *in vitro* and *in vivo* analyses suggest that NTRC is more relevant for 2-Cys Prx reduction than other plastidial Trxs such as CDSP32 and Trx *x*.

enzymes from other eukaryotic organisms and is predominantly reduced by NTRC. The required reducing power in form of NADPH is produced either by the photosynthetic electron transport chain, which occurs during the day, or from sugars by the initial reactions of the oxidative pentose phosphate pathway, which would be the predominant pathway during the night (Spínola et al., 2008; Cejudo et al., 2012). Though not experimentally established, it is expected that Trx *x* and CDSP32 use the reducing power of reduced ferredoxin (Fd) in a reaction catalyzed by Fd-dependent Trx reductase (FTR). Results from our group show that NTRC is unable to reduce plastidial Trxs, such as Trx *x* or CDSP32 (Pérez-Ruiz et al., 2006; Bernal-Bayard et al., 2012). Therefore, it seems that the two different pathways, NTRC and FTR/Trx, for 2-Cys Prx reduction are not connected.

CHLOROPLAST 2-Cys Prxs ARE SENSITIVE TO OVEROXIDATION

The study of the reaction mechanism of chloroplast 2-Cys Prxs revealed that the enzyme may become irreversibly oxidized during the catalytic cycle and shows tendency to form oligomers (Konig et al., 2002). These properties of chloroplast 2-Cys Prxs gained interest when Wood et al. (2003b) proposed the floodgate hypothesis according to which the signaling function of hydrogen peroxide in eukaryotic organisms is due to the overoxidation of the peroxidatic cysteine at the active site of 2-Cys Prxs. As mentioned above, during catalysis the peroxidatic cysteine becomes transiently oxidized to sulfenic acid, which under oxidizing conditions may be overoxidized to sulfinic or even sulfonic acid (**Figure 2**). This overoxidation inhibits the peroxidase activity of the enzyme thus allowing the local accumulation of hydrogen peroxide, which exerts its function as second messenger (Wood et al., 2003b). Though initially it was thought that overoxidation was an irreversible process, it was then found that sulfiredoxin (Srx) is able to reverse the overoxidized form to the reduced form of the enzyme in a reaction that required ATP and Mg^{2+} (Biteau et al., 2003; Woo et al., 2003). Overoxidation favors the formation of the HMW form of 2-Cys Prxs, which promotes the chaperone activity of these enzymes (**Figure 2**). All these data, obtained from analyses with yeast and human enzymes, indicated that the redox status of 2-Cys Prxs is essential to determine their peroxidase or chaperone activity, making them efficient sensors and key components of the response to oxidant conditions (Karplus and Poole, 2012).

In plants, the chloroplast is an essential organelle not only because of photosynthesis, but also because it is the site of synthesis of a variety of compounds, such as hormones, which play a role in signaling. The role of the chloroplast as an important source of hydrogen peroxide is well known (Mubarakshina et al., 2010). Indeed, we have recently shown that restitution of the redox homeostasis exclusively in chloroplasts, by expressing NTRC in the *ntrc* background mutant under the *RbcS* promoter, was necessary and sufficient to recover wild type growth and development of lateral roots regardless of the impaired redox homeostasis in root amyloplasts (Ferrández et al., 2012; Kirchsteiger et al., 2012). Therefore, whether or not chloroplast 2-Cys Prxs undergo overoxidation and the mechanisms controlling the redox status of the enzyme are relevant questions to determine their antioxidant and/or signaling function.

Two-dimensional gel electrophoresis analysis of 2-Cys Prx from wild type and mutants deficient in either 2-Cys Prx A or 2-Cys Prx B from *Arabidopsis* revealed the overoxidation of both enzymes (Kirchsteiger et al., 2009). Surprisingly, the NTRC knock out mutant showed lower level of 2-Cys Prx overoxidation than wild type plants, despite the fact that the deficiency of NTRC may cause oxidative stress. This was a first indication suggesting that the reduction of the enzyme, as a pre-requisite for the formation of the sulfenic acid intermediate, is required for the subsequent overoxidation to sulfinic acid, as outlined in **Figure 2**. The other component affecting the level of 2-Cys Prx overoxidation in chloroplasts is Srx, which is encoded in plants by a single gene, the protein showing dual targeting to chloroplast and mitochondria (Liu et al., 2006; Iglesias-Baena et al., 2011). Chloroplast Srx was shown to effectively reverse 2-Cys Prx overoxidation (Rey et al.,

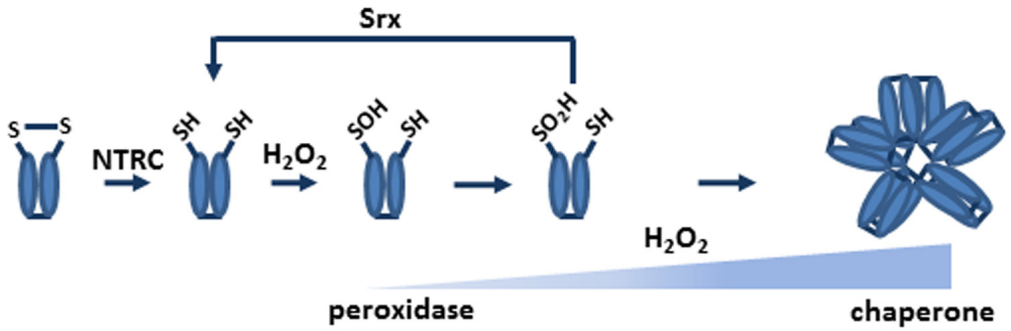

FIGURE 2 | NTRC and Srx determine the redox status of chloroplast 2-Cys Prxs. Under oxidant conditions, the sulfenic acid intermediate of the peroxidatic cysteine residue may be further oxidized to sulfinic acid. The reduction of the enzyme, which is most efficiently performed by NTRC, is a pre-requisite for sulfenic acid formation and, thus, for overoxidation. Srx is able to catalyze the reversion of the overoxidized to the reduced form of the enzyme. Therefore, the redox status of chloroplast 2-Cys Prxs is highly dependent of NTRC and Srx. The quaternary structure of 2-Cys Prxs determines the activity of these enzymes. In the reduced form the enzyme is a dimer and shows peroxidase activity; overoxidation favors the formation of the decameric form, which lacks peroxidase activity and shows chaperone activity.

2007; Iglesias-Baena et al., 2010), though enzyme was also shown to have redox-independent nuclease activity (Chi et al., 2012). The analysis of an *Arabidopsis* Srx knock out mutant revealed a function of the enzyme in the response to photooxidative stress (Rey et al., 2007). In addition, it was shown that the overoxidation of the chloroplast 2-Cys Prxs, like those of other eukaryotic organisms, triggers the oligomerization of the enzyme, which diminishes the peroxidatic activity while it increases the chaperone activity (Barranco-Medina et al., 2009).

Factors affecting 2-Cys Prx overoxidation in chloroplasts are summarized in the scheme outlined in **Figure 2**. The reduction of the enzyme, which is predominantly performed by NTRC, is a pre-requisite for the formation of the sulfenic acid intermediate. At high peroxide concentrations this intermediate may become increasingly overoxidized, which switches the peroxidase to chaperone activity of the enzyme. Reversion of the overoxidized form of 2-Cys Prx is catalyzed by Srx in a reaction that requires ATP and Mg^{2+}. According to this scheme, two enzymes, NTRC and Srx, seem to play a central role in controlling the redox status of 2-Cys Prxs in chloroplasts. It has been proposed that 2-Cys Prx may exert a critical function by balancing antioxidant and signaling activities of chloroplast produced hydrogen peroxide (Dietz et al., 2006). This function is probably essential as suggested by the fact that the double knock out mutant lacking both 2-Cys Prx A and B seems not viable. Nevertheless, much effort is still required to determine the reason why these enzymes have such an essential function for plant survival.

THE CYANOBACTERIAL ORIGIN OF CHLOROPLAST 2-Cys Prx OVEROXIDATION

According to the floodgate hypothesis, the signaling activity of hydrogen peroxide in eukaryotic organisms is based on the inactivation of 2-Cys Prxs by overoxidation, which allows the transient increase in the peroxide necessary to act as second messenger (Wood et al., 2003b). Structural analysis identified the GG(L/V/I)G and YF motifs in sensitive enzymes, and established that the peroxidatic cysteine is 14 Å away from the resolving cysteine, which makes the eukaryotic enzymes about 100-fold more sensitive to overoxidation than the prokaryotic ones (Wood et al., 2003b). Chloroplast 2-Cys Prxs are sensitive to overoxidation (Broin and Rey, 2003; Kirchsteiger et al., 2009; Iglesias-Baena et al., 2010), thus behaving as expected for enzymes of a eukaryotic organelle. Because it is well established that chloroplasts evolved from a prokaryotic endosymbiont (Gould et al., 2008), it arises the question whether 2-Cys Prx sensitivity was already present in the prokaryotic endosymbiont or was a gain-of-function of these enzymes that occurred during chloroplast evolution. To address this question, Pascual et al. (2010) analyzed the presence of the GG(L/V/I)G and YF motifs in the genes encoding 2-Cys Prxs from different sources. This search confirmed the presence of sensitive 2-Cys Prxs, characterized by the presence of both motifs, in eukaryotes. However, it revealed an unexpectedly large number of 2-Cys Prx from prokaryotic organisms containing the GG(L/V/I)G and YF motifs, thus being putatively sensitive to overoxidation. Interestingly, the 2-Cys Prxs from several cyanobacteria, such as *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, were found to contain these motifs. Biochemical analyses revealed that 2-Cys Prx from *Anabaena* sp. PCC7120 shows a level of sensitivity to overoxidation similar to that of the chloroplast enzymes, whereas 2-Cys Prx from *Synechocystis* sp. PCC6803 is less sensitive (Pascual et al., 2010). Moreover, *in vivo* analyses showed different strategies of these cyanobacterial strains to respond to oxidative stress. While *Anabaena* showed high sensitivity, *Synechocystis* survived higher concentrations of hydrogen peroxide. The strategy based on high efficiency of hydrogen peroxide detoxification provides higher resistance though, as it is rapidly reduced, the peroxide cannot be used for signaling. In contrast, the *Anabaena* strategy, based on low capacity of detoxification, causes the increase of hydrogen peroxide required to act as second messenger, though it may have as well a harmful effect. Interestingly, the strategy of chloroplasts, which are equipped with sensitive 2-Cys Prxs and lack catalase, is very similar to the *Anabaena* strategy. This is in agreement with the proposal that chloroplasts originated from cyanobacterial strains similar to present day *Anabaena* species (Deusch et al., 2008).

CONCLUDING REMARKS AND FUTURE PROSPECTS

The inactivation of the peroxidase activity of 2-Cys Prxs, caused by the overoxidation of their peroxidatic cysteines, has been proposed to be essential for the signaling function of hydrogen peroxide in eukaryotic organisms. In chloroplasts, which constitute an important source of hydrogen peroxide and have a prominent signaling function in plants, 2-Cys Prxs are among the most abundant proteins. Despite the prokaryotic origin of the plant chloroplast, the 2-Cys Prxs of this organelle undergo peroxide-mediated overoxidation, thus behaving as eukaryotic-type enzymes. The redox status of chloroplast 2-Cys Prxs, mostly controlled by NTRC and Srx, may balance the antioxidant and signaling functions of chloroplast-produced hydrogen peroxide and, thus, its activity as second messenger. Although much progress has been made on the biochemical properties of 2-Cys Prxs, little is yet known about the mechanisms explaining their function in signaling. The identification of the targets of these enzymes may be of aid to establish these functions.

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Thiol-based redox control of enzymes involved in the tetrapyrrole biosynthesis pathway in plants

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The last decades of research brought substantial insights into tetrapyrrole biosynthetic pathway in photosynthetic organisms. Almost all genes have been identified and roles of seemingly all essential proteins, leading to the synthesis of heme, siroheme, phytochromobilin, and chlorophyll (Chl), have been characterized. Detailed studies revealed the existence of a complex network of transcriptional and post-translational control mechanisms for maintaining a well-adjusted tetrapyrrole biosynthesis during plant development and adequate responses to environmental changes. Among others one of the known post-translational modifications is regulation of enzyme activities by redox modulators. Thioredoxins and NADPH-dependent thioredoxin reductase C (NTRC) adjust the activity of tetrapyrrole synthesis to the redox status of plastids. Excessive excitation energy of Chls in both photosystems and accumulation of light-absorbing unbound tetrapyrrole intermediates generate reactive oxygen species, which interfere with the plastid redox poise. Recent reports highlight ferredoxin-thioredoxin and NTRC-dependent control of key steps in tetrapyrrole biosynthesis in plants. In this review we introduce the regulatory impact of these reductants on the stability and activity of enzymes involved in 5-aminolevulinic acid synthesis as well as in the Mg-branch of the tetrapyrrole biosynthetic pathway and we propose molecular mechanisms behind this redox control.

Keywords: tetrapyrrole biosynthesis, redox regulation, post-translational, plant, ALA synthesis

INTRODUCTION

Regulation of the redox status of processes taking place in plastids, including the tetrapyrrole metabolism, by disulfide bond formation and reduction is one of the crucial post-translational control mechanisms to modulate protein activity, folding, and stability. The exploration of thiol-based control of enzymatic steps in tetrapyrrole biosynthesis has just been initiated. We will report about the enzymes which are proposed to be subjected to thiol-based regulation in Sections "Redox Modifications of ALA Synthesis Enzymes" and "Redox Control in the Mg-Branch." The thiol-reducing systems that control the redox state of enzymes of tetrapyrrole will be introduced in Section "Thiol-Based Redox Regulators of Higher Plants," while the significant role of post-translational redox control in tetrapyrrole biosynthesis will be discussed in Section "The Need for Post-Translational Redox Regulation in Tetrapyrrole Biosynthesis." An initial overview of the tetrapyrrole biosynthetic pathway is given in the following chapter.

THE OVERVIEW OF THE TETRAPYRROLE BIOSYNTHETIC PATHWAY IN PLANTS

Plant tetrapyrrole biosynthesis results in the formation of multiple end products, including chlorophyll (Chl), heme, siroheme, and phytochromobilin. These biomolecules belong to the most abundant macromolecules in the plant kingdom and are formed through a complex metabolic pathway that consists of at least 25 single enzymatic reactions in plastids (Tanaka and Tanaka, 2007; Mochizuki et al., 2010).

The tetrapyrrole metabolism begins with the synthesis of 5-aminolevulinic acid (ALA), the first committed metabolite of the pathway (**Figure 1**). Like most of the prokaryotes, photosynthetic eukaryotes synthesize ALA from glutamate (Glu), while fungi, animals, and α-proteobacteria catalyze the condensation of glycine and succinyl-CoA to ALA by ALA synthase. Plants use Glu which is coupled to glutamyl-transfer RNA ($tRNA^{Glu}$) by glutamyl-tRNA synthase (GluRS). One of the main regulatory enzymes of tetrapyrrole biosynthesis is glutamyl-tRNA reductase (GluTR), which reduces the activated glutamyl-tRNA to Glu-1-semialdehyde (GSA). GSA aminotransferase (GSAT) catalyzes an intramolecular transfer of an amino group of GSA to produce ALA. ALA synthesis is the rate limiting step of the entire pathway and precisely controlled at the transcriptional and post-translational levels by multiple factors (Mochizuki et al., 2010; Tanaka et al., 2011).

In continuation two ALA molecules are merged by ALA dehydratase (ALAD) to form the first monopyrrole porphobilinogen. Hydroxymethylbilane synthase (HMBS) consecutively condenses four molecules of porphobilinogen to its cofactor dipyrromethane, before the final product, the linear tetrapyrrole hydroxymethylbilane (HMB), is released. HMB is converted to uroporphyrinogen III (UroIII) by UroIII synthase (UROS). Siroheme is formed in a three-step reaction from UroIII (not specified in **Figure 1**), while in the main branch of tetrapyrrole biosynthesis UroIII decarboxylase (UROD) and coproporphyrinogen III oxidase (CPOX) catalyze in two sequential reactions the formation of protoporphyrinogen IX (Protogen). Protogen oxidase I (PPOX) catalyzes the removal

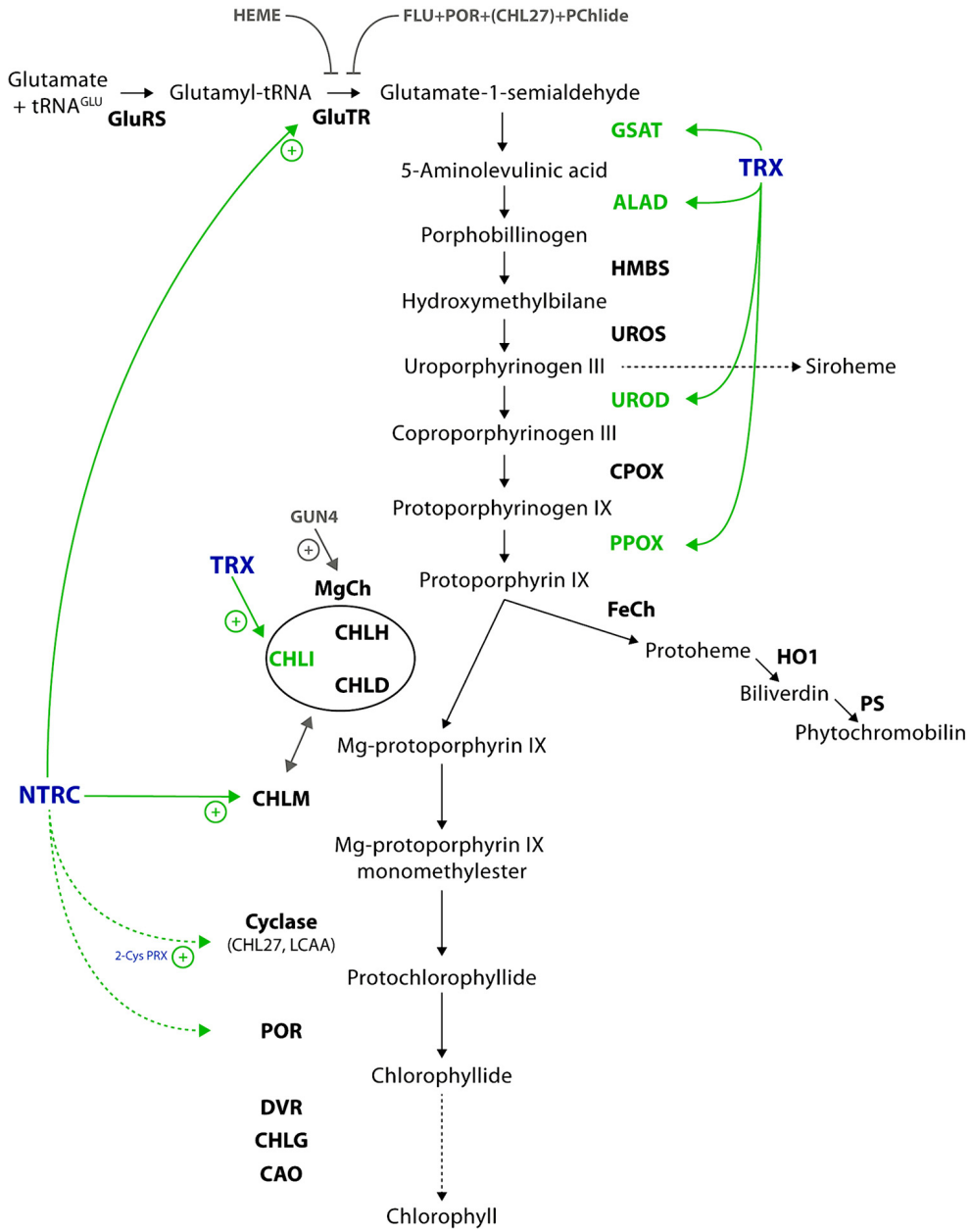

FIGURE 1 | Scheme of the plant tetrapyrrole biosynthetic pathway. The coloring highlights the redox regulation and targets of redox regulation in green and the involved enzymes in blue. Further post-translational regulations are indicated in gray, including the proteins involved. Solid lines refer to verified interactions and dotted lines to potential targets of thiol-based regulation. Further details and the abbreviations of the enzymes are given in the text.

of six electrons of Protogen to form protoporphyrin IX (Proto). At this point, the tetrapyrrole metabolism branches into two distinct pathways, the Fe- and Mg-branch. Whereas the Fe branch leads to the formation of heme and its derivates, the catalytic reactions of the Mg-branch result in Chl formation. Within the Fe branch ferrochelatase (FeCh) catalyzes the insertion of ferrous ion (Fe^{2+}) into Proto. Activities of heme oxygenase (HO) and phytochromobilin synthase (PS) convert Proto into biliverdin IX and phytochromobilin, the chromophore of phytochromes, respectively.

The insertion of an Mg^{2+} ion into the Proto macrocycle by magnesium chelatase (MgCh) is a highly energy-consuming step. MgCh consists of the three subunits CHLI, CHLD, and CHLH. CHLI and CHLD form each a hexameric ring that interacts with the catalytically active CHLH subunit. CHLI possesses ATPase activity (Jensen et al., 1999), while CHLH catalyzes the Mg^{2+}insertion into Proto. This assembly of CHLH with the CHLI/CHLD double ring structure is highly important for the ATP-driven chelation reaction (Hansson and Kannangara, 1997; Jensen et al., 1999). In addition the *in vitro* and *in vivo* activity

of MgCh is stimulated by the regulator protein GUN4, which was originally identified in a screen of mutants with impaired plastid to nucleus signaling (Susek et al., 1993; Larkin et al., 2003; Peter and Grimm, 2009). The product of the chelation reaction is Mg protoporphyrin IX (MgP) which is methylated through the action of MgP methyltransferase (CHLM). Thereby, a methyl group from *S*-adenosyl methionine is transferred to MgP to form MgP monomethylester (MgPME). Subsequently, the aerobic MgPME oxidative cyclase (herein after referred to as cyclase) catalyses the closure of a fifth ring which is specific for the chlorine-type products of the pathway. The cyclase is assumed to consist of three subunits (Rzeznicka et al., 2005), from which CHL27 (Tottey et al., 2003) and LCAA/YCF54 have been already identified and are considered to be essential subunits for the cyclase activity (Albus et al., 2012).

In angiosperms the strictly light-dependent protochlorophyllide oxidoreductase (POR) reduces the D ring of 3,8-divinyl-protochlorophyllide (DV-Pchlide) to 3,8-divinyl-chlorophyllide (DV-Chlide). Gymnosperms, algae, and cyanobacteria contain two structurally distinct PORs, which catalyze either light-dependently or light-independently Chlide formation. The 8-vinyl group of 3,8-DV-Chlide is then reduced by the DV-reductase (DVR) to form monovinyl Chlide. The DVR also catalyzes the DV reduction of other metabolites of the Mg-branch. In the following step, Chlide is esterified at the 17-propionate group of ring D with phytyl pyrophosphate by Chl synthase (CHLG) to form Chl a. Chl(ide) a oxygenase (CAO) catalyses Chl b formation at the end of the Mg-branch of tetrapyrrole biosynthesis (Tanaka and Tanaka, 2011).

THIOL-BASED REDOX REGULATORS OF HIGHER PLANTS

Regulation of the thiol–disulfide exchange reactions in metabolic processes is a decisive post-translational modification of proteins that affects their catalytic activity, folding, and turnover. The thiol-reducing system that controls the redox state of enzymes involved in tetrapyrrole biosynthesis is driven either by light and the ferredoxin-thioredoxin-dependent or the NADPH-thioredoxin-dependent pathway. In the first pathway the electrons flow through thiol–disulfide exchanges from ferredoxin to thioredoxin. This reaction is catalyzed by ferredoxin-thioredoxin reductase. In its reduced state, thioredoxin transfers electrons to the respective target proteins. The reducing state is supported by photosynthetic activity and allows thereby the functional separation of day–night activities. Consequently, in darkness the thiol groups of target proteins can be oxidized by oxygen, reactive oxygen species (ROS), oxidized thioredoxin, and glutathione.

In most cases a light-dependent reduction of enzymes correlates with their enhanced activity. Glucose 6-phosphate dehydrogenase, the entry enzyme into the oxidative pentose phosphate cycle, is an exception, and is inactivated by thiol-reducing activities. Initially, two chloroplastic thioredoxins, the f and m type, were reported as the light-dependent post-translational regulators of metabolic activity. At present, several other types of thioredoxins have been reported, which are classified according to their structure, target proteins, and localization (Chibani et al., 2010). The high multitude of thioredoxin-like proteins in photosynthetic organisms is surprising and challenging in terms of the identification of their specific functions by reverse genetic approaches. Functional redundancy of these thioredoxins in terms of their capacity to reduce thiol groups of the same target proteins most likely explains the repeatedly observed lack of phenotypical effects when single thioredoxin-encoding genes were inactivated. Using thioredoxin-affinity chromatography several proteins of tetrapyrrole biosynthesis have been identified as potential targets of thioredoxin: GSAT, UROD, ALAD, and PPOX (**Figure 1**; Balmer et al., 2003; Marchand et al., 2006). Pheophorbide a oxygenase and red Chl catabolite reductase are two enzymes of the Chl catabolic pathway and were also identified in proteomic studies as thioredoxin targets (Bartsch et al., 2008).

Apart from the ferredoxin-thioredoxin-dependent redox system, a NADPH-dependent thioredoxin reductase (NTR) contributes to the control of tetrapyrrole biosynthesis. Two out of three *Arabidopsis* NTR isoforms, NTRA and NTRB, are dual-targeted to the cytoplasm and mitochondria (Reichheld et al., 2005). The NTRC isoform is localized in plastids and, exceptionally, consists of a NTR module and the thioredoxin module (Serrato et al., 2004). NADPH donates the electrons, which are transferred through FAD to an internal disulfide bond. Following the electron transfer, the reduced cysteines of NTRC serve for the reduction of the thioredoxin domain. NTRC-dependent redox regulation can also occur during dark periods using NADPH generated, e.g., in the oxidative pentose phosphate pathway. Therefore, redox regulation of target enzymes by NTRC can occur independent from photosynthetic activities. It was previously shown that the combined action of NTRC and 2-cysteine-peroxiredoxin (2-Cys PRX) protects the MgPMME cyclase in *in vitro* enzyme assays (Stenbaek et al., 2008). Apart from the initial observations of a NTRC-dependent sensitivity of the cyclase reaction, NTRC deficiency had a strong impact on various proteins in tetrapyrrole biosynthesis. Western blot analysis of the tetrapyrrole biosynthesis enzymatic proteins in a *ntrc* mutant of *A. thaliana* revealed reduced content of GluTR, POR, and CHLM (Richter et al., 2013; see also Redox Modifications of ALA Synthesis Enzymes and Redox Control in the Mg-Branch).

It has been found in recent studies that glutathione participates in a post-translational modification, named glutathionylation, consisting of the reversible formation of mixed disulfides between a thiol group of the target protein and the thiol of glutathione (Dixon et al., 2005; Rouhier et al., 2008; Zaffagnini et al., 2012). This post-translational modification is generally promoted by ROS such as hydrogen peroxide and protects cysteine residues from irreversible oxidation (see also The Need for Post-Translational Redox Regulation in Tetrapyrrole Biosynthesis). But it has also been shown that enzymatic activity of glutathionylated proteins was altered. While glutathionylation can occur via non-enzymatic mechanisms *in vivo*, the reverse reaction (namely deglutathionylation) is catalyzed by glutaredoxins. These are small oxidoreductases also belonging to the thioredoxin superfamily.

Using radioactively labeled glutathione, a proteomic approach in *Chlamydomonas* revealed 25 target proteins, mainly in the chloroplast. Among the identified proteins, the CHLI subunit of MgCh was shown to be glutathionylated (Michelet et al., 2008). Additional targets in tetrapyrrole metabolism cannot be

excluded and glutathione function has to be explored to clarify potential roles in redox regulation, protection, and signaling through glutathionylation and deglutathionylation of specific target proteins.

Apart from post-translational redox modifications, some enzymes of tetrapyrrole biosynthesis contain metal cofactors or iron–sulfur clusters as active redox center in their catalytic centers. These cofactors function in electron transfer reactions to tetrapyrrole substrates and are served with electrons from ferredoxin. Although these reactions do not belong to thiol-dependent redox reactions, these electron distributions are briefly mentioned at the end of this chapter.

Ferredoxin is a [2Fe–2S] cluster containing enzyme that divert electrons to $NADP^+$, but also to other redox-mediating components and is therefore essential in the metabolic reactions including Chl biosynthesis (Hanke and Mulo, 2013). The final enzyme of Chl synthesis, CAO, contains a Rieske-type iron–sulfur cluster[2Fe–2S] and receives electrons from ferredoxin. A structurally related enzyme of Chl degradation, pheophorbide a oxygenase also catalyses the electron transfer via the [2Fe–2S] cluster. Thereby, ring opening of pheophorbide a generates the product red Chl catabolite. Sirohydrochlorin FeCh which belongs to the siroheme biosynthesis branch, as well as Chl b reductase and 7-hydroxymethyl Chl a reductase, two enzymes of the Chl cycle, are all ferredoxin-dependent enzymes (Tanaka and Tanaka, 2011).

Ferredoxin participates also in reactions of heme degradation and synthesis of phytochromobilin, the chromophore of phytochrome (**Figure 1**). Protoheme degradation is initiated by a ring opening reaction catalyzed by HO. This enzyme transfers electrons from ferredoxin to heme and catalyses the formation of biliverdin IX. The successive enzyme, PS, reduces biliverdin IX in a ferredoxin-dependent step to phytochromobilin.

In summary, there are first experimental evidences for physical interactions of potential redox regulated enzymes with redox regulators arguing for a control of the tetrapyrrole biosynthetic pathway in response to the redox poise of chloroplasts. But, despite these experimental hints the need for a specific redox regulation of each enzyme has to be scrutinized in future in its physiological environment.

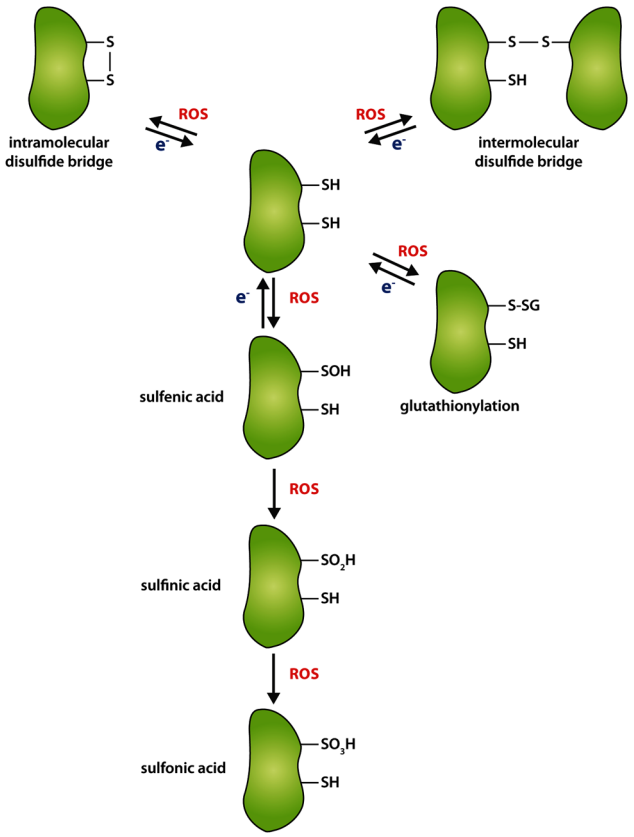

FIGURE 2 | Differential oxidation of thiol groups of cysteine residues. Oxidation of cysteine residues by reactive oxygen species (ROS) can lead to the formation of intramolecular (upper left corner) or intermolecular disulfide bridge (upper right corner), respectively. In the absence of an appropriate interaction partner or a second cysteine for disulfide bridge formation, cysteines can be stepwise oxidized to sulfenic (–SOH), sulfinic ($–SO_2H$), or sulfonic acid ($–SO_3H$). Mixed disulfides can be formed with glutathione (GS). Re-reduction (e^-) of oxidized cysteines is performed by target-specific enzymes, like thioredoxin, NTRC, or GRX.

THE NEED FOR POST-TRANSLATIONAL REDOX REGULATION IN TETRAPYRROLE BIOSYNTHESIS

Cysteine residues are the main redox active components in proteins. Their thiol groups can be reversibly oxidized to stabile intra- or intermolecular disulfide bonds (**Figure 2**). The thiol–disulfide exchanges occur despite the reducing environment in intracellular compartments, such as the cytoplasm, plastidic stroma, or mitochondrial matrix, and in oxidative environment of the extracellular space, including the apoplasm, the vacuole and the endoplasmic reticulum. The thiol group modifications of cysteines are particularly susceptible to oxidation by ROS (*S*-sulfenation) and nitric oxide (NO, *S*-nitrosylation). Dependent on subcellular restricted oxidative conditions, stable intra- and intermolecular disulfide bonds of proteins or mixed disulfide bonds with reduced glutathione (GSH) as well as less stable sulfenic acid side chains can be formed. These thiol switches (thiol–disulfide exchange reactions) lead to structural alterations of proteins indicating that cysteine residues can be critical for protein function and structure.

The need of thiol-based regulation of tetrapyrrole biosynthetic pathway is explained by the regulatory interdependency of a balanced metabolic flow during rapid environmental changes and the high risk of accumulating photosensitizing tetrapyrrole metabolites. One important source of oxidation is the innate production of singlet oxygen ($^1O_2$) in tetrapyrrole biosynthesis, when light-absorbing tetrapyrrole metabolites accumulate as a consequence of a deregulated pathway. Then, these tetrapyrrolic intermediates act as photosensitizers leading to $^1O_2$ formation by transfer of excitation energy from excited triplet-state of these metabolites to the triplet-state of molecular oxygen ($^3O_2$) upon illumination. Another type of ROS production can occur during photosynthesis, when electrons of the linear photosynthetic transfer chain are incurred from oxygen resulting in the formation of superoxide radicals ($O_2^{\bullet-}$; Apel and Hirt, 2004; Asada, 2006). Superoxide is reduced by superoxide dismutase (SOD, in plastids the copper–zinc and iron-dependent SOD) to hydrogen peroxide, which is

reduced to water by peroxidases (e.g., ascorbate peroxidase; Apel and Hirt, 2004; Asada, 2006).

The risk of tetrapyrrole-induced ROS formation drastically increases, when the scavenging activity of ROS-detoxifying enzymes, including SOD or peroxidases, is overstrained (Mock and Grimm, 1997). In addition, excess of highly reactive $^1O_2$ tends to induce lipid peroxidation which can than interfere with various metabolic processes (Vavilin and Vermaas, 2002; Fischer et al., 2013).

The FLU protein of *Arabidopsis* was demonstrated to act as a dark repressor of ALA synthesis. The *flu* mutant accumulates high amounts of non-protein bound Pchlide in the dark (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002; Richter et al., 2010). Upon light irradiation, the excited, free Pchlide in the triplet-state (^{3}Pchlide) can react with $^3O_2$ to produce ROS (Vavilin and Vermaas, 2002; Triantaphylides et al., 2008). It was demonstrated, that in fact all accumulating porphyrin and Mg porphyrin intermediates (and the visible light-absorbing Chl catabolites) enhance ROS production upon light irradiation resulting in phototoxic damage to cellular components and mimicking the effects of photodynamic herbicides (Mock et al., 1995; Kruse et al., 1997; Mock and Grimm, 1997; Lermontova and Grimm, 2000; Peter et al., 2010).

On the whole, the toxicity of all types of ROS depends on their distinct reactivity and signaling activity, half-life and detoxification. Cells are permanently challenged to control and minimize ROS levels in all intracellular compartments by instantaneous detoxification activities, although ROS also act as signaling components for the activation of acclimating antioxidative stress responses. ROS-mediated signaling likely includes reversible modifications of proteins, which ultimately result in adaptation responses. Post-translational modifications of protein thiols, such as thiol–disulfide exchange, sulfenation, glutathionylation, and nitrosylation, could potentially be involved in redox signaling. Singlet oxygen ($^1O_2$) was shown to have an important signaling function in the communication between plastids and the nucleus under certain conditions (Kim et al., 2008, 2012; Kim and Apel, 2013). However, $^1O_2$ has a very short half-life in the cell (∼200 ns; Gorman and Rodgers, 1992) and can be rapidly quenched by the reaction with water, tocopherols or carotenoids (Asada, 2006; Triantaphylides and Havaux, 2009). Accumulation of other ROS, mainly hydrogen peroxide, can modulate the redox signaling pathway and trigger the response of distinct detoxifying enzymes.

One common feature of all ROS is their non-site-specific reactivity which can result in the oxidation of biomolecules, like pigments, proteins, or lipids. Dependently on an oxidative cellular environment and with increasing ROS levels, thiol groups of cysteine residues become oxidized (Dalle-Donne et al., 2009; Triantaphylides and Havaux, 2009). As mentioned above in the introductory paragraph of this chapter due to its potent accessibility of oxidants, cysteines can form adducts with molecular oxygen to sulfenic (–SOH), or to the irreversible forms sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) acids (**Figure 2**). During a less severe oxidative intracellular environment, oxidation of a cysteine thiol group can either lead to the formation of an intra- or intermolecular disulfide bridge between proteins or low-molecular weight mixed disulfides in dependency of the three-dimensional conformation of the protein, the presence of an appropriate interaction partner or other agents with thiol groups (Dalle-Donne et al., 2009). In consequence of the oxidation, active site or regulatory cysteines can influence enzyme activity, conformational integrity, or stability of proteins (Buchanan and Balmer, 2005; Luo et al., 2012; Richter et al., 2013). The re-reduction of disulfide bridges and thus opening of the S–S or reduction of –SOH can be performed by several target-specific redox regulators which transfer electrons to target cysteines.

REDOX MODIFICATIONS OF ALA SYNTHESIS ENZYMES

Section "The Overview of the Tetrapyrrole Biosynthetic Pathway in Plants" introduced the complete pathway of tetrapyrrole biosynthesis of photosynthetic organisms, including ALA biosynthesis. According to previous reports the two first pathway-specific enzymes, GluTR and GSAT, are potential candidates of thiol-based post-translational regulation (Buchanan and Balmer, 2005; Richter et al., 2013). Thus, it is likely that the initial flux-regulating enzymatic steps are controlled in response to the plastid redox modifications by reversible thiol–disulfide exchange reactions of contributing enzymes causing changes in their activity and conformation (**Figure 1**).

Although the GluTR from the archaeon *Methanopyrus kandleri* was found as a tetrameric enzyme when purified from *E. coli* lysates by size exclusion chromatography (Moser et al., 1999). GluTR crystals revealed a homodimerization of two GluTR molecules (Moser et al., 2001). GluTR homodimerization was confirmed for the *Arabidopsis* protein *in planta* using bi-molecular fluorescence complementation assays (Czarnecki et al., 2011). The archaebacterial GluTR dimer forms a V-shaped structure (Moser et al., 2001) and the two monomers interact with each other at their C-terminal dimerization domain (Schubert et al., 2002). Interestingly, the crystallized dimer of GSAT from *Synechococcus* could be modeled into the V-shaped notch of homodimeric GluTR (Hennig et al., 1997; Schubert et al., 2002). However, the direct *in planta* interaction of GluTR and GSAT has still not been proven by affinity chromatography or protein interaction approaches. In addition the high reactivity of GSA, the interim metabolite between both enzymes, makes substrate channeling likely. The proposed tight interaction between GluTR and GSAT would enable a direct contact of the two catalytic sites to ease substrate channeling of GSA between both enzymes.

Based on biochemical analyses and the three-dimensional structure of the archaebacterial GluTR, the cysteine at position 48 (Moser et al., 1999, 2001) nucleophilically attacks the aminoacyl bond of glutamyl-tRNA, leading to the formation of a thioester intermediate, which is subsequently reduced to GSA. This catalytically active cysteine is highly conserved in all GluTR molecules of non- and photosynthetically active organisms (Schubert et al., 2002 and **Figure 3**). Interestingly, GluTRs from higher plants (angiosperms) contain three additional conserved cysteine residues at position 322, 411, and 524 (with the exception of GluTR encoded by *HEMA3*; **Figure 3**). These highly conserved cysteines could be involved in the control of protein–protein interaction, stability, or activity.

First evidences for a functional significance of GluTR cysteine residues were obtained from analysis of an *Arabidopsis NTRC*

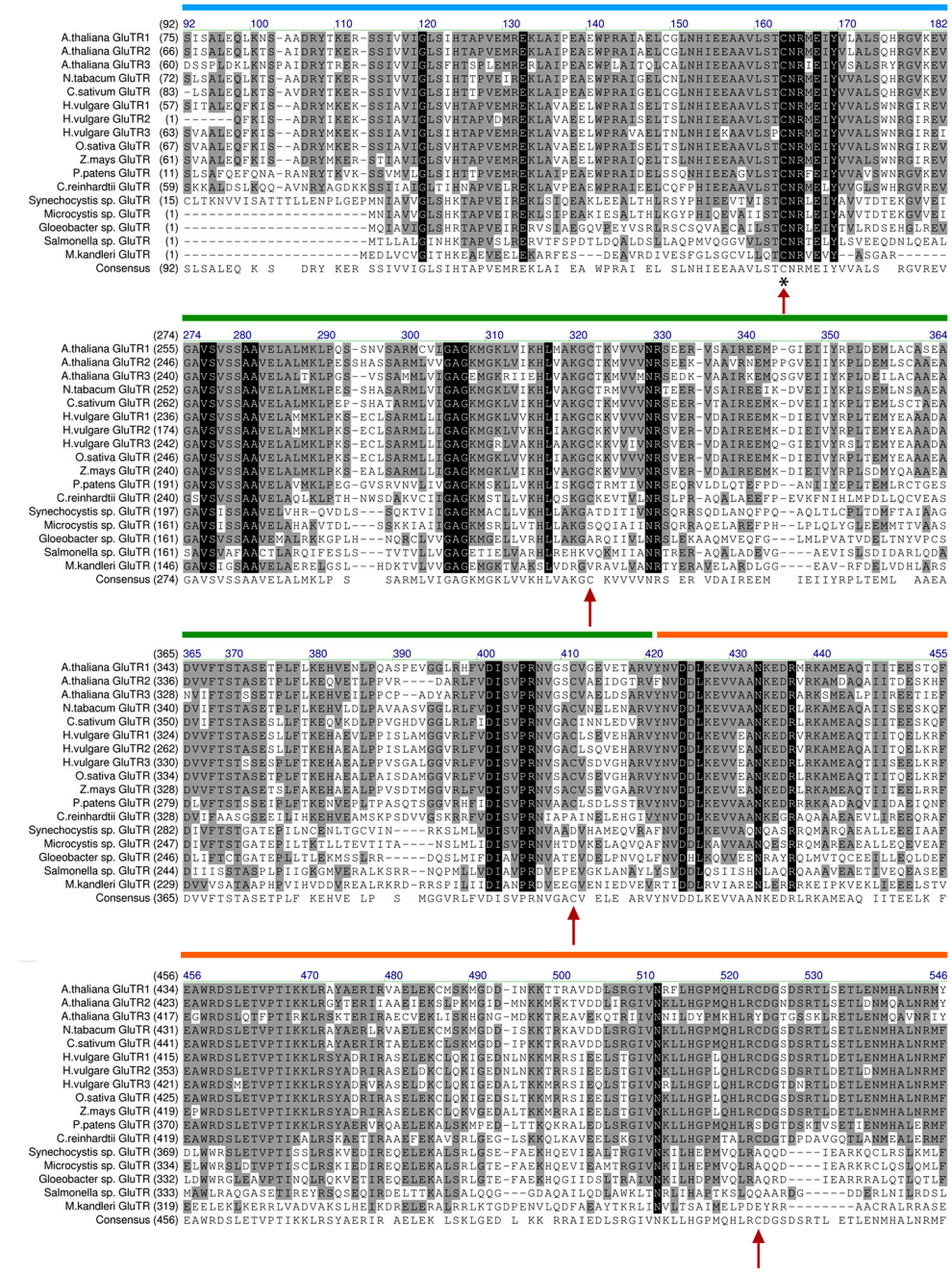

FIGURE 3 | Alignment of GluTR sequences of selected plant and bacterial species. Arrows denote conserved cysteine residues in the GluTR sequence. The cysteine marked with an asterisk (*) was found to perform the nucleophilic attack on $tRNA^{GLU}$ and is thus involved in the catalytic reaction of GluTR (see Moser et al., 1999/therein: Cys48 here: Cys163). Interestingly, only Cys163 is conserved in all analyzed GluTR amino acid sequences. The other cysteines (Cys322, Cys411, and Cys524) are only conserved in GluTR of higher plants (with the exception of *HEMA3* encoded GLUTR). The alignment is depicted without the proposed transit peptide of plant GluTRs and highlights the sequence harboring the conserved cysteines. Colored bars: domains annotated by Schubert et al. (2002). Blue: catalytic domain; green: NADPH-binding domain; orange: dimerization domain.

knock out line. Although the *HEMA1* and *HEMA2* transcript levels encoding GluTR1 and GluTR2 in the *ntrc* mutant are not changed compared to WT, the GluTR levels are significantly reduced. As GluTR evidently interact with NTRC, we conclude post-translational control of NTRC on the redox status of GluTR (Richter et al., 2013). It is suggested that a decreased capacity to reduce oxidized cysteine residues leads to destabilized GluTR. Alteration of the GluTR tertiary structure by oxidized cysteines or the formation of intramolecular disulfide bridges could serve as an explanation for the de-stabilization of oxidized GluTR, as it

was reported for misfolded proteins which are easily susceptible to degradation (Betz, 1993; Hogg, 2003). One of the conserved cysteines (Cys524) is located in the dimerization domain (Schubert et al., 2002; **Figure 3**). Hence, it is not excluded that oxidation of cysteines interferes with the GluTR dimer formation. Then the thiol group of Cys524 in the dimerization domain has to be reduced as a pre-condition of GluTR homodimerization implicating that this process depends on the redox status of GluTR. As described above it is assumed that dimeric GluTR ($GluTR_2$) forms a complex with dimeric GSAT ($GSAT_2$) to facilitate substrate channeling between both enzymes. In terms of a potential redox regulation or a redox-dependent interaction of both $GluTR_2$ and $GSAT_2$ it is worth mentioning that GSAT also contains three highly conserved cysteine residues which are potential targets for thioredoxin-mediated reduction (Buchanan and Balmer, 2005). Future biochemical studies have to elucidate the function of these cysteines for the enzyme activity. But it is also not entirely excluded that interaction between $GSAT_2$ and $GluTR_2$ is mediated through conserved cysteine residues. A putative disulfide bridge between interacting GluTR and GSAT could be formed leading to the stabilization of the multimeric complex $GluTR_2GSAT_2$ (**Figure 4**). Formation of this complex would need a thiol group-reducing activity of enzymes, such as NTRC or thioredoxin. Both proteins are reported to interact with either GluTR or GSAT (Balmer et al., 2003; Richter et al., 2013).

As a key regulatory enzyme of tetrapyrrole biosynthesis, GluTR activity determines the flow rate of intermediates through the entire pathway (summarized in Czarnecki and Grimm, 2012). GluTR encoded by *HEMA1* was found to interact with the membrane-localized FLU protein which down-regulates GluTR activity in response to accumulating Pchlide, the substrate of the light-dependent POR (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002; Richter et al., 2010). Kauss et al. (2012) described that GluTR forms a complex with CHL27 (subunit of the aerobic cyclase), POR, and FLU in darkness. This complex was not found in protein preparations from light-adapted plants. The authors proposed that GluTR is inactivated in darkness through binding to FLU, CHL27, and POR, whereas in light GluTR is released from this complex and becomes active. Hence, ALA synthesis undergoes a switch from "open" (active) in light to "closed" (inactive) in darkness to prevent accumulation of phototoxic intermediates of tetrapyrrole biosynthesis during dark periods. In principle, it is accepted that Pchlide-assembled POR in darkness is the cause for the rapid suppression of ALA synthesizing activity (Kauss et al., 2012). In consistency to the model Pchlide-mediated inactivation of GluTR, seedlings which do not accumulate Pchlide in darkness, do not show the FLU-mediated dark repression of ALA synthesis (Richter et al., 2010).

With regard to the physiological role of GluTR in the control of ALA biosynthesis, a plausible model for redox-dependent interaction of GluTR with other proteins in tetrapyrrole biosynthesis is presented (**Figure 4**). In light, an effective ALA formation is observed most likely due to channeling of substrates from GluTR to GSAT. Hence, high ALA synthesizing activity ensures a high metabolic flow rate in the entire tetrapyrrole biosynthetic pathways. In darkness GluTR interacts with the FLU-complex

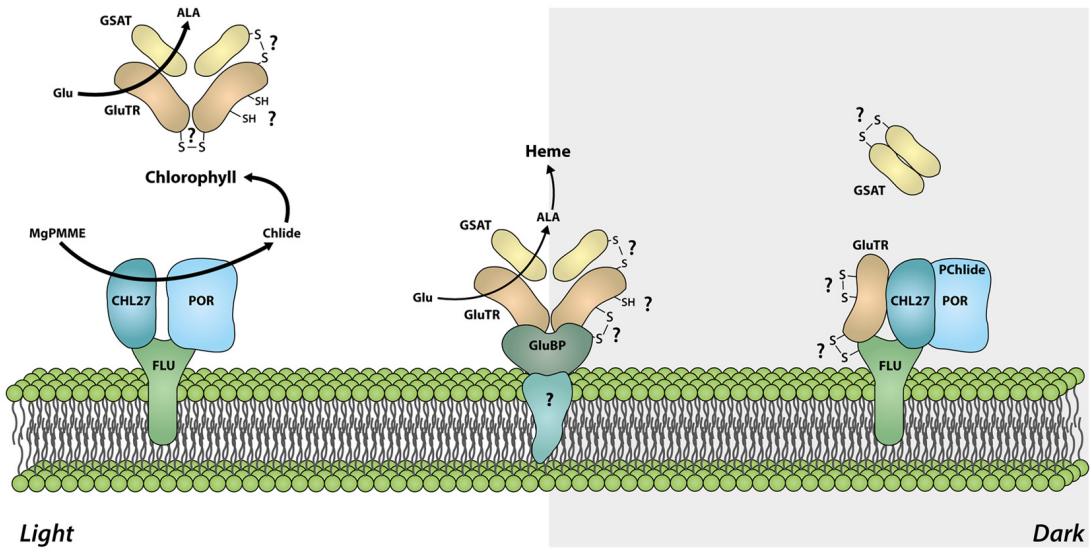

FIGURE 4 | Model of the redox-dependent organization of the ALA synthesizing enzyme complexes. In light, the tetrapyrrole biosynthetic pathway is actively channeling substrates from GluTR to GSAT and ensures high activity of the ALA synthesizing enzymes and allocation of sufficient amounts of the precursors for the synthesis of different end products. In darkness, GluTR interacts with FLU, which is assembled with CHL27 and POR. This protein complex represses ALA synthesis. It is assumed that the reduction of target cysteine residues in intramolecular disulfide bonds of GluTR are the pre-condition for the formation of a heterotetrameric $GluTR_2GSAT_2$ complex or for high activity of ALA synthesizing enzymes in light. Oxidation of cysteine residues or formation of intra-/intermolecular disulfide bonds in darkness could additionally participate in the deactivation of GluTR (e.g., by stabilizing the interaction with FLU) and thus mediating the dark repression of ALA synthesis. Spatial separation of GluTR for heme synthesis by the GluTR-binding protein (GluBP) ensures ALA synthesis for the heme-synthesizing branch, although the bulk of ALA synthesizing activity is repressed by FLU.

thereby repressing ALA synthesis and is perhaps released from the interconnection with GSAT (**Figure 4**). Although the above mentioned assumptions are rather suggestive and were not addressed in direct biochemical experiments yet, there are experimental indications for a light- and redox-dependent modification of GluTR interaction with these later enzymes of tetrapyrrole biosynthesis. Separation of soluble chloroplast protein fractions in blue-native gels revealed that GluTR assembles in high molecular weight protein complexes, including also the protein complex with FLU–POR–CHL27. These electrophoretic separations of protein complexes are performed under non-reducing conditions. When plastid protein extracts were separated under reducing conditions (or after treatment with reductants), at least part of GluTR assembled in the high molecular weight complexes are reduced and separated as monomer (Richter et al., 2013).

Moreover, GluTR is also attached to the thylakoid membrane by a GluTR-binding protein (GluBP; Czarnecki et al., 2011). The GluTR-binding protein attaches GluTR at the thylakoid membrane and spatially separates a small amount of GluTR from a soluble GluTR fraction. This fraction is suggested not to be accessible to the FLU-dependent inactivation in darkness (**Figure 4**). In terms of a putative redox-switch of GluTR between different complexes it is worth mentioning that the GluBP contains three cysteines which could stabilize the interaction of GluBP with GluTR under changing environmental conditions.

Additional studies are required to decipher the molecular processes of inactivation of bulk ALA synthesis in darkness. Among other essential amino acids in the tetratricopeptide repeat (TPR)-domain, a peptide motif mediating protein–protein interactions, FLU harbors also a conserved cysteine at the C-terminal end. The potential role of the cysteine residues of FLU and GluTR has to be explored for a potential redox-dependent shift of GluTR between different protein complexes during the diurnal light–dark transitions and other distinct environmental conditions in future. But a thiol-reducing mechanism for the FLU-dependent GluTR inactivation is plausible.

REDOX CONTROL IN THE Mg-BRANCH

In this chapter we discuss the first reports on redox control in the Mg-branch of tetrapyrrole biosynthesis and emphasize potential thiol-based post-translational regulation of enzymes at these steps of tetrapyrrole biosynthesis. First evidence for a redox-dependent activity of MgCh were obtained, when Fuesler et al. (1984) reported on the sensitivity of MgCh activity from purified plastids to thiol-reacting reagents. Jensen et al. (2000) reported abolished ATPase activity of CHLI under oxidizing conditions and after blocking of conserved cysteines by *N*-ethylmaleimide (NEM). The block of cysteines did not affect the interaction between CHLI and CHLD. Therefore, the authors concluded that among four conserved cysteine residues at least one CHLI cysteine is essential for ATP binding and hydrolysis (Jensen et al., 2000). In the same study it was shown that activity of recombinant NEM-pretreated CHLH is lowered and presence of reducing reagents like DTT favors high MgCh activity. These results gave the first hints that thiol switches modulate activity of CHLI and the complete MgCh complex. Subsequently, the CHLI subunit was found to interact with thioredoxin(s) (Balmer et al., 2003). Further analysis confirmed a stimulatory function of thioredoxin on CHLI ATPase activity *in vitro* and *in vivo* (Ikegami et al., 2007; Luo et al., 2012). Thioredoxin is ferredoxin-dependently reduced suggesting that thioredoxin-mediated redox control of CHLI and the entire MgCh activity directs intermediates into the Mg-branch in response to activity of the photosynthetic apparatus (Luo et al., 2012). Light-dependent activation of MgCh ensures a high activity in day time and a rapid inactivation of MgCh during darkness. Together with the above-described post-translational repression of ALA synthesis (Meskauskiene et al., 2001), an additional inactivation of MgCh by a redox-dependent mechanism in darkness prevents the accumulation of phototoxic intermediates and ensures the bypass of Proto into the iron branch for heme synthesis. In light, thioredoxin accepts electrons from the photosynthesis-driven reduction of NADPH and ferredoxin, reduces disulfide bonds of CHLI and, thereby, activates the MgCh complex. Due to an immediate shift in the photosynthetic redox status in chloroplasts after the transfer from light to darkness, redox-sensitive thiol groups of cysteines in CHLI and CHLH would be accessible for oxidation which results in decreased activity of the MgCh. ALA feeding experiments in darkness indicates that the activity of MgCh and the successive enzymes within the Mg-branch is attenuated, but not dark-dependently blocked (Pontier et al., 2007; Richter et al., 2013). However, the dark-dependent repression of ALA synthesis is still believed to be the main point of action to prevent accumulation of Mg porphyrins in the dark.

Besides the thioredoxin-dependent post-translational activation of MgCh, additional experimental evidences provide insights into the redox-dependent control of enzymes of the Mg-branch. A thiol–disulfide exchange of cysteine residues of MgP methyltransferase was found to modulate CHLM stability and, consequently, its activity (Richter et al., 2013). The stimulatory effect of reduced cysteine residues on CHLM activity was confirmed in *in vitro* assays with recombinant CHLM and NTRC proteins. The *Arabidopsis ntrc* mutant possesses lower CHLM activity than the wild-type seedlings. These investigations conclusively demonstrate that reduction of the cysteines by redox regulators not only leads to stimulation of CHLM activity, but is also essential to maintain the structural integrity of the protein most likely in light and darkness. Further analysis will elucidate which of three highly conserved cysteine residues of CHLM are specific targets of NTRC-mediated redox control and which of these cysteines are essential either for the catalytic activity or the structural integrity of CHLM.

Previous analysis of NTRC suggested also another redox-related regulation of enzyme activities within the Mg-branch (Stenbaek and Jensen, 2010). As mentioned in chapter three NTRC interacts with 2-Cys PRX and provides electrons for the 2-Cys PRX-mediated detoxification of hydrogen peroxide (Perez-Ruiz et al., 2006). Stenbaek et al. (2008) addressed the sensitivity of the oxygen-consuming reaction of aerobic cyclase against ROS-mediated oxidation. They showed that the catalyzing detoxification of hydrogen peroxide by interaction of NTRC with 2-Cys PRX improves the *in vitro* enzymatic activity of the cyclase. This finding was supported by an earlier report of Bollivar and Beale (1996) showing increased cyclase activity in the presence of catalase. Very little is currently known about the catalytic mechanism

and requirement of protein factors for the cyclase reaction. It cannot be excluded that the *in vitro* activity of the aerobic cyclase is inhibited by oxidation of the iron cluster, which is part of the complex catalytic oxidation reaction, resulting in the additional ring formation at MgPMME. It remains open if 2-Cys PRX participates in the *in vivo* reaction of cyclase. However, mutants with a knock down of PRXA (below 5% of WT level) and a parallel *PRXB* knock out do not show any alteration of Chl biosynthesis, although they accumulate high amounts of hydrogen peroxide in comparison to wild-type (Pulido et al., 2010). Future analysis of 2-Cys PRX mutants will help to elucidate the active contribution of 2-Cys PRX as ROS-detoxifying system in Chl biosynthesis.

FINAL CONCLUSION

The thiol-based post-translational modulations serve in tetrapyrrole biosynthesis at multiple levels of oxidation and reductions. It is conceivable that disulfide–thiol exchange reaction affects activity, folding and stability of tightly controlled enzymes. Thus, these regulatory mechanisms contribute to synthesis of the matched amount of end products and facilitate a balanced flow of tetrapyrrole intermediates through the pathway without the risk of metabolite accumulations. It cannot be excluded that more target enzymes of oxidoreductase-mediated reactions will be found in tetrapyrrole biosynthesis. Moreover, the complete regulatory redox network of tetrapyrrole biosynthesis also includes the control of steady state levels of oxidants, which are easily generated in the highly light and oxygen-sensitive pathway of tetrapyrrole biosynthesis, and the supply and activation of a detoxification system against these oxidants. But, we have to admit that many processes are still poorly understood and the entire extent of the interplay between thioredoxins, glutaredoxins, and glutathionylations in tetrapyrrole metabolism cannot currently be envisioned. It remains a challenge to unravel the redox-dependent post-translational control and the redox signaling network of tetrapyrrole biosynthesis in the near future.

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Redox-dependent functional switching of plant proteins accompanying with their structural changes

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Reactive oxygen species (ROS) can be generated during the course of normal aerobic metabolism or when an organism is exposed to a variety of stress conditions. It can cause a widespread damage to intracellular macromolecules and play a causal role in many degenerative diseases. Like other aerobic organisms plants are also equipped with a wide range of antioxidant redox proteins, such as superoxide dismutase, catalase, glutaredoxin, thioredoxin (Trx), Trx reductase, protein disulfide reductase, and other kinds of peroxidases that are usually significant in preventing harmful effects of ROS. To defend plant cells in response to stimuli, a part of redox proteins have shown to play multiple functions through the post-translational modification with a redox-dependent manner. For the alternative switching of their cellular functions, the redox proteins change their protein structures from low molecular weight to high molecular weight (HMW) protein complexes depending on the external stress. The HMW proteins are reported to act as molecular chaperone, which enable the plants to enhance their stress tolerance. In addition, some transcription factors and co-activators have function responding to environmental stresses by redox-dependent structural changes. This review describes the molecular mechanism and physiological significance of the redox proteins, transcription factors and co-activators to protect the plants from environmental stresses through the redox-dependent structural and functional switching of the plant redox proteins.

Keywords: external stress, molecular chaperone, multiple functions, redox proteins, structural and functional switching

INTRODUCTION

Plant cells produce various kinds of reactive oxygen species (ROS) from internal and external sources, such as hydrogen peroxide (H_2O_2), superoxide anions, and hydroxyl radicals. They can damage cellular components or act as important signal transduction molecules to trigger the cellular defense signaling cascades (Baier and Dietz, 2005; D'Autreaux and Toledano, 2007; Schwarzlander and Finkemeier, 2013). Thus, it is crucial for cells to detect the levels of ROS and activate defense signaling pathways (Moller and Sweetlove, 2010). To initiate cellular signaling cascades responding to a myriad of environmental signals, plants generate redox gradient across the plasma membrane, change metabolic activities, and trigger the inactivation of the oxidative burst-generating enzymes (Pignocchi and Foyer, 2003; Suzuki et al., 2012).

The alteration in steady-state level of ROS and subsequent changes of intracellular redox potential are important systems to regulate cellular signaling factors linking external stimuli with intracellular signal transduction pathway in response to stresses (Finkel, 2011). Plants are autotrophic organisms that are capable of undergoing photosynthesis by which they absorbed light energy that generate high electron and transport to chloroplasts, mitochondria, and peroxisomes along a cascade of redox components. During the reactions, ATP, NADPH, and other soluble reducing equivalents of ferredoxin (Fd), and thioredoxin (Trx) are generated (Schurmann and Buchanan, 2008). In addition, the thiol/disulfide state strongly regulates the light-dependent modulation of chloroplast enzyme activities (Scheibe, 1991). On the other hand, both the chloroplast and mitochondria originated as bacterial endosymbionts which retain a specialized genome and electron transport chains (ETCs; Murphy, 2009). The ETCs of plant mitochondria and chloroplast are major generator of ROS that contain flavin, metal centers, and quinones as their electron transport centers. Whereas, in the regulation of nuclear gene expression, plant peroxisomes controls intracellular ROS levels, plant photomorphogenesis, plant development, peroxisomal biogenesis, light signaling, and stress responses (Hu et al., 2002). In the moderate to high rates of photosynthesis, the peroxisome becomes the site of massive light-dependent generation of H_2O_2. They are multi-purpose organelles involved in fatty acid α-oxidation, β-oxidation of very long chain fatty acids, catabolism of purines, and biosynthesis of glycerolipids and bile acids. During the reactions, redox signals play important roles, since peroxisomes produce H_2O_2 at high rates through the reactions of β-oxidation of long chain fatty acid and glycolate oxidation (Jimenez et al., 1997).

To integrate ROS production from major sources of plant cells, they produce large quantities of redox-materials to buffer the intracellular redox changes by expressing many kinds of soluble hydrophilic antioxidants, ascorbate, glutathione, and redox-regulating proteins including Trx, glutaredoxin (Grx), protein

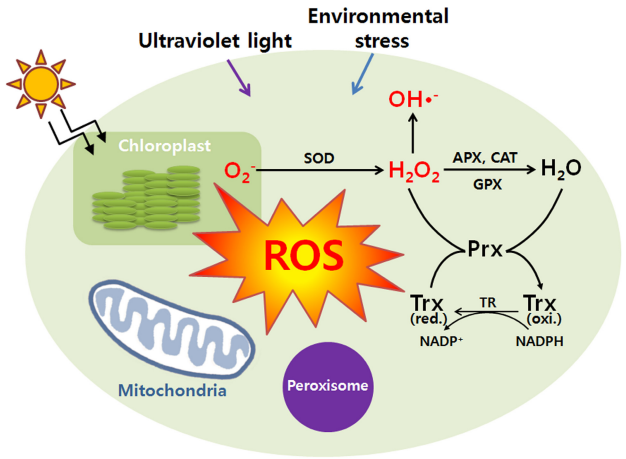

FIGURE 1 | Cellular responses against reactive oxygen species. During a variety of stress conditions (UV, environmental stress, etc), reactive oxygen species (ROS) can be generated. At the start of the reaction, a primary ROS which is superoxide anion (O^{2-}) can be formed by the one electron reduction of molecular oxygen. The superoxide anion (O^{2-}) is dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) which is detoxified by catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and peroxiredoxin (Prx). Once the superoxide anion (O^{2-}) is formed in the presence of H_2O_2, it becomes inevitable. Further reactions may lead to the formation of hydroxyl radicals ($HO^{\cdot}$). Antioxidant proteins protected the damages of ROS leaking from peroxisomes. H_2O_2 can easily permeate the peroxisomal membrane and play an important role in protection of cells from ROS damages. And various ROS transmitted to the mitochondrion play a role in the adaptive response of mitochondrial redox state, especially for the reduction state of respiratory pathways. The redox signals will be transmitted to the nucleus to regulate plant growth and developments. Trx represents thioredoxin.

disulfide reductase (PDI), etc. (**Figure 1**). These compounds are important factors to determine the lifetime of H_2O_2 and redox potential of plant cells (Noctor and Foyer, 1998; Foyer and Noctor, 2013). Changes in the redox state of these components regulate the expression of both plastome- and nuclear-encoded proteins. Furthermore, the redox information co-ordinates the gene expression located in the compartments of chloroplasts, mitochondria, and nucleus (Allen and Pfannschmidt, 2000). Redox signals also leave the chloroplast and mitochondria to provide a decisive input into transcriptional control of the plant nucleus. Thus, redox signaling plays a key role in the coordination between the cytoplasmic genomes in the chloroplast and mitochondria and the nucleus functioning within the global network of whole plants and it may also provide evidence of many cellular proteins and enzyme activities that should be precisely regulated in response to plant growth, development, and differentiation.

STRUCTURAL AND FUNCTIONAL REGULATION OF REDOX-DEPENDENT TRANSCRIPTIONAL FACTOR AND CO-ACTIVATORS

Reactive oxygen species can cause widespread damage to biological macromolecules (Halliwell and Gutteridge, 1990; Alvarez et al., 1998). In order to protect themselves from oxidative stress and ROS-mediated protein unfolding and aggregation, plant cells are equipped with a wide range of antioxidant proteins, including superoxide dismutase, catalase, peroxidases, and diverse forms of molecular chaperones, small heat shock proteins (Hendrick and Hartl, 1993; Dietz, 2003). Furthermore, several moon-light proteins have multiple functions responding to foreign stresses, such as ROS, heat shock, and pathogen attacks, accompanying with their structural changes (Lee et al., 2009; Park et al., 2009; Chae et al., 2013). In this review, we will introduce several representative examples of the proteins which switch their protein structures and functions with a redox-dependent manner in response to external stresses. Redox-regulation is a fine-tuning mechanism for the transcription of plant genomes in nucleus, chloroplast, and mitochondria to co-ordinate with plant development and differentiation along with environmental parameters (Baier and Dietz, 2005; Piippo et al., 2006; Schwarzlander and Finkemeier, 2013). There are several redox sensitive transcriptional factors whose activities are relied on redox- and structure-dependent manner (Tron et al., 2002; Heine et al., 2004; Serpa et al., 2007; Shaikhali et al., 2008, 2012). Among them, the redox-dependent transcriptional factor, Rap2.4a, was isolated and studied as an efficient redox-sensor and transducer of redox status of cells to nucleus to control transcriptional activity of chloroplast antioxidant proteins (Shaikhali et al., 2008). The protein was cloned from a yeast-one-hybrid screening with a *cis*-regulatory element of 2-Cys peroxiredoxin (Prx)-A gene (2CPA) is grouped into activator protein 2 (AP2)-type transcription factor (Srivastava et al., 2009). Rap2.4a transcriptional activity is regulated by dithiol/disulfide transition of regulatory cysteinyl residues (Shaikhali et al., 2008). During the processes, the protein changes its quaternary structures according to redox status. Under oxidizing conditions, Rap2.4a switches its structure from monomer and dimer to polymers, respectively. And the dimeric form of Rap2.4a plays a critical role as a transcription factor to stimulate nuclear gene expression of the photosynthetic chloroplast enzymes (Shaikhali et al., 2008). Thus, the homodimeric structure of Rap2.4a formed by intermolecular disulfide bond is the active form necessary for DNA binding and transcriptional activity. Also the oxidation of the dimer by H_2O_2 or reduction by dithiothreitol (DTT) significantly changes its protein structures to make homopolymer and monomer and reduces its DNA binding and transcriptional activity (**Figure 2**; Shaikhali et al., 2008, 2012).

Another example of transcription factor regulating its activity by structural changes is given in basic leucine zipper (bZIP) transcription factor in *Arabidopsis*, AtbZIP16 (Shaikhali et al., 2012). This protein belongs to the G-group of *Arabidopsis* bZIP type transcription factors whose promoters are responsive to a variety of environmental stimuli (Kleine et al., 2007). bZip16 has a conserved Cys residues that plays a critical role in redox regulation of the target gene expression, which is proven by the transgenic *Arabidopsis* overexpressing the Cys mutated variant of bZIP16 (Shaikhali et al., 2012). Multiple protein bands are detected corresponding to monomer, dimer, and oligomer forms of AtbZIP16 in the non-reducing conditions (Shaikhali et al., 2012). And the oligomeric and dimeric forms are reduced by increasing DTT concentrations, which results in a complete conversion to a monomer. In contrast, increasing the concentration of H_2O_2 produces a gradual loss of the small molecular forms, which suggests that H_2O_2 induces the formation of very high molecular mass complexes

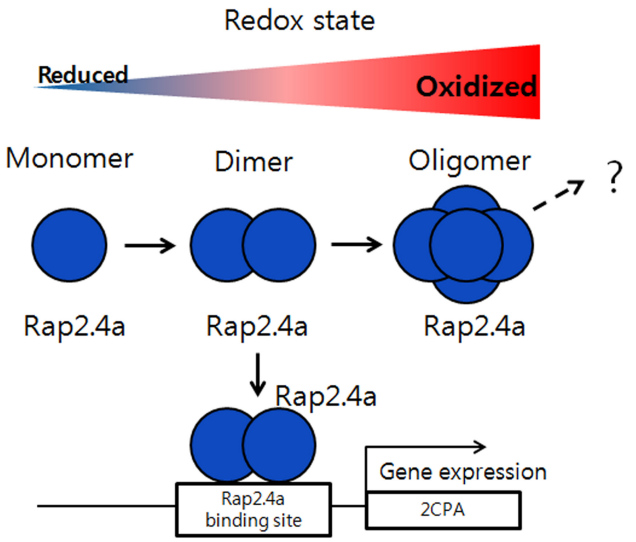

FIGURE 2 | Regulation of gene expression by Rap2.4a through redox changes. Under oxidizing conditions, Rap2.4a changes its structure from monomer, dimer to oligomer. The homodimeric structure of Rap2.4a can bind to the DNA promoter of antioxidant like 2-Cys peroxiredoxin and plays as transcription factor to regulate its gene expression. If the redox balance changes to more oxidized condition, the expression level of Rap2.4a is increased. But, during severe oxidative conditions, Rap2.4a expression is decreased and lost its transcriptional activity due to oligomerization. This model is modified from the reference of Shaikhali et al. (2008).

in a reversible way. The high molecular mass oligomers can be reversibly dissociated by the treatment of reducing agents, such as DTT. Only DTT-mediated dissociated AtbZIP16 can bind to DNA promoter and functions as a transcription factor (Shaikhali et al., 2012). The mutated Cys variant form of bZIP16 has an important physiological significance of its conserved Cys residue in redox regulation of gene expression. Based on these data, the redox-dependent structural changes is highly important to modulate the activity of these transcription factors in response to environmental signals.

A typical redox protein regulating its protein structure and functions against external biotic stress can also be found from the transcriptional co-activator, NPR1 (NON-EXPRESSOR OF PR1; Mou et al., 2003; Tada et al., 2008). The gene was originally cloned from *Arabidopsis* mutant screening to identify the loss of ability to respond to inducers of systemic acquired resistance (SAR) such as salicylic acid (SA), and designated *npr*−1, *nim*−1, and *sai*1, simultaneously. The NPR1 having no DNA-binding domain acts as a co-activator by specifically associated with the specific DNA-binding transcription factor, TGACG sequence-specific binding proteins (TGAs), and plays a critical role in plant pathogen resistance (Grant and Lamb, 2006; Spoel et al., 2009; Maier et al., 2011). NPR1 containing several conserved Cys residues controls the expression of over 2,200 defense response genes in *Arabidopsis* and has stable structure that forms a high molecular weight (HMW) oligomer, which makes the protein confine at the plant cytoplasm (Mou et al., 2003; Wang et al., 2006). The *npr1* mutants neither accumulate pathogen-related (PR) proteins in response to SA nor exhibit resistance against pathogen infection, such as downy mildew. The *NPR1* gene contains a BTB (BR-C, ttk and bab)/POZ (Pox virus and Zinc finger) domain at its N-terminus which facilitates dimerization of NPR1 and ankyrin repeats in the central region that is involved in the interaction with TGA sub-family of bZIP transcription factors (Stogios et al., 2005; Boyle et al., 2009). Under the normal conditions, NPR1 exists as an inactive form of oligomers that are associated by intermolecular disulfide bonds in the cytosol (**Figure 3**). However, the redox changes triggered by SA, a phytohormone produced in response to pathogen attack, induce the release of NPR1 from the oligomers to an active form of monomer by reduction of the intermolecular disulfide bridges (Dong, 2004; Moore et al., 2011; Astier et al., 2012). Then, the NPR1 monomer translocates from the cytosol to nucleus and stimulates the transcriptional activation of defense-related genes through the binding with transcription factors belonging to TGA sub-family (Dong, 2004; Moore et al., 2011; Astier et al., 2012). Particularly, the *S*-nitrosylation of Cys^{156} has been shown to trigger conformational changes of NPR1 facilitating disulfide linkage between NPR1 monomers to form inactive homopolymers. During the redox-mediated switching of its function and structures from the inactive oligomers located in cytosol to the active monomeric NPR1 that will be translocated to the nucleus, Trx-h5 plays an important role in the NPR1 dissociate ion and activation. NPR1 may be the most important gene for the preparation of broad spectrum disease-resistant transgenic crops (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997).

FUNCTIONAL SWITCHING OF REDOX PROTEINS ACCOMPANYING WITH THEIR REDOX-DEPENDENT STRUCTURAL CHANGES

Thioredoxin is a general disulfide oxidoreductase and a ubiquitous redox protein with a single disulfide bridge in all organisms. The function of Trx is involved in numerous redox-dependent cellular processes, such as activation of ribonucleotide reductase, photosynthetic activity of plant cells, modulation of transcription factors, and promotion of a variety of diseases (Aslund and Beckwith, 1999; Balmer et al., 2003; Ravi et al., 2005). Trxs also control several redox-independent cellular reactions including an assembly of T7 DNA polymerase complex and formation of filamentous phage (Feng et al., 1997; Hamdan et al., 2005). The proteins belonging to Trx group share high amino acid sequence similarity and contain a common structural motif, the Trx-fold. The Trx-fold comprises approximately 80 amino acid residues with a central core of five β-strands that are enclosed by four α-helices and two hydrophobic zones (Katti et al., 1990). The interesting point we want to focus in this review is that some of redox proteins harboring the Trx-fold have been shown to behave as molecular chaperones with their endogenous reductase function. The proteins include Trx-like domain containing protein (TDX), protein disulfide isomerase, and 2-Cys-Prxs (Quan et al., 1995; Jang et al., 2004), etc. To be a molecular chaperone, it should interact with target substrates and switch its protein structures in response to external stresses with a reversible fashion (Jang et al., 2004; Lee et al., 2009; Park et al., 2009). The Trx-fold containing proteins interact with substrate proteins through their hydrophobic surfaces around their active sites and reversibly change the protein structures as the following examples.

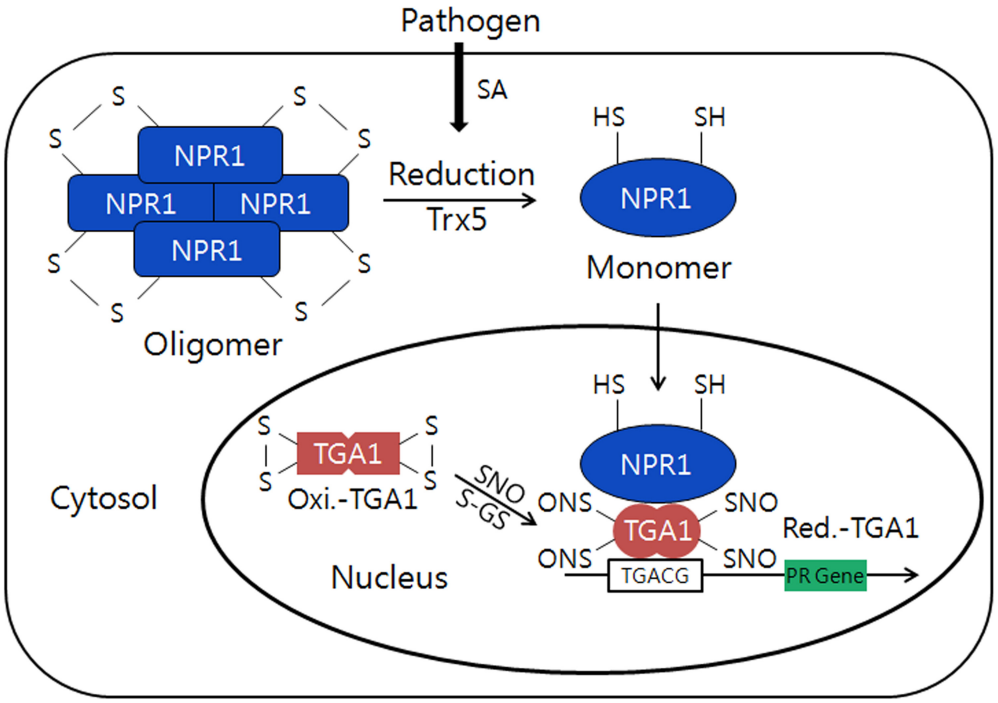

FIGURE 3 | Conformational switching from the oligomers to a monomer of NPR1 by redox changes in plants. Under normal conditions, NPR1forms an inactive oligomer structure and is oxidized by intermolecular disulfide bonds in the cytosol. However, during pathogen attack, the NPR1 changes its structure from inactive to active form of monomer through the reduction of the intermolecular bridges by h5-type thioredoxin (AtTrx-h5). Then, NPR1 will be translocated to the nucleus and interacts with TGA1transcription factor that can induce PR gene expression. TGA1 in normal condition is inactive and oxidized form with intramolecular disulfide bonds in the nucleus. However, in stress condition TGA1 will be reduced and interact with NPR1 and can enhance its DNA binding activity through *S*-nitrosylation (SNO) and *S*-glutathionylation (*S*-GS) by GSNO and glutathione (GSH/GSSG). This model is modified from the reference of Moore et al. (2011).

Among the various kinds of Trx isoforms, a specific type of *Arabidopsis* Trx in cytosol, AtTrx-h3, forms various protein structures ranging from low molecular weight (LMW) protein species to HMW homo-complexes, which are verified by size exclusion chromatography, native-PAGE gel, and electro-microscopic analyses (Park et al., 2009). The AtTrx-h3 performs dual functions, acting as a disulfide reductase and as a molecular chaperone, which are closely associated with its differently sized multiple protein structures. The disulfide reductase function is observed predominantly in the LMW forms, whereas the chaperone function predominates in the HMW complexes. The multimeric structures of AtTrx-h3 are regulated by redox status. The reduction of AtTrx-h3 by DTT changes the HMW structures of AtTrx-h3 into LMW protein species, and subsequent treatment of hydrogen peroxide (H_2O_2) after removal of DTT almost restores the HMW structures of AtTrx-h3. Particularly, the AtTrx-h3 polymeric structures are associated not only by the forces of hydrophobic interaction but also by the redox-dependent disulfide bonds. Two active cysteine residues in AtTrx-h3 are required for disulfide reductase activity, but not for chaperone function. Thus, the active site mutant protein, C39/42S-AtTrx-h3, is not able to reduce disulfide bonds of substrate at all, but has nearly the same chaperone activity as that of native AtTrx-h3 protein. The transgenic lines overexpressing native AtTrx-h3 or C39/42S (DM) mutant AtTrx-h3 having only the chaperone function exhibit enhanced heat shock tolerance compared to wild-type plants. From the results, it can be concluded that the AtTrx-h3 plays a pivotal role in the protection of plant cells from external stresses through its chaperone function (Park et al., 2009).

In addition to the At-Trx-h3, the plant-specific Trx-like protein containing 3 tetratricopeptide repeat (TPR) domains and a Trx motif which is designated AtTDX has a highly heat-stable property. The TPR units in AtTDX are particularly important for protein–protein interaction and formation of multi-protein complexes (Blatch and Lassle, 1999), which are characteristic properties of molecular chaperones. AtTDX has diverse protein structures consisting of monomer, dimer, oligomer, and HMW complexes. The protein also displays multiple functions, acting as a disulfide reductase, foldase chaperone, and holdase chaperone. In particular, the functions of AtTDX are closely associated with its oligomerization status. Like the AtTrx-h3, multimerization of AtTDX enhances its holdase chaperone activity, whereas dissociation promotes its disulfide reductase and foldase chaperone functions. However, when the TPR domains of AtTDX is removed, the truncated protein shows a significant enhancement of its disulfide reductase activity but results in a complete loss of the holdase chaperone function of AtTrx-h3 (Lee et al., 2009). The result suggests the TPR domains of AtTDX completely block the active site of Trx motif and play a critical role in promoting the holdase chaperone function. Moreover, the Cys mutant proteins

(C304S, C307S, and C304/307S) of AtTDX do not exhibit disulfide reductase activity but display a similar activity of holdase chaperone function as native AtTDX. The results suggest that the active site Cys residues critically contribute to the reductase function but not the chaperone function. For the regulation of its multiple functions, protein structure of AtTDX is varied against external conditions. The oligomerization status of AtTDX is reversibly controlled by heat shock and ROS concentrations, which cause a transition from LMW to HMW complexes with a concomitant functional switching from a disulfide reductase and foldase chaperone to a holdase chaperone. It is generally known that the chaperone function contributes resistance to cells against external stresses. Thus, when the heat-stressed *Arabidopsis* of the WT, AtTDX overexpression lines, AtTDX suppression lines, and Cys-mutant (C304/307S) AtTDX overexpression lines having only the holdase chaperone function are returned to their optimal temperature, the transgenic lines overexpressing the native form and C304/307S mutant form of AtTDX recover during the post-stress recovery period. In contrast, AtTDX suppression lines of *Arabidopsis* show a highly sensitive phenotype against heat shock. The results can conclude that the holdase chaperone function of AtTDX plays a major role in the protection of *Arabidopsis* from heat stress during the heat shock and/or recovery period (Lee et al., 2009).

Another redox protein sharing a similar regulation mode with AtTrx-h3 and AtTDX proteins can be found from the C-type of NADPH-dependent Trx reductase (NTRC), which is a new member of the plant-specific NADPH-dependent Trx reductase (NTR) family. During the early evolution of chloroplasts, the NTRC appears to be originated from cyanobacteria by the transfer of this gene into the plant genome. The protein contains an N-terminal Trx reductase (TR) domain and a Trx domain at the C-terminus. The functional role of this fusion of domains in NTRC has been verified as an efficient electron donor to 2Cys-Prx (Moon et al., 2006; Perez-Ruiz et al., 2006). Particularly, *Arabidopsis* NTRC shows enzymatic activity characteristic for each of its separate domains and in a combination of the TR and Trx domains (Moon et al., 2006). The disulfide reductase function of NTRC is coupled with the reducing power, NADPH, which is produced by photosynthetic electron transport systems in the light conditions. The knockout mutant of *Arabidopsis* NTRC exhibits growth inhibition under stress conditions and shows reduced auxin levels (Serrato et al., 2004; Lepisto et al., 2009). In the letter case, the mutant phenotypes are restored by supplementing growth medium with tryptophan and phenylalanine. Interestingly, it has been reported that the protein structures of NTRC have various oligomeric conformations in other species like rice, barrel medic, and barley (Alkhalfioui et al., 2007; Perez-Ruiz and Cejudo, 2009; Wulff et al., 2011). That is, NTRC assembles into homopolymeric structures of varying complexity with functions as a disulfide reductase, a foldase chaperone, and as a holdase chaperone. The multiple functions of NTRC are also associated with its protein structures. Complexes of HMW show stronger activity as a holdase chaperone, whereas the LMW species exhibit weaker holdase chaperone activity with stronger disulfide reductase and foldase chaperone activities (**Table 1**). Heat shock converts LMW proteins into HMW complexes and gradually increases the holdase chaperone function of NTRC. Upon the heat shock treatment, NTRC results in a decrease in its disulfide reductase and foldase chaperone activities. In conclusion, heat shock-mediated oligomeric changes of NTRC are closely associated with a change in its functional switching from a disulfide reductase to a molecular chaperone.

Table 1 | Structural and functional switching of NTRC in *Arabidopsis thaliana* in response to redox state.

NTRC	Redox state	
	Reduction	**Oxidation**
Protein structures	Low molecular weight species • Monomer • Dimer	High molecular weight complexes • Oligomer
Functions	Disulfide reductase Foldase chaperone	Holdase chaperone

CONCLUSION AND PERSPECTIVE

To cope with external stresses, plants regulate their protein functions by employing a number of efficient regulation strategies, such as phosphorylation/dephosphorylation, covalent modification, proteolytic degradation or activation, interacting with partner proteins, and so on. However, at recent, the post-translational modification is identified as one of the most important, rapid and precise methods to respond eukaryotic cells against environmental stresses. The redox-dependent functional switching is a typical scheme for the plant defense systems. Particularly, the functional shift of the redox proteins is accompanied with their structural changes in response to redox changes. In this review, we introduce several examples of the redox proteins to respond environmental circumstances. However, besides the redox proteins in eukaryotes, many redox-independent proteins showing similar regulation pattern with the proteins have also been identified from various sources, such as plant phosphodiesterase, sodium/proton antiporter (NHX), salt overly sensitive 1 (SOS1), mammalian nuclear factor kappa beta (mammalian NF-kB) and AP1, yeast Yap1, bacterial OxyR/S, and so on (Zheng et al., 1998; Toone et al., 2001; Kim et al., 2002, 2012; Hess et al., 2004; D'Autreaux and Toledano, 2007; Salminen et al., 2008; Rodriguez-Rosales et al., 2009; Ji et al., 2013). Thus, these elaborate functional regulation mode allows higher eukaryotic organisms to precisely respond to external stresses and to survive from the harsh and changeable environmental conditions. This review provides valuable insights into how plants can respond to the rapid changes of redox potential induced by biotic/abiotic stresses at the molecular level.

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Overexpression of chloroplast NADPH-dependent thioredoxin reductase in *Arabidopsis* enhances leaf growth and elucidates *in vivo* function of reductase and thioredoxin domains

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Plant chloroplasts have versatile thioredoxin systems including two thioredoxin reductases and multiple types of thioredoxins. Plastid-localized NADPH-dependent thioredoxin reductase (NTRC) contains both reductase (NTRd) and thioredoxin (TRXd) domains in a single polypeptide and forms homodimers. To study the action of NTRC and NTRC domains *in vivo*, we have complemented the *ntrc* knockout line of *Arabidopsis* with the wild type and full-length *NTRC* genes, in which 2-Cys motifs either in NTRd, or in TRXd were inactivated. The *ntrc* line was also transformed either with the truncated NTRd or TRXd alone. Overexpression of wild-type NTRC promoted plant growth by increasing leaf size and biomass yield of the rosettes. Complementation of the *ntrc* line with the full-length *NTRC* gene containing an active reductase but an inactive TRXd, or vice versa, recovered wild-type chloroplast phenotype and, partly, rosette biomass production, indicating that the NTRC domains are capable of interacting with other chloroplast thioredoxin systems. Overexpression of truncated NTRd or TRXd in *ntrc* background did not restore wild-type phenotype. Modeling of the three-dimensional structure of the NTRC dimer indicates extensive interactions between the NTR domains and the TRX domains further stabilize the dimeric structure. The long linker region between the NTRd and TRXd, however, allows flexibility for the position of the TRXd in the dimer. Supplementation of the TRXd in the NTRC homodimer model by free chloroplast thioredoxins indicated that TRXf is the most likely partner to interact with NTRC. We propose that overexpression of NTRC promotes plant biomass yield both directly by stimulation of chloroplast biosynthetic and protective pathways controlled by NTRC and indirectly via free chloroplast thioredoxins. Our data indicate that overexpression of chloroplast thiol redox-regulator has a potential to increase biofuel yield in plant and algal species suitable for sustainable bioenergy production.

Keywords: chloroplast, thioredoxins, NTRC, 3-D model, carbon metabolism, redox regulation, overexpression, biomass yield

INTRODUCTION

Thioredoxins (TRX) are crucial components of the regulatory redox networks in all living cells. In the reduced state, TRXs control functions of cellular proteins by reducing disulphide bridges in the redox active site of a target protein. Subsequently, the oxidized thioredoxins are reduced by thioredoxin reductases (TR). TR-dependent reduction of cellular proteins by TRXs is called a thioredoxin system. Plant chloroplasts have versatile thioredoxin systems including two reductases dependent on ferredoxin (FTR) and NADPH (NTR) as reducing power, respectively, and multiple types of TRXs (f, m, x, y, z, CDSP32; Buchanan and Balmer, 2005; Meyer et al., 2008; Jacquot et al., 2009; König et al., 2012). Plastid-localized NADPH-dependent thioredoxin reductase (NTRC) is a unique NTR enzyme constituting a thioredoxin system in a single polypeptide chain (Serrato et al., 2004; Pérez-Ruiz et al., 2006). In NTRC, a TRX module is fused to the C-terminus of a reductase domain. The protein contains two redox-active 2-Cys motifs, CAIC in its NTR domain (NTRd) and CGPC in the TRX domain (TRXd; Serrato et al., 2004), and it is suggested to function as a dimer (Pérez-Ruiz and Cejudo, 2009; Pérez-Ruiz et al., 2009; Lee et al., 2012). Characterization of knockout lines of *NTRC* (*ntrc*) has indicated that NTRC is a crucial redox-regulator of a number of plastidial processes, including biogenesis of chloroplasts, biosynthetic pathways and ROS metabolism in chloroplasts (Pérez-Ruiz et al., 2006; Stenbaek et al., 2008; Michalska et al., 2009; Lepistö et al., 2009, 2012;

Pulido et al., 2010; Kirchsteiger et al., 2012; Chae et al., 2013; Richter et al., 2013). 2-Cys-peroxiredoxins and ADP -glucose pyrophosphorylase, the H_2O_2-detoxification enzymes and the key enzyme in starch synthesis, respectively, are the most conclusively documented target proteins of NTRC (Pérez-Ruiz et al., 2006; Michalska et al., 2009; Pulido et al., 2010).

According to the reaction mechanism model of the NTRC dimer (Pérez-Ruiz and Cejudo, 2009; Lee et al., 2012), the redox-active site of the NTRd of one subunit reduces the disulphide bridge of TRXd in the second subunit, which subsequently reacts with NTRC target proteins. *In-vitro* assays with purified NTRC and its target proteins support the model, demonstrating that the NTRd of NTRC primarily reduces its own TRXd, whereas it has a poor capability of reacting with other chloroplast TRXs (Pérez-Ruiz and Cejudo, 2009; Bohrer et al., 2012; Lee et al., 2012). However, recombinant NTRC protein forms oligomeric aggregates *in vitro* (Wulff et al., 2011) that may inhibit the interactions of NTRC with other chloroplast TRXs. To study the action of the NTRC domains *in vivo*, we have complemented the *ntrc* knockout line with a wild type *NTRC* gene and with full-length genes, in which the redox-active Cys motif in the NTRd (C217S/C220S) or in the TRXd (C454S/C457S) was inactivated. The *ntrc* line was also independently transformed either with a truncated NTRd or TRXd domain. Here we show that overexpression of the full-length NTRC with inactivated redox active Cys residues either in the NTRd or the TRXd partly complemented the *ntrc* mutant phenotype in *Arabidopsis*. The mutated NTRC proteins were capable of dimerization *in vivo*. Modeling of the three-dimensional structure of NTRC dimers indicates extensive interactions at the dimeric interface. It is also likely that the mutated NTRC acted via other chloroplast thioredoxin systems in restoring chloroplast development and the activity of metabolic pathways. We demonstrate here that thioredoxin f (TRXf) is the most prominent partner to interact with NTRC *in vivo*. Finally, overexpression of wild type NTRC promoted leaf expansion and dry weight accumulation of *Arabidopsis* rosettes, especially under increased light intensity.

MATERIALS AND METHODS

PLANT TRANSFORMATION AND DNA ANALYSIS

An *NTRC* coding sequence of *Arabidopsis thaliana* (At2g41680) containing a chloroplast signal sequence was used as a template in PCR to amplify a full-length *NTRC* (*OE-NTRC*), a truncated N-terminal NTRd encoding the amino acids 1–400 of NTRC (*OE-NTRd*), and a truncated TRXd encoding the amino acids 401–529 of NTRC (*OE-TRXd*; **Figure 1**; **Table 1**). A putative transit peptide of *NTRC* consisting of 67 amino acids (ChloroP-program, Emanuelsson et al., 1999) was fused into an N-terminus of a truncated TRXd. The sequences were cloned as *Nco*I/*BamH*I fragments into the pGWR8 plasmid (Rozhon et al., 2010) under control of the cauliflower mosaic virus (CaMV) 35S promoter. The calculated molecular masses of mature NTRC, NTRd and TRXd (without chloroplast transit peptide) are 50890, 35796, and 15100 Da, respectively.

Full-length *NTRC* was used as a template to generate monocysteine NTRC mutants with a C220S mutation in the NTRd or a C457S mutation in the TRXd by using QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Stratagene, Santa Clara, CA, USA; **Table 1**). These constructs were used as templates to make double cysteine mutants, C217S/C220S in the NTRd, or C454S/C457S in the TRXd (**Figure 1**), which were then named as *OE-SAIS* and *OE-SGPS* constructs, respectively. All plasmids were sequenced.

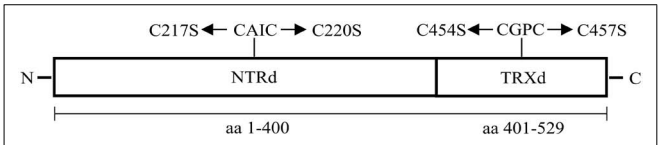

FIGURE 1 | Schematic representation of the NTRd and TRXd and the active site motifs of *Arabidopsis* NTRC. The amino acid sequence presented in the figure includes a chloroplast transit peptide of 67 amino acids at the N-terminus. Mutations of Cys residues in the redox active site of NTRd and TRXd are indicated in the figure.

Overexpression constructs *OE-NTRC*, *OE-SAIS*, *OE-SGPS*, *OE-NTRd*, and *OE-TRXd* were introduced to electrocompetent *Agrobacterium tumefaciens* strain GV3101 by standard electroporation protocol using Gene Pulser cuvettes (Bio-Rad, CA, USA). Transformed agrobacterium cells were selected by growing for two days at 29°C on LB agar plates in the presence of 20 μg/ml of rifampicin, 50 μg/ml of gentamicin sulfate, 5 μg/ml of tetracycline hydrochloride and 50 μg/ml of kanamycin. All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transformation of *ntrc* knockout plants (SALK_096776[1], Alonso et al., 2003; Lepistö et al., 2009) was done according to standard floral dipping procedures (Clough and Bent, 1998). Plants treated with agrobacterial suspension were grown in a growth chamber under long day conditions (16 h light/8 h dark) until seeds were collected. Selection of transformed seeds was carried out in 0.6% agar containing 0.5× Murashige and Skoog basal salt mixture (MS; Sigma-Aldrich) and 50 μg/ml of kanamycin. Resistant seedlings were transferred into soil and plants were grown in a growth chamber under long day conditions until the harvest of seeds (T2 seeds).

MATERIALS AND GROWTH CONDITIONS

Seeds of the T2 or T3 generations of two independent transgenic lines (*OE-NTRC-18 and 22, OE-SAIS-57 and 58*, *OE-SGPS-11 and 12*, *OE-NTRd-13 and 1*4, and *OE-TRXd-15 and 27)* in *ntrc* background were used in the experiments, except in **Table 3**, in which the biomass yield of the five additional independent transgenic *OE-NTRC* lines indicated in the table was measured. Seeds were first germinated on agar plates containing 50 μg/ml kanamycin and the resistant seedlings were transferred into soil. Wild type *Arabidopsis* ecotype Columbia (WT), T-DNA insertion mutant of NTRC (*ntrc*) and transgenic lines overexpressing wild type or mutant NTRC proteins in *ntrc* background were grown on a mixture of soil and vermiculite (1:1) under 130 and 600 μmol of photons m^{-2} s^{-1} at 23°C under short day (8-h light/16-h dark) or long day (16-h light/8-h dark) conditions as indicated in figures and tables.

[1]http://signal.salk.edu/

Table 1 | Forward and reverse primers for overexpression (OE) and yeast two-hybrid (Y2H) bait and prey constructions.

	Sequence	Constructs used for
OE-primers		
OE.NTRC		
Frw	5′-CTGCCATGG*ATG*GCTGCGTCTCCCAAGA-3′	OE-NTRC, OE-SAIS, OE-SGPS
Rev	5′-CGGGATCCTCATTTATTGGCCTCAATGAAT-3′	
OE.NTRd		
Frw	5′-CTGCCATGG***ATG***GCTGCGTCTCCCAAGA-3′	OE-NTRd
Rev	5′-CGGGATCC***TCA***AAATTCAACAAGAAGATTGTT-3′	
SS_NTRC		
Frw	5′-CTGCCATGG***ATG***GCTGCGTCTCCCAAGA-3′	Cloning of SS to TRXd
Rev	5′-GGAGACTCTGAGGCGGAGTCCCCCCGGG-3′	
OE.TRXd		
Frw	5′-TCCCCCCGGGCACCAGCCTCAAACTGAAGA-3′	OE-TRXd
Rev	5′-CGGGATCC***TCA***TTTATTGGCCTCAATGAAT-3′	
Y2H primers		
Y2H.NTRC		
Frw	5′-CGCAT***ATG***GCTGCGTCTCCCAAGA-3′	pGBKT7.NTRC, pGADT7.NTRC, pGBKT7.NTRC^{CAIS}, pGADT7.NTRC^{CAIS},
Rev	5′-CGGGATCC***TCA***TTTATTGGCCTCAATGAATT-3′	pGBKT7.NTRC^{CGPS}, pGADT7.NTRC^{CGPS}, pGBKT7.NTRC^{SGPS}
Y2H.NTRd		
Frw	5′- GGGAATTC CAT***ATG***GCCACCGCCAATTCTCCG-3′	pGBKT7.NTRd, pGADT7.NTRd, pGBKT7.NTRC^{SAIS}
Rev	5′-CGGGATCC***TCA***AAATTCAACAAGAAGATTGTT-3′	
Y2H.TRXd		
Frw	5′-GGGAATTCCAT***ATG***CACCAGCCTCAAACTGAAGA-3′	pGBKT7.TRXd, pGADT7.TRXd, pGADT7.TRXd^{SGPS}
Rev	5′-CGGGATCC***TCA***TTTATTGGCCTCAATGAATT-3′	
Mutagenesis primers		
NTRC^{C217S}		
Frw	5′-GATAAGTGCTAGTGCTATCAG-3′	OE-SAIS, pGBKT7.SAIS
Rev	5′-CTGATAGCACTAGCACTTATC-3′	
NTRC^{C454S}		
Frw	5′-TCACCAACAAGTGGCCCCA-3′	OE-SGPS, pGBKT7.NTRC^{SGPS}, pGADT7.TRXd^{SGPS}
Rev	5′-TGGGGCCACTTGTTGGTGA-3′	
NTRC^{C220S}		
Frw	5′-GATAAGTGCTTGTGCTATCAG-3′	OE-SAIS, pGBKT7.NTRC^{CAIS}, pGBKT7.NTRC^{SAIS}, pGADT7.NTRC^{CAIS},
Rev	5′-CTGATAGCACAAGCACTTATC-3′	pGADT7.NTRC^{SAIS}
NTRC^{C457S}		
Frw	5′-ATGTGGCCCCAGTAGGACTC-3′	OE-SGPS, pGBK7.NTRC^{CGPS}, pGADT7.NTRC^{CGPS}, pGBKT7.NTRC^{SGPS},
Rev	5′-GAGTCCTACTGGGGCCACAT-3′	pGADT7.TRXd^{SGPS}

Restriction enzyme sites are underlined and translational start and stop codons are marked by bold italic. NTRd = NTR domain and TRXd = TRX domain without any mutations. CAIS and SAIS = Second or both redox cysteines in the NTRd of the full-length NTRC are switched to serine. CGPS and SGPS = Second or both redox cysteines in the TRXd of the full-length NTRC are switched to serine. SS = chloroplast signal sequence of NTRC. Frw = forward primer, rev = reverse primer.

DETERMINATION OF ROSETTE DRY WEIGHT, CHLOROPHYLL CONTENT, PHOTOCHEMICAL EFFICIENCY OF PHOTOSYSTEM II, AND STARCH CONTENT OF LEAVES

Leaf number of five rosettes of WT, *ntrc* and transgenic lines overexpressing wild-type or mutated NTRC was counted, the rosettes were then dried at 60°C for 24 h and weighed thereafter.

Five leaf disks, 5 mm in diameter were incubated in 1 ml of 100% dimethylformamide (DMF) (Mallinckrodt Inc.) overnight at 4°C in darkness. Total content of chlorophyll and chlorophyll a/b ratio (Chl a/b) of the solutions were measured at wavelengths 646.6, 663.6 and 750 nm with Lambda 25 UV/VIS Spectrometer (Perkin Elmer, MA, USA). Chlorophyll concentrations were calculated according to Porra et al. (1989).

The photochemical efficiency of Photosystem II in intact leaves illuminated under growth light intensity for 2 h was measured with a Hansatech PEA fluorometer after a 30-min dark incubation and recorded as the ratio of variable to maximal fluorescence (Fv/Fm), where Fv is the difference between maximal fluorescence (Fm) and initial fluorescence (Fo).

For detection of starch content leaves were detached from rosettes after 4 h of illumination and incubated in DMF until chlorophyll was bleached. Plants were rinsed with water, stained with Lugol solution (5% I2 and 10% KI) in distilled water with total iodine content of 130 mg/ml for 2 min, destained with water for 1 h and photographed (Lepistö et al., 2009).

EXTRACTION OF SOLUBLE LEAF PROTEINS, SDS-PAGE AND WESTERN BLOTTING

Arabidopsis leaves were frozen in liquid N_2, and proteins were extracted in buffer containing 50 mM HEPES (Fisher Scientific, UK), 5 mM NaCl (Mallinckrodt Inc., Phillipsburg, NJ, USA), 10 mM $MgCl_2$ (Sigma-Aldrich) and with and without 2 mM DTT (Lepistö et al., 2009). Soluble protein extracts were solubilized with and without mercaptoethanol at 0°C or heated at 100°C for 1 min (Laemmli, 1970). The amounts of protein indicated in the figures were loaded on a gel containing 10% (w/v) acrylamide in the separation gel. After separation in SDS-PAGE the proteins were subsequently electroblotted to a PVDF membrane (Millipore[2]; Lepistö et al., 2009). Wild type and mutated NTRC as well as the truncated TRXd of NTRC were detected by NTRC-specific antibody that was raised against the amino acids 475–488 in the TRXd (Lepistö et al., 2009). Because the anti-NTRC antibody does not recognize the truncated NTRd, its overexpression in transgenic lines was detected by anti-NTRB antibody raised against wheat NTRB enzyme (kindly provided by prof. F.J. Cejudo, Institute of Plant Biochemistry, University of Sevilla). Anti-Rubisco antibody (Agrisera), cross-reacting with the large subunit of Rubisco, was used for the detection of Rubisco content in leaf extracts. Molecular mass markers were purchased from New England Biolabs (NEB, MA, USA).

Y2H BINARY ASSAYS

Full length and truncated *NTRC* sequences described in Section "Plant Transformation and DNA Analysis" and **Figure 1** were subcloned as DNA fragments between the NdeI(5′) and BamHI(3′) restriction sites of both pGAD.T7 and pGBK.T7 yeast two-hybrid vectors (Clontech, TAKARA BIO INC., Japan). These constructs were used as templates for site-directed mutagenesis to generate single (CAIS, CGPS) and double (SGPS) mutants. All plasmids were checked by sequencing.

Y2H experiments were conducted in the yeast reporter strain CY306 (Vignols et al., 2005), a Y2H strain designed for stabilizing redox interactions between TRXs and their target proteins (Vignols et al., 2005). CY306 strain was cultured in YNB with 0.7% yeast extract w/o amino acids (BD Difco, NJ, USA) and 2% glucose monohydrate (Mallinckrodt) supplemented with required amino acids and bases (Sigma-Aldrich), and co-transformed with different sets of pGAD and pGBK constructs carrying *NTRC* and derived sequences using lithium acetate as previously described (Vignols et al., 2005; Lepistö et al., 2013; **Table 1**). CY306 double transformants were selected as cells growing in the absence of leucine and tryptophan, and further assayed for Y2H interaction in the absence of tryptophan, leucine and histidine. Interaction between the viral genome-linked protein VPg and eukaryotic translation initiation factor eIF4G (Hébrard et al., 2010) was used as a positive control, while empty pGADT7 or pGBKT7 vectors served as negative controls in the Y2H tests.

TRANSMISSION ELECTRON MICROSCOPY

Leaves of 6-week-old plants were collected after the dark period, fixed with 3% glutaraldehyde in 100 mM sodiumphosphate buffer, pH 7.0, placed under a vacuum for 2.5 h, thereafter fixed and stained according to Pätsikkä et al. (2002), and examined with

[2]http://www.millipore.com

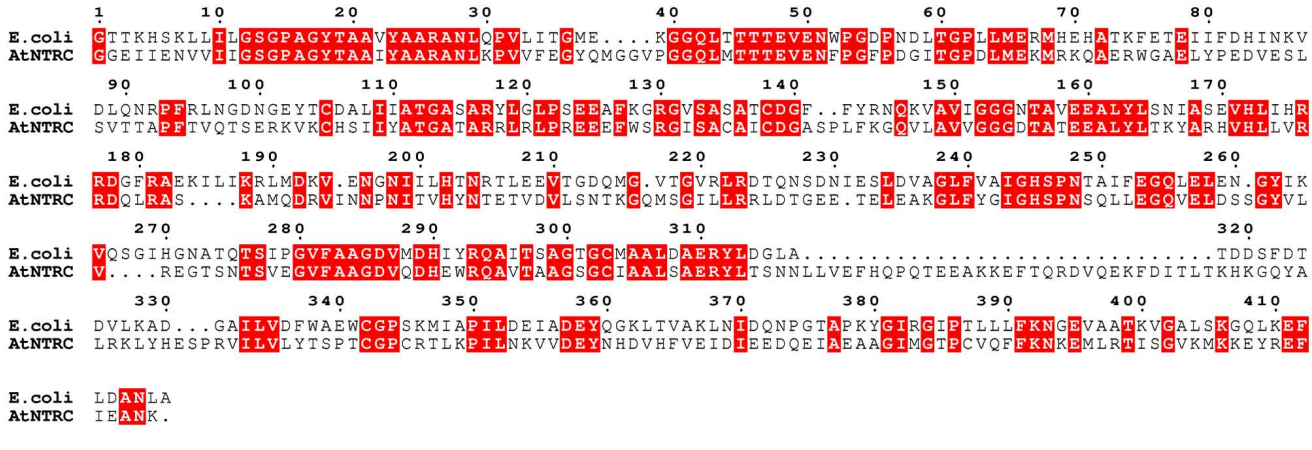

FIGURE 2 | Sequence alignment used for modeling. The sequence alignment used for modeling shows the *E. coli* NTR and the TRX sequences aligned to the NTRC sequence. Conserved residues are shown in red boxes.

a transmission electron microscope (JEM-1400 Plus TEM, JEOL Ltd., Tokyo 196-8558, Japan).

STRUCTURAL MODELING OF NTRC

To search for related sequences and a crystal structure that could be used as a template for modeling, the *Arabidopsis* NTRC sequence was used as bait to search UniProtKB and Protein Data Bank (PDB) with the Basic Local Alignment Search Tool (BLAST) at NCBI[3]. A homology model of NTRC (residues 79–529) was constructed based on the crystal structure of the NTR–TRX complex from *Escherichia coli* (PDB code 1F6M; Lennon et al., 2000). MALIGN (Johnson et al., 1996) in the BODIL modeling environment (Lehtonen et al., 2004) was used to align NTRC with similar sequences and the *E. coli* NTR sequence, which shares 43% sequence identity to NTRC (**Figure 2**). A separate alignment was made for the TRXd of NTRC and the *E. coli* TRX sequence, together with several other known TRX structures. For modeling, all sequences except NTRC and the *E. coli* sequences were excluded from the alignments. The NTRd and the TRXd sequence alignments were then combined into one alignment, which was used for modeling (**Figure 2**). The linker region connecting the NTRd to the TRXd was determined based on sequence alignment with barley NTRC, which has an approximately 35 amino acids long linker between residues 341 and 374 (Wulff et al., 2011). *Arabidopsis* NTRC shares 81 % identity to barley NTRC, indicating a similar linker region. Based on the alignment, the *Arabidopsis* NTRC linker is located between amino acids 396 and 429, i.e., 34 amino acids long. Residues 406 to 421 of the linker were restrained to an α-helix as predicted by the JPred (Cole et al., 2008) and PSIPred (Jones, 1999) secondary structure prediction programs. A set of ten models was created with MODELLER (Sali and Blundell, 1993), and the model with the lowest value of the MODELLER objective function was analyzed and compared to the crystal structure of the *E. coli* NTR–TRX complex by superimposition with VERTAA (Johnson and Lehtonen, 2000) in BODIL. The quality of the final model was assessed with PROCHECK (Laskowski et al., 1993) and QMEAN (Benkert et al., 2009). The same was done for all ten different TRXs found in *Arabidopsis* chloroplasts, which were modeled to the NTR domain of NTRC. The APBS tool in PyMOL (Version 1.4, Schrödinger, LLC) was used to calculate electrostatic surfaces. PyMOL was also used to prepare pictures of the 3D model and ESPript (Gouet et al., 1999) for alignment pictures.

RESULTS

NTRC CONTENT IN TRANSGENIC OVEREXPRESSION LINES

To study the function of NTRC domains *in vivo*, we complemented the *ntrc* line with a wild type *NTRC* gene (*OE-NTRC* lines) and with full-length genes, in which the redox-active 2-Cys motifs of the NTRd (*OE-SAIS* lines) or TRXd (*OE-SGPS* lines) were inactivated by site-directed mutations (**Figure 1**). The *ntrc* line was also independently transformed either with a truncated NTRd (*OE-NTRd* lines) or TRXd (*OE-TRXd* lines). The expression levels of transgenic genes were detected by immunoblotting with both an NTRC-specific antibody and with an antibody raised against the NTRB enzyme from wheat. In comparison to WT, the content of NTRC protein was ten to forty times higher in the leaves of transgenic lines except in the *OE-TRXd* line, in which the accumulation of truncated TRXd equalled the content of NTRC in WT leaves (**Figure 3**). Despite high overexpression of transgenic genes no degradation products of NTRC were detected in leaf extracts indicating that the mutated NTRC protein was stable in transgenic lines. The phenotype complementation of transgenic lines in *ntrc* background was not caused by the leaking of the original T-DNA insertion in endogenous *NTRC* gene, since no full-length NTRC protein was detected in the transgenic lines expressing truncated NTRC domains (**Figure 3**).

OVEREXPRESSION OF NTRC IN *ARABIDOPSIS*

Complementation of the *ntrc* line with the wild-type *NTRC* gene fully recovered the green phenotype of seedlings, the wild-type growth rate of rosettes and the ultrastructure of chloroplasts (**Figures 4** and **5**; **Table 2**). Yellowish leaves typical for *ntrc* mutant lines were not detected at any developmental stage of leaves. In comparison to WT, the rosette dry weight of OE-NTRC lines was about 40% higher in 7-weeks-old *OE-NTRC* lines grown at 130 μmol photons m^{-2} s^{-1} (**Table 2**). Overexpression of *NTRC* did not increase the number of leaves in these plants, but the size of the fully expanded *OE-NTRC* leaves was substantially larger (**Figure 4**). Increase in light intensity further stimulated the biomass yield of transgenic *OE-NTRC* plants in comparison to WT (**Table 2**). For validation of positive effect of *NTRC* overexpression on biomass yield we measured the dry weight of six independent transgenic *OE-NTRC* lines grown under 600 μmol photons m^{-2} s^{-1} for 6 weeks (**Table 3**). The data showed that overexpression of *NTRC* generally increased the biomass yield of rosettes albeit the extent of growth stimulation varied between lines.

Chlorophyll content of *OE-NTRC* leaves in plants grown at 130 μmol photons m^{-2} s^{-1} was about 25% lower than in WT leaves, whereas slightly higher chlorophyll content was detected in plants grown at 600 μmol photons m^{-2} s^{-1} (**Table 2**). Increase in light intensity particularly reduced the amount of chlorophyll per WT leaf area. Overexpression of NTRC did not change the chlorophyll a/b ratio in leaves. Complementation of the *ntrc* line with wild type *NTRC* also fully recovered the photochemical efficiency of Photosystem II, which was significantly reduced in *ntrc* lines at growth light intensity (**Figure 6**; Lepistö et al., 2009). Overexpression of *NTRC* also increased the accumulation of starch in illuminated leaves (**Figure 7**).

The *ntrc* lines show most severe growth defects under short day conditions that promote the vegetative growth of *Arabidopsis* (Pérez-Ruiz et al., 2006; Lepistö et al., 2009). Therefore we studied, whether the overexpression of NTRC also promotes *Arabidopsis* growth under long day conditions that induce early flowering in *Arabidopsis*. Similarly to short day conditions, three weeks-old *OE-NTRC* plants grown at 600 μmol photons m^{-2} s^{-1}showed substantial increase in rosette dry weight and a higher number and larger size of leaves (**Figure 4**; **Table 3**). We conclude that overexpression of NTRC significantly stimulates leaf expansion in *Arabidopsis* and the increase in light intensity promotes the gain of leaf biomass in *OE-NTRC* transgenic lines.

[3]http://blast.ncbi.nlm.nih.gov/

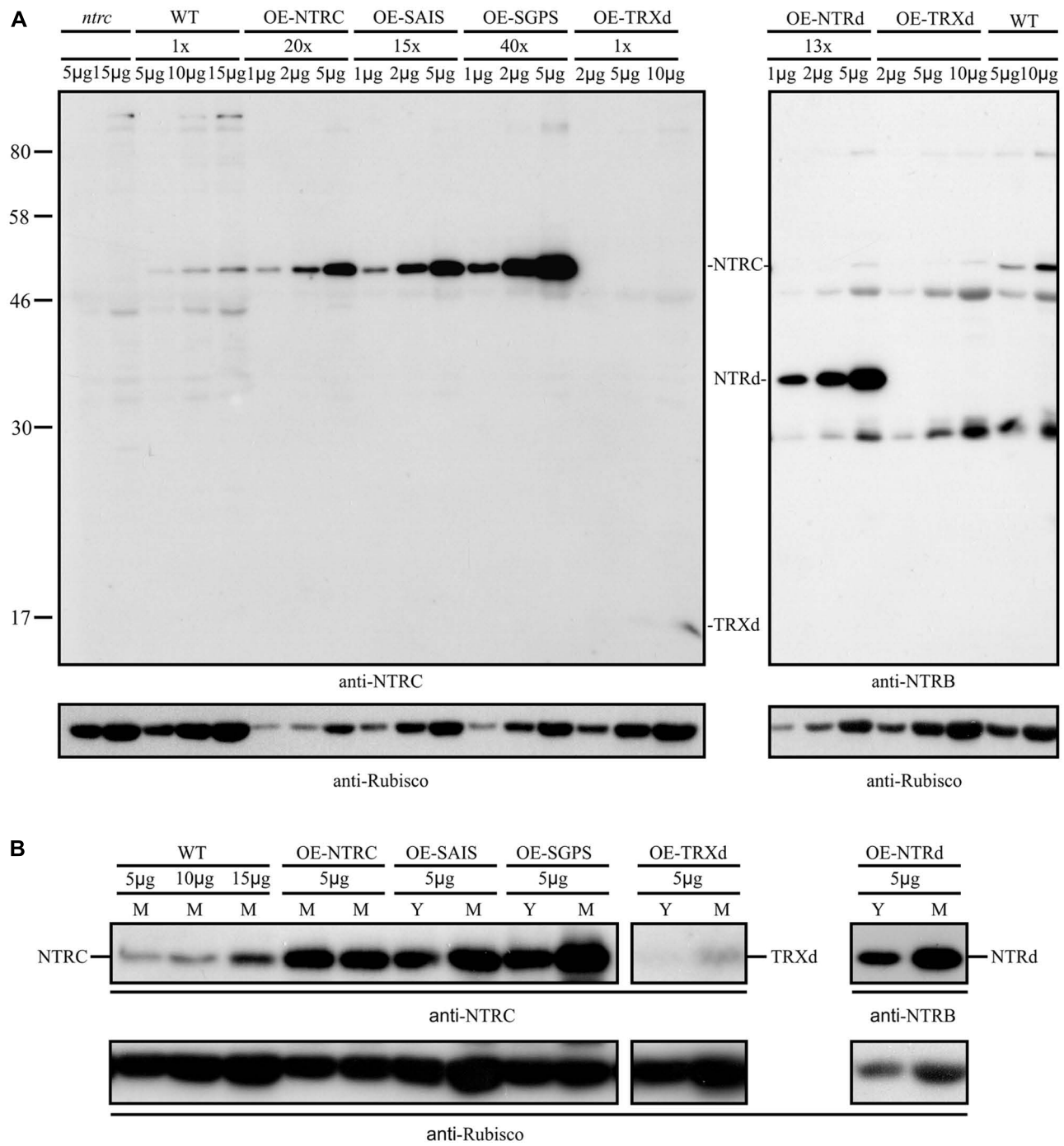

FIGURE 3 | (A) NTRC content in the wild type (WT) and in the transgenic lines overexpressing wild-type, mutated NTRC, or truncated NTRC domains. Different amounts of leaf extracts (µg soluble protein, upper panel) were loaded on SDS-gel and the proteins were separated in SDS-PAGE before Western blotting with anti-NTRC antibody, anti-NTRB antibody, and anti-Rubisco antibody. The content of mutated NTRC and truncated NTRC domains (NTRd and TRXd) in leaf extracts of transgenic lines were normalized to Rubisco content and presented in the upper panel relative to the amount of NTRC in wild type (WT). Molecular mass standard (kDa) is presented in left side of the blot. OE-NTRC, OE-SAIS, OE-SGPS, OE-NTRd, and OE-TRXd represent the overexpression lines of *NTRC*, *NTRC* with inactive reductase or thioredoxin active sites, and the transgenic lines overexpressing truncated NTRd or TRXd, respectively. *ntrc* is a knockout line of *NTRC*. In addition to NTRC, anti-NTRB antibody cross-reacts with *Arabidopsis* proteins of the apparent molecular masses of 80, 46, and 30 kDa. **(B)** Increase in accumulation of NTRC protein during maturation of transgenic leaves overexpressing mutated NTRC, or truncated NTRC domains. Amounts of leaf extracts (µg soluble protein, upper panel) were loaded on SDS-gel and the proteins were separated by SDS-PAGE before Western blotting with anti-NTRC antibody, anti-NTRB antibody, and anti-Rubisco antibody. Y and M; soluble proteins were extracted from yellowish and green leaves, respectively.

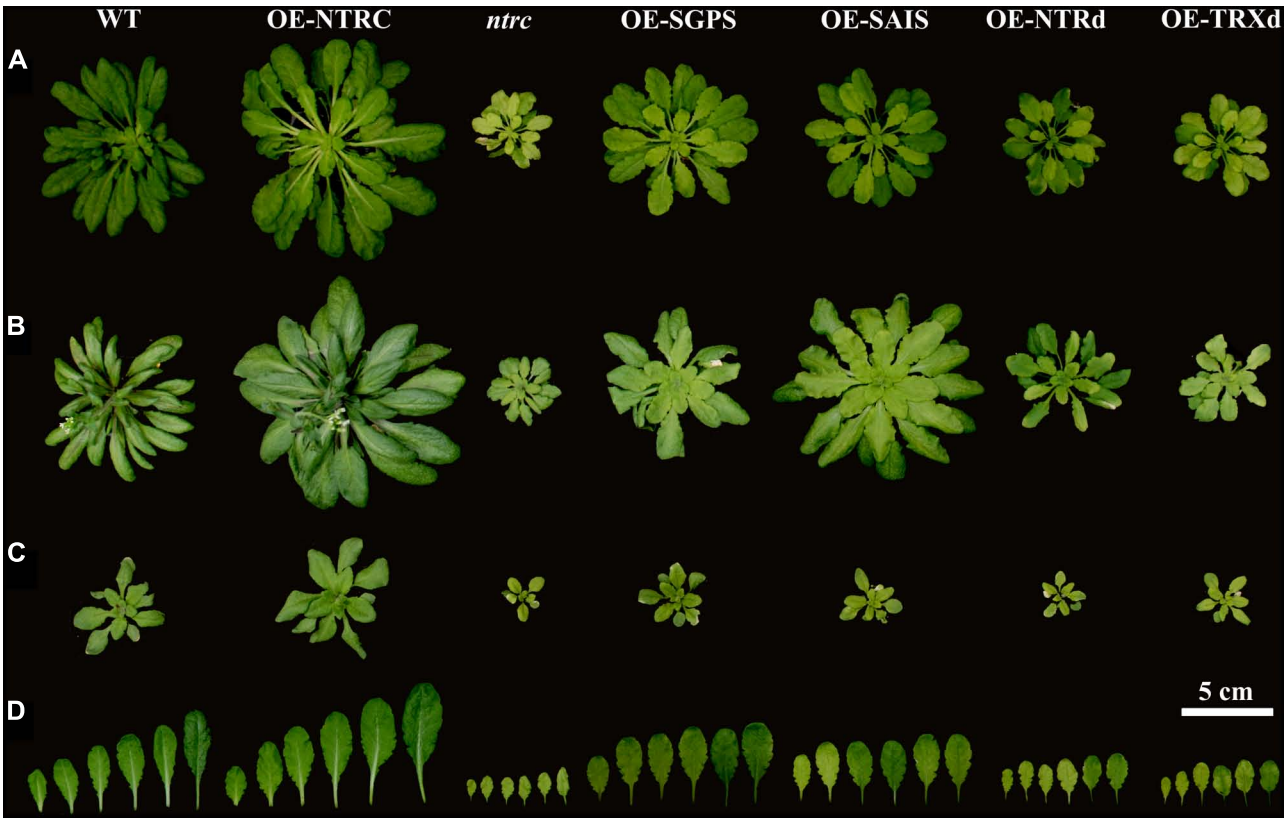

FIGURE 4 | Phenotypes of transgenic lines overexpressing wild-type, mutated NTRC, or truncated NTRC domains. Wilt type (WT), knockout line of *NTRC* (*ntrc*), and the overexpression lines of mutated and truncated *NTRC* are grown under short day conditions **(A)** at 130 μmol of photons m^{-2} s^{-1}, or **(B)** at 600 μmol of photons m^{-2} s^{-1}, and **(C)** under long day conditions at 600 μmol of photons m^{-2} s^{-1}. **(D)** Largest rosette leaves of plants presented in **(A)**. For abbreviations see the legend of the **Figure 3**.

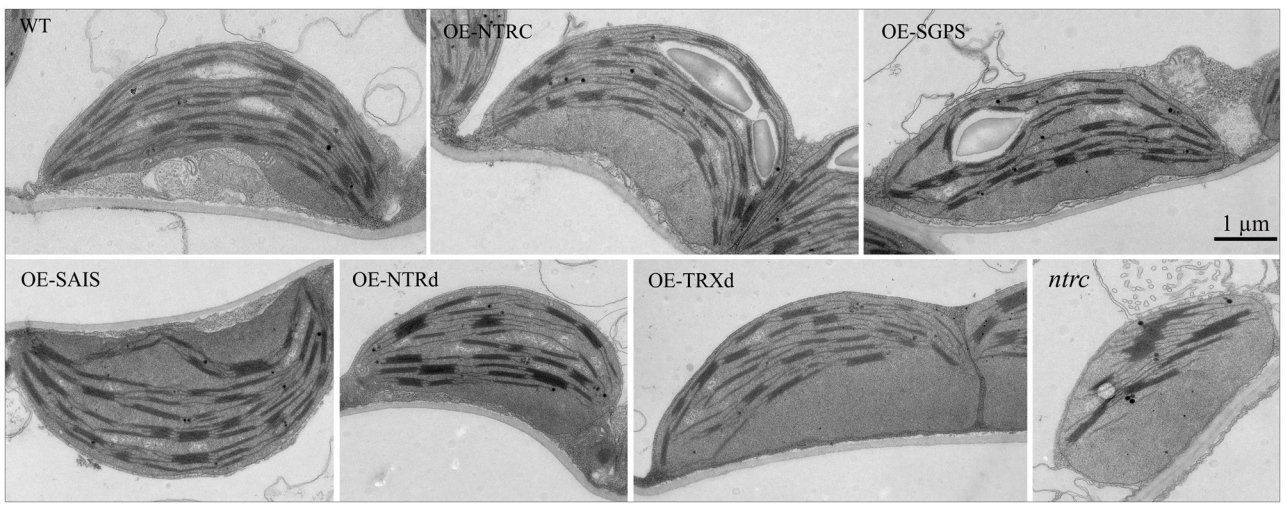

FIGURE 5 | Chloroplast ultrastructure in transgenic lines overexpressing wild-type, mutated NTRC or truncated NTRC domains. Plants were grown at 600 μmol of photons m^{-2} s^{-1} under short day conditions for 6 weeks before sampling of the leaves. Scale bar is 1 μm. For abbreviations see the legend of the **Figure 3**.

Table 2 | Leaf number, chlorophyll content and dry weight of transgenic rosettes overexpressing wild-type, mutated NTRC, or truncated NTRC domains.

Line	Chl a + b (μg cm^{-2} ± SE) (% of WT)	Chl a/b ± SE	Dry weight (mg ± SE) (% of WT)	Leaf number ± SE
SD 130 μmol m^{-2} s^{-1}				
WT	26.3 ± 1.2 (100)	3.13 ± 0.08	197 ± 18 (100)	38 ± 1
OE-NTRC	19.9 ± 1.8 (76)*	3.13 ± 0.08	280 ± 46 (142)	39 ± 1
ntrc	3.0 ± 0.5 (11)***	2.61 ± 0.09**	24 ± 5 (12)***	22 ± 1***
OE-SGPS				
Young	6.0 ± 0.2 (23)***	2.78 ± 0*	108 ± 12 (55)**	37 ± 1
Mature	16.4 ± 1.6 (63)**	3.09 ± 0.04		
OE-SAIS				
Young	8.3 ± 0.6 (32)***	2.98 ± 0.07	102 ± 7 (52)**	34 ± 1*
Mature	14.1 ± 0.7 (54)***	3.18 ± 0.07		
OE-NTRd				
Young	5.9 ± 0.5 (22)***	2.85 ± 0.10	51 ± 3 (26)***	30 ± 1***
Mature	10.9 ± 0.7 (41)***	3.13 ± 0.04		
OE-TRXd	ND	ND	56 ± 2 (28)***	28 ± 1***
SD 600 μmol $m^{-2}s^{-1}$				
WT	13.5 ± 0.4 (100)	3.90 ± 0.04	334 ± 13 (100)	ND
OE-NTRC	14.9 ± 0.2 (110)*	3.90 ± 0.08	877 ± 57 (263)***	ND
ntrc	7.5 ± 0.5 (55)***	3.35 ± 0.09***	33 ± 4 (10)***	ND
OE-SGPS				
Young	7.9 ± 0.5 (58)***	3.93 ± 0.11	176 ± 16 (53)**	ND
Mature	14.5 ± 1.1 (107)	4.01 ± 0.10		
OE-SAIS				
Young	8.6 ± 0.8 (63)***	3.75 ± 0.08	354 ± 47 (106)	ND
Mature	14.9 ± 0.7 (110)	4.09 ± 0.05*		
OE-NTRd				
Young	9.0 ± 1.0 (67)**	3.93 ± 0.06	112 ± 18 (34)***	ND
Mature	16.7 ± 1.1 (124)*	4.02 ± 0.06*		
OE-TRXd				
Young	7.7 ± 0.4 (57)***	3.86 ± 0.10	45 ± 5 (13)***	ND
Mature	10.4 ± 0.7 (77)**	4.08 ± 0.08		

*Plants were grown under short day conditions (SD) for 7 weeks in the light intensity indicated in the table. Leaf number, chlorophyll content (Chl a+b), chlorophyll a/b ratio (Chl a/b) and rosette dry weight were measured as described in Section "Materials and Methods." Each value is the mean ± SE of four to five independent determinations. Chlorophyll content of transgenic lines overexpressing mutated NTRC was measured from both young yellowish and green (mature) leaves. ND, not determined. *P, **P, ***P < 0.05, 0.01, 0.001 (student's t-test), respectively, compared with WT plants. For the other abbreviations, see the legend for* **Figure 3**.

PHENOTYPES OF PLANTS OVEREXPRESSING MUTATED NTRC GENES AND TRUNCATED NTRC DOMAINS

Complementation of the *ntrc* line with the full-length *NTRC* gene containing an active reductase but inactive thioredoxin domain (*OE-SGPS*), or vice versa (*OE-SAIS*), substantially accelerated the growth of transgenic plants in comparison to the *ntrc* line (**Figure 4**; **Tables 2** and **3**). In plants grown at 130 μmol photons m^{-2} s^{-1} for 7 weeks the rosette dry weight of the *ntrc* line was less than 15% of the wild type, whereas transgenic plants with an active reductase or Trx catalytic site produced about 50% of the wild type dry mass. Overexpression of the *OE-SGPS* and *OE-SAIS* mutant genes in *ntrc* background partially recovered the chloroplast ultrastructure (**Figure 5**) as well as increased the chlorophyll content (**Table 2**) and restored the photosynthetic function of mature leaves (**Figure 6**). The young leaves, particularly in the *OE-SAIS* transgenic lines, were yellowish in color but became green during expansion and aging of leaves (**Figure 4**; **Table 2**). The greening of the leaves correlated with the accumulation of transgenic NTRC protein in the leaves (**Figure 3B**), suggesting that the mutated NTRC protein was less competent in the complementation of the knockout of the endogenous NTRC, but high accumulation of mutated NTRC proteins promoted greening and

Table 3 | Dry weight and leaf number of transgenic rosettes overexpressing wild-type, mutated NTRC, or truncated NTRC domains.

Line	Dry weight mg ± SE (% of WT)	Leaf number±SE
LD 600 μmol m^{-2}s^{-1}		
WT	129 ± 17 (100)	13 ± 1
OE-NTRC	257 ± 17 (200)***	17 ± 1**
ntrc	29 ± 1 (23)***	10 ± 1*
OE-SGPS	89 ± 7 (69)*	12 ± 1
OE-SAIS	67 ± 5 (52)**	12 ± 1
OE-NTRd	28 ± 2 (22)***	9 ± 1**
OE-TRXd	34 ± 4 (26)***	11 ± 1*
SD 600 μmol m^{-2}s^{-1}		
WT	235 ± 13 (100)	33 ± 2
OE-NTRC 4	286 ± 14 (122)*	36 ± 1
OE-NTRC 6	316 ± 21 (135)*	35 ± 2
OE-NTRC 8	334 ± 7 (142)***	35 ± 1
OE-NTRC 9	379 ± 55 (162)*	38 ± 1
OE-NTRC 10	446 ± 38 (190)**	41 ± 3
OE-NTRC 18	336 ± 33 (143)*	39 ± 3

*Plants were grown at 600 μmol of photons m^{-2} s^{-1} under long day conditions (LD) for 3 weeks or under short day conditions (SD) for 6 weeks. Leaf number and rosette dry weight were measured as described in Section "Materials and Methods." The numbers in OE-NTRC lines represents independent transgenic lines. Each value is the mean ± SE of four to five independent determinations. ND, not determined. *P, **P, ***P < 0.05, 0.01, 0.001 (student's t-test), respectively, compared with WT plants. For the other abbreviations, see the legend of* **Figure 3**.

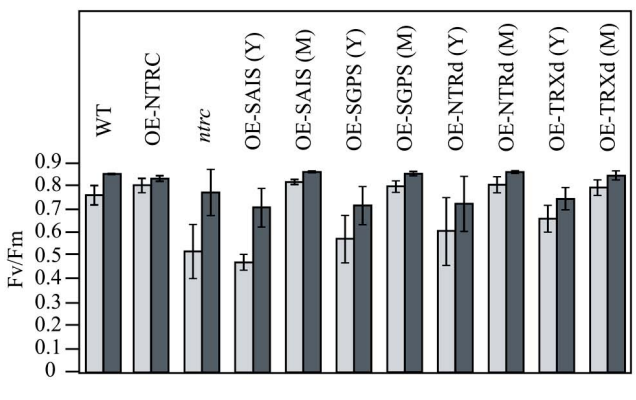

FIGURE 6 | Recovery of photochemical efficiency of Photosystem II in transgenic lines overexpressing wild-type, mutated NTRC, or truncated NTRC domains. Plants were grown under short day condition at 130 μmol of photons m^{-2} s^{-1} (light gray bars) or at 600 μmol of photons m^{-2} s^{-1}(dark gray bars) for 6 weeks and the photochemical efficiency of Photosystem II was measured after illumination for 2 h at growth light intensity. Each value is the mean ± SE (bars) of five independent determinations. Y and M; the photochemical efficiency of Photosystem II was measured both for young yellowish (Y) and green (M) leaves of the transgenic lines. For other abbreviations see the legend of the **Figure 3**.

recovery of leaf photosynthetic activity. The slow greening rate of young leaves reduced the growth rate of the *OE-SGPS* and the *OE-SAIS* transgenic lines, resulting in substantial reduction of rosette dry mass in comparison to WT under all growth light conditions tested. The *OE-SAIS* transgenic lines also accumulated less starch in light than the *OE-NTRC* or the *OE-SGPS* lines (**Figure 7**). The *OE-SGPS* and *OE-SAIS* transgenic lines demonstrate that the full-length NTRC with an active reductase but inactive TRXd, or vice versa, is capable of regulating chloroplast processes to some extent.

Overexpression of truncated forms of NTRC containing either NTRd or TRXd sequences in *ntrc* background complemented only poorly the mutant phenotype of *ntrc* (**Figures 3** and **4**; **Tables 2** and **3**). The content of truncated NTRd in the *OE-NTRd* transgenic lines was high and no proteolytic degradation products were detected by Western blotting (**Figure 3**), indicating that the lack of phenotypic complementation was due to low catalytic activity of the NTRd in the absence of full-chain NTRC polypeptide. The truncated TRXd is likely unstable, since high accumulation of the TRXd was not detected in *OE-TRXd* lines. An increase in the content of truncated NTRC proteins during leaf expansion induced slow greening of the leaves with increased amount of chlorophyll per leaf area and resulted in the recovery of the photosynthetic activity of old green leaves (**Figures 3–6**; **Table 2**). However, due to the inefficient complementation of chloroplast function by the truncated forms of NTRC, the growth rate of these transgenic plants was only slightly accelerated in comparison to the *ntrc* line. The *OE-NTRd* and *OE-TRXd* lines demonstrate that the NTRd and TRXd alone can only poorly form a catalytically competent enzyme capable of interacting with plastidial proteins.

DIMERIC STRUCTURE OF NTRC

The phenotype of transgenic lines overexpressing mutated full-length NTRC proteins indicates that the intact catalytic site of NTRC remains active in chloroplasts, albeit less efficient in regulation of chloroplast proteins than the WT enzyme. Furthermore, removing either the NTRd or TRXd from the full-length NTRC abolished the activity of the remaining domain. Eukaryotic NTRs are strictly homodimeric proteins (Hirt et al., 2002), which led us to ask if the reduced catalytic activity of NTRC in transgenic lines overexpressing the mutated NTRC or truncated NTRC domains is due to an inability to form homodimers *in vivo*.

The 3D model of the NTRC homodimer is presented in **Figure 8A**. The overall flavin reducing structure of the model shows the fold required for the reaction mechanism of the NTRC dimer (Pérez-Ruiz and Cejudo, 2009; Lee et al., 2012). The linker that connects the C-terminus of the NTRd and the N-terminus of the TRXd is ∼34 amino acids long and is likely to form a short α-helix. In the dimer, the TRXd of one subunit interacts with the NTRd of the other subunit. In addition to this arrangement, the FAD binding domains also strengthen the dimer interactions. The inter-monomeric interactions are formed by eleven hydrogen bonds and five possible salt bridges, three of which are at an optimal distance from each other (∼2.7 Å; **Figure 8B**). Additionally, there are four phenylalanines interacting with each other (pi-pi stacking) in a square.

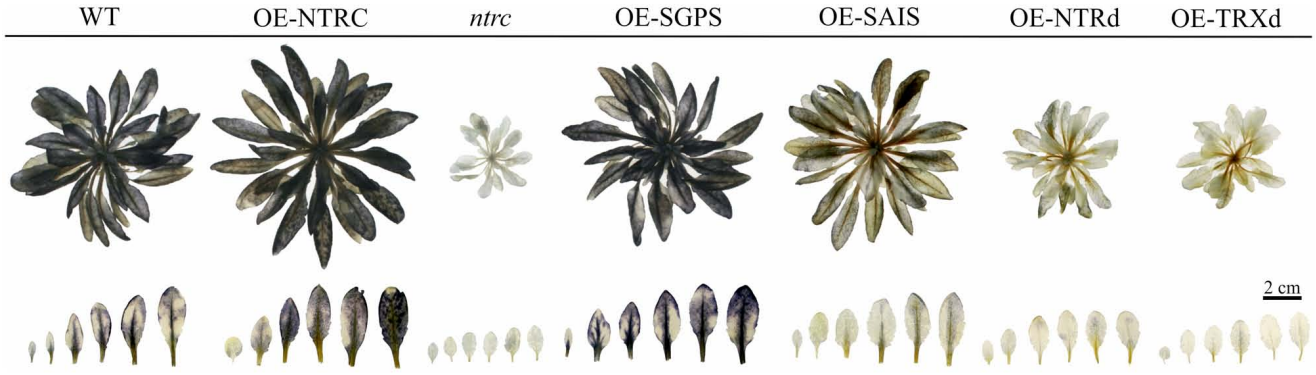

FIGURE 7 | Accumulation of starch in transgenic lines overexpressing wild-type, mutated NTRC, or truncated NTRC domains. Seven-weeks-old rosettes were grown at 600 μmol of photons m^{-2} s^{-1} under short day condition and five rosettes were harvested after illumination for four hours. For starch staining rosettes were treated with Lugol solution as described in Section "Materials and Methods" and photographed. Blue color indicates the amount of starch in leaves. For abbreviations, see the legend of the **Figure 3**.

To analyze the oligomeric form of NTRC *in vivo*, soluble proteins were extracted from WT, *ntrc* and transgenic lines overexpressing mutated and truncated NTRC proteins in the presence or absence of thiol reducing chemical. The protein samples were solubilized with SDS either with or without mercaptoethanol and kept on ice or heated as described in Section "Materials and Methods" before the separation of proteins in SDS-PAGE. While the NTRC band of heated samples corresponded to the monomeric full-length NTRC with an apparent molecular mass of 50 kDa, the molecular mass of the major NTRC band in unheated samples was approximately 100 kDa, representing the homodimer of NTRC (**Figure 9**). Dimeric NTRC was detected both with NTRC-specific and anti-NTRB-antibodies and it was totally absent in protein extract from *ntrc* lines. The dimeric form of NTRC structure was very stable because it was present in the samples treated with SDS and mercaptoethanol and kept on ice before SDS-PAGE, and it did not monomerize until heating of the protein extract. The heat-induced monomerization of polypeptides took place also without addition of thiol-reducing compounds, although extraction of proteins without DTT caused slight smearing of NTRC polypeptides in the SDS gel (**Figure 9**). The monomerization of NTRC can be explained by the absence of disulphide bridges at the dimer interface of NTRC (**Figure 8B**). Therefore, the inter-monomeric aromatic interactions, together with the salt bridges, and the interlocking arrangement of the TRXds, account for the strong dimeric interactions we observed. This is further supported by the fact that the salt bridges of optimal length are conserved in the alignment of NTRC like sequences (**Figure 8C**). F137 is also conserved in most sequences, but F134 is sometimes substituted by tryptophan or tyrosine, both of which retain the aromatic stacking property. K, Q or N occasionally replaces D139, but these substitutions retain the hydrogen bonding capacity at this position.

The Western blots demonstrated that WT and the mutated full-length NTRC proteins formed homodimers *in vivo* (**Figure 9**). The unheated protein samples extracted from the *OE-NTRd* lines also gave two bands, which cross-reacted with the anti-NTRB antibody. The apparent molecular mass of the band with lower mobility was less than 50 kDa, which is much lower than the estimated molecular mass of a homodimeric NTRd (72 kDa). Heating of the protein extract removed the upper band and concomitantly increased the intensity of the band of monomeric NTRd (36 kDa), indicating that both bands consisted of truncated NTR polypeptides (**Figure 9**). The anomalous migration of NTRd in SDS-gel and the high content of monomeric form in unheated protein sample suggest that the truncated NTRd has reduced capability of forming dimers *in vivo*. No homodimer of TRXd was detected in transgenic lines overexpressing truncated TRXd.

The self-interaction of the wild-type NTRC proteins was confirmed by binary Y2H test (**Figure 10**). Neither NTRC nor NTR polypeptide without TRXd (NTRd) interacted with a truncated wild-type (TRXd) or with mutated TRX polypeptide ($TRXd^{SGPS}$) in the Y2H test, whereas truncated NTRd did interact with itself. To study the effect of altered redox state of NTRC active sites on the interactions of polypeptides, the second Cys in the catalytic site either of the NTRd ($NTRC^{CAIS}$) or TRXd ($NTRC^{CGPS}$) was mutated to Ser. The CAIS-form of the NTRd active site reacts with the disulphide of a target TRX resulting in a stable mixed disulphide between the NTRd active site and TRX. The CGPS-form of the TRXd active site is permanently reduced and thus cannot react with the NTRd active site of NTRC. The self-interaction of the NTRC proteins was detected, when wild-type NTRC was tested with $NTRC^{CAIS}$ or $NTRC^{SGPS}$, and when $NTRC^{CAIS}$ was tested with $NTRC^{CGPS}$, whereas neither $NTRC^{CAIS}$ nor $NTRC^{CGPS}$ was capable of self-interaction. Neither NTRC with a monothiol in the TRXd active site ($NTRC^{CGPS}$) interacted with a truncated TRX polypeptide (TRXd) in the Y2H test.

DISCUSSION

Thioredoxin reductases are homodimeric enzymes that catalyze the NADPH-dependent reduction of cellular thioredoxins (Hirt et al., 2002; Bernal-Bayard et al., 2012). We report here that the full-length NTRC polypeptides extracted from *Arabidopsis* leaves in the presence or absence of DTT (**Figure 9**) exist as homodimers that are resistant to detergent treatment without heating, suggesting that the dimeric structure of chloroplast NTRC is stable without any disulphide bridges between the monomers

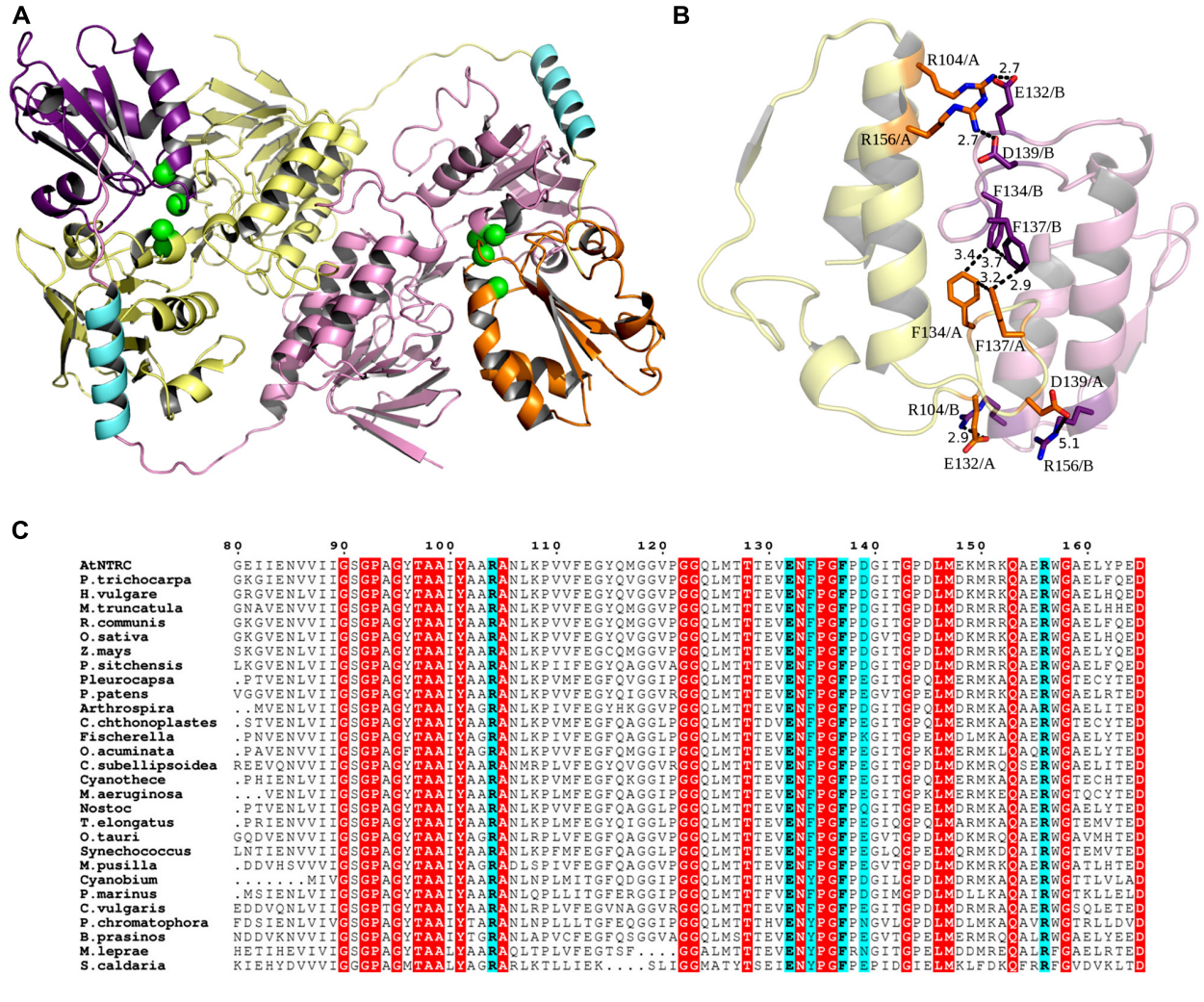

FIGURE 8 | (A) Dimer model of NTRC. In the dimer, the TRX domain (orange and purple) of one subunit binds to the NTR domain (yellow and pink) of the other subunit and, thus, tightens the inter-monomeric interactions. Within one subunit, a long linker region that starts from the C-terminus of the NTR domain and forms a short α-helix (cyan) connects to the N-terminus of the TRX domain, suggesting possible flexibility for the position of the TRX domain. Active site cysteines are shown as green spheres. **(B)** Dimer interactions. In addition to the arrangement of subunits and the linker-mediated interactions, five possible salt bridges are formed between the FAD binding domains. Four of these are shown as sticks in the picture (orange and purple), and three of these are at an optimal distance (~2.7 Å) from each other. Additionally, four phenylalanines in the middle of the interface interact with each other (pi-pi stacking) in a square. Altogether, these special interactions can account for the experimentally observed strong dimeric interactions. **(C)** NTRC like sequences. A part of the multiple sequence alignment of NTRC like sequences from *Arabidopsis thaliana* (UniProtKB O22229), *Populus trichocarpa* (UniProtKB B9H9S9), *Hordeum vulgare* (UniProtKB B0FXK2), *Medicago truncatula* (UniProtKB A6XJ27), *Ricinus communis* (UniProtKB B9SU44), *Oryza sativa Japonica Group* (UniProtKB Q70G58), *Zea mays* (GenBank DAA63960.1), *Picea sitchensis* (UniProtKB B8LPW6), *Pleurocapsa* sp. *PCC 7327* (UniProtKB K9T492), *Physcomitrella patens* subsp. *patens* (UniProtKB A9U311), *Arthrospira* sp. *PCC 8005* (UniProtKB H1WE73), *Coleofasciculus chthonoplastes PCC 7420* (UniProtKB B4VQ87), *Fischerella* sp. *JSC-11* (UniProtKB G6FX47), *Oscillatoria acuminata PCC 6304* (UniProtKB K9TN60), *Coccomyxa subellipsoidea C-169* (UniProtKB I0Z4C2), *Cyanothece* sp. *PCC 7822* (UniProtKB E0U9J8), *Microcystis aeruginosa PCC 7941* (UniProtKB I4GKJ6), *Nostoc* sp. *PCC 7120* (UniProtKB Q8YYV6), *Thermosynechococcus elongatus BP-1* (UniProtKB Q8DHM2), *Ostreococcus tauri* (UniProtKB Q00SZ2), *Synechococcus* sp. *PCC 6312* (UniProtKB K9RZN2), *Micromonas pusilla CCMP1545* (UniProtKB C1N9L2), *Cyanobium* sp. *PCC 7001* (UniProtKB B5INL4), *Prochlorococcus marinus* subsp. *marinus str. CCMP1375* (UniProtKB Q7VB53), *Chlorella vulgaris* (UniProtKB B9ZYY5), *Paulinella chromatophora* (UniProtKB B1X3G8), *Bathycoccus prasinos* (UniProtKB K8EJ46), *Mycobacterium leprae TN* (UniProtKB P46843) and *Spirochaeta caldaria DSM 7334* (UniProtKB F8F2K7) show that the residues forming salt bridges of optimal length (cyan) are mostly conserved between species. Red boxes indicate other conserved residues.

(**Figure 8B**). This conclusion is further confirmed by the observation that the mutation of both Cys residues either in the NTRd or in the TRXd active site does not abolish the formation of NTRC homodimers (**Figure 9**; Pérez-Ruiz et al., 2009). The high conservation rate of the aromatic amino acids and those involved in salt bridges in the inter-monomeric interface of the NTRC domains (**Figure 8B**), suggests that these residues are important for forming stable homodimers. The interlocked arrangement of the NTRd and TRXd together with the NTR-TRX linker region further stabilizes the homodimeric architecture of *Arabidopsis* NTRC. This

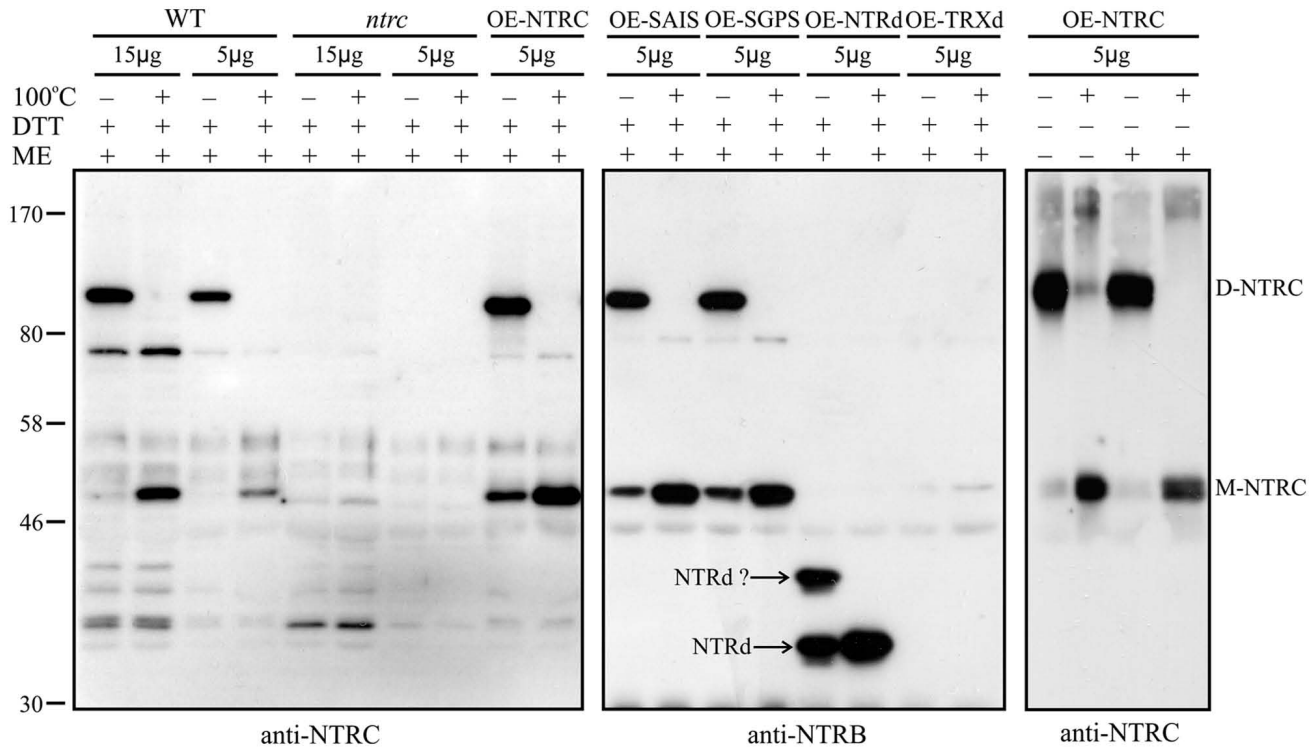

FIGURE 9 | Heat-induced monomerization of NTRC homodimers. Proteins were extracted from leaves in the presence (+) or absence (−) of DTT and solubilized in SDS-buffer with (+) or without (−) mercaptoethanol (ME; Laemmli, 1970) on ice (−) or heated at 100°C for 1 min (+). Amounts of leaf extracts (µg soluble protein, upper panel) were loaded on SDS-gel and the proteins were separated in SDS-PAGE before Western blotting with anti-NTRC antibody or anti-NTRB antibody. D-NTRC and M-NTRC, dimeric and monomeric NTRC proteins, respectively. NTRd?, truncated NTRd with anomalous movement in SDS-gel. For other abbreviations, see the legend for **Figure 3**.

conclusion is supported by the observation that no homodimers were present in transgenic lines overexpressing truncated NTRd (**Figure 9**), although the Y2H test indicated that the NTRd could interact with itself (**Figure 10**). Furthermore, the linker region may facilitate the correct folding of NTRd, since an anomalous form of truncated NTRd was detected in SDS-PAGE (**Figure 9**). The position of the linker is still uncertain, since it could fold on the side of the NADPH-binding domain as in our models (**Figure 8A**) or into the groove in front of the protein as Lee et al. (2012) suggested. The linker placement could also differ depending on the state and conformation of the protein, which is determined by the rotation of the NADPH-binding domain. Similarly to the crystal structure of AhpF (PDB code 1HYU; Wood et al., 2001), which has a short α-helical linker between the NTRd and TRXd, the relatively long linker (∼34 amino acids) in *Arabidopsis* NTRC is likely to form an α-helix as predicted.

The binary Y2H tests showed that the TRX active site of NTRC has to be intact or both Cys residues mutated to Ser in order to support interactions of NTRC polypeptides, since a monothiol form of the TRXd active site repulsed the self-interactions of NTRC polypeptides in Y2H tests (**Figure 10**; NTRC + $NTRC^{CGPS}$, $NTRC^{CGPS}$ + $NTRC^{CGPS}$). The repulsion was not detected when the interaction of $NTRC^{CAIS}$ was tested with $NTRC^{CGPS}$ (**Figure 10**). In this case two thiols instead of three exist in the contacting region of mutated TRXd and NTRd in a dimer that mimics the conditions in wild-type NTRC during reduction of TRXd by NTRd. Furthermore, $NTRC^{CAIS}$ failed to interact with itself, which may be caused by artificial aggregation of $NTRC^{CAIS}$ proteins. $NTRC^{CAIS}$ makes a stable mixed-disulphide with the active site of the TRXd in the second NTRC polypeptide, which may be linked with the third polypeptide by disulphide formation resulting in an oligomerization of polypeptides. Such an aggregated molecule is likely incapable of activating the reporter gene system in yeast cells. Poor complementation of *ntrc* phenotype in *OE-NTRd* and *OE-TRXd* lines further support our conclusion that only full-length NTRC is capable of forming a stable dimeric, functional NTRC enzyme.

NADPH-dependent thioredoxin reductase has been shown to form aggregates *in vitro*, although contradictory conclusions have been drawn on the generation and activity of oligomeric forms of NTRC. Pérez-Ruiz et al. (2009) demonstrated that rice recombinant NTRC protein or NTRC extracted from *Arabidopsis* chloroplasts form oligomeric aggregates that dissociate into the dimeric form in the presence of NADPH, whereas NADPH did not influence the dissociation of barley NTRC aggregates *in vitro* (Wulff et al., 2011). Chae et al. (2013) reported a heat-shock induced aggregation of NTRC and that the oligomeric NTRC acts as a chaperone preventing stress-induced aggregation of chloroplast proteins. They also demonstrated that only homodimeric NTRC has a disulphide reductase activity, while Wulff et al.

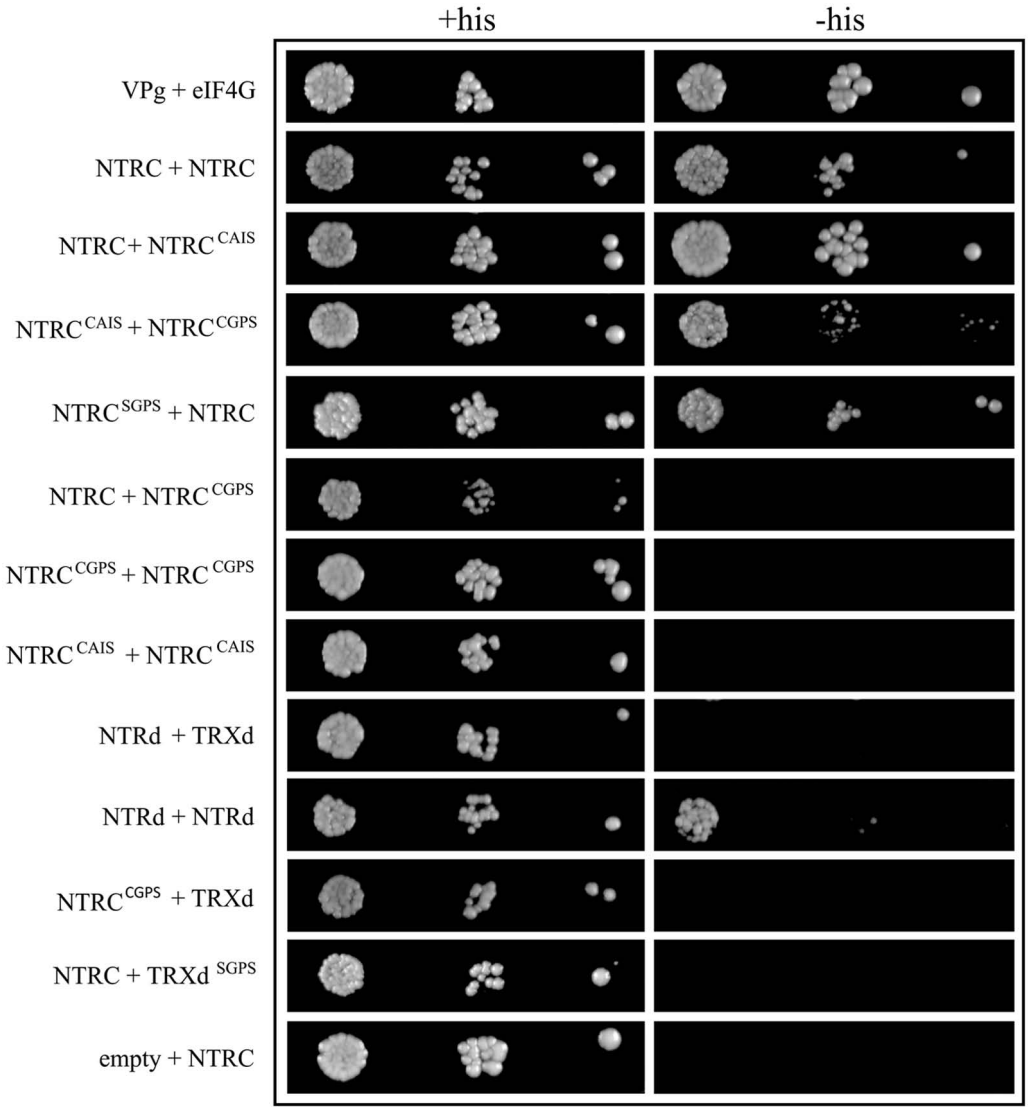

FIGURE 10 | Interactions of the wild-type, mutated, and truncated NTRC in Y2H tests. Yeast CY306 cells were transformed with NTRC, truncated NTRd or TRXd, and with mutated NTRC constructs with both or the second Cys of the redox active site of NTRd (CAIS construct), or TRXd (SGPS and CGPS constructs, respectively) replaced by Ser. The cells were grown into stationary phase and adjusted to an OD_{600} of 0.05, 0.005 and 0.0005 before spotting onto plates containing histidine (+His) or without histidine (-His). Yeast cells were photographed after four days growth at 28°C. VPg + eIF4G and empty vector was used as positive and negative controls, respectively (see Materials and Methods).

(2011) reported that neither reductase nor thioredoxin activity was affected by oligomerization of NTRC. These studies indicate that NTRC has a strong tendency to form oligomeric aggregates but it is still technically difficult to prove, whether oligomeric NTRC is the dominant form of the enzyme *in vivo*. Pérez-Ruiz et al. (2009) demonstrated that stromal fraction of chloroplasts contained oligomeric NTRC aggregates with different masses that mostly dis-aggregated into dimers and monomers in the presence of DTT. We also detected slight smearing of NTRC polypeptides in the SDS-gel, if leaf proteins were extracted in DTT-free buffers (**Figure 9**). Furthermore, it has been shown that heat-shock-induced oligomerization of NTRC depended on the active site cysteines *in vitro* (Chae et al., 2013). These observations suggest that oxidation of thiols in NTRC redox active sites may induce oligomerization during extraction or purification of the NTRC protein.

Overexpression of the full-length NTRC with an inactivated NTRd or TRXd active site in *ntrc* background partially complemented the mutant phenotype of *Arabidopsis* rosettes. Both mutated NTRC constructs promoted growth and recovery of photosynthetic function of transgenic lines. NTRC controls chloroplast biogenesis (Lepistö et al., 2012) and regulates the activity of chlorophyll biosynthesis enzymes (Richter et al., 2013). Greening of the *OE-SAIS* and *OE-SGPS* transgenic leaves suggests that also the mutated NTRC is to some extent capable of promoting chloroplast biogenesis. However, greening of the *OE-SAIS* lines was delayed (**Figure 4**) and these lines accumulated less starch

in light than *OE-SGPS* lines (**Figure 7**), indicating that mutated NTRC with an active NTRd was more efficient in complementing the thiol redox network in chloroplasts than mutated NTRC with an active TRXd. Reduction of free chloroplast TRXs by the NTRd of NTRC would explain the partial recovery of wild-type phenotype of *OE-SGPS* lines. The 3-D model of the NTRC dimer (**Figure 8A**) showed that the TRXd is connected to the NTRd by a long linker region that allows some flexibility for the position of the TRXd. Recently, Bernal-Bayard et al. (2012) suggested that a conformational change takes place in the NTRC dimer after reduction of TRXd active site that allows the reaction between TRXd and its target protein. This structural change also exposes the NTR active site (Bernal-Bayard et al., 2012, see also the location of 2-Cys motifs of NTRC in **Figure 8A**), which may promote the interaction between NTRd of NTRC and free thioredoxins. The Y2H test failed to show any interactions of NTRC or NTRd with TRXd (**Figure 10**). However, Y2H assay might not be suitable to test interactions between dimeric NTRC and truncated TRXd, since the bulky transcription factor domain fused to the N-terminus of NTRd may interfere with the interaction (**Figure 8A**).

To determine whether free chloroplast TRXs are capable of compensating for a non-functioning TRXd in the transgenic *OE-SGPS* lines, the TRXd of NTRC was supplemented by the 10 different chloroplast TRXs (TRXf1, TRXf2, TRXm1, TRXm2, TRXm3, TRXm4, TRXy1, TRXy2, TRXx, and TRXz) in the 3-D model of NTRC (**Figure 11**). Compared to the dimeric NTRC model (**Figure 8**), NTRd with TRXf1, TRXf2 and TRXm1 have only one major difference in the interacting amino acids. This residue is K466 (A-chain) in the TRXd of NTRC, which interacts with Y162 (B-chain) of the NTRd. In the other free TRX models, K466 is replaced by alanine (TRXf1, TRXx), glutamate (TRXf2, TRXm1, TRXm3, TRXy1, TRXy2), aspartate (TRXm2), glutamine (TRXm4), or methionine (TRXz). None of these is able to interact with Y162, due to their shorter length and different properties compared to K466. The models with the TRXd of NTRC replaced by TRXm2, TRXm3, TRXm4, TRXy1, TRXy2, TRXx, and TRXz have at least two additional major differences in the interacting amino acids. Electrostatic surface calculations show that the TRX contact surface on the NTRd of NTRC is strongly negatively charged, and correspondingly, the interaction site on the TRXd of NTRC is strongly positively charged (**Figure 11**). Both TRXf isoforms are similar to the TRXd of NTRC in terms of charge distribution, while the shape of TRXf1 is more similar to the TRXd than that of TRXf2. Therefore, the TRXf isoforms are the most likely candidates for supplementing the inactive TRXd of NTRC. An *in-vitro* test has shown that both NADPH-dependent thiol reductases (NTRC and NTRA) can donate electrons toTRXf1, although the reduction of TRXf was less efficient than the reduction of TRXh3, which is a natural substrate of NTRA (Bohrer et al., 2012). In these *in-vitro* assays, however, the concentration of TRXf1 was 500 times higher than the concentration of NTR enzymes (Bohrer et al., 2012), whereas the content of NTRC protein in the transgenic lines used in this article was ten to forty times higher than in WT line (**Figure 3**). Thereby the high concentration of NTRC-SGPS protein in stroma may facilitate the reduction of TRXf by NTRC that consequently mediates the redox regulation of chloroplast proteins in *OE-SGPS* lines.

Of the other chloroplast thioredoxins, TRXm1 and TRXm2 have a more neutral surface, while TRXm3 has a positively charged patch on the contact area (**Figure 11**). TRXm4, on the other hand, is weakly positively charged, while TRXy1 has a neutral surface and TRXy2 is weakly negatively charged. Hence, these are not likely to compensate for a non-functional TRXd in NTRC. TRXx and TRXz have a positively charged surface and a negatively charged surface, respectively. The negative charge on the surface of TRXz probably prevents it from interacting with the negatively charged surface on the NTRd of NTRC. TRXx, on the other hand, has a very different overall surface shape compared to the TRXd of NTRC, which makes an interaction unlikely.

The recovery of chloroplast development and function in *OE-SAIS* lines demonstrates a presence of a reducing system in chloroplasts, likely FTR, that can donate electrons to the oxidized TRXd of NTRC in the absence of an active NTRd (**Figure 12**). The slightly delayed development and low accumulation of starch in *OE-SAIS* lines may be due to inefficient reduction of the TRXd of NTRC by FTR.

Recently, it has been proposed that the oligomeric NTRC, TRXf and TRXm have foldase (assists protein folding) and holdase (buffers proteins against aggregation) chaperone activities that are independent of the disulphide reductase activity of these proteins (Sanz-Barrio et al., 2012; Chae et al., 2013). Thus the complementation of the mutant phenotype in transgenic lines overexpressing mutated NTRC can be due to the chaperone functions of the oligomeric NTRC and be independent of disulphide reductase activity. Some experimental observations, however, speak against the phenotype of the *OE-SGPS* and *OE-SGPS* lines being largely explained by NTRC chaperone activities. Like discussed in the previous chapter, the proportion of NTRC existing as oligomers *in vivo* is not known. Furthermore, mutation in the redox active cysteines in NTRC likely reduces probability to form oligomers since heat-shock-induced oligomerization of NTRC depended on the active site cysteines *in vitro* (Chae et al., 2013). Neither chaperone activities explain why the complementation of the *ntrc* line with an *OE-SAIS* gene differs from that with an *OE-SGPS* gene (**Figures 4** and **7**). We conclude that chaperone activities of thioredoxins may only partly explain the complementation of *ntrc* line with mutated NTRC constructs.

Overexpression of wild-type *NTRC* in *ntrc* background fully recovered the wild-type structure and function of *Arabidopsis* rosettes. Moreover, high content of NTRC in leaves promoted dry mass production of rosettes, expansion of leaves, and accumulation of starch in light. Stimulation of growth was pronounced by increased growth light intensity. It has been estimated that the concentration of TRXf, TRXm and NTRC is in the range of 0.01–0.1 μM in the stroma (Peltier et al., 2006; König et al., 2012). *OE-NTRC* lines accumulated about twenty times more NTRC in leaves in comparison to WT *Arabidopsis*. Accordingly, the NTRC content in the overexpressing lines was in the range of 1 μM, which is still lower or equals to the estimated concentrations of NTRC-target enzymes in chloroplasts (enzymes in tetrapyrrole and starch synthesis, 2-Cys peroxiredoxins, Peltier et al., 2006). Interestingly, overexpression of TRXf, but not of TRXm increased biomass yield and specific leaf weight and highly stimulated the accumulation of starch and sugars in tobacco leaves (Sanz-Barrio et al., 2013).

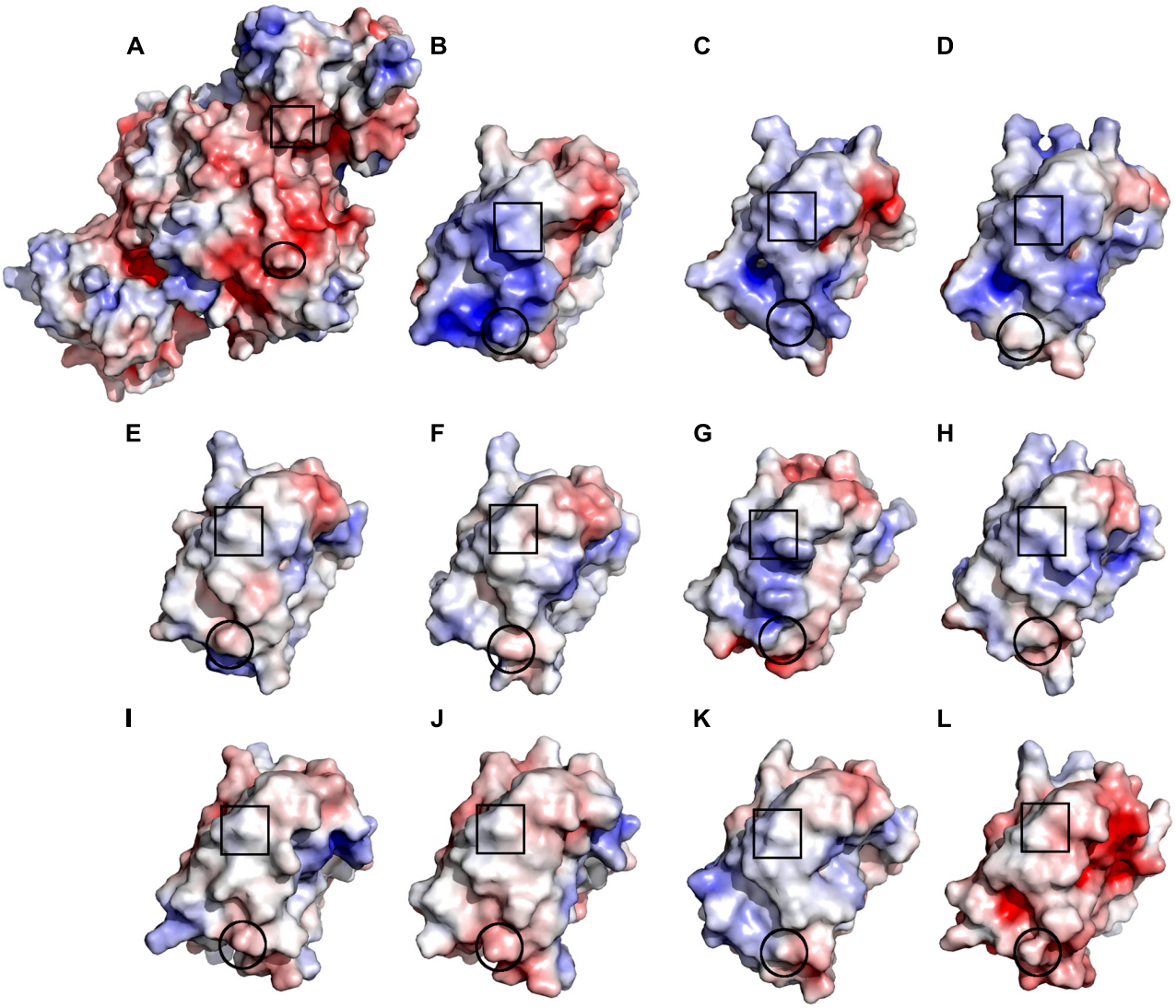

FIGURE 11 | Electrostatic surface of *Arabidopsis* NTRd and TRXd of NTRC and the free chloroplast TRXs. (A) In the flavin reducing conformation, the NTRd of *Arabidopsis* NTRC has a negative surface charge (red) at the TRXd interaction site. **(B)** The corresponding site on the TRXd of NTRC is strongly positively charged (blue), facilitating electrostatic interactions with the oppositely charged surface of the NTRC domain. Ten free TRXs are found in *Arabidopsis* chloroplasts: TRXf1 **(C)**, TRXf2 **(D)**, TRXm1 **(E)**, TRXm2 **(F)**, TRXm3 **(G)**, TRXm4 **(H)**, TRXy1 **(I)**, TRXy2 **(J)**, TRXx **(K)** and TRXz **(L)**. Of these, TRXf1, TRXf2 and TRXm3 share a positively charged patch at the same site as TRXd of NTRC. TRXf1 and TRXf2 have only one amino acid difference in the interaction surface and a similar overall shape, indicating that these could compensate for a non-functional TRXd of NTRC. The active site cysteines are marked by boxes, while Y162 on the NTRd and position 466 on the different Trx forms are circulated. Surface charges were calculated with the APBS tool in PyMOL and the color ranged from −7 to 7.

Like in the *OE-NTRC* lines of *Arabidopsis* the extent of growth stimulation in tobacco plants depended on light intensity. The overexpression of either TRXf or NTRC did not change the steady state rate of photosynthesis in tobacco (Sanz-Barrio et al., 2013) or the chlorophyll content per leaf area in *Arabidopsis* (**Table 2**). Thus the extra sugars in overexpression lines of *NTRC* and *TRXf* are likely used to expand the total leaf area (number and leaf size) per plant, which eventually increases the total photosynthesis per plant and promotes growth. Overexpression lines of *TRXf* and *NTRC* clearly prove that the plant benefits from an increase in thiol reducing systems in chloroplasts.

Specificity of TRXs to their target proteins has been under debate since the discovery of numerous TRX-types in chloroplasts (see Capitani et al., 2000; Buchanan and Balmer, 2005; Meyer et al., 2008). The observation that overexpression of TRXf, but not of TRXm (Sanz-Barrio et al., 2013), increased the biomass yield and sugar content of leaves corroborates the concept that TRXf and TRXm have different target proteins in chloroplasts. It also rules out the hypothesis that growth stimulation of transgenic lines overexpressing thioredoxins is mainly due to the chaperone activities of thioredoxins, since both TRXf and TRXm were reported to function as chaperones in tobacco plants

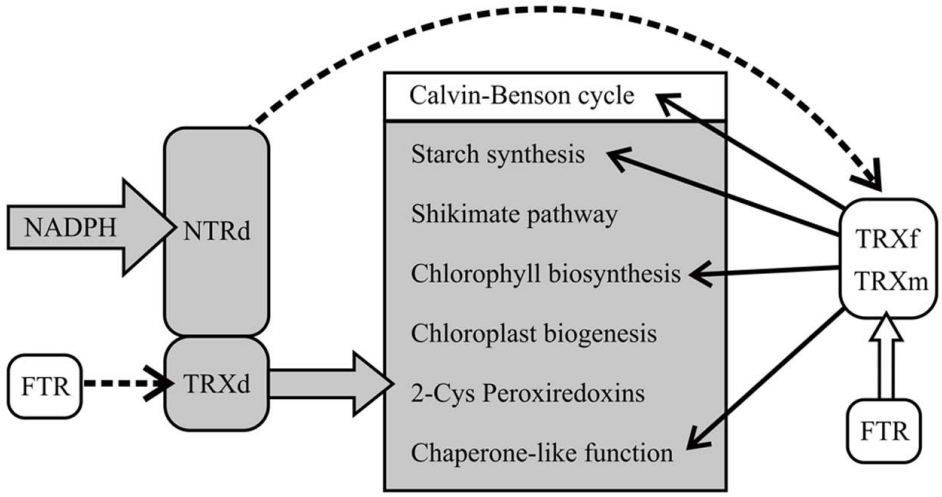

FIGURE 12 | The hypothetical model of NTRC function in transgenic lines overexpressing wild-type and mutated NTRC in chloroplasts. Overexpression of wild-type NTRC in *Arabidopsis* stimulates chloroplast metabolism both by direct reduction of NTRC target proteins by thioredoxin domain (TRXd) of NTRC and indirectly via TRXf function. The mutated NTRC with an intact reductase domain (NTRd) regulates chloroplast metabolism via free chloroplast thioredoxins, preferably TRXf. Ferredoxin-dependent thioredoxin reductase (FTR) may reduce the TRXd in the mutated enzymes with an inactive NTRd. The dashed lines indicate an inefficient reduction of the thioredoxin by the thioredoxin reductase.

(Sanz-Barrio et al., 2012). We demonstrate here that the electrostatic surface of the TRXd of NTRC resembles best the charges in TRXf (**Figure 11**), suggesting that NTRC and TRXf may have overlapping targets in chloroplasts. Indeed, both NTRC and TRXf have been demonstrated to activate ADP glucose pyrophosphorylase, the key enzyme of starch synthesis (Michalska et al., 2009; Thormählen et al., 2013) and the enzymes in chlorophyll biosynthesis pathway (Ikegami et al., 2007; Stenbaek et al., 2008; Richter et al., 2013). However, the growth stimulation in transgenic lines overexpressing NTRC or TRXf is hardly due to the activation of a single metabolic reaction in chloroplast. For example, redox activation of AGPase was not changed in tobacco plants overexpressing TRXf (Sanz-Barrio et al., 2013) and *Arabidopsis* transgenic lines expressing permanently reduced AGPase had an excess-starch phenotype but no stimulation of growth was reported (Hädrich et al., 2011), suggesting that stimulation of starch synthesis alone does not explain the enhanced growth of transgenic lines overexpressing NTRC or TRXf. Thereby, we propose that the increase in biomass yield is due to general promotion of chloroplast development and broader stimulation of carbon metabolism (**Figure 12**). Stimulation of growth by overexpressing TRXf and NTRC is likely based on the control of multiple biosynthetic and protective pathways in chloroplasts. Besides starch and chlorophyll biosynthesis, TRXf is a key regulator for thioredoxin-dependent enzymes in the Calvin-Benson cycle (Buchanan and Balmer, 2005; Meyer et al., 2008). Knockout of NTRC induces production of plastids with anomalous ultrastructure (Lepistö et al., 2012) suggesting that NTRC is essential for the correct biogenesis of chloroplasts. Moreover, NTRC regulates the synthesis of aromatic amino acids (Lepistö et al., 2009; Lepistö, 2011), which are precursors for the biosynthesis pathways of auxin, plant flavonoids and phenolics (Tzin and Galili, 2010). It has also been reported to protect against oxidative stress in chloroplasts (Pérez-Ruiz et al., 2006) and to increase heat-tolerance of plants (Chae et al., 2013), which may facilitate the growth of the *NTRC* overexpression line in high light. Therefore, the overexpression of NTRC may stimulate the biomass yield of plants by overall stimulation of chloroplast biosynthetic reactions, biogenesis of chloroplasts, activation state of the enzymes in the Calvin-Benson cycle via TRXf, the synthesis of aromatic amino acids and the compounds derived from them (including auxin, Lepistö et al., 2009) and finally by protecting against stresses induced by high light intensity (**Figure 12**). The chaperone-like function of chloroplast TRXs (Sanz-Barrio et al., 2012; Chae et al., 2013) may further promote metabolic homeostasis in the chloroplast.

We and Sanz-Barrio et al. (2013) have shown that overexpression of a chloroplast-localized regulatory protein stimulates biomass yield without harmful side effects on plant growth and welfare under controlled growth conditions. If the overexpression of a thiol redox-regulator turns out to permanently stimulate biomass production and the accumulation of primary carbon compounds in plants grown under various conditions, it has a potential to increase biofuel yield in plant and algal species suitable for sustainable bioenergy production.

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Dissecting the integrative antioxidant and redox systems in plant mitochondria. Effect of stress and *S*-nitrosylation

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Mitochondrial respiration provides the energy needed to drive metabolic and transport processes in cells. Mitochondria are a significant site of reactive oxygen species (ROS) production in plant cells, and redox-system components obey fine regulation mechanisms that are essential in protecting the mitochondrial integrity. In addition to ROS, there are compelling indications that nitric oxide can be generated in this organelle by both reductive and oxidative pathways. ROS and reactive nitrogen species play a key role in signaling but they can also be deleterious via oxidation of macromolecules. The high production of ROS obligates mitochondria to be provided with a set of ROS scavenging mechanisms. The first line of mitochondrial antioxidants is composed of superoxide dismutase and the enzymes of the ascorbate-glutathione cycle, which are not only able to scavenge ROS but also to repair cell damage and possibly serve as redox sensors. The dithiol-disulfide exchanges form independent signaling nodes and act as antioxidant defense mechanisms as well as sensor proteins modulating redox signaling during development and stress adaptation. The presence of thioredoxin (Trx), peroxiredoxin (Prx) and sulfiredoxin (Srx) in the mitochondria has been recently reported. Cumulative results obtained from studies in salt stress models have demonstrated that these redox proteins play a significant role in the establishment of salt tolerance. The Trx/Prx/Srx system may be subjected to a fine regulated mechanism involving post-translational modifications, among which *S*-glutathionylation and *S*-nitrosylation seem to exhibit a critical role that is just beginning to be understood. This review summarizes our current knowledge in antioxidative systems in plant mitochondria, their interrelationships, mechanisms of compensation and some unresolved questions, with special focus on their response to abiotic stress.

Keywords: abiotic stress, ascorbate-glutathione cycle, mitochondria, peroxiredoxin, signaling, *S*-nitrosylation, sulfiredoxin, thioredoxin

INTRODUCTION

Plant mitochondria host some of the most important biological processes, i.e, oxidative phosphorylation, citric acid cycle and fatty acid oxidation. Based on their physiological relevance, mitochondria are involved in underpinning cellular proliferation, plant growth, development and death (Millar et al., 2011). Although chloroplasts and peroxisomes are the major ROS producers in plant cells under light periods (Foyer and Noctor, 2003), mitochondrial metabolism significantly accounts for the total ROS generation (Noctor et al., 2007). Overall, complexes I and III of the electron transport chain (ETC) are the main sites of ROS production and about 1–5% of the total consumed oxygen is converted into hydrogen peroxide (H_2O_2; Moller, 2001).

Initially, mitochondrial ROS were considered as an undesirable by product with deleterious effects. Higher ROS amounts resulting from uncontrolled ROS generation can cause oxidative stress by damaging cellular components and affecting organelle integrity. A growing number of publications now recognize the implication of ROS in many other cellular processes, including its proposed role as signaling molecules under oxidative conditions (Dat et al., 2000; Mittler et al., 2011). The condition of signaling molecules implies a tight control of ROS-antioxidants' interplay in the different cell compartments, and the activation of signaling pathways by ROS responsive regulatory genes has been suggested as contributing to plant tolerance toward different stresses (Schwarzländer and Finkemeier, 2013). Therefore, the response of plants to ROS is dose dependent (Veal et al., 2007). Under stress conditions, the presence of ROS is not always a symptom of cellular dysfunction, but rather a signal to modulate transduction pathways through mitogen-activated protein kinases (MAPK) and transcription factors (Jaspers and Kangasjärvi, 2010). In mammals, this signaling process is present in several diseases and shows the crosstalk between multiple transcription factors and the redox-regulating protein Trx (Burke-Gaffney et al., 2005). In plants, a much less studied system, the involvement of Trx in redox signaling is being considered (Zaffagnini et al., 2012b).

Besides ROS, plant mitochondria have also emerged as an important site for nitric oxide production by two main pathways: a mitochondrial nitrite reducing activity whose site of NO• generation remains uncertain (Planchet et al., 2005), and the oxidation of L-arginine by an elusive nitric oxide synthase (NOS; Guo and Crawford, 2005). Formation of ROS in junction with NO• may present a danger in the mitochondria. To maintain the cellular redox homeostasis and avoid an oxidative stress that could cause molecular damage, plant mitochondria possess a set of antioxidant enzymes such as manganese superoxide dismutase (Mn-SOD), enzymes of the ascorbate-glutathione cycle and enzymes of the Trx/Prx/Srx system (Sevilla et al., 1982; Jiménez et al., 1997; Barranco-Medina et al., 2008b). These antioxidant scavengers respond to the stress situations (Martí et al., 2011) by regulating the level of ROS and modulating the redox signaling.

Along with ROS, reactive nitrogen species (RNS) are critical factors in signaling, by working as second messengers. The signaling process can be indirectly exerted by molecules that have suffered the oxidative damage by a reversible change in the redox state. Post-translational modifications (PTMs) of redox cysteine residues of targets proteins constitute a secondary mitochondrial retrograde regulation (MRR) and can modulate ROS and RNS signaling (Hartl and Finkemeier, 2012). Among them, *S*-glutathionylation and *S*-nitrosylation have emerged as novel regulators in cell signaling and response to stress conditions (Zaffagnini et al., 2012a; Camejo et al., 2013a). Protein oligomerization and reversible overoxidation of cysteine residues add a further step into the redox regulation (Barranco-Medina et al., 2009; Iglesias-Baena et al., 2010).

In this work we dissect the different aspects of the redox regulation of plant mitochondria, with special emphasis on the ascorbate-glutathione cycle and Trx/Prx/Srx system under stress.

MITOCHONDRIA ARE ESSENTIAL SOURCES OF ROS AND RNS

Mitochondria are highly dynamic, metabolically active cell organelles. From a functional point of view, ETC in plant mitochondria differs from its animal counterpart in two additional pathways: alternative NAD(P)H dehydrogenases (type II NDH) and alternative oxidase (AOX). Both of these non-proton-pumping pathways could function as "safety valves" to limit ROS production by maintaning the ETC relatively oxidized (Moller, 2001; Rasmusson and Wallström, 2010; Millar et al., 2011). Plants ETC consists of four main complexes, some of them organized into supracomplexes (Dudkina et al., 2006). Supplementary to the NADH dehydrogenase, complex I and the flavoprotein complex II, the inner mitochondrial membrane contain type II NDH that bypass complex I and supply electrons to the ubiquinone pool and do not contribute to the generation of the proton motive force needed for ATP synthesis. Besides the usual cytochrome c oxidase (complex IV), a non-phosphorylating AOX is present. This enzyme bypasses the electron flow from complex III and IV, coupling the oxidation of ubiquinol with the reduction of oxygen to water, dissipating the energy as heat and lowering the ADP/O ratio. Shunting electrons through this pathway is important in energy-rich plants cells for primary and secondary metabolism, as well as for oxidation of excess carbohydrate (Rasmusson and Wallström, 2010). The expression of AOX and type II NDH, both of nuclear encoding, is increased during ETC inhibition by mitochondria to nucleus signaling (Van Aken et al., 2009a; Hartl and Finkemeier, 2012; Leister, 2012). In this process, organellar redox state and ROS metabolism have been poproposed as sources for retrograde signals which could trigger gene expression responses and provide a metabolic flexibility which, during stress conditions, play an important role in the acclimation of plants (Rhoads and Subbaiah, 2007; Woodson and Chory, 2008)

ROS PRODUCTION

A key feature of mitochondrial biochemistry is the unavoidable production of ROS, with complex I and complex III being the major sites (Noctor et al., 2007). Under specific conditions ROS may be produced at complex II site, in the course of reverse electron transport (Turrens, 2003). ROS production is enhanced under conditions of high matrix $NADH^{+}$/NAD. On the other hand, increased membrane potential correlates with more highly reduced ETC components, so raising the probability of single electron leak to oxygen and of $O_2^{\bullet -}$. This superoxide can, in turn, act as substrate for the generation of secondary ROS such as H_2O_2 and hydroxyl radical (•OH). The magnitude of membrane potential is dependent on the activity of the energy-dissipating systems, and on the oxidative phosphorylation. Hence, when ADP is being actively phosphorylated, membrane potential and ROS are lower than when ADP is limiting. Increased energy dissipation can similarly be achieved by artificial uncouplers, uncoupling proteins (UCPs; Moller, 2001; Finkel, 2011; Collins et al., 2012) and by the plant mitochondria potassium channel ($PmitoK_{ATP}$) which can be stress-activated through several mechanisms, including activation by ROS, so indicating the fine regulation of this biochemical pathway. Dissipation of membrane potential directly by these components may be important in tissues with low AOX expression and/or activities (Trono et al., 2004). Similarly, in mammalian, H_2O_2 treatment of myoblast and cardiomyocyte mouse cells, increased the expression of the transcription factor Nrf2 that promoted the expression of the UCP, UCP3 decreasing ROS production and preventing cell death (Anedda et al., 2013).

Reactive oxygen species accumulation in mitochondria could also be influenced by PTM of respiratory complexes (Taylor et al., 2003; Beer et al., 2004), activity of alternative NADPH dehydrogenases (Rasmusson and Wallström, 2010) and modification of antioxidant systems and oxygen concentration (Jiménez et al., 1998). The relative importance of the different factors could be tissue specific (Noctor et al., 2007).

NO• PRODUCTION

In plants, two major enzymatic pathways are proposed to participate in NO• formation: oxidation of L-arginine to L-citruline by a NOS like enzyme and reduction of nitrite to NO• by a nitrate reductase (NR; Neill et al., 2003; Fröhlich and Durner, 2011; Gupta et al., 2011). In the past decade, the presence of NOS-like activity in plant peroxisomes was demonstrated. However, the characterization of such an enzyme is unresolved (del Río et al., 2002). To date, in contrast with mammalian tissue, the production of NO• by a NOS-like enzyme in plant mitochondria remains elusive (Gupta and Kaiser, 2010). The reduction of nitrite to NO• by the mitochondrial ETC contributes to ATP production under

hypoxic conditions. NO• production by a mitochondrial nitrite reducing activity has yet been detected in different photosynthetic sources and mitochondria isolated from roots of diverse plants species. These activities depend on the expression and/or activity of NR, since this enzyme is the main source of nitrite in plants (Wulff et al., 2009). Pharmacological evidences based on inhibitor sensitivity, suggests that complex III, cytochromec oxidase (COX) and AOX are all involved in nitrite to NO• reduction, although a clear mechanism is established only for cytochrome oxidase under hypoxia. However, this may become increasingly important as partial pressures of oxygen are reduced from the ambient level (Gupta and Igamberdiev, 2011).

Nitric oxide can react immediately with superoxide originated from ETC, to form peroxynitrite ($ONOO^-$). Through this reaction, superoxide probably plays a role in regulating free NO• level (Leitner et al., 2009). The protonated form of $ONOO^-$, the peroxynitrous acid ONOOH (pKa 6.8) is involved in many deleterious reactions, such as oxidation of DNA, lipids: protein thiols and iron clusters (Vandelle and Delledonne, 2011). Paradoxically, in systems where the toxicity comes predominantly from more toxic molecules such as peroxides, NO• may elicit protective activity against them (Van Breusegem et al., 2001).

REDOX REGULATION IS AN ESSENTIAL FEATURE OF PLANT MITOCHONDRIAL FUNCTION

Mitochondrial ROS generation can be perpetuated throughout a broad number of reactions yielding different reactive species that serve as substrates for the specific antioxidant enzymes. The mitochondrial antioxidant system, through superoxide and peroxides detoxification, has a pivotal role affecting redox signaling.

Mn-SOD AND ENZYMES OF THE ASCORBATE-GLUTATHIONE CYCLE

Mn-SOD

In plants, Mn-SOD (**Figure 1**) appears as a tetrameric isoenzyme initially purified and characterized in *Pisum sativum* leaves (Sevilla et al., 1982), and located in both, mitochondria and peroxisomes (del Río et al., 1992). Numerous proteins have been identified as being dual targeted, mainly to plastids and mitochondria although around ten-twelve have been described as nuclear and plastidial, or mitochondrial and peroxisomal as Mn-SOD (Duchêne and Giegé, 2012). Mitochondrial and peroxisomal Mn-SOD expression is regulated differently in processes like leaf senescence, where post-translational events may regulate the enzymatic activity of the peroxisomal enzyme (del Río et al., 2003; Palma et al., 2006). Mn-SOD is important in providing protection against oxidative stress in these organelles, so avoiding the formation of more dangerous •OH radicals and controlling H_2O_2 production. Defects in mitochondrial function are associated to a large number of different phenotypes. It has been reported that the lack of mitochondrial SODs in *Caenorhabditis elegans* mutants, in contrast to that reported in yeast or animals (Kirby et al., 2002), reduces not longevity but growth (Van Raamsdonk and Hekimi, 2009). In this case, a reduction in the metabolic energy observed could afford different explanations like the reported induction of uncoupling mechanisms, which reduced ROS generation in mitochondria, the decrease of the membrane potential and/or activity of the ETC. A similar reduction in growth has been described for Mn-SOD mutants in plants; in this case the respiration rate was not affected but the mitochondrial redox balance and some of the tricarboxylic acid (TCA) cycle enzymes were altered. Unexpectedly, Mn-SOD mutants displayed an increased antioxidant capacity, suggesting the existence of a retrograde pathway trying to compensate the lack of this antioxidant enzyme (Morgan et al., 2008). Reduction in growth is a general phenotypic characteristic in mitochondrial dysfunction and it may exhibit the interconnection established between mitochondrial metabolism and photosynthetic carbon assimilation. A complementary hypothesis has adduced the crosstalk between redox signaling and hormonal pathways regulating growth inhibition (Schwarzländer and Finkemeier, 2013).

ASC-GSH cycle

As a result of the $O_2^{\bullet-}$ dismutation, the newly formed H_2O_2 can be decomposed by the mitochondrial peroxidase activities dependent on the antioxidants: (I) ascorbate (ASC) for the hemo-containing enzyme ascorbate peroxidase (APX; **Figure 1**), (II) the thiol reductant glutathione (GSH) for the glutathione peroxidases (GPX) and (III) the thioredoxin/peroxiredoxin system (Trx/Prx). The generated oxidized forms of ASC are then reduced by the FAD-containing monodehydroascorbate reductase (MDHAR) in an NAD(P)H-dependent manner and dehydroascorbate reductase (DHAR) using GSH as electron donor. Oxidized GSSG is reduced by the flavoprotein glutathione reductase (GR) and oxidized by thioredoxin reductase (NTR), both in an NADPH-dependent manner (Noctor and Foyer, 1998; Barranco-Medina et al., 2007; Martí et al., 2009). Accordingly, the antioxidant and redox systems in mitochondria depend on an adequate supply of NAD(P)H that is maintained by transhydrogenases in the mitochondrial membrane, as well as the enzymes isocitrate dehydrogenase and malate dehydrogenase in the matrix (Rasmusson and Moller, 1991).

The first publications reporting the presence of the some components of the so-called ASC-GSH cycle in mitochondria (**Figure 1**) appeared in 1981 and 1990 with MDHAR and GR of potato and pea mitochondria, respectively (Arrigoni et al., 1981; Edwards et al., 1990). The final proof of principle of a complete cycle in plant mitochondria, similar to that in chloroplast (Foyer and Halliwell, 1976), was later described in pea leaves (Jiménez et al., 1997). Using enzymatic latency assays, APX activity was located outside of the inner mitochondrial membrane whereas MDHAR was highly latent in intact mitochondria and was membrane-bound. These findings suggested that the electron acceptor and donor sites of this redox protein are not on the external side of the mitochondrial membrane. DHAR and GR were found in the mitochondrial matrix and the antioxidants ASC and GSH were present as demonstrated by chromatographic techniques. Biochemical data also indicated that the mitochondrial APX activity resulted in at least two isoezymes with different substrate specificity and sensibility to inhibitors when compared to that found in peroxisomes and chloroplasts (Jiménez et al., 1998). The possible presence of the isoenzymes linked to the inner face of the external membrane was described by Chew et al. (2003). The membrane location of APX and MDHAR suggested a dual complementary function for both enzymes: they could reoxidize endogenous NADH to maintain a constant supply of NAD^+ for

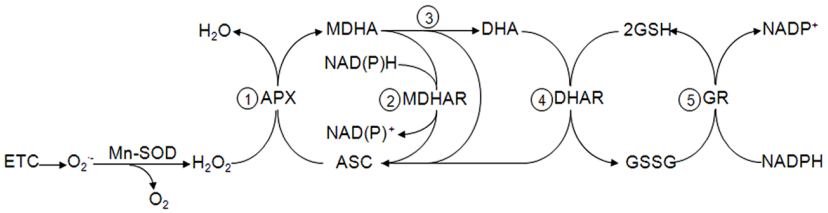

FIGURE 1 | Mitochondrial ascorbate-glutathione cycle. The hydrogen peroxide in the mitochondria produced by ETC is reduced by APX at the expense of ASC to produce MDHA (step 1) that is either reduced to ASC (step 2) or disproportionated to DHA and ASC (step 3). DHAR reduces DHA using GSH as electron donor (step 4), which is regenerated by GR and NADPH (step 5).

mitochondrial metabolism (Douce et al., 2001) and protection against H_2O_2 (del Río et al., 1998; Chew et al., 2003). Thus, both enzymes also contribute to the signal transduction processes that lead to specific gene expression by regulating the mitochondrial and cytosolic concentration of the diffusible signaling molecule H_2O_2 (del Río et al., 1996). The presence of the ASC-GSH cycle in nitrogen-fixing legumes root nodules has been proved as well as its protective activity toward mitochondrial-derived radicals in sensitive spots like the hemo o leghemoglobin groups (Puppo et al., 2005).

Pea GR and *Arabidopsis* MDHAR were described as dual-targeted proteins in plant cells (Creissen et al., 1995; Obara et al., 2002). In *Arabidopsis*, two genes encode GR, called GR1 encoding a cytosolic and peroxisomal protein, and GR2, found in chloroplast and mitochondria, which is lethal when it is inactivated at an early stage of embryo formation (Meyer et al., 2012). Using biochemical, targeting and proteomic assays, the presence of the ASC-GSH cycle was corroborated in mitochondria of *Arabidopsis* by Chew et al. (2003). These authors proposed an integrative coordination between chloroplast and mitochondria through the dual targeting of proteins such as APX, MDHAR, and GR gene products to both organelles, while DHAR only had a mitochondrial localization. It was postulated that the coordination between plastids and mitochondria might occur by the dual targeting rather than subtle retrograde signaling (Millar et al., 2001; Chew et al., 2003).

The presence of one isozyme of APX on the intermembrane space side of the inner membrane is convenient for the use of the ASC generated in this location. ASC is produced by the terminal enzyme L-galactono-1,4-lactone dehydrogenase (GalLDH) also attached to the inner membrane and located in the mitochondrial complex I, and its presence is required for the stability of the complex (Pineau et al., 2008). GalLDH activity is highly dependent on the availability of oxidized cytochrome c from the mitochondrial respiratory chain and is also regulated by redox controls such as glutathionylation (Bartoli et al., 2000; Millar et al., 2003; Leferink et al., 2009). In addition to the reductive GSH dependent DHA reduction, ASC regeneration may also be attributed to the respiratory ETC (Szarka et al., 2007) or linked to other redox compounds as glutaredoxin (Grx) and Trx systems (Potters et al., 2002; Meyer et al., 2012).

Levels and redox state of ASC have been shown to be involved in the modulation of photosynthesis by mitochondrial metabolism and a complementation has been suggested between AOX pathway and ASC to protect photosynthesis against photoinhibition. Respiration-dependent changes in mitochondrial ASC synthesis could regulate retrograde signaling as a common signal from both mitochondria and chloroplasts (Talla et al., 2011). A good example of such an inter-organelle communication is the ASC produced in the mitochondria and then transported into the apoplast. In contrast to GSH, ASC appears to exert its greatest influence by setting thresholds for apoplastic and cytoplasmic signaling (Munné-Bosch et al., 2013).

The second abundant antioxidant in plant tissues is the thiol compound GSH, participating in the detoxification of ROS, heavy metals and xenobiotics and in the cell cycle regulation (Rouhier et al., 2008; Foyer and Noctor, 2009; Diaz Vivancos et al., 2010). GSH is synthesized in plastids and cytosol and then transported to mitochondria, although the nature and regulation of these transporters is still unclear. The dicarboxylate/2-oxoglutarate transporter in the inner mitochondrial membrane has been proposed as a potential candidate, as reported in animals (Wilkins et al., 2012). Immunolabelling studies have proved the presence of GSH in both, mitochondria and chloroplast containing about 15–25% and 62–75% respectively of the total pool of GSH (Fernández-García et al., 2009).

Under non-stress conditions, GSH is presented mainly in its reduced form, but stress conditions and/or senescence and detoxification of ROS can lead to its oxidation impacting in the cellular redox state (Jiménez et al., 1998; Vanacker et al., 2006; Noctor et al., 2012). GSH is also emerging as a player in the intracellular redox potential regulation, protection and signaling through PTMs such as glutathionylation of specific target proteins (Zaffagnini et al., 2012b) involving Cys residues. A link between complex I (CI) activity and GSH has also been shown in CI *Arabidopsis* mutants insensitive to a GSH biosynthesis inhibitor and with higher levels of GSH, implying an as yet unexplained effect of mitochondrial respiration on GSH homeostasis (Koprivova et al., 2010).

Mn-SOD, AOX AND ENZYMES OF THE ASCORBATE-GLUTATHIONE CYCLE IN STRESS RESPONSE AND SIGNALING

Abiotic stress can produce contradictory effects depending on the specie, tissue analyzed and the developmental stage of the plant. The plant acclimation also depends on the application time and strength of the treatment. Mitochondria are central organelles in setting cellular redox balance and homeostasis (Noctor et al., 2007). Increased ROS production in the mitochondria along with

the antioxidant defense orchestrating the cellular stress response, including salinity, has been well documented (Hernández et al., 2000; Mittova et al., 2003; Taylor et al., 2009). ROS production in mitochondria has been reported to increase under salinity and drought conditions. A stimulation of $O_2^{\bullet-}$ generation dependent on NADH- and succinate has been reported in plants under salinity, with a higher increase in sensitive cultivars than in tolerant plants (Hernández et al., 1993; Pastore et al., 2007). Furthermore, oxidative damage induced by NaCl stress can affect different cellular targets selectively: complex I of the ETC was found to be damaged via oxidative stress while complex II directly by salt (Hamilton and Heckathorn, 2001). In this context, changes in ROS levels caused by the perturbation of the respiratory complex I: have been proposed to trigger a mitochondrial retrograde signal (Rhoads and Subbaiah, 2007).

The adaptive response of plants induced by salt stress is well documented; in *Arabidopsis*, of 300 salt stress-induced genes, more than half had a predicted mitochondrial localization (Heazlewood et al., 2007). In general, an induced expression of antioxidant defense genes is usually correlated with enhanced salt stress tolerance (Hernández et al., 2000; Attia et al., 2008) although the molecular mechanisms involved in the regulation of this induction remains unrevealed (Foyer and Noctor, 2005). Moreover, changes at a transcript level did not usually correlate well with changes in protein responsive to stress, and post-transcriptional mechanisms are believed to play an important role in defining the mitochondrial stress response (MSR; Van Aken et al., 2009b).

Alternative oxidase in one of the components of MSR and has been used as a model system to study MRR (Van Aken et al., 2009b). *Arabidopsis AOX1a* mutant plants have been described as exihibiting altered antioxidant transcripts of both chloroplasts and mitochondria, when exposed to a combination of drought and light stress (Filipou et al., 2011). Interestingly, the ABI4 transcription factor involved in the chloroplast-nucleus signaling is responsible for the transcriptional regulation of *AOX1a* (Giraud et al., 2009). Transcripts encoding AOX genes, mainly *AOX1a* and *AOX1d*, are highly responsive to stress including salinity. In fact, plants constitutively over-expressing *Ataox1a*, with increased AOX capacity, showed lower ROS formation and improved growth in salinity conditions (Smith et al., 2009). Yet, discrepancies in AOX expression and *in vivo* activity have also been reported, and recently discussed (Ribas-Carbó et al., 2005, Rasmusson et al., 2009). Overall, the current knowledge in AOX attributes it an important role in stress adaptation in plants while its participation in cell re-programming under salinity stress has been proposed (Clifton et al., 2006).

Mitochondrial Mn-SOD, has also been reported to modulate its expression in response to salinity stress (Kaminaka et al., 1999; Dai et al., 2009; Rubio et al., 2009), undergoing an overexpression in tolerant cultivars while decreasing in salt sensitive ones (Hernández et al., 2000). This seminal observation has gained additional support by the fact that the overexpression of Mn-SOD in transgenic *Arabidopsis*, poplar, rice and tomato plants showed increased salt tolerance (Tanaka et al., 1999; Wang et al., 2004, 2007, 2010a). The study of the changes in Mn-SOD protein revealed that, in tolerant pea plants, this protein was maintained with the duration of the salt treatment (Camejo et al., 2013a), while proteomic studies have shown that Mn-SOD of *Arabidopsis* accumulated during NaCl stress (Jiang et al., 2007). Also, mitochondria from salt-tolerant and salt-sensitive wheat cultivars subjected to salinity stress showed augmented levels of Mn-SOD and AOX, along with changes in cysteine synthase required for GSH formation. The coordinated increase in Mn-SOD and AOX proteins is thought to prevent the over-reduction of the mitochondrial ubiquinone pool, so lowering the content of superoxide in this organelle. The marked overexpression of these enzymatic systems responds to the specific adjustment of the cells in response to the oxidative stress. The fact that the vast majority of the non-redox proteome remained unchanged under saline stress strengthens this hypothesis (Jacoby et al., 2010). These authors suggest that the differences in proteomes of wheat varieties correlated with whole-plant salinity tolerance.

The heterogeneity of the antioxidant systems response under stress is manifested in numerous occasions. Each isoform of the same antioxidant enzyme in the different cell compartments can present a specific profile activity in lines/cultivars differing in salt tolerance (Olmos et al., 1994; Ashraf, 2009). A correlation between expression, protein and activity levels is usually found for Mn-SOD. Salt-tolerant tomato, pea and wheat cultivars have shown higher activity of mitochondrial Mn-SOD compared with a salt-sensitive cultivar (Hernández et al., 1993; Sairam and Srivastava, 2002; Mittova et al., 2003). This is not the case of peroxisomal Mn-SOD isoform since it was not induced in response to salt stress either in the tolerant or in the sensitive pea plants (Corpas et al., 1993).

As previously noted, GSH and ASC have a strong influence in gene expression (Munné-Bosch et al., 2013). The balance of reduced to oxidized forms of both antioxidants is crucial for the cell to sense oxidative stress and to respond accordingly (Mullineaux and Rausch, 2005; Foyer and Noctor, 2009, 2011). Consequently, the ASC/GSH pathway plays an essential role to cope the oxidative stress imposed by environmental stress including salinity (Hernández et al., 2000, 2001; Pallanca and Smirnoff, 2000; Gómez et al., 2004; Sharma and Dubey, 2005; Hefny and Abdel-Kader, 2009; Noctor et al., 2012). The existence of balance mechanisms to maintain ASC and/or GSH-dependent processes and related signaling response in specific compartments, when their respective contents are depleted, has been well established (Foyer and Noctor, 2011). In contrast, information on mitochondrial ASC and GSH contents and redox state is scarcely reported and their accurate role in mitochondria under abiotic stress is not well stated.

Information on the enzymes responsible to maintain and regulate the reduced/oxidized state of mitochondrial ASC and GSH, shows that the regulation of their gene expression presents high plasticity, and is an important component in the response of plants to stressful conditions.

The expression of *APX* encoding genes is modulated by various environmental stimuli, such as drought and salt (Hernández et al., 2000; Menezes-Benavente et al., 2004; Gill and Tuteja, 2010; Bonifacio et al., 2011). Very scarce information has been published on the APX mitochondrial isoform. In mitochondria from *Oriza sativa*, Δ*OsAPX6* expression remained unchanged against

salt stress (Teixeira et al., 2006), while other works have reported an induction for the same isoenzyme in rice (Yamane et al., 2010). The discrepancy in regulation for this and other *APX* genes might be due to the absence of standardized conditions of measurements, since each group used different cultivars, organs, plant age and growth conditions which, as related above, have an important contribution in plant stress response. The beneficial effects of APX have been documented in plants overexpressing this enzyme in chloroplast, peroxisomes and cytosol displaying an enhanced plant tolerance to salt and water deficit and ameliorating induced-oxidative injury (Badawi et al., 2004; Lu et al., 2007; Wei-Feng et al., 2008). A compensatory mechanism in rice mutant double silenced for cytosolic APXs by other antioxidant enzymes has been described, making the mutants able to cope with salt, heat, high light and methyl viologen stress, similar to non-transformed plants (Bonifacio et al., 2011).

Enzyme activity comparisons have proved that mitochondrial APX and GR are constitutively higher in salt-tolerant wheat cultivar than in sensitive plants although none responded to salinity (Sairam and Srivastava, 2002). This response was different in mitochondria from tolerant pea plants, in which APX and MDHAR activities appeared early increased at mild salt stress and progressively increased under high salt concentrations, whereas GR and Mn-SOD were induced only after severe salinity. In chloroplasts and peroxisomes, these isoenzymes responded differently than in mitochondria, although stromatic APX, but not thylakoidal, was significantly and progressively increased, together with DHAR in response to the severity of the salt stress (Corpas et al., 1993; Gómez et al., 1999, 2004). The study in tomato revealed a decreased oxidative stress in a tolerant salt cultivar which, in part, was attributed to induced activities of Mn-SOD and mitochondrial APX, as previously commented in pea, as well as to increases of both ASC and GSH content in mitochondria, by a yet-unexplained mechanism (Mittova et al., 2004). Scarce information exists on the possible relation of these activities with the mitochondrial MDHAR and DHAR expression. Nonetheless, a compensative overexpression of different cytosolic and chloroplastic MDHAR and DHAR can enhance plant tolerance against various abiotic stresses (Gill and Tuteja, 2010) including salinity in tobacco, potato and *Arabidopsis* (Eltayeb et al., 2007, 2011; Wang et al., 2010b).

Similarly, the regulation of the GR has been proved to efficiently respond to different stresses (Creissen et al., 1994). A cytosolic GR gene was found induced in a pea salt tolerant, but not in the salt-sensitive, cultivar (Hernández et al., 2000) and the induction of the symplastic GR activity was higher in the tolerant plants, at the same time as increased DHAR and MDHAR activities. A putative role for all these enzymes in the control of symplastic/apoplastic ASC content was described (Hernández et al., 2001). The overexpression of GR has been shown to improve tolerance to oxidative stress, leading in tobacco and poplar to a higher ASC content in leaves (Aono et al., 1993; Foyer et al., 1995).

All together, these results suggest a fine-tuning for chloroplasts and mitochondrial signaling mechanisms to coordinate the response of these antioxidant enzymes for the acclimation of plants to salinity conditions.

Trx/Prx/Srx SYSTEM

Thioredoxins

Thioredoxins (Trxs) are ubiquitous small proteins involved in the reduction of disulphide bonds of other proteins through a dithiol-disulfide exchange. They have a conserved active site WCG/PPC with reductive properties to regulate specifically target proteins. Plants, unlike bacteria and animals, contain several nuclear encoded *Trx* genes. In *Arabidopsis thaliana*, at least 20 *Trx* genes have been reported with different location (Collin et al., 2004; Meyer et al., 2012). The presence of Trx in plant mitochondria was shown by Laloi et al. (2001) in *Arabidopsis*, and was classified as Trx*o* type (AtTrx*o*1), although an additional mitochondrial *h*-type Trx was also localized in poplar (Gelhaye et al., 2004). More recently, a pea Trx*o*1 was described in both mitochondria and nucleus under normal conditions (Martí et al., 2009) while the localization of a nuclear Trx type *h* had been shown only under oxidative conditions in germinating wheat seeds (Serrato et al., 2001; Serrato and Cejudo, 2003; Pulido et al., 2009). Mitochondrial and cytosolic Trxs are reduced by a homodimeric FAD-NTR that utilizes NADPH (**Figure 2**), while chloroplastic ones use a ferredoxin-NTR system (Gelhaye et al., 2005). Two genes encoding NTR have been found in *Arabidopsis*: *AtNTRB*, which expresses the mitochondrial form and *AtNTRA*, expressing the cytosolic one (Reichheld et al., 2005; Tovar-Méndez et al., 2011). A new NADPH NTR (NTRC) has been demonstrated to exist in chloroplasts and non-photosynthetic plastids (Serrato et al., 2004; Kirchsteiger et al., 2012). NTRC have both, NTR and Trx, domains in the same polypeptide chain and reduces chloroplast 2-Cys Prx without the assistance of Trx (Pérez-Ruiz et al., 2006; Pulido et al., 2010). As far as we know, the high abundance of different Trx types in the cell as well as the redundant coexistence of different Trxs within the same organelle may reflect the presence of differential redox pathways for each. The specific function, protein–protein interaction and redox-network implication for the cited Trxs is far from being elucidated. Moreover, the high diversity in plants Trxs when compared with humans might add an additional antioxidant support in plants.

Although the extensive research in the last two decades has revealed diverse aspects of Trxs in plants, very little is known about the mitochondrial Trx function. It has been suggested that it is related to mitochondrial redox regulation and AOX (Balmer et al., 2004; Gelhaye et al., 2004; Martí et al., 2009; Yoshida et al., 2013) and, the detoxification of ROS via a mitochondrial PrxIIF has also been proposed (Barranco-Medina et al., 2008b). Application of the mutant affinity column approach by using cytoplasmic or

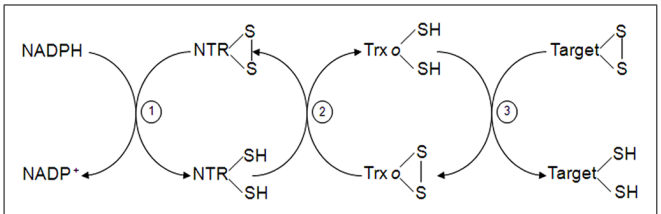

FIGURE 2 | Trx system in mitochondria. Mitochondrial Trx*o* is reduced by NADPH-dependent TR (steps 1 and 2). Reduced Trx*o* can reduce in turn mitochondrial target proteins (step 3).

chloroplastic forms of mutated Trxs, led to a systematic screening of Trx targets and thus, Balmer et al. (2004) were able to identify 50 potential Trx targets in mitochondria that covered major metabolic pathways. However, mutant PsTrx*o*1C37S in a proteomic assay with pea mitochondria only identified nine potential PsTrx*o*1 targets (Martí et al., 2009). Among the PsTrx*o*1-linked proteins there are components of the glycine decarboxylase complex and serine hydroxymethyl transferase (SHMT), key enzymes in photorespiration, and the alpha-subunit of the mitochondrial ATP synthase, which links Trx*o*1 with the control of ATP synthesis. Besides, the elongation factor Tu, that promotes the GTP-dependent binding of aminoacyl-tRNA to the ribosome, thiosulfate sulfurtransferase, mercaptopyruvate sulfurtransferase involved in sulfur metabolism and the drought stress related short-chain alcohol dehydrogenase were also identified.

Biochemical characterizations have reported that PsTrx*o*1 is able to activate two additional enzymes, the antioxidant PrxIIF (see below) and the respiratory enzyme AOX (Martí et al., 2009). Recently, Yoshida et al. (2013), using a similar methodology have found 101 Trx targets in mitochondria. Among them, the enzymes cited before have also been reported. A more detailed confirmation analysis by additional approaches is required to evaluate all these proteins as "true" targets, helping to understand the *in situ* functional significance of these Trx–target interactions.

Alternative oxidase has been identified in *Arabidopsis* as a protein of the inner mitochondrial membrane with an intramolecular disulfide bond (Winger et al., 2007). This protein is encoded by a small gene family, whose members have been shown to be both tissue-and development specific. AOX has not been identified as a Trx target using Trx-linked resins, although it can be both reduced and activated by mitochondrial thioredoxin PtTrx*h*2 by using its effector pyruvate (Gelhaye et al., 2004; Umbach et al., 2006). Similarly, PsTrx*o*1 specifically reduced pea mitochondrial AOX homodimers and produced the activation of oxygen consumption by this AOX pathway, using a NADPH/NTR system (Martí et al., 2009). Our comparative study of the published literature reveals the higher ability of PsTrx*o*1 to activate AOX in pea mitochondria compared with that in soybean organelle, presenting NADPH/NTR/PsTrx*o*1 as a highly effective system in the activation of the AOX pathway in pea. As reported, AOX plays an important role in preventing or minimizing ROS formation in cells (Maxwell et al., 1999; Yip and Vanlerberghe, 2001; Millar et al., 2011). Thus, we hypothesize that Trx*o*1, through the control of the reduced levels of AOX, might regulate respiratory metabolism and associated reactions. Trx*o*1 through activation of PrxIIF and AOX, could also play a role in linking ROS and redox signaling in mitochondria.

Several proteins have a dual localization to mitochondria and nucleus (Duchêne and Giegé, 2012) and a signaling function for mitochondrial biogenesis has been speculated. In pea leaves, PsTrx*o*1 was also found in nuclei with an apparent molecular mass of 20.6 kDa, corresponding to the protein translated and driven to the nuclei without the removal of the mitochondrial N-terminal targeting signal (Martí et al., 2009). Some plant Trxs have been found in the nucleus under stress conditions, i.e., Trx h typically located in the cytosol, has been reported to accumulate in the nucleus of aleurone and scutellum cells during germination (Serrato and Cejudo, 2003; Pulido et al., 2009). The function of PsTrx*o*1 in the nucleus is unknown although could be related to transcriptional regulation through oxidation protection of heterochromatin as proposed for the mammalian PRDX5 (Kropotov et al., 2006), the regulation of activity of several transcription factors (Hirota et al., 1997) and/or the control of apoptosis signal-regulated kinase 1 activity (Saitoh et al., 1998). Further studies seeking to identify functional targets for PsTrx*o*1 in the nucleus are needed to learn more about new physiological roles of this Trx*o*1 in plant cell.

Peroxiredoxins

Peroxiredoxins (Prxs) are thiol-based peroxidases involved in peroxide detoxification and play an important role in signaling (Wood et al., 2003). Prxs share a common catalytic mechanism where, by reducing peroxide, the catalytic active site Cys is oxidized to a sulfenic acid, which then forms a disulphide bond with a resolving Cys that is reduced by the Trx-NTR and NADPH system. Prxs reduce hydrogen peroxide and alkyl hydroperoxides to water and the corresponding alcohol, respectively. They were initially identified in yeast (Kim et al., 1988) and then in mammals and humans, with six different human Prxs (PrxI-VI) grouped in three types (Chae et al., 1994). The presence of plant Prxs was first discovered by Baier and Dietz (1996) and their classification does not correspond with the nomenclature established for human Prxs. Plant Prxs are divided into four subgroups based on the number and position of the conserved cysteine residues, namely 2-Cys Prx, type II Prx, Prx Q, and 1-Cys Prx, with different subcellular locations.

Type II Prxs are dimeric enzymes with varying molecular mass, isoelectric points and subcellular localization and have been proposed as primary sensors for hydrogen peroxide (Rhee et al., 2005). They were discovered in mammalian as a type of Prxs that forms an intramolecular disulfide as a reaction intermediate. In mammals, only one type II Prx (Prx V), with mitochondrial localization, has been found (Seo et al., 2000). In plants, three type II Prxs have a cytosolic (PrxIIB, C and D), one a chloroplastic (PrxIIE) and one a mitochondrial localization (PrxIIF; Horling et al., 2002). Plant mitochondrial PrxIIF is highly conserved between different species and contains the two cysteine-residues characteristic of type II Prx at positions 59 (peroxidatic Cys) and 84 (resolving Cys) of the mature protein (Finkemeier et al., 2005; Barranco-Medina et al., 2007).

While the disulfide bridge formed in typical 2-Cys Prx, after hydroperoxide reduction, is intermolecular, in atypical type II Prx it is intramolecular (Seo et al., 2000). The catalytic cycle of PrxIIF consists of three steps (**Figure 3**): (1) the nucleophilic attack of the peroxide by the conserved peroxidatic Cys (Cys-S_PH) that is oxidized to sulfenic acid (Cys-S_POH), (2) the formation of the disulfide by attack of the free thiol of the resolving Cys (Cys-S_RH) to release water, and (3) the regeneration of the thiol form by mitochondrial Trx*o,* Grx and GSH as electron donors (Finkemeier et al., 2005; Gama et al., 2007; Barranco-Medina et al., 2008b). Rouhier et al. (2002) proposed a reaction mechanism for a cytosolic type II Prx from poplar in which only one of the two cysteinyl residues is involved in catalysis. Furthermore, Barranco-Medina et al. (2007), using two mutated variants, demonstrated that both Cys residues are essential for efficient catalysis. The interaction between Trxo

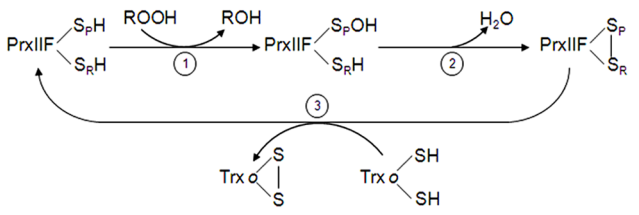

FIGURE 3 | Reaction mechanism of mitochondrial PrxIIF. PrxIIF is oxidized to its sulfenic form in the reduction of peroxides (step 1). PrxIIF-SOH forms an intramolecular disulfide bridge (step 2) that is reduced by the mitochondrial Trx*o* system (step 3).

and PrxIIF has been demonstrated with recombinant proteins and using C36S Trx*o* variant (Barranco-Medina et al., 2008b; Martí et al., 2009). The catalytic efficiency of plant PrxIIF (Rouhier and Jacquot, 2005; Barranco-Medina et al., 2007) is significantly higher than of 2-Cys Prx (König et al., 2003; Bernier-Villamor et al., 2004).

Structural studies of atypical Prxs have shown that PrxIIF dimerizes like typical 2-Cys Prx, but its dimerization is based on A-type, instead of B-type, interfaces (Echalier et al., 2005; Karplus and Hall, 2007). Moreover, the presence of high molecular weight species has been established (Evrard et al., 2004). Unlike 2-Cys Prx, that occurs as decamers, pea mitochondrial PrxIIF crystallizes as hexamers (Barranco-Medina et al., 2006) which are favored in oxidant conditions, but dissociate to dimers upon reduction (Barranco-Medina et al., 2008b). The presence of peroxidatic Cys was critical for hexamer formation whereas substitution of resolving Cys did not impact the oligomeric pattern (Barranco-Medina et al., 2007). By analogy with the dimer-decamer transition of the typical 2-Cys Prx (König et al., 2002; Wood et al., 2003; Bernier-Villamor et al., 2004; Barranco-Medina et al., 2008a, 2009), the dimer-hexamer transition in atypical PrxIIF displays a functional switch that could be involved in signaling (Barranco-Medina et al., 2008b).

Mitochondrial PrxIIF was one of the last identified antioxidants to be discovered in this organelle with functions in the reduction of hydrogen peroxide, playing also a chaperone-like activity (Finkemeier et al., 2005; Barranco-Medina et al., 2008b). In spite of the fact that mitochondria are one of the major sites of ROS generation in plant cells, and in contrast to other cellular compartments, PrxIIF is the only Prx type present in mitochondria. Its comparable activity with other Prxs and the presence of other efficient antioxidants in mitochondria bear witness to the auxiliary function of PrxIIF as H_2O_2 scavenger (Finkemeier et al., 2005). The recently reported signaling/chaperone functions of PrxIIF are no longer trivial and deserve special attention.

Recently, the overoxidized form of PrxIIF has been shown to work as a non-transcriptional rhythmic marker. The circadian clock is an endogenous 24 h oscillator regulating many critical biological processes in plants. One of the key characteristics of the circadian clock is that it is buffered against temperature, maintaining an approximately 24 h rhythm over a broad physiological temperature range. The existence of overoxidized PrxIIF and its retroreductive sulfiredoxin (Srx) systems raises the question as to whether or not this plant mitochondrial antioxidant could work as circadian clock (O'Neill and Reddy, 2011). This feature might be crucial to plants growing in a constantly changing environment. This unaddressed hypothesis is a challenge to future investigations to elucidate new functions of plant Prxs.

Sulfiredoxins

Under oxidative conditions, Prxs undergo a transient oxidation of their cysteine residues from thiol to sulfenic acid and further stable disulfide bridges, which are regenerated to the thiolic forms by Trxs interaction (**Figure 4**). Under severe oxidative stress, Prxs rapidly overoxidize to the sulfinic (Cys-S_PO_2H) and sulfonic (Cys-S_PO_3H) form, locking the enzyme in a permanent inactive state which was primarily hypothethized to serve as an internal indicator of the hyperoxidative conditions inside the cells. The oxidation of the sulfenic acid to sulfinic acid was thought to be an irreversible step (Yang et al., 2002) until Woo et al. (2003) reported that the sulfinic form, produced under high levels of H_2O_2, was reduced to the catalytically active thiol form. These seminal observations served to suggest the presence of an enzyme able to retroreduce the overoxidized form of Prxs. These results were further confirmed by studies of different mammalian 2-Cys Prxs (Chevallet et al., 2003), but the identification of the proposed enzyme was carried out by Biteau et al. (2003). They observed how yeast treated with H_2O_2 induced overexpression of a new protein that they called Srx. Concurrently, deletion of the Srx gene reduced the tolerance of yeast to H_2O_2. Since its discovery in yeast (Biteau et al., 2003; Vivancos et al., 2005), Srxs have been studied, in mammals (Chang et al., 2004; Woo et al., 2005; Jeong et al., 2006), plants (Liu et al., 2006; Rey et al., 2007; Iglesias-Baena et al., 2010) and cyanobacteria (Pascual et al., 2010; Boileau et al., 2011).

The almost concomitant discovery of Srxs (Biteau et al., 2003) together with other redox active protein family called sestrins, described in prokaryote and animal systems (Budanov et al., 2004), as novel enzymes able to regenerate the overoxidized forms of Prxs brought several implications. Firstly, a new enzyme was added to the redox network of Prx adding a new level of regulation. Secondly, the span life of Prxs in the cell increased as a direct implication of their regeneration by Srx and sestrins. Consequently, the concept of a constant rate for "de novo Prx synthesis" needs to be reevaluated. Regeneration of Prxs partially challenges the idea of overoxidized Prxs as cellular indicators of overoxidation: assuming that only the sulfinic form of Prx can be retroreduced, only the sulfonic overoxidized form could work as permanent cell markers as long as they last inside the cell and before being degraded by the cell scavengers. The controversial capability of sestrins to retroreduce overoxidized Prxs (Woo et al., 2009) has drastically diluted their impact in the redox literature, while Srxs have emerged as their clear regenerators, establishing the triade Trx-Prx-Srx.

Sulfiredoxin are a special type of ATP-dependent reductase containing a conserved C-terminal cysteine critical for their antioxidant function (Jönsson and Lowther, 2007). Originally, Srxs were thought to be exclusively involved in the reduction of the sulfinic form of typical 2-Cys Prxs (Woo et al., 2005). Subsequent studies carried out by Iglesias-Baena et al. (2011) demonstrated a

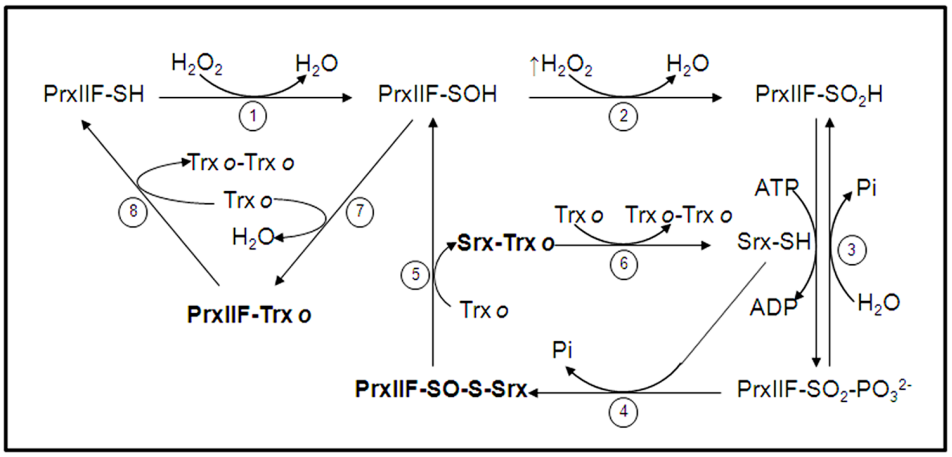

FIGURE 4 | Catalytic cycle of mitochondrial PrxIIF overoxidation and regeneration by Srx. In physiological conditions mitochondrial PrxIIF is oxidized to its sulfenic form in the reduction of peroxides (step 1). At high concentration of H_2O_2 PrxIIF may be overoxidized to the inactive sulfinic form (PrxIIF-SO_2H; step 2) that is phosphorilated, through a reversible step, in the presence of Srx and ATP (step 3). The phosphoril ester (PrxIIF-SO_2-PO_3^{2-}) is converted into sulfinate (PrxIIF-SO-S-Srx) with Srx and Pi is released (step 4). A reducing agent (mitochondrial Trx*o*) reduces the heterocomplex to release PrxIIF-SOH and Srx-Trx*o* (step 5). The complex Srx-Trx*o* is subsequently reduced to Srx-SH by Trx*o* (step 6). The sulfenic form of PrxIIF is reduced by Trx*o* that forms the intermolecular complex PrxIIF-Trx*o* (step 7) and the active PrxIIF-SH is released by another Trx*o* (step 8) that forms the dimer Trx*o*-Trx*o*. The binary complexes between the three proteins in the cycle (sulfinic PrxIIF, Srx and Trx*o*) are in bold type. (cif. ref. Iglesias-Baena et al., 2011).

broader specificity toward the inactive sulfinic forms of atypical plant PrxIIF and atypical human PrxV. These encouraging results stimulate future investigations to establish a general mechanism of retroreduction for the broad diversity of plants Prxs, which could, presumably, respond to the Prx type as well as the subcellular localization. Although mammal Srx are cytosolic, the sulfinic form of mitochondrial human 2-Cys PrxIII can be reduced by hSrx (Woo et al., 2005). Recently, Noh et al. (2009) have reported the hSrx translocation from cytosol to mitochondria under oxidative stress to reduce overoxidized hPrxIII. These results reinforce the hypothesis of a general mechanism of Srx assisting in the regeneration of a broad battery of Prxs. However, the fine mechanism of chemiotaxis targeting Srx to different compartments as a response to the redox conditions needs to be addressed. An aggressive oxidative stress would lead to an increment in the protein concentration and detection in mitochondria. A different scenario have been reported in plants (pea and *Arabidopsis*) in which Srxs were found in chloroplasts and mitochondria regardless of the redox state (Liu et al., 2006; Rey et al., 2007; Iglesias-Baena et al., 2010, 2011). Additional works with Srxs and Prxs from different organisms are needed to tackle the ambiguous localization and substrate specificity of Srx.

The mitochondrial Srx retroreduces the inactive sulfinic form of atypical PrxIIF, employing a mechanism similar to that proposed for other Srxs (Jönsson et al., 2008). One oxygen atom on the sulphinic moiety of the oxidized PrxIIF functions as a nucleophile and attacks the γ-phosphate of ATP at the Srx to yield a sulphinic acid phosphoryl ester intermediate that is resolved by the nucleophilic attack of the Cys from the Srx (**Figure 4**). This mechanism involves two binary complexes, namely PrxIIF-Srx and Srx-Trx*o*. Only the sulfinic form of PrxIIF interacts with Srx (Iglesias-Baena et al., 2011). A secondary complex Srx-Trx has been isolated through formation of a mixed disulfide between Srx and C36STrx (Iglesias-Baena et al., 2011). Roussel et al. (2009) have also demonstrated that Trx forms an efficient complex with Srx. Both complexes, PrxIIF-Srx and Trx-Srx strengthen the proposed mechanism for sulfinic PrxIIF reduction by Srx.

Arabidopsis srx (AtSrx) gene codes for a protein bearing a transit peptide in the N-terminus with the characteristics of dual import to chloroplast and mitochondria (Pujol et al., 2007; Mitschke et al., 2009; Iglesias-Baena et al., 2010, 2011). The mature Srx has a catalytic cysteine (Cys72) involved in the activity. Plant Srxs have an additional non-catalytic cysteine (Cys88; Iglesias-Baena et al., 2010) and, similar to mammalian Srxs, display low efficiency as retroreducing enzymes (Jönsson and Lowther, 2007). Unlike human Srx, only able to retroreduce typical 2-Cys-Prx, AtSrx has a lower substrate specificity showing activity toward typical and atypical Prxs, in different cellular compartments and in different organisms. The concentration of AtSrx was estimated as 0.2% of the total chloroplast protein (Iglesias-Baena et al., 2010, 2011).

Systematic site-directed mutagenesis and molecular modeling suggest that plant Srx has special characteristics that differentiate it from its counterparts in humans (Iglesias-Baena et al., 2010). Although this singularity of plant Srx does not change its reaction mechanism, the structural differences with mammalian Srx can be related with a broad specificity, including atypical Prxs.

Trx/Prx/Srx SYSTEM IN STRESS RESPONSE AND SIGNALING

The involvement of Trxs, Prxs and Srxs in plant tolerance to abiotic stress including salinity is not widely reported in the literature (Barranco-Medina et al., 2007; Pulido et al., 2009; Tripathi et al., 2009). The existing data have allowed the attribution to the Trx/Prx/Srx system of a redox sensing and signal transduction function (Rouhier and Jacquot, 2005) as well as its participation in the repair of oxidized proteins during environmental

stress (Dos Santos and Rey, 2006). Leaf transcriptome results of salt-tolerant and salt-sensitive poplar, revealed that Trx members including chloroplast and cytosolic Trxs, displayed an inconsistent response to salt stress in the leaves, with the majority of the genes unchanged, whereas others showed up- or down-regulation under salinity conditions (Ding et al., 2010). Regarding mitochondrial Trx*o*1, an early induction in its gene expression at short salt treatments (five days at 150 mM NaCl) was described in pea leaves, pointing to an adaptive behavior. Under long salt stress (15 days 150 mM NaCl), a parallel increase in Trx*o*1 activity and protein levels were found with an unexpected down regulation of the gen (Martí et al., 2011). At this long stress, the induction of Trx*o*1 activity was correlated with the *in vivo* activity of the alternative pathway (AP) and with an increase in its capacity, reflecting the presence of the sustainable active form of AOX. PsTrx*o*1 could then have a role through the regeneration of oxidized AOX to the functional reduced enzyme. Under salt stress, increasing amounts and activity of *Trxo1* might correlate also with either, the higher demand to regenerate the oxidized PrxIIF in mitochondria, or the interactions with other target proteins such as those of the photorespiration (Martí et al., 2011). More substantial biological information could be derived from the comparison between the overexpression of Trx*o*1 with its mammalian analog Trx2. Higher amounts of mitochondrial mammalian Trx-2, have been correlated with protection against *t*-butylhydroperoxide and etoposide-induced apoptosis (Chen et al., 2002) and cells deficient in Trx-2 had increased ROS production and exacerbated apoptosis (Tanaka et al., 2002).

Studies on the response of plant PrxIIF toward abiotic stress describe this mitochondrial antioxidant as a constitutive or responsive gen depending on the plant species and stress situation. No changes were described in PrxIIF mRNA levels in *Arabidopsis* leaves under NaCl, H_2O_2, light or ozone treatments (Horling et al., 2002, 2003; Dietz et al., 2006). However, transcript and protein levels were up-regulated in *Arabidopsis* roots after cadmium treatment (Finkemeier et al., 2005), and in poplar leaves after exposure to chilling and water deficit (Gama et al., 2007). In pea plants exposed to salinity, cold and cadmium stress, an up-regulation in PrxIIF mRNA transcript and protein levels was reported in leaves, but not in roots (Barranco-Medina et al., 2007), and the most recent work on pea leaf PrxIIF regulation adds a new time-dependent variable; PrxIIF presented a biphasic response toward salt stress increasing its transcript level after five days of treatment and decreasing after 14 days (Martí et al., 2011). Strikingly, PrxIIF protein content remained constant throughout the salt treatment but a PTM was detected at long time (see below; Camejo et al., 2013a).

Although PrxIIF is involved in acclimation under salinity stress, the enzyme is not essential for plant survival. Lack of PrxIIF in knock-out lines of *Arabidopsis* does not worsen the cellular redox state under optimal conditions and its absence might be compensated by increased mitochondrial APX activity (Finkemeier et al., 2005) or by the presence of the ASC-GSH cycle in mitochondria (**Figure 5**). Notwithstanding the compensatory mechanisms, significant changes in expression of both nuclear and mitochondrial genes were described in the mutants, suggesting that, together with its antioxidant function, PrxIIF is an important candidate for perception of changes in the redox-state in the mitochondria (Finkemeier et al., 2005).

Regulation of Srxs under abiotic stress is not conclusive yet, in part due to their recent discovery and the few works addressing this topic. In *Arabidopsis*, an induction of the chloroplast Srx transcript level has been reported in plants responding to cold treatment (Liu et al., 2006) as observed with mitochondrial PrxIIF (Barranco-Medina et al., 2007). In *Arabidopsis*, the absence of Srx in knock-out lines (ΔAt*Srx*) produced an accumulation, not only of the inactive chloroplastic sulfinic form of 2-Cys Prx, but also of the mitochondrial sulfinic PrxIIF, which is in accordance with its dual location. Besides, the deletion of Srx yielded into more sensitive ΔAt*Srx* plants against high concentration of H_2O_2 when compared with AtWT plants (Iglesias-Baena et al., 2010, 2011). Although Srx is not essential for plant viability, it protects chloroplast and mitochondria depending on the intensity of the oxidative stress to regenerate the inactive sulfinic Prxs (**Figure 5**; Vivancos et al., 2005).

The sulfinic PrxIIF lacking of peroxidase activity can exhibit signaling functions in the cell. Therefore, Srxs, by controlling the reversion of the sulfinic form of PrxIIF, could indirectly regulate the signaling process (**Figure 5**). Herein, we propose an integrative model of signaling/antioxidant function taking into account ROS and antioxidants. On allowing H_2O_2 to carry out its signaling function, its level must increase rapidly above a threshold (Rhee, 2006). To maintain this concentration, some antioxidant enzymes must remain inactive; among them, mAPX and sulfinic PrxIIF could help in this aim. When the fast signaling function of H_2O_2 has finished, the peroxidase activities of mAPX and PrxIIF can be recovered. Furthermore, high levels of ROS in the mitochondria can lead to PrxIIF overoxidation contributing to its oligomerization and a switch of activity from peroxidase to chaperone (Barranco-Medina et al., 2006). The link between activity and oligomerization is well correlated and seems to establish a general mechanism for Prxs (see the review by Barranco-Medina et al., 2009).

All the reported changes of the antioxidant and redox systems imply that stress tolerance seems to require the induction of specific isoforms in the different cell compartments or a constitutively higher content of antioxidants, depending on the species, variety or strength and duration of stress. In this context, mitochondrial Mn-SOD, APX, MDHAR, AOX, Trx*o*1 and PrxIIF appear as key enzymes in the ROS network, functioning in both, salt adaptation and signaling pathways (**Figure 5**).

The miss-correlation existing between gene expression, protein level and activity evidences a complex regulation in the response of plants to changing environments, and points out the relevance of post-transcriptional and PTMs in the tolerance mechanisms involving MRR.

POST-TRANSLATIONAL MODIFICATIONS: THE IMPORTANT ROLE OF *S*-NITROSYLATION IN MITOCHONDRIAL PROCESSES

EFFECTS OF NO• AND ITS DERIVATES ON MITOCHONDRIA

Mitochondria are exposed to NO• on activation of enzymatic emission associated to ETC and arginine or diffused from surrounding cell compartments (del Río et al., 2002; Blokhina and

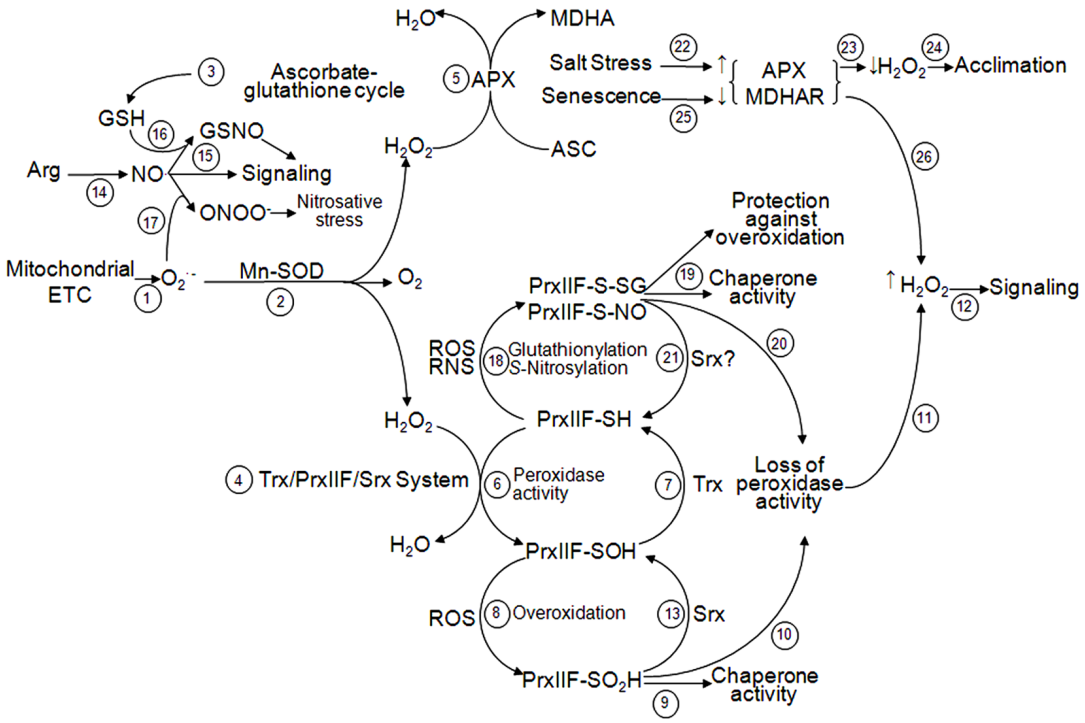

FIGURE 5 | Interaction between ascorbate–glutathione cycle and Trx/PrxIIF/Srx system in ROS and RNS signaling in plant mitochondria. Superoxide radicals ($O_2^{\bullet -}$) produced by the mitochondrial ETC (step 1) are dismutated to molecular O_2 and H_2O_2 by the activity of Mn-SOD (step 2). H_2O_2 is reduced by two different systems: ascorbate-glutathione cycle (step 3) and Trx/PrxIIF/Srx system (step 4). In the ascorbate-glutathione cycle, H_2O_2 is reduced by APX (step 5) and throughout the cycle as indicated in **Figure 1**. In the Trx/PrxIIF/Srx system, H_2O_2 is reduced by the peroxidase activity of PrxIIF (step 6) that produces its sulfenic form (PrxIIF-SOH) and is reduced by the Trx system (step 7) as indicated in **Figures 2** and **3**. Under oxidative stress, PrxIIF-SOH can be overoxidized to the sulfinic form (PrxIIF-SO_2H; step 8) that gains chaperone activity (step 9) losing its peroxidase activity (step 10). This loss of activity increases H_2O_2 concentration in the mitochondria (step 11) allowing the signaling (step 12). PrxIIF-SO_2H can be regenerated to the reduced form by the action of Srx (step 13) and Trx (step 7) as indicated in **Figure 4**. The generation of NO$^\bullet$ in the mitochondria (step 14) allows signaling (step 15) in addition of forming GSNO by reduction with GSH from ascorbate-glutathione cycle (step 16). NO$^\bullet$ can react with $O_2^{\bullet -}$, produced by the mitochondrial ETC, to form $ONOO^-$ that bursts nitrosative stress (step 17). Under oxidative or nitrosative stress, PrxIIF-SH can be glutationylated or *S*-nitrosylated (step 18) in order to protect the enzyme against overoxidation and to gain chaperone activity (step 19). PrxIIF-S-SG and PrxIIF-S-SNO lose the peroxidase activity (step 20), allowing the signaling by H_2O_2 (step 11 and 12). These post-translational modifications could be reverted to PrxIIF-SH by the Srx activity (step 21) as it happens with the 2-Cys Prx. On the other hand, salt stress induces an increase of APX and MDHAR (step 22), which produces a decrease in the concentration of H_2O_2 (step 23) allowing acclimation (step 24). During the oxidative mechanism of senescence there are decreases of APX and MDHAR activities (step 25) which produce an increase in H_2O_2 (step 26) allowing signaling (step 12).

Fagerstedt, 2010). NO$^\bullet$ can affect mitochondrial metabolism involving oxidation of metals in proteins complexes and reduction of free metal ions. NO$^\bullet$ can also react with oxygen to form oxidized NO$^\bullet$, which interacts with mitochondrial GSH to form *S*-nitrosoglutahione (GSNO; **Figure 5**) or with thiol-containing molecules to yield low molecular weight *S*-nitrosocysteine and *S*-nitrosocystein glicyne in a process called *S*-nitrosylation (Hess and Stamler, 2012). GSNO is considered to be the most abundant low-molecular mass *S*-nitrosothiol (SNO) and also a vehicle of NO$^\bullet$ throughout the cell, which enables NO$^\bullet$ activity to expand. GSNO has been located in pea mitochondria, together with cytosol, peroxisomes and chloroplasts (Corpas et al., 2013).

Prime targets of NO$^\bullet$ and its derivates in plants, are the mitochondrial electron transport components and enzymes, producing an inhibition of cytochrome c pathway whereas alternative respiration via AOX is only partially inhibited (Day et al., 1996; Martí et al., 2012). These inhibitions may potentially be involved in the regulation of energy metabolism, generation of ROS, cell death and response to stress. From a pharmacological approach, mitochondrial Mn-SOD has been found to be not inactivated by NO$^\bullet$ (Martí et al., 2012), although this enzyme binds and stimulates NO$^\bullet$ decay under both anaerobic and aerobic conditions (Filipovic et al., 2007). However, $O_2^{\bullet -}$ can react with NO$^\bullet$ three times faster than with mitochondrial Mn-SOD (Yamakura et al., 1998), so generating $ONOO^-$ (**Figure 5**; Wilson et al., 2008), which represents a mechanism for NO$^\bullet$ consumption by mitochondria. Thus, when mitochondria respiration is inhibited by NO$^\bullet$, the formation of peroxynitrite contributes to NO$^\bullet$ degradation, reactivation of COX and restoration of oxygen consumption (Poderoso et al., 1996). The observed insensitivity of AOX to NO$^\bullet$also represents another mechanism to prevent its deleterious effects on respiratory activity (Millar and Day, 1996; Martí et al., 2012). Recent results highlight the importance of AOX in the control of NO$^\bullet$ level in plants; mitochondrial NO$^\bullet$ content is increased in the absence of AOX (Cvetkovska and Vanlerberghe, 2012), and curiously a NO-dependent up-regulation of *AOX* gene has been

described (Huang et al., 2002). Furthermore the mitochondrial heme-enzyme APX (mAPX) was found to be reversibly inhibited by NO• in an ascorbate dependent manner, which could have physiological relevance during oxidative and/or nitrosative stress conditions where ASC depletion may occur (de Pinto et al., 2006; Martí et al., 2012). Hence, mAPX could be part of a NO• redox signaling pathway in mitochondria, through the H_2O_2 and NO• signaling cross-talk (Bright et al., 2006). Like Mn-SOD, mitochondrial MDHAR, DHAR and GR enzymes were not inhibited under continuous fluxes of NO•, which may contribute significantly to prevent a build-up of ROS, and also to allow the recycling of ASC and GSH from its oxidized forms, thus reducing the risk of RNS accumulation (Martí et al., 2012).

Overall the results probably indicate that not only NO• resistant AOX but also mAPX, may be important components of the H_2O_2-signaling pathways under conditions inducing the production of NO• in this organelle (Martí et al., 2012). New studies are needed to further elucidate the physiological relevance of the relation between mitochondrial NO• and derivates, and the ASC-GSH cycle enzymes under normal and stressful conditions.

MITOCHONDRIAL TARGETS OF *S*-NITROSYLATION

Cellular functions of NO• are carried out in part through *S*-nitrosylation, a dinamic PTM for the regulation of the protein function (Hess et al., 2005). Several mechanisms of *S*-nitrosylation have been proposed, but those operating in plant cells have not yet been elucidated (Kovacs and Lindermayr, 2013). Biological *S*-nitrosylation can take place by transnitrosylation, which involves the transfer of NO• onto a cysteine thiol. Recent findings underscore the importance of subcellular compartimentation in determining when and where proteins are *S*-nitrosylated during signal transduction. In recent years, a long list of plant proteins undergoing *S*-nitrosylation and cellular processes affected has been identified from proteome-wide analysis by using NO• donors as *S*-nitrosylating agents or by biotic and abiotic stressors, in cultured cells, whole leaves and cellular organelles (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Astier et al., 2011; Fares et al., 2011). Thus, more than 50 *S*-nitrosylated candidate proteins were identified in *Arabidopsis* leaves, including cytosolic PrxIIB, chloroplast Trxf1 and a chloroplast PrxIIE, among others (Lindermayr et al., 2005). *S*-nitrosylation in PrxIIE was demonstrated to inhibit its peroxidase and peroxynitrite reductase activity. Authors suggested a model where this PTM regulates the transduction of NO• and ROS-linked signals during infection by *P. syringae*, highlighting a key role for PrxIIE in controlling the endogenous level of $ONOO^-$ (Romero-Puertas et al., 2007, 2008). Recent studies have demonstrated that the Trx system may be involved in regulating the *S*-nitrosylation status of target proteins in different systems (Wu et al., 2011) through the transnitrosylation or denitrosylation, thus modulating their biological activities (Benhar et al., 2008; Kornberg et al., 2010). In mammalian systems, a subpopulation of caspase-3 in the mitochondria is constitutively *S*-nitrosylated and, as such, is inhibited, and mitochondrial Trx2 has been involved in this *S*-nitrosylation (Mitchell and Marletta, 2005), although the exact intramolecular mechanism of Trx denitrosylation remains ill-defined, as does the denitrosylation target specificity (Benhar et al., 2009). To the date, no information on the putative *S*-nitrosylation capacity for plant mitochondrial Trx*o*1 and its transnitrosylating/denitrosylating activity is available. In addition to Trx, *S*-nitrosoglutathione reductase (GSNOR) plays a predominant role in protein denitrosylation (Benhar et al., 2009). In mitochondria from *Arabidopsis* plants treated with GSNO, 11 proteins were identified as possible targets for *S*-nitrosylation (Lindermayr et al., 2005). Some of these proteins were mETC constituents and three of them were subunits of the glycine decarboxylase complex (GDC H1, T and P proteins), a key enzyme of the photorespiratory C2 cycle. The activity of this enzyme complex was inhibited by *S*-nitrosylation, and appears to be involved in the regulation of NO• dependent signal transduction, although the underlying signaling pathway remains elusive (Palmieri et al., 2010).

It has been shown that AOX is co-expressed with GDC and the decrease of GDC amount in mitochondria also results in very low AOX levels (Bykova et al., 2005). GSNO treatment also inhibits complex I, and as a result, increases ROS in mitochondria, which could later affect chloroplastic ROS through reduced photorespiratory capacity (Gupta, 2011). Endogenous *S*-nitrosylation protein pattern in mitochondria from *Pisum sativum* leaves was reported as being similar or even higher than that found for mitochondrial proteins in other sources, including *Arabidopsis* (Tanou et al., 2009; Fares et al., 2011). A differential pattern of target proteins was identified during plant development, with a minor number of *S*-nitrosylated proteins in older plants, specifically some key enzymes related with respiration and photorespiration, including GDC T and GDC P subunits, NAD-MDH, succinate dehydrogenase, NADH ubiquinone oxidoreductase and ADP/ATP carrier, which disappeared as *S*-nitrosylated targets, while elongation factor Tu and protein kinase appeared as new targets of *S*-nitrosylation. Similar to GDC, *S*-nitrosylation of peroxisomal isozyme NAD-MDH also decreased its activity (Ortega-Galisteo et al., 2012). The differential *S*-nitrosylation pattern during development may be a mechanism for avoiding malfunction of the photorespiratory cycle and that of the respiratory components, which can further increase mitochondrial and chloroplastic ROS levels and affect redox signaling during this process (Camejo et al., 2013a). To date, the significance of the regulation of both processes by *S*-nitrosylation/denitrosylation during plant development remains undefined.

S-NITROSYLATION UNDER SALT STRESS

The participation of NO• in plants in response to biotic and abiotic stress including drought and salt stress has been demonstrated. The real role of NO• in the cell is not exempt of controversy. While some authors consider NO• as a stress-inducing agent, others have reported its protective task (Huang et al., 2002; Neill et al., 2003; del Río et al., 2004; Leitner et al., 2009; Rodríguez-Serrano et al., 2009). NO• was recently proposed in the mediation of the response to salinity in different plant systems and varieties (Gould et al., 2003), not only for its harmful reactivity and toxicity but also for its involvement as signal molecule able to mitigate the damage associated with salt stress, either in plants and germinating seeds (Zheng et al., 2009). In pea plants, the increase observed in NO• content by long-term salt treatments (14 days) was, in part, associated to mitochondria, although the contribution of

other organelles, like peroxisomes and cytosol was not discarded (Rodríguez-Serrano et al., 2009; Camejo et al., 2013a).

In salt-stressed plants, the information for *S*-nitrosylation in mitochondria is quite limited, despite the significant developments in proteomics analysis allowing the identification of mitochondrial proteins suffering changes in abundance under salinity (Taylor et al., 2009; Jacoby et al., 2010). Recently 49 proteins including mitochondrial enzymes were identified as targets of *S*-nitrosylation in response to NaCl-stress in citrus plants (Tanou et al., 2009). In pea leaf mitochondria, at least 9 *S*-nitrosylated proteins were described under salt-stress, but as reported during pea development, as the stress became longer, so the number of identified protein targets decreased. This affected some enzymes related to NADH metabolism, as MDH, the photorespiratory GDC P and T subunit, and also Mn-SOD and aminomethyltransferase, identified as *S*-nitrosylated at short salinity period (5 days). Interestingly, mitochondrial PrxIIF and a heat shock Hsp90 protein appeared as new *S*-nitrosylation targets during the salt stress progression. Others enzymes involved in respiration were Succinate dehydrogenase, NADH ubiquinone oxidoreductase, and in photorespiration SHMT were found *S*-nitrosylated in control pea mitochondria but not under salt-stress. Thus, the denitrosylation of the respiratory activities may not limit ETC transport, at least through the AP which was not decreased with salt stress, as previously reported (Martí et al., 2011). The denitrosylation of the three key enzymes of photorespiration, was not accompanied by changes in its protein content and may allow this process to be functional after long period of salt stress. All these changes may contribute to the elevated NADH/NAD^+ and the maintained NAD(P)H/$NADP^+$ produced in pea mitochondria inducing a modification in the mitochondrial matrix or ETC redox state. Several mitochondrial dehydrogenases are described to be affected by changes in the NADH/NAD^+ ratio depending on their kinetics characteristics (Noctor et al., 2007). Another possible destination for reducing equivalents in mitochondria is the Trx system. In pea plants, the expression, content and activity of Trx*o*1 were increased under salt stress (Martí et al., 2011), so Trx*o*1 could play a pivotal role in sensing the local redox environment and regulating the activity of its target enzymes through reduction of their disulfide bridges. Consequently, functional photorespiration can prevent important photooxidative damage as results of the more intense salt-induced reduction of stomatal conductance at long-term salt stress (Martí et al., 2012). A functional role for the cooperation between the mitochondria, chloroplast and peroxisomes to modulate cell redox homeostasis under salinity and drought stress has been described (Noctor et al., 2007; Pastore et al., 2007).

The increase in $NO^\bullet$ content under salt conditions was not related to an enhanced mitochondrial protein *S*-nitrosylation (Camejo et al., 2013a), raising the question of whether $NO^\bullet$ could exhibit different actions. GSNOR activity was also induced under salt conditions and might constitute a mechanism of the degradation of SNOs, so influencing $NO^\bullet$ level in mitochondria by preventing $NO^\bullet$ kidnapping.

As a new target of *S*-nitrosylation under short salt stress, Mn-SOD does not seem to be affected in its activity, either under salt conditions or after GSNO treatment of mitochondria (Martí et al., 2012; Camejo et al., 2013a). These results corroborated the previously reported antioxidant function of this important enzyme under salt stress, as a ROS scavengers and possible $NO^\bullet$ sink (Hernández et al., 2000; Wang et al., 2004; Jacoby et al., 2010). Only during long-term salt stress, PrxIIF was found as *S*-nitrosylated (**Figure 5**) parallel to an increase of $NO^\bullet$, while the protein amount did not change. *S*-nitrosylation of PrxIIF was not previously described, although a strong posttranslational regulation to explain its response to high H_2O_2 concentration was suggested (Finkemeier et al., 2005). This modification may inhibit its peroxidase activity during salt stress, as indicated after treatment of the recombinant pea PrxIIF with GSNO (Camejo et al., 2013a). This inhibition suggests a role for PrxIIF as a signaling component more than as an antioxidative enzyme during long NaCl stress (**Figure 5**). Recently, a change in PrxIIF peroxidase activity to chaperone by *in vitro S*-nitrosylation of recombinant protein has been observed (Camejo et al., 2013b). It could be important to address whether this process is also taking place and to what intensity, under salt stress, when endogenous PsPrxIIF appears as *S*-nitrosylated.

CONCLUSIONS AND PERSPECTIVES

Redox regulation and ROS metabolism are interlinked and involved in optimizing the function of cell organelles. The mitochondrial antioxidant system has a key role in the detoxification of $O_2^{\bullet-}$ and peroxides and thus plays a crucial role in controlling redox signaling. Redox proteome and *in vitro* recombinant protein studies have shown that many of the mitochondrial proteins undergo different redox PTMS, so modulating their antioxidant activity. A good example is Prx, including mitochondrial PrxIIF and Prx 3 in plants and mammalian, respectively. Thus, PrxIIF can be partially inactivated by hyperoxidation, glutathionylation and *S*-nitrosylation or by the extent of its oligomerization. To relate these modifications to events in situ it is important to distinguish between protective and signaling purposes under physiological and abiotic stress responses. Also, to understand how these redox PTMs regulate mitochondrial redox signaling it would be necessary to know how the different types of PTMs are coordinated and/or interconnected to allowing specific proteins to respond not only to different stimuli, but also to their intensity and duration.

Many thiols in proteins are susceptible to redox modifications, but only a few are important in signaling pathways. Some studies also highlight the concept that some protein cysteine residues are differently susceptible to NO-modifications and the functionality of reactive Cys as NO-sensor "*in vivo*" and their regulation by NO affecting biological processes is still little known. The study of these aspects would help to clarify the true significance of redox signaling.

Post-translational modifications on plant mitochondrial ASC-GSH cycle enzymes have not yet been studied in detail and although the reversible inhibition of APX by NO and its irreversible inactivation by $ONOO^-$ have been reported, there is scarce information about the "*in vivo*" NO's effects on these antioxidant enzymes. The link between $NO^\bullet$ and the ASC-GSH cycle enzymes is an essential target to clarify $NO^\bullet$ participation in redox signaling that awaits further functional characterization.

Similarly, the redox state of the Trx*o*1 can reversibly affect the activity of target proteins. Moreover, given that some Trxs can reduce sulfenic acids, SNOs and glutathionylated cysteines and could promote trans-nitrosylation reactions, the putative involvement of Trx*o*1 in some of these reactions should be analyzed in future studies. Additionally, the mechanism of protein deglutathionylation catalyzed by Srx still needs to be explored as well as its functional significance.

Thus, another interesting question will be to assess the functional aspect of AOX redox regulation by Trx*o*1 in controlling mitochondrial $NO^{\bullet}$ levels. This aspect may help to reinforce the model of cross-talk between NO/ROS in mitochondria. It would be also advisable to study the putative interaction between mAPX and Trx*o*1 for a better understanding of the mitochondrial response to oxidative stress.

Finally, an understanding of the above proposed aspects of the cross-talk between signaling pathways linked to ROS and RNS is also a major issue to elucidate the mechanisms underlining plant abiotic stress tolerance.

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Kinetic analysis of the interactions between plant thioredoxin and target proteins

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Thioredoxin is a critical protein that mediates the transfer of reducing equivalents *in vivo* and regulates redox sensitive enzymes in several cases. In addition, thioredoxin provides reducing equivalents to oxidoreductases such as peroxiredoxin. Through a dithiol–disulfide exchange reaction, the reduced form of thioredoxin preferentially interacts with the oxidized forms of targets, which are immediately released after this reaction is complete. In order to more thoroughly characterize these interactions between thioredoxin and its target proteins, a mutant version of thioredoxin that lacked the second cysteine was synthesized and interactions were monitored by surface plasmon resonance. The binding rates of thioredoxin to its targets were very different depending on the use of reducing equivalents by the targets: the enzymes whose activity was controlled by reduction or oxidation of a cysteine pair(s) in the molecule and the enzymes that used reducing equivalents provided by thioredoxin for their catalysis. In addition, thioredoxin revealed a stronger preference for an oxidized target. These results explain the reason for selective association of thioredoxin with oxidized targets for reduction, whereas immediate dissociation from a reduced target when the dithiol–disulfide exchange reaction is complete.

Keywords: cysteine, protein–protein interaction, redox regulation, surface plasmon resonance, thioredoxin

INTRODUCTION

Thioredoxin (Trx) is a small, ubiquitous protein that contains a pair of redox-sensitive cysteine residues within its catalytic domain. This domain comprises the highly conserved sequence motif Trp-Cys-Gly-Pro-Cys-[Lys/Arg] (Holmgren, 1985; Buchanan, 1991; Balmer et al., 2006). The cysteines allow Trx to reduce disulfide bonds on target proteins by catalyzing a dithiol–disulfide exchange reaction, which regulates target activity in several cases. On the basis of the reduction process mediated by Trx studies, the cysteine toward the N-terminal end of the Trx catalytic domain first attacks disulfide bond in a target protein to form an intermolecular disulfide complex, often described as a mixed disulfide intermediate complex (Brandes et al., 1993). Subsequently, the cysteine toward the C-terminal end of the Trx catalytic domain attacks this intermolecular disulfide bond, which results in an oxidized form of Trx and a reduced target.

Trx was first identified as a reducing equivalent donor for the deoxyribonucleotide reductase in *E. coli* (Laurent et al., 1964). In contrast to bacteria and animals, numerous isoforms of Trx have been identified in plants, although their specific roles are poorly understood (Balmer et al., 2006). Recent proteomics studies revealed that these Trx isoforms could react with a large number of candidate target proteins *in vitro* (Motohashi et al., 2001; Yano et al., 2001; Balmer et al., 2003; Yamazaki et al., 2004) and *in vivo* (Hall et al., 2010). For example, approximately 400 proteins have been described as potential targets of the plant Trx system (Montrichard et al., 2009), although only a small portion of these have been confirmed to be *bona fide* target proteins by biochemical analyses.

With regard to the target specificity and the Trx-mediated reaction process, a number of questions remain unanswered. For example, the mechanism of disulfide bonds recognition on the target proteins by Trx and, during the reaction process, the mechanism by which reduced form of Trx react with the oxidized form of the target protein and release it immediately after the dithiol–disulfide exchange reaction is completed. The target proteins have been categorized into two groups on the basis of their mode of interaction with Trx.

One group is the "switch" type proteins that are predominantly found within the chloroplast such as the chloroplast ATP synthase γ subunit (McKinney et al., 1979; Nalin and Mccarty, 1984), four Calvin cycle enzymes (glyceraldehyde 3-phosphate dehydrogenase, fructose 1,6-bis-phsophatase, sedoheptulose 1,7-bis-phosphatase, and phosphoribulokinase) (Jacquot et al., 1997), and malate dehydrogenase (MDH) (Scheibe and Anderson, 1981). The activities of the target proteins in this group are regulated through the oxidation/reduction of the Trx-targeted disulfide bond located within the molecule. Once reduced by Trx, these target proteins no longer require reducing equivalents provided by Trx.

The second group comprises the "catalytic" types such as peroxiredoxin (Dietz, 2003) and methionine sulfoxide reductase (MSR) (Tarrago et al., 2009). Because "catalytic" type target

enzymes use reducing equivalents provided by Trx as an integral part of their catalytic process, they must repeatedly interact with Trx during the catalytic cycle of a target enzyme.

During the dithiol–disulfide exchange reaction, the reduced form of Trx associates with and reduces the oxidized form of the target protein, and then the interchange in redox conditions results in oxidized and reduced forms, respectively. Moreover, when a disulfide bond on the target protein is reduced by the reduced form of Trx, the target protein and Trx immediately dissociate for re-reduction of the oxidized form of Trx by Trx-reductase. This suggests that these proteins must drastically change their affinity for Trx before and after the dithiol–disulfide exchange reaction.

Till date, several attempts regarding the interactions between Trx and the target proteins have been reported. The key residues on the surface of a Trx-*f* molecule involved in target specificity were revealed by mutation analyses (Geck et al., 1996; Geck and Hartman, 2000). The altered $S_{0.5}$ and V_{max} values for target enzyme activation caused by a Trx mutation indicated the importance of protein–protein interaction in addition to the dithiol–disulfide exchange reaction. In the case of the interaction between MSR and Trx from *E. coli*, the rate constants for the chemical reaction steps were determined by fluorescence stopped-flow measurements (Antoine et al., 2003; Olry et al., 2004). The authors concluded that the rate limiting step for regeneration of the reduced form of MSR by Trx was the dissociation of oxidized Trx from a reduced MSR complex.

Considering the Trx reduction reaction, there are two interaction modes between Trx and the target protein that depend on their redox states. One is the interaction between the reduced form of Trx and the oxidized form of target protein, and the other is the interaction between the oxidized form of Trx and the reduced form of target protein. Clarifying the difference between these interactions would be helpful for understanding Trx-mediated reduction mechanisms. However, conventional approaches are difficult to use for revealing the changes in affinity between Trx and the target proteins, in particular with regard to the redox states of Trx and its target proteins.

For characterization of these enigmatic but fundamental phenomena that occur within the Trx catalytic domain, we used surface plasmon resonance (SPR) to directly determine the association and dissociation rate constants of Trx to its target proteins. *Arabidopsis thaliana* cytosolic MDH and chloroplast peroxiredoxin Q (PrxQ) were used as "switch" and "catalytic" type target model proteins, respectively. Cytosolic MDH is a homodimeric enzyme that contains a Cys330–Cys330 intermolecular disulfide bond, and reduction of this disulfide bond by Trx has a strong effect on its activity (Hara et al., 2006). PrxQ is a chloroplast localized Trx-dependent peroxiredoxin, the activity of which is exhibited efficiently by the cytosolic Trx rather than the chloroplast Trx (Rouhier et al., 2004). Moreover, PrxQ is a monomeric protein that contains a Cys45–Cys50 intramolecular disulfide bond. Because MDH and PrxQ each have a disulfide bond, we selected them as target model proteins and Trx*h* as the reductant for this study.

MATERIALS AND METHODS

PROTEINS

Cloning and purification of *A. thaliana* cytosolic thioredoxin *h*1 (Trx*h*1) (AT3G51030), cytosolic MDH (AT1G04410), and chloroplast PrxQ (AT3G26060) were performed as described by Motohashi et al. (2001); Yamazaki et al. (2004); and Hara et al. (2006), respectively. roGFP1-iL was prepared as described by Lohman and Remington (2008) with minor modifications. This protein was used as the redox sensitive GFP derivative (roGFP) for this study. In brief, roGFP expressed in *E. coli* strain BL21(DE3) was purified by anion exchange chromatography using a DEAE-Toyopearl 650 M and by hydrophobic interaction chromatography using a Butyl-Toyopearl 650 M.

A Trx*h*1 mutant with a C43S substitution (Trx$h1_{CS}$) and a Trx*h*1 mutant with a C40S/C43S substitution (Trx$h1_{SS}$) were prepared by the mega-primer method (Sarkar and Sommer, 1990). These were expressed and purified using the same procedures as for the wild-type Trx*h*1 with minor modifications. The Trx$h1_{CS}$ and Trx$h1_{SS}$ mutants were purified using a solution containing 0.5 mM DTT in all purification procedures to prevent unexpected disulfide bond formation.

The oxidized MDH was prepared by incubation with 50 μM $CuCl_2$ for 1 h at 30°C. Purified PrxQ and roGFP were obtained as oxidized forms. To obtain reduced PrxQ, oxidized PrxQ was incubated with 5 mM DTT and then dialyzed against well degassed H buffer (10 mM HEPES-NaOH, 150 mM NaCl, and 50 μM EDTA, pH 7.4). The redox states of all proteins were confirmed by the AMS-labeling method with non-reducing SDS-PAGE (Motohashi et al., 2001). Protein concentrations were determined using a BCA assay with BSA as standard.

SPR MEASUREMENTS

SPR measurements were carried out using a Biacore X system (GE Healthcare, Piscataway, NJ). All experiments were performed at 25°C with the indicated flow rates. All proteins were dialyzed against H buffer before the measurements. Ligand proteins were immobilized on a CM5 sensor chip at a flow rate of 5 μl/min using amino coupling methods. After activation of the CM5 sensor chip with 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysulfosuccinimide (NHS) for 7 min, ligand proteins were diluted with 10 mM sodium acetate (pH 4.5) and immediately injected into the flow cell (Fc.2) onto the activated CM5 sensor chip using the manual injection mode. When sufficient amounts of ligand proteins were immobilized (equivalent to 800–900 RU signals on SPR), residual active NHS esters were blocked by injecting 1 M ethanolamine–HCl (pH 8.5) for 7 min. The reference flow cell (Fc.1) was treated in the same way without ligand proteins.

Experiments were performed after adding 0.005% Surfactant P20 (GE Healthcare) to H buffer. For repeat measurements, the surface of the sensor chip was regenerated with 5 mM DTT and 500 mM NaCl in H buffer for 2 min. The association and dissociation phase data (signal from Fc.2–signal from Fc.1) were used to determine kinetic parameters. A 1:1 binding model was used to obtain the rate constants for MDH. For PrxQ, data were fit to a double exponential model that had two individual pairs of

association and dissociation rate constants. These analyses were done using a BIAevalution version 4.1 (GE Healthcare).

DISULFIDE BOND REDUCTION ON roGFP BY Trx*h*1

Disulfide reduction on roGFP was fluorometrically evaluated using the excitation ratio of 395 nm/475 nm with emission at 510 nm. Oxidized roGFP (15 μM) was incubated with 0.5 mM NADPH, 0.5 μM of NADPH-Trx reductase from *A. thaliana* (AtNTR), and 1 μM Trx*h*1 at 25°C for 30 min in H buffer. The excitation spectra were then measured using a FP-8500 spectrofluorometer (JASCO, Tokyo, Japan).

RATE OF DISULFIDE BOND REDUCTION ON MDH MEDIATED BY Trx*h*1

The extent of disulfide bond reduction was determined from the proportion of reduced forms in total MDH proteins. To reduce Trx*h*1, 0.5 mM NADPH, 0.5 μM AtNTR, and 0.2–5 μM Trx*h*1 were incubated at 25°C for 5 min in H buffer in advance. The reaction was initiated by adding 1 μM oxidized MDH to this mixture, and at the indicated times, proteins in the aliquot were precipitated by adding 5% TCA (w/v). SDS-sample buffer containing 10 mM N-ethylmaleimide (NEM) was then added to these precipitants and proteins were separated by non-reducing SDS-PAGE. The band intensities (I_{red} and I_{ox} for reduced and oxidized proteins, respectively) were determined using a Scion Image software and the reduced proportion was calculated by the following equation: Reduced proportion (%) $= I_{red}/(I_{red} + I_{ox}) \times 100\%$.

DISULFIDE BOND REDUCTION RATE ON PrxQ BY Trx*h*1

The rate of disulfide bond reduction on PrxQ was estimated from the H_2O_2 reduction activity of PrxQ. This activity was determined using a coupling assay system that contained AtNTR and Trx. The decrease in absorbance at 340 nm due to NADPH oxidation was monitored (Motohashi et al., 2001). The reaction was initiated by adding 3 μM Trx to a reaction mixture that contained 0.5 mM NADPH, 1 μM AtNTR, 0.5 μM PrxQ, and 0.5 mM H_2O_2 in H buffer.

GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography analysis was performed using a TSK-G2000SW_{XL} (Tosoh, Tokyo), which had been equilibrated with H buffer. Eluted proteins were monitored at 280 nm.

RESULTS

REDUCTION OF DISULFIDE BOND CONTAINING PROTEINS BY WILD TYPE Trx*h*1

We used the following three proteins for this study: MDH, PrxQ, and roGFP, each of which contained a disulfide bond. We first determined the rates of disulfide bond reduction in these proteins by wild type Trx*h*1 (**Table 1**). Although roGFP has a surface exposed disulfide bond, it is barely reduced by Trx*h*1 (Meyer et al., 2007). We previously reported that the MDH and PrxQ were reduced by Trx (Motohashi et al., 2001; Hara et al., 2006), although the reduction rates of these proteins were very different. This difference was probably caused not only by the different chemical reaction rates of disulfide bond reduction but also by the differences in protein–protein interactions between Trx and these target proteins.

TARGET PREFERENCES REVEALED BY SPR MEASUREMENTS

We first attempted SPR measurement using a combination of wild type Trx*h*1 as a ligand and oxidized MDH as the analyte; however, no SPR signals could be detected using this combination. Thus, we used the Trx$h1_{CS}$ mutant as the ligand in subsequent experiments. The Trx$h1_{CS}$ lacks the second cysteine in its catalytic domain and cannot catalyze the complete exchange of disulfide bond pairs with the target proteins. Thus, this appeared to be a good experimental model protein to capture a snapshot of the dithiol–disulfide exchange reaction mediated by Trx. Although this mutant cannot be used to imitate the actual affinity due to the formation of a mixed disulfide bond, this mutant exhibited association and dissociation activity in our SPR measurements. Therefore, we describe the data obtained in this study as apparent affinities for Trx and the target proteins.

Using the Trx$h1_{CS}$, we attempted to observe the dissociations and associations between Trx and three proteins, roGFP, PrxQ, and MDH, as SPR signals. These proteins have redox-sensitive disulfide bonds in their oxidized states. Thus, the resulting SPR signals should have been due to the formation of mixed disulfide intermediate complexes. When these proteins were injected into the Trx$h1_{CS}$ immobilized on a CM5 sensor chip for SPR measurements, the sensorgrams for the association phase were very different (**Figure 1**). When roGFP with a surface-based Trx irreducible disulfide bond was used as the analyte, the SPR signals

Table 1 | Rate constants of target proteins interacting with Trx*h*1.

Method	Rates	roGFP	MDH	PrxQ	
				Rapid	Slow
SPR[a]	k_{on} (M^{-1} s^{-1})	N.D.	247	7.97×10^4	765
	k_{off} (s^{-1})	N.D.	3.01×10^{-4}	0.62	5.62×10^{-4}
Reduction reaction	Reduction rate (s^{-1})	N.D.	6.5×10^{-3}[b]	0.67[c]	

[a] *Association and dissociation rate constants were determined by using Trxh1$_{CS}$ mutant.*

[b] *Reduction rate of MDH was determined from the reduction extent of MDH by wild type Trxh1 measured at the various reaction periods (for detail, see Materials and Methods).*

[c] *Reduction rate of PrxQ was determined from the amount of NADPH consumption in the presence of AtNTR, wild type Trxh1, PrxQ and hydrogen peroxide. The amounts of PrxQ was compensated to be a rate limiting step (for detail, see Materials and Methods).*

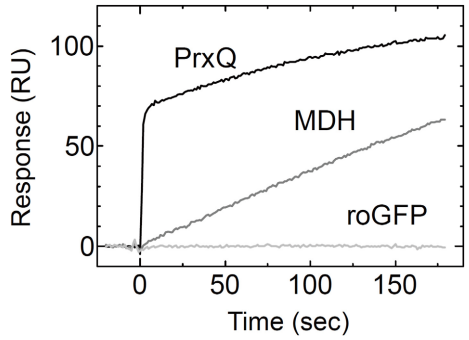

FIGURE 1 | Associations of model proteins with the Trx*h*1$_{CS}$. PrxQ, MDH, or roGFP at 5 μM were injected onto the Trx*h*1$_{CS}$ immobilized on a CM5 sensor chip. Experiments were performed at a flow rate of 30 μl/min for PrxQ and 10 μl/min for MDH and roGFP.

were barely detectable. This suggested that the Trx*h*1$_{CS}$ did not associate non-specifically with this protein, even if the protein contains disulfide bond(s) on its surface. In contrast, PrxQ and MDH provided more significant SPR signals. These sensorgrams clearly revealed that the binding rate of PrxQ to the Trx*h*1$_{CS}$ was much faster than that of MDH. These results were in accordance with the significant differences in their reduction rates of the disulfide bonds located on MDH (6.5×10^{-3} s^{-1}) and PrxQ (0.67 s^{-1}) by the wild type Trx*h*1 (**Table 1**).

KINETIC ANALYSIS OF THE INTERACTION BETWEEN MDH AND Trx

To thoroughly investigate the interaction between the MDH and Trx*h*1$_{CS}$, we determined the rate constants for association and dissociation with the Trx*h*1$_{CS}$ using SPR (**Figure 2A**). These data were fit to a 1:1 binding model, from which we obtained the association rate constant, $k_{on} = 2.5 \times 10^2$ M^{-1} s^{-1}, and dissociation rate constant, $k_{off} = 3.0 \times 10^{-4}$ s^{-1} (**Table 1**). MDH dissociated from the immobilized Trx*h*1$_{CS}$ during these measurements, although the increase in the SPR signals that were observed indicated the formation of an irreversible disulfide bond.

We then used gel filtration chromatography to determine the cause for MDH dissociation from the Trx*h*1$_{CS}$ mutant. To form a mixed disulfide intermediate complex between the Trx*h*1$_{CS}$ and MDH, 44 μM of the oxidized form of MDH was incubated with 160 μM Trx*h*1$_{CS}$ for 30 min at 25°C. This protein mixture was then applied to the TSK-G2000SW$_{XL}$ column and the desired peak with a retention time of 13.5 min that contained the mixed disulfide intermediate complexes was fractionated (orange colored portion in **Figure 2B**). The thiol alkylation reagent NEM was immediately added to this collected fraction and chromatography was repeated (**Figure 2B**).

When NEM was added to this fraction, the mixed disulfide intermediate complexes were maintained during the second round of chromatography and the protein peak on the second chromatography run was observed at the same retention time. In contrast, the intermediate protein complexes readily dissociated and MDH dimers were observed when the second round of chromatography was performed without NEM treatment in advance. These results clearly indicated that the dissociation of the

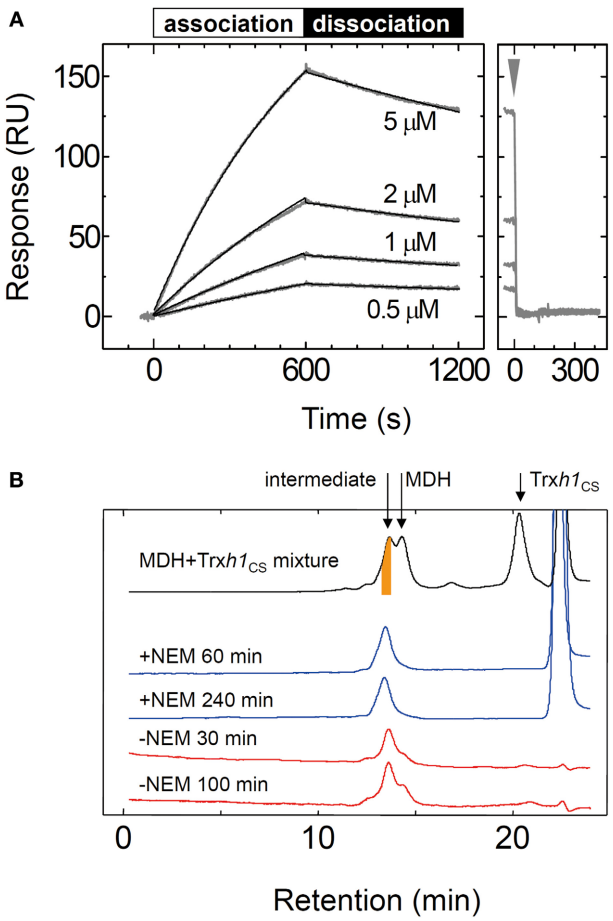

FIGURE 2 | Interaction between the oxidized form of the MDH and Trx*h*1$_{CS}$ proteins. (A) To investigate the association and dissociation phases, the indicated concentrations of MDH were injected for 600 s, followed by injection by buffer for additional 600 s (left panel) and the SPR signals were recorded. Fitted curves are indicated by the black lines on the SPR sensorgram. The association and dissociation phases are indicated by bars on the sensorgram. The arrow indicates the time when 5 mM DTT was injected (right panel). **(B)** Mixed disulfide intermediate complexes formed by the Trx*h*1$_{CS}$ and oxidized MDH were separated by gel filtration chromatography (TSK-G2000SW$_{XL}$) (black trace). The intermediate complexes (orange colored portion) were fractionated and then applied to the same column chromatography after incubation for the indicated period in the presence (blue trace) or the absence (red trace) of 10 mM NEM.

mixed disulfide intermediate proteins occurred following nucleophilic attack on the disulfide bond by the thiol residue on the target protein, presumably observed as dissociation on the SPR sensorgram.

KINETIC ANALYSIS OF THE INTERACTION BETWEEN PrxQ AND Trx

The sensorgram for the interaction between the Trx*h*1$_{CS}$ and PrxQ is presented in **Figure 3A**. Both association and dissociation phases revealed a biphasic interaction. Thus, these data were fit to a double-exponential equation, from which we obtained two rate constant pairs (**Table 1**). To identify the protein complexes involved in these observed biphasic reactions, 50 μM

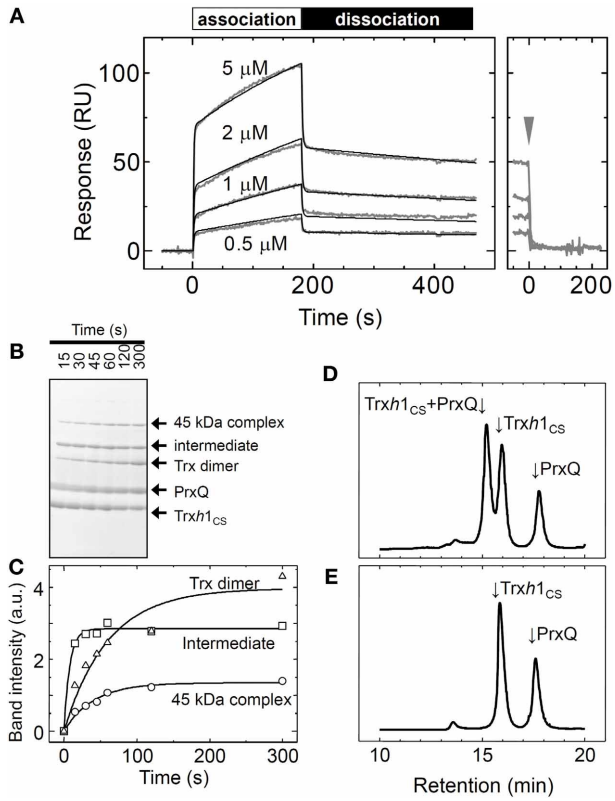

FIGURE 3 | Interaction between the oxidized form of the PrxQ and Trx*h*1$_{CS}$ proteins. (A) Same experiment as presented in **Figure 2A**, except using PrxQ as the analyte. **(B)** Trx*h*1$_{CS}$ (50 μM) and PrxQ (50 μM) were incubated for the periods indicated. The reaction was then quenched with 5% TCA (w/v). Proteins were separated by non-reducing SDS-PAGE. **(C)** Band intensities of the mixed disulfide intermediate complexes (intermediate), 45 kDa complexes, and the Trx*h*1$_{CS}$ dimers (Trx dimer) obtained from SDS-PAGE presented in panel **(B)** were determined using a Scion image software and plotted. a.u.; arbitrary units. **(D)** Trx*h*1$_{CS}$ (50 μM) and oxidized PrxQ (50 μM) were incubated for 3 min at room temperature, and the complexes that formed were then incubated with 10 mM NEM. The protein mixture was then loaded onto G2000SW$_{XL}$ and separated. **(E)** Same experiment as in **(D)** except for incubation in the absence of NEM.

Trx*h*1$_{CS}$ and 50 μM PrxQ were incubated for the periods indicated (**Figures 3B,C**). Mixed disulfide intermediate complex formation reactions were then quenched by adding 5% TCA (w/v) and proteins were separated by non-reducing SDS-PAGE.

From N-terminal amino acid sequence analysis, we confirmed that the two bands labeled "45 kDa" and "intermediate" were composed of the Trx*h*1$_{CS}$ and PrxQ through intermolecular disulfide bonds (**Figure 3B**). Although the stoichiometry for these proteins in this complex remains to be determined, the 45 kDa protein could be composed of one Trx*h*1$_{CS}$ and two PrxQ molecules linked through the intermolecular disulfide bonds. The apparent formation rate for the one-to-one mixed disulfide intermediate complex between the Trx*h*1$_{CS}$ and PrxQ was sufficiently fast and formation of this complex reached a plateau in less than 15 s.

The Trx*h*1$_{CS}$ dimer formation and 45 kDa band formation occurred after this mixed disulfide intermediate complex was formed (**Figure 3C**). Because the SPR sensorgram additionally revealed two binding phases, faster and slower, we concluded that the faster phase most possibly corresponded to the formation of the mixed disulfide intermediate complex and the latter corresponded to the formation of the 45 kDa protein complex (**Figure 3C**).

We then used gel filtration chromatography to determine whether the rapid dissociation rate ($k_{off-rapid} = 0.62\ s^{-1}$) observed with SPR measurements corresponded to the release of the target protein from the mixed disulfide intermediate complex. For this purpose, 50 μM Trx*h*1$_{CS}$ and 50 μM PrxQ were incubated for 3 min at 25°C to prepare mixed disulfide intermediate complexes, and then this protein mixture was treated with 10 mM NEM. Proteins were then loaded onto the TSK-G2000SW$_{XL}$. Mixed disulfide intermediate complexes were only observed when prior treatment with NEM was used (**Figures 3D,E**). This indicated that mixed disulfide intermediate complexes comprising the Trx*h*1$_{CS}$ and PrxQ would completely dissociate into monomeric proteins during gel filtration chromatography, similar to the MDH/Trx*h*1$_{CS}$ complexes when the second cysteines on PrxQ were functional.

These data suggested that the rate constants obtained for the rapid phase presented in **Table 1** corresponded to the formation and reverse dissociation reactions of mixed disulfide intermediate complexes.

REDOX STATE OF THE TARGET PROTEINS IS REQUIRED FOR EFFICIENT INTERACTION

Trx obviously recognizes the redox state of the target proteins *in vivo* to efficiently reduce them. We aimed to determine whether the redox state of the target protein affects the affinity between the target protein and Trx. We measured the binding of MDH and PrxQ as analytes to the Trx*h*1$_{CS}$ by SPR. When the reduced form of MDH was injected onto the Trx*h*1$_{CS}$ immobilized on a CM5 sensor chip, no SPR signals were detected, although sufficient binding could be detected when oxidized MDH was used (**Figure 4A**).

In contrast, the reduced form of PrxQ could partially associate with the Trx*h*1$_{CS}$, although the sensorgram clearly revealed a quantitative difference as compared to the oxidized form of PrxQ (**Figure 4B**). In fact, the reduced form of PrxQ exhibited immediate association and dissociation with the Trx*h*1$_{CS}$ in a manner similar to that of the oxidized form of PrxQ. However, the SPR signals with the reduced form of PrxQ disappeared immediately when buffer was injected onto the sensor chip, which indicated that the affinity between the Trx*h*1$_{CS}$ and the reduced form of PrxQ was very weak.

SIGNIFICANCE OF PROTEIN–PROTEIN INTERACTIONS

Finally, to investigate the contribution of the surface properties of Trx for its interaction with the target proteins, we examined the association of Trx*h*1$_{SS}$ with PrxQ by substitution of both the cysteines with serine. As presented in **Figure 5A**, the Trx*h*1$_{SS}$ interacted with the oxidized form of PrxQ. This interaction between the Trx*h*1$_{SS}$ and the oxidized form of PrxQ was not affected by alkylation of the additional cysteine at position 11 of the immobilized Trx*h*1$_{SS}$ using 10 mM NEM (**Figure 5B**). This suggested that

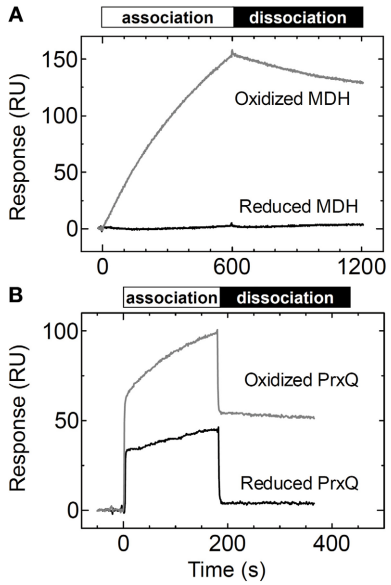

FIGURE 4 | Changes in affinity based on the redox states of the target proteins. (A) Reduced or oxidized MDH (5 μM) was injected onto the Trx*h*1$_{CS}$ immobilized on a sensor chip and SPR signals were recorded. **(B)** Reduced or oxidized PrxQ (5 μM) was injected onto the Trx*h*1$_{CS}$ immobilized on a sensor chip and SPR signals were recorded.

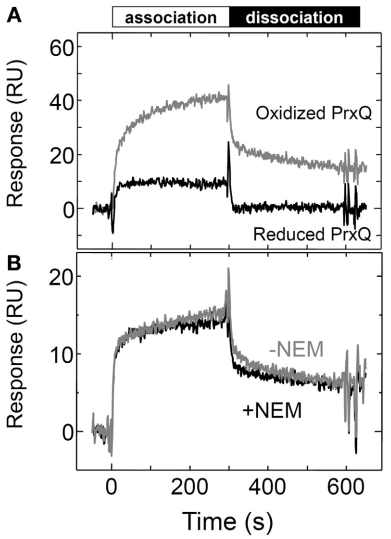

FIGURE 5 | Interactions between the Trx*h*1$_{SS}$ and the reduced and oxidized forms of PrxQ. (A) Reduced or oxidized PrxQ (5 μM) was injected onto the Trx*h*1$_{SS}$ immobilized on a sensor chip and SPR signals were recorded. **(B)** The oxidized form of PrxQ (2 μM) was injected onto Trx*h*1$_{SS}$ immobilized on a CM5 sensor chip before (gray line) and after (black line) thiol alkylation. Thiol alkylation was accomplished by injecting 10 mM NEM for 10 min onto the sensor chip.

the observed binding of the oxidized form of PrxQ to the immobilized Trx*h*1$_{SS}$ was due to a protein–protein interaction without intermolecular disulfide bond formation. The observed binding rate was slower than that for the Trx*h*1$_{CS}$.

DISCUSSION

Several studies attempted to evaluate the affinities of Trx to its target proteins or the reduction efficiencies of the target proteins by determining K_m or k_{cat}/K_m values obtained from the results of complete reduction of the target proteins using Trx (Collin et al., 2003; Perez-Perez et al., 2009; Maeda et al., 2010). However, a rather enigmatic behavior of Trx remained unresolved; the reduced form of Trx preferentially interacts with the oxidized form of the target protein and the oxidized form of Trx must release the reduced form of the target in order to ensure an efficient reduction cycle. To address this issue, we applied the direct binding measurements to detect the associations and dissociations of Trx with its target proteins.

For this purpose, MDH, PrxQ, and roGFP were selected as model target proteins. Cytosolic MDH is reportedly a target protein for Trx*h*1 (Hara et al., 2006) whose activity is regulated by its reduction/oxidation. Therefore, we defined this type of target protein as a "switch" type target for Trx. In contrast, PrxQ uses reducing equivalents provided by Trx for catalysis; thus, we defined it as a "catalytic" type target. Although PrxQ is localized in the chloroplasts of higher plants, cytosolic Trx can be an efficient reductant for this peroxiredoxin *in vitro*, as previously described (Rouhier et al., 2004). In addition, the reduction rate of PrxQ by Trx*h*1 ($0.67\ s^{-1}$) obtained in this study was comparable to or higher than the reduction rate of PrxQ reduced by chloroplast type Trx (Collin et al., 2004; Perez-Perez et al., 2009). In this study, we selected a combination of PrxQ and Trx*h*1 for our binding study because PrxQ has only one pair of cysteines in its monomeric molecule and its reaction mode is the simplest among peroxiredoxins.

When the wild type Trx*h*1 was used as a ligand, no SPR signals were detected with MDH as the analyte. Based on the reaction mechanism of Trx-mediated reduction of a target disulfide bond, the target protein should be immediately released from Trx when the reduction reaction is complete. This implies that the number of bound target proteins on immobilized Trx should be statistically limited by the duration of the reaction period from the formation of a mixed disulfide bond to its reduction. The time during which the target protein remains on immobilized Trx may be too short to allow for detecting the bound molecules on the gold surface of an SPR sensor chip. In addition, when the reduction reaction is completed by the dithiol–disulfide exchange reaction, immobilized Trx*h*1 adopts its oxidized form and can no longer reduce the target protein because there is no way to simultaneously reduce immobilized Trx*h*1 alone. Thus, a direct analysis of the binding affinity of Trx to its target protein is difficult.

Therefore, we used the immobilized Trx*h*1$_{CS}$, which had been used for Trx affinity chromatography (Yamazaki et al., 2004), as the ligand in our SPR measurements. Because the Trx*h*1$_{CS}$ could efficiently capture the target protein molecules through intermolecular disulfide bond formation, we expected that this mutant Trx could be used for observing the formation of a mixed disulfide intermediate complex, which is the initial contact of Trx and its target protein during the entirety of a reduction reaction. In contrast, a Trx*h*1$_{SC}$ mutant, a Trx*h*1 mutant with a C40S substitution,

was not used for these measurements because this mutant could not capture any specific target proteins when used for Trx affinity chromatography (Yamazaki et al., 2004).

As presented in **Figure 1**, SPR signals for the association of the analyte protein with the ligand were successfully obtained by using the Trx*h*1$_{CS}$ as the ligand. In contrast, the Trx*h*1$_{CS}$ did not associate with roGFP, which contains a disulfide bond that cannot be reduced by Trx. This indicated that the immobilized Trx*h*1$_{CS}$ maintained specificity for the target proteins. In addition, the remarkable differences between the binding time courses for MDH and PrxQ (**Figure 1**) suggested that the method applied in this study was useful for our purposes.

Because the fast dissociation rate of the analyte from the mixed disulfide intermediate complex by the reverse reaction ($k_{off-rapid} = 0.62\ s^{-1}$) appear to pose difficulty in accurate determination of the association rate constant when performing SPR analysis using PrxQ, we concluded that the association rate constant for the formation of the mixed disulfide intermediate complex was in the order of $10^4\ M^{-1}\ s^{-1}$. In addition, the association rate constant for PrxQ was higher than that for MDH by two orders of magnitude. These results were comparable to the reported differences in the reduction rates of PrxQ and MDH by the wild type Trx*h*1; $0.67\ s^{-1}$ and $6.5 \times 10^{-3}\ s^{-1}$, respectively (**Table 1**).

Why did Trx (Trx*h*1$_{CS}$) that mimics the reduced form of Trx interact with the oxidized form of MDH but not with the reduced form as presented in **Figure 4A**? A significant conformational change during the transition between the oxidized form and the reduced form of an MDH dimer has been suggested by size exclusion chromatography and analytical ultracentrifugation (Hara et al., 2006). This large conformational change must be critical for forming an intermolecular disulfide bond between Cys330–Cys330. In addition, the structure or the molecular surface of MDH for its interaction with Trx may only be exposed in its oxidized form but not its reduced form because on the basis of its reported crystal structure, the two Cys330 residues are located at the opposite sides in an MDH dimer molecule. This may be the reason for interaction of the Trx*h*1$_{CS}$ with the oxidized form of MDH but not with the reduced form as presented in **Figure 4A**. In addition, a redox-dependent large conformational change has been reported for the other switch type target protein, HSP33. For HSP33, which is known to be a redox-dependent chaperone that contains four redox responsive cysteines, the domain that contains Trx-targeted disulfide bonds is largely unfolded in the oxidized form, although the reduced form of HSP33 is completely folded and coordinates zinc in this domain (Ilbert et al., 2007).

In contrast, the Trx*h*1$_{CS}$ could bind to the reduced form of PrxQ as presented in **Figure 4B**. On the basis of the reported structures of the reduced and oxidized forms of PrxQ from *Aeropyrum pernix* (*Ap*PrxQ), a redox-dependent conformational change was clearly observed at the region that contained two active cysteines (Perkins et al., 2012). In contrast, molecular modeling for BCP1, the homolog of PrxQ in *Sulfolobus solfataricus*, and its reductase, *Ss*PDO (Limauro et al., 2010), revealed that the stable regions such as the end of β3 and α3 were involved in the molecular interaction, although these regions do not change their conformations in *Ap*PrxQ in a redox-dependent manner. These reports suggest that a certain stable region on a PrxQ protein can interact with its partner protein irrespective of their redox states. The reduced form of PrxQ may have a similar interaction region on its molecular surface, and it may have caused the relatively weak association between the Trx*h*1$_{CS}$ and the reduced form of PrxQ in our SPR experiments.

In this study, we conclusively confirmed that the redox state of the target protein was the significant determinant for its affinity to Trx. As presented in **Figure 4**, MDH and PrxQ altered their affinities for Trx*h*1. As previously noted, the Trx*h*1$_{CS}$ cannot associate with the reduced form of MDH. The amount of the reduced form of PrxQ that associated with Trx was definitely less than that of its oxidized form. These results strongly suggest that the interaction between Trx and an oxidized target protein exhibits a higher affinity than that between Trx and the reduced form, a prerequisite for efficient association and subsequent dissociation.

In addition, the Trx*h*1$_{SS}$ exhibited a preference for the oxidized target protein, even though its active domain lacks two cysteines (**Figure 5**). This indicates that the protein–protein interaction defined by the molecular surfaces of Trx and its target proteins is an important determinant for the redox-dependent selectivity of Trx, in addition to the cysteines in the catalytic domain of Trx and the rate of formation of the mixed disulfide intermediate complex. Similarly, the interaction between a Trx mutant that lacked two cysteines and its target ATP synthase complex was previously reported (Stumpp et al., 1999).

Considering together, these data reveal that the efficient reduction of the target proteins by Trx is accomplished by a remarkable change in the affinity between Trx and its target protein before and after the dithiol–disulfide exchange reaction. This selectivity is defined by these protein–protein interactions. The key molecular determinants of Trx that confer these interesting properties need to be elucidated by structural analysis of the Trx–target protein co-complexes.

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Plant cytoplasmic GAPDH: redox post-translational modifications and moonlighting properties

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous enzyme involved in glycolysis and shown, particularly in animal cells, to play additional roles in several unrelated non-metabolic processes such as control of gene expression and apoptosis. This functional versatility is regulated, in part at least, by redox post-translational modifications that alter GAPDH catalytic activity and influence the subcellular localization of the enzyme. In spite of the well established moonlighting (multifunctional) properties of animal GAPDH, little is known about non-metabolic roles of GAPDH in plants. Plant cells contain several GAPDH isoforms with different catalytic and regulatory properties, located both in the cytoplasm and in plastids, and participating in glycolysis and the Calvin-Benson cycle. A general feature of all GAPDH proteins is the presence of an acidic catalytic cysteine in the active site that is overly sensitive to oxidative modifications, including glutathionylation and S-nitrosylation. In Arabidopsis, oxidatively modified cytoplasmic GAPDH has been successfully used as a tool to investigate the role of reduced glutathione, thioredoxins and glutaredoxins in the control of different types of redox post-translational modifications. Oxidative modifications inhibit GAPDH activity, but might enable additional functions in plant cells. Mounting evidence support the concept that plant cytoplasmic GAPDH may fulfill alternative, non-metabolic functions that are triggered by redox post-translational modifications of the protein under stress conditions. The aim of this review is to detail the molecular mechanisms underlying the redox regulation of plant cytoplasmic GAPDH in the light of its crystal structure, and to provide a brief inventory of the well known redox-dependent multi-facetted properties of animal GAPDH, together with the emerging roles of oxidatively modified GAPDH in stress signaling pathways in plants.

Keywords: cysteine thiols, glycolytic glyceraldehyde-3-phosphate dehydrogenase, moonlighting protein, redox modifications, S-nitrosylation

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous enzyme involved in glycolysis that catalyzes the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglyceric acid (BPGA) in the presence of nicotinamide adenine dinucleotide (NAD^+) and inorganic phosphate. Whereas animal cells contain only one isoform of GAPDH, plants contain several isoforms of GAPDH that are encoded by different types of genes (*gapA*, *gapB*, *gapC* and *gapCp*) and are located in different subcellular compartments. The products of *gapA* and *gapB* genes give rise to A_2B_2- and A_4-GAPDH isozymes of chloroplasts that participate to the Calvin-Benson cycle (Michelet et al., 2013, submitted). Genes *gapC* and *gapCp* code for the subunits of glycolytic GAPDH isoforms, located either in the cytoplasm (GAPC) or in plastids (GAPCp; Petersen et al., 2003; Muñoz-Bertomeu et al., 2009, 2010). Depending in the species, each type of *gap* gene may be further duplicated, for example Arabidopsis contains two *gapA*, two *gapC* and two *gapCp* genes, but only a single *gapB*. All these GAPDH isoforms are defined phosphorylating, because they all catalyze *in vitro* the phosphorylation of the substrate G3P. However, phosphorylating GAPDHs of the Calvin-Benson cycle (A_2B_2- and A_4-GAPDH) physiologically act in the opposite direction, and therefore catalyze the dephosphorylation of the substrate BPGA. In addition, the cytoplasm of plants also contains a non-phosphorylating GAPDH (GAPN) that oxidizes G3P directly to 3-phosphoglycerate and can bypass the GAPC-catalyzed reaction (Rius et al., 2006). GAPN belongs to the aldehyde dehydrogenase superfamily, and has no close functional/structural relationships with phosphorylating GAPDHs (Michels et al., 1994). Moreover, GAPN catalyses an NADP-specific irreversible reaction that represents an additional source of NADPH for the cytoplasm, whereas the reaction catalyzed by GAPC and GAPCp isozymes is reversible and strictly specific for NAD(H).

Glyceraldehyde-3-phosphate dehydrogenase, however, is not only an enzyme, but also a moonlighting protein. In animal cells, the concept of moonlighting proteins was introduced after the discovery of proteins that were able to perform additional

functions in respect to their originally assigned ones (Jeffery, 1999; Kim and Dang, 2005). Often, additional and original functions were completely unrelated. A prototype of moonlighting proteins in animal cells is GAPDH that, besides acting as a glycolytic enzyme, is involved in several alternative functions including, among many others, DNA stability, control of gene expression and apoptosis (Sirover, 2012 and references therein). Several additional roles of GAPDH are linked to redox modifications of its catalytic cysteine that besides blocking its catalytic activity, have also profound effects on the capacity of GAPDH to interact with other proteins and eventually change its subcellular localization.

In contrast with the well established moonlighting properties of animal GAPDH, little is known on the multifunctional roles of plant cytoplasmic GAPDH, here referred as GAPC. Nonetheless, GAPC was identified as a potential target of diverse redox modifications and these modifications appear to be related to changes in subcellular localization in plant cells as well. Emerging evidence clearly show that, similar to animal GAPDH, plant cytoplasmic GAPDH may also perform alternative functions that are completely unrelated to its catalytic activity, strongly suggesting that plant GAPDH may also behave as a moonlighting protein.

The aim of this review is to provide an overview of what is known on plant cytoplasmic GAPDH, starting from its crystal structure and the structural features that determine its pronounced redox sensitivity. A comprehensive description of the molecular mechanisms underlying redox regulation of plant GAPCs will be provided, and a brief inventory of the redox-dependent multi-facetted properties of animal GAPDH will constitute a framework for the emerging role of oxidatively modified GAPDH in stress signaling pathways in plant cells.

STRUCTURE AND REACTION MECHANISM OF GAPDH: AN ABUNDANT PROTEIN WITH A HIGHLY REACTIVE CATALYTIC CYSTEINE

All types of phosphorylating GAPDHs, both in plants and non-photosynthetic organisms, either involved in glycolysis or in the Calvin-Benson cycle, share a similar structure with variations on the theme. GAPDH is essentially a tetramer of identical or similar subunits, with the same overall organization in which the subunits are related to three symmetry axis. Subunits are identical in glycolytic GAPDHs (GAPC, GAPCp) and in A_4-GAPDH of oxygenic phototrophs, and similar in the main photosynthetic isoform of land plants (A_2B_2-GAPDH). B-subunits share a high sequence homology with A-subunits but contain an extra sequence of 30 amino acids (C-terminal extension, CTE) that is essential for thioredoxin-based regulation and required for the association of A_2B_2-GAPDH isoforms into tetramers of tetramers (A_8B_8-GAPDH; Trost et al., 2006). In all types of GAPDH, each subunit contains the binding sites for the coenzyme (NAD(P)(H)) and for the substrates/products (G3P, P_i, BPGA). This latter site hosts also the intermediates formed during the catalytic cycle via covalent modification of the active site cysteine.

Several structures of GAPDH isoforms from animal, microbial and plant sources are available (**Table 1**), although cytoplasmic GAPDH from rice (*Oryza sativa*; OsGAPC) is the only known crystal structure of a glycolytic GAPDH from a photosynthetic organism (PDB code 3E5R, Tien et al., 2012). Rice GAPC shares a very high sequence homology with GAPC from flowering plants, e.g., 85% identical to *A. thaliana* GAPC, and GAPDH genes of the GAPC-type are generally quite well conserved in eukaryotes and eubacteria (e.g., OsGAPC is 69% identical to *H. sapiens* GAPDH, and 50% identical to *B. stearothermophilus* GAPDH). Moreover, the crystal structure of OsGAPC can be nicely superimposed to known GAPDH structures of animals or eubacteria. Plastidic GAPCp forms are also quite similar in sequence to cytoplasmic counterparts from which they arose by gene duplication in early chloroplasts evolution (Petersen et al., 2003), but no crystal structure of GAPCp is yet available. Sequence identity decreases to about 40% when GAPC is compared to GAPA and GAPB subunits of chloroplastic GAPDH isozymes, but still the overall structure of these proteins remains closely related.

This review is focused on GAPC-type GAPDH and a brief overview of the only known GAPC structure from plants is given. OsGAPC is a tetramer of four identical subunits related by three symmetry molecular axis (**Figure 1A**). The homotetramer is stabilized by several hydrogen bonds between monomers (or subunits). The monomer structure consists of two domains: a cofactor-binding domain comprising residues 1–153 and 318–337, and a catalytic domain composed of residues 154–317 (**Figure 1B**). The cofactor binding domain shows an α/β folding pattern typical

Table 1 | List of selected GAPDH isoforms from diverse organisms for which the three-dimensional structure has been solved.

Pdb code	Organism	Nomenclature	Metabolic pathway	Reference
1GD1	*Bacillus stearothermophilus*	GAPDH	Glycolysis/gluconeogenesis	Skarzyński et al. (1987)
1GPD	*Homarus americanus*	GAPDH	Glycolysis/gluconeogenesis	Moras et al. (1975)
1ZNQ	*Homo sapiens*	GAPDH	Glycolysis/gluconeogenesis	Ismail and Park (2005)
3E5R	*Oryza sativa*	GAPC	Glycolysis/gluconeogenesis	Tien et al. (2012)
1JN0	*Spinacia oleracia*	A_4-GAPDH	Calvin-Benson cycle	Fermani et al. (2001)
2PKQ	*Spinacia oleracia*	A_2B_2-GAPDH	Calvin-Benson cycle	Fermani et al. (2007)
2EUH	*Streptococcus mutans*	GAPN	Glycolysis	Cobessi et al. (1999)

Only GAPDH isoforms that are cited in the text are listed.

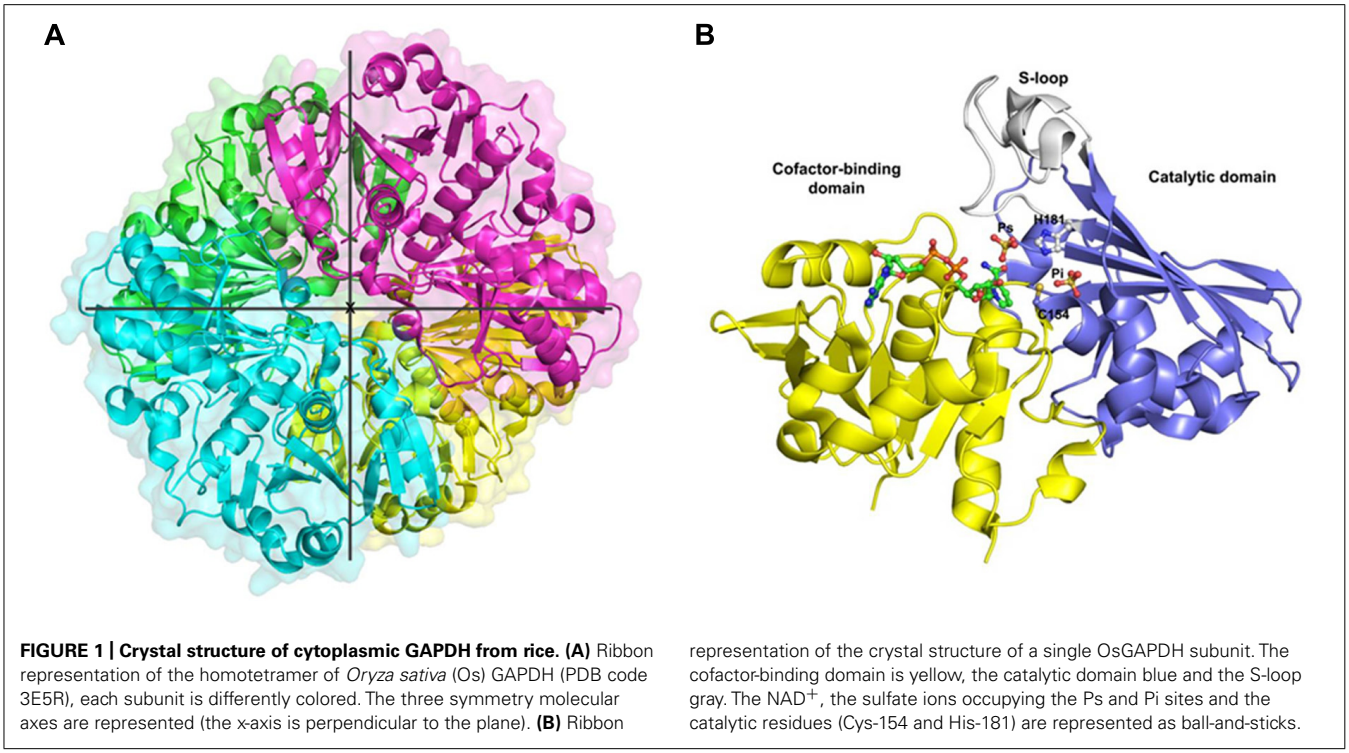

FIGURE 1 | Crystal structure of cytoplasmic GAPDH from rice. (A) Ribbon representation of the homotetramer of *Oryza sativa* (Os) GAPDH (PDB code 3E5R), each subunit is differently colored. The three symmetry molecular axes are represented (the x-axis is perpendicular to the plane). **(B)** Ribbon representation of the crystal structure of a single OsGAPDH subunit. The cofactor-binding domain is yellow, the catalytic domain blue and the S-loop gray. The NAD^+, the sulfate ions occupying the Ps and Pi sites and the catalytic residues (Cys-154 and His-181) are represented as ball-and-sticks.

of the Rossmann fold (Rossmann et al., 1975) with a central β-sheet composed of six parallel strands plus two extra antiparallel β-strands and five α-helices on both sides of the sheet. The additional segment of the NAD-domain (residues 318–337) folds in a long α-helix corresponding to the C-terminal region of the monomer. The catalytic domain is formed by three α-helices and eight β-strands and contains a long ordered loop called S-loop, stretching from residue 181 to 209, which extends toward the adjacent subunit in close proximity to the cofactor nicotinamide moiety (**Figure 1B**).

A NAD^+ molecule is bound to each enzyme subunit and anchored by several hydrogen bonds, either directly or through water molecules (Tien et al., 2012). In particular, the backbone atoms of the highly conserved N-terminal segment (Asn-9 to Arg-16: NGFGRIGR; **Figure 2**) interacts with the pyrophosphate moiety of the coenzyme, and the carboxyl group of Asp-35 (another strictly conserved residue of glycolytic GAPDHs, **Figure 2**) makes two hydrogen bonds with two adjacent hydroxyl groups (in position 2′ and 3′) of the ribose molecule linked to the adenine of NAD^+. Other residues from the same subunit, and Asp-191 from the catalytic domain of the adjacent subunit further contribute to stabilize the cofactor. The correct holding of the adenine moiety of bound NAD^+ is also determined by the conformation of Phe-37 side chain (conserved in glycolytic GAPDHs from higher plants and animals, but not in prokaryotes, **Figure 2**), that is 90°-rotated in apo-OsGAPC to allow the access of NAD^+ to the coenzyme binding site (Tien et al., 2012).

The structure of the active site of cytoplasmic OsGAPDH is very similar to that of other glycolytic and also chloroplastic GAPDHs. Sequence alignments show a highly conserved region between residues 150 and 161 (**Figure 2**), corresponding to the substrate binding site. The two most important catalytic residues are Cys-154 (corresponding to Cys-149 in *H. americanus* and *B. stearothermophilus* GAPDHs, the first solved GAPDH structures, **Table 1**) and His-181 (corresponding to His-176 in *H. americanus* and *B. stearothermophilus* GAPDHs; **Figure 2**). The side-chain of Cys-154 points toward His-181 and the distance between the sulfur atom of the first residue and the closest nitrogen atom (NE2) of the second residue is 4.03 Å on average (**Figure 3A**). The minimum distance of Cys-154 from the coenzyme nicotinamide ring is 3.69 Å (**Figure 3A**).

The presence of basic residues as histidines, arginines or lysines located in a close proximity with a cysteine residue generally at a distance lower or equal to 6 Å, enhances the deprotonation of the thiol (-SH) to the nucleophilic thiolate ($-S^-$), making the cysteine quite reactive to oxidative modifications. The positioning of the catalytic cysteine at the N-terminal side of an α-helix (α6), might further increase its reactivity due to the helix overall dipole moment (Roos et al., 2013). According to these observations, it has been proposed that the histidine acts as an acid-base catalyst, extracting a proton from the catalytic cysteine and thus favoring a nucleophilic attack of the thiolate species on the substrate carbonyl group that generates a hemithioacetal intermediate (Moras et al., 1975). The subsequent oxidation step leads to the formation of the acyl-enzyme intermediate as a result of a hydride transfer (assisted by the nearby histidine) from the hemithioacetal to the nicotinamide ring of the coenzyme. NADH is then released and rapidly replaced by a new NAD^+ molecule (**Figure 4**).

With the aim of evaluating the reactivity of the catalytic cysteine as a function of its chemical environment, the pK_a values for this residue were determined in GAPDHs

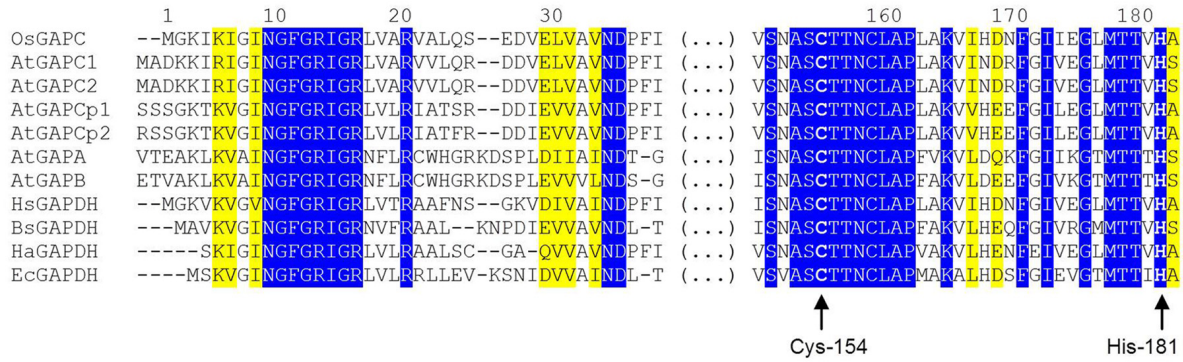

FIGURE 2 | Partial sequence alignment of GAPDH isoforms from different organisms. The alignment is focused on two highly conserved regions of GAPDH, one belonging to the coenzyme-binding domain and one belonging to the catalytic domain. The first (N-terminal) region includes the strictly conserved sequence NGFGRIGR and other important residues for the binding of the pyridine nucleotide cofactor (Asp-35, Phe-37). The second region, located in the central part of GAPDH sequences, contains several residues involved in substrate binding including the catalytic cysteine (Cys-154), but also a second cysteine close to the active site (Cys-158) and His-181, essential for activating Cys-154. Residues are numbered according to the sequence of *Oryza sativa* (Os) cytoplasmic GAPDH (GAPC). Abbreviation and accession numbers: OsGAPC, *Oryza sativa* GAPC, Q0J8A4.1; AtGAPC1, *Arabidopsis thaliana* GAPC1, AEE74039.1; AtGAPC2, *Arabidopsis thaliana* GAPC1, AEE29016.1; AtGAPCp1, *Arabidopsis thaliana* GAPCp1, Q9SAJ6.1; AtGAPCp2, *Arabidopsis thaliana* GAPCp2, Q5E924.1; AtGAPA, *Arabidopsis thaliana* GAPA, AEE77191.1; AtGAPB, *Arabidopsis thaliana* GAPB; HsGAPDH, *Homo sapiens* GAPDH, P04406.3; BsGAPDH, *Bacillus stearothermophilus* GAPDH, PDB code 2DBV; HaGAPDH, *Homarus americanus* GAPDH, P00357; EcGAPDH, *Escherichia coli* GAPDH, ACI83895.1. Invariant residues are on a blue background while yellow background indicated residues with strongly similar properties. Catalytic Cys-154 (corresponding to Cys-149 and Cys-156 in BsGAPDH and AtGAPCs, respectively) and His-181 (corresponding to His-176 and His-183 in BsGAPDH and AtGAPCs, respectively) are highlighted by arrows. The sequences were aligned with the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

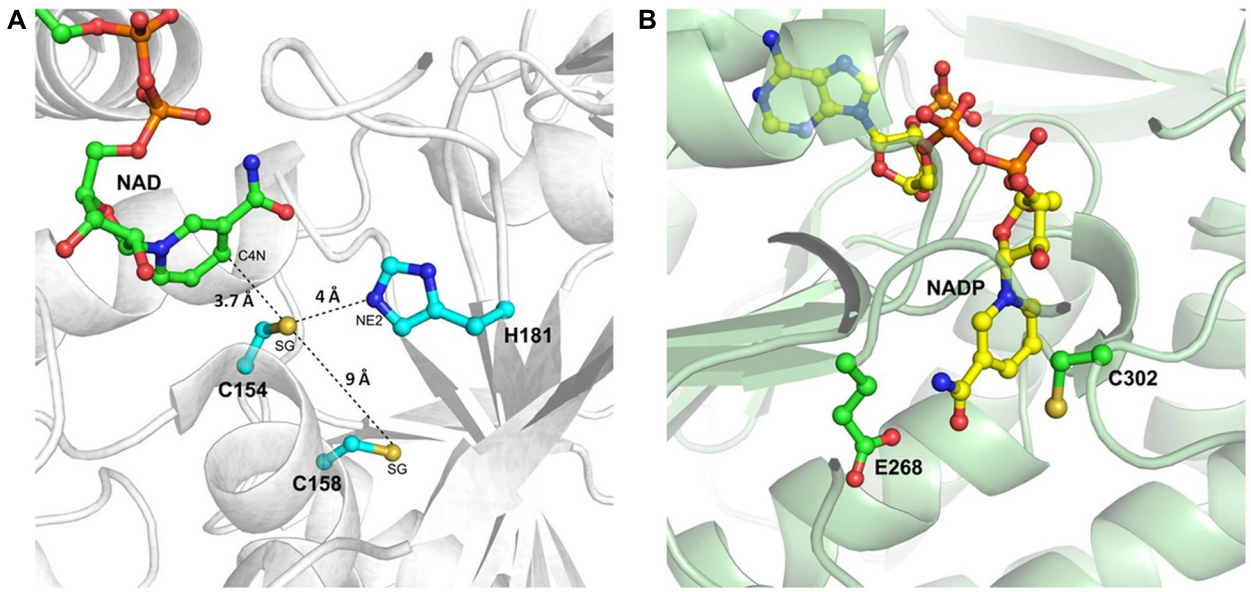

FIGURE 3 | The catalytic sites of rice cytoplasmic GAPDH and non-phosphorylating GAPDH from *Streptococcus mutants*. (A) Magnified representation of OsGAPC catalytic site (PDB code 3E5R). Important catalytic residues, the cofactor NAD^+ and residue Cys-158 are shown as ball-and-sticks. The distances of the sulfur atom (SG) of catalytic Cys-154 from the basic residue His-181 (atom NE2), the sulfur atom of Cys-158 and the cofactor (atom C4N) are indicated by dashed lines. **(B)** Catalytic site representation of *Streptococcus mutans* non-phosphorylating GAPDH (SmGAPN; PDB code 2EUH). The catalytic residues Cys-302 and Glu-268, and the cofactor $NADP^+$ are in ball-and-sticks representation.

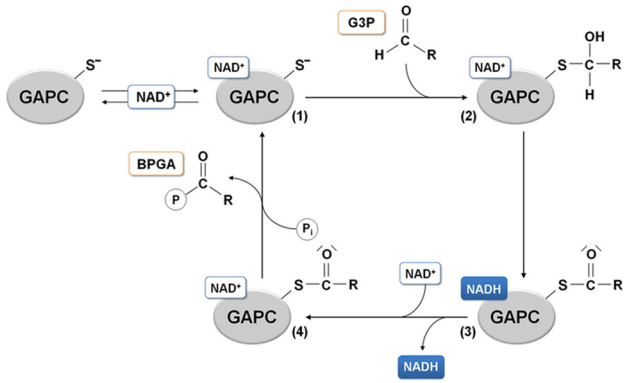

FIGURE 4 | GAPDH catalytic mechanism. In the glycolytic direction, the binding of the substrate glyceraldehyde-3-phosphate (G3P) to the holo-enzyme containing NAD^+ (1) leads to the formation of a hemithioacetal intermediate (2). In the next redox step, hydride transfer from the covalently bound substrate to the coenzyme allows formation of NADH, that can leave the acyl-enzyme (3) and be substituted by a molecule of NAD. The NAD-acyl-enzyme (4) can finally accept a free inorganic phosphate to release the product 1,3-bisphosphoglycerate (BPGA).

from different sources. Values of pK_a, estimated with different methods (cysteine titration with iodoacetamide or 2,2′-dipyridyl disulfide, or measurement of thiol ionization by UV absorbance), have been reported for pig liver GAPDH (5.5, Polgar, 1975), *E. coli* GAPDH (∼5.7, Talfournier et al., 1998), *B. stearothermophilus* GAPDH (5.9, Talfournier et al., 1998), and *A. thaliana* GAPC (5.7, Bedhomme et al., 2012). The corresponding pK_a measured for the activating histidine (His-181) is around 8.0 (Talfournier et al., 1998). These values are very similar among different GAPDHs and reflect similar distances between the catalytic cysteine and the histidine, as observed in all GAPDH crystal structures available (e.g., 3.6 Å in *B. stearothermophilus* GAPDH and 3.4 Å in *A. thaliana* A_4-GAPDH; average distance in different subunits). Overall, these values indicate that at physiological pH values the thiol group of the catalytic cysteine is deprotonated (i.e., thiolate, $-S^-$) and involved in an ion pair with the protonated histidine. Additional features such as the positioning at the N-terminus of an α-helix, or the tridimensional arrangement of amino acids in the active site, further contribute to the acidic character of the catalytic cysteine.

In the OsGAPC structure, a second cysteine (Cys-158 corresponding to Cys-153 in *H. americanus* and *B. stearothermophilus* GAPDHs) is located, four residues away, in the same α-helix of the catalytic cysteine (**Figures 2, 3A**). The thiol groups of these two cysteines are oriented in opposite directions at a distance of about 9 Å, indicating that the formation of a disulfide bridge between these two cysteines, without significant distortion of the α-helix, is unlikely. Consistently, no disulfide bond was observed for *A. thaliana* GAPC1 under conditions promoting disulfide formation such as in the presence of oxidized thioredoxins (Bedhomme et al., 2012).

As already mentioned, plant cells contain a cytoplasmic non-phosphorylating NADP-GAPDH (GAPN), with low sequence homology with classical phosphorylating GAPDH (∼20% with OsGAPC). The crystal structure of a GAPN from *Streptococcus mutans* (Sm) has been reported (**Table 1**; Cobessi et al., 1999). The catalytic mechanism comprises, similarly to GAPDHs, the formation of a covalent intermediate via the nucleophilic attack of the invariant Cys-302 on the aldhehydic function of G3P. However, the active site of SmGAPN has no basic amino acids with an equivalent role to the histidine found in GAPDHs (**Figure 3B**). Titration of Cys-302 in the apo-enzyme shows a pK_a of 8.5 that decreases to 6.1 upon $NADP^+$ binding (Marchal and Branlant, 1999). Indeed, the binding of the coenzyme permits the chemical activation of Cys-302 and the formation of an efficient ternary complex intermediate (GAPN plus G3P and $NADP^+$), through a local rearrangement of the active site and repositioning of the nicotinamide ring of the cofactor (D'Ambrosio et al., 2006). This suggests that GAPN uses a Cys activation mechanism alternative to the one observed for phosphorylating GAPDHs to achieve a similar catalytic pathway.

PLANT GLYCOLYTIC GAPDH: A PERFECT PROTEIN TOOL TO INVESTIGATE REDOX POST-TRANSLATIONAL MODIFICATIONS

Reactive oxygen and nitrogen species (ROS and RNS, respectively) play an important role in redox signaling mainly through a set of reversible post-translational modifications (PTMs) of cysteine thiols on proteins (Hess et al., 2005; Besson-Bard et al., 2008; Dalle-Donne et al., 2009; Zaffagnini et al., 2012a).

The reaction between ROS and RNS with protein thiols mainly involves cysteine residues with unique physico-chemical properties. These residues are considered reactive when their ionization constant (pK_a) is lower than that of free cysteines (8.3, Klomsiri et al., 2011), often ranging between 3 and 7. This implies that at physiological pH, these residues are predominantly found as thiolates (i.e., deprotonated, $-S^-$), and thiolates are much stronger nucleophiles than thiol counterparts (-SH). Protein containing reactive cysteines can undergo different types of redox modifications in response to different ROS- and RNS-dependent redox signals. Physiologically, the most relevant ones are disulfide bond formation (S-S, intra- or inter-molecular), glutathionylation (S-SG), S-nitrosylation (S-NO) and oxidation to sulfenic acid (S-OH). Due to its unstable nature, sulfenic acids can generate disulfide bonds by reacting with a second cysteine nearby, or evolve to irreversible oxidized forms such as sulfinic and sulfonic acids ($S-O_2H$ and $S-O_3H$, respectively). These redox PTMs are mostly controlled by oxidoreductases named thioredoxins (TRXs) and glutaredoxins (GRXs; Zaffagnini et al., 2012a).

Since 2001, numerous proteomic approaches have been developed to identify plant proteins putatively regulated by TRXs via dithiol/disulfide interchange or undergoing glutathionylation or S-nitrosylation (Besson-Bard et al., 2008; Lindahl et al., 2011; Zaffagnini et al., 2012a). Altogether, these studies allowed the identification of more than 500 putative targets involved in a wide array of cellular processes, including carbon metabolism, amino acid biosynthesis, redox control and stress responses (Lindahl et al., 2011; Michelet et al., this issue and references therein). However, compared to the large number of putative targets, very few studies

reported a detailed biochemical investigation of the functional and structural effects of these redox modifications on each target protein and the molecular mechanisms involved.

In redox proteomic studies, plant glycolytic GAPDH (GAPC) has been identified as a potential target of TRX regulation (i.e., dithiol/disulfide interchange) and glutathionylation (Marchand et al., 2004; Dixon et al., 2005; Zaffagnini et al., 2012b). GAPC was also found to undergo S-nitrosylation in rice plants with increased NO levels (Lin et al., 2012) and in protein extracts from Arabidopsis leaves or cell suspensions treated with artificially released nitric oxide (NO; Lindermayr et al., 2005). In another study, GAPC from *A. thaliana* was identified as the prominent target of H_2O_2-dependent oxidation in plant protein extracts (Hancock et al., 2005).

The identification of plant GAPC as a target of multiple redox modifications is consistent with the fact that GAPC contains a reactive catalytic cysteine (referred in the following as Cys-149) with a low pK_a (e.g., 5.7 in *A. thaliana* GAPC1, Bedhomme et al., 2012; see previous section). At physiological pH, this residue is largely deprotonated (thiolate) and thus highly sensitive to ROS- and RNS-dependent oxidation with consequent inhibition of protein activity. Based on these considerations, the recombinant GAPC protein from *A. thaliana* has been extensively used as a protein tool to determine the molecular mechanisms underlying the different types of redox modifications and to investigate the enzymes and/or molecules involved in the control of these modifications.

PLANT GAPC AS A TARGET OF PROTEIN SULFENATION

Protein sulfenation consists of the formation of a sulfenic acid after the reaction between a reactive cysteine and an oxidant molecule such as H_2O_2 through a two-electron oxidation process. The sensitivity of GAPC toward H_2O_2 has been tested by several research groups and in all cases a strong inhibition of enzyme activity was observed, suggesting that the highly reactive catalytic Cys-149 was indeed the target of H_2O_2 oxidation (Hancock et al., 2005; Bedhomme et al., 2012; Guo et al., 2012; Piattoni et al., 2013). The inhibitory effect of $H_2O_2$was also tested in the presence of GAPC substrates (BPGA in the reverse reaction and G3P in the forward reaction) and cofactor (NAD^+). Whereas the cofactor is not able to protect GAPC from H_2O_2, a full protection of enzyme activity was observed in the presence of either substrates, consistent with the fact that either BPGA or G3P can covalently bind GAPC catalytic cysteine (Bedhomme et al., 2012; see **Figure 4**).

Beyond sulfenation, a modification that is fully DTT-reversible, cysteine thiolates may be further oxidized to sulfinic and sulfonic acids (Poole et al., 2004; Zaffagnini et al., 2012a). These oxidized forms are not reduced back by DTT and are generally considered irreversible although, in the case of 2-Cys-peroxiredoxins, the over-oxidation of the catalytic cysteine to sulfinic acid can be reversed by reduction by sulfiredoxins. Sulfiredoxins, first discovered in yeast (Biteau et al., 2003), are common to eukaryotes including plants (Rey et al., 2007). However, no other proteins than 2-Cys-peroxiredoxin have been shown to be reduced by sulfiredoxins. In the case of GAPC, the reversibility of H_2O_2treatments was examined after incubating with DTT or GSH, either soluble extracts from Arabidopsis suspension cells or the purified recombinant protein (Hancock et al., 2005; Bedhomme et al., 2012; Guo et al., 2012). Although a partial recovery of protein activity was observed after GSH/DTT treatment of soluble extracts, H_2O_2 induced a nearly irreversible inhibition of purified recombinant GAPC (Bedhomme et al., 2012; Guo et al., 2012), suggesting that Cys-149 sulfenate is unstable and can evolve into over-oxidized forms at rates that depend on experimental conditions (e.g., factors present in crude extracts, such as glutathione (GSH), may modulate over-oxidation; **Figure 5**).

PLANT GAPC AS A TARGET OF PROTEIN GLUTATHIONYLATION

Protein glutathionylation, consisting in the formation of a mixed-disulfide between a protein thiol and a molecule of GSH, can act as a mechanism of protection against irreversible oxidation of reactive cysteine thiols but has also a role in modulating protein functions (Zaffagnini et al., 2012a). Although the precise mechanism leading to glutathionylation is still unclear *in vivo*, two major mechanisms may be envisioned: a GSSG-dependent thiol/disulfide exchange or a ROS-dependent sulfenic acid formation followed by reaction with reduced GSH. The role of glutathionylation for the protection of GAPC from over-oxidation was recently investigated (Bedhomme et al., 2012). The activity of GAPC was inhibited with similar kinetics by either H_2O_2 alone or by H_2O_2plus GSH, but only in the latter case DTT could totally recover the activity of the enzyme, indicating a reversible modification of catalytic Cys-149. A rational interpretation of this result is that GSH reacts with short-living Cys-149 sulfenates, generating a stable S-glutathionylated form that DTT can reduce, thus regenerating the protein thiol (**Figure 5**). Indeed, glutathionylation of GAPC by GSH plus H_2O_2 treatments was confirmed by MALDI-TOF mass spectrometry and immunodetection of biotin-labeled reduced GSH (BioGSH) (Bedhomme et al., 2012).

Glutathionylation of GAPC *in vitro* was also obtained with treatments with GSSG (Holtgrefe et al., 2008; Bedhomme et al., 2012). However, the effect on enzyme activity was limited because the K_{ox} of GAPC glutathionylation (i.e., the GSH/GSSG ratio at which 50% of the protein is glutathionylated) is very low (0.3 ± 0.05) compared with proteins that are easily glutathionylated by GSSG, like plastidial glutaredoxin S12 from poplar ($K_{ox} = 300$; Zaffagnini et al., 2012c). Therefore, the reaction of GAPC with GSSG might not have a significant role *in vivo*, since it requires GSH/GSSG ratios that are far lower than ever observed (Zaffagnini et al., 2012d and references therein). Moreover, the inhibitory effect of GSSG is completely blocked by either BPGA or NAD^+ (Bedhomme et al., 2012). BPGA protection is in agreement with its covalent binding to Cys-149 (**Figure 4**), and is different from the effect of NAD^+ that, although not linked to Cys-149, is close enough to shield Cys-149 from the attack of bulky GSSG, but not of smaller molecules like H_2O_2 and GSH. Overall, glutathionylation of GAPC is more likely to occur *in vivo* by reaction with both H_2O_2 and GSH, and can be considered either a mechanism of protection against over-oxidation or a mechanism of down-regulation of enzyme activity under oxidative stress conditions.

Removal of GSH from glutathionylated proteins (namely deglutathionylation) is, in most cases, a reaction that GSH cannot perform by itself with any significant efficiency. Glutathionylated

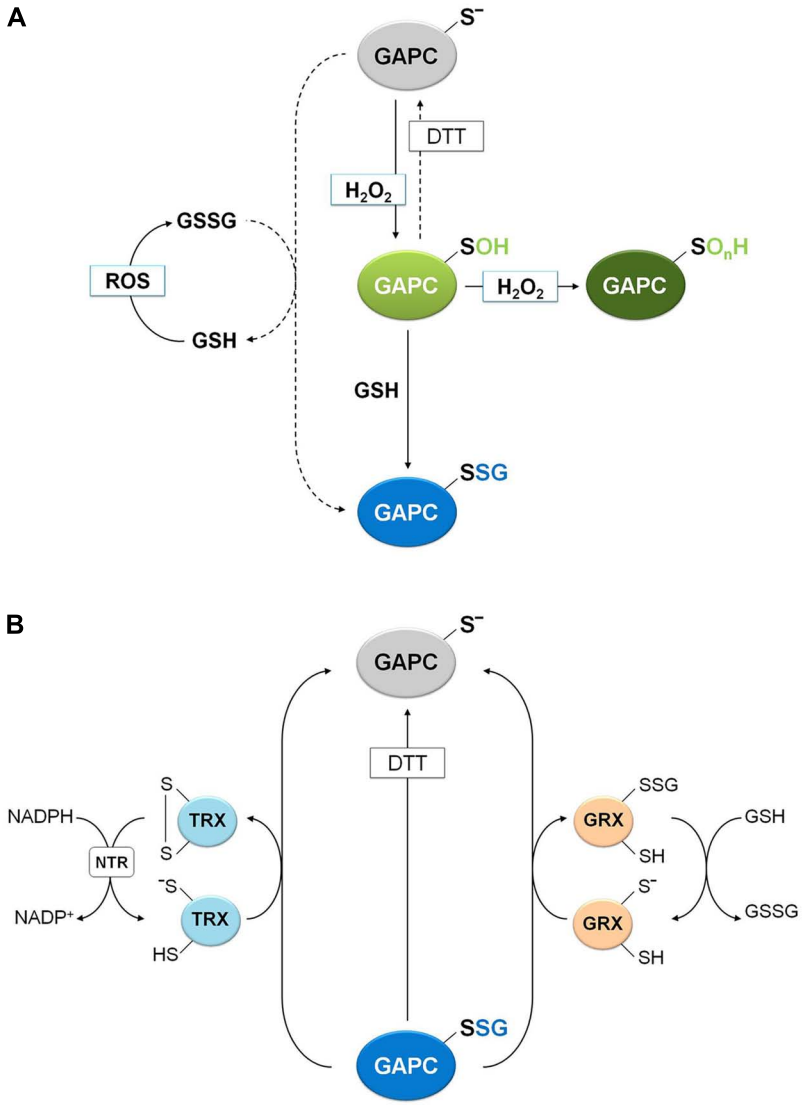

FIGURE 5 | Molecular mechanisms of plant GAPC glutathionylation and deglutathionylation. (A) Oxidation and glutathionylation of plant GAPC. Plant GAPC can undergo primary oxidation to sulfenic acid (GAPC-SOH) in the presence of H_2O_2 that can subsequently react with GSH leading to the glutathionylated form (GAPC-SSG) and protecting the enzyme from irreversible oxidation (GAPC-SO_nH). In principle, GAPC glutathionylation may also be performed by GSSG with release of GSH, but this reaction does not occur under physiological GSH/GSSG ratios (dashed arrow). GSSG can be formed by the reaction of GSH with ROS. **(B)** GRX- and TRX-dependent deglutathionylation mechanism of plant GAPC. The most reactive cysteine of both GRX and TRX (the N-terminal active-site cysteine) performs the nucleophilic attack on the glutathione-mixed disulfide on GAPC, resulting in the release of the reduced GAPC and the formation of a glutathionylated GRX intermediate (right side) or an oxidized TRX (left side). Subsequently, the mixed disulfide on GRX is reduced by a GSH molecule to form GSSG and reduced GRX and the oxidized TRX is reduced by NADPH-TRX reductase (NTR) in the presence of NADPH.

GAPC (GAPC-SSG) and GRXs were recently used to investigate the molecular mechanisms of deglutathionylation reactions in a plant system (Bedhomme et al., 2012). Reactivation tests of GAPC-SSG were conducted in the presence of cytoplasmic class I GRXC1 from poplar, which contains a -CysGlyTrpCys- active site, and two GRXC1 mutants, each lacking one of the two active site cysteines (Bedhomme et al., 2012). These analyses revealed that GRXC1 efficiently catalyzes the deglutathionylation of GAPC-SSG following a monothiol mechanism that only requires the N-terminal active site cysteine (**Figure 5**). The first step of this mechanism consists in the nucleophilic attack performed by the most N-terminal active site cysteine of GRX on the mixed disulfide of GAPC-SSG. This reaction releases reduced, i.e., active, GAPC and a glutathionylated GRXC1 intermediate. In a second step, GSH removes the glutathionyl moiety from glutathionylated GRXC1, regenerating the reduced (i.e., active) GRXC1 (**Figure 5**). Similar results were described for other class I GRXs such as mammalian GRX1 and GRX2 (Gallogly et al., 2008), yeast GRX1 (Discola et al., 2009) and Arabidopsis GRXC5 (Couturier et al., 2011).

Glutathionylated GAPC was also used to investigate the deglutathionylating activity of different plant TRXs. These enzymes are known to control the activity of a large number of proteins through dithiol/disulfide exchange reactions (Lemaire et al., 2007; Schürmann and Buchanan, 2008; Lindahl et al., 2011), but recently, they have been proposed as important factors controlling the glutathionylation state of yeast proteins (Greetham et al., 2010; Tan et al., 2010). Interestingly, deglutathionylation of GAPC-SSG was found to be catalyzed by different plant cytoplasmic h-type TRX isoforms, although much less efficiently than GRXC1 (Bedhomme et al., 2012; **Figure 5**). TRXs contain two active site cysteine residues and the N-terminal one performs the nucleophilic attack on the mixed disulfide (**Figure 5**). In principle, the mechanism of TRX-mediated deglutathionylation could either involve an intermediate in which GAPC and TRX are disulfide-linked, or a glutathionylated TRX intermediate, as in the GRX-catalyzed mechanism. Although this point could not be resolved, in both cases TRX ends up with a disulfide that requires NADPH and NADPH-thioredoxin reductase (NTR) to regenerate the original thiols (**Figure 5**).

PLANT GAPC AS A TARGET OF PROTEIN S-NITROSYLATION

Protein S-nitrosylation, consisting in the formation of nitrosothiols (S-NO) by reaction of protein thiols with NO, can be triggered chemically by RNS such as NO or by trans-nitrosylation reactions mediated by small nitrosothiols (e.g., nitrosoglutathione, GSNO) or by other S-nitrosylated proteins (Besson-Bard et al., 2008; Benhar et al., 2009; Astier et al., 2011; Yu et al., 2012). In the case of GAPC, NO-donors such as GSNO and sodium nitroprusside (SNP) were found to inhibit the NAD(H)-dependent GAPDH activity of total extracts of *A. thaliana*, and DTT was found to restore the activity almost completely (Lindermayr et al., 2005; Hancock et al., 2005). The mechanism of GSNO inhibition required further investigation since GSNO, besides acting as NO-donor, can also induce glutathionylation (Giustarini et al., 2005). Moreover, the inhibitory effect of SNP is controversial since the breakdown of this molecule (composed of a ferrous center surrounded by five tightly bound cyanide ligands and one linear NO ligand) releases one molecule of NO and five cyanide ions. Being cyanide reactive with protein thiols, it might contribute to inhibition. Recently, two papers addressed the question and demonstrated by the biotin switch method that GSNO induces S-nitrosylation of GAPC on its catalytic cysteine with concomitant inhibition of enzyme activity (Holtgrefe et al., 2008; Zaffagnini et al., 2013). Moreover, MALDI-TOF analysis demonstrated that S-nitrosylation of GAPC by GSNO largely prevailed over glutathionylation, both reactions being partially prevented by NAD^+ (Zaffagnini et al., 2013). S-nitrosylation of GAPC was further confirmed by using a specific NO-donor such as DEA-NONOate (Zaffagnini et al., 2013), indicating that S-nitrosylation of GAPC may occur either through direct reaction with NO or GSNO-dependent trans-nitrosylation (**Figure 6**). The structural determinants that allow GSNO to act as a specific nitrosylating agent of GAPC remain to be understood, although the existence of specific binding motifs for GSNO, composed of acidic and hydrophobic residues has been proposed (Hess et al., 2005).

The reduction of nitrosothiols on proteins, i.e., denitrosylation, entails two possible mechanisms, either dependent on reduced GSH or on the TRX system (NADPH, NADPH: TRX reductase and TRX; Benhar et al., 2009; Sengupta and Holmgren, 2011). The relative contribution of these two mechanisms was recently investigated using S-nitrosylated GAPC (GAPC-SNO) as a protein substrate (Zaffagnini et al., 2013). Cytoplasmic TRXs (h-type from either poplar or *Chlamydomonas reinhardtii*) were found to have little or no ability to denitrosylate GAPC-SNO in the presence of a complete TRX reducing system (TRXs plus NADPH and NTR b from *A. thaliana*; **Figure 7**). In contrast, GSH at physiological concentrations (2 mM) allowed a rapid and complete recovery of GAPDH activity, comparable with the reactivation kinetics observed with DTT. Interestingly, GSH-dependent denitrosylation activity was sensitive to the GSH/GSNO ratio but independent of the GSH/GSSG ratio, in agreement with GSNO being released in the denitrosylation reaction (Zaffagnini et al., 2013; **Figure 6**).

POSSIBLE METABOLIC CONSEQUENCES OF PLANT GAPC OXIDATION

Since cytoplasmic GAPDH is sensitive to oxidative stress due to its reactive catalytic cysteine, oxidative modification of this residue invariably inhibits enzyme activity and, in animal cells at least, also glycolysis (Ralser et al., 2007). The inhibition of the glycolytic pathway might increase the flux of glucose through the oxidative pentose phosphate (OPP) pathway, a metabolic re-routing that ensures high $NADPH/NADP^+$ ratios in the cytoplasm and is believed to play a fundamental role in counteracting the oxidative stress (**Figure 7**). While GAPDH-dependent down-regulation of the glycolytic pathway has been demonstrated in animal cells (Ralser et al., 2007), it remains to be demonstrated in plants, which contain non-phosphorylating GAPN in the cytoplasm. This enzyme generates NADPH in an irreversible reaction that could in principle bypass the GapC-catalyzed reaction (Rius et al., 2006). Furthermore, GAPN appears to be more resistant to H_2O_2-dependent oxidation/inactivation than plant GAPCs (Piattoni et al., 2013). While creating a potential bypass for the blocked glycolytic flux under oxidative stress, GAPN might also provide an alternative source of NADPH for the antioxidant enzymes (**Figure 7**).

ANIMAL GAPDH: REDOX-DEPENDENT NON-GLYCOLYTIC (MOONLIGHTING) FUNCTIONS

Although mammalian GAPDH clearly retains its fundamental role as a glycolytic housekeeping enzyme in the cytoplasm, mounting evidence indicates that different redox PTMs can induce or regulate additional non-glycolytic functions of GAPDH. In this sense, GAPDH is considered as a moonlighting protein. Besides inhibiting enzyme activity, redox PTMs of GAPDH can induce or affect the interaction with other proteins or nucleic acids (Tristan et al., 2011; Sirover, 2011, 2012) or the covalent binding with its cofactor NAD (Mohr et al., 1996). Two major moonlighting functions of GAPDH in animal cells will be described. The first deals with the apoptotic cascade that is triggered by GAPDH S-nitrosylation; the second concerns the role of GAPDH in the post-transcriptional regulation of endothelin, a moonlighting function of GAPDH that is not induced, but regulated by redox PTMs.

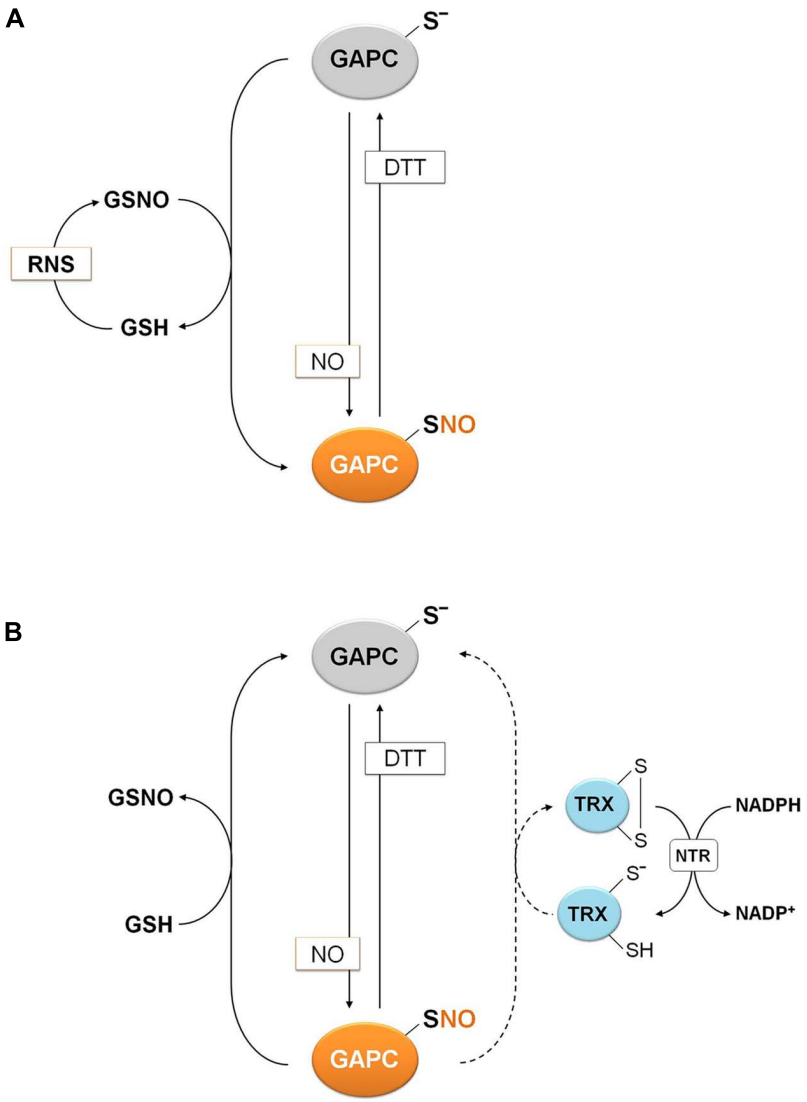

FIGURE 6 | Molecular mechanisms of plant GAPC nitrosylation and denitrosylation. (A) Nitrosylation of plant GAPC. Plant GAPC undergoes reversible S-nitrosylation in the presence of NO donors or trans-nitrosylation by GSNO with concomitant release of GSH. GSNO can be generated by the reaction of GSH with RNS. **(B)** Denitrosylation mechanism of plant GAPC. Denitrosylation of plant nitrosylated GAPC (GAPC1-SNO) is not catalyzed by plant cytoplasmic TRXs (dashed arrow), but it is efficiently catalyzed by GSH with formation of GSNO.

S-NITROSYLATION-DEPENDENT GAPDH ROLE IN APOPTOSIS

Animal GAPDH is involved in the signaling cascade that governs NO-dependent apoptosis. After apoptotic stimuli, GAPDH is found in the nucleus in a number of cell systems, including neurons (Nakamura and Lipton, 2013). A fundamental prerequisite for GAPDH relocalization from the cytoplasm to the nucleus appears to be the S-nitrosylation of its catalytic cysteine. Diverse apoptotic stimuli induce NO biosynthesis in animal cells and S-nitrosylated GAPDH (SNO-GAPDH) acquires the ability to bind Siah1 (seven in absentia homologue 1), an E3-ubiquitin ligase with a nuclear localization signal (**Figure 8**). The SNO-GAPDH/Siah1 interaction is mediated by the C-terminal catalytic domain of GAPDH and has the effect of increasing the stability of Siah1. The complex SNO-GAPDH/Siah1 translocates to the nucleus and mediates cell death (**Figure 8**; Hara et al., 2005). Different mechanisms are involved in SNO-GAPDH/Siah1-mediated apoptosis (**Figure 8**). Diverse nuclear proteins are ubiquitinated by Siah1, including the nuclear co-repressor N-CoR (Hara et al., 2005), and SNO-GAPDH/Siah1 can also bind to acetyltransferase p300/CBP and in turn be acetylated on Lys-160. Interestingly, this interaction (in which Lys-160 of GAPDH plays an essential role) stimulates the autoacetylation of p300 and activates the acetylation of downstream targets like tumor suppressor p53 and others that, upon activation, induce cell death (Sen et al., 2008).

Siah1-mediated GAPDH translocation to the nucleus is counteracted by GOSPEL (GAPDH's competitor Siah1 protein enhances life; **Figure 8**), a cytoplasmic protein that is more

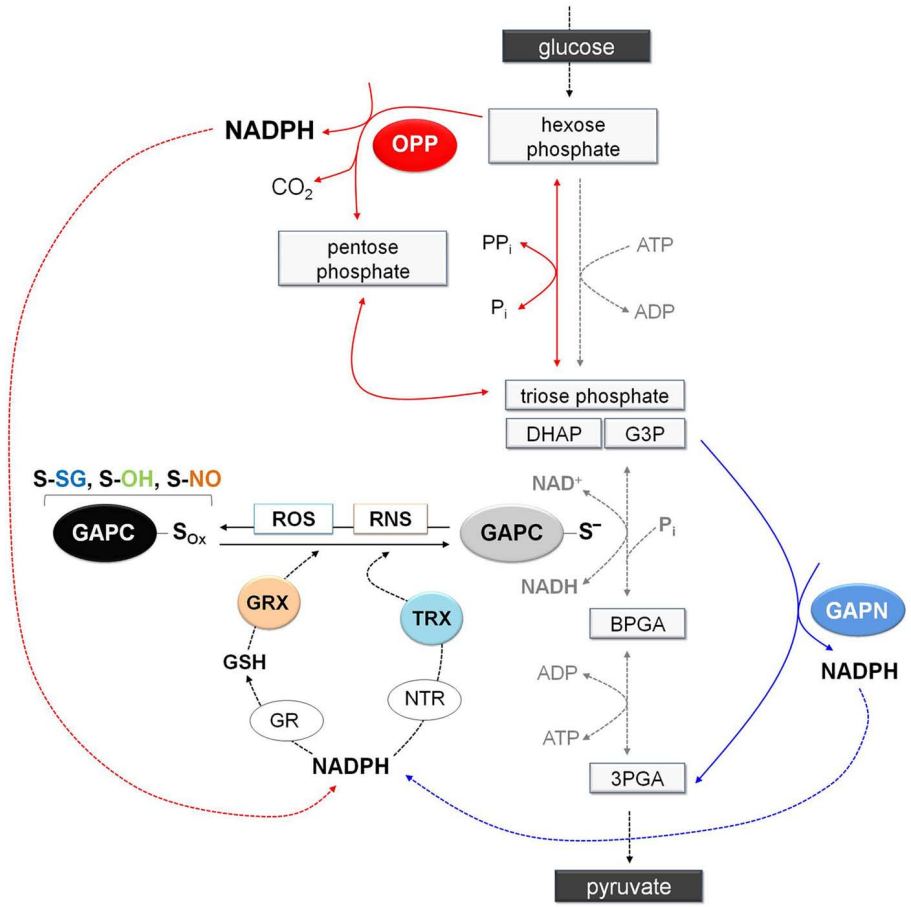

FIGURE 7 | Schematic representation of glycolysis showing the NADPH-producing systems in a situation of oxidative modification of GAPC. Under stress conditions, GAPC might undergo different type of oxidative modifications (GAPC-S_{ox}: sulfenation, S-OH; glutathionylation, S-SG; or nitrosylation, S-NO) with important effects on cytoplasmic primary metabolism. Indeed, inhibition of GAPC activity and the consequent down-regulation of glycolysis pathway would promote entry of glucose equivalents into the OPP pathway leading to the generation of NADPH (red arrows). Although inhibition of GAPC would down-regulate the glycolytic pathway, plant cells also contain a non-phosphorylating GAPDH (GAPN) that can by-pass the GAPC-catalyzed reaction providing an alternative source of NADPH for the antioxidant enzymes (blue arrows). Glutathione reductase and thioredoxin reductases (GR and NTR, respectively) are major antioxidants enzymes in the cytoplasm of plant cells. Glutathione reductase, using NAPDH as electron donor, can keep the glutathione pool reduced providing the reductant (GSH) for the efficient reduction of nitrosylated GAPC or the deglutathionylation via cytoplasmic glutaredoxins (GRXs). Alternatively, GAPC may be also deglutathionylated by a GSH-independent system involving NADPH, NTR and cytoplasmic thioredoxins (TRXs). Overall, redirection of primary metabolism in stressed plant cells would allow reinforcing the antioxidant systems and creating the conditions for recovery (e.g., reduction/reactivation of redox-modified proteins such as GAPC). 3PGA, 3-phosphoglycerate; BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phospate.

abundant in energy-demanding tissues in which GAPDH is also abundant. GOSPEL physiologically binds GAPDH and competes with Siah1 binding. GOSPEL is itself a target of S-nitrosylation (Cys-47) and this modification enhances GOSPEL ability to bind GAPDH. Under moderate stress conditions, GOSPEL efficiently protects GAPDH against the cytotoxic interaction with Siah1, but under harsher stress conditions the interaction of SNO-GAPDH with Siah1 prevails and apoptosis is consequently induced (Sen et al., 2009). Besides GOSPEL, other mechanisms can compete with Siah1-mediated nuclear translocation of GAPDH. Notably, the catalytic cysteine of GAPDH is physiologically acylated by its substrate G3P during the catalytic cycle (see Structure and Reaction Mechanism of GAPDH: an Abundant Protein with a Highly Reactive Catalytic Cysteine) and since acylated GAPDH cannot be S-nitrosylated no interaction with Siah1 can occur. Conditions of increased intracellular levels of G3P were reported to maintain cell survival by protecting GAPDH from S-nitrosylation (Lee et al., 2012). Actually, it may be speculated that any type of covalent modification of GAPDH catalytic cysteine, other than S-nitrosylation (e.g., glutathionylation), might in principle suppress the GAPDH-dependent apoptotic cascade.

Another mechanism counteracting the Siah1-mediated nuclear translocation of GAPDH is the formation of aggregates that immobilize GAPDH in the cytoplasm and may evolve into cytotoxic amyloid-like aggregates (**Figure 8**; Nakajima et al., 2007, 2009). In neurons, cytoplasmic GAPDH aggregates can form under oxidative stress conditions and are stabilized by

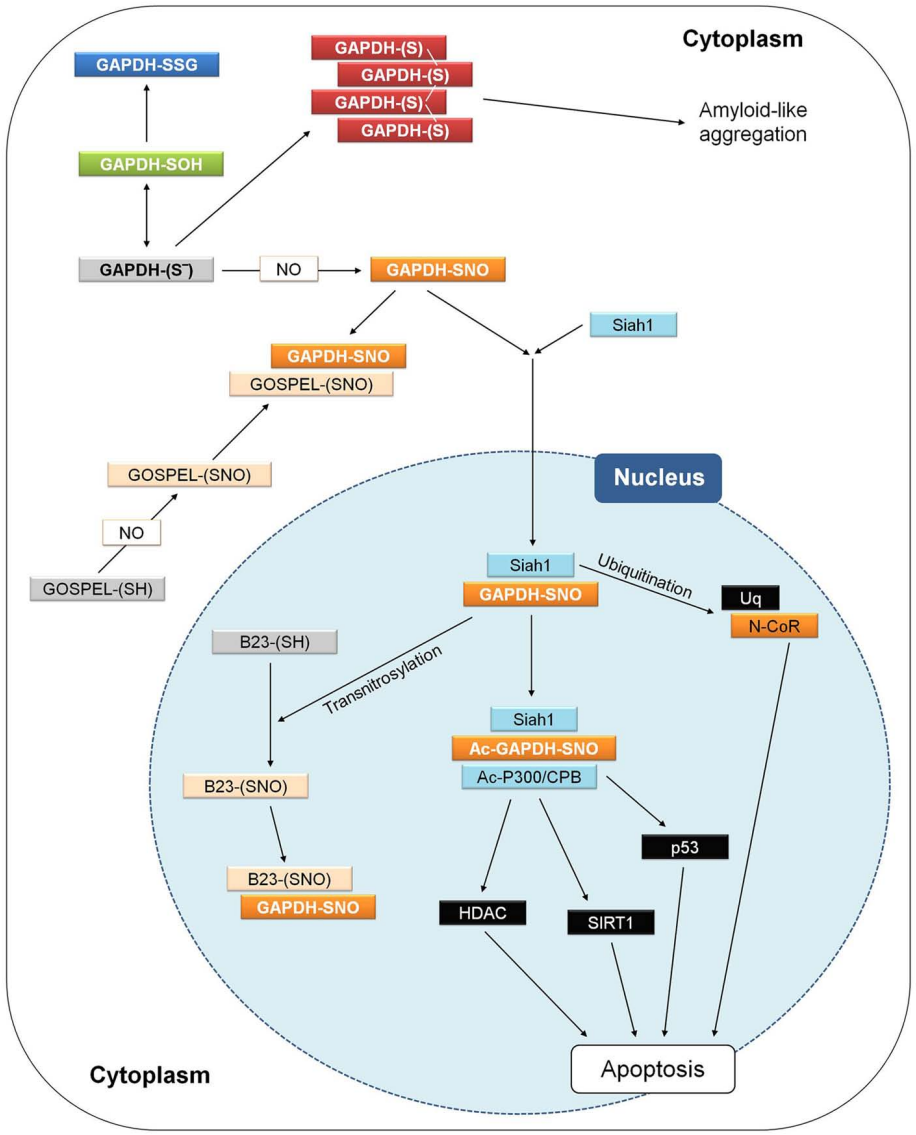

FIGURE 8 | GAPDH and apoptosis in animal cells. Interactions and processes that have been observed in different studies and cell types are here summarized in a single hypothetical animal cell. Different apoptotic stimuli may induce NO biosynthesis and the figure shows how apoptosis in animal cells may be triggered by the initial nitrosylation of GAPDH, and counteracted by several processes. For a detailed explanation, see text (Animal GAPDH: redox-dependent non-glycolytic (moonlighting) functions). GOSPEL, GAPDH's competitor Siah1 protein enhances life; N-CoR, nuclear co-repressor; Siah1, seven in absentia homologue 1.

intermolecular disulfides between catalytic cysteines and conformational changes of the protein with a concomitant enrichment of β-sheets (Nakajima et al., 2007, 2009). Formation of such abnormal GAPDH aggregates also promotes cell death, although through mechanisms independent of Siah1-dependent nuclear translocation.

The interaction between SNO-GAPDH and Siah1 can be counteracted also in the nucleus. This mechanism of regulation of the SNO-GAPDH/Siah1-dependent pro-apoptotic cascade depends on the ability of SNO-GAPDH to function as a trans-nitrosylase, i.e., to transfer its nitroso-group to a receiving thiol on a different protein (Kornberg et al., 2010; **Figure 8**). The neuroprotective nuclear protein B23/nucleophosmin is trans-nitrosylated by SNO-GAPDH and in its S-nitrosylated form binds to the SNO-GAPDH/Siah1 complex, thereby favoring the release of GAPDH from the complex and inhibiting the ubiquitinating activity of Siah1 (Lee et al., 2012). The neuroprotective role of B23 in the nucleus is reminiscent of that of GOSPEL in the cytoplasm: both proteins regulate cell survival by preventing SNO-GAPDH/Siah1 death-signaling under oxidative stress conditions.

The trans-nitrosylating activity of SNO-GAPDH in the nucleus is not restricted to B23. Other nuclear proteins that are similarly trans-nitrosylated include deacetylases such as SIRT1 and HDAC

(Kornberg et al., 2010; **Figure 8**). However, S-nitrosylation of these targets is envisioned in the framework of normal processes of neuronal development rather than apoptosis and neurodegeneration (Nakamura and Lipton, 2013).

GAPDH AND POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

GAPDH can bind to RNA and several examples of post-transcriptional regulation mechanisms involving GAPDH can be found in the literature. Binding of GAPDH to messenger RNA may have contrasting effects: in some cases GAPDH increases mRNA stability (e.g., CSF1), in others stimulates mRNA degradation (ET-1) or causes inhibition of translation (AT1R; Sirover, 2011, 2012). In several systems, including plants, GAPDH was also found to bind viral RNA and strongly affect, either positively or negatively, viral replication (Prasanth et al., 2011; see Plant Glycolytic GAPDH: Metabolic and Non-Metabolic Functions). At least in the case of human endothelin (ET-1) discussed below, binding of GAPDH to mRNA was experimentally demonstrated to be redox-regulated (i.e., inhibited by redox PTMs of GAPDH itself), but there are reasons to believe that redox PTMs may have a more general effect on GAPDH-RNA interactions.

Post-transcriptional regulation of the vaso-constricting peptide endothelin (ET-1) is based on the ability of human GAPDH to bind AU-rich elements (ARE) of mRNA. AREs are common cis-regulatory elements of 3′-untranslated regions (UTR) of eukaryotic messenger RNAs and are important determinants of mRNA stability in mammals. Proteins binding to these elements can either increase or decrease mRNA stability and thus affect protein expression levels (Ahmed et al., 2011). The mRNA of the endothelin ET-1 contains in its 3′-UTR an ARE forming a secondary structure. By binding to this ARE, GAPDH causes mRNA unwinding and favors a faster degradation through ribonucleases. The mRNA binding activity of GAPDH occurs in its nucleotide binding domain and competes with the binding of NAD^+ required for enzymatic activity (Nagy and Rigby, 1995). Interestingly, GAPDH-mRNA binding activity also depends on glycolytic GAPDH catalytic cysteine (the catalytic cysteine mutant does not bind ET-1 mRNA) and is prevented by treatments with GSSG or GSNO, suggesting that conditions leading to oxidative modifications of GAPDH (see Plant Glycolytic GAPDH (GAPC): a Perfect Protein Tool to Investigate Redox Post-Translational Modifications) may contribute to ET-1 post-transcriptional regulation *in vivo* (Rodríguez-Pascual et al., 2008).

Thanks to its capability to interact with AREs via its Rossmann fold, GAPDH was shown to bind many other types of mRNA, and may well exert a more general role in post-transcriptional regulation. Although only in the case of ET-1, GAPDH-mRNA interaction was experimentally shown to be redox-regulated, it is plausible that this property may also apply to other GAPDH-mRNA combinations, as long as different mRNAs may bind to GAPDH in a similar fashion.

PLANT GLYCOLYTIC GAPDH: METABOLIC AND NON-METABOLIC FUNCTIONS

Compared to mammalian cells in which a single gene codes for GAPDH, Arabidopsis contains two cytoplasmic isozymes, GAPC1 (At3g04120) and GAPC2 (At1g13440), 98% identical in amino acid sequence, and both similarly contributing to the total NAD-specific GAPDH activity of the cytoplasm (Guo et al., 2012; Vescovi et al., 2013). The presence of more than one GAPC isoform is common in higher plants.

METABOLIC ROLE OF GLYCOLYTIC GAPDH IN PLANTS

In spite of its role in a fundamental metabolic pathway such as glycolysis, reduction of GAPC protein levels in transgenic plants generally leads to relatively mild phenotypes under normal growth conditions, in agreement with the notion that plants contain possible alternatives to cytoplasmic GAPC, e.g., non-phosphorylating GAPN and plastidic GAPDHs (A_4- and A_2B_2-GAPDH and GAPCp).

Partial suppression by antisense technology of cytoplasmic GAPC was obtained in transgenic potato (Hajirezaei et al., 2006). In these plants, NAD(H)-dependent GAPDH activity was reduced in both leaves, stems and tubers, but no clear phenotypic alterations were detected. In Arabidopsis, delayed growth, reduction of fertility and alterations of seed and fruit development were instead observed in both knock-out and antisense mutants of GAPC1. These traits were associated with a reduced respiratory rate, suggesting a metabolic-related phenotype (Rius et al., 2006). However, in another study, an Arabidopsis double mutant lacking both GAPCs (Guo et al., 2012) showed no particular phenotypic alterations under normal growth conditions, though defects in stomatal regulation made these plants overly sensitive to water stress. This effect was elegantly explained by a proposed role of GAPC1/GAPC2 in sensing H_2O_2 and promoting phosphatidic acid (PA) production in guard cells (Guo et al., 2012). As discussed below this function of GAPC does not rely on GAPDH enzymatic activity and is actually a moonlighting activity of the plant enzyme.

Quite unexpectedly, a strong metabolic-related phenotype is displayed by GAPCp double mutants in Arabidopsis. These plants are dwarf and suffer of arrested root development and sterility. In spite of the little contribution, in quantitative terms, of GAPCp to the total glycolytic activity of plant tissues, GAPCp (together with phosphoglycerate kinase) is essential in providing 3-phosphoglyceric acid (3-PGA) precursors for serine biosynthesis in plastids through the phosphorylating pathway (Ho and Saito, 2001). Lack of serine in non-photosynthetic tissues was shown to be the major reason for root growth inhibition, but also for defects in pollen and embryo development (Muñoz-Bertomeu et al., 2009, 2010).

NON-METABOLIC ROLES OF PLANT GLYCOLYTIC GAPDH

Besides the multitude of examples in mammalian cells, emerging evidence for non-metabolic functions of glycolytic GAPDHs (GAPCs) are also coming from research carried out in plants (**Figure 9**). Here we will present some recent discoveries in which plant GAPCs have started to reveal their functional plasticity.

Arabidopsis mutants in which both GAPC isoforms were knocked out are impaired in ABA-induced stomatal closure, and consequently suffer of a higher transpirational water loss than wild type plants under drought stress (Guo et al., 2012). Drought stress and ABA are known to induce ROS production by NADPH

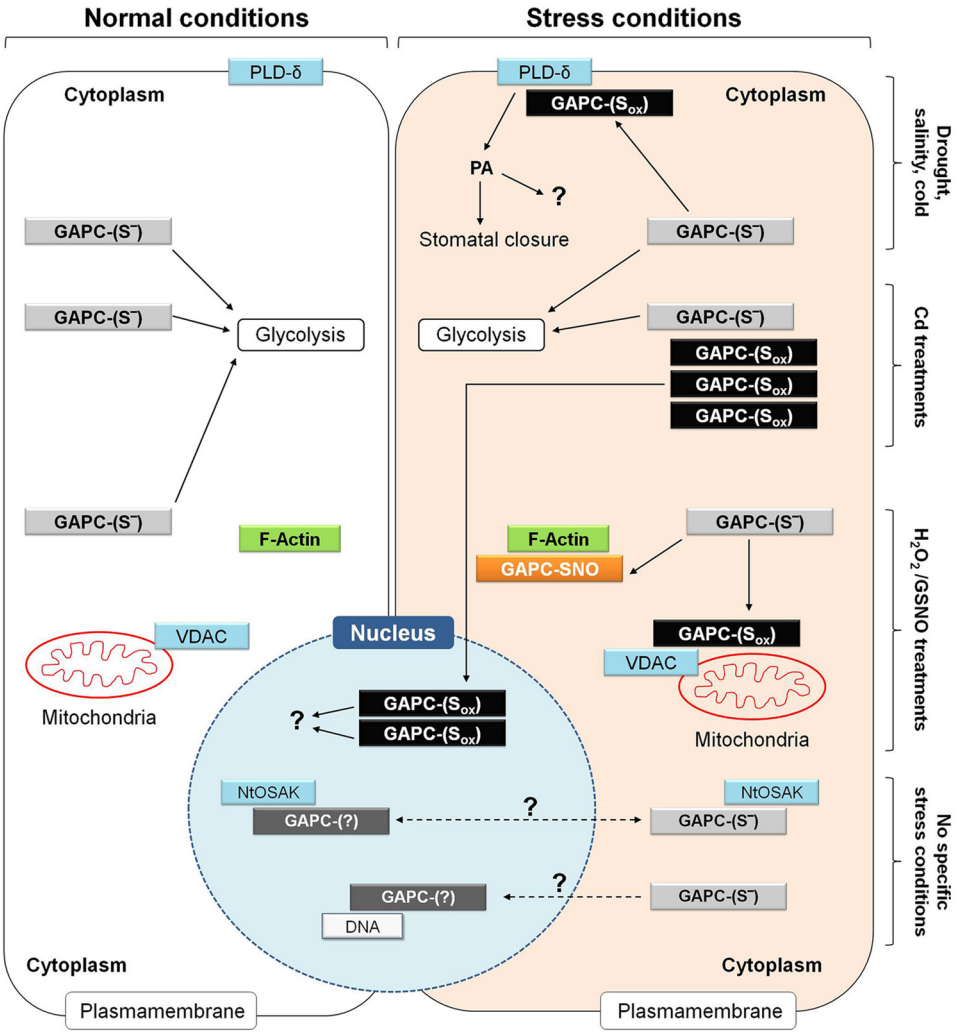

FIGURE 9 | Metabolic and non-metabolic functions of GAPC in plant cells under normal and different stress conditions. Functional roles of GAPC that have been observed in different studies and cell types are here summarized in a single hypothetical plant cell. Under normal conditions, GAPC fulfills metabolic function by participating to glycolysis (left side of the figure) while it can play non-metabolic functions under specific stress conditions (e.g., drought, salinity and cold stress, treatments in the presence of cadmium (Cd), hydrogen peroxide (H_2O_2) and nitrosoglutathione (GSNO; right side of the figure). Overall, these conditions are supposed to induce different type of redox modifications of GAPC (GAPC-S_{ox}) and stimulate interaction with several partner proteins or to induce protein expression, accumulation of inactive protein and relocalization to the nucleus. Additional functions of GAPC are not clearly related to either specific stress conditions or redox modification or sub-cellular localization of the GAPC protein and are indicated by dashed arrows. For a detailed explanation, see text (Plant Glycolytic GAPDH: Metabolic and Non-Metabolic Functions). NtOSAK, *Nicotiana tabacum* osmotic stress activated kinase; PA, phosphatidic acid; PLD-δ, phospholipase D-δ; VDAC, voltage-dependent anion channels.

oxidase in guard cells and GAPC is an established target of H_2O_2 (Hancock et al., 2005), probably also in guard cells. It was demonstrated in Arabidopsis leaves, that oxidation of GAPCs inhibits enzyme activity but stimulates the physical interaction between GAPCs and phospholipase D-δ (PLD-δ), which is then activated (**Figure 9**). PLD-δ is bound to the plasma membrane and once activated releases PA from phospholipids, with preference for phosphatidylethanolamine. Synthesis of PA in plants is triggered in response to various biotic and abiotic stresses, including pathogen infection, drought, salinity, wounding and cold, and is performed by many different PLD-isoforms (Testerink and Munnik, 2005). However, oxidized GAPC interacts specifically with PLD-δ, and PA synthesized by PLD-δ appears to induce stomatal closure through activation of mitogen-activated protein kinases, though other mechanisms may also be operative. Intriguingly, GAPC itself is also a target of PA (McLoughlin et al., 2013) and GAPC2 undergoes a proteolytic cleavage after binding to PA *in vivo* (Kim et al., 2013). Although the function of this process is not clear yet, it may represent a feed-back mechanism to down-regulate the GAPC-mediated PA-signaling (Guo et al., 2012). In any case, the activation of PLD-δ by oxidized GAPC is a clear example of a non-metabolic function of GAPDH in plants.

Similar to mammalian cells, also in plant cells GAPDH was detected both in the cytoplasm and in the nucleus (Bae et al., 2003;

Anderson et al., 2004; Holtgrefe et al., 2008; Waver et al., 2010; Kim et al., 2013; Vescovi et al., 2013). The nuclear localization of a glycolytic enzyme is a good hint for a moonlighting function, but at difference with mammalian GAPDH for which the mechanism of nuclear translocation and the function in apoptosis has been well established (Hara et al., 2005; **Figure 8**), studies on nuclear GAPDH in plants are still in their infancy.

In a recent study (Vescovi et al., 2013), cadmium (Cd) treatments of Arabidopsis seedlings were found to induce the translocation of GAPC1-YFP chimeric protein from the cytoplasm to the nucleus in root tip cells (**Figure 9**). In these experiments, the GAPC1-YFP chimeric protein was expressed under the control of the GAPC1 endogenous promoter. This proved essential to avoid artifacts, as stronger constitutive promoters (e.g., CaMV-35S) may cause nuclear localization of GAPC as a mere consequence of its extremely high intracellular concentration (Vescovi et al., 2013). In Arabidopsis plants transformed with the endogenous GAPC1 promoter, translocation and accumulation of GAPC-YFP in the nucleus was thus a genuine effect of Cd treatments of Arabidopsis roots, that was found to correlate, in the same cells, with NO accumulation and oxidation of the GSH pool, as detected by the redox probe roGFP2. Inhibitors of GSH biosynthesis or catalase inhibitors, both treatments causing cytoplasmic oxidation, induced GAPC1 protein accumulation in a similar way as Cd treatments, suggesting that GAPC1 promoter activation may be mediated by unknown oxidized elements of the cytoplasm, rather than Cd itself (Vescovi et al., 2013). Independent works have also reported the up-regulation of GAPC (particularly GAPC1) under other stress conditions, including salt stress (Jiang et al., 2007), and oxidative stress by menadione (Lehmann et al., 2009). Interestingly, in the case of Cd-treated Arabidopsis roots, nuclear-accumulated GAPC1 was apparently inactive as an enzyme (**Figure 9**), consistent with the high sensitivity of GAPC to inactivation by oxidants like NO, H_2O_2, GSNO or GSSG (Lindermayr et al., 2005; Holtgrefe et al., 2008; Bedhomme et al., 2012; Guo et al., 2012; Vescovi et al., 2013; Zaffagnini et al., 2013). Therefore, it is concluded that Cd treatments may impose cellular oxidized conditions that, on one side stimulate GAPC1 gene expression, but on the other favor its inactivation and nuclear translocation (**Figure 9**).

Cadmium-induced nuclear translocation of GAPC1 in Arabidopsis reminds the nuclear translocation of mammalian GAPDH upon apoptotic stimuli. However, in this latter case translocation is triggered by the S-nitrosylation of GAPDH catalytic cysteine and inhibited by its mutation to serine [Hara et al., 2005; see Animal GAPDH: Redox-Dependent Non-Glycolytic (Moonlighting) Functions], while in Cd-treated Arabidopsis the nuclear relocalization of GAPC1 was stimulated by the substitution of the catalytic cysteine with a serine (Vescovi et al., 2013). This result seemed to rule out the involvement of a redox modification of the catalytic cysteine for the nuclear translocation of GAPDH in Arabidopsis. Recently, a mechanism based on the acetylation of three lysine residues was alternatively proposed for the nuclear translocation of GAPDH after the apoptotic stimulus in animal cells (Ventura et al., 2010). Whether a mechanism of this type may apply to Cd-induced nuclear translocation of GAPC in plants is still an open question.

In BY-2 tobacco cells, cytoplasmic GAPDH isoforms NtGAPCa and NtGAPCb were found to interact, both in the cytoplasm and the nucleus, with an osmotic stress activated kinase (NtOSAK; **Figure 9**), belonging to the SnRK2 family (sucrose non-fermenting 1-related protein kinase 2, Waver et al., 2010). This kinase is activated by phosphorylation under osmotic stress and NO treatments, and by the interaction with GAPC isoforms that were found partially S-nitrosylated under these conditions. Interestingly, GAPC mutants with no cysteines in the active site (because converted to serines) interacted with NtOSAK only in the cytoplasm and no complexes were detected in the nucleus, suggesting that GAPC S-nitrosylation was required for either nuclear translocation of GAPC itself or for the interaction with NtOSAK within the nucleus. Anyhow, GAPC/NtOSAK complexes were neither stimulated by salt stress nor by NO-donors, and therefore the function of the whole system still remains obscure (**Figure 9**).

Whatever the mechanism required for nuclear localization, a clear role of plant GAPDHs in the nucleus is still missing. Preliminary evidence that GAPC may bind to a DNA sequence coding for NADP-malate dehydrogenase (Holtgrefe et al., 2008; **Figure 9**) confirmed the results of a previous yeast one-hybrid screen (Hameister et al., 2007), but the effect of such interaction is unknown. An interesting, alternative scenario for the role of nuclear GAPC might come from the demonstration that mammalian and also chloroplastic GAPDHs display uracil glycosylase activity (Wang et al., 1999; Mazzola and Sirover, 2003). The uracil glycosylase activity of mammalian GAPDH is associated with its monomeric form present in the nucleus (Mazzola and Sirover, 2003). Plant cytoplasmic GAPDH has not yet been tested in this regard. However, bearing in mind that the uracil glycosylase activity is important for the maintenance of DNA integrity and preventing mutagenesis by eliminating uracil from DNA molecules, it is possible that the GAPC nuclear accumulation under oxidative stress in plants may play a role in DNA protection. In support of this hypothesis, Cd, a major inducer of GAPC nuclear translocation, has been reported to induce not only oxidative stress (De Michele et al., 2009; Vescovi et al., 2013), but also DNA damage and mutations in Arabidopsis plantlets (Liu et al., 2012).

The capability of GAPDH to bind nucleic acids does also explains its recently discovered role in regulating virus replication in plants. Indeed GAPC interacts with the replicase complex of different phytopathogenic viruses with a single-stranded, positive-sense RNA genome. The effect of GAPC binding consists in either repressing or stimulating virus replication. In *Nicotiana benthamiana* infected with the Bamboo Mosaic Virus (BaMV), GAPC binds the 3′-untranslated region of both BaMV genomic and satellite RNA and negatively regulates virus replication. Consistently, in GAPC-overexpressing plants, BaMV replication is further inhibited, whereas GAPC-silenced plants are more susceptible to the virus (Prasanth et al., 2011). For Tomato Bush Stunt Virus (TBSV), again infecting *Nicotiana benthamiana*, the effect of GAPC was opposite. In this case, GAPC is incorporated into the viral replicase complex and binds to the 3′-UTR of the negative-sense RNA of the virus, in correspondence of an AU-pentamer. This binding favors retention of the (-)RNA by

the replication complex, such that synthesis of positive strands are favored. Moreover, by binding the viral polymerase subunit $p92^{pol}$, GAPC help recruiting the RNA-dependent RNA-polymerase on the negative strand, again favoring positive RNA strands production. Although the N-terminal part of GAPC, including the catalytic cysteine, is required for binding viral RNA, the C-terminal catalytic domain is also involved in regulation as GAPC fragments with no catalytic domain may still bind negative-strand RNA, but repress rather than stimulate viral replication (Huang and Nagy, 2011). Since the catalytic cysteine of GAPC is close to its putative RNA-binding domains, the capability of GAPC to affect viral replication is likely, though not demonstrated, to be redox regulated.

Moonlighting properties of GAPC, possibly shared with other glycolytic enzymes, are also suggested by the specific and dynamic binding to sub-cellular structures such as membranes and the cytoskeleton. A portion of all glycolytic enzymes, including GAPC, is associated to the cytoplasmic face of the outer membrane of plant mitochondria (Giegé et al., 2003). Binding of glycolytic enzymes to the organelle is dynamic and favored by conditions of high respiratory demands. Organelle-bound glycolytic enzymes may support respiration by directly providing pyruvate to mitochondria and restricting the use of glycolytic intermediates by alternative metabolic pathways (Graham et al., 2007). However, mitochondrial association of Arabidopsis GAPCs was also increased by H_2O_2- treatments (Sweetlove et al., 2002), i.e., under conditions in which GAPDH activity may be inhibited. The interaction between GAPCs and mitochondria is probably mediated by voltage-dependent anion channels (VDAC; Graham et al., 2007; **Figure 9**), and *in vitro* the binding of GAPC1 to VDAC3 was actually found to be inhibited by DTT (Wojtera-Kwiczor et al., 2013). Under oxidative stress conditions, it is thus possible that GAPC, and perhaps other glycolytic enzymes like aldolase, are induced to associate to mitochondria in an oxidized and inactive state that may play an alternative function to respiration. In mammals, binding of GAPDH to VDAC was also found to be inhibited by DTT and suggested to induce mitochondrial membrane permeabilization and apoptosis (Tarze et al., 2007). Whether a similar role might also be played by plant GAPC remains to be investigated in detail.

Plant glycolytic GAPDH can also interact with the cytoskeleton and promote actin bundling, again a function of GAPDH that is stimulated by oxidizing conditions (Wojtera-Kwiczor et al., 2013). GSNO causes both glutathionylation and S-nitrosylation of GapC (Zaffagnini et al., 2013) and GSNO-treated GAPC1 binds F-actin in a DTT-reversible manner (Wojtera-Kwiczor et al., 2013; **Figure 9**). Generally speaking, the complex relationships between the cellular redox state, the GAPDH redox state and enzyme activity, and its subcellular localization (cytoplasm, nucleus, plasma membrane, mitochondria, cytoskeleton) speaks in favor of a possible role as an oxidative stress sensor, but clearly our comprehension of the system is still fragmental.

CONCLUSION

Glyceraldehyde-3-phosphate dehydrogenase is an abundant protein of animal and plant cells, whose catalytic role in glycolysis is based on a highly reactive catalytic cysteine. GAPDH is also a moonlighting, multifunctional protein with non-glycolytic roles that often involve oxidative modifications of the catalytic cysteine. Plant cytoplasmic GAPDH (GAPC) proved an excellent tool to study the different types of redox PTMs that may affect a plant protein (Holtgrefe et al., 2008; Guo et al., 2012; Bedhomme et al., 2012; Piattoni et al., 2013; Zaffagnini et al., 2013), and it is now established that also GAPC, like animal GAPDH, can be translocated to the nucleus under particular, oxidizing conditions (Waver et al., 2010; Kim et al., 2013; Vescovi et al., 2013). However, neither the mechanism of translocation nor the function of GAPC in the plant nucleus are currently understood. In spite of its popularity, the role of GAPC in plant cell death is still speculative, whereas it is well established that the nuclear translocation of S-nitrosylated GAPDH can transmit an apoptotic stimulus in animal cells (Hara et al., 2005; Sen et al., 2009; Kornberg et al., 2010; Nakamura and Lipton, 2013). Nonetheless, plant GAPC is a moonlighting protein and other non-glycolytic roles have been recently established, including the control of transpiration in Arabidopsis plants (via ROS-dependent activation of phospholipase D-δ; Guo et al., 2012), and the regulation of the replication of phytopathogenic viruses (via binding of viral RNA; Prasanth et al., 2011). Interestingly both functions are, or likely to be, redox-regulated and thus affected by perturbations of the cellular redox homeostasis.

Every function of GAPDH that depends, or is regulated by a redox modification that blocks its catalytic activity is by definition alternative to its role in glycolysis. However, alternative functions of GAPC, signaling functions in particular, may well involve only a small fraction of the large pool of GAPC in plant cells. Moreover, plant cells contain metabolic alternatives to keep the glycolytic flux operative under conditions in which GAPC is oxidatively inactivated. Overall, GAPC appears to have suitable properties to behave as an oxidative stress sensor in plant cells and this function may not be in contrast with its classical glycolytic role.

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Toward a refined classification of class I dithiol glutaredoxins from poplar: biochemical basis for the definition of two subclasses

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Glutaredoxins (Grxs) are small oxidoreductases particularly specialized in the reduction of protein-glutathione adducts. Compared to other eukaryotic organisms, higher plants present an increased diversity of Grxs which are organized into four classes. This work presents a thorough comparative analysis of the biochemical and catalytic properties of dithiol class I Grxs from poplar, namely GrxC1, GrxC2, GrxC3, and GrxC4. By evaluating the *in vitro* oxidoreductase activity of wild type and cysteine mutated variants and by determining their dithiol-disulfide redox potentials, pK_a values of the catalytic cysteine, redox state changes in response to oxidative treatments, two subgroups can be distinguished. In accordance with their probable quite recent duplication, GrxC1 and GrxC2 are less efficient catalysts for the reduction of dehydroascorbate and hydroxyethyldisulfide compared to GrxC3 and GrxC4, and they can form covalent dimers owing to the presence of an additional C-terminal cysteine (Cys_C). Interestingly, the second active site cysteine (Cys_B) influences the reactivity of the catalytic cysteine (Cys_A) in GrxC1 and GrxC2 as already observed with GrxC5 (restricted to *A. thaliana*), but not in GrxC3 and C4. However, all proteins can form an intramolecular disulfide between the two active site cysteines (Cys_A-Cys_B) which could represent either a protective mechanism considering that this second cysteine is dispensable for deglutathionylation reaction or a true catalytic intermediate occurring during the reduction of particular disulfide substrates or in specific conditions or compartments where glutathione levels are insufficient to support Grx regeneration. Overall, in addition to their different sub-cellular localization and expression pattern, the duplication and maintenance along evolution of several class I Grxs in higher plants can be explained by the existence of differential biochemical and catalytic properties.

Keywords: cysteine oxidation, deglutathionylation, disulfide bond, glutaredoxin, redox potential

INTRODUCTION

Glutaredoxins (Grxs) are oxidoreductases sharing a conserved 3D structure with members of the thioredoxin (Trx) superfamily. They are present in most living organisms except in some bacterial and archaeal phyla (Rouhier et al., 2008; Alves et al., 2009). The primary function of Grxs was long thought to be the reduction of disulfide bonds and more particularly those formed between reduced glutathione (GSH) and a protein cysteinyl residue, a process known as glutathionylation, but as explained below, specific Grx members could rather serve as iron-sulfur (Fe-S) cluster transfer proteins (Rouhier, 2010). Glutathionylation is a post-translational modification which potentially fulfills several functions. It can constitute an intermediate of some reaction mechanisms, but it can also modulate protein function and serve as a signaling mechanism or protect cysteine residues from irreversible oxidation in plants (Zaffagnini et al., 2012b) and in animals (Dalle-Donne et al., 2009). Although a few additional proteins, as some specific thioredoxins, can catalyze deglutathionylation reactions, Grxs are likely the major deglutathionylation system, at least in plants (Bedhomme et al., 2012; Chibani et al., 2012). They are usually regenerated via a NADPH/glutathione reductase (GR)/GSH system, but a few Grxs can be reduced by ferredoxin- or NADPH-dependent thioredoxin reductases (Johansson et al., 2004; Zaffagnini et al., 2008). Depending on the Grx isoforms and target proteins or substrates considered, Grxs can use different catalytic mechanisms, called monothiol and dithiol mechanisms (Rouhier et al., 2008). The monothiol mechanism is used for the reduction of glutathionylated proteins and requires *a priori* only the catalytic cysteine (the first or more N-terminal of the two active site cysteines, referred to as Cys_A). It performs a nucleophilic attack on the protein-glutathione adduct, the Grx becoming glutathionylated. This oxidized Grx is regenerated by reduction via a GSH molecule. The dithiol mechanism requires the catalytic cysteine but also a recycling cysteine which could be either the second active site cysteine (Cys_B) or an additional extra active site cysteine (Cys_C). If the target protein is glutathionylated, the first step is similar to the monothiol mechanism, but the glutathione-adduct formed on

Grx is solved by one of these recycling cysteines instead of GSH. The resulting disulfide is then either reduced by two molecules of GSH or by a thioredoxin reductase. If the target protein has an intra or inter-molecular disulfide, the mechanism is comparable to the one used by Trxs. The catalytic cysteine forms a transient mixed disulfide with one of the cysteines of the target protein, which is then resolved by the recycling cysteine. As above, final regeneration occurs via two molecules of GSH or via a thioredoxin reductase.

Based on the active site sequence, Grxs were initially categorized into two classes, a dithiol (CP[Y/F]C motif) and a monothiol (CGFS motif) class (Rodriguez-Manzaneque et al., 1999) that were subsequently renamed classes I and II, respectively (Couturier et al., 2009a). Analyses of Grx distribution and evolution in archaea, bacteria, and eukaryotes suggested that Grx domains of classes I and II have evolved through duplication and divergence from one initial gene present in the last common ancestor of all organisms (Alves et al., 2009). Two additional classes are found in land plants (Couturier et al., 2009a). The Grx isoforms belonging to class III are specific to land plants and those from class IV are also present in few algae and animals. There is no biochemical or functional information on the latter Grxs yet. From genetic analyses, it appears that class III Grx isoforms also named CC-type Grxs or ROXY play a role in plant development and in pathogen defense mechanisms, reviewed in (Meyer et al., 2012). These functions are related to their ability to interact with TGA transcription factors, likely regulating their redox states. However, the biochemical properties of these Grxs have not been studied in detail yet, because of the difficulty to produce soluble recombinant proteins (Couturier et al., 2010). Only one study demonstrated that *Arabidopsis thaliana* GrxS10 is able to regenerate *in vitro* the mitochondrial type IIF peroxiredoxin (Finkemeier et al., 2005). The Grxs from class II are particular considering their involvement in the maturation of Fe-S clusters and in the regulation of iron homeostasis (Rouhier et al., 2010), which is likely related to their capacity to bind labile Fe-S clusters and to transfer them to target proteins (Bandyopadhyay et al., 2008). On the other hand, though many studies performed with class II Grxs from various sources indicated that they have no or very poor efficiency for the reduction of the traditionally tested glutathionylated substrates using a GR/GSH regeneration system, at least one study indicated that they have a reductase activity. Indeed, *Chlamydomonas reinhardtii* Grx3 is able to reduce *in vitro* a glutathionylated A_4-glyceraldehyde-3-phosphate dehydrogenase (Zaffagnini et al., 2008). Interestingly, it can be regenerated by the ferredoxin-thioredoxin reductase but not by the GSH/GR system. The existence of alternative regeneration systems may explain why the characterization of such activities for class II Grxs has been retarded. Overall, the demonstrated capacity of several class I Grxs to provide electrons to a battery of enzymes, as peroxiredoxins and methionine sulfoxide reductases, enzymes that go through a catalytic cycle which involves glutathionylation of their catalytic cysteine (Rouhier et al., 2001; Vieira Dos Santos et al., 2007; Tarrago et al., 2009), suggests that they are the central and favorite deglutathionylating agents in cells.

In plants, class I regroups six different glutaredoxin members called GrxC1, C2, C3, C4, S12, and C5, the latter being restricted to Brassicaceae (Couturier et al., 2011). The cytoplasmic GrxC1 and the plastidial GrxC5 are able to bind an $[Fe_2S_2]$ cluster, at least *in vitro* into recombinant proteins (Rouhier et al., 2007; Couturier et al., 2011). The biochemical and structural characterization of the two plastidial isoforms, GrxS12 and GrxC5, confirmed that the apoforms of these Grxs reduce glutathionylated proteins with catalytic properties quite similar to other plant and non-plant class I Grxs (Couturier et al., 2009b, 2011). Moreover, poplar GrxS12 was shown to be sensitive and temporarily inactivated after treatments with oxidizing compounds such as hydrogen peroxide (H_2O_2), nitrosoglutathione (GSNO), and oxidized glutathione (GSSG) (Zaffagnini et al., 2012a). Considering the very electronegative redox potential of the glutathione adduct formed on the catalytic Cys_A (−315 mV at pH 7.0), the reduction of GrxS12 necessitates a quite low redox potential of the GSH/GSSG couple in the chloroplast. Thus, GrxS12 may act as a stress-related redox regulator allowing glutathione to play a signaling role in some oxidizing conditions by maintaining the glutathionylation of its target proteins over the duration of the stress period. Several class I Grxs, *C. reinhardtii* Grx1 and Grx2 (Gao et al., 2010), *A. thaliana* GrxC1, C2 (Riondet et al., 2012) and GrxC5 (Couturier et al., 2011) and poplar GrxC1, GrxC4, and GrxS12 (Rouhier et al., 2002, 2007; Couturier et al., 2009b; Zaffagnini et al., 2012a) have been partially or extensively characterized at the biochemical level, but some members have not yet been studied and there is no thorough and comparative study allowing to understand if the existence of several members is only related to their sub-cellular localization or to their expression pattern or whether some biochemical properties can partially explain their diversity.

From the biochemical analyses performed here with the four dithiol class I Grxs existing in poplar, GrxC1 to GrxC4 (the fifth member, GrxS12, has a monothiol active site and was characterized previously), two sub-groups with distinct properties emerged. In addition, this work provides new insights on the existence of different oxidation forms involving the active site cysteines (Cys_A and Cys_B) or an additional C-terminal cysteine (Cys_C) and on their potential role for the reaction mechanism.

MATERIALS AND METHODS

SITE-DIRECTED MUTAGENESIS

The construction of pET3d expression plasmids containing poplar GrxC1, C2, C3, and C4 was described previously (Rouhier et al., 2002, 2007). All cysteine residues of GrxC2, C3 were individually substituted into serines using two complementary mutagenic primers (listed in **Table S1**). The corresponding mutated proteins are called GrxC2 C23S, C26S, or C80S, GrxC3 C37S or C40S. The recombinant proteins GrxC1 C31S, C34S, C88S, and GrxC4 C27S and C30S were prepared previously (Rouhier et al., 2002, 2007). Two additional mutants, in which only the catalytic cysteine is remaining, GrxC1 C34/88S and GrxC2 C26/80S, have also been cloned.

HETEROLOGOUS EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS IN *E. coli*

For protein expression, cultures of the *E. coli* BL21(DE3) strain, containing the pSBET plasmid and co-transformed with the

different recombinant pET3d plasmids, were successively amplified up to 2.4 L in LB medium at 37°C supplemented with 50 μg/mL of ampicillin and kanamycin. Induction of protein expression was performed at exponential phase by adding 100 μM isopropyl β-D-thiogalactopyranoside for 4 h at 37°C. After centrifugation (20 min at 4400 g), the cell pellets were resuspended in about 20 mL of TE NaCl buffer (30 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl) and eventually conserved at −20°C. Cell lysis was performed by sonication and the soluble and insoluble fractions were separated by centrifugation for 45 min at 27,000 g.

Purification of proteins was carried out in three steps. The soluble fraction was first precipitated by ammonium sulfate from 0 to 40% and then to 80% of the saturation. The 40–80% ammonium sulfate-precipitated fraction was subjected to gel filtration chromatography (ACA44 gel) equilibrated with TE NaCl buffer. After dialysis against TE (30 mM Tris-HCl pH 8.0, 1 mM EDTA) buffer and concentration, the interesting fractions were loaded to a DEAE (diethylaminoethyl) sepharose column equilibrated in TE buffer. GrxC1 WT, GrxC2 WT and their corresponding cysteinic mutants passed through the column, whereas GrxC3 WT, GrxC4 WT and their respective cysteinic mutants were retained and eluted using a linear 0–0.4 M NaCl gradient. The purest fractions, as judged by SDS-PAGE analysis, were pooled and dialyzed against TE buffer by ultrafiltration in Amicon cells equipped with a YM10 membrane. Finally, the fractions were concentrated and stored at −20°C until further use. Protein purity was checked by SDS-PAGE, and protein concentrations were determined spectrophotometrically using the corresponding molar extinction coefficients at 280 nm of 10,095 M^{-1} cm^{-1} for GrxC1 WT and its monocysteinic mutants, 9970 M^{-1} cm^{-1} for GrxC1 C34/88S, 8605 M^{-1} cm^{-1} for GrxC2 WT and its monocysteinic mutants, 8480 M^{-1} cm^{-1} for GrxC2 C26/80S, 7575 M^{-1} cm^{-1} for GrxC3 WT, 7450 M^{-1} cm^{-1} for GrxC3 C37S and C40S, 4595 M^{-1} cm^{-1} for GrxC4 WT and 4470 M^{-1} cm^{-1} for GrxC4 C27S and C30S.

ENZYMATIC ACTIVITIES

The thioltransferase activities [2-hydroxyethyl-disulfide (HED) and dehydroascorbate (DHA) assays] were measured as described previously (Couturier et al., 2011) using as purified non-reduced proteins. Briefly, measurements were performed at 25°C in steady-state conditions by following NADPH oxidation at 340 nm in the presence of a Grx reducing system which is composed of NADPH, GR, and GSH. The reaction was started by adding Grx after a 3-min pre-incubation time and Grx activity was corrected by subtracting the spontaneous reduction rate observed in the absence of Grx. Because of the spontaneous reaction of GSH with the substrates, we can generally not work at saturating concentrations of all substrates using these assays. Enzyme and substrate concentrations used are indicated in the legend of figures and tables. The activity was expressed as nmol of NADPH oxidized/nmol of Grx/s using a molar extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm for NADPH. Three independent experiments were performed at each substrate concentration, and k_{cat} and apparent K_m values were calculated by non-linear regression using the Michaelis–Menten equation using the program GraphPad Prism 4.

PREPARATION AND ANALYSIS OF OXIDIZED Grxs

Around 10 mg of protein was reduced using 40 mM DTT in 500 μl of 30 mM Tris-HCl pH 8.0 buffer for 2 h at 25°C followed by desalting on G25 column pre-equilibrated with 30 mM Tris-HCl pH 8.0 buffer. Oxidized proteins were prepared by incubating 500 μM reduced poplar Grxs in *ca* 200 μL with a 10 fold excess GSSG or H_2O_2 or a 5 fold excess GSNO for 2 h at 25°C before desalting on G25 columns. The protein oxidation state was then analyzed by electrospray ionization mass spectrometry analysis as described previously (Couturier et al., 2011).

For alkylation assays, reduced Grxs treated or not with oxidants were diluted to 10 μM into 50 μl of 30 mM Tris-HCl pH 8.0 buffer and precipitated on ice for 30 min with one volume of 20% trichloroacetic acid (TCA). After centrifugation (10 min at 13,000 *g*) and washing with 100% acetone, the pellet was resuspended into 10 μl of 100 mM Tris-HCl pH 8.0, 1% SDS containing 2 mM of methoxyl-PEG maleimide of 2 kDa (mPEG maleimide) which alkylates free thiol groups. The protein mixture was then separated on non-reducing 15% SDS-PAGE.

For midpoint redox potential titrations, Cys_A glutathionylated forms were obtained by the above described GSNO treatment of the double cysteinic mutants (GrxC1 C34/88S, GrxC2 C26/80S) and of the monocysteinic mutants (GrxC3 C40S and GrxC4 C30S). The intramolecular disulfide bond (Cys_A-Cys_B) containing proteins were obtained by the above described H_2O_2 treatment but using GrxC1 C88S, GrxC2 C80S and GrxC3 and GrxC4 WT isoforms.

pK_a DETERMINATION OF THE CATALYTIC CYSTEINE

The pK_a measurements of N-terminal active site cysteines (Cys_A) have been performed with WT Grx isoforms following a procedure described in (Gallogly et al., 2008). Briefly, 3 μM reduced Grx was incubated with or without 300 μM iodoacetamide (IAM) in 100 mM sodium citrate or phosphate buffers ranging from pH 2.0 to 7.0. Following this pre-incubation step, Grx activity was determined by adding an aliquot of the pre-incubation mixture to the HED assay described above. The percentages of remaining activity at each pH were determined by comparing the activity of the enzyme incubated with and without IAM and an adaptation of the Henderson–Hasselbach equation (Gallogly et al., 2008) was used for pK_a value determination using the program GraphPad Prism 4.

MIDPOINT REDOX POTENTIAL (E_m) DETERMINATION

Oxidation-reduction titrations using the fluorescence of the adduct formed between protein free thiols and monobromobimane (mBBr) were carried out at ambient temperature in 200 μl of 100 mM HEPES pH 7.0 buffer containing 10 μM of oxidized proteins, either glutathionylated or with an intramolecular disulfide-bond, and defined mixtures of oxidized and reduced DTT or glutathione for more positive values to set the ambient potential (E_h). Total concentration of DTT or glutathione was 2 mM. After 2 h incubation, mBBr was added at a final concentration of 2 mM and the reaction was carried out in the dark for 20 min. The reaction mixture was then precipitated on ice for 30 min with one volume of 20% TCA. After centrifugation

(10 min at 13,000 g) and washing with 100% acetone, the pellet was resuspended into 400 µl of 100 mM Tris-HCl pH 8.0, 1% SDS. Fluorescence emission of the resulting solution was measured at 472 nm after excitation at 380 nm using a variant Cary Eclipse (Agilent). Values were transformed into percentages of reduced protein and fitted to the Nernst equation using non-linear regression for E_m value determination.

RESULTS

KINETIC COMPARISON OF POPLAR CLASS I Grxs

An amino acid sequence alignment of the predicted mature forms of all poplar class I Grxs reveals several conserved residues, particularly those around the active sites and those involved in glutathione binding (**Figure 1**). However, GrxC1 and GrxC2 present CGYC and CPFC active site motifs, respectively, different from the CPYC active site found in GrxC3 and GrxC4. Interestingly, contrary to GrxC3 and GrxC4, GrxC1 and GrxC2 isoforms possess an additional cysteine (Cys_C) located in their C-terminal part in a IGGCD motif, as in human Grx1, *E. coli* Grx3 and the two other class I Grxs found in plants, GrxC5 and GrxS12 (**Figure 1**). With the aim of comparing the redox and kinetic properties of all dithiol Grxs (GrxC5 is not found in poplar) and understanding why these duplicated genes have been retained during evolution, we have performed a thorough biochemical analysis of the corresponding recombinant proteins.

The catalytic properties of each isoform were first determined by measuring their reductase activity using the classical HED and DHA assays, which measure the reduction of glutathionylated-β-mercaptoethanol or of dehydroascorbate, respectively. All proteins display a better catalytic efficiency (k_{cat}/K_m) in the HED test with a difference of two orders of magnitude (**Table 1**). In both assays, the kinetic parameters determined for each protein follow the same tendency. GrxC3 and C4 have quite comparable turnover numbers, with k_{cat} values about 10 fold higher than those of GrxC1 and C2. In terms of catalytic efficiencies, the dichotomy between GrxC1/C2 and GrxC3/C4 is clear but it is often below this factor of 10 because GrxC3 and C4 have higher apparent K_m values for both substrates. Overall, GrxC1 always exhibited the lowest catalytic efficiency and GrxC4 the highest. Concerning the apparent affinity for GSH, although there

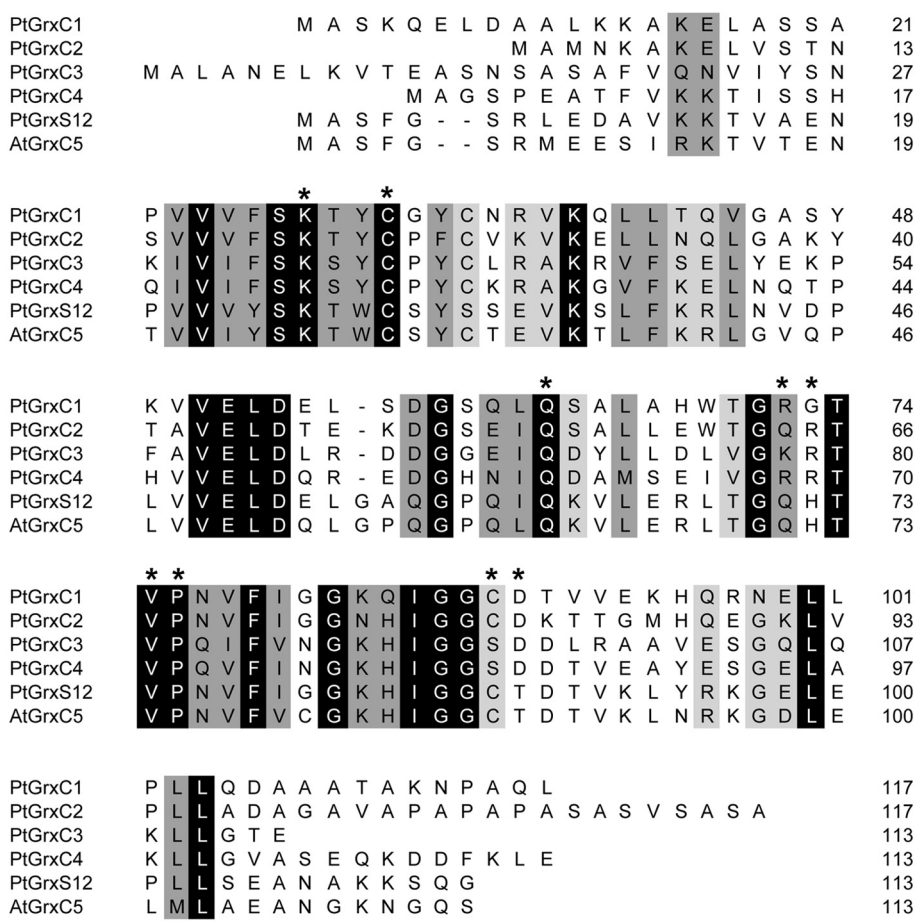

FIGURE 1 | Amino acid alignment of class I glutaredoxins. The alignment was done using ClustalW from sequences of the recombinant proteins that are deprived of putative targeting sequences. The strictly conserved amino acids are depicted in white on black. Other conservative amino acid changes are indicated in black on gray. Residues involved in GSH binding based on the 3D structures of poplar GrxC4 and GrxS12 and Arabidopsis GrxC5 are marked by an asterisk.

Table 1 | Kinetic parameters of poplar dithiol class I glutaredoxins.

		PtGrxC1	PtGrxC2	PtGrxC3	PtGrxC4	PtGrxS12[a]	AtGrxC5[a]
β-ME-SG	K_m (mM)	0.10 ± 0.01	0.06 ± 0.01	0.50 ± 0.06	0.32 ± 0.04	0.31 ± 0.04	0.20 ± 0.02
	k_{cat}(s^{-1})	7.9 ± 0.2	11.7 ± 0.1	77.0 ± 4.4	90.3 ± 4.9	23.1 ± 0.9	1.21 ± 0.03
	k_{cat}/K_m(x $10^5 M^{-1}.s^{-1}$)	0.79	1.95	1.54	2.82	0.745	0.06
DHA	K_m (mM)	0.55 ± 0.06	0.37 ± 0.03	1.14 ± 0.13	0.60 ± 0.09	0.38 ± 0.19	0.21 ± 0.03
	k_{cat}(s^{-1})	0.6 ± 0.1	1.3 ± 0.1	6.5 ± 0.4	8.2 ± 0.6	1.7 ± 0.2	0.23 ± 0.01
	k_{cat}/K_m(x $10^3 M^{-1}.s^{-1}$)	1.09	3.51	5.70	13.67	4.6	1.1
GSH	K_m (mM)	2.76 ± 0.26	3.87 ± 0.53	3.60 ± 0.42	1.96 ± 0.28	4.0 ± 0.5	nd
	k_{cat}(s^{-1})	13.8 ± 0.6	30.1 ± 2.3	131.3 ± 8.3	102.9 ± 6.5	92.8 ± 5.3	nd
	k_{cat}/K_m(x $10^3 M^{-1}.s^{-1}$)	5.00	7.78	36.47	52.50	23.20	nd

The apparent K_m values for β-ME-SG (glutathionylated β-mercaptoethanol) were determined using a HED concentration range of 0.1–1 mM in presence of 2 mM GSH and 100 nM enzyme for GrxC1, 50 nM for GrxC2, 10 nM for GrxC3 and GrxC4. The apparent K_m values for DHA were determined using a DHA concentration range of 0.1–1 mM in presence of 2 mM GSH and 2 μM enzyme for GrxC1, 1 μM for GrxC2, 200 nM for GrxC3, and 150 nM for GrxC4. The apparent K_m values for GSH were determined using a GSH concentration range of 0.25–5 mM in the presence of 0.7 mM HED and 100 nM enzyme for GrxC1, 50 nM for GrxC2, 10 nM for GrxC3 and GrxC4. The k_{cat} and K_m values were calculated by non-linear regression using the Michaelis–Menten equation. Values are the means ± SD of three replicates. [a]*Data extracted from the following references Couturier et al. (2009b, 2011), Zaffagnini et al. (2012a).* nd, *not determined.*

are differences in the K_m values for GSH between the four isoforms, ranging from ∼2.0 mM for GrxC4 to ∼3.9 mM for GrxC2, the GrxC1/C2 and GrxC3/C4 dichotomy is not visible using this parameter.

Then, the involvement for the catalytic mechanism of the different cysteine residues present in each isoform was investigated by comparing the activity of WT proteins to their corresponding cysteinic mutants using similar assays at fixed substrate concentrations (**Figure 2**). As expected, all monocysteinic mutants for the catalytic cysteine Cys_A (GrxC1 C31S, GrxC2 C23S, GrxC3 C37S, and GrxC4 C27S) were inactive, confirming that it is indispensable for the reductase activity. On the contrary, mutating Cys_C, in positions 88 and 80 in GrxC1 and GrxC2, respectively, did not affect catalytic activity. Finally, the mutation of Cys_B has a different impact depending on the isoforms. The protein variants for this cysteine in GrxC1 and C2 (GrxC1 C34S and GrxC2 C26S) were 5 fold more efficient and 2–3 fold more efficient than the corresponding WT proteins in the HED and DHA assays, respectively. On the other hand, the mutation of this cysteine in GrxC3 and GrxC4 has almost no impact, since, only a slight decrease was observed in the HED assay for the GrxC3 C40S variant.

OXIDATIVE MODIFICATIONS OF CYSTEINE RESIDUES

Several observations prompted us to analyze the sensitivity of all dithiol class I Grxs to oxidizing treatments and the resulting changes in their redox state. First, the mutation of Cys_B increased the catalytic efficiency of GrxC1 and C2 but not of GrxC3 and C4 suggesting possible difference in the reaction mechanisms. Second, although Cys_C does not influence their catalytic activity, the migration of the purified proteins in non-reducing SDS PAGE gels indicated the formation of covalent linkages for GrxC1 and C2 that are not visible for GrxC3 and C4 (data not shown).

For this purpose, reduced proteins have been treated for 2 h with a 10 fold excess of GSSG or H_2O_2, or a 5 fold excess of GSNO. The resulting redox state was analyzed by mass spectrometry and by protein migration on non-reducing SDS PAGE with or without thiol alkylation by 2 kDa mPEG maleimide, which allows distinguishing reduced and oxidized forms of Grxs. Since reduced mPEG-alkylated proteins and covalent dimers of GrxC1 and GrxC2 migrate roughly at the same position, controls without alkylation have been included (**Figure 3**). With non-alkylated proteins, we confirmed that an H_2O_2 treatment, but not the GSSG and GSNO treatments, promoted the formation of covalent dimers of GrxC1 and GrxC2 (**Figures 3A,B**) but not of GrxC3 and GrxC4 (**Figures 3C,D**). Only a small part of GrxC2 dimer is visible after these 2 h GSSG and GSNO treatments. From the procedure that included an alkylation step, we can further conclude that all treatments led essentially to a complete oxidation of all thiol groups present on all Grxs since almost no shift of migration was observed. A minor form with one free thiol group is visible for GrxC2 after a GSSG treatment. It is even more discrete upon GSNO treatment. For GrxC3 and C4, these oxidized monomeric forms are interpreted as containing intramolecular disulfides between active site cysteines (Cys_A-Cys_B) (**Figures 3C,D**). For GrxC1 and C2, these two cysteines should also form this intramolecular disulfide, and Cys_C is either modified by a glutathione or a nitroso (in the case of GSNO) adduct as confirmed by mass spectrometry analyses (see below) or it is involved in an intermolecular disulfide upon H_2O_2 treatment. From similar experiments performed with the GrxC1 C88S and GrxC2 C80S mutants (**Figures 3E,F**), the absence of covalent dimers indicated that Cys_C is indeed involved in dimerization. Moreover, in the context of these variants, after GSSG and GSNO treatments, the proteins mainly contain an active site disulfide bond whereas a small part of the proteins has one remaining free thiol, likely corresponding to glutathionylated intermediates.

In order to identify the type of protein modification formed by these oxidative treatments, all proteins have been analyzed by mass spectrometry (**Table 2** and **Figures S1–S6**). First, all reduced proteins gave a single species with a mass decrease of *ca* 131 Da compared to the calculated theoretical molecular masses. It does undoubtedly correspond to the cleavage of the first methionine as expected from the presence of an alanine in second position. The results obtained with GrxC3 and GrxC4 are more easily

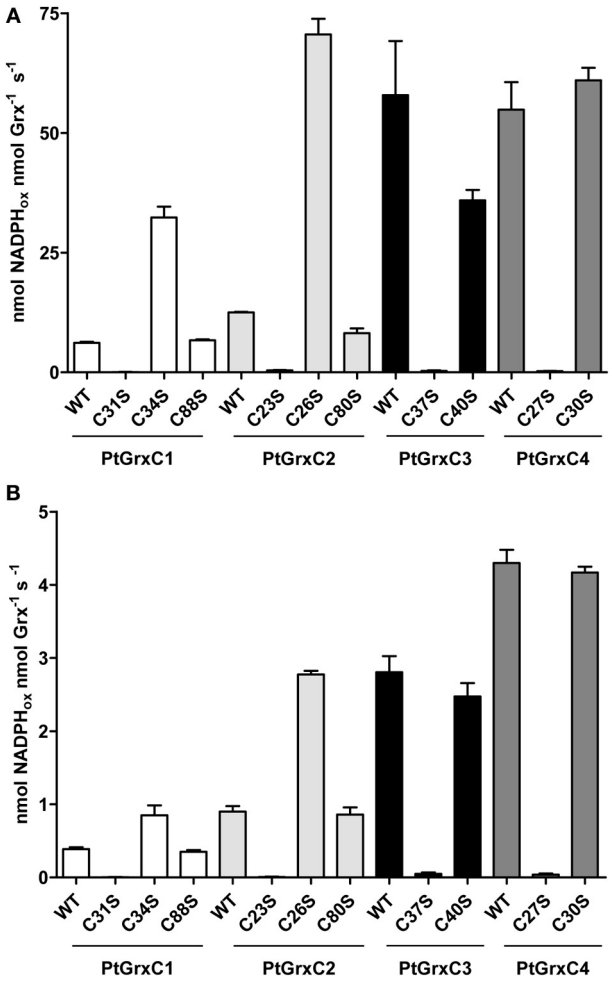

FIGURE 2 | Reductase activity of GrxC1, C2, C3, and C4 and of cysteine-to-serine variants. (A) HED reduction was measured in the presence of 2 mM GSH and 0.7 mM HED using either 100 nM GrxC1, 20 nM for GrxC1 C34S, 50 nM GrxC2, 10 nM for GrxC2 C26S, 10 nM GrxC3, or 10 nM GrxC4. **(B)** DHA reductase activity was measured in the presence of 2 mM GSH and 1 mM DHA using either 2 μM GrxC1, 1 μM for GrxC1 C34S, 1 μM GrxC2, 250 nM GrxC2 C26S, 200 nM GrxC3, or 150 nM GrxC4. The data are expressed as nmol NADPH oxidized per nmol enzymes per s and represent means ± SD of three separate experiments.

interpreted since these proteins contain only two cysteines. In the presence of H_2O_2, and in accordance with the absence of detected free thiols in the previous experiments (**Figures 3C,D**), GrxC3 and GrxC4 are exclusively detected as a monomeric oxidized form with a decrease of ∼2 Da confirming the formation of a Cys_A-Cys_B intramolecular disulfide bond. The same forms were primarily obtained upon GSSG and GSNO treatments but a minor monomeric form with a mass increment of 305 Da corresponding to one glutathione adduct was also detected (**Table 2**). It was not detected on gels presumably because of the presence of minute amounts. Nevertheless, this suggests that the glutathionylated form represents an intermediate for the formation of the intramolecular disulfide.

The results obtained for GrxC1 and GrxC2 are slightly more complex. In response to an H_2O_2 treatment, two different species are formed for both proteins. In accordance with the results from alkylation experiments, a major form corresponds to a disulfide-bridged dimer having also an intramolecular Cys_A-Cys_B disulfide bond. The detected monomeric forms, which have no free thiols or a very small fraction as observed for GrxC2, exhibited a mass increment of 29.5 Da for GrxC1 and 32.2 Da for GrxC2, suggesting the presence of two oxygen atoms. Considering that this overoxidation is not observed for GrxC1 C88S and GrxC2 C80S variants which only form monomeric proteins with an intramolecular disulfide (as indicated by the mass decrease of 2 Da), the results obtained for the WT proteins are best interpreted as the formation of an intramolecular disulfide between the active site cysteines (Cys_A-Cys_B) and of a sulfinic acid on Cys_C. In the presence of GSSG or GSNO, GrxC1 and GrxC2 exist only under monomeric forms but with variable oxidation states. Except for the GSSG-treated GrxC1, there is a minor form with a mass decrease of about 2 Da consistent with the formation of an intramolecular disulfide. Another minor form, found in all samples except the GSNO-treated GrxC2, exhibited a mass increment of 610.5–611 Da, which corresponded to the presence of two glutathione adducts. However, the major species formed by these two treatments is a monomer with a mass increment of about 303.5 Da which is consistent with the presence of one glutathione adduct on Cys_C in addition to the Cys_A-Cys_B disulfide bond. Accordingly, the major species detected in protein variants mutated for Cys_C (Grx C1 C88S and GrxC2 C80S) is the one with an intramolecular disulfide (decrease of 2 Da upon GSSG or GSNO treatments). The glutathionylated forms detected by mass spectrometry were visible on gels (see **Figures 3E,F**).

The *in vitro* observation of intermolecular disulfide bonds in GrxC1 and C2, involving Cys_C, raised the question of their possible reduction. Thus, we tested the ability of DTT but also of a physiological GSH reducing system composed of NADPH, GR, and GSH to reduce this intermolecular disulfide, the formation of which was initiated by a 2h H_2O_2treatment. As shown in **Figure 4**, both DTT and GSH are able to reduce GrxC1 and GrxC2 dimers to a similar extent. Whether minute amounts of reduced monomeric Grx initially present or formed can catalytically increase the reduction of dimeric Grxs in the presence of GSH cannot be excluded. Overall, it indicates that the formation of these disulfide-bridged forms of GrxC1 and GrxC2 isoforms is reversible, notably by GSH, a relevant cellular physiological reductant.

REDOX PROPERTIES OF CLASS I GLUTAREDOXINS

As in other thiol-dependent oxidoreductases, the catalytic efficiency of class I Grxs should be at least partially governed by thermodynamic parameters as the redox midpoint potentials of dithiol-disulfide couples and by the reactivity of the catalytic cysteine which is dependent on its pK_a. Hence, we determined the Cys_A pK_a of the four Grx isoforms using a method relying on iodoacetamide which is an alkylating reagent reacting with thiolates but not thiols. The pH-dependent inactivation of WT Grx by iodoacetamide was followed by comparing the activity of WT proteins in the HED assay, after pre-incubation of the protein in

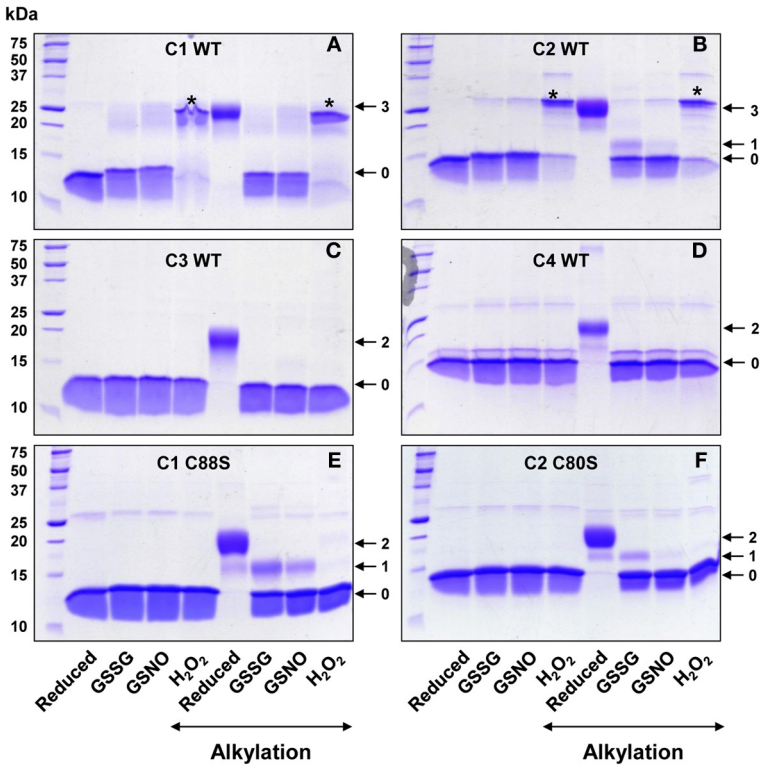

FIGURE 3 | Sensitivity of poplar Grxs to oxidative treatments. Pre-reduced Grxs were subjected to 2 h oxidative treatments with H_2O_2, GSSG, and GSNO as described in the "materials and methods" section. Prior to separation on SDS-PAGE gels in non-reducing conditions, GrxC1 WT **(A)**, GrxC2 WT **(B)**, GrxC3 WT **(C)**, GrxC4 WT **(D)**, GrxC1 C88S **(E)**, and GrxC2 C80S **(F)** were alkylated or not with 2 kDa mPEG maleimide. The shift observed following the alkylation of the free thiol groups is larger than expected (ca. 4 kDa instead of 2 kDa), but this has been already observed previously (Chibani et al., 2011). The numbers on the right correspond to the number of alkylated thiols that remained free after reducing or oxidizing treatment of Grxs. The stars in panels **(A)** and **(B)** indicate the position of covalent dimer.

different buffers ranging from pH 2.0 to 7.0 in the presence or not of iodoacetamide. From these titration curves, we obtained pK_a values of 5.3 ± 0.1 for GrxC1, 5.0 ± 0.1 for GrxC2, 4.6 ± 0.1 for GrxC3, and 4.6 ± 0.1 for GrxC4 (**Figure 5**). These results indicate first that at physiological pH, the thiolate ion will be the dominant species for all Grxs. Although the differences are not huge, it also confirmed the existence of two subgroups, GrxC1 and C2 having higher p$K_a$compared to GrxC3 and C4.

Two disulfide bond forms may be catalytically relevant for all these Grxs, the one with a glutathionylated Cys_A and the one with a Cys_A-Cys_B intramolecular disulfide bond. For measuring the midpoint redox potentials (E_m) of each of these disulfide/dithiol couples, we used different protein variants and two distinct oxidative treatments in order to discriminate between these two oxidized forms. To obtain proteins containing an intramolecular disulfide bond, reduced Grxs possessing only the two active site cysteines (GrxC1 C88S, GrxC2 C80S, GrxC3 WT, and GrxC4 WT) have been treated with H_2O_2 before desalting on G25 columns. On the other hand, glutathionylated proteins have been obtained by treating reduced proteins in which only Cys_A was remaining (GrxC1 C34/88S, GrxC2 C26/80S, GrxC3 C40S, and GrxC4 C30S) with GSNO before desalting. The E_m values determined at pH 7.0 for the intramolecular disulfide range from −233 mV for GrxC3 to −263 mV to GrxC1 (**Figure 6**). On the other hand, only little variations were visible when measuring E_m values for the glutathione adduct formed on the catalytic cysteine. They varied from −242 mV for GrxC3 to −254 mV for GrxC1. Overall, the intramolecular active site disulfide bond and glutathionylated forms of GrxC2 and GrxC4 present similar E_m values. A 10 mV difference was measured between both forms for GrxC1 and C3, the more electronegative redox potential being the active site disulfide in GrxC1 and the glutathione adduct in GrxC3. However, the differences are small and should not explain the observed differences in catalytic efficiencies. In reducing cellular environments, all these oxidation forms should be compatible with glutathione reduction. Considering their possible targeting to secretory pathways, this may not be the case for Grx3 and C4 if they are in the endoplasmic reticulum or in the apoplasm.

DISCUSSION

DUPLICATION AND NEO-FUNCTIONALIZATION INTO CLASS I GLUTAREDOXINS

Among all living organisms, higher plants have the highest number of genes coding for Grxs. In addition to specific isoforms such as those belonging to the class III, land plants also possess

Table 2 | Electrospray ionization mass spectrometry analysis of the redox states of poplar class I Grxs.

Protein	Theoretical size (Da)	Theoretical size without Met (Da)	DTT_{red} treatment	GSSG treatment	Relative percentage values (%)	GSNO treatment	Relative percentage values (%)	H_2O_2 treatment	Relative percentage values (%)	Deduced redox state		
										Cys_A	Cys_B	Cys_C
PtGrxC1 WT	12,514.3	12,383.1	12,382.7	**12,686.3 (+303.6)**	**76.4**	12,380.0 (−2.7)	5.7	**12,412.2 (+29.5)**	**72.1**	SH	SH	SH
				12,993.8 (+611.1)	23.6	**12,686.3 (+303.6)**	**81.8**	24,756.5 (×2)	27.9			
						12,993.4 (+610.7)	12.5					
										S-S		SH
										S-S		SO_2H
										S-S		SSG
										SSG	SH	SSG
										S-S		S-S (interchain)
PtGrxC2 WT	12,178.9	12,047.7	12,047.3	12,045.0 (−2.3)	11.1	12,044.9 (−2.4)	23.7	12,077.2 (+32.2)	50	SH	SH	SH
				12,351.5 (+304.2)	**68.4**	**12,350.7 (+303.4)**	**76.3**	24,088.9 (×2)	50			
				12,657.9 (+610.6)	20.5							
										S-S		SH
										S-S		SO_2H
										S-S		SSG
										SSG	SH	SSG
										S-S		S-S (interchain)
PtGrxC3 WT	12,516.2	12,385.0	12,384.7	**12,382.7 (−2.0)**	**66.3**	**12,382.9 (−1.8)**	**75.9**	12,382.7 (−2.0)	100	SH	SH	
				12,690.0 (+305.3)	33.7	12,690.0 (+305.3)	24.1					
										S-S		
										SSG	SH	
PtGrxC4 WT	12,526.1	12,394.9	12,394.4	**12,392.0 (−2.4)**	**62.5**	**12,392.1 (−2.5)**	**90.8**	12,392.3 (−2.7)	100	SH	SH	
				12,699.6 (+305.2)	37.5	12,699.2 (+304.8)	9.2					
										S-S		
										SSG	SH	
PtGrxC1 C88S	12,498.2	12,367.0	12,366.6	**12,364.7 (−1.9)**	**64.0**	**12,364.5 (−2.1)**	**79.6**	12,364.6 (−2.0)	100	SH	SH	
				12,671.8 (+305.2)	36.0	12,672.0 (+305.4)	20.4					
										S-S		
										SSG	SH	
PtGrxC2 C80S	12,162.8	12,031.7	12,030.6	**12,029.3 (−1.3)**	**81.5**	12,028.8 (−1.8)	100	12,028.7 (−1.9)	100	SH	SH	
				12,336.5 (+305.9)	18.5							
										S-S		
										SSG	SH	

Reduced proteins and proteins treated with GSNO, GSSG, or H_2O_2 were analyzed by mass spectrometry. The mass differences are indicated between parentheses. The mass accuracy is generally ±0.5 Da. Comparing mass differences and migration profiles of corresponding alkylated proteins under non-reducing conditions enabled to deduce the redox state of each cysteine residue. When several oxidized forms are detected and with clear different abundance, the major form is indicated in bold. The relative percentage of each form has been deduced from the spectra assuming equal ionization of all GRX forms.

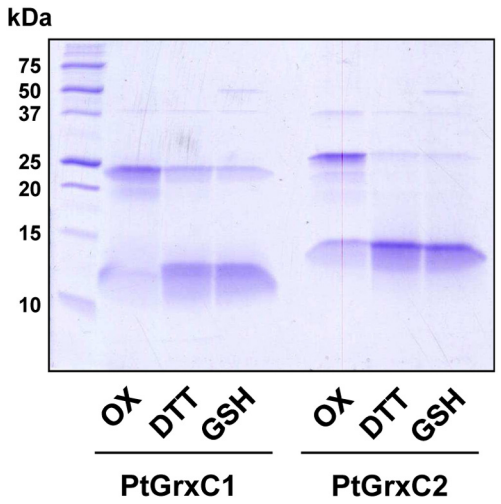

FIGURE 4 | GSH-dependent reduction of the intermolecular disulfide in poplar GrxC1 and GrxC2. The ability of GSH to reduce poplar disulfide-bridged dimer GrxC1 and disulfide-bridged dimer GrxC2 generated by a 2 h H_2O_2 treatment was evaluated in the presence of 1 mM DTT and 1 mM GSH coupled to NADPH and GR. The lanes Ox represent the initial oxidized proteins. Proteins were separated on SDS-PAGE gels under non-reducing conditions.

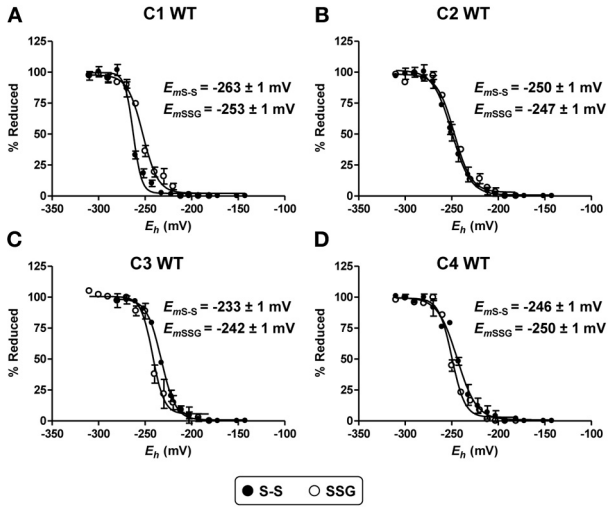

FIGURE 6 | Determination of redox potential of oxidized poplar GrxC1, C2, C3, and C4. Representative curve of oxidation-reduction titration of Cys_A-Cys_B intramolecular disulfide forms (black circles) and glutathionylated Cys_A (white circles) forms of poplar GrxC1 **(A)**, GrxC2 **(B)**, GrxC3 **(C)**, and GrxC4 **(D)** at pH 7.0. The titration was carried out using a total DTT or GSH concentration of 2 mM for 2 h and resulting free thiol groups were labeled by mBBr. Values in the text are the means ± SD of three replicates.

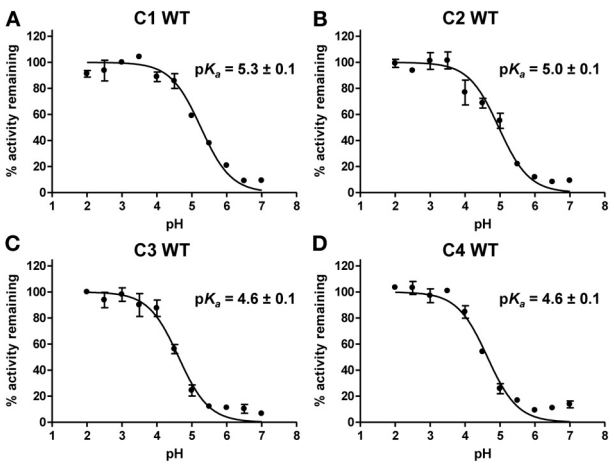

FIGURE 5 | pK_a determination of the catalytic cysteine of poplar GrxC1, C2, C3, and C4. Reduced GrxC1 **(A)**, GrxC2 **(B)**, GrxC3 **(C)**, and GrxC4 **(D)** WT proteins were incubated in different buffers ranging from pH 2.0 to 7.0 in presence or not of iodoacetamide (IAM) prior to measurement of their activity by using the HED assay. The percentages of remaining activity at each pH were determined by comparing the activity of the enzyme incubated with and without IAM and an adaptation of the Henderson–Hasselbach equation was used for pK_a value determination. The data are represented as mean ± SD of three separate experiments.

an expanded class I, usually with 5–6 isoforms (Couturier et al., 2009a). From previous phylogenetic analyses, it was quite clear that, there are three Grx couples, GrxC1/C2, GrxC3/C4, and GrxC5/S12 that probably evolved by more or less recent duplication (Couturier et al., 2009a). Interestingly, GrxC1 and C2 proteins are cytoplasmic proteins (Riondet et al., 2012), GrxC3 and C4 have N-terminal extensions predicted to target the proteins to secretory pathways but the final destination is not known, and GrxC5 and S12 are chloroplastic (Couturier et al., 2009b, 2011). The case of GrxC1 and C2 is interesting. Whereas GrxC2 prototypes are found in all land plants (mosses, lycophytes, monocots, dicots), GrxC1 is specifically found in dicots. A redundancy between both isoforms is suggested in *A. thaliana* by the observation that single knock-out mutants in *grxc1* or *grxc2* are aphenotypic, whereas a double mutant is lethal at an early stage after pollinization (Riondet et al., 2012). Considering that GrxC1, but not GrxC2, is able to bind an Fe-S cluster (at least *in vitro*), this would rather suggest that the essential nature of these two paralogs is linked to their reductase activity. These results point to the need of investigating at the genetic level a possible redundancy among the GrxC3/C4 and GrxC5/12 couples. This study primarily focused on the biochemical properties of dithiol class I Grxs since a detailed structure-function analysis was previously achieved on the two other prototypes of class I Grxs, the poplar monothiol GrxS12 and the Arabidopsis dithiol GrxC5 (Couturier et al., 2009b, 2011). GrxC5 isoforms are only found in species of the Brassicaceae family. We have demonstrated in particular that, although being very similar in sequence due to their recent duplication, a single substitution in the active site sequence (from WCSYS in GrxS12 to WCSYC in GrxC5) explains the capacity of GrxC5, but not GrxS12, to bind an [Fe_2-S_2] cluster into a dimer. Among other class I Grxs, only GrxC1 can also exist under a holoform binding an Fe-S cluster (Rouhier et al., 2007). In this case, the mutational analysis showed that the presence of the glycine in the CGYC active site instead of a proline in other Grxs (CPFC in GrxC2 or CPYC in GrxC3 and C4) was sufficient to explain this property. Overall, this indicates that subtle sequence differences

affecting key residues may provide different properties to otherwise quite close proteins. This prompted us to further analyze the various glutaredoxins of this class as GrxC2 and GrxC3 have been only poorly studied so far.

TWO SUBGROUPS IN DITHIOL Grxs EXHIBIT DISTINCT KINETIC PROPERTIES

Considering the divergence observed in the amino acid sequences, the first aim of this work was to perform a thorough comparative analysis of the biochemical properties of GrxC1, GrxC2, GrxC3, and GrxC4 from poplar. All these proteins exhibit deglutathionylation or reductase activities using classical HED and DHA assays with catalytic efficiencies (10^5 M^{-1} s^{-1} for HED and 10^3–10^4 M^{-1} s^{-1} for DHA) in the same range as those previously reported for plant and non-plant class I Grxs (**Table 3**). Two subgroups can however be distinguished, GrxC1 and GrxC2 appeared to be less efficient catalysts than GrxC3 and GrxC4. The difference is particularly clear when considering the turnover numbers (k_{cat}) and the k_{cat}/K_{DHA} and k_{cat}/K_{GSH}. This is not visible with $k_{cat}/K_{\beta-ME-SG}$ because the differences in the turnover number are compensated in this case by differences in apparent affinity in favor of GrxC1 and C2. An observation that may be interesting to understand these differences is that GrxC1/C2 and GrxC3/C4 should have quite different charge distribution at the protein surface that could account for substrate recognition and thus for the observed differences in catalytic efficiency. Indeed, their behavior on ion exchange chromatography indicates that GrxC1 and GrxC2 should be more basic having a pI close or higher than 8, whereas GrxC3 and GrxC4 should be more acidic having a pI value below 8 (theoretical pI estimations are indeed comprised between 5 and 6).

In order to further explain the differences between poplar class I Grxs, we determined the pK_a value of their catalytic cysteine, as it was proposed that reactivity of human and Chlamydomonas class I Grxs is partially related to the pK_a of N-terminal active site cysteine (Gallogly et al., 2008; Gao et al., 2010). The values obtained for poplar Grxs are globally close to those reported for other plant and non-plant class I Grxs (**Table 3**), including the value of 3.9 determined for poplar GrxS12 using the same procedure (Zaffagnini et al., 2012a; Roos et al., 2013). Interestingly, a difference of ca 0.5 unit is visible between GrxC1/C2 (pK_a around 5) and GrxC3/C4 (pK_a around 4.6). Such differences between paralogs have been described already since *C. reinhardtii* Grx1, *S. cerevisiae* Grx2, and human Grx1 exhibit higher turnover numbers often translated into higher catalytic efficiencies compared to *C. reinhardtii* Grx2, *S. cerevisiae* Grx1, and human Grx2, respectively (**Table 3**). For the human and *C. reinhardtii* enzymes, it is also associated to a difference in pK_a values of *ca* 1 unit, the enzymes with the lowest pK_a being the most efficient. According to the Brønsted theory, in a thiol-disulfide exchange reaction, each unit decrease in the pK_a value of the leaving group is responsible for a 4 fold increase in the second-order rate constant (Szajewski and Whitesides, 1980). This could fit with the conclusion that differences in pK_a values between class I poplar Grx isoforms are at least partially responsible for the differences in catalytic efficiencies observed. On the other hand, only small differences were observed for the oxidation-reduction midpoint potentials of both the glutathione adduct or the intramolecular disulfide and they are not discriminating GrxC1/C2 vs. GrxC3/C4. The measured values (from −230 to −260 mV) are in the same range than those reported for *E. coli* Grx1 (−233 mV) and human Grx1 and 2 (−232 and −221 mV) (**Table 3**) (Aslund et al., 1997; Sagemark et al., 2007).

THE ROLE OF THE RECYCLING Cys_B: MONOTHIOL OR DITHIOL REACTION MECHANISM?

Interestingly, the differences in protein activities between GrxC1 or C2 and GrxC3 or C4 are attenuated when Cys_B of GrxC1 or C2 is removed (see **Figure 2**). This has been reported also for Arabidopsis GrxC5, yeast Grx1, human Grx1 and Grx2, and pig Grx (Yang and Wells, 1991; Yang et al., 1998; Johansson et al., 2004; Gallogly et al., 2008; Discola et al., 2009; Couturier et al., 2011). In clear contrast, mutating this cysteine in yeast Grx2, *E. coli* Grx1 and Grx3 led to a decrease of Grx activity (Bushweller et al., 1992; Nordstrand et al., 1999; Peltoniemi et al., 2006; Discola et al., 2009; Saaranen et al., 2009). For *E. coli* Grx1 and by extension for Grxs for which mutation of this cysteine decreased their activity, this decrease was attributed to a change in glutathione recognition, binding, or affinity, because Cys_B would determine the glutathione specificity of the glutathionylated Grx reduction step (Saaranen et al., 2009). For GrxC1 and GrxC2 and other eukaryote Grxs mentioned above in which the mutation of this cysteine increased their activity, the data indicates that the presence of Cys_B slows down the reaction for some reason. It may eventually modify the pK_a of Cys_A. This has been experimentally confirmed for a pig Grx, for which the pK_a of the catalytic Cys22 is 3.8 in a WT protein but 4.9 and 5.9 in C25S and C25A variants (Yang and Wells, 1991). Another possibility is that a Cys_A-Cys_B intramolecular disulfide is formed during activity measurements either because it constitutes a regular step of the reaction cycle in some situations, especially for dithiol substrates or because it is a side reaction reflecting the competition between Cys_B and GSH for the reduction of the glutathionylated Cys_A (Gallogly et al., 2009). Then, the reduction of this Cys_A-Cys_B intramolecular disulfide intermediate would require two molecules of GSH vs. one molecule of GSH for the glutathionylated form and add two steps in the reaction mechanism. Thus, it seems unfavorable in terms of efficiency and energetic cost.

We have previously analyzed possible variations in the redox state of the dithiol AtGrxC5 (WCSYC active site) in response to 15 min treatments with GSSG or oxidized DTT by measuring the tryptophan intrinsic fluorescence of the protein, since the oxidation of the catalytic cysteine quenched the fluorescence of the adjacent tryptophan (Couturier et al., 2011). It was concluded that the dithiol AtGrxC5 cannot form a Cys_A-Cys_B intramolecular disulfide first because only the GSSG treatment led to the formation of a glutathionylated protein and to fluorescence quenching and second because the WT protein was crystallized with a glutathionylated adduct. However, at that time, we did use neither H_2O_2 and GSNO treatments, nor prolonged incubation, nor alkylation assays. In fact, reconsidering the results of mass spectrometry showing, in addition to the peaks with one or two glutathione adducts, a peak with a decrease of 2 Da between the reduced protein and the GSSG-treated protein, it is very likely

Table 3 | Kinetic and biochemical parameters of selected plant and non-plant class I Grxs.

Name	Active site sequence	HED			DHA			GSH			pK_a	E_m (pH7.0) (mV)	References
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m			
AtGrxC1	YCGYC	0.74 ± 0.01	38.91 ± 0.43	5.3×10^4	0.08 ± 0.01	3.03 ± 0.82	3.8×10^4	nd	nd	nd	nd	nd	Riondet et al., 2012
AtGrxC2	YCPYC	0.34 ± 0.02	18.44 ± 0.57	5.4×10^4	0.08 ± 0.01	3.26 ± 0.03	4.1×10^4	nd	nd	nd	nd	nd	Riondet et al., 2012
ScGrx1	YCPYC	0.14	3.5	2.5×10^4	nd	Nd	nd	6.2	17.1 (HED)	2.75×10^3	3.2 to 4.0 ± 0.2	nd	Discola et al., 2009
ScGrx2	YCPYC	0.6	85	1.4×10^5	nd	nd	nd	0.9	129 (HED)	1.43×10^5	3.1 to 3.5 ± 0.2	nd	Discola et al., 2009
CrGrx1	HCPYC	0.34 ± 0.04	30.40 ± 4.31	8.9×10^4	0.39 ± 0.03	1.67 ± 0.09	4.3×10^3	2.6 ± 0.5	$161.6 \pm$ 15.0 (HED)	6.1×10^4	3.9 ± 0.1	nd	Zaffagnini et al., 2008
CrGrx2	YCPYC	0.20 ± 0.04	7.10 ± 0.27	3.5×10^4	0.17 ± 0.05	0.84 ± 0.06	4.9×10^3	3.7 ± 0.7	26.5 ± 4.1 (HED)	7.1×10^3	4.8 ± 0.1	nd	Gao et al., 2010
HsGrx1	TCPYC	1.07	8.16	7.6×10^3	nd	Nd	nd	2.2	293 (HED)	1.4×10^5	3.6	−232	Johansson et al., 2004; Jao et al., 2006; Sagemark et al., 2007
HsGrx2	SCSYC	0.11	1.3	1.2×10^4	nd	Nd	nd	5.9	71.3 (HED)	1.2×10^4	4.6	−221	Jao et al., 2006; Sagemark et al., 2007; Gallogly et al., 2008

nd, not determined.

that a small fraction of the protein contained an intramolecular disulfide formed between active site cysteines (Couturier et al., 2011). Here, we have clearly observed that a prolonged oxidative treatment of reduced class I Grxs with H_2O_2, GSSG, or GSNO led preferentially to the formation of an intramolecular disulfide bond between Cys_A and Cys_B. For the H_2O_2 treatment, it likely goes first through the formation of a sulfenic acid on Cys_A before Cys_B performs a nucleophilic attack on it. For the GSSG or GSNO treatments, it is very likely that a nucleophilic attack of Cys_A onto these oxidized glutathione species leads to the formation of a glutathione adduct on Cys_A which is then slowly reduced by a Cys_B nucleophilic attack leading to the formation of the intramolecular disulfide and to the concomitant release of a GSH molecule.

It is worth mentioning here that these *in vitro* experiments have been done in particular with the aim of exploring all possible post-translational modifications but the conditions are not exactly physiological first because the concentrations of the oxidants may not be realistic and more importantly because this is done in the absence of GSH, which is normally found at high amounts and is highly reduced in most cell compartments. The presence of GSH would certainly prevent to a large extent the formation of these intramolecular disulfides because it may preferentially reduce either the sulfenic acids or the glutathione adducts over the Cys_B recycling cysteine. This is certainly the case for GrxC3 and GrxC4 since mutating CysB has no effect on the activity. The formation of an intramolecular disulfide in these proteins could thus constitute uniquely a side reaction, protecting the cysteine or inactivating the proteins only under specific conditions. On the contrary, if we consider that the increase of protein activity observed for CysB mutated GrxC1 and GrxC2 in HED and DHA assays in the presence of a fully reduced GSH pool reflects the formation of this intramolecular disulfide, it may be physiologically relevant for GrxC1 and GrxC2. This is interesting in several respects. First, while the current dogma is that the preferential substrates of Grxs are glutathionylated proteins, recent studies performed with mammalian Grx2 indicated that they can reduce dithiol substrates, e.g., proteins with intra- or intermolecular disulfide (Hanschmann et al., 2010; Schutte et al., 2013). In this case, the Grxs should employ a reaction mechanism similar to the one used by Trxs involving both Cys_A and Cys_B. Second, based on the observation that some Grxs can be reduced by thioredoxin reductases (Johansson et al., 2004; Zaffagnini et al., 2008; Couturier et al., 2013), another possibility could be that in specific physiological situations or sub-cellular compartments where glutathione is depleted, absent or oxidized, the formation of an intramolecular disulfide may be determinant either to favor the use of an alternative reducing system as the one constituted by NADPH and thioredoxin reductase or to simply constitute a protective mechanism, preventing the irreversible overoxidation of this residue and thus protein inactivation.

In conclusion, the role of Cys_B in dithiol Grxs is yet uncertain and may depend on the Grx isoform as this residue influences differentially the protein activity. The fact that the formation of an intramolecular disulfide bond between Cys_A and Cys_B is possible in all cases lets open the possibility to use both monothiol and dithiol catalytic mechanisms, contrary to Grxs possessing only one cysteine residue.

THE ROLE OF THE SEMI-CONSERVED Cys_C IN GrxC1 AND GrxC2 ISOFORMS

The Cys_C cysteine residue, present in the IGGCD motif (at position 88 and 80 in GrxC1 and C2, respectively), is found in several other class I Grxs, including plant GrxC5 and S12 as well as in human Grx1 and *E. coli* Grx3. Moreover, it is also present in many class II Grxs. It was shown for instance that a Cys_A-Cys_C intramolecular disulfide bond can be formed in *S. cerevisiae* Grx5 and *C. reinhardtii* Grx3 (Tamarit et al., 2003; Zaffagnini et al., 2008), suggesting that Cys_C can serve as a recycling cysteine in the absence of Cys_B. Reduced glutathione does not seem to be an efficient reductant for this intramolecular disulfide bond and in the case of CrGrx3, it is reduced by a ferredoxin-thioredoxin reductase system (Zaffagnini et al., 2008). Although GrxS12 has a monothiol WCSYS active site sequence, a Cys_A-Cys_C intramolecular disulfide has not been observed whatever the oxidizing conditions tested. Moreover, in the crystal structure of a glutathionylated GrxS12, the sulfur atom of Cys87 is 9.6Å away from the Cys27-SG adduct (Couturier et al., 2009b). Hence, this cysteine may not serve as a recycling cysteine in class I Grxs and does not *a priori* participate to the reaction mechanism. Consistently, its mutation in GrxC1 and C2 did not influence protein activity, as previously observed for poplar GrxS12, Arabidopsis GrxC5, or *E. coli* Grx3 (Nordstrand et al., 1999; Couturier et al., 2009b, 2011).

However, considering its position very close to the active site residues and to residues involved in GSH binding an effect on activity could have been observed. Indeed, it is positioned between the so-called GG kink which is important in determining the backbone geometry of the following amino acids, and a Thr or an Asp which forms hydrogen bonds with GSH (see **Figure 1**) (Couturier et al., 2009b; Li et al., 2010). In the GrxS12 structure, the backbone amino group of Cys_C also directly forms a hydrogen bond with the glutamyl group of GSH (Couturier et al., 2009b), but the substitution of the cysteine into serine would not disrupt this interaction, preventing to definitely rule out a GSH stabilizing effect of this cysteine. On the other hand, it is probable that modifying the redox state of this cysteine might have more pronounced effects influencing for example GSH binding. It has been shown for example that, under non-reducing conditions, *E. coli* Grx3 exists under an expected monomeric form but also forms disulfide-bridged dimer or multimers via Cys_C (Aslund et al., 1994, 1997). In addition, previously reported oxidative treatments of human Grx1, which contains three extra active site cysteines including Cys_C, led to the identification of several possible post-translational modifications, i.e., intramolecular disulfide, disulfide-bonded dimers and oligomers, glutathione adducts, nitrosylation, some of them inhibiting its activity (Hashemy et al., 2007). Depending on the conditions, Cys_C was found to be either nitrosylated or glutathionylated or involved into a disulfide. Similarly, we have observed redox changes of GrxC1 and C2 upon treatment with oxidants that could further provide clues about the possible function of this cysteine, if any. In the presence of H_2O_2, this cysteine was found to be either overoxidized or involved in disulfide-bridged homodimers.

Since this dimerization only occurs in presence of H_2O_2, the formation of an intermediate sulfenic acid is likely required before intermolecular disulfide bond formation which is consistent with the presence of a small part of overoxidized proteins. Interestingly, GSH can efficiently reduce these disulfide-bridged dimers, making this reaction physiologically reversible. In the presence of GSSG or GSNO, Cys_C is also prone to oxidative modification, but in the form of a glutathione adduct. Although we cannot test the impact of these modifications on Grx activity (we cannot selectively modify Cys_C without also oxidizing Cys_A and Cys_B), we can speculate that the different reversible oxidation forms of Cys_C observed here might represent regulatory or signaling intermediates transiently modifying the functions of these Grxs by affecting for example the nature or the time-course reduction of the target proteins.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00518/abstract

Figure S1 | Electrospray ionization mass spectrometry analysis of PtGrxC1 WT. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B)**, GSNO **(C)**, or H_2O_2 **(D)** as described in the Methods section.

Figure S2 | Electrospray ionization mass spectrometry analysis of PtGrxC2 WT. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B)**, GSNO **(C),** or H_2O_2 **(D)** as described in the Methods section.

Figure S3 | Electrospray ionization mass spectrometry analysis of PtGrxC3 WT. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B)**, GSNO **(C),** or H_2O_2 **(D)** as described in the Methods section.

Figure S4 | Electrospray ionization mass spectrometry analysis of PtGrxC4 WT. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B)**, GSNO **(C),** or H_2O_2 **(D)** as described in the Methods section.

Figure S5 | Electrospray ionization mass spectrometry analysis of PtGrxC1 C88S. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B)**, GSNO **(C),** or H_2O_2 **(D)** as described in the Methods section.

Figure S6 | Electrospray ionization mass spectrometry analysis of PtGrxC2 C80S. Spectra of whole protein were determined for reduced protein before **(A)** and after treatment with GSSG **(B),** GSNO **(C),** or H_2O_2 **(D)** as described in the Methods section.

Table S1 | Primers used for site-directed mutagenesis.

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Glutaredoxins are essential for stress adaptation in the cyanobacterium *Synechocystis* sp. PCC 6803

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Glutaredoxins are small redox proteins able to reduce disulfides and mixed disulfides between GSH and proteins. *Synechocystis* sp. PCC 6803 contains three genes coding for glutaredoxins: *ssr2061* (*grxA*) and *slr1562* (*grxB*) code for dithiolic glutaredoxins while *slr1846* (*grxC*) codes for a monothiolic glutaredoxin. We have analyzed the expression of these glutaredoxins in response to different stresses, such as high light, H_2O_2 and heat shock. Analysis of the mRNA levels showed that *grxA* is only induced by heat while *grxC* is repressed by heat shock and is induced by high light and H_2O_2. In contrast, *grxB* expression was maintained almost constant under all conditions. Analysis of GrxA and GrxC protein levels by western blot showed that GrxA increases in response to high light, heat or H_2O_2 while GrxC is only induced by high light and H_2O_2, in accordance with its mRNA levels. In addition, we have also generated mutants that have interrupted one, two, or three glutaredoxin genes. These mutants were viable and did not show any different phenotype from the WT under standard growth conditions. Nevertheless, analysis of these mutants under several stress conditions revealed that single *grxA* mutants grow slower after H_2O_2, heat and high light treatments, while mutants in *grxB* are indistinguishable from WT. *grxC* mutants were hypersensitive to treatments with H_2O_2, heat, high light and metals. A double *grxAgrxC* mutant was found to be even more sensitive to H_2O_2 than each corresponding single mutants. Surprisingly a mutation in *grxB* suppressed totally or partially the phenotypes of *grxA* and *grxC* mutants except the H_2O_2 sensitivity of the *grxC* mutant. This suggests that *grxA* and *grxC* participate in independent pathways while *grxA* and *grxB* participate in a common pathway for H_2O_2 resistance. The data presented here show that glutaredoxins are essential for stress adaptation in cyanobacteria, although their targets and mechanism of action remain unidentified.

Keywords: glutaredoxin, stress, redox regulation, cyanobacteria, high light, heat shock, oxidative stress, metal resistance

INTRODUCTION

Glutaredoxins are small redox proteins that were first discovered as alternatives to thioredoxin as electron donors for ribonucleotide reductase (Holmgren, 1976). Glutaredoxin structures are closely related to those of thioredoxins, the so-called thioredoxin fold, and they also catalyze disulfide reduction through a dithiol mechanism, as thioredoxins, though some glutaredoxins use a monothiol mechanism (Fernandes and Holmgren, 2004; Meyer et al., 2009). Furthermore, glutaredoxins are able to catalyze protein glutathionylation/deglutathionylation, a function initially ascribed for these enzymes (Rouhier et al., 2008; Meyer et al., 2009), but recent data suggest that thioredoxins could also contribute to deglutathionylating reaction in yeast and plants (Greetham et al., 2010; Bedhomme et al., 2012). Glutaredoxins generally use the GSH/glutathione reductase system for reduction, although some of them can also accept electrons directly from thioredoxin reductases or can be reduced by thioredoxins or other glutaredoxins (Zaffagnini et al., 2008; Couturier et al., 2009a; Meyer et al., 2009). Glutaredoxins can function as alternative electron donors for ribonucleotide reductase in *E. coli* (but not in yeast), in sulfate assimilation, as electron donors for 3′-phosphoadenosine 5′-phosphosulfate synthase or for methionine sulfoxide reductases (Meyer et al., 2009; Hanschmann et al., 2013; Lillig and Berndt, 2013; Toledano et al., 2013). Moreover, some glutaredoxins present peroxidase activity and/or are able to reduce some peroxiredoxins thus contributing to resistance to oxidative stress (Rouhier et al., 2001, 2002; Finkemeier et al., 2005; Hanschmann et al., 2010; Pedrajas et al., 2010). In addition, it has also been shown that some glutaredoxins are able to bind Fe-S clusters and are involved in some aspects of Fe-S cluster biogenesis and its regulation (Muhlenhoff et al., 2010; Rouhier, 2010; Couturier et al., 2011; Kumar et al., 2011; Li and Outten, 2012; Boutigny et al., 2013).

Six different classes of glutaredoxins have been described in photosynthetic organisms (Couturier et al., 2009a), where class I and II correspond to classical dithiolic and monothiolic glutaredoxins, respectively, that are present in all organisms. Class IV is restricted to photosynthetic eukaryotes and class III is specific to land plants. On the other hand class VI seems to be restricted to cyanobacteria and class V is only present in cyanobacteria and some proteobacteria (Benyamina et al., 2013). In photosynthetic eukaryotes the repertoire of glutaredoxin proteins is

larger than in other organisms, which suggests that they could play critical roles regulating processes related to photosynthesis (Rouhier et al., 2008; Couturier et al., 2009a). The exact roles of glutaredoxins in photosynthetic organisms remain unclear mainly because several paralogs of each have been identified in many plant genomes and only a few mutants are available (Couturier et al., 2009a; Meyer et al., 2009, 2012). In *Arabidopsis* only a few mutants in glutaredoxin genes have been analyzed. GrxS14 and GrxS17 class II glutaredoxins have been shown to be essential for oxidative stress tolerance and heat tolerance, respectively, while class III glutaredoxins have been shown to be essential for flower development and pathogen resistance (Li et al., 2009; Murmu et al., 2010; Zander et al., 2011). In contrast, several of these proteins have been characterized biochemically indicating that glutaredoxins could play multiple roles, including functions as electron donors, deglutathionylation of target proteins or as Fe-S cluster sensors (Rouhier et al., 2001; Gelhaye et al., 2003; Rouhier et al., 2003, 2005; Feng et al., 2006; Vieira Dos Santos et al., 2007; Bandyopadhyay et al., 2008; Zaffagnini et al., 2008; Couturier et al., 2009b, 2011; Gao et al., 2011; Bedhomme et al., 2012).

In cyanobacteria much less is known about glutaredoxins and their functions. All cyanobacteria contain at least two different glutaredoxins: one from the classical dithiolic subgroup (class I) and one from the monothiolic subgroup (class II) (Couturier et al., 2009a). Moreover, some cyanobacteria contain genes encoding for additional dithiolic glutaredoxins (class I) or for proteins from classes V and VI. *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) contains 3 genes coding for glutaredoxins: *ssr2061* (*grxA* also known as *grx2*) and *slr1562* (*grxB* also known as *grx1*) code for dithiolic glutaredoxins that belong to class I (Couturier et al., 2009a), while *slr1846* (*grxC* also known as *grx3*) codes for a monothiolic glutaredoxin that belongs to class II (Couturier et al., 2009a). The *Synechocystis* glutaredoxins have been implicated in responses to metals and metalloids (Lopez-Maury et al., 2009; Marteyn et al., 2009; Kim et al., 2012; Marteyn et al., 2013) and oxidative stress (Li et al., 2007; Marteyn et al., 2009). Moreover, putative targets of GrxA have been identified using a monocysteinic glutaredoxin mutant (Li et al., 2007). GrxC has been shown to be a dimer and to bind a 2Fe-2S cluster that is ligated by one cysteine from each subunit of the dimer and two molecules of GSH (Picciocchi et al., 2007; Iwema et al., 2009). In contrast, the *in vivo* functions of GrxC have not been explored in cyanobacteria.

Here we have characterized mutant strains lacking one, two, or all glutaredoxin genes, in all possible combinations, in response to several stresses in *Synechocystis*. These have showed that GrxA and GrxC play a prominent role in protecting cells from stress. Analysis of double and triple mutants showed a complex pattern of genetic interactions, suggesting that they can be involved in different pathways which are all interconnected. Furthermore, expression of *grxA* and *grxC* genes was induced by some of these stresses, while the *grxB* gene was expressed constitutively under all conditions analyzed. GrxA and GrxC proteins levels did not always correspond to their mRNA levels under these conditions suggesting that expression of glutaredoxins could be controlled also post-transcriptionally.

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS

Synechocystis cells were grown photoautotrophically on BG11C (Rippka et al., 1979) at 30°C under continuous illumination ($50\,\mu E{\cdot}m^{-2}{\cdot}s^{-1}$) and bubbled with a stream of 1% (v/v) CO_2 in air. For plate cultures, the medium was supplemented with 1% (wt/vol) agar. Kanamycin, chloramphenicol and spectinomycin were added to a final concentration of $50\,\mu g\ mL^{-1}$, $20\,\mu g\ mL^{-1}$, and $5\,\mu g\ mL^{-1}$, respectively. BG11C medium was supplemented with different concentrations of $CuSO_4$, $NiSO_4$, $CdCl_2$, Na_2SeO_4, and Na_2SeO_3 when indicated. Experiments were performed using cultures from the mid-logarithmic phase (3–5 μg chlorophyll mL^{-1}) cultivated without antibiotics. For high light conditions, cultures were illuminated with white light at an intensity of $500\,\mu E{\cdot}m^{-2}{\cdot}s^{-1}$, and the temperature was kept at 30°C by applying a 5-cm-thick water filter. For heat shock conditions, cells were grown in a water bath at 42°C. For oxidative stress conditions, 1 mM hydrogen peroxide was added. *Synechocystis* strains and their relevant genotypes are described in **Table 1**.

RNA ISOLATION AND NORTHERN BLOT ANALYSIS

Total RNA was isolated from 30 mL samples of *Synechocystis* cultures in the mid-exponential growth phase (3–5 μg chlorophyll mL^{-1}). Extractions were performed by vortexing cells in the presence of phenol-chloroform and acid-washed baked glass beads (0.25–0.3 mm diameter) as previously described (Garcia-Dominguez and Florencio, 1997). Five microgram of total RNA was loaded per lane and electrophoresed in 1.2% agarose denaturing formaldehyde gels (Sambrook et al., 1989) and transferred to nylon membranes (Hybond N-Plus; Amersham). All probes were

Table 1 | Strains used in this work.

Strain	genotype	ORF mutated	Antibiotic resistance	References
WT	*Synechocystis* sp. PCC 6803			Lab stock
SGRXA	*grxA::C.C1*	*ssr2061*	Cm	Lopez-Maury et al., 2009
SGRXB	*grxB::SpΩ*	*slr1562*	Sp	Lopez-Maury et al., 2009
SGRXC	*grxC::C.K1*	*slr1846*	Km	Lopez-Maury et al., 2009
SGRXAB	*grxA::C.C1* *grxB::SpΩ*	*ssr2061* *slr1562*	Cm Sp	Lopez-Maury et al., 2009
SGRXAC	*grxA::C.C1* *grxC::C.K1*	*ssr2061* *slr1846*	Cm Km	Lopez-Maury et al., 2009
SGRXBC	*grxB::SpΩ* *grxC::C.K1*	*slr1562* *slr1846*	Sp Km	Lopez-Maury et al., 2009
SGRXABC	*grxA::C.C1* *grxB::SpΩ* *grxC::C.K1*	*ssr2061,* *slr1562* *slr1846*	Cm Sp Km	Lopez-Maury et al., 2009

synthesized by PCR and oligonucleotide pairs used are described in **Table 2** and were ^{32}P-labeled with a random-primer kit (Amersham Biosciences) using α-[^{32}P] dCTP (3000 Ci/mmol). Prehybridization, hybridization, and washes were in accordance with Amersham instruction manuals. All filters were stripped and re-hybridized with the constitutively expressed *rnpB* gene from *Synechocystis* as loading control (Vioque, 1992). Hybridization signals were quantified with a Cyclone Phosphor System (Packard).

ANTI-Grx ANTIBODY PRODUCTION, WESTERN BLOTTING AND PREPARATION OF CRUDE EXTRACTS FROM *Synechocystis* CELLS

Anti-GrxA and anti-GrxC antisera were obtained according to standard immunization protocols by injecting GrxA and GrxC purified proteins in rabbits. For analysis of GrxA and GrxC protein levels in *Synechocystis* cells grown under different conditions, crude extracts were prepared using glass beads in 50 mM Tris HCl pH 8.0, 50 mM NaCl. Protein concentration in cell-free extracts or purified protein preparations was determined by the method of Bradford, using ovalbumin as a standard and the specified amounts of proteins were resolved using SDS-PAGE gels. Proteins were thereafter electroblotted onto nitrocellulose membranes that were blocked in PBS containing 0.1% tween 20 and 5% of skimmed milk and incubated with anti-GrxA (1:1000) or anti-GrxC (1:3000). Anti-GSI (1:50.000) was used as loading control.

RESULTS

GLUTAREDOXINS ARE NOT ESSENTIAL IN *Synechocystis*

We have previously generated *Synechocystis* mutants lacking each or all of the glutaredoxin genes but their phenotypes were not analyzed in detail (Lopez-Maury et al., 2009). All mutant strains were viable in all possible combinations and were fully segregated when grown on solid media. Here, we have further analyzed their phenotypes in our standard growth conditions (BG11C, 30°C, 1% CO_2 bubbled in air, 50 $\mu E \cdot m^{-2} \cdot s^{-1}$) during the growth curve. Growth of the mutants was analyzed in cultures inoculated at $OD_{750nm} = 0.1$ (which is equivalent to 0.5 μg chl mL^{-1}) and growth was monitored following both chlorophyll content and OD_{750nm} for 5 days when it leveled off around 30 μg chl mL^{-1} (**Figure 1A**) and an $OD_{750nm} = 6$ (Figure S1). None of the strains presented a substantial reduced growth rate during the lag, exponential, linear or stationary phases of the growth curve. We have also studied expression of the glutaredoxin genes during the growth curve. Signals for all three mRNAs could be detected during all growth phases although *grxC* was induced slightly (2 to 3-fold) during the early exponential growth phase and both *grxA* and *grxB* remained almost constant during all phases (**Figures 1B,C**). Furthermore, we have also analyzed GrxA and GrxC protein levels during the growth phases, using specific antibodies raised against the corresponding proteins, and the proteins levels did not change substantially (**Figure 1D**). We have further analyzed expression of the three glutaredoxins genes during the growth curve in single mutants lacking one of the other genes in order to study if there were any compensatory effects in their expression. None of the mutant strains presented changes in mRNA levels of the remaining glutaredoxins when compared to the WT strain (**Figures 2A–C**, S2). We have also analyzed protein levels in exponentially growing cells (3–5 μg chl ml^{-1}) of single mutant strains although neither GrxA or GrxC protein levels changed (**Figure 2D**). These data suggest that glutaredoxins play a less prominent role than thioredoxins under non-stress conditions and contrast with data from yeast or *E. coli* in which at least one glutaredoxin is essential for growth (Draculic et al., 2000; Fernandes and Holmgren, 2004; Ortenberg et al., 2004; Buchanan and Balmer, 2005; Toledano et al., 2013).

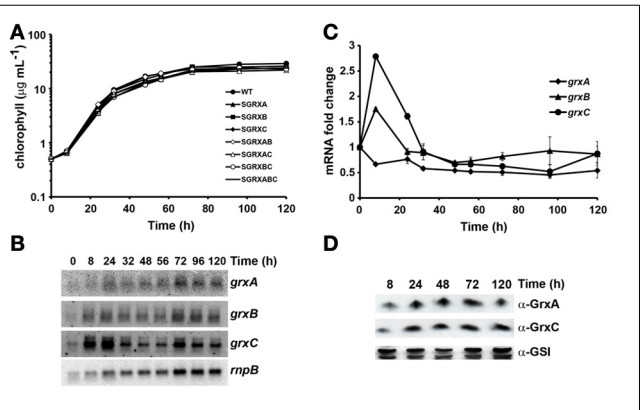

FIGURE 1 | Glutaredoxins are not essential under standard growth conditions. (A) Semi-logarithmic representation of growth of *Synechocystis* glutaredoxin mutants strains under standard conditions. WT (●), SGRXA (▲), SGRXB (■), SGRXC (♦), SGRXAB (◇), SGRXAC (△), SGRXBC (○), and SGRXABC (–) strains were inoculated at 0.5 μg chlorophyll mL^{-1} and growth was monitored by measuring chlorophyll content. **(B)** Northern blot analysis of *grxA*, *grxB*, and *grxC* expression along the growth curve. Total RNA was isolated from WT cells grown in BG11C at the indicated times. The filter was hybridized with *grxA*, *grxB*, and *grxC* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(C)** Quantification of relative mRNA levels of *grxA*, *grxB*, and *grxC* during the growth curve. Radioactive signals were quantified and normalized to the *rnpB* signal. Plots of relative mRNA levels vs. time were drawn; data represent average of 3 independent experiments and error bars represent SE. *grxA* (♦), *grxB* (▲), and *grxC* (●). **(D)** Western blot analysis of GrxA and GrxC levels during the growth curve. WT cells were grown in BG11C medium and cells were harvested at the indicated times. Fifteen microgram of total protein from soluble extracts were separated by 15% SDS-PAGE and subjected to western blot to detect GrxA, GrxC, and GSI.

Table 2 | Oligonucleotides used in this work.

Oligonucleotide	Sequence
GrxA250F	GGCTGTCTCGGCAAAAATTG
GrxA250R	GGTCCAACTTGCCTGCACCATC
GrxB331F	GGCTAATTTGTTCAACTGGC
GrxB331R	CTAGGCTGGGTTAGGAGGAG
GrxC295F	GCAAGAATTGATCAGTTGGTC
GrxC295R	GCCACTTCTAACATTTCCTGC
isiAF	CATAGGTCTCGGGTGGAC
isiAR	TAAAGCTGATGGCTAATG
pgr5_F	GGAGTCACTCATATGTTCGCCC
pgr5_R	CTCAGTTTCTCGAGAATTATTG
hspA_F	CCACACATCAGGAGTTAACAT
hspA_R	TTGATCATCTAGGGTCAGGAGC

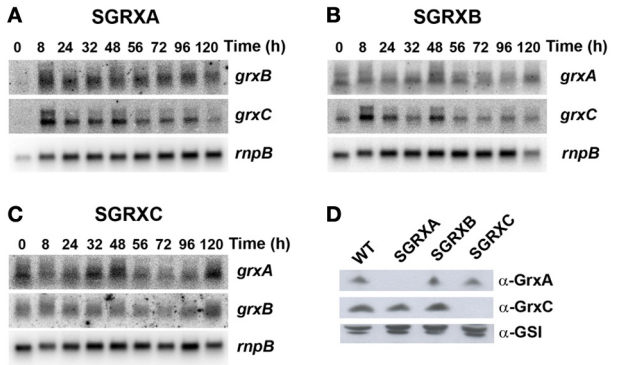

FIGURE 2 | Glutaredoxin gene expression is not altered in single glutaredoxin mutants. (A) Northern blot analysis of *grxB* and *grxC* expression during different stages of the growth curve in SGRXA. Total RNA was isolated from SGRXA cells grown in BG11C at the indicated times. The filter was hybridized with *grxB* and *grxC* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(B)** Northern blot analysis of *grxA* and *grxC* expression during different states of the growth curve in SGRXB. Total RNA was isolated from SGRXB cells grown in BG11C at the indicated times. The filter was hybridized with a *grxA* and *grxC* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(C)** Northern blot analysis of *grxA* and *grxB* expression during different states of the growth curve in SGRXC. Total RNA was isolated from SGRXC cells grown in BG11C at the indicated times. The filter was hybridized with a *grxA* and *grxB* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(D)** Western blot analysis of GrxA and GrxC levels in glutaredoxin mutants. WT, SGRXA, SGRXB, and SGRXC cells were grown in BG11C medium and cells were harvested at exponential phase (3–5 μg chl mL^{-1}). Fifteen microgram of total protein from soluble extracts were separated by 15% SDS-PAGE and subjected to western blot to detect GrxA, GrxC, and GSI.

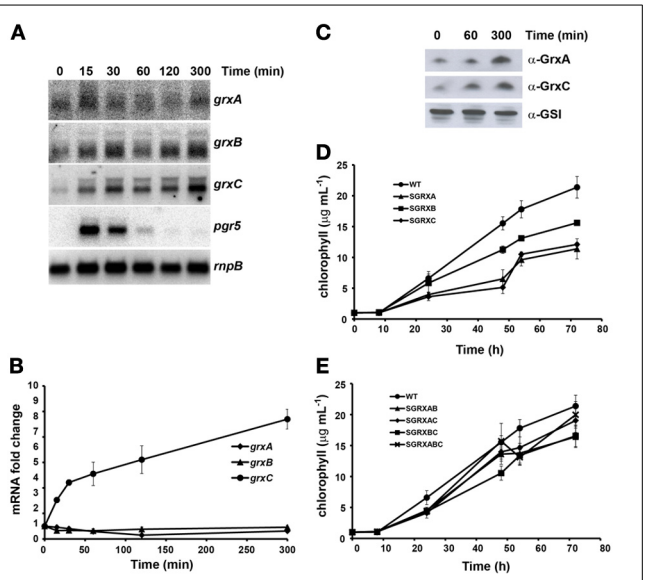

FIGURE 3 | Effect of high light on glutaredoxin gene expression and growth. (A) Northern blot analysis of *grxA*, *grxB*, *grxC*, and *pgr5* expression in response to a shift from 50 to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity. Total RNA was isolated from exponentially growing WT cells at the indicated times after the shift. The filter was hybridized with *grxA*, *grxB*, *grxC*, and *pgr5* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(B)** Quantification of relative mRNA levels of *grxA*, *grxB*, and *grxC* in response to a shift from 50 to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity. Radioactive signals were quantified and normalized to the *rnpB* signal. Plots of relative mRNA levels vs. time were drawn; data represent average of 3 independent experiments and error bars represent SE. *grxA* (♦), *grxB* (▲), and *grxC* (●). **(C)** Western blot analysis of GrxA and GrxC levels in response to a shift from 50 to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$. WT cells were grown in BG11C medium and samples were collected at the indicated times after the shift. 15 μg of total protein from soluble extracts were separated by 15% SDS-PAGE and subjected to western blot to detect GrxA, GrxC, and GSI. **(D)** Growth of glutaredoxin single mutants strains after a shift to high light. WT (●), SGRXA (▲), SGRXB (■), SGRXC (♦) were grown until the exponential phase, diluted to 1 μg chlorophyll mL^{-1} and shifted to high light intensity. Growth was monitored by measuring chlorophyll content. **(E)** Growth of glutaredoxin double and triple mutants strains after a shift to high light. WT (●), SGRXAB (▲), SGRXAC (♦), SGRXBC (■), and SGRXABC (x) were grown until the exponential phase, diluted to 1 μg chlorophyll mL^{-1} and shifted to high light intensity. Growth was monitored by measuring chlorophyll content.

RESPONSES TO DIFFERENT STRESS CONDITIONS

In order to further characterize the glutaredoxin mutant strains we have analyzed both the growth and expression of glutaredoxins in response to different stress treatments, namely high light intensity, heat shock, hydrogen peroxide stress, and metal stress.

High light intensity

In order to study the effect of high light treatment we transferred exponentially growing cells (3–5 μg chl mL^{-1}) from our standard light intensity (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ and followed glutaredoxin gene expression in response to this change in light intensity. This light intensity was chosen because it did not induce photoinhibition (**Figure 3**) and in fact WT cells grew faster under this illumination, but was high enough to reveal a phenotype in light sensitive mutants (Perez-Perez et al., 2009a). First, we analyzed expression of glutaredoxin genes in response to this treatment; as a marker gene we have used *pgr5*, the mRNA of which has been described to be induced by high light treatment (Allakhverdiev et al., 2002). *pgr5* was transiently induced in our experiment, with a peak induction at 15 min (**Figures 3A**, S3A). After the shift to high light, *grxC* mRNA levels increased linearly during the 5 h of the treatment, while levels of *grxA* and *grxB* did not significantly change during the treatment (**Figures 3A,B**). In contrast, both GrxA and GrxC protein levels increased after this treatment (**Figure 3C**).

In order to clarify the roles of the three different glutaredoxins we analyzed growth of the different mutants after a shift from normal to high light. To this end, exponentially growing cells (3–5 μg chl mL^{-1}) were diluted to 1 μg chl mL^{-1}, shifted to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ and growth of the strains was monitored measuring chlorophyll content (**Figures 3D,E**) and OD_{750nm} for 72 h (Figure S3). Growth of the SGRXA (*grxA*) and the SGRXC (*grxC*) mutant strains was markedly retarded after the shift to high light while that of SGRXB (*grxB*) was only slightly reduced (**Figures 3D**, S3). Double and triple mutant strains behaved more similarly to SGRXB and WT strains (**Figure 3E**). These results suggest that both GrxA and GrxC play a role in adaptation to high light and that *grxB* mutation was able to suppress mutations in both *grxA* and *grxC*.

Heat shock

We have performed experiments by shifting cells grown at 30–42°C, that is, a non-lethal heat shock treatment. As for the experiments involving high light treatment, we analyzed expression of glutaredoxins both at mRNA and protein levels. As a control we analyzed expression of the *hspA* mRNA, which was induced rapidly after the shift from 30 to 42°C and returned to almost initial levels after 5 h as previously described (**Figures 4A**, S4; Fang and Barnum, 2004; Suzuki et al., 2005; Tuominen et al., 2006). *grxA* mRNA levels decreased slightly during the first hour after the shift to 42°C and increased afterwards reaching more than 2 fold induction after 5 h of the treatment. In contrast *grxC* mRNA decreased 2–3 times and remained at this level along the course of the experiment, while *grxB* mRNA did not change significantly during this treatment (**Figure 4B**). In accordance with mRNA levels GrxA protein levels increased appreciably after 5 h (**Figure 4C**). However, GrxC protein levels remained constant despite the fact its mRNA levels were repressed. These results suggest that either GrxC was stabilized and/or that *grxC* mRNA translation was enhanced after heat shock.

To analyze the growth of the different strains exponentially growing cells (3–5 μg chl mL^{-1}) were diluted to 2 μg chl mL^{-1}, shifted from 30 to 42°C and growth of the strains was monitored measuring chlorophyll content (**Figures 4D,E**) and OD_{750nm} (Figures S4B,C). Analysis of mutant strains at elevated temperatures revealed that most strains showed some degree of growth inhibition. Strikingly, the SGRXA and SGRXC were completely unable to grow under this condition (**Figure 4D**). Surprisingly, SGRXABC, SGRXAB, SGRXBC strains, and to a lesser extent SGRXAC strain, were less sensitive to heat stress than SGRXA and SGRXC, suggesting again that mutations in *grxB* could partially suppress *grxA* and *grxC* mutations and that *grxA* and *grxC* mutation suppress partially each other. All the above results suggest that both GrxA and GrxC could play a very important role in protecting cells from heat shock.

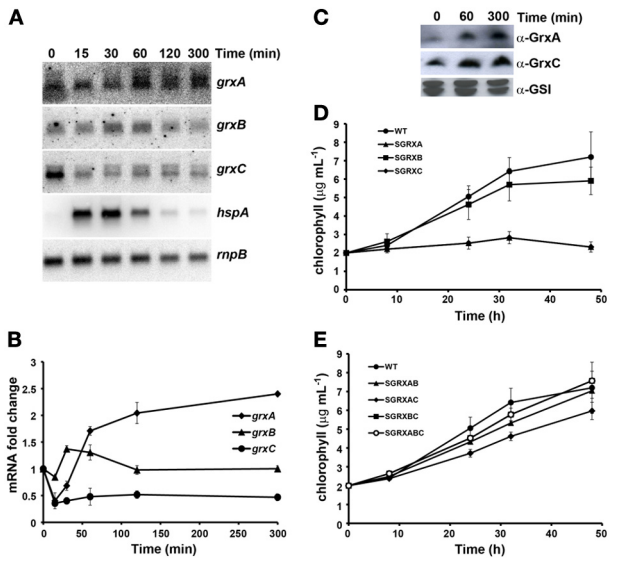

FIGURE 4 | Effect of heat shock on glutaredoxin gene expression and growth. (A) Northern blot analysis of *grxA, grxB, grxC,* and *hspA* expression in response to heat shock. Total RNA was isolated from exponentially growing WT cells at the indicated times after the shift from 30 to 42°C. The filter was hybridized with *grxA, grxB, grxC,* and *hspA* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(B)** Quantification of relative mRNA levels of *grxA, grxB,* and *grxC* in to response to heat shock. Radioactive signals were quantified and normalized to the *rnpB* signal. Plots of relative mRNA levels vs. time were drawn; data represent average of 3 independent experiments and error bars represent SE. *grxA* (♦), *grxB* (▲), and *grxC* (●). **(C)** Western blot analysis of GrxA and GrxC levels in response to heat shock. WT cells grown in BG11C and samples were collected at the indicated times after a shift from 30 to 42°C. Fifteen microgram of total protein from soluble extracts were separated by 15% SDS-PAGE and subjected to western blot to detect GrxA, GrxC, and GSI. **(D)** Growth of glutaredoxin single mutants strains after heat shock. WT (●), SGRXA (▲), SGRXB (■), and SGRXC (♦) were grown until the exponential phase, diluted to 2 μg chlorophyll mL^{-1} and shifted from 30 to 42°C. Growth was monitored by measuring chlorophyll content. **(E)** Growth of glutaredoxin double and triple mutant strains after heat shock. WT (●), SGRXAB (▲), SGRXAC (♦), SGRXBC (■), and SGRXABC (○) were grown until the exponential phase, diluted to 2 μg chlorophyll mL^{-1} and shifted from 30 to 42°C. Growth was monitored by measuring chlorophyll content.

Hydrogen peroxide stress

We have analyzed the effect of addition of 1 mM of hydrogen peroxide (H_2O_2) both at mRNA and protein levels and on growth of the mutant strains. As a marker gene we used the *isiAB* operon, which has been reported to be induced after this treatment (Li et al., 2004a; Kanesaki et al., 2007), with a peak at 30 min in our experiments (**Figures 5A**, S5). *grxC* mRNA was induced transiently with a peak at 1h after the treatment and decreased afterwards. In contrast, both *grxA* and *grxB* mRNA levels remained nearly constant during the time course (**Figures 5A,B**). However, both GrxA and GrxC protein levels increased during the experiment with maximum levels reached after 5 h of treatment (**Figure 5C**). These results suggest that GrxA is stabilized in response to oxidative stress or, alternatively, that its translation is increased, while GrxC accumulation followed its mRNA induction pattern but with a delay in protein accumulation.

In order to study the sensitivity of the different strains, cells were grown until they reached the exponential growth phase (3–5 μg chl mL^{-1}), then cultures were diluted to 1 μg chl mL^{-1} and 1 mM of H_2O_2 was added to the cultures. Growth was monitored for 4 days by measuring chlorophyll content (**Figures 5D,E**). Both SGRXA and SGRXC mutant strains presented a clear sensitivity to the H_2O_2 treatment and were unable to grow under this condition (**Figures 5D,E**). However, in contrast to high light and heat shock treatments, after addition of H_2O_2 the SGRXAC double mutant strain presented an enhanced sensitivity, suggesting that GrxA and GrxC participate in parallel pathways that cooperate in response to H_2O_2 (**Figures 5D,E**). Furthermore, mutation of *grxB* partially suppresses the sensitivity of *grxA* mutants (SGRXAB was less sensitive than SGRXA) but had almost no effect on *grxC* mutation (compare SGRXC and SGRXBC which were almost identical). The triple mutant SGRXABC showed a degree of sensitivity comparable to that of SGRXAC, which suggested that the *grxB* mutation was not able to suppress the stress sensitivity of the SGRXAC strain.

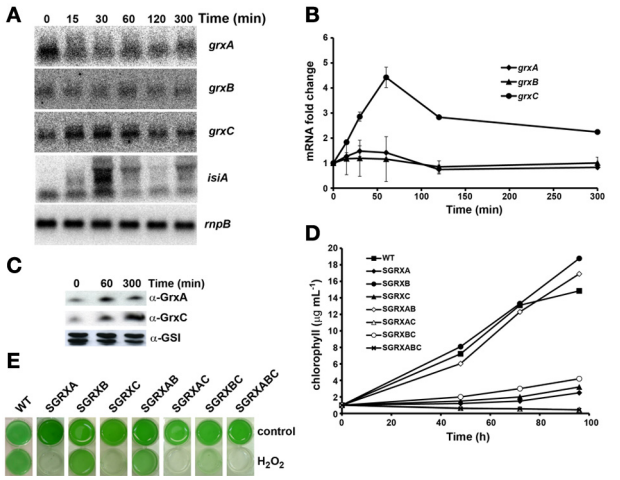

FIGURE 5 | Effect of H_2O_2 addition on glutaredoxin gene expression and growth. (A) Northern blot analysis of *grxA*, *grxB*, *grxC*, and *isiA* expression in response to H_2O_2 addition. Total RNA was isolated from WT cells grown in BG11C at the indicated times after a 1 mM H_2O_2 addition. The filter was hybridized with *grxA*, *grxB*, *grxC*, and *isiA* probes and subsequently stripped and re-hybridized with an *rnpB* probe as a control. **(B)** Quantification of relative mRNA levels of *grxA*, *grxB*, and *grxC* in response to H_2O_2 addition. Radioactive signals were quantified and normalized to the *rnpB* signal. Plots of relative mRNA levels vs. time were drawn; data represent average of 3 independent experiments and error bars represent SE. *grxA* (♦), *grxB* (▲), and *grxC* (●). **(C)** Western blot analysis of GrxA and GrxC levels in response to hydrogen peroxide. WT cells grown in BG11C and samples were collected at the indicated times after 1mM H_2O_2 addition. Fifteen microgram of total protein from soluble extracts were separated by 15% SDS-PAGE and subjected to western blot to detect GrxA, GrxC, and GSI. **(D)** Growth of glutaredoxin mutants strains after H_2O_2 addition. WT (■), SGRXA (♦), SGRXB (●), SGRXC(▲), SGRXAB (◊), SGRXAC (Δ), SGRXBC (○), and SGRXABC (x) were grown until the exponential phase, diluted to 1 μg chlorophyll mL^{-1} and 1 mM H_2O_2 was added. Growth was monitored by measuring chlorophyll content. **(E)** Photograph of glutaredoxin mutants grown in liquid BG11C or BG11C supplemented with 1 mM H_2O_2 for 5 days.

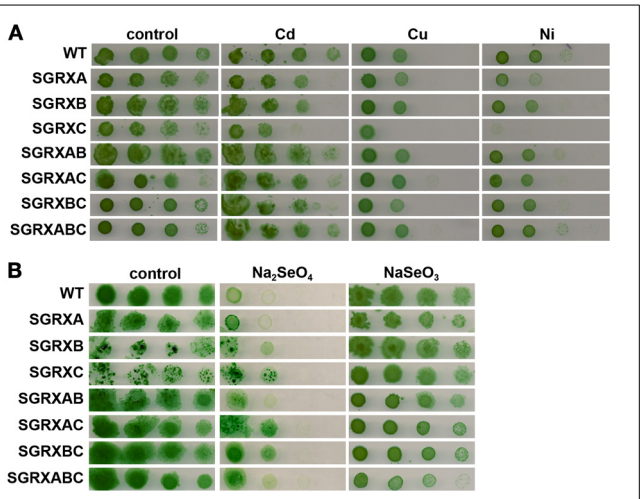

FIGURE 6 | Effect of different metals on glutaredoxin mutants growth. (A) Sensitivity of glutaredoxin mutants to metals. Tolerance of WT, SGRXA, SGRXB, SGRXC, SGRXAB, SGRXAC, SGRXBC, and SGRXABC strains to cadmium, copper, and nickel was examined. Tenfold serial dilutions of a 1 μg chlorophyll mL^{-1} of exponentially growing cells suspension were spotted onto BG11C supplemented with 2 μM Cd, 3 μM Cu, and 5 μM Ni. Plates were photographed after 5 days of growth. **(B)** Sensitivity of glutaredoxin mutants to selenium. Tolerance of WT, SGRXA, SGRXB, SGRXC, SGRXAB, SGRXAC, SGRXBC, and SGRXABC strains to selenate (Na_2SeO_4) or selenite (Na_2SeO_3) was examined. Ten-fold serial dilutions of a 1 μg chlorophyll mL^{-1} of exponentially growing cells suspension were spotted onto BG11C supplemented with 30 μM Na_2SeO_3 or 30 μM Na_2SeO_4. Plates were photographed after 5 days of growth.

Metal stress

We have previously shown that GrxA was essential for arsenate resistance as mutants lacking *grxA* were sensitive to arsenate in the media and GrxA was the best electron donor *in vitro* for the main arsenate reductase from *Synechocystis* (Lopez-Maury et al., 2009). Furthermore, GrxA (Grx2) was suggested to be involved in selenate resistance (Marteyn et al., 2009) and recently GrxB has been implicated in mercury and uranium resistance (Marteyn et al., 2013). In order to further analyze the sensitivity of glutaredoxin mutants to different metals, we have analyzed the growth of all mutants in the presence of selenate, selenite, cadmium, copper, and nickel. All mutant strains, except SGRXC, grew as well as the WT in the presence of 2 μM of Cd, 3 μM of Cu, 5 μM of Ni and 30 μM Na_2SeO_3 or 30 μM Na_2SeO_4 (**Figure 6**). The SGRXC strain presented hypersensitivity to nickel and copper, and to a lesser extent to Cd (**Figure 6A**), while the double mutants SGRXAC and SGRXBC (lacking *grxA* or *grxB* in addition to *grxC*) and triple mutants (lacking all glutaredoxin genes) strains behaved like the WT. This suggests that compensatory mechanisms were activated in double and triple mutant strains that protect against the toxic effect of these metals. However, although these mutations were able to suppress *grxC* mutation, they did not enhance resistance in a WT background (**Figure 6A**).

DISCUSSION

Here we have extensively characterized the complete set of glutaredoxins in the cyanobacterium *Synechocystis*, a model photosynthetic prokaryote. The characterization of the different glutaredoxin mutant strains have revealed that none of the glutaredoxin genes are required under normal growth conditions but that they play an important role in protecting cells from environmental stresses. Our data also indicated that there are complex genetic interactions between glutaredoxins genes and that these interactions are stress dependent. The fact that we have not observed any compensatory effects on expression of glutaredoxin genes or GrxA and GrxC protein levels in mutants lacking other glutaredoxin genes, would suggest that the phenotypes we have observed are most probably linked to downstream processes regulated by glutaredoxins and not to dosage-dependent effects. Furthermore, analysis of the expression of the glutaredoxin genes has shown that these genes were not all under the same regulatory system because their patterns of expression were not coordinated. In fact, the *grxB* mRNA level did not change significantly under any of the conditions tested, while *grxA* was only induced by heat shock and *grxC* was induced after H_2O_2 and during high light treatment but repressed by heat shock. Although *grxC* is induced by high light intensities and H_2O_2, this gene is not under the

control of any of the regulatory proteins described to respond to these stress conditions in *Synechocystis* (Hsiao et al., 2004; Kobayashi et al., 2004; Suzuki et al., 2005; Jantaro et al., 2006; Tuominen et al., 2006; Kanesaki et al., 2007; Tuominen et al., 2008; Horiuchi et al., 2010; Singh et al., 2010; Muramatsu and Hihara, 2012). On the other hand, GrxA protein levels increased under all stress conditions, while the GrxC protein levels were induced only after high light and H_2O_2, following the changes of its mRNA levels, and was maintained constant after heat shock, despite repression of its mRNA levels. This suggests that in addition to a transcriptional regulation there are also other levels of regulation at the protein level for both glutaredoxins.

Analysis of growth the mutant strains showed that two of the glutaredoxins, GrxA and GrxC, play a prominent role in cell protection against stress, as the single mutants lacking *grxA* or *grxC* were sensitive to several stresses. Both GrxA and GrxC were necessary for oxidative stress defense, since mutants lacking one of them were extremely sensitive to the presence of external H_2O_2 and the mutant lacking both genes showed and additive phenotype (**Figure 5**). The single mutant lacking *grxA* (the SGRXA strain) proved to be hypersensitive to H_2O_2, which is in agreement with phenotypes previously described for this mutant (Li et al., 2007; Marteyn et al., 2009), but the mechanism for this sensitivity and the targets of GrxA are still unknown. GrxA is able to decompose H_2O_2 *in vitro* although it is much less efficient than most peroxiredoxins, which are in fact reduced by thioredoxins and not by glutaredoxins in *Synechocystis* (Perez-Perez et al., 2009b). Therefore, it is unlikely that this activity could account for the protection of cells against H_2O_2. Besides that, GrxA has been shown to interact with both catalase and peroxiredoxin II (PrxII), although the catalase activity was inhibited by GrxA in *Synechocystis* extracts (Li et al., 2007) and PrxII eventually did not accept electrons from GrxA (Perez-Perez et al., 2009b). However, it can not be ruled out that glutathionylation is needed for activation and/or protection of these two proteins under oxidative stress conditions. The SGRXA strain was also sensitive to high light and GrxA protein levels increased after this treatment suggesting that GrxA could play a role in high light acclimation. During the high light treatment the excess of light absorbed can generate reactive oxygen species (ROS) leading to oxidative damage and this ROS might affect the growth of the SGRXA strain. Furthermore, the SGRXA strain was also extremely sensitive to heat shock which suggests that GrxA could also play an important role during heat stress. In this regard both GroEL and DnaK1 (two chaperones that are essential for heat adaptation) were also identified as GrxA targets (Li et al., 2007), which suggests that GrxA could be needed for activation or as a helper of both chaperones to function in response to heat shock. Similar phenotypes with respect to oxidative stress sensitivity and heat shock sensitivity have been reported for glutaredoxin mutants in other bacteria (Prinz et al., 1997; Fernandes and Holmgren, 2004; Li et al., 2004b; Benyamina et al., 2013) and in yeast (Luikenhuis et al., 1998; Draculic et al., 2000; Chung et al., 2004), suggesting that dithiolic glutaredoxins could have conserved roles in microorganisms. Finally, we have tested whether SGRXA strains were sensitive to metals and selenium compounds. Any of the tested metals affected significantly the growth of *grxA* mutant strains (**Figure 6**), including selenate or selenite which is in disagreement with previous data (Marteyn et al., 2009). Sensitivity to metals is highly influenced by growth conditions, especially light conditions, and this could explain the differences in selenium sensitivity between our results and previously published ones (Marteyn et al., 2009).

The SGRXC strain is unique in that it showed hypersensitivity to all stresses tested. GrxC is a prototypical monothiolic glutaredoxin which has been shown to contain an oxygen labile 2Fe-2S cluster (Picciocchi et al., 2007), and the *grxC* gene is able to complement the defects of a *grx5* yeast mutant (Molina-Navarro et al., 2006). In several organisms it is well-documented that monothiolic glutaredoxins impact Fe-S cluster synthesis and/or assembly although their specific roles are still under discussion. Yeast Grx5 is involved in mitochondrial Fe-S cluster assembly and mutants lacking this protein showed symptoms of oxidative stress under standard growth conditions, accumulation of apoproteins lacking Fe-S clusters in the mitochondria and were unable to grow under aerobic conditions (Rodriguez-Manzaneque et al., 1999, 2002; Belli et al., 2004). A similar phenotype has also been described for a monothiolic glutaredoxin mutant in *Sinorhizobium meliloti* (Benyamina et al., 2013). Furthermore, in yeast it has been shown that GSH is essential for Fe-S cluster assembly but in this case it affects both cytoplasmic and mitochondrial Fe-S clusters, suggesting a more global impact in Fe-S cluster assembly. This is probably related to its role in binding Fe-S clusters in all monothiolic glutaredoxins in yeast cells (Kumar et al., 2011). In photosynthetic organisms the *in vivo* role of monothiolic glutaredoxins has been analyzed only in *Arabidopsis* which has four class II monothiolic glutaredoxins (AtGrxS14–17; Couturier et al., 2009a). Of these AtGrxS14 and AtGrxS16 have been localized to the chloroplast, AtGrxS15 is localized in the mitochondria and chloroplast and AtGrxS17 is a cytosolic protein (Cheng et al., 2006; Bandyopadhyay et al., 2008; Cheng, 2008). It was also shown that both AtGrxS14 and AtGrxS15 mutants are hypersensitive to H_2O_2 (Cheng et al., 2006; Cheng, 2008), while the AtGrxS17 mutant is sensitive to heat shock and has higher ROS contents under this condition (Cheng et al., 2011). These proteins also present a labile 2Fe-2S cluster (like GrxC) which *in vitro* could be transferred to *Synechocystis* ferredoxin (Bandyopadhyay et al., 2008; Liu et al., 2013). Furthermore, these proteins are also able to complement the yeast *grx5* mutant strain, like *grxC*, suggesting that they are functionally conserved (Bandyopadhyay et al., 2008; Liu et al., 2013). Recently it has been shown that the *E. coli* monothiolic glutaredoxin (GrxD) is able to transfer its Fe-S cluster to ferredoxin (Yeung et al., 2011) but not to MiaB, a radical AdoMet enzyme containing two 4Fe-4S clusters, despite holo-GrxD being able to interact with MiaB. This led these authors to propose that GrxD is not involved in Fe-S cluster assembly but in Fe-S cluster repair (Boutigny et al., 2013). This role in repair and/or synthesis of Fe-S cluster could explain SGRXC sensitivity to different stresses, because Fe-S clusters are vulnerable to many stresses, especially H_2O_2 and metal stress (Imlay, 2006, 2013). In fact destabilization of Fe-S clusters in essential enzymes constitute the main mechanism for metal toxicity in several organisms, including *Synechocystis*, suggesting that this is a common problem when cells are challenged with an excess of metals

(Ranquet et al., 2007; Macomber and Imlay, 2009; Chillappagari et al., 2010; Fantino et al., 2010; Tottey et al., 2012; Xu and Imlay, 2012). The target proteins which require GrxC for Fe-S cluster assembly and/or repair in *Synechocystis* remain unidentified, but because the SGRXC strain is viable and did not show any growth defects under non-stressed conditions, its targets are unlikely to be essential. Alternatively GrxC could be involved only in Fe-S cluster repair under stress conditions. This is further supported by GrxC induction after several stress treatments, suggesting that cellular requirements for GrxC under these conditions may increase.

In contrast the SGRXB strain did not display any obvious phenotype, neither were the *grxB* mRNA levels altered under any of the conditions tested, suggesting that this protein has a minor role and/or a very specialized role. This is supported by the recent data, which showed that *grxB* mutants were sensitive to mercury and uranium. GrxB regulates the activity of MerA, a mercury reductase, via glutathionylation of one of its active cysteines (Marteyn et al., 2013) and mutation of both *merA* or *grxB* confers sensitivity to these compounds. Surprisingly, our results showed that *grxB* mutation was able to partially suppress most of the phenotypes of *grxA* mutant strains suggesting that it could be negatively controlling functions activated by GrxA. This is further supported by data showing physical interaction between GrxA and GrxB, which has been observed by two different groups using different experimental approaches (Li et al., 2007; Marteyn et al., 2009). This suggests that these interactions are *bona fide* interactions and that these proteins could regulate each other's activities. Furthermore, GrxA and GrxB have been proposed to be reduced by *Synechocystis* NADPH-thioredoxin reductase (encoded by *slr0600;* NTR), which present homology to bacterial NADPH thioredoxin reductases. Which redoxins are reduced by NTR is controversial because both dithiolic glutaredoxins, GrxA and GrxB, and TrxA have been reported to be reduced by NTR (Li et al., 2007; Hishiya et al., 2008; Marteyn et al., 2009). In addition, a *Synechocystis* mutant lacking GSH also presented stress sensitivity, although its phenotypes were more severe (Cameron and Pakrasi, 2011) than phenotypes of glutaredoxin mutants described here. This suggests that, as described in yeast (Kumar et al., 2011), GSH could have additional roles in *Synechocystis* which are not mediated solely by glutaredoxins. One possibility is that the NTR-Grx1/2 or the NTR-TrxA system could work as alternative pathways to reduce GSH in *Synechocystis*, because this strain lacks a gene for a canonical glutathione reductase (GR) and NADPH dependent GR activity is not detected in extracts (Marteyn et al., 2009; Cameron and Pakrasi, 2011). Similar results have been obtained in yeast in which mutant lacking GR can use both Grx2 and Trx2 to reduce GSH (Morgan et al., 2013). All the above data suggest that there could be a cross-regulation between the thioredoxin and glutaredoxin systems in *Synechocystis* as it has been shown in other organisms including plants and algae (Gelhaye et al., 2003; Michelet et al., 2005; Rouhier et al., 2008; Zaffagnini et al., 2008; Meyer et al., 2009, 2012; Lillig and Berndt, 2013; Toledano et al., 2013). Finally, analysis of double mutants with *grxC* shows that *grxB* was also able to suppress totally or partially the sensitivity phenotype of *grxC* single mutants to high light, heat shock and metal stress but not to oxidative stress. The relation between these two proteins is completely unknown but is possible that *grxB* affects GSH metabolism which will probable also alter GrxC function.

In conclusion, we have shown that glutaredoxins are essential for adaptation to several stresses in *Synechocystis* and that GrxA and GrxC levels increase in response to stress treatments. This induction is mediated mainly by transcriptional induction for GrxC and by posttranscriptional mechanisms in the case of GrxA. Analysis of the different genetic interactions between the glutaredoxin genes suggest the existence of an interconnected genetic network that is perturbed in response to environmental changes. In this regard, it would be interesting to analyze mutants lacking both glutaredoxins and other components of antioxidative system, such as the thioredoxins, as some of the phenotypes we have observed are shared between them.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00428/abstract

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Utility of *Synechocystis* sp. PCC 6803 glutaredoxin A as a platform to study high-resolution mutagenesis of proteins

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Glutaredoxin from the cyanobacterium *Synechocystis* sp. PCC 6803 is a small protein, containing only 88 amino acids, that participates in a large number of redox reactions, serving both as an electron donor for enzyme-catalyzed reductions and as a regulator of diverse metabolic pathways. The crystal structures of glutaredoxins from several species have been solved, including the glutaredoxin A isoform from the cyanobacterium *Synechocystis* sp. PCC 6803. We have utilized the small size of *Synechocystis* glutaredoxin A and its propensity to form protein crystals that diffract to high resolution to explore a long-standing question in biochemistry; i.e., what are the effects of mutations on protein structure and function? Taking advantage of these properties, we have initiated a long-term educational project that would examine the structural and biochemical changes in glutaredoxin as a function of single-point mutational replacements. Here, we report some of the mutational effects that we have observed to date.

Keywords: glutaredoxin A, educational platform, mutagenesis, protein structure, tertiary, synechocystis sp. PCC 6803

INTRODUCTION

Glutaredoxin, a member of the glutaredoxin/thioredoxin superfamily, was originally discovered during the search for the electron donor for *Escherichia coli* ribonucleotide reductase (Holmgren, 1976, 1979). Later, it was determined that glutaredoxins serve not only as the electron donor for a variety of reductant-requiring enzymes, but also as essential components in other cellular processes. For example, glutaredoxins play a critical role in restoring protein function following damage by oxidative stress, as well as mediating the response to heavy metal ion induced damage (Lundstrom-Ljung and Holmgren, 1995). Plant glutaredoxins play strategic roles in signaling pathways, as they participate in the regulation of diverse metabolic pathways. They are also involved in the regulation of gene expression (Couturier et al., 2013). In the case of the glutaredoxin that is the focus of this study, glutaredoxin A from the cyanobacterium *Synechocystis* sp. PCC 6803 serves as the preferred electron donor for the 2-electron reduction of arsenate to arsenite, catalyzed by arsenate reductase (Li et al., 2003; Lopez-Maury et al., 2003).

As part of the thioredoxin superfamily, glutaredoxin A shares the characteristic thioredoxin fold. This motif is most prominent in prokaryotic glutaredoxins, including glutaredoxin A from *Synechocystis* sp. PCC 6803, while the thioredoxin fold only exists as a substructure or domain in eukaryotic glutaredoxins (**Figure 1**) (Eklund et al., 1984). The small size, the ease of purification, and the physiological importance of this protein in both prokaryotes and eukaryotes make *Synechocystis* sp. PCC 6803 glutaredoxin A an ideal platform on which to base the study of protein mutations.

We previously reported the high-resolution crystal structure of glutaredoxin A from *Synechocystis* sp. PCC 6803 (**Figure 2**) (Kim et al., 2012). The structure of the wild-type protein is comprised of the major protein secondary structure elements: four α-helical segments, and four mixed parallel: anti-parallel β-strands that form a single β-pleated sheet. The assortment of secondary structural elements and the oxidation-reduction activity of glutaredoxin are two characteristics that establish this protein as an ideal template for students to study protein structure/function in detail. The glutaredoxin that we crystallized possesses an N-terminal hexa-His affinity tag to facilitate purification of the protein. The resulting crystal structure is unusual in that the entire N-terminal His-tag extension is completely resolved and each of the histidine residues in the affinity tag is visible in the electron density. The final crystal structure includes two sulfate anions from the crystallization medium. One of the sulfate ions is coordinated at the positive end of the helix dipole of helix 3, while the other sulfate ion is coordinated to the N-terminal hexa-histidine affinity tag. The pH of the crystallization buffer is 8.0; therefore, there is likely very little charge contributed by the histidine side chains that are expected to have pK_a values near 6.0. The affinity of the negatively charged sulfate ion for this particular aspect of the protein probably results from a combination of the amide backbone charge interactions and shape complementarity of the N-terminus with the sulfate anion.

Another marked difference between the *Synechocystis* sp. PCC 6803 glutaredoxin A structure and other glutaredoxin structures is the lack of a disulfide bond between the two cysteine

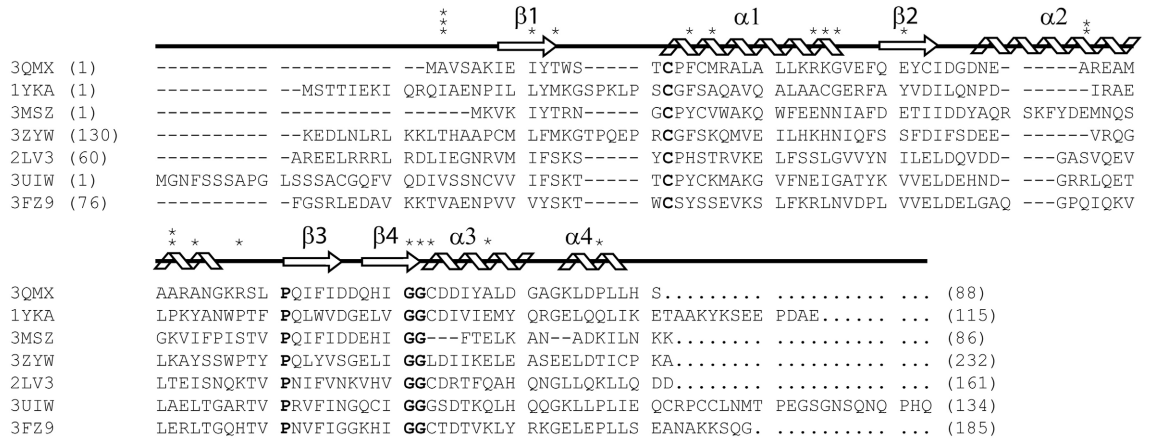

FIGURE 1 | ClustalX primary sequence alignment of *Synechocystis* sp. PCC 6803 glutaredoxin A (3QMX) Kim et al., 2012, Grx4 from *E. coli* (1YKA) (Fladvad et al., 2005), Grx1 from *Francisella tularensis* (3MSZ), glutaredoxin domain from human glutaredoxin 3 (3ZYW), Grx domain from *Mus* thioredoxin reductase (2LV3) (Dobrovolska et al., 2012), Zebrafish Grx2 (3UIW) (Brautigam et al., 2013), glutaredoxin S12 from Poplar (3FZ9) (Couturier et al., 2009). The "∗" above the sequence corresponds to mutations made and characterized within the protein. The arrow shapes represent beta-strand secondary structure; coils are regions of the protein in alpha-helical configuration. Letters in **BOLD** correspond to highly conserved features in all glutaredoxins proteins. Numbers in parentheses correspond to the residue numbers in the native protein sequence. Non-native residues were excluded from this alignment.

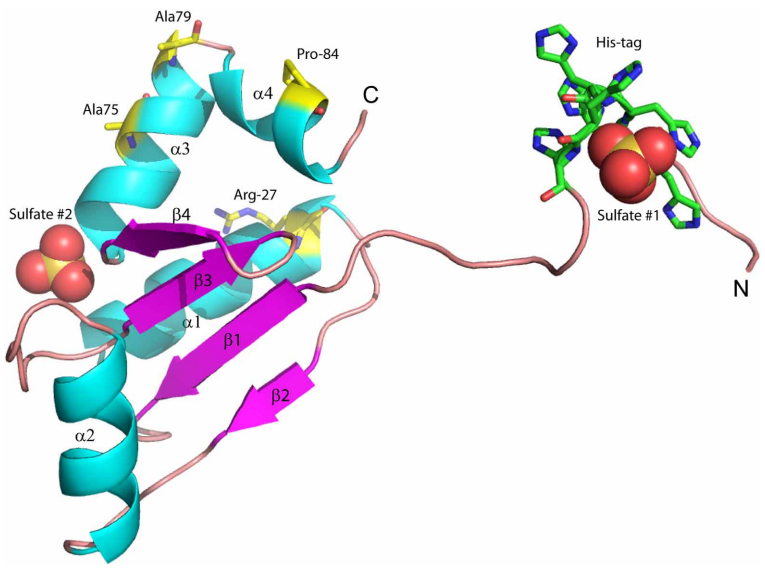

FIGURE 2 | Structure of wild-type glutaredoxin from *Synechocystis* sp. PCC 6803. Magenta arrows correspond to β-strands; cyan coils represent α-helices. Sulfate anions are shown in the space filling representation. Also shown as sticks, are the six histidine residues used for affinity purification. The amino (N) and carboxy (C) termini are labeled.

residues at the catalytically-active site, i.e., Cys15 and Cys18. The oxidation-reduction midpoint potential of the active site disulfide/dithiol couple in *Synechocystis* glutaredoxin A has been measured at −225 mV at pH 7.0 (Kim et al., 2012). Typically, glutaredoxins and thioredoxins with redox potentials in this range are found with the active site in the oxidized disulfide form at the conclusion of the purification protocol. However, the X-ray structure of *Synechocystis* glutaredoxin A unambiguously shows that the active site is fully reduced with no sign of any disulfide formation (Kim et al., 2012). The reasons for this unexpected result are not yet clear.

Site-directed mutagenesis has been used for many years to investigate the detailed function of genes and proteins (Muller et al., 1978). In this technique, the codon of a single amino acid is modified by the introduction of a mutagenic primer in a PCR amplification protocol to produce genes with alternative nucleotide sequences. When translated in a suitable expression system, these mutated genes will produce proteins with an altered

primary sequence. In most mutagenesis experiments, the selection of the amino acids to be replaced is logically determined. For example, the technique is typically used to focus on specific features of a protein, such as the active sites of enzymes. In contrast, the selection of amino acids for our purposes involves choosing the mutations by random chance, without any preconceived assumption about function or location within the protein. Upon completion of this project, we will have compiled a comprehensive database of biochemical and biophysical information that includes, but is not limited to, the effects of point mutations on protein stability and activity, effects of mutation on solubility, effects of mutation on three-dimensional structure, and effects of mutation on protein crystal formation.

RESULTS AND DISCUSSION

Widespread mutagenesis studies have previously been carried out with proteins other than glutaredoxins. For example, extensive analysis of the T4 lysozyme protein using a rational selection of sites, uncovered valuable information on the biophysics of protein folding and stability (Baase et al., 2010). However, complete coverage of mutagenesis space has not yet been accomplished for any single protein. This is largely because complete mutagenesis of a protein would require a great deal of labor, and many of the results would be scientifically trivial. In the case of glutaredoxin A, there are 88 amino acids in the complete protein, making it one of the smallest redox proteins known. If each site were mutated to one of the other 19 amino acids, 1672 new genes will have to be prepared. To accomplish this herculean task, we have integrated the research component of this project into an educational framework as an adjunct to a traditional biochemistry or protein-engineering course. A further advantage arises from the fact that the task can be accelerated by involving students from many different schools who are linked by a common database from a centralized campus via the Internet. A pilot version of this collaborative project has already collected information on protein solubility, crystallization, and structure. We have completed 26 random mutations in the three consecutive semesters that we have offered this course. Four new crystal structures have been generated.

EFFECTS OF RANDOMLY SELECTED MUTATIONS

Given that our mutations are randomly selected, at least four possible outcomes are expected. First, the mutated gene yields a protein that is unable to fold, and thus, would exhibit very low solubility. In this case, the protein could be sequestered in inclusion bodies in the bacteria, it could be completely proteolyzed, or it would yield only extremely low amounts of soluble protein. Second, the protein may be soluble, but is unable to crystallize under our current crystallization conditions. Third, the protein may crystallize, but the resulting crystals are too small for rapid analysis. Fourth, large, single-protein crystals are formed, and are amenable for crystallographic analysis.

We assume that many of the randomly selected mutations in the glutaredoxin gene will result in protein misfolding. For example, mutations within the hydrophobic core of most proteins are not well tolerated (Guo et al., 2004). Of the 27 mutations that we have characterized, three—T11Y, L25S, and G68Y—have resulted in the formation of an insoluble protein. The Gly68-Gly69 sequence is highly conserved within the glutaredoxin family (**Figure 1**). This di-glycine motif makes a bend in a loop between β-strand 4 and α-helix 3; hence, this sequence is likely to be an essential determinate of the native structure. The introduction of bulky tyrosine residues may impose considerable steric hindrance on this loop, thus, resulting in misfolding and this may explain the insolubility of the G68Y variant. In contrast, the G69F mutation is soluble, but did not crystallize. The T11Y mutation occurs at the apex of β-strand 2, adjacent to critical cysteine residues in the molecule. The introduction of a bulky side chain at this point may destabilize the core packing at this critical position.

MUTANTS OF GLUTAREDOXIN A THAT PRODUCE PROTEIN CRYSTALS

Four of our mutant proteins produced crystals suitable for X-ray structure determination (**Tables 1**, **2**). At least 10 of the 28 mutations that we have examined have produced crystalline material (**Table 1**). We define "crystalline material" as protein crystals that form either thin, fibrous crystals or protein crystals that are too small to justify further effort. Proteins that form fibrous crystals typically add protein molecules to the crystal lattice preferentially along one growth axis. In our case, A2I, A2T, I9L, R27V, K28C, A43I, A43T, A49H, A49W, and R55A variants each produced sub-optimal protein crystals. Interestingly, both hydrophobic and polar variants replacing Ala2 (A2I, A2T, and A2Y) produced soluble protein, but only A2I and A2T grew protein crystals, albeit crystals of poor quality. This particular locus on the molecule is in a flexible loop at the beginning of the polypeptide chain and is not involved in any direct crystal contacts; however, there are residues from symmetry-related molecules within 5 Å of this locus. The introduction of a bulky mutation such as Tyr could add as many as 6 new amino acid neighbors within a 5 Å sphere that could interfere with crystal packing (**Table 1**).

The I9L replacement should be a conservative change in the protein, as the physio-chemical properties and side chain volumes of isoleucine and leucine are very similar. Mutational studies of other proteins have concluded that these two amino acids are largely interchangeable and that replacement of one by the other generally has little or no effect on a protein's biophysical properties (Betts and Russell, 2003). Accordingly, one would predict that such a minor change should be tolerated by crystal packing. However, this mutation did affect crystal formation. The reason for this change is still unclear. K28C is another interesting mutation. In wild-type glutaredoxin, Lys28 is involved in a complex salt-bridge between α-helices 1, 2, and 3. One would expect that the substitution of an essential, conserved residue like Lys28 with a slightly polar side chain like Cys, should interfere with the biophysical properties of the molecule. However, this mutation yields soluble protein and small protein crystals.

FOUR NEW MUTANT STRUCTURES

Our random mutagenesis approach has yielded four new crystal structures of glutaredoxin A, one for each of the following variants: R27L, A75I, A79S, and P84R (**Table 2**). Each of these crystal structures provides information about the structure and

Table 1 | List of currently studied mutations in *Synechocystis* sp. PCC 6803 glutaredoxin A.

Mutation	Soluble	Insoluble	Crystallize	Δ #Neighbors	Pseudo ΔΔG kcal/mol|prediction		Final structure
WT	✓		✓	–	–		Yes
A2Y	✓		–	+6	−0.02	n	
A2I	✓		✓	+1	−0.30	n	
A2T	✓		✓	+1	−0.6	sd	
I9L	✓		✓	0	−0.59	sd	
T11Y	X	✓	–	+3	+1.39	s	
F17N	✓		–	−4	−1.89	d	
M19A	✓		–	−2	+0.91	ss	
A23N	✓		–	0	−3.62	hd	
L25S	X	✓	–	−3	−4.56	hd	
R27L	✓		✓	−2	−0.08	n	Yes
R27V	✓		✓	−1	−0.82	sd	
K28C	✓		✓	−1	+0.56	ss	
G29C	✓		–	+2	−0.44	n	
E34L	✓		–	0	+1.00	ss	
A43G	✓		–	−1	−3.54	hd	
A43I	✓		✓	+2	+0.27	n	
A43T	✓		✓	+3	−2.29	hd	
A49H	✓		✓	+2	−2.10	hd	
A49W	✓		✓	+4	−2.32	hd	
A51E	✓		–	+4	−2.84	hd	
R55A	✓		✓	−4	+0.11	n	
G68Y	X	✓	–	+1	+3.18	hs	
G69F	✓		–	+3	+1.83	s	
C70S	✓		–	0	−3.69	hd	
A75I	✓		✓	0	−1.19	d	Yes
A79S	✓		✓	0	−0.58	ds	Yes
P84R	✓		✓	+3	+1.65	s	Yes

Soluble = mutant protein could be purified from bacterial lysate.

Insoluble = no mutant protein could be purified from bacterial lysate.

Crystallize = were crystals of any quality grown?

Δ#Neighbors = residues which could cause potential collisions in this crystallographic setting.

Pseudo ΔΔG (kcal/mol) n, neutral; sd, slightly destabilizing; s, stabilizing; d, destabilizing; hd, highly destabilizing; ss, slightly stabilizing; hs, highly stabilizing.

Structure = Was a refined crystal structure produced?

function of the glutaredoxin protein, in addition to information about protein crystal formation and protein structure in general.

The overall structure of the R27L variant is virtually indistinguishable from that of the wild-type protein. The RMSD (root-mean-square deviation) between both structures is 0.099 Å over all atoms, and the Leu27 substitution was confirmed in the refined experimental electron density (**Figure 3**). Arg27 occurs at the C-terminal end of α-helix 1 of the wild-type protein and potentially forms a H-bond with Tyr74. In addition, Arg27 occurs in a cluster of other basic residues (...K-R-K...), so it is likely that it is part of a localized positively charged patch on the surface of the protein. The leucine substitution would obviously disrupt this patch, but little else would be predicted to occur. Nevertheless, the diffraction resolution of the crystals of the R27L variant was significantly increased compared to the data obtained with wild-type glutaredoxin A. Diffraction resolution is an optical property of crystals, which is dictated by the inherent order of a crystal lattice to diffract X-rays. The lower the value for the resolution, the better the crystal is ordered and the better it can provide X-ray diffraction data that can resolve the measured distance between atoms. Crystals of the wild-type protein diffract to 1.7 Å resolution, while the R27L mutation produced crystals that diffract X-rays to 1.2 Å resolution. The sizes of the crystals of the wild-type and R27L variants were similar, so the increase in diffraction cannot be explained by larger crystals. Furthermore, as all data were collected at the same X-ray source, increased X-ray intensity cannot explain this increase in resolution. Therefore, it is likely that a property of the mutation contributed to this effect. There is a possibility that more efficient hydrophobic packing between symmetry-related molecules, mediated by the leucine replacement, contributed to more efficient crystal packing. This observation is essentially what has been described as surface entropy reduction (SER). SER is an *in silico* technique that predicts mutants, which could possibly facilitate

Table 2 | Data collection and refinement statistics.

PDB code:	R27L	A75I	A79S	P84R
	4MJE	4MJA	4MJB	4MJC
DATA COLLECTION				
Wavelength (Å)	0.9795	1.2320	1.2830	1.1270
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
CELL DIMENSIONS				
a, b, c (Å)	37.3, 39.1, 50.6	37.2, 38.4, 51.6	37.2, 38.1, 51.6	37.2, 38.8, 50.7
α, β, γ (o)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	30.95-1.2	30.81-2.0	30.68-2.1	30.83-1.4
Mosaicity (°)	0.35	0.67	0.4	0.79
R_{sym} or R_{merge}	7.4 (30.4)	7.3 (19.2)	4.5 (13.1)	6.7 (31.9)
$I/\sigma I$	11.8 (3.3)	16 (7.0)	20.2 (2.2)	9.6 (3.2)
Completeness (%)	99.5 (99.8)	99.9 (99.7)	99.3 (87.5)	98.8 (99.8)
Redundancy	13.3 (13.2)	6.3 (6.4)	6.8 (6.6)	3.3 (3.3)
REFINEMENT				
Resolution (Å)	30-1.2*	30-2.0**	30.7-2.11	26.8-1.4
No. reflections	23433	5603	4418	14533
R_{work}/R_{free} (%)	17.87/19.78	20.57/22.92	19.02/22.81	17.81/21.57
NO. ATOMS				
Protein	1654	846	838	883
SO_4	10	–	10	5
Water	106	62	51	93
***B*-FACTORS (Å^2)**				
Protein	9.8	20.50	22.60	15.8
SO_4	20.0	–	99.60	16.8
Water	20.0	26.40	26.90	24.1
R.M.S. DEVIATIONS				
Bond lengths (Å)	0.009	0.03	0.004	0.009
Bond angles (o)	1.27	2.13	0.96	1.298
RAMACHANDRAN PLOT				
% Ideal	97	94	95	97
% Allowed	3	6	5	3
Outliers	0	0	0	0

**Coordinates refined with "riding" hydrogen atoms.*

***No sulfate ions were present in this crystal structure.*

more productive contacts among protein molecules in a crystal lattice (Goldschmidt et al., 2007). More efficient crystal packing would then result in higher X-ray scattering angles and higher overall resolution. Such a result, if it were to turn out to be a general effect, could provide information that would allow the engineering of better diffracting protein crystals. Potentially, if a low-resolution structure revealed critical crystal contacts, then mutations could be introduced to optimize those crystal contacts and increase overall diffraction without compromising the structure or activity of the protein.

Due to the location of Ala75, one might have predicted that changes in structure and function observed for the A75I and the A79S variants would be subtle. These replacements are located at the extreme C-terminus of the protein in α-helix 4 (**Figure 2**), and both are exposed to the outside surface of the helix. Neither is involved in crystal packing interactions nor core packing interactions, and therefore, these positions are predicted to be highly tolerant of modification. However, in the case of the A79S mutant, there are minor structural changes observed in the geometry of α-helix 4. There is a measurable decrease in the twist of the helix, a decrease in the helix-bending angle of α-helix 4 (**Table 3**). The significance of these minor changes remains to be explored.

Table 3 | Changes in helix properties in helix α-4 of glutaredoxin (Bansal et al., 2000; Kumar and Bansal, 2012).

Mutation	Twist (°)	*n*	height (Å)	Bending angle (°)
Wild-type	102.2	3.52	1.67	7.3
R27L	101.1	3.56	1.76	7.8
A75I	101.1	3.60	1.65	7.6
A79S	101.3	3.55	1.68	5.4
P84R	101.8	3.54	1.66	11.8

n = amino acids/turn.

h (Å) = unit height of the helix.

Bending angle (°) = Bending angle between successive local helix axes.

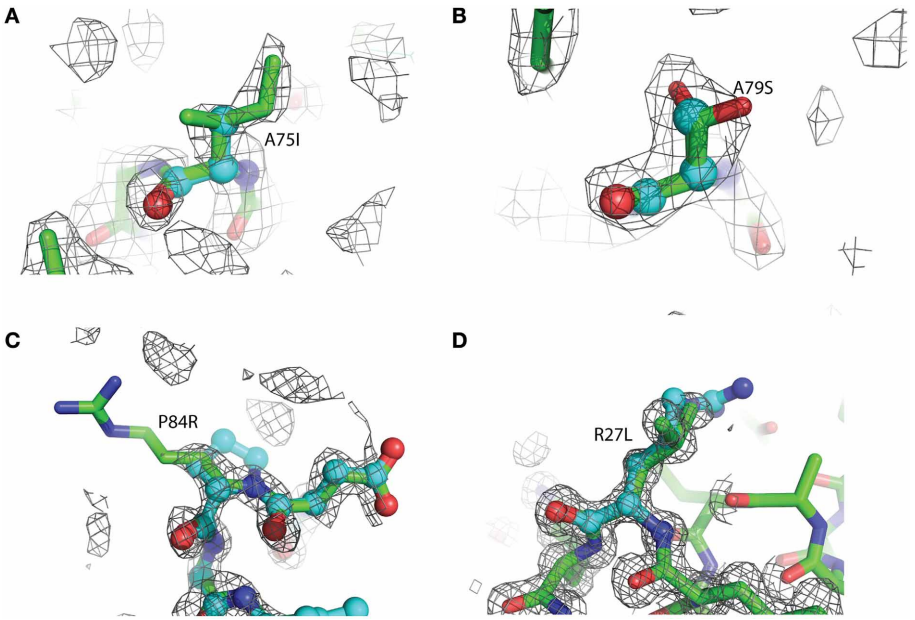

FIGURE 3 | Representative 2Fo-Fc electron density maps, contored at the 1σ level, of each of the four mutations described. (A) A75I: The wild-type Ala is shown as light blue ball-and-stick, while the mutant Ile is shown as green sticks. The chicken wire shape around the amino acid represents the electron density carved from the final refined structure. **(B)** A79S: Ala79 is shown as blue balls-and-sticks. The mutant Ser is shown as green sticks. In this case, two conformations of Ser79 can be modeled. Both rotamers were used at 50% occupancy in the refinement of this structure. **(C)** P84R: wild-type Pro 84 is shown as blue balls-and-sticks. Mutant Arg79 is shown as green sticks. It is common for the more flexible amino acids, such as Arg or Lys, to exhibit abbreviated electron density due to the rotary motion of the side chain. **(D)** R27L: The Arg27 present in the wild-type protein is shown as blue balls-and-sticks. The structure of the leucine replacement at this position has been superimposed on top of the wild-type and is shown as green sticks.

The P84R mutation is interesting from a structural perspective. Firstly, Pro84 occurs at the beginning of an α-helix that is bent by almost 90° relative to a neighboring α-helix in the structure. The only impetus for this dramatic bend is the maintenance of a protected hydrophobic core. A proline is present at this locus in other glutaredoxin molecules, but not all. A Blast search using the *Synechocystis* sp. PCC 6803 glutaredoxin A sequence revealed that 48% of 98 glutaredoxin sequences utilize proline at a homologous position in the primary sequence (data not shown). As proline does not provide a free backbone amide hydrogen to form an H-bond to the $i + 4$ backbone carbonyl, it rarely occurs within a helix (Kim and Kang, 1999). In the wild-type glutaredoxin structure, there is only one i, $i + 4$ backbone H-bond interaction to stabilize α-helix 4 (**Figure 2**). While mutation of Pro84 to arginine does not restore the complete backbone H-bond pattern typical of α-helices, it does appear to alleviate strain on the peptide backbone so an additional i, $i + 4$ backbone H-bond interaction can form between the backbone carbonyl of Asp83 and the backbone amine of Ser88(**Figure 4**).

CHANGES IN PROTEIN STABILITY

Many of the mutations that we introduce using our random selection method could compromise protein stability in many different ways. For example, secondary structure may be disrupted by introducing a residue incompatible with β-sheet formation. The resulting misfolded protein could result in an insoluble protein in *E. coli*. To predict changes in stability, we submitted our point mutants to the Site Directed Mutator (SDM) server for analysis (**Table 1**). This procedure returns a prediction on whether the point mutation is neutral, stabilizing, or destabilizing based on environment-specific substitution tables and the resulting change in free energy (Worth et al., 2011). Large negative ΔΔG correlate with varying degrees of instability, while large positive ΔΔG values correlate with stabilizing energies. In general, the glutaredoxin A mutations that yielded soluble protein were predicted to be either neutral, stabilizing or destabilizing, but the range in free energies was small. The mutants at the extremes of the free energy distribution tended to yield insoluble protein. The exception to this is the C90S mutant, where the calculated free energy difference was negative (−3.69 kcal/mol), and therefore, predicted to be highly destabilizing (hd), yet the purified protein is soluble. The energetics of point mutations is clearly a complex problem; however, with more mutations, it is possible that the predictive abilities of the free energy algorithms will correlate more closely with our observations.

INCONSISTENCIES WITH THE ORTHORHOMBIC CRYSTAL LATTICE

Ideally, we would like to screen each soluble, purified glutaredoxin A mutation for its own unique crystallization condition, but we are limited in resources and by the time our students can spend with this research project. Therefore, we have concentrated on a single crystallographic condition. This restriction obviously imposes limitations on our method, but it also addresses the

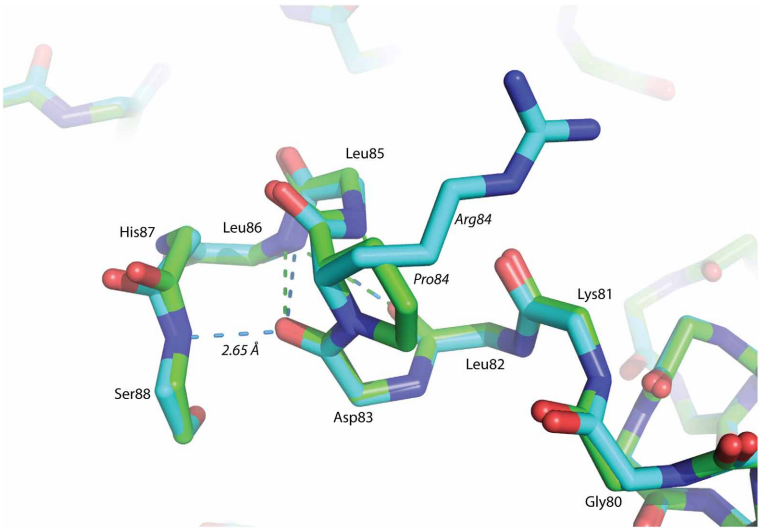

FIGURE 4 | Superposition of helix α -4 from Glutaredoxin A. The green structure is wild-type, while the blue structure is mutant P84R. The dashed-lines are backbone H-bonds that stabilize α-helix 4. One additional consensus H-bond has been included in the P84R structure.

question about how variable or plastic a protein can be yet pack into a given lattice. Most of our mutants form suboptimal protein crystals. It is possible that the modifications we make to the glutaredoxin protein produces changes that cannot form productive crystal contacts, and therefore, disfavors efficient crystal packing. To assess this, we studied the change in the number of neighboring inter-molecular residues (within 5 Å) between mutant and wild-type glutaredoxin, assuming orthorhombic crystal packing (**Table 1**). More inter-molecular neighbors would likely correlate with potential clashes; whereas, fewer contacts could correlate with missing interactions. One would predict that extremes in both would make the protein less likely to crystallize in the orthorhombic setting we have measured in the wild-type glutaredoxin. Interestingly, in the case of the mutations that produced crystal structures, there is no obvious correlation between either adding or subtracting inter-molecular neighbors. The R27L crystal structure lacks two neighbors; the A75I and A79S mutant have no net differences, while the P84R mutant adds three possible interactions (**Table 1**). This result could reflect the plastic nature of proteins that allows them to adopt new shapes. As we have also observed that most of our soluble glutaredoxin mutants can form crystalline material, it is therefore, possible that changing the number of clashes either could make nucleation more probable or alternatively, could hinder crystal growth. As our dataset increases in size with time, we will be able to make more definitive conclusions.

SULFATE BINDING

Since ammonium sulfate is used in the crystallization medium of these glutaredoxin A crystals, there are two sulfate anions coordinated to the wild-type and to the mutated glutaredoxin A structures. Interestingly, the A75I mutation does not coordinate sulfate ions in its crystal structure. In the wild-type structure, sulfate #2 (**Figure 2**) is 10.6 Å from the Cα atom of Ile75, while sulfate #1 (**Figure 2**) is 35.2 Å distant. The large distance between the site of the mutation and the ligand binding site discounts any direct effect on sulfate binding. A cogent rationale for the absence of sulfate anions in the electron density is still unknown; however, we can draw conclusions about the effects of sulfate binding to the A75I crystal structure. As there is no sulfate anion bound to the N-terminus, the His-tag of A75I is considerably more disordered that the other glutaredoxin structures that do coordinate sulfate. The temperature factor is a measure of thermal motion within a crystal structure; atoms with higher temperature factors are thought to be more disordered. The average refined temperature-factors for the artificial N-terminus of wild-type glutaredoxin A (the loop including the His-tag residues) including the coordinated sulfate anion is 17.7 $Å^2$. The same residues in the A75I structure without a coordinated sulfate anion show an average temperature factor of 32.6 $Å^2$ (**Figure 5**). While the elevated temperature factors can be explained by the lack of sulfate coordination, the rationale for the lack of this anion in this mutation is still unknown.

CONCLUSION

Our random point mutant selection scheme will ultimately cover all of the 1672 possible mutations of *Synechocystis* sp. PCC 6803 glutaredoxin A, while simultaneously teaching students about biotechnology and protein structure. There are, however, several weaknesses to this method. Due to time constraints, we have to restrict our crystallization experiments to one specific condition; one that had originally been identified during crystallization trials for the wild-type protein. While we will clearly learn how mutation affects crystal growth under these conditions, a broader-ranging, independent approach would be more informative. Another weakness of this project is that we seek to involve students from different institutions with a wide range of backgrounds and training. As such, it introduces considerable variability in the skill

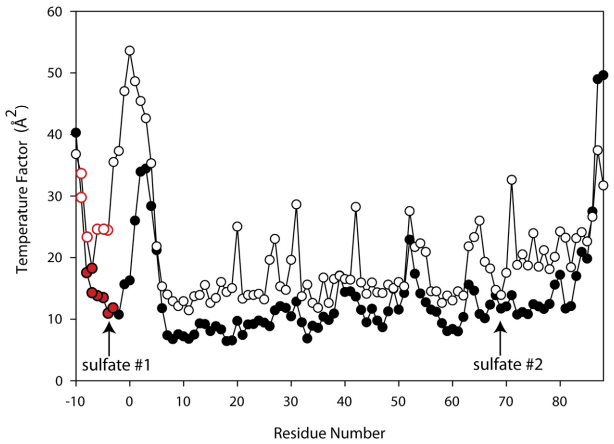

FIGURE 5 | Superimposed Temperature Factor plot of wild-type glutaredoxin (solid circles) (3QMX) vs. A75I (white circles). The red color depicts the His-residues that make up the His-tag of the molecule. The arrows show the location of the shortest sulfate:amino acid distance.

with which different amino acid replacements will be carried out and analyzed. While only the most diligent students will be able to make significant headway in this project, the rewards of involving a large number of students and potentially exciting them about science surely outweighs the negative aspects.

It is hoped that we will ultimately compile an extensive database that will include information to stabilize protein folds or, perhaps, to reveal techniques that improve protein crystal diffraction. However, in the limited coverage of mutational space that we have already explored, it is clear that not all mutations can be immediately understood, and their effects are still reasonably unpredictable. This is one of the primary concepts that we try to instill in students from the beginning.

MATERIALS AND METHODS

HETEROLOGOUS EXPRESSION

The mutagenesis protocol that we use is described in detail (Kim et al., 2012). A full description of the plasmid is described in Kim et al. (2012).

1.0 μg of plasmid DNA was added to 100 μl of chemically competent BL-21(DE3) *E. coli* in a 1.5 ml microfuge tube. This mixture was incubated on ice for 30 min. The cells were then heat-shocked at 37°C in a water-bath for 45 s. 650 μl of SOC medium were added to each microfuge tube. The plasmid DNA/BL-21(DE3) mixture and the SOC medium were placed in a 37°C incubator, shaken at 225 r.p.m. for a 1 h and subsequently plated onto an agar substrate containing 100 μg ml 87,221 ampicillin. The plates were then placed in a stationary incubator at 37°C for 12 h. Start-up cultures were prepared by inoculating individual colonies from the plasmid transformation plate into 25 mL of Luria-Bertani medium (LB) and 100 μg ml^{-1} of ampicillin. This culture was incubated at 37°C while shaking at 225 r.p.m. overnight. The start-up LB cultures were added to 1 L Terrific broth (TB) to maximize bacterial cell yield. TB cultures were incubated at 37°C, with shaking at 225 r.p.m. until OD600 reached 1.3, at which point 400 μl of isopropyl β-D-1-thiogalactopyranoside (IPTG) was introduced to induce protein expression. Prior to IPTG induction, a 500 μl sample of un-induced cells was set aside for PAGE electrophoresis. TB cultures were incubated for a 12 h period at 20°C for a 3–4 h period at 37°C, with shaking at 225 r.p.m. and subsequently spun down at 7200 r.p.m. in a centrifuge at 4°C. A post-induction gel electrophoresis sample was taken prior to centrifugation to confirm heterologous expression. Cell pellets were collected, flash-frozen in liquid nitrogen, and stored at −80°C until lysis.

PURIFICATION

Following SDS-PAGE verification of heterologous protein induction in bacteria, cell cultures were thawed at 0°C, suspended in a lysis buffer (30 mM Tris buffer, pH 8.0, containing 500 mM NaCl), vortexed, and then lysed at 20,000 psi in a Microfluidics M-100EH microfluidizer. Lysed cells were centrifuged at 42,000 r.p.m. for 45 min at 4°C; the supernatant was subsequently collected and bound to Ni-NTA resin for 12 h. Glutaredoxin-bound Ni-NTA resin was washed with 30 mM Tris buffer, pH 8.0, containing 500 mM NaCl plus 30 mM imidazole until the OD_{280} of the eluent was ≤0.010. Glutaredoxin A was eluted from the column with 40–50 ml of 30 mM Tris buffer, pH 8.0, containing 300 mM imidazole, plus 500 mM NaCl. Post-elution protein purity was confirmed by SDS-PAGE using a Phastgel apparatus (GE Healthcare). Protein concentrations were determined either using the BioRad Protein Assay with bovine serum albumin as a standard (Bradford, 1976), or by using reference parameters calculated from the molecular weight and calculated extinction coefficient of the mutant in question (Gill and Von Hippel, 1989). Glutaredoxin A mutants were concentrated to approximately 6 mg/ml and further purified by gel filtration on a Superdex 75 column (5 × 300 mm). Protein was re-concentrated for crystallization using a 10 K cutoff Amicon spin concentrator.

CRYSTALLIZATION

Crystallization trials of all mutant glutaredoxin proteins utilized the hanging-drop vapor-diffusion method. Noting previously identified glutaredoxin A wild-type crystallization conditions, 24 well trays were set up with a horizontally varying salt gradient (1.1–1.5 M ammonium sulfate), and a vertically varying pH gradient (pH 7.0–8.2 HEPES buffer). 1% PEG400 (w/v) was included throughout. Crystal droplets consisting of 2 μl protein solution and 2 μl reservoir solution were added and trays were placed in 23°C for crystallization. Long, rod-like structures displaying the typical morphology of glutaredoxin crystals generally appeared after 24 h; however, some mutants grew crystals in as much as 2 weeks.

STRUCTURE SOLUTION

The crystals were captured on nylon loops and flash frozen in liquid N_2. Initial data sets were collected on a Rigaku ScreenMachine. Subsequent data sets were collected at SLAC beamline 7-1. The data were collected at 90 K. X-ray data was processed with imosflm (Battye et al., 2011) and the data were scaled using SCALA as a part of the CCP4 package (Winn et al.,

2011). 10% of the data were allocated for R-free cross validation. A summary of the crystal statistics are presented in **Table 2**.

Structures of all mutants were solved by molecular replacement using 3QMX as the target structure. We used the Phaser module as implemented in the Phenix package for this calculation. Subsequent electron density for the protein was manually fit using Coot (Emsley et al., 2010). Electron density assigned as water molecules was automatically assigned by Phenix during the final stages of refinement. Each water molecule was checked manually in Coot, and in cases that were missed by Phenix, waters were added manually to density that met the H-bonding geometry and inter-atomic distance criteria established by Phenix. Structural refinement was also performed using the Phenix package (Adams et al., 2010).

MUTANT ANALYSIS

The thermodynamic stability for each single site mutant in our data set was predicted using the SDM web server (Worth et al., 2011). This website calculates changes in stability that likely result from single site mutations in proteins. Factors such as the physiochemical differences between the wild-type and mutant protein within the known protein structure are used to compute a free energy term (Worth et al., 2011). A significant change in effective free energy correlates with either with a stabilizing or a destabilizing prediction (**Table 1**).

Potential conflicts with the wild-type crystal packing were analyzed in Pymol (Schrodinger, 2013). First, the wild-type residue of interest was selected in Pymol. Then, complete symmetry-related molecules in crystal lattice were expanded in a sphere 5 Å around that amino acid. All of the residues within 5 Å of the amino acid were selected and counted. Simulated mutants were computed using the Pymol mutagenesis wizard. The most common rotamer was selected without any subsequent energy minimization. The same symmetry expansion and residue selection was performed as was described from the wild-type protein. The net difference between the number of neighboring residues in the wild-type protein and the mutant protein was reported as Δ # Neighbors (**Table 1**).

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Missing links in understanding redox signaling via thiol/disulfide modulation: how is glutathione oxidized in plants?

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Glutathione is a small redox-active molecule existing in two main stable forms: the thiol (GSH) and the disulphide (GSSG). In plants growing in optimal conditions, the GSH:GSSG ratio is high in most cell compartments. Challenging environmental conditions are known to alter this ratio, notably by inducing the accumulation of GSSG, an effect that may be influential in the perception or transduction of stress signals. Despite the potential importance of glutathione status in redox signaling, the reactions responsible for the oxidation of GSH to GSSG have not been clearly identified. Most attention has focused on the ascorbate-glutathione pathway, but several other candidate pathways may couple the availability of oxidants such as H_2O_2 to changes in glutathione and thus impact on signaling pathways through regulation of protein thiol-disulfide status. We provide an overview of the main candidate pathways and discuss the available biochemical, transcriptomic, and genetic evidence relating to each. Our analysis emphasizes how much is still to be elucidated on this question, which is likely important for a full understanding of how stress-related redox regulation might impinge on phytohormone-related and other signaling pathways in plants.

Keywords: oxidative stress, hydrogen peroxide, dehydroascorbate, glutathione *S*-transferase, glutaredoxin

INTRODUCTION

Arabidopsis cannot develop past the embryonic stage without glutathione (Cairns et al., 2006), a multifunctional tripeptide thiol found in the cells of most organisms. Although this small molecule has diverse roles in defense and metabolism, a key function is in redox homeostasis (Foyer and Noctor, 2011). This protective role notably involves acting in the metabolism of oxidants such as reactive oxygen species (ROS) and buffering protein thiol groups against excessive oxidation. Like most other biological roles of glutathione, these functions depend on the redox-active cysteine residue.

While the cysteine sulphur can exist in several redox states, the key stable forms are the thiol found in reduced glutathione (GSH) and the disulphide found in GSSG. Other factors remaining constant, the concentrations of the two forms modulate the effective redox potential of the couple according to the relationship $[GSH]^2/[GSSG]$ (Meyer, 2008). When plants are growing in unchallenging conditions, in which GSH-oxidizing compounds are kept relatively low by cellular antioxidant systems, the glutathione redox potential in subcellular compartments such as the cytosol, chloroplast, and mitochondria is maintained at highly reducing values. Redox-sensitive green fluorescent proteins (roGFP) have been developed as *in vivo* probes for cell thiol-disulfide status. Although it cannot be completely excluded that other thiols may influence their status *in vivo*, the *in vitro* specificity of roGFP oxidoreduction suggests that they report mainly on the glutathione redox potential (Meyer et al., 2007). Analyses using these probes have measured redox potentials in the cytosol, chloroplasts and mitochondria that are close to −320 mV, the midpoint potential of NADPH (Meyer et al., 2007; Schwarzländer et al., 2008; Jubany-Mari et al., 2010). If these values faithfully reflect the glutathione redox potential, they imply that the GSH:GSSG ratio is well over 1000. Thus, while total glutathione concentrations are typically in the 1–10 mM range (Queval et al., 2011), GSSG concentrations in unchallenging conditions may be 10^3–10^6 times lower in compartments that contain significant activities of GR.

When plants are subject to suboptimal conditions, GSSG can accumulate to higher levels. This phenomenon is observed in extracts of plants exposed to various abiotic and biotic stresses (Edwards et al., 1991; Sen Gupta et al., 1991; Vanacker et al., 2000; Bick et al., 2001; Gomez et al., 2004). Based on studies of plants deficient in enzymes such as ascorbate peroxidase (APX) and catalase (CAT), accumulation of GSSG is quite closely related to the intracellular availability of H_2O_2 (Rizhsky et al., 2002; Mhamdi et al., 2010a), which is expected to be enhanced in stress conditions.

[a] GSH and GSSG are used here to refer specifically to the thiol and disulphide forms of glutathione. Where both forms may be referred to, "glutathione" is used.

[b] GPX is used to refer to a specific family of genes encoding enzymes that may catalyse only low rates of this activity *in vivo*. To avoid confusion, the biochemical activity of glutathione peroxidation (e.g., catalysed by some glutathione *S*-transferases) is denoted by the abbreviation "GSH peroxidase."

The principal reactions and proteins responsible for reducing GSSG to GSH in plants are relatively well characterized. In Arabidopsis, two genes each encode dual-targeted glutathione reductases (GR), and this is sufficient to explain the presence of GR activity in the chloroplasts, mitochondria, cytosol, and peroxisomes (Chew et al., 2003; Kataya and Reumann, 2010). Given that GR has a K_M value for NADPH below 10 μM (Edwards et al., 1990), conversion of GSSG to GSH is unlikely to be limited by reductant. Moreover, loss of function of *GR1*, encoding the cytosol/peroxisome enzymes, causes only moderate GSSG accumulation in leaf tissue, an observation explained by the existence of an auxiliary GSSG-reducing activity ensured by cytosolic NADPH-thioredoxin (TRX) systems (Marty et al., 2009). However, GR1 becomes more important in oxidative stress conditions (Mhamdi et al., 2010b; Dghim et al., 2013).

In contrast to GSSG reduction, the reactions that are most important in converting GSH to GSSG are less clear and, potentially, more complex. Since GSSG accumulation is not only a useful biochemical marker for oxidative stress in plants, but may also be of functional importance in transmitting signals triggered by increased H_2O_2 (Han et al., 2013a,b), the aim of the discussion below is to present an overview of current knowledge on the reactions that could be responsible for this phenomenon.

CANDIDATE PATHWAYS

ENZYME-INDEPENDENT OXIDATION

Because GSH oxidation is strongly dependent on deprotonation to the thiolate form (GS−), electron transfer is pH-dependent. The p*K*a of the GSH thiol is about 9.0. Thus, only about 1% of GSH thiols will be deprotonated at any one moment in the cytosol (pH 7.2). This percentage will be even less in more acidic compartments such as the vacuole or apoplast, although GSH concentrations are relatively low at these locations. In the chloroplast, the chemical reactivity of GSH will be favored in the light compared to the dark because photosynthetic electron transport drives alkalinisation of the stroma. Chemical oxidation of GSH can therefore be influenced by physiologically relevant changes in pH. Decreases in proton concentration will also decrease the glutathione redox potential for a given value of $[GSH]^2/[GSSG]$.

The nucleophilic properties of GS- mean that it can react with a wide spectrum of electrophiles. In some cases, this will not lead to oxidation to GSSG or other disulfide but rather formation of a stable *S*-conjugate with various compounds (Dixon and Edwards, 2010). Such GS-conjugates are generally transported by ATP-dependent pumps (ABCC proteins) to the vacuole, where the constituent amino acids of the glutathione moiety are recycled (Martinoia et al., 1993; Lu et al., 1998; Grzam et al., 2006). Among the molecules able to oxidize GSH to produce GSSG are ROS and dehydroascorbate (DHA), the stable non-radical product of ascorbate oxidation. Rate constants for reactions with some of these compounds are shown in **Table 1**. Glutathione reacts with singlet oxygen and superoxide at rates similar to other molecules with recognized antioxidant properties, such as ascorbate and phenolic compounds. However, both glutathione and ascorbate react appreciably slower with singlet oxygen than tocopherols and, especially, carotenoids (**Table 1**). While the reaction between GSH and the hydroxyl radical is very fast, this powerful oxidant also reacts rapidly with numerous other metabolites that are present in the cellular environment at higher concentrations than GSH, such as ascorbate and sugars (**Table 1**). Thus, other compounds might be expected to compete effectively with GSH in the scavenging of both singlet oxygen and the hydroxyl radical. As for other non-enzymatic thiols, and ascorbate, the chemical reaction with H_2O_2 is very slow. Superoxide and DHA are therefore the most likely of the molecules shown in **Table 1** to contribute to uncatalyzed production of GSSG *in vivo*. This conclusion receives some support from kinetic modeling studies (Polle, 2001).

Table 1 | Rates of nonenzymatic reactions between glutathione and various oxidants.

Oxidant	Metabolite	k ($M^{-1}s^{-1}$)
Singlet oxygen	Glutathione[a]	2×10^6
	Ascorbate[a]	1×10^7
	β-Carotene[b]	1.4×10^{10}
	α-Tocopherol[b]	3×10^8
Superoxide	Glutathione[a]	7×10^5
	Ascorbate[a]	2×10^5
	Kaempferol[c]	5.5×10^5
	Quercetin[c]	0.9×10^5
Hydroxyl radical	Glutathione[f]	8.1×10^9
	Ascorbate[f]	1.5×10^9
	Kaempferol[c]	4.6×10^9
	Quercetin[c]	4.3×10^9
	Glucose[f]	4.0×10^9
	Sucrose[f]	8.9×10^9
	Galactinol[f]	7.8×10^9
Hydrogen peroxide	Glutathione[d]	0.9
	Ascorbate[e]	2
	Cysteine[d]	2.9
	Thioredoxin[d]	1.1
Dehydroascorbate	Glutathione[g]	1×10^5

For comparative purposes, rate constants are also shown for ascorbate and other metabolites.

References: [a] Asada and Takahashi, 1987; [b] Di Mascio et al., 1989; [c] Bors et al., 1990; [d] Winterbourn, 2013; [e] Polle, 2001; [f] Nishizawa et al., 2008; [g] Hausladen and Kunert, 1990. Values for thioredoxin are for the E.coli protein.

THE ASCORBATE-GLUTATHIONE PATHWAY

The slow chemical reaction of GSH with H_2O_2 contrasts with the rapid reaction with DHA (**Table 1**). This is the key observation underlying the importance ascribed to the close redox coupling of ascorbate and glutathione pools *in vivo*, which allows glutathione to play an indirect role in H_2O_2 reduction as part of a reaction sequence that ultimately depends on electrons derived from NAD(P)H and/or ferredoxin (**Figure 1**). Thus, APX reduces H_2O_2 to water, yielding monodehydroascorbate (MDHA) as an unstable initial product. MDHA that is not rapidly reduced can dismutate to ascorbate and DHA, which can then be reduced by GSH with the concomitant production of GSSG (**Figure 1**).

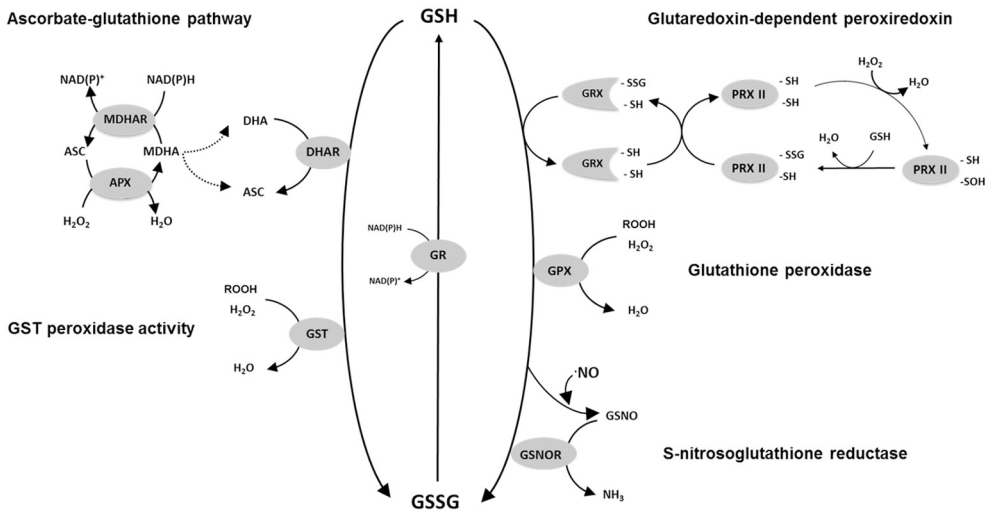

FIGURE 1 | Scheme of some of the possible reactions involved in GSH oxidation in plants. Not all possible reactions or reaction mechanisms are shown. For reasons of clarity, stoichiometries are not depicted. For PRXII a proposed regeneration mechanism of Prx IIE using the GSH/Grx system is shown according to Gama et al. (2008). Abbreviations: GSH, reduced glutathione; GSSG, glutathione disulphide; GR, glutathione reductase; DHAR, dehydroascorbate reductase; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; ASC, ascorbate; DHA, dehydroascorbate; MDHA, monodehydroascorbate; H_2O_2, hydrogen peroxide; GST, glutathione *S*-transferase, GRX, glutaredoxin; PRX, peroxiredoxin; -SH, sulfhydryl (thiol) group; -SSG, glutathionylated protein Cys residue; GSNO, S-nitrosoglutathione; GSNOR, GSNO reductase; GPX, glutathione peroxidase.

While GSH can chemically reduce DHA at high rates, the reaction is accelerated significantly by DHA reductases (DHAR; Foyer and Halliwell, 1977). These enzymes have been purified from several species (Hossain and Asada, 1984; Dipierro and Borranccino, 1991; Urano et al., 2000). In Arabidopsis, at least three *DHAR* genes are expressed. Although they are not able to catalyze glutathione *S*-conjugation reactions at significant rates, they are considered to belong to the glutathione *S*-transferase (GST) superfamily (Dixon and Edwards, 2010). The difference in the activities of DHARs and most classes of GST is explained by the presence of a cysteine in place of serine at the active sites of DHARs (Dixon et al., 2002).

GLUTATHIONE PEROXIDASE (GPX)

Although GSSG could be generated as a product of GSH-dependent DHA reduction, genomics has revealed the complexity of the plant antioxidative system and identified several GSH-dependent enzymes that may play more direct roles in peroxide metabolism. GPX has long been known to be a player in H_2O_2 metabolism in mammalian cells, but only began to be seriously studied in plants following the description of sequences homologous to the animal enzymes in the 1990s. Described as glutathione hydroperoxidases, the plant GPXs are distinguished from animal GPXs in having an active site cysteine in place of selenocysteine (Eshdat et al., 1997), even though both selenocysteine- and cysteine-dependent GPXs are found in unicellular algae such as *Chlamydomonas* (Dayer et al., 2008). Genome sequencing has shown that plant GPXs are encoded by several genes (8 in Arabidopsis). Despite their current nomenclature, several independent studies have shown that the encoded enzymes prefer TRX as reductant and have comparatively low activity against GSH (Herbette et al., 2002; Iqbal et al., 2006; Navrot et al., 2006). Thus, they might be considered to be TRX-dependent peroxiredoxins, and so not strong candidates to account for GSSG accumulation *in vivo*. However, they are included in **Figure 1** because it cannot as yet be discounted that they make some contribution to the GSH oxidation that occurs during stress.

GLUTATHIONE S-TRANSFERASES (GST)

The *GST* superfamily is composed of 55 genes in Arabidopsis, including the *DHAR* sequences mentioned above (Dixon and Edwards, 2010). As well as the DHARs, the family is divided into several classes (zeta, theta, TCHQD, phi, tau, lambda), with the last three being specific to plants. The most numerous are the phi and tau classes, composed of 13 and 28 genes, respectively (Supplemental Table 1). Proteins that catalyze the classical conjugase reaction using GSH are found in several classes. At least some GSTs can also use GSH to reduce organic hydroperoxides (Cummins et al., 1999; **Figure 1**). Studies on the Arabidopsis proteins have revealed that several classes of GST include enzymes with both conjugase and peroxidase activities (Dixon et al., 2009; Dixon and Edwards, 2010). Enzymes of the lambda class are unusual in that they do not catalyze conjugase reactions. Like DHARs, they have an active-site cysteine and function as monomers (Dixon et al., 2002). They may generate GSSG by catalyzing the reduction of small molecules or, possibly, the deglutathionylation of protein cysteine residues (Dixon et al., 2002; Dixon and Edwards, 2010).

PEROXIREDOXINS (PRX)

These enzymes are classed into several types: 2-cys PRX, 1-cys PRX, type II PRX, and PRX Q (Dietz et al., 2002). The first

to be studied in plants was chloroplastic 2-Cys PRX, which can be regenerated by specific thioredoxins or by an NADPH-thioredoxin reductase (Dietz, 2003; Collin et al., 2004; Pulido et al., 2010). However, plants also contain several type II PRX that, once oxidized by peroxides or other compounds, can oxidize glutathione, particularly *via* glutaredoxins (GRX; **Figure 1**). Interactions between PRXII and GRX have been studied at the biochemical level in poplar and *Arabidopsis* (Rouhier et al., 2002; Bréhélin et al., 2003; Couturier et al., 2011; Riondet et al., 2012). PRXII are encoded by five expressed genes in Arabidopsis. While information is emerging, the identification of the GRXs that couple their re-reduction to GSH oxidation *in vivo* remains incomplete (Rouhier, 2010).

OTHER POSSIBILITIES

The reactions outlined above are not intended to be exhaustive. Numerous other routes could allow oxidation of GSH to GSSG. A comprehensive treatment of all of these is beyond the scope of this discussion. Among other possibilities of note is the reaction catalyzed by *S*-nitrosoglutathione (GSNO) reductase (**Figure 1**), which can produce GSSG from GSH and GSNO. Although this enzyme is receiving considerable attention for its role in various physiological functions (Sakamoto et al., 2002; Díaz et al., 2003; Barroso et al., 2006; Kwon et al., 2012), its capacity relative to enzymes such as DHAR is unclear. Adenosine phosphosulfate reductase (APR), a key chloroplastic enzyme in sulphate reduction, uses GSH as electron donor (Bick et al., 1998). The capacity of this enzyme is relatively low, although the activity may be stimulated by enhanced expression and post-translational activation in oxidative stress conditions, notably to produce cysteine for glutathione synthesis (Bick et al., 2001; Queval et al., 2009).

At least one type of plant methionine sulphoxide reductase activity may be coupled to GSH oxidation *via* glutathione-linked GRX (Tarrago et al., 2009). This enzyme regulates the oxidation state of protein methionine residues but based on its turnover rates (Tarrago et al., 2009), it is unlikely to make an appreciable contribution to increases in GSSG during oxidative stress. One interesting mechanism that could potentially contribute has been described as "proteome-dependent glutathione peroxidase" (Zaffagnini et al., 2012). This process, which could be stimulated under conditions of stress, envisages a chloroplastic sequence of reactions involving H_2O_2-triggered *S*-glutathionylation of diverse available protein cysteine residues, followed by regeneration of the free cysteines by glutathione-dependent GRX (Zaffagnini et al., 2012). The net result would be reduction of H_2O_2 to two water molecules with oxidation of 2 GSH to GSSG, i.e., a GSH peroxidase reaction. Such a sequence may share mechanistic features with the PRXII-GRX pathway shown in **Figure 1**, a principal difference being that H_2O_2 would not react with a specific catalytic cysteine but rather in a more general way with free and reactive chloroplast protein cysteines. As yet, the physiological significance of this process is difficult to evaluate, although it has been noted that the abundance of potentially reactive cysteines in chloroplast proteins is far from negligible (Zaffagnini et al., 2012).

SUBCELLULAR COMPARTMENTATION

Supplemental Table 1 presents a list of Arabidopsis genes involved in the pathways shown in **Figure 1**. Given the relative concentrations of different ROS, the battery of H_2O_2-metabolizing enzymes potentially linked to glutathione, and the marked changes in glutathione status when other H_2O_2-metabolizing enzymes such as catalase are down-regulated, the main focus of the following discussion concerns the enzymes that could be important in linking H_2O_2 or related peroxides to GSH oxidation to GSSG. Based on the above discussion, we suggest that the major candidates to perform this function are (1) DHARs, (2) GSTs, and (3) GRX-PRXII. The subcellular compartmentation of Arabidopsis proteins within these families is summarized in **Figure 2**.

While DHAR1 has been localized to the peroxisomes, DHAR2 is cytosolic and *DHAR3* encodes a dual-addressed chloroplast/mitochondrial enzyme (**Figure 2**). These three genes are therefore sufficient to explain the presence of the ascorbate-glutathione cycle in these compartments (Foyer and Halliwell, 1977; Jiménez et al., 1997). Three type II PRX (PRXIIB, C, D) are found in the cytosol, with PRXIIE and PRXIIF located in the chloroplast and mitochondrion, respectively (**Figure 2**; Rouhier and Jacquot, 2005; Tripathi et al., 2009).

Many GSTs are located in the cytosol, but several of these have also been detected in other compartments such as the chloroplast (**Figure 2**; Supplemental Table 1). The only types of GST that are not thought to be found in the cytosol are the theta class, encoded by three genes that direct the proteins to the peroxisome (Dixon et al., 2009). The GFP-fusion proteins of GSTU12 and GSTT3L were found to be localized in the nucleus (Dixon et al., 2009).

EVIDENCE FOR THE IMPORTANCE OF THE DIFFERENT PATHWAYS

BIOCHEMICAL DATA

DHA as oxidant

On the basis of modeling of ROS metabolism in the chloroplast, it was suggested that the ascorbate pool could operate largely

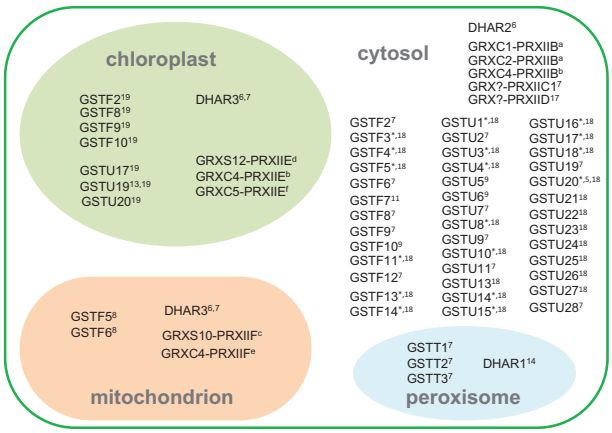

FIGURE 2 | Subcellular compartmentation of GSH-oxidizing enzymes, based on available information. Numbers and "∗" indicate references given in Supplemental Table 1 except for the following, indicated in the figure by letters in superscript: [a]Riondet et al., 2012; [b]Bréhélin et al., 2003; [c]Finkemeier et al., 2005; [d]Gama et al., 2008; [e]Rouhier et al., 2005; [f]Couturier et al., 2011. Abbreviations as in **Figure 1** legend. GRX-PRX partnerships are indicated for proteins shown to functionally interact in peroxidase activity, but these proteins may also interact with other partners.

independently of glutathione because most of the MDHA produced would be efficiently reduced to ascorbate, entailing the formation of little DHA (Polle, 2001). It was concluded that the chemical reaction with GSH would suffice to reduce the small amounts of DHA produced (Polle, 2001). It is possible that this situation is specific to the illuminated chloroplast.

Both the chemical and enzyme-dependent reduction of DHA can be easily detected *in vitro* by following the GSH-dependent production of ascorbate in the absence and presence of crude protein extract. The total enzyme-dependent leaf activity that can be measured in standard assay conditions is around 0.2–0.5 μmol.mg^{-1} total protein.min^{-1}, which is typically about twice the total leaf GR activity but somewhat lower than extractable APX (e.g., Sen Gupta et al., 1993; Mhamdi et al., 2010b).

A protein purified from spinach by classical biochemical procedures, considered to be a cytosolic DHAR, had K_M values of 2.5 mM and 70 μM for GSH and DHA, respectively (Hossain and Asada, 1984). Analysis of a purified chloroplast protein produced similar values, although with a somewhat higher affinity for GSH (Shimaoka et al., 2000). Subsequent studies of recombinant DHARs from Arabidopsis, poplar, and rice produced similar maximal activities and K_M values to these preparations, although the organellar forms in Arabidopsis have a significantly lower affinity for GSH (**Table 2**). With the exception of values reported for the Arabidopsis enzymes, the K_M values for GSH are below or close to *in vivo* concentrations (Queval et al., 2011). Much of the DHA that can be measured in unstressed plant tissues is probably apoplastic (Foyer and Noctor, 2011) and concentrations are probably low in compartments that contain GSH (Polle, 2001). If so, oxidative stress-induced changes in DHA could be a major factor contributing to accelerated activity *in vivo*, at least in some compartments. Under conditions favoring accumulation of H_2O_2, a second factor that could come into play is increases in DHAR abundance. The expression of some *DHAR* genes is increased by oxidative stress (discussed further in the next section).

Table 2 | Kinetic properties of dehydroascorbate reductases from several plant species.

Name/ Subcellular localization	Max activity	GSH			DHA		
		K_M	k_{cat} (/10^4)	k_{cat}/K_M (/10^4)	K_M	k_{cat} (/10^4)	k_{cat}/K_M (/10^4)
PURIFIED FROM SPINACH LEAVES							
Cyt[a]	370	2.5	–	–	0.07	0.01	–
Chp[b]	360	1.1	–	–	0.07	0.03	–
Chp[c]	–	1.1	–	0.52	0.05	0.05	0.9
RECOMBINANT ARABIDOPSIS ENZYMES[d]							
DHAR1 Per	936	10	–	–	0.26	–	–
DHAR2 Cyt	120	–	–	–	–	–	–
DHAR3 Chp/Mit	264	10	–	–	0.50	–	–
RECOMBINANT POPLAR ENZYMES[e]							
DHAR1 Chp	53	3.8	9.9	2.7	0.07	1.3	18.8
DHAR2 Cyt	50	2.3	5.4	2.4	0.23	1.8	7.7
DHAR3 Cyt	38	2.5	4.1	1.7	0.48	2.1	4.4
RECOMBINANT RICE ENZYME[f]							
DHAR1 Cyt	350	1.0	–	–	0.35	–	–

Values are expressed in mM (K_M), s^{-1} (k_{cat},), $mM^{-1}s^{-1}$ (k_{cat}/K_M), and μmol. $min^{-1}mg^{-1}$protein (maximal activity). Chp, chloroplast. Cyt, cytosol. Mit, mitochondrion. Per, peroxisome.

References: [a]Hossain and Asada, 1984; [b]Shimaoka et al., 2000; [c]Shimaoka et al., 2003; [d]Dixon et al., 2002; [e]Tang and Yang, 2013; [f]Amako et al., 2006.

GST activities

Although they have hydroperoxide activity, GSTs generally use H_2O_2 only at low rates (Mannervik, 1985). The physiological oxidants used by the different GSTs in plants remain in many cases to be identified, and studies of their biochemical activities frequently use the artificial substrate, cumene hydroperoxide. Specific GSTs have been purified from several species and their activities as conjugases or peroxidases compared (DeRidder et al., 2002; Cummins et al., 2003; Park et al., 2005; Nutricati et al., 2006; Yang et al., 2009). Such studies reveal that the K_M values of GSTs for GSH in conjugase reactions are generally below 2 mM. Some studies have compared the peroxidatic competence of several GSTs from the same species (Wagner et al., 2002; Dixon et al., 2009). Most notably, the detailed study of Dixon et al. (2009) reported that GSTs showing peroxidase activity are numerous and not limited to any class. Of 38 theta, phi, and tau class GSTs tested for GSH peroxidase activity against short-chain organic peroxides, only six were found to have undetectable activity (**Table 3**). Of the 32 with detectable peroxidase activity, most were also able to catalyze GSH conjugation to one or both of two model substrates (Dixon et al., 2009).

Table 3 | Arabidopsis recombinant glutathione *S*-transferases shown to have GSH peroxidase activity *in vitro*.

PEROXIDATIC ACTIVITY					
T1 > U25 > T3 > T2 > U8 > U17 > U24 > F6 > F8 > U6 > U16 > U5 > F2 > F9 > U18 > U3 > U19 > U1 > U22 > F7 > U4 > F3 > U20 = U23 > U10 = U26 > U28 = U2 > U9 > U7 > U21 > U13					
PEROXIDATIC ACTIVITY NOT DETECTED					
F5	F14	U11	U12	U14	U27
PEROXIDATIC ACTIVITY NOT TESTED					
F4	F13	F10	F11	F12	U15

The enzymes for which peroxidatic activity was detected are listed in descending order of the activity measured on a unit protein basis against cumene hydroperoxide (Dixon et al., 2009).

Although 32 GSTs showed peroxidase activity *in vitro*, their specific activities varied considerably, the most active (GSTT1) having rates ~600-fold higher than the least active (GSTU13). The relatively small theta class was the only one whose members were all found to be competent in GSH peroxidation. Together with the tau-type U25, these three GSTs showed the highest specific peroxidase activity (**Table 3**). Moreover, the theta class enzymes were active not only against cumene hydroperoxide but also against long-chain (C_{18}) fatty acid peroxides. This contrasted with U25, which was highly active only against the model peroxide (Dixon et al., 2009). Interestingly, the theta GSTs were shown to be localized in the peroxisomes (**Figure 2**), organelles that can have high rates of both peroxide generation and fatty acid metabolism.

Glutaredoxin-linked peroxiredoxins

The activity of recombinant poplar mitochondrial PRXIIF against H_2O_2 and organic peroxides was measured in the presence of GSH and/or GRX (Gama et al., 2007). With *tert*-butyl hydroperoxide as oxidant, a K_M of 260 μM was obtained for GSH. However, the activity was considerably stimulated by the additional presence of poplar GRX C4 (Gama et al., 2007). Analysis of the chloroplastic PRXIIE (Gama et al., 2008) revealed fairly similar properties, except that lower K_M peroxide and higher k_{cat} values produced somewhat higher catalytic efficiencies than for the mitochondrial protein (**Table 4**). In fact, the kinetic properties against peroxide of the glutathione-linked PRXIIE were very similar to 2-cys PRX, which is also chloroplastic but glutathione-independent (Horling et al., 2003; Bernier-Villamor et al., 2004; Rouhier et al., 2004a,b; Gama et al., 2007). A study of the mitochondrial PRXIIF from pea reported a similar K_M for H_2O_2 but significantly higher turnover values (Barranco-Medina et al., 2007). The K_M values for H_2O_2 of PRXII, 2-cys PRX and APX are quite similar, but the turnover rates of both types of PRX are significantly lower. Thus, k_{cat}/K_M values for PRX are about 100-fold below those measured for chloroplastic APX or, in the case of the pea PRXIIF, over 10-fold lower (**Table 4**).

In terms of the capacity for GSH oxidation through GRX-PRXII compared to the ascorbate-glutathione pathway, it is interesting to compare the k_{cat} and k_{cat}/K_M values of PRXII and DHAR obtained when the respective oxidants (peroxide and DHA) were varied. Based on available data, the parameters for DHAR are about 20–1000-fold higher (compare **Tables 2**, **4**). For equal amounts of protein, this indicates that DHAR should be more efficient, although the actual *in vivo* rates will be influenced by several factors, most obviously the relative abundance of the proteins and the *in vivo* concentrations of the respective oxidants in the compartments where the proteins are located.

Glutaredoxins can catalyze reduction of DHA *in vitro* (Wells et al., 1990). Arabidopsis GRXC1 and C2, which are competent in regeneration of PRXIIB, can also catalyze DHA reduction (Riondet et al., 2012). K_M values of the GRX for DHA were similar to DHAR but the k_{cat} values were only about 3 s^{-1} (Riondet et al., 2012), several orders of magnitude lower than the DHARs (**Table 2**).

GENE EXPRESSION DURING OXIDATIVE STRESS

There have been many transcriptomic analyses of plants undergoing oxidative stress, conditions in which oxidation of GSH is expected to be accelerated. However, the number of such studies that have included data on glutathione status is more limited. These notably include Arabidopsis mutants deficient in the major leaf form of catalase (CAT2), in which conditionally increased H_2O_2 availability through photorespiration drives reproducible changes in GSSG:GSH (Mhamdi et al., 2010a). These increases in leaf GSSG are also observed, to a lesser extent, in knockout mutants for GR1, and are particularly marked in *cat2 gr1* double mutants lacking both enzymes (**Figure 3**). However, the processes that are responsible for GSSG accumulation when CAT2 function is lost remain unclear.

As a first step to investigating this question, we mined two Arabidopsis *cat2* microarray datasets for genes encoding the enzymes listed in Supplemental Table 1. The data on *cat2* were compared with responses to external H_2O_2, ozone, and paraquat (**Figure 4C**) and to results obtained for the *flu* mutant, which generates excess singlet oxygen in the chloroplast (**Figure 4D**). Experimental details for all these microarray studies are given in Supplemental Table 2. The two datasets in **Figures 4A,B** come from different experiments performed on leaf rosette material from plants of different age (Mhamdi et al., 2010b; Queval et al., 2012). They were also obtained using two different microarray chips, and some probe sets were present on only one of the chips (**Figure 4**: absence of corresponding probe sets is indicated by white rows). Moreover, key factors determining the measured response to oxidative stress are likely to be exposure time and stress intensity. As well as other differences in plant growth conditions, the data shown in the different parts of **Figure 4** were obtained after different exposure times, ranging from 1 h (H_2O_2; **Figure 4C**) to 4 days (**Figures 4A,B**, right column).

For all the above reasons, some divergence is expected between responses observed in the different datasets. Despite this, a number of genes within each family responded quite similarly to

Table 4 | Kinetic chacteristics of glutaredoxin-dependent peroxiredoxins from poplar and pea.

Enzyme		Substrate	Peroxide			GSH	GrxC4
			K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_M (mM)	K_M (μM)
Poplar PRXIIF[a]	Mit	H_2O_2	0.07	0.38	5.3		
		t-BOOH	0.02	0.51	31.5	0.260	1.3
		CuOOH	0.33	0.39	1.2		
Pea PRXIIF[b]	Mit	H_2O_2	0.02	10.6	560	ND	ND
Poplar PRXIIE[c]	Chp	H_2O_2	0.02	0.57	26		
		t-BOOH	0.01	0.90	104	ND	0.51
Pea 2-Cys PRX[d]	Chp	H_2O_2	0.03	0.69	25	NA	NA
Spinach APX[e]	Chp	H_2O_2	0.03	290	9667	NA	NA

For comparison, data are shown for 2-cys peroxiredoxin from pea (Pisum sativum) and ascorbate peroxidase (APX) from spinach (Spinacia oleracea). t-BOOH, tert-butyl hydroperoxide. CuOOH, cumene hydroperoxide. ND, not determined. NA, not applicable.

References: [a] Gama et al., 2007; [b] Barranco-Medina et al., 2007; [c] Gama et al., 2008; [d] Bernier-Villamor et al., 2004; [e] Nakano and Asada, 1987.

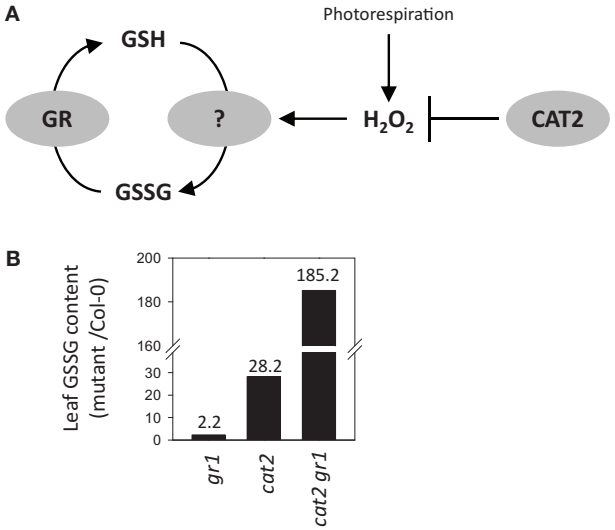

FIGURE 3 | Accumulation of GSSG relative to Col-0 in Arabidopsis knockout mutants for glutathione reductase 1 (*gr1*), catalase 2 (*cat2*), or both (*cat2 gr1*). (A)Simple scheme showing the interplay between GR and H_2O_2-triggered oxidation in determining the balance between GSH and GSSG in the mutant systems. **(B)** Leaf GSSG contents in the three mutant lines relative to Col-0 controls. Fold-change compared to Col-0 GSSG contents are indicated above the bars.

the different oxidative stresses. Among the three DHARs, *DHAR2* was the most obviously responsive, being clearly induced in *cat2*, *flu*, and by external H_2O_2 or paraquat. Induction was less evident following ozone exposure (**Figure 4**). PRXIIC was the only GRX-linked PRX that was induced, though only in response to ozone. None of the *PRXII* genes were induced alongside GSSG accumulation in *cat2* (**Figures 4A,B**). In contrast, marked up-regulation of several *GST*s was observed in most of the datasets. GSTs that responded to most of the treatments included F6 and F7, as well as many tau types, with U1, U4, U5, U6, U7, U8, U24, and U25 showing a particularly clear induction. Indeed, U24 and U25 are among the most strongly *cat2*-induced genes on a fold-change basis (Queval et al., 2012). Interestingly, U11, U12, and U13 were induced by all treatments except in *cat2* and *cat2 gr1* (**Figure 4**, compare A and B with C and D). This could indicate that these GSTs are involved in early responses to oxidative stress. In contrast to several phi and tau *GST* genes, none of the theta types were induced in any condition, despite their documented high peroxidase activity (**Table 3**). Little or no induction of *GSNOR* was apparent, while among the *GPX* sequences, *GPX5*, *GPX6*, and *GPX7* were most obviously responsive, although some variation was observed between the experiments, including the two *cat2* datasets.

This comparison points to numerous candidates that could play a role in oxidizing glutathione. For several reasons, however, the expression data remain at best indicative. First, increased transcripts may not feed through to an increase in protein abundance. Second, transcriptomic analyses do not identify other possible regulatory mechanisms that may operate during oxidative stress at the post-transcriptional or post-translational levels. Third, the data of **Figure 4** show fold-changes compared to wild-type: even if strongly induced at the protein level, low abundance enzymes may not make a marked contribution to GSH oxidation within the cellular context. Inversely, it is not possible to discount a role for an enzyme on the basis of lack of induction. Finally, even if transcript up-regulation feeds through to enhanced protein, some of the enzyme activities encoded by responsive genes shown in **Figure 4** may not involve GSH oxidation. At least some of the GSTs are probably induced in connection with a conjugase function. For example, GSTF6 has been implicated in the synthesis of camalexin (Su et al., 2011), a phytoalexin that can accumulate strongly in *cat2* in oxidative stress conditions (Chaouch et al., 2010). As noted above, GPXs may mainly if not exclusively catalyze peroxidation using TRX, not GSH.

With the above caveats in mind, useful pointers can be obtained by combining information from biochemical competence and efficiency (**Tables 2–4**) with responsiveness at the transcript level (**Figure 4**). Some examples of possible candidates identified according to these criteria would include *DHAR2*, *GSTU8*, *GSTU24*, and *GSTU25*. Interestingly, the proteins encoded by these genes are cytosolic (**Figure 2**), even though the initial location of oxidant generation in several of the studies shown in **Figure 4** is expected to be mainly peroxisomal (*cat2*) or chloroplastic (paraquat, *flu*). The potential importance of cytosolic pathways in determining glutathione status in *cat2* has been previously noted (Mhamdi et al., 2010b).

GENETIC STUDIES

While the data discussed above provide useful information on biochemical properties and expression patterns, establishing the physiological importance of specific components will require other approaches such as reverse genetics. Gene-specific loss-of-function studies will be required to establish the contributions of particular enzymes to GSSG production. As yet, there is relatively little information on the response of glutathione pools in plants specifically deficient in the genes discussed above. A few studies in Arabidopsis have generated potentially relevant data. A preliminary analysis of a *dhar2* mutant reported that although DHA was somewhat increased compared to Col-0, GSSG levels were similar in the two lines (Yoshida et al., 2006). Loss of *PRXIIF* function had slight effects on root glutathione pools in control conditions, but little difference from wild-type was observed during stresses involving exposure to cadmium or to an inhibitor of the mitochondrial alternative oxidase (Finkemeier et al., 2005). Knockout Arabidopsis *gstu17* mutants that showed altered stress responses were reported to have increased root and shoot glutathione contents in unstressed conditions, although no data for glutathione redox state or glutathione contents during stress were presented (Chen et al., 2012).

While loss-of-function studies are a more incisive approach to establishing the roles of specific genes, overexpression studies can also provide useful indications of the potential importance of a given enzyme. Several studies have overexpressed genes encoding GSTs, GPX, or DHAR, often with the objective of improving plant stress tolerance. A comprehensive discussion of these studies is beyond the scope of the present review. Hence, we limit ourselves here to reports that have included effects on glutathione status in the overexpressing lines.

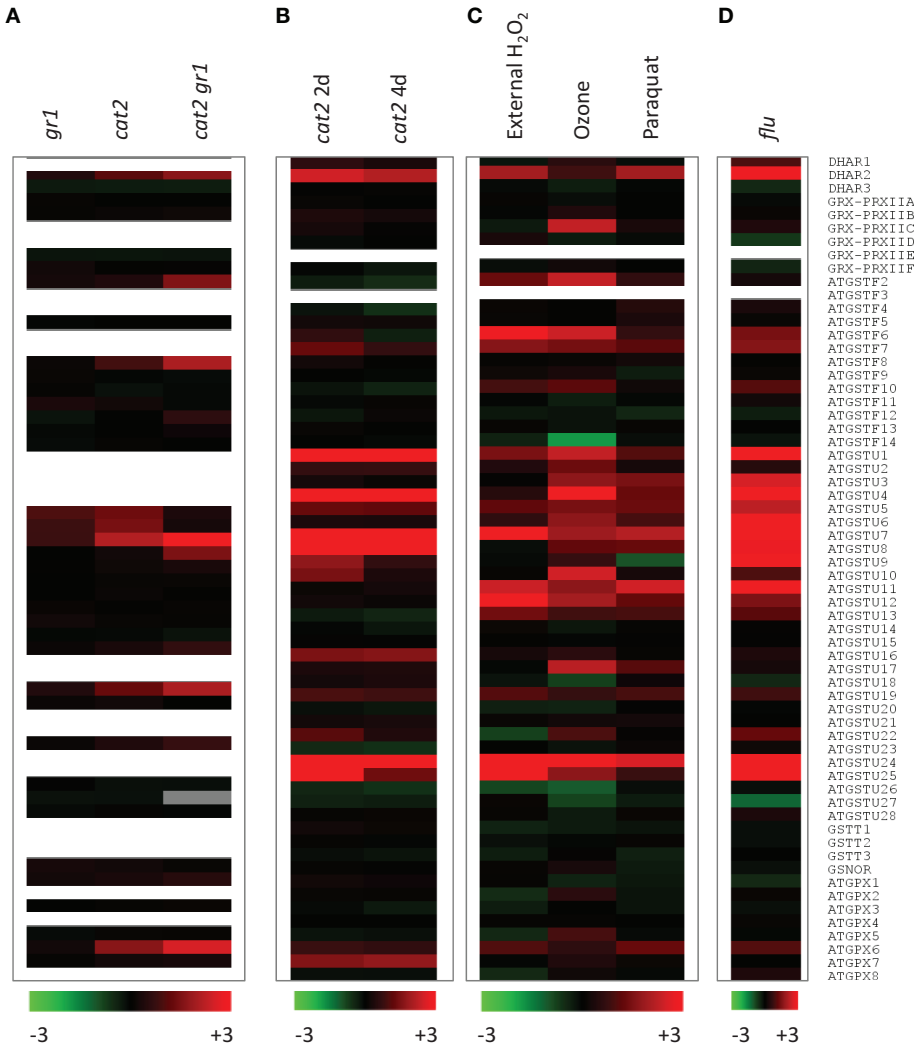

FIGURE 4 | Expression of candidate genes involved in glutathione oxidation in GSSG-accumulating and/or oxidative stress mutants and in conditions promoting oxidative stress. Transcript abundance are shown as $\log_2$ values compared to control (wild-type or untreated). Experimental details are given in Supplemental Table 1. Red and green indicate induction and repression according to the scales shown at the bottom. White rows indicate absence of a corresponding probe set from the array chip. "GRX-PRXII" denotes the signals for the indicated PRX. **(A)** GSSG-accumulating lines shown in **Figure 2** (*gr1*, *cat2*, *cat2 gr1*). Oxidative stress was induced in 3 week old plants (Mhamdi et al., 2010b). **(B)** Two independent datasets for *cat2* at two timepoints after onset of oxidative stress (2 and 4 days). Oxidative stress was induced in 5 week old plants (Queval et al., 2012). **(C)** Genevestigator data for chemically and environmentally induced oxidative stress (H2O2, paraquat and ozone). **(D)** Genevestigator data for the singlet oxygen-accumulating *flu* mutant (Laloi et al., 2007).

Tobacco knockdown and overexpressor DHAR lines showed a substantial decrease and increase in extractable foliar activity, respectively (Chen and Gallie, 2005). While the knockdown lines had decreased ascorbate and increased DHA, the opposite effect was observed in the overexpressors (Chen and Gallie, 2005). The GSH:GSSG ratio was lower in the knockdowns and higher in the overexpressors, with overexpression increasing GSH:GSSG from about 4 in the control plants to almost 20, an effect largely due to increased GSH rather than decreased GSSG (Chen and Gallie, 2005). Another study in tobacco also reported that overexpression of Arabidopsis *DHAR2* increased the reduction state of the ascorbate pool, an effect that was observed both in control conditions and in response to aluminum stress (Yin et al., 2010). However, no difference in glutathione redox state between the control and overexpressors was observed in either condition. The authors concluded that GSH was not limiting for DHAR activity (Yin et al., 2010). Arabidopsis lines homologously overexpressing DHAR have also been described (Wang et al., 2010). Alongside improved stress tolerance, these plants had increased total pools of ascorbate and glutathione, both in control and stress conditions (high temperature, paraquat; Wang et al., 2010).

The picture that emerges from these studies is complex. If the reaction is considered in isolation, a negative relationship between the GSH:GSSG ratio and DHAR activity would be

predicted, because the enzyme consumes GSH and produces GSSG. So far, there is little evidence that this relationship is observed in plants with genetically altered DHAR capacity. The reported increases in total glutathione and GSH:GSSG in tobacco and Arabidopsis overexpressor lines could be partly related to the need for enhanced GSH to support increased DHAR activity (Chen and Gallie, 2005; Wang et al., 2010). Thus, the effects of genetic manipulation of DHAR may not be limited to direct effects on GSH:GSSG ratios, e.g., because the plant may also respond by increasing glutathione synthesis. Another complication could be that altering the capacity of a single antioxidative enzyme may produce indirect effects on ROS availability that then alter the flux through other pathways that oxidize GSH, thus masking more direct effects.

Glutathione status was also assessed in tobacco lines overexpressing a GST with GSH peroxidase activity (Roxas et al., 2000). These lines showed enhanced tolerance to salt. Both in control and salt-stressed conditions, GST overexpression caused a more than three-fold increase in GSSG relative to control plants. This study therefore revealed that enhanced GST expression, which is a feature of oxidative stress responses (**Figure 4**), is able to decrease the reduction state of glutathione *in vivo*. It also reported the operation of secondary effects within the antioxidant system. Increased GSSG was associated with enhanced activities of ascorbate-glutathione pathway enzymes such as APX and MDHAR (Roxas et al., 2000).

MODIFICATIONS OF GLUTATHIONE STATUS ASSOCIATED WITH GSSG ACCUMULATION

Why does GSSG accumulate during oxidative stress? Given the existence of opposing GSH oxidation and GSSG reduction activities, such accumulation is unlikely to be a simple result of conversion of GSH to GSSG. Rather, it is probably more accurately viewed as the net outcome of oxidation outpacing reduction, even if only slightly. As a simple hypothetical example: if enhanced H_2O_2 drives oxidation of 2 GSH to GSSG at 20 nmol.g^{-1}FW min^{-1} (which even if one considers only DHAR as a source of GSSG is no more than 1% of typical capacities measured *in vitro*) but the *in vivo* GR activity is 1% slower (19.8 nmol.g^{-1}FW min^{-1}; about 2% of typical *in vitro* capacities), the two rates would entail a net accumulation of about 0.3 µmol.GSSG g^{-1} FW in 24 h. GSSG accumulation of this magnitude can be observed in catalase-deficient plants following transfer to oxidative stress conditions. The above calculation is obviously simplistic as the rates of the two reactions will vary as a function of changes in substrate concentrations following the onset of stress, and kinetic modeling would be required to examine the question more closely. It is intended merely to illustrate that slightly lower activity of GSSG reduction compared to GSH oxidation may be one factor driving a drop in the GSH: GSSG ratio *in vivo*.

In many cases, stress-induced GSSG accumulation in plants does not occur at the expense of decreased GSH. Rather, the GSH pool remains rather constant while even marked increases in total glutathione are almost entirely due to the accumulation of GSSG (Smith et al., 1985; Willekens et al., 1997; Mhamdi et al., 2010a). Available data suggest that this involves at least two processes additional to redox cycling between GSH and GSSG. The first is increased GSH neosynthesis as a result of processes that probably involve activation of cysteine and glutathione production at transcriptional and post-translational levels (Bick et al., 2001; Hicks et al., 2007; Gromes et al., 2008; Queval et al., 2009). The second is a marked change in glutathione compartmentation, with GSSG accumulation occurring particularly in the vacuole (Queval et al., 2011). This second process means that the GSH:GSSG ratio measured in tissue or whole cell extracts does not report on the actual glutathione status in specific compartments but is rather a composite value of GSH:GSSG ratios, which may differ widely between different subcellular locations (Noctor et al., 2013). For example, the cytosolic glutathione pool may well be less oxidized than that measured in extracts because a substantial amount of the GSSG generated by oxidative stress is shipped to the vacuole, possibly by ABCC transporters (Martinoia et al., 1993; Lu et al., 1998). This does not necessarily invalidate the GSSG:GSH ratio as a useful oxidative stress marker, because accumulation at sites such as the vacuole is predicted to be dependent on GSSG accumulation in other compartments caused by the oxidant-driven imbalance discussed above.

Figure 5 presents an overview that attempts to integrate some of the different processes that are likely to be involved in oxidative stress-driven changes in glutathione status. As well as GR activity, oxidation by the three main pathways we have discussed in this review is situated within the context of glutathione synthesis and the vacuolar sequestration of a substantial part of the GSSG that is generated. It is noteworthy that the vacuole is not the only compartment in which GSSG is highly enriched during oxidative stress. Marked accumulation of GSSG also seems to occur in

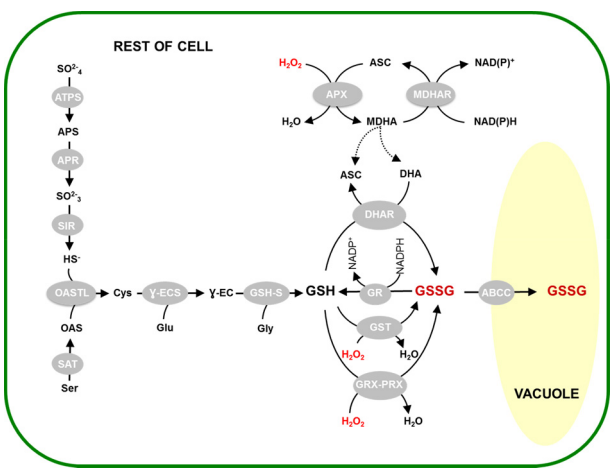

FIGURE 5 | Activation of glutathione synthesis and vacuolar sequestration linked to GSSG accumulation. H_2O_2-triggered oxidation of GSH is considered to occur through the activities of DHAR (top), GST (center) or GRX-PRX (bottom). This is accompanied by activation of glutathione synthesis and GSSG sequestration in the vacuole, through the reactions shown on the left and right, respectively. Abbreviations as in **Figure 1** legend, or as follows: ATPS, ATP sulphurylase; APS, adenosine phosphosulfate; APR, adenosine phosphosulphate reductase; SIR, sulphite reductase; OAS, *O*-acetylserine; OASTL, O-acetylserine(thiol)lyase; SAT, serine acetyltransferase; γ-ECS, γ-glutamylcysteine synthetase; GSH-S, glutathione synthetase; ABCC, sub-class C of the ATP-binding cassette transporters.

the chloroplast. This has been reported in two independent studies of catalase-deficient plants using different techniques, despite the fact that the initial increase in H_2O_2 availability in these systems is expected to be extra-chloroplastic (Smith et al., 1985; Queval et al., 2011). While GSSG import from the cytosol cannot be completely discounted, the most influential process may be oxidation of GSH within the chloroplast (Noctor et al., 2013). Whatever the mechanisms, oxidation of GSH and/or accumulation of GSSG in the chloroplast may have consequences for thiol-dependent reactions in this compartment. These include regulation of chloroplast proteins by *S*-glutathionylation reactions or the TRX system (Dixon et al., 2005; Michelet et al., 2005; Zaffagnini et al., 2012) as well as activation of synthesis pathways that contribute to the accumulation of total glutathione in these conditions (**Figure 5**).

CONCLUSIONS AND PERSPECTIVES

The post-genomics era has witnessed a dramatic increase in our understanding of plant antioxidative systems. Work over the last decade has also underscored their complexity. The present discussion has attempted to emphasize that a multiplicity of reactions may contribute to GSH oxidation during oxidative stress, leading to modifications in the status of this potentially important cellular redox signal. Whatever the reactions involved, the resulting changes in GSH:GSSG triggered by oxidants such as H_2O_2 may be signaled to sensitive proteins by catalysts such as certain GRX. Alternatively, the oxidation of certain thiol-dependent peroxidases, including some we have mentioned here, may itself act as a signal or signal relay independent of "bulk-phase" changes in GSH:GSSG.

While DHAR function in the ascorbate-glutathione pathway remains an outstanding candidate as a GSSG-generating enzyme, data from biochemical, transcriptomic and reverse genetics studies all suggest that other enzymes may contribute. The potential for redundancy between different enzyme classes is evident. It is also possible that considerable genetic redundancy exists within enzyme classes, both in peroxide removal and in GSH oxidation. This is most obviously apparent for the large GST family. Moreover, identification of the enzymes specifically involved in GSH oxidation using targeted loss-of-function studies may be complicated by the existence of different pathways that are able to replace or compensate for one another.

Despite these complexities, establishing the importance of the pathways able to generate GSSG from GSH *in planta* should be favored by the wide range of gene-specific mutants now available in Arabidopsis and other species. Kinetic modeling could also be useful to evaluate interactions between different reactions, and to define the limits of a system in which numerous components may act in parallel. A key issue could be (sub)cellular compartmentation, both of the reactions that cause GSH oxidation and of the GSSG that accumulates as a result. GSH-oxidizing processes may be condition-specific, with some reactions being more important in certain stresses than in others. Other issues that will need to be taken into account are the stage of plant development as well as modulating environmental conditions such as photoperiod, factors that may impose different patterns of gene expression in response to oxidative stress.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00477/abstract

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A phenomics approach to the analysis of the influence of glutathione on leaf area and abiotic stress tolerance in *Arabidopsis thaliana*

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Reduced glutathione (GSH) is an abundant low molecular weight plant thiol. It fulfills multiple functions in plant biology, many of which remain poorly characterized. A phenomics approach was therefore used to investigate the effects of glutathione homeostasis on growth and stress tolerance in *Arabidopsis thaliana*. Rosette leaf area was compared in mutants that are either defective in GSH synthesis (*cad2*, *pad2*, and *rax1*) or the export of γ-glutamylcysteine and GSH from the chloroplast (*clt*) and in wild-type plants under standard growth conditions and following exposure to a range of abiotic stress treatments, including oxidative stress, water stress, and high salt. In the absence of stress, the GSH synthesis mutants had a significantly lower leaf area than the wild type. Conversely, the *clt* mutant has a greater leaf area and a significantly reduced lateral root density than the wild type. These findings demonstrate that cellular glutathione homeostasis exerts an influence on root architecture and on rosette area. An impaired capacity to synthesize GSH or a specific depletion of the cytosolic GSH pool did not adversely affect leaf area in plants exposed to short-term abiotic stress. However, the negative effects of long-term exposure to oxidative stress and high salt on leaf area were less marked in the GSH synthesis mutants than the wild type. These findings demonstrate the importance of cellular glutathione homeostasis in the regulation of plant growth under optimal and stress conditions.

Keywords: abiotic stress tolerance, glutathione synthesis, root architecture, lateral root density, leaf area

INTRODUCTION

Environmental stresses severely limit plant growth and decrease the predictability of crop yields for the farmer. Abiotic stress often has a greater impact on crop productivity than genotypic effects. Enhancing stress tolerance is therefore a major second-generation trait target for crop improvement programs. Plant stress responses are complex traits regulated by large numbers of genes and quantitative trait loci (QTL). This complexity has restricted the success of conventional breeding approaches. Similarly, transgenic approaches to enhancing tolerance to complex stresses such as drought have not as yet significantly reduced environmentally related yield losses under field conditions (Lawlor, 2013). A greater understanding of the mechanisms that restrict the growth of plants in response to the imposition of abiotic stress is required to facilitate development and molecular breeding of crop varieties with enhanced stress tolerance traits.

Enhanced cellular oxidation is a common feature of the plant response to stress. Oxidative signaling underpins plant responses to stress and is intimately associated with hormone signaling pathways that regulate plant growth, senescence, and cell death responses. The thiol tripeptide, glutathione (GSH; γ-glutamyl-L-cysteinylglycine) is an important component of the plant antioxidant system that protects against the harmful effects of uncontrolled oxidation (Noctor and Foyer, 1998; Noctor et al., 2013). Moreover, GSH acts downstream of hydrogen peroxide in mediating the stress responses of phytohormones such as jasmonate and salicylate (Mhamdi et al., 2010). Many studies have implicated GSH in biotic and abiotic stress tolerance (Noctor and Foyer, 1998; Ogawa, 2005).

The pathway of GSH synthesis involves two ATP-dependent steps catalyzed by g-glutamate-cysteine ligase (GCL; also called γ-glutamylcysteine (γ-EC) synthetase), which is considered to be the rate-limiting enzyme of GSH production, and GSH synthetase (GSHS; also called GSH synthase). In *A. thaliana*, the GCL protein is found only in chloroplasts and other plastids, whereas the GSH-S is found in both the chloroplasts and cytosol (Wachter et al., 2005).

Plant homologs of the malaria chloroquine-resistance transporter *Pf*CRT (CLTs) are thiol transporters required for transport of γ-EC and GSH across the plastid envelope membranes and interconnect the plastidic and cytosolic thiol pools (Maughan et al., 2010). *Arabidopsis* mutants lacking these transporters (*clt1clt2clt3*) show enhanced sensitivity to cadmium and to the fungal pathogen, *Phytophthora brassicae*, as well as a failure to activate appropriate pathogen defense responses despite having wild-type levels of GSH in the leaves (Maughan et al., 2010). The

clt1clt2clt3 mutants have an altered partitioning of GSH between plastid and cytosol, with a marked decrease in the cytosolic GSH levels but not in the chloroplast GSH pool in the leaves (Maughan et al., 2010). The cytosolic GSH pool is important in the mediation of systemic acquired resistance responses linked to salicylic acid signaling, as demonstrated by defects in pathogen-resistance and the expression of pathogenesis-resistance protein (PR)1 in the *clt1clt2clt3* triple mutants (Maughan et al., 2010) and in mutants that are defective in the cytosolic/peroxisomal form of NADPH-dependent glutathione reductase (*gr1*). These mutants accumulate less salicylic acid with lower *PR1* transcripts under oxidative stress conditions (Mhamdi et al., 2010). Mutants lacking the chloroplast/mitochondrial *GR2* are embryo-lethal (Tzafrir et al., 2004) but the *gr1* knockout mutants do not show a marked phenotype (Marty et al., 2009; Mhamdi et al., 2010). However, crossing the *gr1* knockout mutants with a mutant that is defective in the photorespiratory form of catalase *cat2,* led to a large accumulation in GSSG relative to the parent lines (Mhamdi et al., 2010). The *cat2 gr1* double mutants that are deficient in both the major leaf catalase isoform and GR1 have altered responses to pathogens and expression of genes involved in jasmonate and salicylate signaling pathways (Mhamdi et al., 2010).

Glutathione synthesis and accumulation are increased in response to oxidative stress (Queval et al., 2009) because of direct effects of oxidation on the GCL protein, which is most active in its homodimeric form requiring linkage through two disulfide bonds (Hothorn et al., 2006). Reducing conditions disrupt one of the two disulfide bonds (Cys178–Cys398) altering the dimer interface and shifting the protein to the less active monomeric form (Jez et al., 2004; Hothorn et al., 2006; Galant et al., 2011). Oxidation-dependent decreases in cellular GSH/GSSG ratios also favor increased synthesis of cysteine, which is also considered to be a limiting factor for GSH synthesis (Noctor et al., 2012).

Arabidopsis mutants, with defects in the pathway of GSH synthesis, have been particularly useful in the characterization of GSH functions in plants. A number of mutants with defects in the *GSH1* gene that encodes GCL have been identified and these were often first characterized in terms of effects on stress tolerance. For example, the *cad2-1* mutant that has 15–30% of wild-type GSH was identified by its enhanced sensitivity to cadmium (Cobbett et al., 1998), the *rax1-1* mutant, where the leaf GSH pool is decreased by between 50 and 80% relative to the wild type, was identified by the altered expression of the gene encoding the cytosolic ascorbate peroxidase (Ball et al., 2004) and the *pad2-1* mutant, where leaf GSH is decreased by 80% compared to the wild type, shows enhanced sensitivity to fungal pathogens such as *P. brassicae* and *Pseudomonas syringae* because of decreased camalexin content (Parisy et al., 2007). Mutations in the *GSH2* gene that encodes GSHS have also been very useful in elucidating the functions of glutathione in plants (Pasternak et al., 2008; Au et al., 2012). The morphology of the endoplasmic reticulum is altered and protein export is perturbed when γ-glutamylcysteine accumulates as a result of limitations in GSHS activity (Au et al., 2012). This may explain why the levels of γ-glutamylcysteine are very low in plant cells (Noctor et al., 1998).

In addition, to its antioxidant and signaling functions, GSH is also required for plant growth and development. GSH deficiency leads to an arrest in cell proliferation and root meristem formation (Vernoux et al., 2000; Diaz-Vivancos et al., 2010). GSH also has roles in flower development and vernalization responses (Bashandy et al., 2010; Hatano-Iwasaki and Ogawa, 2012). Despite this, the role of GSH in the control of plant growth under abiotic stress conditions is largely unknown.

Phenomics technologies allow accurate measurements of leaf area in large numbers of plants grown in either the absence or presence of abiotic stress over periods of days to weeks. This approach was therefore used to explore the effects of GSH deficiency on rosette leaf area in different GSH synthesis mutants (*cad2-1*, *rax1-1*, and *pad2-1*) and in the *clt1clt2clt3* triple mutants, which have altered intracellular partitioning of GSH between the chloroplasts and cytosol, under either standard (optimal) growth conditions and under abiotic stress conditions. The findings show that in contrast to the GSH synthesis mutants, which have a lower leaf area in the absence of stress, leaf area was increased in the *clt1clt2clt3* triple mutants relative to wild-type controls. The abiotic stress-induced decreases in leaf area were similar in all genotypes in short-term experiments. However, in the longer term stress treatments, the negative impacts of some abiotic stresses on leaf area were less marked in the GSH synthesis mutants than in the wild-type plants or in the *clt1clt2clt3* mutants.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds for wild-type *A. thaliana* accession Columbia 0 (Col-0), *cad2-1* (Cobbett et al., 1998), *pad2-1* (Parisy et al., 2007), *rax1-1* (Ball et al., 2004), *rml1-1* (Cheng et al., 1995; Vernoux et al., 2000), and *clt1clt2clt3* triple mutants (Maughan et al., 2010), were sown on plates containing half strength Murashige and Skoog medium plus 1.2% glucose. Plants were grown in controlled environmental cabinets under an irradiance of 100 μmol m^{-2} s^{-1} with a photoperiod of 16 h, a constant temperature of 22 ± 2°C, and a relative humidity of 60%, for up to 17 days. For high light treatments, seedlings were grown for 10 days as above and then transferred to 400 μmol m^{-2} s^{-2} irradiance conditions for a further 4 days. The seedlings were then transferred back to 100 μmol m^{-2} s^{-1} for a further 3 days. Each experiment consisted of four plates (32 seeds per plate) per genotype and per stress treatment. Each experiment was repeated at least three times.

SHORT STRESS TREATMENTS

For these experiments, seeds were sown on a sterile 1 μm filter mesh, which was placed on the media prior to sowing. Seedlings were grown for 10 days under the 100 μmol m^{-2} s^{-1} irradiance conditions and then transferred with the mesh to plates containing media alone (control) or growth media plus either hydrogen peroxide (4 mM), sodium chloride (75 mM), *N,N'*-dimethyl-4,4'-bipyridinium dichloride (paraquat; 1 μM), or sorbitol (100 mM). Seedlings were then grown for a further 7 days under these conditions and growth analysis performed as described below.

LONG STRESS TREATMENTS

For these experiments, seeds were sown on plates in media alone (control), or media containing paraquat (0.1 μM), sodium

chloride (75 mM), or sorbitol (100 mM). Seedlings were grown for 14 days under these conditions. Lower concentrations of paraquat were used in the long-term stress treatments than in the short-term stress treatments because preliminary experiments had shown that the higher concentrations of paraquat were lethal in long-term experiments. In contrast, the lower concentrations of paraquat had little effect on leaf area in the short-term experiments.

LEAF AREA DETERMINATION

Leaf area measurements were performed as described by Schulz et al. (2012). Photographs were taken with a Canon EOS 450 D (Canon Inc., Tokyo, Japan) on successive days after sowing. Data presented here are taken from measurements made 14 and 17 days after sowing only. Total rosette surface area (hereafter called leaf area) was measured and analyzed using Fiji ImageJ[1] as described by Schindelin et al. (2012). Data was processed using Microsoft Excel 2010 and statistical analysis was performed with program R[2] (Hornik, 2013). The ImageJ analysis used here to calculate leaf area measures the total rosette (leaf) surface, which is highly correlated with the fresh weigh of the plant (usually $r = 0.9$–0.95).

ROOT GROWTH AND ROOT ARCHITECTURE DETERMINATION

Primary root length and number of lateral roots were measured on 7-day-old seedlings. Root measurements were determined using Fiji ImageJ software, as above. Lateral root density was calculated from these values as the ratio between the number of visible lateral roots and the primary root length.

METABOLITE ANALYSIS

For these experiments, the wild-type and *clt1clt2clt3* plants were grown in pots containing compost (Levington, Bramford, UK) in controlled environment chambers (16/8 h light/dark regime with photosynthetic photon flux of 250 μmol m^{-2} s^{-1}). The whole rosettes of 5-week-old plants were harvested and assayed for ascorbate, glutathione, and pyridine nucleotides as described by Pellny et al. (2009).

RESULTS

The open-source platform for biological-image analysis used in these studies involves ImageJ analysis measurements of the total rosette (leaf) surface area (from hereon called leaf area), a parameter which is highly correlated to the fresh weigh of the plant (usually $r = 0.9$–0.95). Total leaf surface area was compared in *rax1-1*, cad2-*1*, and *pad2-1* mutants with that of wild-type *A. thaliana* seedlings at 14 days (**Figure 1**). The genotypes that are deficient in GSH synthesis were visibility smaller (**Figure 1A**) and they had a significantly lower leaf area than the wild type (**Figure 1B**).

The effects of short-term (7 days) exposure to different abiotic stress treatments on leaf area was measured (**Figure 2**). Exposure to oxidative stress (4 mM hydrogen peroxide or 1 μM paraquat), high salt (75 mM sodium chloride), or osmotic stress (100 mM sorbitol) led to a visible shoot phenotype (**Figure 2A**) and a decrease in leaf area in all genotypes (**Figures 2B–E**). All treatments except

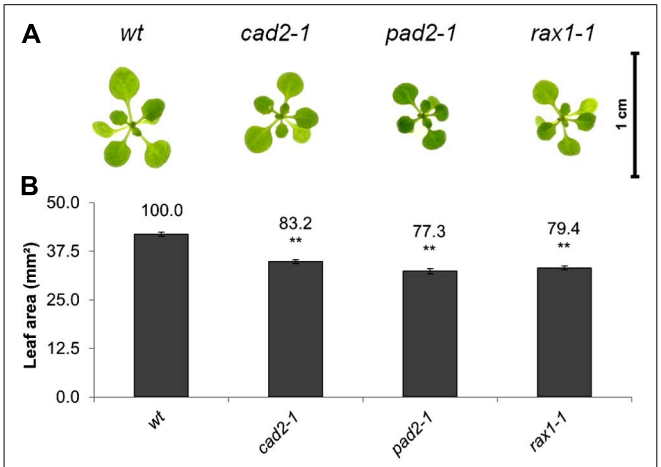

FIGURE 1 | A comparison of rosette leaf area in the *rax1-1*, cad2-*1*, and *pad2-1* mutants relative to wild type, Col-0 (wt). Phenotypes **(A)** and leaf area of seedlings at 14 days **(B)**. The asterisks indicate significant differences ($p < 0.05$; ANOVA).

exposure to hydrogen peroxide led to a significant decrease in leaf area in all genotypes (**Figures 2B–E**). The genotypes that were defective in GSH synthesis were not more sensitive to the treatments in relation to leaf area than the wild type except for the *rax1-1*, which was more sensitive to the paraquat treatment (**Figure 2E**). However, the *pad2-1* mutants were less sensitive to the paraquat treatment relative to the wild type (**Figure 2D**). The *cad2-1* and *pad2-1* mutants were also less sensitive to the high salt treatment than the wild type (**Figure 2C**). Short-term (4 days) exposure to a relatively high light (400 μmol m^{-2} s^{-1}) treatment visibly stimulated increases in leaf area (**Figure 2A**) and led to a significant increase in leaf area in all genotypes (**Figure 2F**). Moreover, under these conditions the *pad2-1* mutants had a similar leaf area to the wild type, whereas the leaf area in the *cad2-1* and *rax1-1* plants was significantly smaller than the wild type (**Figure 2F**).

The effects of long-term (14 days) exposure to the different abiotic stress treatments such as oxidative stress (paraquat), salt stress (sodium chloride), or osmotic stress (sorbitol) on leaf area was measured in all genotypes (**Figure 3**). All treatments led to a visible decrease in the rosettes of all genotypes (**Figure 3A**) and significant decrease in leaf area in all cases (**Figures 3B–E**). In contrast to the osmotic stress treatment, which led to similar decrease in leaf area in all genotypes except the *pad2-1* mutants, the GSH synthesis mutants had a significantly greater leaf area than the wild type in the oxidative stress treatment (paraquat) and in the high salt treatment (**Figures 3B–E**).

The ascorbate and glutathione contents of the *clt1clt2clt3* leaves were similar to that of the wild type (**Table 1**). The rosettes of the *clt1clt2clt3* triple mutants were visibly larger than those of the wild type (**Figure 4A**) and they had significantly greater leaf area (**Figure 4B**). However, root architecture was markedly different in the *clt1clt2clt3* triple mutants relative to the wild type (**Figure 5A**). The primary root length was significantly shorter than that of the wild type (**Figure 5B**) and there were significantly fewer lateral roots (**Figure 5C**). The lateral root density was

[1]http://fiji.sc/

[2]www.r-project.org

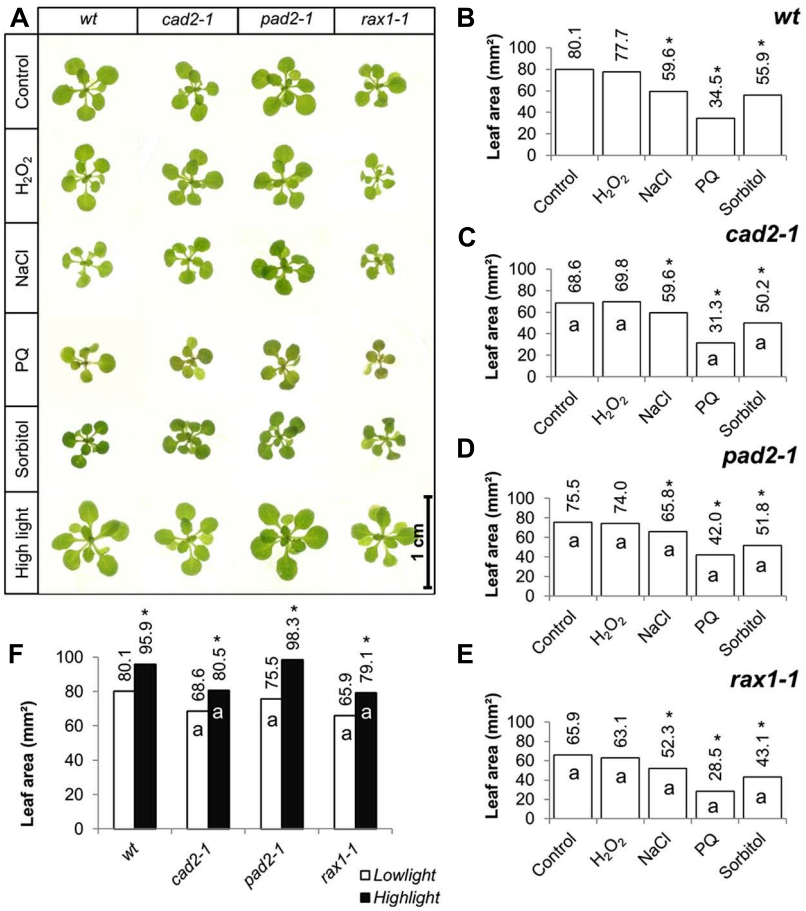

FIGURE 2 | A comparison of the effects of short-term (4 days) exposures to different abiotic stresses treatments on rosette leaf area in the *rax1-1*, cad2-*1*, and *pad2-1* mutants relative to wild type, Col-0 (wt). In all cases seedlings were grown for 10 days under optimal conditions and then transferred to different abiotic stress treatments for a further 4 days. Phenotypes **(A)** and leaf area **(B–F)** of seedlings measured at 14 days either in the absence (control) or presence of the oxidative stress caused by the addition of hydrogen peroxide (H_2O_2) or paraquat (PQ), or high salt (sodium chloride, NaCl), or osmotic stress (sorbitol). For the high light treatment [closed bars, **(F)**] seedlings were either grown under 150 μmol m^{-2} s^{-1} irradiance for 14 days (open columns) or they were grown under 150 μmol m^{-2} s^{-1} irradiance for 10 days and then transferred to 400 μmol m^{-2} s^{-1} irradiance for 4 days prior to measurement. The asterisks indicate significant differences ($p < 0.05$; ANOVA).

markedly decreased in the *clt1clt2clt3* triple mutants relative to the wild type (**Figure 5D**).

The effects of short-term (7 days) exposure to abiotic stress on leaf area were compared in the *clt1clt2clt3* triple mutants and wild-type plants (**Figure 6A**). Exposure to low levels of hydrogen peroxide (4 mM) had no effect on leaf area in either genotype (**Figure 6**). Salt stress caused a significant decrease in leaf area in both genotypes, but the salt-induced decrease in leaf area was greater in the wild type than the *clt1clt2clt3* triple mutants (**Figures 6B,C**). Similarly, exposure to paraquat caused a significant decrease in leaf area in both genotypes, but the oxidative stress-induced decrease in leaf area was greater in the wild type than the *clt1clt2clt3* triple mutants (**Figures 6B,C**). Moreover, exposure to osmotic stress caused a significant decrease in leaf area in both genotypes, but the osmotic stress-induced decrease in leaf area was greater in the wild type than the *clt1clt2clt3* triple mutants (**Figures 6B,C**). Short-term (4 days) exposure to a high light resulted in visibly larger rosettes (**Figure 6A**) and led to a significant increase in leaf area in all genotypes (**Figure 6D**).

The effects of long-term (14 days) exposure to the different abiotic stress treatments were compared in the *clt1clt2clt3* triple mutants and wild-type plants (**Figure 7A**). All treatments led to a visible decrease in the size of the rosettes of both genotypes (**Figure 7A**). A significant decrease in leaf area was observed following exposure to oxidative stress, high salt, and osmotic stress in both genotypes (**Figures 7B,C**).

The total leaf ascorbate or glutathione contents were similar in the *clt1clt2clt3* triple mutants and the wild type (**Table 1**). While the NAD^+/NADH ratios were also similar in the leaves of both genotypes, the *clt1clt2clt3* triple mutant leaves had higher $NADP^+$/NADPH ratios than those of the wild type (**Table 1**).

DISCUSSION

Genetic evidence has demonstrated links between glutathione redox state and shoot and root meristem activity (Vernoux et al.,

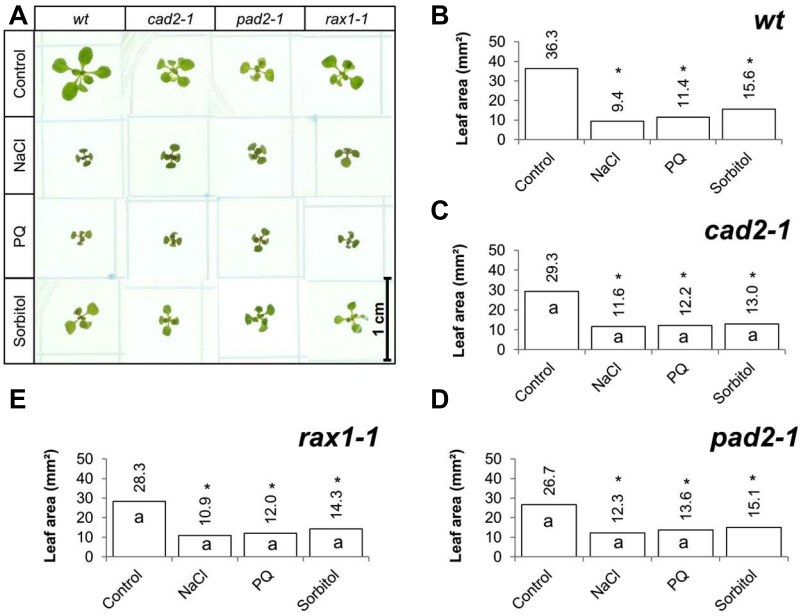

FIGURE 3 | A comparison of the effects of long-term (14 days) exposures to different abiotic stresses treatments on rosette leaf area in the *rax1-1*, cad2-*1*, and *pad2-1* mutants relative to wild type, Col-0 (wt). Phenotypes **(A)** and leaf area **(B–E)** of seedlings measured at 14 days either in the absence (control) or presence of the oxidative stress caused by the addition of paraquat (PQ) or high salt (sodium chloride, NaCl), or osmotic stress (sorbitol). The asterisks indicate significant differences ($p < 0.05$; ANOVA).

Table 1 | A comparison of the major redox metabolites in the rosette leaves of the *clt1clt2clt3* triple mutants and wild-type (Col-0) plants.

Metabolite	Genotype	
	Col-0	*clt1clt2clt3*
Ascorbate (μmol mg^{-1} Chl)	3.48 ± 0.17[a]	4.20 ± 0.18[a]
Dehydroascorbate (μmol mg^{-1} Chl)	1.34 ± 0.14[a]	1.01 ± 0.16[a]
Ascorbate/dehydroascorbate	2.59[a]	3.82[a]
GSH (μmol mg^{-1} Chl)	228.33 ± 30.10[a]	264.67 ± 30.64[a]
GSSG (μmol mg^{-1} Chl)	7.51 ± 0.46[a]	10.73 ± 0.81[a]
GSH/GSSG	31.23[a]	20.10[a]
NADH (μmol mg^{-1} Chl)	2.73 ± 0.48[a]	2.44 ± 0.16[a]
NAD (μmol mg^{-1} Chl)	7.12 ± 1.12[a]	5.61 ± 0.77[a]
NADH/NAD	0.38[a]	0.43[a]
NADPH (μmol mg^{-1} Chl)	25.59 ± 5.42[a]	16.45 ± 2.50[b]
NADP (μmol mg^{-1} Chl)	1.77.88 ± 0.09[a]	4.22 ± 0.40[b]
NADPH/NADP	15.02[a]	3.90[b]

Values represent the mean values ± SE (n = 6). Values within a single row that were significantly different as estimated by Fishers protected LSD test ($p < 0.05$) are indicated by different letters.

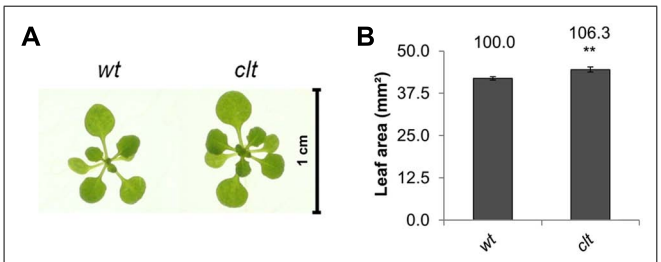

FIGURE 4 | A comparison of rosette leaf area in the *clt1clt2clt3* triple mutants (*clt*) relative to wild type, Col-0 (wt). Phenotypes **(A)** and leaf area of seedlings at 14 days (**B**; $^{**}p < 0.01$).

2000; Reichheld et al., 2007; Bashandy et al., 2010; Koprivova et al., 2010). However, mechanisms by which GSH participate in the control of growth particularly under stress conditions remain to be characterized. The *rml1-1* mutant, which has less than 5% of the wild-type GSH levels is unable to establish a post-embryonic root meristem because of cell cycle arrest at G1 (Vernoux et al., 2000). Similarly, a pharmacological approach has demonstrated that inhibition of GSH synthesis also leads to an arrest of root growth (Koprivova et al., 2010). Under similar growth conditions to those used here the *cad2-1*, *pad2-1*, and *rax1-1* mutants had a lower number of lateral roots leading to a lower lateral root density in all the GSH deficient mutant genotypes compared to the wild type (Marquez-Garcia et al., 2013). While visual inspection failed to establish a clear phenotype under routine growth conditions (Maughan et al., 2010), the results presented here clearly demonstrate that like the GSH synthesis mutants, the *clt1clt2clt3* triple mutants have significantly lower lateral root densities than the wild type. These data indicate that a high cytosolic GSH pool is required in the control of root architecture. Root growth may be regulated in part by the shoot; the decrease in the cytosolic GSH levels in *clt1clt2clt3* leaves leading to GSH depletion in the roots, which rely, at least in part, on GSH transport from the

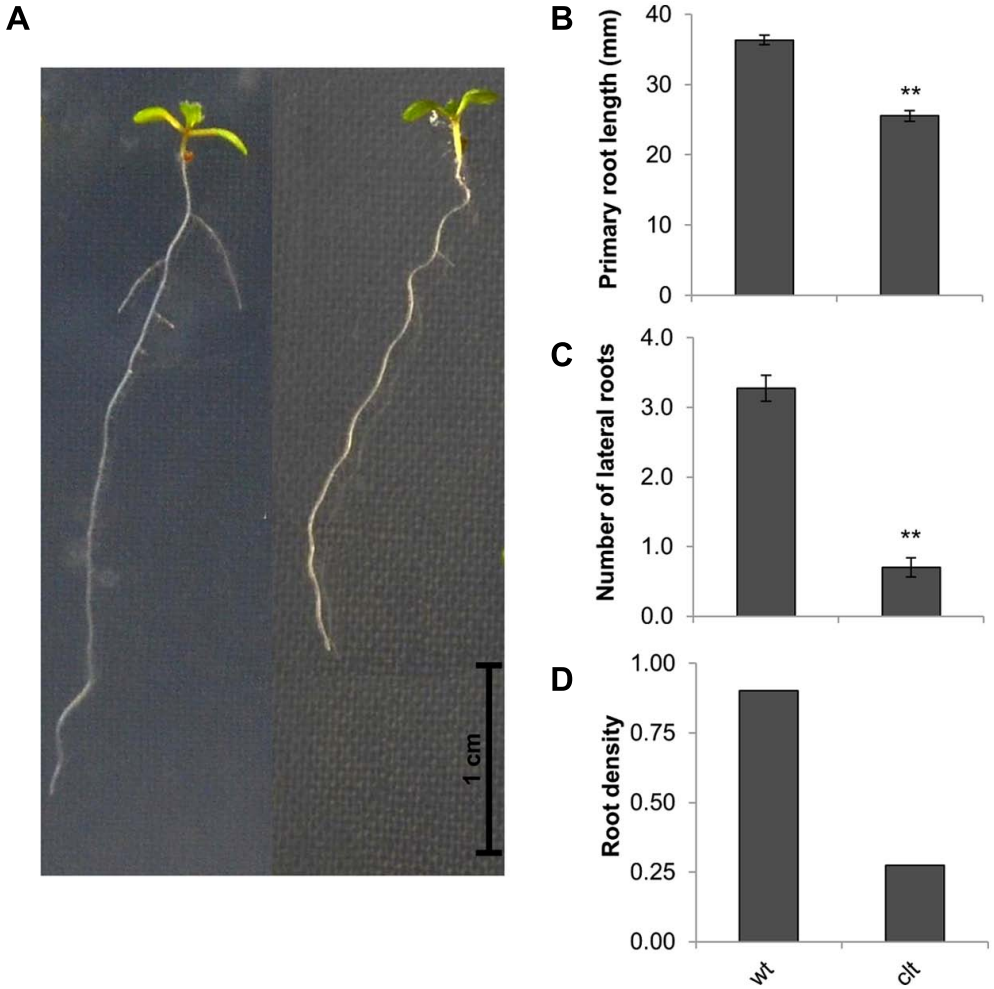

FIGURE 5 | Root architecture the *clt1clt2clt3* triple mutants (*clt*) relative to wild type Col-0 (wt). The phenotype of 14-day-old seedlings **(A)**, primary root length **(B)**, number of visible lateral roots **(C)**, and lateral root density **(D)**. The asterisks indicate significant differences ($p < 0.05$; ANOVA).

leaves (Noctor et al., 1998; Li et al., 2006). Moreover, whereas the *cad2-1*, *rax1-1*, and the *pad2-1* mutants have decreased leaf area relative to the wild type, leaf area shows a small but significant increase in the *clt1clt2clt3* triple mutants compared to the wild type. These data may implicate chloroplast GSH pool in the regulation of leaf area. However, rosette size was significantly enhanced by high light, which led to a significant increase in leaf area in all genotypes. Under these conditions the *pad2-1* mutants performed better than the *cad2-1* and *rax1-1* mutants, achieving leaf areas that were similar to or even slightly higher than the wild type, suggesting that high light can overcome the adverse influence of low GSH in signaling that controls leaf area.

The results presented here show that the leaves of the *clt1clt2clt3* mutants had a similar redox status to the wild-type plants, under optimal growth conditions, with comparable ascorbate and glutathione levels and similar ascorbate/dehydroascorbate, GSH/GSSG, and NAD/NADH ratios, even though the partitioning of GSH between the cytosol and chloroplasts was changed in the *clt1clt2clt3* mutants relative to the wild type (Maughan et al., 2010). The observed decreases in the leaf NADPH/$NADP^+$ ratios in the *clt1clt2clt3* mutants linked to the altered intracellular partitioning of GSH between these compartments, might be explained by in terms of increased demand for NADPH for cytosolic redox processes linked to thioredoxin, as a result of GSH depletion (Marty et al., 2009).

Short-term exposures to oxidative stress (paraquat), salt stress, and osmotic stress resulted in a decrease in leaf area in all genotypes. However, the stress effects were similar in the GSH synthesis mutants and in the *clt1clt2clt3* triple mutants to the wild type, the only exception being the *rax1-1* mutant, which was slightly more sensitive to the paraquat treatment. Longer term abiotic stress treatments caused larger decreases in leaf area in all genotypes. However, in contrast to the *clt1clt2clt3* triple mutants, which showed a similar response to the abiotic stresses to the wild-type plants, the GSH synthesis mutants with the exception of *pad2-1*, had a significantly greater leaf area than the wild type under the oxidative stress and the high salt treatments. These results suggest that impaired GSH synthesis capacity may therefore serve to

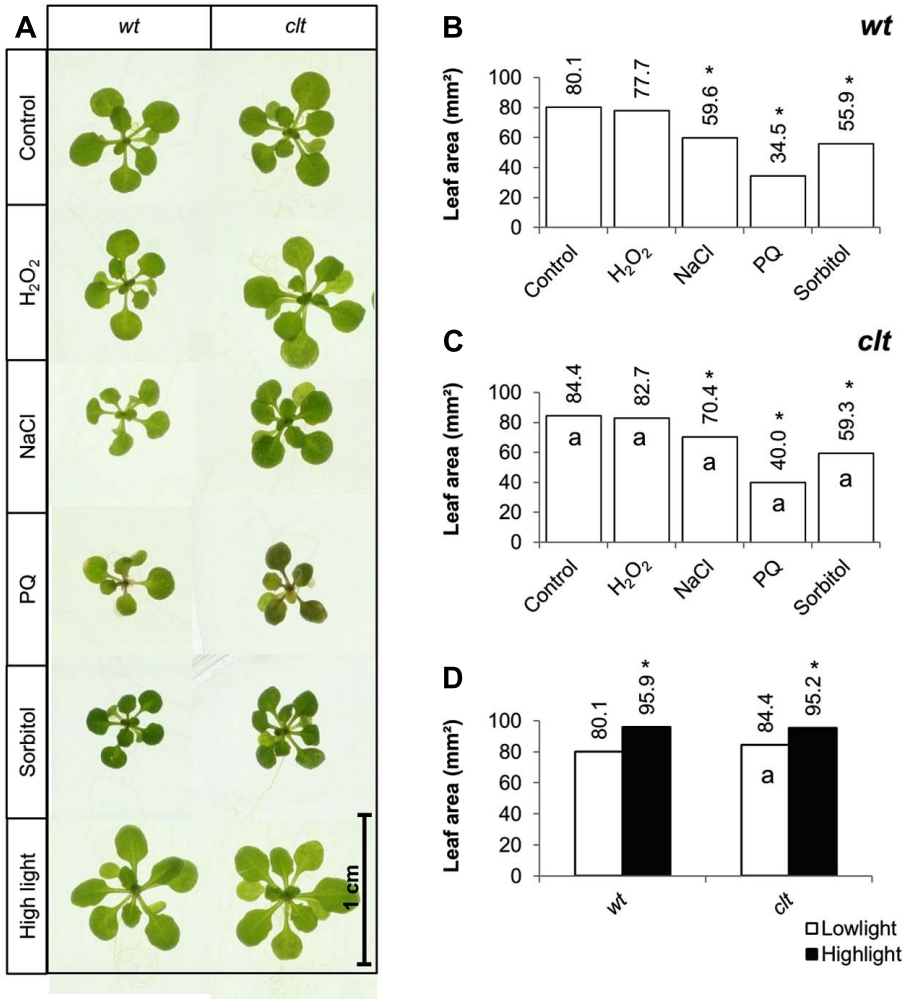

FIGURE 6 | A comparison of the effects of short-term (4 days) exposures to different abiotic stresses treatments on rosette leaf area in the *clt1clt2clt3* triple mutants relative to wild type, Col-0 (wt). In **(A–C)** seedlings were grown for 10 days under optimal conditions and then transferred different abiotic stress treatments for a further 4 days. Phenotypes **(A)** and leaf area **(B,C)** of seedlings measured at 14 days either in the absence (control) or presence of the oxidative stress caused by the addition of hydrogen peroxide (H_2O_2) or paraquat (PQ), or high salt (sodium chloride, NaCl), or osmotic stress (sorbitol). For the high light treatment (closed bars, **(D)**) seedlings were either grown under 150 μmol m^{-2} s^{-1} irradiance for 14 days (open columns) or they were grown under 150 μmol m^{-2} s^{-1} irradiance for 10 days and then transferred to 400 μmol m^{-2} s^{-1} irradiance for 4 days prior to measurement. The asterisks indicate significant differences ($p < 0.05$; ANOVA).

mitigate the adverse effects of some abiotic stresses such as salt stress and dehydration on leaf growth but not others such as heavy metal stress, where GSH is required for other pathways such as phytochelatin biosynthesis.

The observation that GSH deficiency limits the adverse effects of salt stress and dehydration on leaf growth may be related to the central role of glutathione in the regulation of gene expression linked to oxidative stress signaling (Noctor et al., 2013). Accumulating evidence supports the concept that glutathione status is involved in the cross talk between oxidative signaling and hormone signaling (Mhamdi et al., 2010; Han et al., 2013a,b). Crucially, glutathione status also influences the auxin signaling pathways that control growth (Bashandy et al., 2010; Gao et al., 2013). The oxidative signals that limit growth under oxidative stress are therefore likely to be transmitted at least in part via modulation of the redox status of the glutathione pool. The greater leaf area observed here in the GSH synthesis mutants relative to the wild type under oxidative and high salt stresses may therefore be linked to a requirement for GSH in the cross talk between redox and hormone-mediated signaling processes that serve to restrict growth in plants exposed to abiotic stress.

CONCLUSION

Abiotic stress tolerance is an important factor determining plant growth and productivity, and is the subject of ever-intensifying interest in relation to crop improvement. The importance of antioxidants such as glutathione in abiotic stress tolerance is well documented, particularly with regard to its antioxidant functions in protection against stress-induced oxidation. In addition to its potential usefulness as a stress marker, glutathione status is

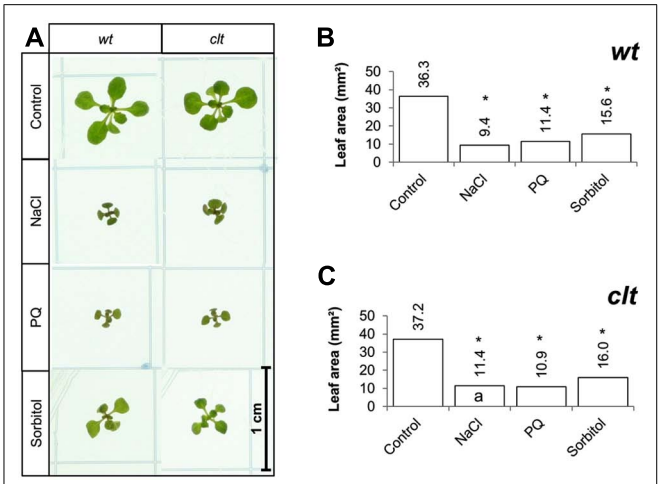

FIGURE 7 | A comparison of the effects of long-term (14 days) exposures to different abiotic stresses treatments on rosette leaf area in the *clt1clt2clt3* triple mutants relative to wild type, Col-0 (wt). Phenotypes **(A)** and leaf area **(B,C)** of seedlings measured at 14 days either in the absence (control) or presence of the oxidative stress caused by the addition of paraquat (PQ) or, or high salt (sodium chloride, NaCl), or osmotic stress (sorbitol). The asterisks indicate significant differences ($p < 0.05$; ANOVA).

important in the control of growth and oxidative stress signaling (Noctor et al., 2013). Within this context, the results presented here demonstrates that the intracellular compartmentalization of glutathione influences plant growth, a depletion in the cytosol in the *clt1clt2clt3* triple mutants leading to significant decreases in lateral root density and increases in rosette leaf area under non-stressed conditions. However, in contrast to biotic stress tolerance, which is impaired in the *clt1clt2clt3* triple mutants (Maughan et al., 2010), depletion of the cytosolic GSH pool had no effect on the stress-induced decreases in leaf area in plants experiencing short or long periods of abiotic stress. Conversely, while decreases in GSH synthesis capacity resulted in significant decreases in lateral root density (Marquez-Garcia et al., 2013), this change in root architecture was accompanied by decreased rosette leaf area under non-stressed conditions. Moreover, limitations on GSH synthesis capacity favored larger leaf areas in plants experiencing long (but not short) periods of abiotic stress. Taken together, these findings shed new light on the functions of glutathione in plant growth and abiotic stress tolerance, showing that unexpectedly limitations on GSH synthesis enhance abiotic stress tolerance in the longer term as determined by leaf area. Moreover, while the intracellular partitioning of glutathione is important in the regulation of root architecture, it has little impact on leaf area and hence abiotic stress tolerance.

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Thiol-based redox signaling in the nitrogen-fixing symbiosis

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In nitrogen poor soils legumes establish a symbiotic interaction with rhizobia that results in the formation of root nodules. These are unique plant organs where bacteria differentiate into bacteroids, which express the nitrogenase enzyme complex that reduces atmospheric N_2 to ammonia. Nodule metabolism requires a tight control of the concentrations of reactive oxygen and nitrogen species (RONS) so that they can perform useful signaling roles while avoiding nitro-oxidative damage. In nodules a thiol-dependent regulatory network that senses, transmits and responds to redox changes is starting to be elucidated. A combination of enzymatic, immunological, pharmacological and molecular analyses has allowed us to conclude that glutathione and its legume-specific homolog, homoglutathione, are abundant in meristematic and infected cells, that their spatio-temporally distribution is correlated with the corresponding (homo)glutathione synthetase activities, and that they are crucial for nodule development and function. Glutathione is at high concentrations in the bacteroids and at moderate amounts in the mitochondria, cytosol and nuclei. Less information is available on other components of the network. The expression of multiple isoforms of glutathione peroxidases, peroxiredoxins, thioredoxins, glutaredoxins and NADPH-thioredoxin reductases has been detected in nodule cells using antibodies and proteomics. Peroxiredoxins and thioredoxins are essential to regulate and in some cases to detoxify RONS in nodules. Further research is necessary to clarify the regulation of the expression and activity of thiol redox-active proteins in response to abiotic, biotic and developmental cues, their interactions with downstream targets by disulfide-exchange reactions, and their participation in signaling cascades. The availability of mutants and transgenic lines will be crucial to facilitate systematic investigations into the function of the various proteins in the legume-rhizobial symbiosis.

Keywords: (homo)glutathione, legume nodules, reactive nitrogen species, reactive oxygen species, redox regulation, symbiosis

SYMBIOTIC NITROGEN FIXATION AND ANTIOXIDANT DEFENSES

Legumes are unique among crop plants in their ability to establish symbiotic associations with soil bacteria known collectively as rhizobia. As a result of a molecular dialog between the legume cells and rhizobia, nodules are formed on the roots or, in a few cases, on the stems. Nodules are organs specialized in dinitrogen (N_2) fixation, a biological process in which atmospheric dinitrogen is reduced to ammonia by the nitrogenase enzyme complex of the bacteroids (for a review, see Vance, 2008). The energy required for N_2 fixation derives ultimately from sucrose transported from the leaves to the nodules. In return, the ammonia produced by the bacteroids is assimilated into organic compounds to fulfill the nitrogen demand of both the bacteria and the plant.

Nodules contain abundant metalloproteins, including leghemoglobin and nitrogenase, which are prone to oxidation generating reactive oxygen and nitrogen species (RONS). Some RONS, such as the superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO), perform signaling functions and have been detected in nodules using cytochemical staining, specific fluorescent probes or electron paramagnetic resonance (Santos et al., 2001; Rubio et al., 2004; Sánchez et al., 2010; del Giudice et al., 2011). However, these RONS are potentially cytotoxic, giving rise to highly oxidizing hydroxyl radicals ($^{\bullet}OH$), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) if their concentrations are not tightly controlled by antioxidant enzymes and metabolites. Nodule antioxidants include ascorbate, thiol tripeptides, superoxide dismutases, catalases, thiol peroxidases and the enzymes of the ascorbate-glutathione pathway (Becana et al., 2010). Here, we will focus on those antioxidants of nodules whose protective and regulatory functions entail thiol groups, paying special attention to the contribution of their redox activities to the lifespan of the symbiosis, from root cell infection to nodule senescence.

BIOSYNTHESIS OF THIOL TRIPEPTIDES IN LEGUMES

THIOL COMPOUNDS AND THE THIOL BIOSYNTHETIC PATHWAY

The thiol tripeptide glutathione (GSH; γGlu-Cys-Gly) and ascorbate are the major water-soluble antioxidants and redox buffers of plants. In addition, GSH performs multiple and diverse functions, including regulation of cell cycle, sulfur transport and

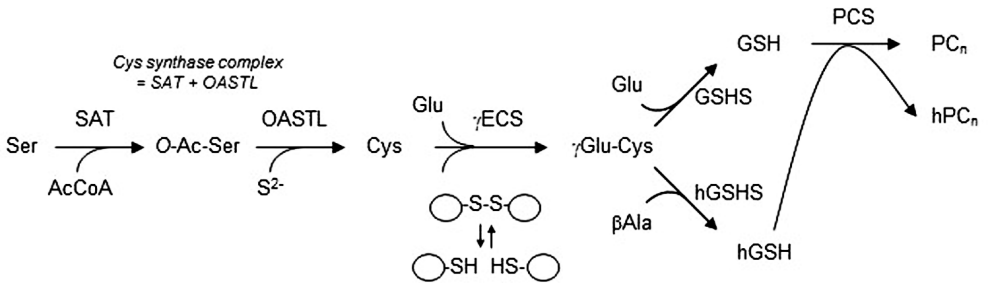

FIGURE 1 | Schematics of the (h)GSH biosynthetic pathway. Depicted are also the enzymes forming the cysteine synthase complex, namely, serine acetyltransferase (SAT) and *O*-acetylserine(thiol)lyase (OASTL), as well as those involved in phytochelatin (PC_n) and homophytochelatin (hPC_n) synthesis. For the γECS enzyme, the redox switch is drawn as an equilibrium between the more active (oxidized) dimeric form and the less active (reduced) monomeric form. Other abbreviations not used in the text: *O*-Ac-Ser, *O*-acetylserine; γGlu-Cys, γ-glutamylcysteine.

storage, stress responses and detoxification of heavy metals and xenobiotics (Rausch et al., 2007; Foyer and Noctor, 2011). In legumes, the structural homolog, homoglutathione (hGSH; γGlu-Cys-βAla), may partially or completely replace GSH (Frendo et al., 2001; Matamoros et al., 2003). Both compounds can be found at concentrations of 0.5–1.5 mM in nodules (Matamoros et al., 1999b), similar to the estimated ranges of 1–3 mM GSH and 0.4–0.7 mM hGSH in the chloroplasts (Bergmann and Rennenberg, 1993) or 2–3 mM GSH in the cytosol of root cells (Fricker et al., 2000).

The synthesis of GSH in plants and other organisms is accomplished in two sequential reactions (**Figure 1**) catalyzed by γ-glutamylcysteine synthetase (γECS) and GSH synthetase (GSHS), both showing a strict requirement for ATP and Mg^{2+} (Bergmann and Rennenberg, 1993). In legumes, the synthesis of hGSH is also carried out in two steps, involving the same γECS enzyme and a specific hGSH synthetase (hGSHS), which exhibits a much higher affinity for β-alanine than for glycine (Macnicol, 1987; Klapheck et al., 1988; Frendo et al., 2001; Iturbe-Ormaetxe et al., 2002). Detailed work using site-directed mutagenesis of hGSHS has conclusively shown that only two contiguous amino acid residues in the active site (Leu-534 and Pro-535 in *Medicago truncatula* hGSHS, and Leu-487 and Pro-488 in soybean (*Glycine max*) hGSHS) determine the substrate preference for β-alanine over glycine (Frendo et al., 2001; Galant et al., 2011).

The *GSHS* and *hGSHS* genes share high homology (∼70% amino acid identity) and are located in tandem on the same chromosome in the model legumes *M. truncatula* (Frendo et al., 2001) and *Lotus japonicus* (Matamoros et al., 2003). These findings are consistent with the proposal that the *hGSHS* gene derives from the *GSHS* gene by a duplication event occurred after the divergence between the Fabales, Solanales and Brassicales (Frendo et al., 2001). Despite this close relationship, the two genes are differentially regulated in plant organs and in response to stressful conditions or signal compounds such as hormones and RONS. This can be examplified with studies performed on the two model legumes. Thus, *M. truncatula* produces exclusively GSH in the leaves and both GSH and hGSH in the roots and nodules (Frendo et al., 1999), whereas *L. japonicus* produces almost exclusively hGSH in the roots and leaves, but more GSH than hGSH in the nodules (Matamoros et al., 2003). In legumes, the thiol contents are positively correlated with the GSHS and hGSHS activities and in general with their mRNA levels (Frendo et al., 1999; Matamoros et al., 1999b, 2003). In *M. truncatula* roots, the expression of *γECS* and *GSHS* but not of *hGSHS* is induced by NO (Innocenti et al., 2007). In *L. japonicus* roots, NO, cytokinins and polyamines up-regulated *GSHS* but not *hGSHS*, whereas hGSHS mRNA and activity were induced by auxins (Clemente et al., 2012). Taken together, these observations suggest the presence of gene-specific *cis*-acting regulatory elements in the *GSHS* and *hGSHS* promoters. However, despite the long time elapsed since the discovery of hGSH in legumes, the reason why this thiol replaces GSH in some legume species and tissues is still a mistery.

Other thiol compounds can be found in plants, but little is known about their roles in the symbiosis and hence they will be only very briefly described here. The GSH and hGSH precursors, cysteine and γ-glutamylcysteine, are found in nodules at concentrations considerably lower (<15%) than GSH or hGSH, in the range of 30–120 μM. As occurs for the tripeptides, both precursors are more abundant in nodules than in roots and leaves, pointing out an active thiol metabolism in N_2-fixing nodules (Matamoros et al., 1999b). This conclusion is reinforced by the high transcript levels of the two enzymes involved in cysteine synthesis, serine acetyltransferase and *O*-acetylserine(thiol)lyase (**Figure 1**), that can be found in nodules (*M. truncatula* Gene Expression Atlas[1] and *L. japonicus* Gene Expression Atlas[2]). Another thiols that can be found in legume nodules and other plant organs are (homo)phytochelatins. These are cysteine-rich polypeptides of general structure $(\gamma Glu\text{-}Cys)_{2-11}$-Gly or $(\gamma Glu\text{-}Cys)_{2-11}$-βAla, which are synthesized from (h)GSH only in the presence of certain metals and metalloids, such as selenium, cadmium, mercury or lead (**Figure 1**). It has been shown that these polypeptides form complexes with cadmium, which is then sequestrated to the vacuoles, avoiding poisoning of cellular metabolism. Interestingly, three functional phytochelatin synthase genes were found in *L. japonicus*, which differ in their cadmium response and are all expressed in nodules (Ramos et al., 2007).

[1]http://mtgea.noble.org/v3/

[2]http://ljgea.noble.org/v2/

REGULATION AND LOCALIZATION OF THIOL SYNTHESIS

The *γECS*, *GSHS* and *hGSHS* genes can be transcriptionally regulated in response to RONS and hormones, as mentioned above. A notable case of this type of regulation is the coordinated induction of the *γECS* and *GSHS* genes of *Arabidopsis thaliana* (Xiang and Oliver, 1998) and of the three genes of *L. japonicus* (Clemente et al., 2012) exposed to jasmonic acid. However, it has also been demonstrated that the (h)GSH biosynthetic pathway can be controlled at the translational and post-translational levels by modulation of the γECS mRNA stability and enzyme activity, respectively (Rausch et al., 2007; Galant et al., 2011). The post-translational regulation of plant γECS enzymes would occur *via* a conserved intermolecular disulfide bond that is likely to operate *in vivo* as a redox switch, in such a way that oxidation shifts the equilibrium toward the more active, dimeric form (Galant et al., 2011; **Figure 1**).

An additional, but by no means less important, mechanism of regulation may rest on the compartmentation of the thiol biosynthetic pathway. In nodules, subcellular fractionation and immunogold labeling studies have shown that γECS is localized in plastids, whereas GSHS and hGSHS are localized in both the plastids and cytosol (Moran et al., 2000; Clemente et al., 2012). A localization of GSHS in cowpea (*Vigna unguiculata*) nodule mitochondria needs to be confirmed by electron microscopy and examined in other legume nodules (Moran et al., 2000). Similar subcellular localizations have been reported for the enzymes of *A. thaliana*, where γECS is confined to the plastids and GSHS is predominantly located to the cytosol (Rausch et al., 2007; Galant et al., 2011). Because γ-glutamylcysteine needs to be exported from the plastids to the cytosol, where most (h)GSH synthesis takes place, subcellular compartmentation provides a potential conduit for transmitting redox signals out of the chloroplasts and probably of other plastids (Mullineaux and Rausch, 2005).

ROLES OF THIOLS IN NODULE FORMATION AND FUNCTIONING

A recent electron microscopy study of pea (*Pisum sativum*) nodules with a GSH-specific antibody revealed that this thiol is present in the bacteroids, mitochondria, cytosol and nuclei of infected cells (Matamoros et al., 2013). Furthermore, as nodules progress from the young to mature stage, total glutathione (reduced + oxidized) decreases in the mitochondria but increases in the bacteroids, cytosol and nuclei, which indicates differential turnover of the thiol or its redistribution between nodule compartments. The finding of GSH in nuclei of infected cells suggests that the thiol performs additional functions to the regulation of the cell cycle, which will be more important in meristematic cells (Diaz Vivancos et al., 2010). These functions may include DNA antioxidative protection or redox regulation of transcription factors (Matamoros et al., 2013).

At the tissue level, careful dissection of nodules has shown that, in general, the (h)GSH content and the γECS, GSHS and hGSHS activities are particularly high in the meristematic and infected zones of legume nodules (Matamoros et al., 1999b). Remarkably, hGSHS is very active in the cortex of bean nodules. The reasons of this specific distribution are unknown, but could be related to a function of this protein in the vascular bundles or in the O_2 diffusion barrier, which are localized to the nodule cortex. These observations have been recently corroborated by using promoter-GUS fusions. Thus, El Msehli et al. (2011) have determined the spatio-temporal gene expression of the (h)GSH synthesis pathway in *M. truncatula*. The expression of *γECS* appears to be higher in the meristematic and infection zones of nodules, whereas the *hGSHS* mRNA is more abundant in the cortex and the *GSHS* mRNA in the cortex and in the N_2-fixing zone.

The concentration of (h)GSH and the N_2-fixing activity of nodules are positively correlated during nodule development (Dalton et al., 1993). The two parameters decline with advancing age (Evans et al., 1999; Groten et al., 2005) as well as during stress-induced senescence (Escuredo et al., 1996; Gogorcena et al., 1997; Matamoros et al., 1999a; Marino et al., 2007; Naya et al., 2007). These findings suggest that (h)GSH is important for nodule activity, a hypothesis that was tested by modulating the nodule content of (h)GSH by pharmacological and genetic approaches. The application of a specific inhibitor of (h)GSH biosynthesis (buthionine sulfoximine) or the expression of *(h)GSHS* in antisense orientation caused depletion of (h)GSH in *M. truncatula* roots (Frendo et al., 2005). The deficiency of (h)GSH synthesis in roots decreased substantially the number of nascent nodules and the expression of some early nodulin genes (Frendo et al., 2005). These results, along with the proposed role of GSH in meristem formation in *A. thaliana* (Vernoux et al., 2000; Reichheld et al., 2007), suggest that (h)GSH is required for the initiation and maintenance of the nodule meristem. The transcriptomic analysis of (h)GSH-depleted plants during early nodulation revealed down-regulation of genes implicated in meristem formation and up-regulation of salicylic acid-related genes after infection with *Sinorhizobium meliloti* (Pucciariello et al., 2009). The potentially enhanced expression of defense genes provides a partial explanation for the negative effects of (h)GSH depletion on the symbiosis. Likewise, the reduction of (h)GSH content in transgenic roots led to a significantly lower N_2-fixing activity, which was related to a smaller nodule size (El Msehli et al., 2011). Conversely, the overexpression of γECS resulted in an elevated (h)GSH content, which was associated with enhanced N_2 fixation. All these data underpin the importance of (h)GSH in nodule development and functioning.

Although the precise roles of (h)GSH in the onset and life-span of symbiosis are still to be defined, a central role of (h)GSH in the regulation of symbiotic activity *via* hormone transduction pathways can be already anticipated (Bashandy et al., 2010; Clemente et al., 2012). Moreover, GSH and hGSH act as substrates for key antioxidant enzymes, such as glutathione reductases, glutathione-*S*-transferases and glutaredoxins (Grxs), and hence both thiols probably participate in the regulation of symbiosis *via* modulation of enzyme activities (Dalton et al., 2009).

THIOL PEROXIDASES AND OTHER REDOXINS OF NODULES

Glutathione peroxidases (Gpxs), peroxiredoxins (Prxs) and thioredoxins (Trxs) are protein components of a regulatory network system (**Figure 2**) that perceives, modulates and transmits information of the cellular redox state *via* thiol-disufide exchange (Dietz et al., 2006; Meyer et al., 2009). Although phylogenetically distant, plant Gpxs and Prxs catalyze similar biochemical reactions (Rouhier and Jacquot, 2005). During their catalytic mechanism,

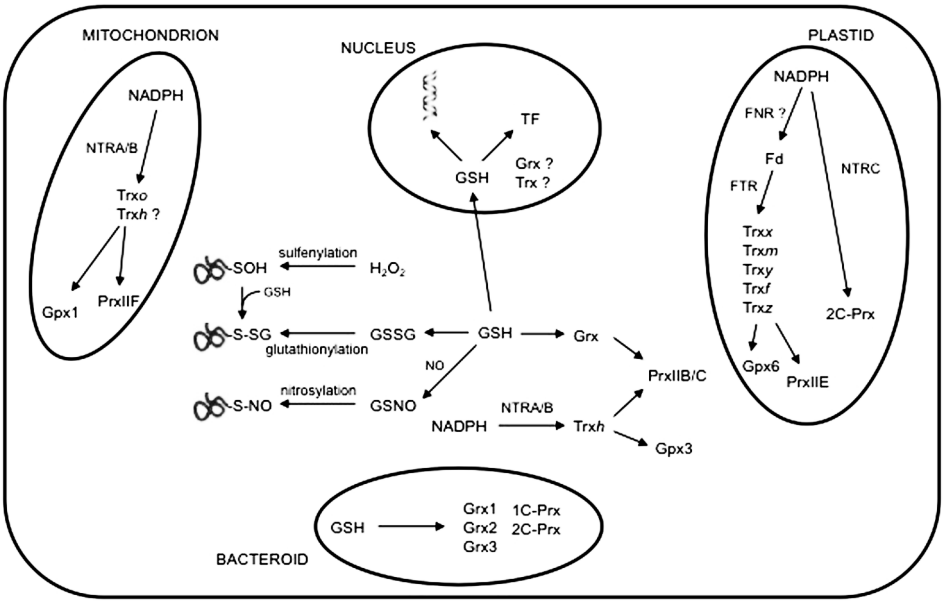

FIGURE 2 | A simplified overview of the thiol-based redox network in legume nodules. Representative cellular compartments of an infected cell showing their contents of proteins, such as Gpxs, Prxs and other redoxins, that contain active cysteine residues. The figure includes also the NTRA/B-Trx-Prx redox systems in the mitochondria and cytosol, and the NTRC-Prx and FTR-Trx redox systems in the plastids. Post-translational modifications involving protein cysteine residues, such as sulfenylation (oxidation of the cysteine thiolate to sulfenic acid), glutathionylation (incorporation of a glutathione moiety) and nitrosylation (incorporation of a nitrosyl group), are shown. GSH in the nucleus may protect DNA from oxidative damage by RONS and modulate activity of transcription factors (TF), activating or inactivating defense and stress-related genes. For simplicity, GSH in the mitochondria or plastids is not indicated. Other abbreviations not used in the text: Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ reductase; FTR, ferredoxin-thioredoxin reductase.

both types of thiol peroxidases reduce H_2O_2, lipid hydroperoxides or, in some cases, $ONOO^-$, with formation of a sulfenic acid (–SOH) or a disulfide bond on the proteins. Cysteine thiol groups are regenerated by Trxs, which are in turn reduced by NADPH-thioredoxin reductases (NTRs) with the consumption of NADPH. It has been also shown that Grxs can substitute for Trxs as electron donors of some Prxs (Rouhier and Jacquot, 2005). Oxidized Grxs are reduced by GSH and glutathione reductase using the reducing power of NADPH.

Thiol peroxidases are widely distributed in all organisms and are encoded by small multigenic families. The *A. thaliana* and poplar (*Populus trichocarpa*) genomes contain eight and six Gpxs, respectively, that are differentially regulated at the transcriptional level in plant organs and in response to stress conditions and growth regulators (Rodriguez Milla et al., 2003; Navrot et al., 2006). The *L. japonicus* genome contains six *Gpx* genes encoding cytosolic, plastidial and mitochondrial isoforms (Ramos et al., 2009). In nodules, the *Gpx3* gene showed a remarkable up-regulation relative to uninfected roots (Colebatch et al., 2002; Ramos et al., 2009). Interestingly, prediction programs of subcellular localization suggest that Gpx3 is targeted to the secretory pathway and/or cytosol and could thus participate in the perception and transduction of redox changes in the nodule apoplast *via* thiol-disulfide exchange reactions with membrane proteins. An unequivocal localization of Gpx3 is necessary to clarify its role in nodule cells. In a recent immunolocalization study, Gpxs were found in the amyloplasts of nodules of *L. japonicus*, alfalfa (*Medicago sativa*) and *Sesbania rostrata*. In most cases, labeling was associated to starch grains, which hints to a role of Gpxs in the regulation of starch metabolism (Ramos et al., 2009).

In plants, Prxs are grouped into four classes (PrxQ, PrxII, 2-CPrx and 1-CPrx) that differ in their catalytic mechanisms and subcellular locations (Dietz et al., 2006). The *L. japonicus* genome encodes eight Prxs, which are localized to the chloroplasts (PrxQ, 2C-PrxA, 2C-PrxB and PrxIIE), mitochondria (PrxIIF), cytosol (PrxIIB/C) and nucleus (1C-Prx). These proteins show specific organ distribution. Thus, 1C-Prx localizes mainly to the embryo and PrxQ levels are very high in leaves compared to other organs. Nodules contain PrxIIB/C in the cytosol, PrxIIF in the mitochondria and low levels of 2C-Prx and PrxIIE in the plastids (Tovar-Méndez et al., 2011). These proteins are part of Prx-Trx-NTR systems that are operative in the cytosol, plastids and mitochondria (**Figure 2**). Trxs form a complex family of disulfide oxidoreductases involved in the redox regulation of cell metabolism. In plant tissues, several groups of Trxs have been identified. Trx*f*, Trx*m*, Trx*x*, Trx*y* and Trx*z* are localized in the plastids, Trx*h* in the cytosol and Trx*o* in the mitochondria. In addition, some Trx*h* isoforms have been found in the mitochondria, nucleus and endoplasmic reticulum (Meyer et al., 2009). In legumes, the Trx protein family has been analyzed in detail in *M. truncatula* (Alkhalfioui et al., 2008) and *L. japonicus* (Tovar-Méndez et al., 2011). In nodules, the cytosolic Trx*h* mRNAs are very abundant, whereas mitochondrial Trx*o* is moderately expressed and plastidial Trx mRNAs are poorly represented. The Trx*h* and Trx*o* isoforms

are maintained in reduced form by cytosolic and mitochondrial NTRs, whereas in the plastids this function is probably performed by the sequential action of ferredoxin-NADP and ferredoxin-Trx reductases (Tovar-Méndez et al., 2011). Nodule plastids also contain low levels of a singular NTR protein, NTRC, characterized by the presence of a Trx domain that enables the enzyme to reduce directly 2C-Prxs with high catalytic efficiency (Pulido et al., 2010). On the other hand, *M. truncatula* contains two novel Trx isoforms, Trx*s1* and Trx*s2*, which are associated with symbiosis (Alkhalfioui et al., 2008). The function of these proteins in the nodules awaits clarification. Notably, no orthologs were found in *A. thaliana*, *L. japonicus*, soybean and pea, suggesting that the Trx*s* isoforms could be unique to certain legume species. The information on Grxs in legume nodules is still very scarce. Proteomic analyses identified two Grxs (GrxC2 and GrxC4) in *L. japonicus* nodules (Tovar-Méndez et al., 2011). As in other plant organs, nodule Grxs probably constitute an alternative reducing system to Trxs (**Figure 2**). Moreover, biochemical studies suggest that Grxs could perform specific functions, such as deglutathionylation, more efficiently than Trxs (Meyer et al., 2009).

The presence of multiple isoforms of thiol peroxidases, Trxs and Grxs in cell compartments and plant organs reflects the necessity of a tight thiol-based control of redox homeostasis for plant function. Furthermore, these proteins probably fulfill redox-independent specific roles due to their differential ability to interact with other proteins. The crucial role of Trxs in nodules was demonstrated by the finding that soybean hairy roots with RNAi-suppressed levels of a Trx*h* isoform showed a severe impairment of nodule formation and development (Lee et al., 2005). Nevertheless, the precise functions of the thiol-based regulatory network in the N_2-fixing symbiosis remain largely undefined.

Early work has established that Gpxs, Prxs and Trxs are involved in the plant's response to environmental constraints (Dietz et al., 2006; Navrot et al., 2006; Meyer et al., 2009). However, direct evidence for a similar function in legume nodules is still lacking. The thiol redox system has been recently investigated during the natural senescence of common bean (*Phaseolus vulgaris*) nodules (Matamoros et al., 2013). Contrary to cytosolic PrxIIB/C, the content of mitochondrial PrxIIF remains constant in aging nodules (Groten et al., 2006; Matamoros et al., 2013). The mitochondrial redox status influences nuclear gene expression and cell fate *via* retrograde signaling (Rhoads and Subbaiah, 2007), and PrxIIF might participate in this process. On the other hand, nodule mitochondria are an early target of oxidative modifications in aging nodules and have increased levels of oxidized lipids and proteins (Matamoros et al., 2013). PrxIIF may protect mitochondria from excessive oxidative damage and thus warrant mitochondrial activity late in nodule development.

THIOL-BASED REDOX REGULATORY NETWORK IN BACTEROIDS

The intracellular redox state of the bacterial partner appears to play an important signaling role in the establishment and functioning of symbiosis. Bacteroids are endowed with multiple antioxidant enzymes, including peroxidases, catalase, Prxs and Grxs, to modulate or detoxify RONS (**Figure 2**). Alteration of RONS levels in mutant strains have profound effects at different stages of symbiosis (Puppo et al., 2013). We will briefly refer to some recent advances concerning thiol-dependent redox signaling in bacteroids.

THE IMPORTANCE OF BACTERIAL GLUTATHIONE DURING SYMBIOSIS

The first direct evidence that the GSH pathway of the bacterial symbiotic partner is important for nodulation and N_2 fixation was obtained by using *S. meliloti* mutants impaired in GSH synthesis. In *S. meliloti*, as in other organisms, GSH is synthesized by γECS and GSHS, encoded, respectively, by the *gshA* and *gshB* genes. An *S. meliloti* mutant deficient in *gshA* was unable to grow under non-stress conditions, precluding any nodulation on alfalfa. Conversely, a *gshB* mutant was able to grow and nodulate alfalfa, indicating that γ-glutamylcysteine can partially compensate for GSH deficiency. The *gshB* strain showed nevertheless a delayed-nodulation phenotype coupled to abnormal development and early senescence of nodules. Both *gshA* and *gshB* mutants exhibited higher catalase activity than the wild-type, suggesting that the two mutants were experiencing oxidative stress (Harrison et al., 2005). Furthermore, *gshB* mutants of *Rhizobium tropici* and *Rhizobium etli* were affected in their ability to compete during nodulation of common bean, and nodules induced by *gshB* mutants displayed early senescence (Riccillo et al., 2000; Tate et al., 2012). A deficiency in GSH was associated with increased levels of $O_2^{\bullet}$ radicals in nodules infected with the *gshB* mutant of *R. tropici*, and thus antioxidant mechanisms dependent on bacterial GSH might be impaired (Muglia et al., 2008). In *R. etli*, GSH deficiency was linked to a reduction of glutamine uptake in growing cultures, suggesting a complex GSH-glutamine metabolic relationship that may be important for symbiotic efficiency (Tate et al., 2012). Finally, the mutation in the *gshA* gene of *Bradyrhizobium* sp. 6144-S7Z appears to affect the ability of the bacterium to compete during peanut (*Arachis hypogaea*) nodulation, but not its capacity to form effective nodules (Sobrevals et al., 2006). Taken together, these results show that the bacterial GSH pool plays a critical role in the rhizobia-plant interaction and that different cellular processes are regulated by, or are dependent on, GSH in free-living rhizobia and in N_2-fixing bacteroids.

THE ROLE OF BACTERIAL GRX AND TRX PATHWAYS

An *in silico* analysis of the *S. meliloti* genome led to the identification of three genes that putatively encode Grxs from different classes (**Figure 2**): Grx1, containing the dithiol CGYC active site of class I Grxs; Grx2, containing the monothiol CGFS active site of class II Grxs; and Grx3, carrying two domains, an N-terminal Grx domain with a CPYG active site and a C-terminal domain with a methylamine utilization protein motif (Benyamina et al., 2013). Inactivation of one gene or the other showed that Grx1 and Grx2 play different roles, Grx1 in protein deglutathionylation and Grx2 in regulation of iron metabolism. Both *grx1* and *grx2* mutants were impaired in bacterial growth and in nodule functioning. On one hand, *grx1* inactivation led to nodule abortion and absence of bacteroid differentiation; on the other, *grx2* inactivation decreased nodule development without modifying bacteroid differentiation. Therefore, both Grx1 and Grx2 appear to be critical proteins for optimal development of the N_2-fixing symbiosis. The incapacity of the *grx1* mutant to differentiate is remarkably similar to the

phenotype of a mutant (*katB katC*) affected in catalase activity (Jamet et al., 2003). This observation emphasizes the importance of a fine tuning of the RONS balance in bacteroid differentiation and the key role of *S*-glutathionylation in modulating the function of proteins essential for this process.

In *Escherichia coli*, the Grx and Trx pathways, the two branches of the thiol-redox system, are functionally redundant. Whereas the simultaneous inactivation of the two pathways is non-viable, inactivation of either of them is viable, indicating that each pathway can fully carry out the essential function of reducing disulfides in the absence of the other (Toledano et al., 2007). This does not appear to be the case in *S. meliloti*, where *grx1* and *grx2* mutants were affected in both free-living bacteria and plant-hosted bacteroids, which points out that Grx1 and Grx2 have more specific roles than the corresponding *E. coli* enzymes. Consistent with the notion of poor redundancy, Trx-like proteins are required for optimal N_2-fixation efficiency in *S. meliloti* and *Rhizobium leguminosarum* (Vargas et al., 1994; Castro-Sowinski et al., 2007).

POST-TRANSLATIONAL MODIFICATIONS AND REDOX SIGNALING IN NODULES

Several lines of evidence indicate that RONS are key signals that regulate the establishment of symbiosis (Ramu et al., 2002; del Giudice et al., 2011; Puppo et al., 2013). Differences in the intensity, duration and localization of RONS might be perceived and transmitted by thiol-dependent mechanisms. Transcription factors that respond to redox changes by interaction with Trxs have been described in yeast, plants and animals (Buchanan and Balmer, 2005). In legumes, the expression of several transcription factors involved in the onset of symbiosis seems to be regulated by RONS (Andrio et al., 2013). Moreover, NO is required for the transcriptional control of genes involved in nodule development (del Giudice et al., 2011). Consequently, it is expected that the thiol-based regulatory network plays relevant functions in the transcriptional reprogramming that takes place during the initial stages of the symbiotic interaction.

The important signaling role of NO can be modulated by the reaction of this short-lived free radical with GSH and presumably hGSH, in the presence of O_2, to form the corresponding *S*-nitrosothiols. In particular, nitrosoglutathione (GSNO) is considered a carrier and reservoir of NO in plant cells, where it participates in transnitrosylation reactions, delivering NO to protein cysteine residues (Corpas et al., 2011). In turn, the bioactivity of GSNO can be regulated by the enzyme *S*-nitrosoglutathione reductase (GSNOR), also termed class III alcohol dehydrogenase, which catalyzes the NADH-dependent reduction of GSNO to glutathione disulfide and ammonia (Corpas et al., 2011). The concentrations of GSNO or nitrosohomoglutathione (hGSNO) have not been determined yet in nodules. However, treatment of *M. truncatula* seedlings with GSNO in hydroponic medium causes a massive induction of genes involved in key processes, such as primary metabolism and defense response, in roots and in young and mature nodules, pointing out that this compound may perform important functions *in vivo* during rhizobial infection and nodule development (Ferrarini et al., 2008). Likewise, the GSNOR transcript is clearly detectable in nodules, roots, leaves and seeds, suggesting that the enzyme is functional in all these plant organs (*M. truncatula* Gene Expression Atlas[1] and *L. japonicus* Gene Expression Atlas[2]).

The regulation of protein function *via* oxidative modification has emerged as an important molecular mechanism that modulates various biological processes. Protein cysteine residues are sensitive targets of glutathione disulfide and RONS (**Figure 2**), leading to post-translational modifications such as glutathionylation, nitrosylation and sulfenylation (Couturier et al., 2013; Kovacs and Lindermayr, 2013). These cysteine-based modifications regulate protein function, localization and/or turnover (Klomsiri et al., 2011; Spoel and Loake, 2011). Proteomic analysis brought to light a hundred of sulfenylated proteins in the *M. truncatula-S. meliloti* symbiosis (Oger et al., 2012). The major functional group of sulfenylated proteins identified at 2 days post-inoculation was represented by redox-active proteins, but this was not the case for mature nodules, in which the main targets of sulfenylation were proteins related to primary metabolism. Thus, RONS-induced modifications of proteins occur during N_2 fixation and may be involved in the onset and functioning of symbiosis (Oger et al., 2012). Eighty proteins have also been identified in mature nodules by proteomics as targets of *S*-nitrosylation (Puppo et al., 2013). Twenty-seven proteins were found to be sensitive to both sulfenylation and *S*-nitrosylation. As occurs for sulfenylation, a large part of nitrosylated proteins was found to be related to carbon, nitrogen or energy metabolism, which strengthens the potential role of the cysteine redox state of proteins in the regulation of nodule metabolism. In addition, highly oxidizing NO derivatives, such as $ONOO^-$, NO_2 or nitrosonium cation (NO^+), may also participate in post-translational modifications such as tyrosine and heme nitration, as demonstrated for glutamine synthetase (Melo et al., 2011) and leghemoglobin (Navascués et al., 2012), respectively.

Redox regulation *via* cysteine residues is also important for the bacterial partner of the symbiosis, and this will be briefly illustrated here with a few examples. In *Bradyrhizobium japonicum*, the cellular pool of active FixK2, a crucial regulator of genes required for the micro-oxic lifestyle, is partly controlled at the post-translational level (Mesa et al., 2009). The FixK2 activity is modulated by an oxidative-dependent inactivation involving a critical cysteine residue near the DNA-binding domain. This post-translational modification might be a strategy to prevent the detrimental activation of the FixK2 regulon depending on the cellular status. The expression of the *fixK2* gene itself is activated by the FixLJ system in response to a moderate decrease of O_2 tension. Particularly, in bacteroids, where ROS are assumed to be generated as a side product of the high respiration turnover, the FixK2 transient inactivation could prevent the generation of more ROS and guarantee an adequate balance between the beneficial and detrimental effects of respiration.

Thiol-dependent redox sensing also modulates the activity of antioxidant enzymes such as Prxs. Interestingly, an atypical 2C-Prx of *R. etli* is involved in the defense of bacteroids against H_2O_2 stress and could require the Trx system as a source of reducing power (Dombrecht et al., 2005). An *S. meliloti 1C-Prx* gene is predominantly expressed in alfalfa root nodules and the protein was detected by proteomic analysis (Puppo et al., 2013). Among

the twenty sulfenylated enzymes detected in bacteroids, proteins related to carbohydrate and nitrogen metabolism are largely represented, showing that sulfenylation may regulate the activity of crucial proteins for nodule functioning (Oger et al., 2012).

In indeterminate nodules, bacteroid differentiation is mediated by nodule-specific cysteine-rich (NCR) peptides, which are defensin-type antimicrobial peptides (Mergaert et al., 2003; Kereszt et al., 2011). These NCRs are targeted *via* the plant cell secretory pathway to the symbiosomes, where they trigger bacterial differentiation and/or membrane damage and permeabilization (Van de Velde et al., 2010; Wang et al., 2010). Modifying the cysteine residues or the disulfide configuration of an NCR has been shown to influence its activity against *S. meliloti*, suggesting that a tight control of NCR redox status is a prelude to bacteroid terminal differentiation (Haag et al., 2010).

CONCLUSIONS

Over the last two decades, many advances have been made on the characterization, regulation and localization of the enzymes of the thiol biosynthetic pathway in model and crop plants. Notable accomplishments include the findings that the pathway is compartimentalized in plant cells, with export of γ-glutamylcysteine from the plastids to the cytosol; the crystallization and subsequent structure elucidation of γECS, GSHS and hGSHS; the role of thiol compounds and associated enzymes in redox signaling and in controlling the cell cycle; the transcriptional regulation of γECS, GSHS and hGSHS by RONS and hormones; and the post-translational regulation of γECS by a redox switch involving conserved cysteine residues. Importantly also in the case of legumes, the use of enzyme inhibitors and transgenic plants has demonstrated that thiol tripeptides are essential for the functioning of the rhizobia-legume symbiosis.

Several important questions need, however, to be solved. Further research will be required to establish if GSH and hGSH perform distinct functions, especially in redox homeostasis and signaling during nodule development. Our knowledge on the function of other components of the thiol regulatory network in legume nodules is still at its infancy. This may be due to the amazingly high number of Prx, Gpx, Trx and Grx isoforms, which are present in multiple cellular compartments and differ in biochemical properties. Also, it will be necessary to assess the role of thiol peroxidases and other redoxins during rhizobial infection and to identify their target nodule proteins. Redox-dependent post-translational modifications constitute a versatile adaptive mechanism to changing conditions. The recent development of redox proteomics permits the large-scale identification of proteins that are modified in response to specific stimuli. To shed light on the signaling events that take place in response to RONS, it will be important to characterize nodule proteins that undergo oxidation, nitrosylation or glutathionylation of critical cysteines, and to investigate the impact of these modifications on their biological activities. The generation of mutants and/or transgenic lines will be most helpful to establish the function of individual proteins and metabolites in the rhizobia-legume symbiosis. Finally, a comparative study of the thiol-based signaling mechanisms underpinning the symbiotic and pathogenic interactions and the plant responses to environmental cues will provide critical information to enhance nitrogen nutrition in crop legumes as well as their tolerance to abiotic and biotic stress. The improvement of the N_2 fixation efficiency is expected, in turn, to have direct beneficial consequences for sustainable agriculture and the environment as this biological process will eventually lead to a reduction in the input of costly and contaminating nitrogen fertilizers.

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Thiol-based redox regulation in sexual plant reproduction: new insights and perspectives

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The success of sexual reproduction in plants involves (i) the proper formation of the plant gametophytes (pollen and embryo sac) containing the gametes, (ii) the accomplishment of specific interactions between pollen grains and the stigma, which subsequently lead to (iii) the fusion of the gametes and eventually to (iv) the seed setting. Owing to the lack of mobility, plants have developed specific regulatory mechanisms to control all developmental events underlying the sexual plant reproduction according to environmental challenges. Over the last decade, redox regulation and signaling have come into sight as crucial mechanisms able to manage critical stages during sexual plant reproduction. This regulation involves a complex redox network which includes reactive oxygen species (ROS), reactive nitrogen species (RNS), glutathione and other classic buffer molecules or antioxidant proteins, and some thiol/disulphide-containing proteins belonging to the thioredoxin superfamily, like glutaredoxins (GRXs) or thioredoxins (TRXs). These proteins participate as critical elements not only in the switch between the mitotic to the meiotic cycle but also at further developmental stages of microsporogenesis. They are also implicated in the regulation of pollen rejection as the result of self-incompatibility. In addition, they display precise space-temporal patterns of expression and are present in specific localizations like the stigmatic papillae or the mature pollen, although their functions and subcellular localizations are not clear yet. In this review we summarize insights and perspectives about the presence of thiol/disulphide-containing proteins in plant reproduction, taking into account the general context of the cell redox network.

Keywords: redox regulation, sexual plant reproduction, thioredoxin, glutaredoxin, pollen, pistil, pollen-pistil interaction, stigma

INTRODUCTION

Sexual Plant Reproduction involves complex biochemical pathways under a tight genetic control, which lead to drastic architectural changes at developmental and cellular levels (Franklin-Tong, 1999). These changes begin with the generation of the haploid gametes in specialized structures know as the mega- and microgametophytes: the embryo sac in the pistils and the pollen grain in the anthers. Afterwards, mature pollen grains land over a receptive stigma, hydrate and germinate, emerging a pollen tube, which enlarges at its apical region at exceptionally high growing rates. This pollen tube penetrates throughout the style toward the embryo sac in order to deliver the male gametes, which fertilize the egg cell and the polar nuclei generating an embryo and the endosperm, respectively, and ultimately the offspring (Sprunck et al., 2013).

Plants, like other eukaryotes, have evolved dedicating enormous resources and efforts to guarantee sexual reproduction. Among others, they have developed molecular mechanisms, which allow a tight regulation of all developmental events underlying the process. These mechanisms not only have contributed to assure the emergence of genetically-improved progenies, but also allowing plants to tune this process according to challenging environmental conditions, which has let flowering plants evolve dominating almost every terrestrial ecosystem (Hiscock, 2011). Redox regulation has recently emerged as a crucial mechanism able to manage significant stages during sexual plant reproduction where ROS, nitric oxide (NO) and other classical antioxidant protein and molecules are critically involved (Feijó et al., 2004; Prado et al., 2004; Dresselhaus and Franklin-Tong, 2013). In addition, several isoforms of plant redoxins like TRXs or GRXs seem to be specifically associated with reproductive tissues according to their precise space-temporal expression patterns (**Table 1**), although no clear functions have usually been assigned in this context.

TRXs and GRXs are redox proteins whose activity depends on conserved cysteine (Cys) residues present in their active centers (Meyer et al., 2012). These Cys residues are usually maintained in their reduced state within the cell (Foyer and Noctor, 2005a). TRXs and GRXs carry out oxide-reductive reactions on essential Cys residues of a variety of plant proteins. The *Arabidopsis* genome contains about 40 genes encoding TRXs or TRX-related proteins grouped in different clusters and subclusters according to several aspects like sequence similarity, subcellular localization or intron position, which have been described as putatively involved in a plethora of plant roles (Buchanan and Balmer, 2005; Meyer et al., 2012). GRXs share important similarities with the TRX family like their protein structures (both protein types belong to the TRX superfamily) and the fact that higher plants also possess

Table 1 | Described redoxins and other related proteins involved in sexual plant reproduction.

	Protein / Gene	Accession number	Organism	Localization associated with sexual plant reproduction	Potential role	Reference(s)
TRXs	AtTRXh1	At3g51030	*A. thaliana*	Gene expressed in style Protein detected in pollen tube	Unknown	Reichheld et al., 2002; Ge et al., 2011
	AtTRXh4	At1g19730	*A. thaliana*	Gene expressed in pollen and pollen tube	Unknown	Reichheld et al., 2002
	AtTRXh5	At1g45145	*A. thaliana*	Protein detected in pollen tube	Unknown	Ge et al., 2011
	PsTRXh1	AJ310990	*P. sativum*	Gene expression and protein localized in pollen and stigma	Unknown	Traverso et al., 2007
	TRX h (subgr. 1)	CI000057:1 (Contig)	*C. sativus* (Saffron)	Gene highly expressed in stigma	Unknown	D'Agostino et al., 2007
	THL-1	AF273844	*B. rappa*	Protein in pollen coat and stigma	Unknown (pollen) and Self-Incompatibility response (stigma)	Toriyama et al., 1998; Cabrillac et al., 2001; Ivanov and Gaude, 2009a
	TRX h (subgr. 3)	AF159388	mono and dicot	Conserved genes expressed in pollen	Unknown	Juttner et al., 2000
	NaTRXh	AAY42864	*N. alata*	Protein secreted into the extracellular matrix (stylar transmitting tract)	Self-Incompatibility?	Juárez-Díaz et al., 2006
	Protein S	X81992	*P. coerulescens*	Gene expressed in mature pollen	Self-Incompatibility (grass model)	Li et al., 1997
	ACHT3	At2g33270	*A. thaliana*	Gene highly expressed in pollen	Unknown	Becker et al., 2003; Lee and Lee, 2003
	PsTRXf and PsTRXm	X63537 and X76269	*P. sativum*	Gene expressed in pollen grains, anthers, style and ovules	Unknown; Photosynthesis regulation in style?	de Dios Barajas-López et al., 2007
	AtNTRC	At2g41680	*A. thaliana*	Gene expressed in style	Unknown	Kirchsteiger et al., 2012
GRXs	CC-type GRX	GRX; patent US2009/0038028A1	Maize	Gene expressed in anthers	Anther development	Chaubal et al., 2003; Timofejeva et al., 2013
	CC-type GRX (ROXY1)	At3g02000	*A. thaliana*	Gene expressed in anthers	Petal and anther development	Xing et al., 2005
	CC-type GRX (ROXY2)	At5g14070	*A. thaliana*	Gene expressed in anthers	Anther development (gametogenesis?)	Xing and Zachgo, 2008
	CC-type GRX	LOC_Os07g05630	rice	Gene and protein expression in anthers	Male gametogenesis and anther development	Hong et al., 2012
	AtGrxC2	At5g40370	*A. thaliana*	Extracellular protein secreted from germinated pollen tube	Unknown	Ge et al., 2011
	GRX	G4XH75_9POAL	Triticale (*Triticum* spp x *Secale* spp)	Extracellular protein released from pollen coat upon pollen hydration	Unknown	Zaidi et al., 2012
OTHERS	CBSX1	At4g36910	*A. thaliana*	Gene and protein expressed in anthers	Anther dehiscence	Yoo et al., 2011
	CP12-1	At2g47400	*A. thaliana*	Gene expressed in style	Unknown; Photosynthesis regulation in style?	Singh et al., 2008
	CP12-2	At3g62410	*A. thaliana*	Gene expressed in style	Unknown; Photosynthesis regulation in style?	Singh et al., 2008
	PRX (TPx2)	At1g70950	*A. thaliana*	Gene highly expressed in pollen	Unknown	Lee and Lee, 2003

(Continued)

Table 1 | Continued

Protein / Gene	Accession number	Organism	Localization associated with sexual plant reproduction	Potential role	Reference(s)
AtPRXII-C	At1g65970	*A. thaliana*	Gene expressed in mature pollen	Unknown	Bréhélin et al., 2003
AtPRXII-D	At1g60740	*A. thaliana*	Gene expressed in pollen and pollen tube	Unknown	Bréhélin et al., 2003
AtPRDII-E	At3g52960	*A. thaliana*	Gene expressed in immature anthers and ovules	Unknown	Bréhélin et al., 2003

a larger number of GRX genes per genome when compared to other organisms (about 50 genes encoding for GRXs or GRX-related proteins in *Arabidopsis*) (Couturier et al., 2009; Meyer et al., 2012). The physiological roles of plant GRXs are less-known than those of TRXs, although they have also been involved in a variety of functions (Meyer et al., 2012).

Oxidized TRXs and GRXs are physiologically reduced by dedicated systems according to their subcellular localizations (for an extended review see Meyer et al., 2012). Cytosolic and mitochondrial TRXs are mainly reduced by NADPH in a reaction catalyzed by the enzyme NADPH-TRX-Reductase (NTR) (Arner and Holmgren, 2000; Laloi et al., 2001), while the isoforms from plastids are reduced by ferredoxin via the enzyme Ferredoxin-TRX-Reductase (FTR) (Shürmann and Jacquot, 2000; Balmer et al., 2006) and the GRXs are generally described as reduced by reduced glutathione (GSH). However, alternative crosstalk between both TRX and GRX systems has also been demonstrated in addition to these classical schemes, which reveals a high plasticity of the thiol-based redox regulation in plants (Gelhaye et al., 2003; Balmer et al., 2006; Reichheld et al., 2007; Bandyopadhyay et al., 2008; Marty et al., 2009).

TRXs and GRXs are described as the main protein families responsible for the redox status of protein Cys residues within the cell. These Cys are particularly susceptible to oxidations by reactive species, this fact being usually identified by researchers as a regulatory mechanism of the protein activity, as well as a protective or redox signaling mechanism (Couturier et al., 2013). These thiol-based regulations have been interpreted as a sensing mechanism of the cellular redox state, which acts between stress perception and plant response against environmental challenges (Foyer and Noctor, 2005a).

In this review we summarize, discuss and hypothesize about the occurrence of these thiol/disulfide containing proteins in reproductive tissues, pointing out an increased importance of the thiol-based redox regulation and signaling mechanisms in sexual plant reproduction.

REDOX REGULATION BY ROS/RNS IN SEXUAL PLANT REPRODUCTION

Reactive species are produced in living cells as an unavoidable consequence of their own metabolism (Foyer and Noctor, 2005a). Apart from their activity damaging different macromolecules, they have been shown to act as secondary messengers, reason why the concept of oxidative stress has been re-evaluated (Foyer and Noctor, 2005b). Taking into account that protein Cys residues are particularly affected by reactive species, we review in this chapter the most important results involving ROS and RNS at different stages of the sexual plant reproduction.

Reactive species have been shown to increase in a rapid and transient manner by specific molecular mechanisms for a proper plant growth and development, including sexual plant reproduction (Foreman et al., 2003; Potocký et al., 2007). ROS and NO have been involved in redox signaling taking place previously and during pollen-pistil interactions (**Figure 1**; Sharma and Bhatla, 2013). A high production of H_2O_2, exclusively confined to receptive stigmatic papillae was suggested to serve as a redox signal promoting pollen-pistil interaction and/or germination, as well as a defense mechanism against microbe attack (**Figure 1A**; McInnis et al., 2006a; Wilson et al., 2009; Zafra et al., 2010). In parallel to ROS production, a Stigma-Specific Peroxidase (SSP) was shown to be active only during a short period of stigma receptivity in *Senecio squalidus* (**Figures 1A,B**; McInnis et al., 2005, 2006b). *In vitro* experiments also revealed that mature pollen grains produce a high level of NO, which inhibits ROS production in the stigmatic papillae (**Figure 1B**; McInnis et al., 2006a; Bright et al., 2009; Zafra et al., 2010).

In addition, physiological mechanisms of ROS generation have been indicated to be required for normal pollen tube development (**Figures 1B,C**; Cardenas et al., 2006; Potocký et al., 2007, 2012). ROS production has been described to be mainly originated by the activity of specific isoforms of NADPH oxidases (NOX) localized at the tip of tobacco pollen tube (**Figure 1B**), whose activity was found to be increased by Ca^{2+} (Potocký et al., 2007). This represents a conserved mechanism of polarized cell tip growth (Bushart and Roux, 2007; Konrad et al., 2011).

In cucumber germinated pollen, ROS and NO production was specifically tip-localized during the initial germination steps, although was extended along the pollen tubes and the pollen grain later during germination (**Figure 1B**) (Šírová et al., 2011).

Both redox chemical agents and external conditions have shown to alter the production of these reactive species (Šírová et al., 2011). The addition of a reducer like ascorbate abolishes this production probably due to its capacity to effectively scavenge the intracellular ROS. The treatment with NO donors inhibits pollen germination and growth, and the addition of NO scavengers increases pollen germination rates (Prado et al., 2004; Šírová et al., 2011). NO seems also to be involved in the inhibition of germination and tube growth after pollen exposure to UV-B (Feng et al., 2000; He et al., 2007; Wang et al., 2010a,b). Curiously, NO exerts just the opposite effect in gymnosperm pollens, since it was shown that Pine pollen tube growth rate is accelerated by NO donors, whereas NO scavengers affect contrary (Wang et al., 2009a,b).

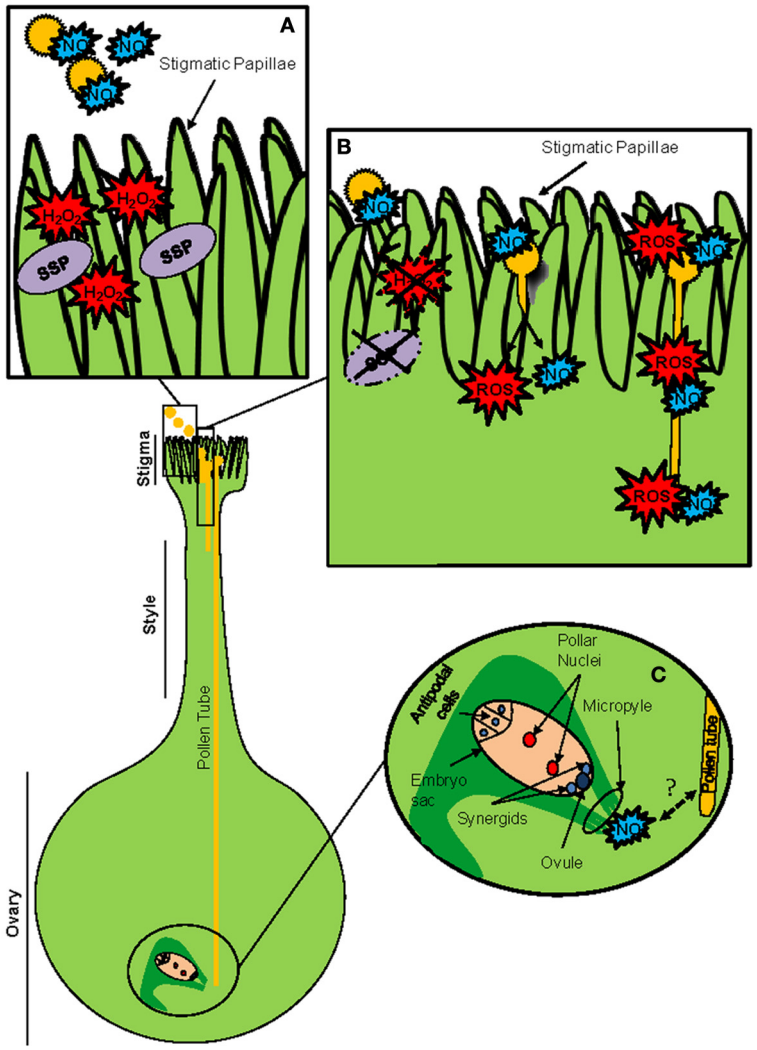

FIGURE 1 | ROS/NO are implicated in plant reproduction signaling. Model gynoecium of an Angiosperm. **(A)** Receptive stigma at high magnification, before pollen-pistil interaction. Pollen grains are active NO producers, while the receptive stigma papillae generate large quantities of H_2O_2 and display enhanced peroxidase activity, in some cases in the form of stigma-specific peroxidase (SSP). **(B)** Stigma and pollen grains during diverse phases of their interaction. Both the enhanced peroxidase activity and the high levels of H_2O_2 become significantly reduced after pollen landing on the stigma and pollen germination, likely through pollen-produced NO signaling. The germinating pollen grains and the elongating pollen tubes produce ROS and NO, particularly at their tips. **(C)** The embryo sac is reached by the pollen tube tip trough the micropylar end. NO produced in micropyle could be involved in pollen tube guidance.

Although the mechanisms controlling pollen tube guidance and pollen-pistil interaction are still unknown (Boavida et al., 2005; Higashiyama and Hamamura, 2008; Márton and Dresselhaus, 2008) there is also evidence about the involvement of NO as a key molecule at this regard (Prado et al., 2004). Prado et al. (2008) showed that a Ca^{2+} specific response to NO induces pollen tube re-direction toward the ovule. Prado's work also describes the detection of NO production in the micropyle, where it was suggested to participate in pollen tube guidance to the ovules (**Figure 1C**; Prado et al., 2008). The generation of reactive oxygen species has also been involved in microsporogenesis, usually throughout programmed cell death (Jiang et al., 2007; Wan et al., 2007). In addition, it was recently shown that the male germ cell fate critically depends on H_2O_2 levels of the precursor cells (Kelliher and Walbot, 2012). Moreover, the molecular models developed in order to explain self-incompatibility (SI) in plants, usually include important roles for ROS or NO (McClure and Franklin-Tong, 2006; McInnis et al., 2006a,b; Wilkins et al., 2011).

Several of the detailed molecular mechanisms through which ROS and NO exert these functions are beginning to be outlined, and some of them involve thiol modifications. At this regard, Cys residues in proteins are particularly affected by these reactive species, and Cys-based signaling by ROS and/or RNS is a well-described feature affecting an increasing number of proteins, some of them from plants (Couturier et al., 2013; Corpas

and Barroso, 2013). In bacteria, fungi or mammals, Cys modification by ROS and/or RNS has been described to affect DNA binding properties of some transcription factors (D'Autreaux and Toledano, 2007). In plant cells, the Cys-based actions of RNS (S-nitrosylation) and ROS (oxidation) on NPR1 and TGA1 proteins regulate plant systemic defense (Tada et al., 2008; Lindermayr et al., 2010). Protein S-nitrosylation is produced by the interaction of specific Cys residues with NO generated by different types of RNS, and this modification is emerging as a crucial regulatory mechanism involved in several aspect of plant physiology (Corpas et al., 2013). As an example, the activity of NADPH oxidases can be controlled by the S-nitrosylation of a C-terminal Cys (Yun et al., 2011). NOX proteins are highly involved in pollen tube growth and the subject of further regulation mechanisms (Potocký et al., 2007, 2012).

S-nitrosoglutathione (GSNO), an abundant molecule in plant tissues (Airaki et al., 2011) originated by S-nitrosylation of reduced glutathione (GSH) (Broniowska et al., 2013) is considered a reservoir, vehicle and biological donor of NO in plant cells (Corpas et al., 2013). Protein S-nitrosylation by GSNO (S-transnitrosation) also seems to be a feasible physiological mechanism for post-translational modification of proteins (Begara-Morales et al., 2013), however, not yet sufficiently described in plant reproductive tissues.

According to all these data, further experiments must be carried out to identify key proteins involved in the regulation of sexual plant reproduction via ROS or NO-mediated Cys oxidations. The specific presence of redoxins at these stages (**Table 1**; **Figure 3**) together with the importance of these reactive species suggests a more critical thiol-based regulation of several stages of the sexual plant reproduction than initially thought.

SPECIFIC REDOXINS INVOLVED IN ANTHER DEVELOPMENT AND MALE GAMETOGENESIS

Successful sexual reproduction depends on the proper formation of specialized complex structures in the flower: anthers and pistils. Initially, a group of somatic cells must switch from the mitotic to the meiotic pathway to generate the haploid gametes. All processes are developed according to both environmental and developmental signals (Bhatt et al., 2001). Later, anther dehiscence will produce the release of mature pollen grains. Anther development and male gametogenesis processes are known to be critically influenced by the redox activity of specific thiol-based redox proteins. CC-type GRXs and the redox chloroplastidial system including CBSX (single cystathionine β-synthase domain–containing proteins)/TRX/PRX proteins play important roles in redox homeostasis and development in male reproductive tissues (**Figure 2**) (Wang et al., 2009a,b; Yoo et al., 2011).

CC-type GRXs, (also named ROXY proteins), are conserved plant-specific GRXs involved in anther and male gamete differentiation and flower development (**Figure 2A**; Xing et al., 2006; Wang et al., 2009a,b). The first evidence about such involvement was described by Chaubal et al. (2003) during the characterization of the maize mutant *msca1*. In this mutant, all anther cell layers were transformed into non-differentiated vegetative tissues. This phenotype was associated later with the lack of a GRX (Xing et al., 2011), and recently corroborated during the screening of male sterile lines in maize (Timofejeva et al., 2013). Culture of the *msca1* mutant under hypoxia conditions (low oxygen / H_2O_2) allows a rescue of the differentiation of the germinal line in the mutant flowers (Kelliher and Walbot, 2012).

However, probably the most important data concerning the role of ROXY proteins in anther development was provided by studies based on *A. thaliana*. Initially, the redox activity of the GRX ROXY1 was identified as a major regulator of early petal organ initiation and further steps of floral morphogenesis (Xing et al., 2005). Afterwards, the functionally redundant GRXs ROXY 1 and 2 were described to perform essential redox-dependent activities in early steps of anther and tapetum differentiation (**Figure 2A**; see anther structure in **Figure 2B**) by affecting the expression of a large variety of anther genes supporting critical roles (Xing and Zachgo, 2008). During anther development, they act via the redox activation of TGA9 and 10 transcription factors, probably among other protein targets (Murmu et al., 2010). *Arabidopsis* ROXY proteins were also suggested to be involved in male gametogenesis (Xing and Zachgo, 2008). In fact, this involvement has been recently evidenced in monocots (Hong et al., 2012). These authors have shown that the rice *MIL1* gene encodes for a CC-type GRX which is not only involved in the differentiation of the surrounding somatic layer of the anthers, but also in the switch of microsporocytes from mitosis to meiosis (**Figure 2A**). According to these results, pollen mother cells contain specific meiosis-initiation machinery in which this nuclear GRX (MIL1) plays preponderant roles, probably acting also via TGA-type transcription factors. In this context, the results from Kelliher and Walbot (2012), demonstrating that changes in the redox status critically control the male germ lineage fate in maize, suggest a master or integrator role of these types of GRXs in the redox regulation associated with anther and gamete differentiation.

In the anthers, the chloroplast redox system comprising CBSXs, TRXs and peroxiredoxins (PRXs) is involved in anther dehiscence and therefore pollen release via the control of H_2O_2 (Ok et al., 2012), which ultimately allows connecting plant nutritional information and pollen release (**Figure 2B**). CBSX are redox proteins characterized by sharing only a single pair of Cystathione β-Synthase domains (CBS) in their structures that belong to the CBS-containing protein (CDCPs) superfamily. *Arabidopsis* genome contains six genes encoding CBSX proteins (CBSX1-6), which have recently been described as cellular sensors involved in the control of plant redox homeostasis and development (Yoo et al., 2011). They act interacting and increasing the activity of TRXs by sensing cellular changes of adenosine nucleotides. CBSX1 is a member of this family in *Arabidopsis*, which is preferentially expressed in the chloroplast of the anther. This protein is able to interact and increase the activity of all four types of plastidial TRXs (*f*, *m x* and *y*). This augmentation is favored by the presence of AMP, but not by ADP or ATP (**Figure 2B**; Yoo et al., 2011; Ok et al., 2012). The overexpression of CBSX1 or CBSX2 in *Arabidopsis* transgenic plants yields plants showing a severe sterility as a consequence of the inhibition of their anther dehiscence, which prevents the liberation of mature pollen grains (Yoo et al., 2011; Jung et al., 2013). This sterility is due to a decrease of H_2O_2 in the anthers, which causes

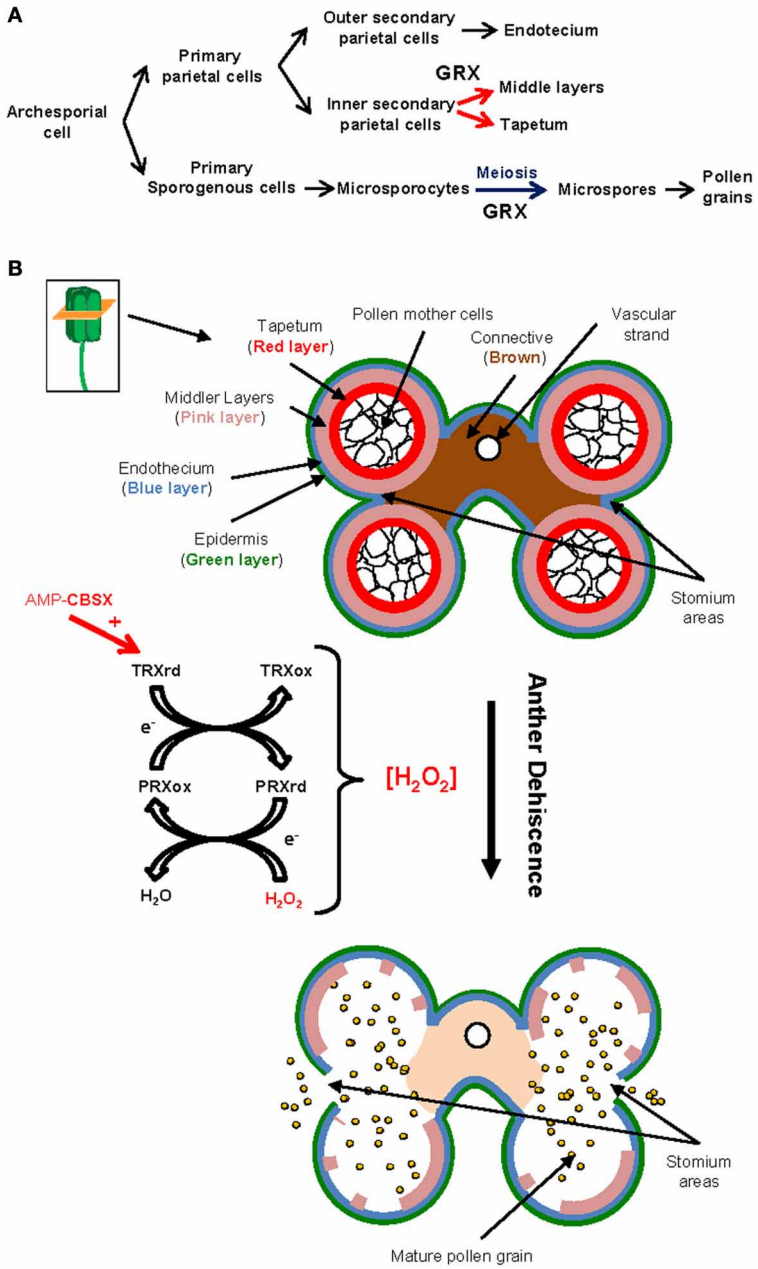

FIGURE 2 | Thiol-based redox proteins are critically involved in male gametogenesis, anther development and dehiscence. (A) Ontogenic development of the male germline. CC-type GRXs are essential for the switch from mitosis to meiosis in the microsporocytes (blue arrow), which ultimately originate the haploid pollen grains. They also participate in the development of the anther layers surrounding the microsporocytes (red arrows). **(B)** Diagram representing the histological structure of an anther (transversal section) and major changes occurring after dehiscence. H_2O_2 is required for cell wall lignification, which induces a thickening of the endotecium leading to anther dehiscence. CBSXs can regulate the level of H_2O_2 via their ability to active TRXs, which ultimately reduce PRXs in the plastids. This activity is enhanced by the presence of AMP, thus connecting the nutrition state with anther development.

a lignin deficiency that originates a failure in the secondary wall thickening of the endothecium layer, and subsequently a very narrow crevice in the stomium area (region of the anther where dehiscence occurs and pollen grains leave the anthers; **Figure 2B**). Male sterility caused for this same reasons (a limitation of H_2O_2) have previously been reported (Karlsson et al., 2005; Villarreal et al., 2009). According to these authors, CBSX1 regulates the level of H_2O_2 via the activation of plastid TRXs, which reduce and activate peroxiredoxins (PRX) directly detoxifying this radical (**Figure 2B**). In our opinion, an evaluation of the roles of other enzymes or non-enzymatic systems known to be involved in the homeostasis of H_2O_2 would be of great interest at this regard.

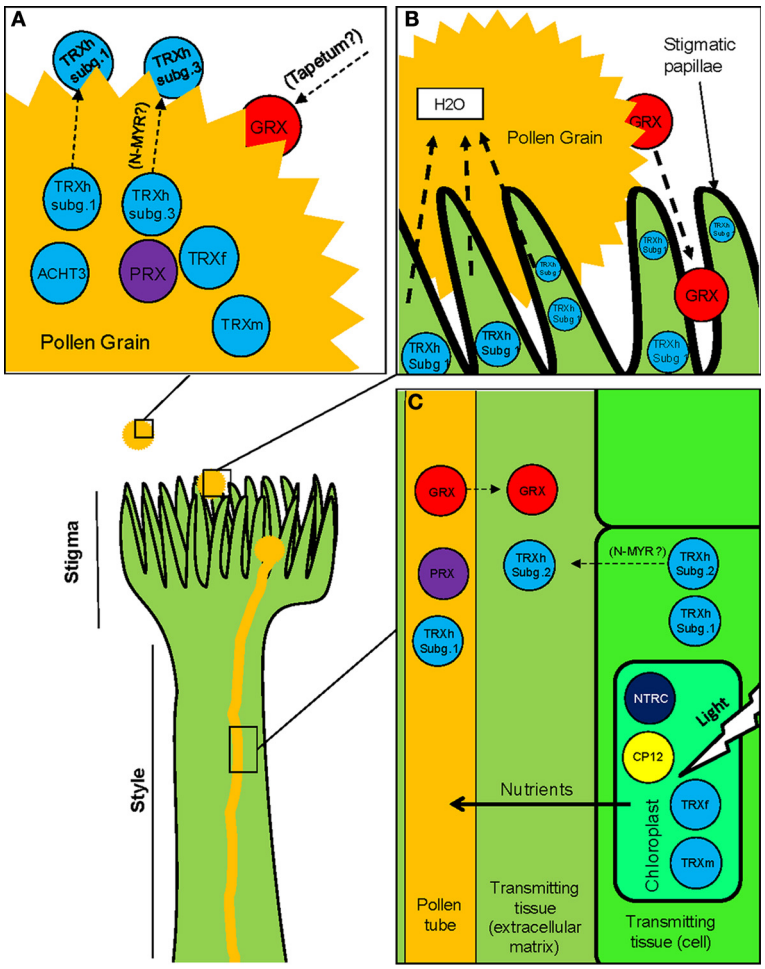

FIGURE 3 | Redoxins and related proteins are critical for pollen-pistil interactions. Illustration of the stigma and style during pollen-pistil interaction. **(A)** Representation of a pollen grain and the pollen coat. The presence of redoxins has been described both within the pollen grain and in the pollen coat. It is particularly remarkable the specific and conserved occurrence of h-type TRXs in the pollen grains, which can be secreted to the pollen coat. We hypothesize that N-terminal lipidations may play a role in this mechanism of secretion. Also, some redoxins synthesized in the tapetum may become integrated into the pollen coat after tapetum degeneration **(B)** Image representing the initial stages of the pollen-stigma interaction. Upon pollen arrival, pollen starts to hydrate, rapidly releasing GRXs present in the pollen coat. Also, the stigmatic papillae are rich in subgroup 1 TRXs. These TRXs have been involved in SI processes in *Brassica* although their occurrence has also been described for self-compatible species. **(C)** Representation of a pollen tube growing throughout the transmitting tissues of the style. Different types of redoxins are present in the pollen tube, some of them being secreted to the extracellular matrix of the transmitting tissue. Other redoxins are mainly expressed in stylar cells and may also be secreted to the extracellular matrix. Several redoxins related to photosynthesis are specifically expressed in the cells of the stylar tissues, which suggest that high photosynthetic rates are probably supporting pollen tube growth.

For example, the NADPH-TRX-Reductase C (NTRC) protein has been shown to be the main reducer of type-2 PRXs in chloroplasts (Kirchsteiger et al., 2009; Pulido et al., 2010). Apoplastic type III peroxidases (POXs), which are involved in cell wall polymerization via H_2O_2 regulation, should be assessed into their participation in this process (Hiraga et al., 2001). It is also well known the role of the ascorbic acid as main redox buffer in apoplast compartments (Foyer and Noctor, 2005a). Finally, overexpression of ROXY GRXs has also been shown to alter the level of H_2O_2 (Wang et al., 2009a,b).

Plant genomes contain higher number of genes encoding redoxins (like TRXs or GRXs) than other species, allowing them assigning specific isoforms to precise plant metabolic functions. This includes anther development and male gametogenesis. Remarkably, a similar case is found in mammals, where male germ cells are endowed with three testis-specific thioredoxins named Sptrx-1, Sptrx-2, and Sptrx-3, which are specially involved in spermatogenesis (Jiménez et al., 2004; Miranda-Vizuete et al., 2004).

UNEXPECTED AND SPECIFIC OCCURRENCE OF REDOXINS IN PLANT REPRODUCTIVE TISSUES SUGGEST KEY FUNCTIONS IN POLLEN-PISTIL INTERACTIONS

Once a mature and dehydrated pollen grain lands on the appropriate stigma, it rapidly hydrates and incorporates nutrients from the stigma exudates (wet stigmas) or the stigma papillae (dry

stigmas). This fact transforms the pollen grain into a polarized cell, which organizes its cytoplasm and cytoskeleton to support the extension of a tube within minutes after hydration (Edlund et al., 2004). This tube must grow to enter the transmitting tract along the style and finally reach the embryo sac where it will deliver the sperm nuclei that will participate of the double fertilization. At these stages, it is required a continuous interchange of both physical and chemical signals between partners (pollen, stigma, ovules…), which has to take place in a tight time frame (Dresselhaus and Franklin-Tong, 2013). Redox regulation and signaling by reactive species (**Figure 1**; see part 2) and probably by some redoxins (**Table 1**; **Figure 3**), might be critically involved in these signal exchanges.

Redoxins in pollen grain

TRXs *h* from subgroup 1 (Gelhaye et al., 2004), and GRXs are among the most abundant redoxins present in the pollen grain and the pollen coat (**Figure 3**). THL-1 is an h-type TRX that was immunodetected as a *B. rapa* extracellular pollen protein (Toriyama et al., 1998). However, although THL-1 is a well-known stigmatic protein involved in SI (Cabrillac et al., 2001), there is no clear data about its function in the pollen coat. The *Arabidopsis* h-type TRX*h*4 was shown to be expressed in the pollen grain and the pollen tube (Reichheld et al., 2002). Their counterparts AtTRX*h*1 and AtTRX*h*5, and the GRX AtGrxC2 proteins were detected in the pollen tube but not in the mature pollen grain. AtGrxC2 was also identified as a secreted protein (**Figures 3A,C**; Ge et al., 2011). Molecular and proteomic approaches also identified a GRX from triticale as a major pollen protein rapidly released upon pollen hydration on the stigma papillae (**Figure 3B**; Zaidi et al., 2012). This GRX contains a Gly in position 2, predicted to be co-translationally modified by N-myristoylation (N-MYR), a type of lipidation assisting protein anchoring to membranes, which therefore could account for secretion (**Figure 3B**; Denny et al., 2000; Utsumi et al., 2005; Martinez et al., 2008). The occurrence of this N-terminal modification has been recently evidenced *in vitro* for a similar GRX, AtGrxC1 from *Arabidopsis* (At5g63030) (Traverso, Pers. Commun.). It must be mentioned here that some of the proteins of the pollen coat are originated in the tapetum, and then incorporated into the pollen coat after the degeneration of this layer (**Figure 2B**), a mechanism that could be also suggested for the GRX released after hydration (**Figures 2A,B**; Zaidi et al., 2012).

The h-type TRXs belonging to the subgroup 3 (Gelhaye et al., 2004) were initially identified as a highly conserved group of pollen-expressed TRXs from both mono and dicots, featured by the presence of a N-terminal extension which contains conserved Gly and Cys residues in positions 2 and 4, respectively (Juttner et al., 2000). Curiously, these N-terminal extensions have been recently identified as a substrate for N-terminal myristoylation (N-MYR) as well as N-terminal palmitoylation (N-PAL). This last is another type of lipidation, usually identified in plasma membrane proteins (Traverso et al., 2008, 2013). No clear information is available concerning the specific roles or the subcellular localizations of these TRXs in pollen, although a member of this subgroup in *A. thaliana* (AtTRXh9) was shown to move from cell to cell via its N-terminal extension (Meng et al., 2010). According to these results, we hypothesized that these lipidations can be involved in the release of this subgroup of TRXs to the extracellular matrix, where others TRXs and redoxins have already been identified (**Figure 3A**; Ge et al., 2011; Zaidi et al., 2012).

Other types of redoxins have been identified as highly expressed in pollen. Two independent transcriptomic analyses (Becker et al., 2003; Lee and Lee, 2003) have shown ACHT3 TRX to be highly expressed in *Arabidopsis* pollen. However, no functional data are associated with this presence. In addition, PRXs are also among the redoxins displaying specific localization in pollen grains (**Figure 3A**). Transcriptomic analysis of *Arabidopsis* revealed two PRXs among the 50 most expressed genes in pollen. One of them (TPx2) showed increased expression under cold treatment (Lee and Lee, 2003). In addition, some type II PRXs from *Arabidopsis* showed specific expression patterns associated with male reproductive tissues (**Figures 3A,C**) (Bréhélin et al., 2003). The cytosolic AtPRXII-C is almost exclusively expressed in mature pollen, whereas its counterpart AtPRXII-D is detected in mature pollen, germinating pollen and pollen tubes, where both proteins could be reduced by AtTRXh4 (Reichheld et al., 2002; Bréhélin et al., 2003). Finally, the plastid-addressed AtPRDII-E has been described as mainly expressed in immature anthers and ovules (Bréhélin et al., 2003).

Considering that no clear functional data are associated with this specific occurrence in pollen grains, further work is necessary to better understand the precise redox mechanisms underlying pollen function. It is well-known that when pollen grains reach the appropriated stigma, they release a number of proteins from pollen coat, which together with the proteins released from pistil surrounding tissues, seem to play important roles during pollen-pistil interaction, adhesion, germination or pollen tube growth as well as providing protection against pathogen attack (Andersson and Lidholm, 2003; Grote et al., 2008; Zaidi et al., 2012). In a different context, redoxins from the pollen coat have been attributed with some allergic potential (Toriyama et al., 1998). However, there is no evidence supporting this fact, with the exception of the description of a 1-Cys PRXs and a h-type TRX as respiratory wheat flours allergens from maize (Fasoli et al., 2009; Pahr et al., 2012).

Occurrence of GR/GRX and NTR/TRX systems during pollen germination and pollen tube growth

Several works based on the characterization of *A. thaliana* mutant lines have evidenced that GRXs and TRXs are specifically involved in pollen germination and pollen tube growth. The *Arabidopsis* double mutant *ntra ntrb*, lacking NTR activity to reduce cytosolic h-type TRXs, showed a reduced fitness due to defects in pollen functions (Reichheld et al., 2007). Under this genetic background, GRXs are also able of directly reduce h-type TRXs although in lesser extension, thus revealing a more complex *in vivo* interplay between the TRX and glutathione pathways (Reichheld et al., 2007). In fact, the additional disruption of the glutathione reductase 1 gene (*GR1*) under this double mutant background (triple mutant *ntra ntrb gr1*) led to a pollen lethal phenotype (Marty et al., 2009). Noteworthy, the characterization of the single mutant *gr1* demonstrated that a residual reduction of GSSG could be directly attributed to h-type TRXs, reason why this NTR/TRX

system was described as a functional backup for the activity of GR1 (Marty et al., 2009).

The characterizations of the double (*ntra ntrb*) and triple mutant (*ntra ntrb gr1*) headed to other important conclusions. Defects in pollen grains were not associated with gametogenesis, since mature pollen grains inside the anthers were viable in both mutants (Reichheld et al., 2007; Marty et al., 2009). Then it is important to elucidate, in our opinion, in which subsequent process these redox systems are critically involved: (i) pollen germination, (ii) pollen tube growth, (iii) polarity or guidance, or (iv) pollen tube–embryo sac interactions and fertilization.

A second conclusion is that the lack of both redox systems drastically affected male gametophyte functions, contrary to what occurred in the female haploid gametophyte. The unpaired NTR-TRX System (NTS) in the double *ntra ntrb* mutant yielded reduced fitness in pollen grains, probably derived from a limited reduction of h-type TRXs, although the diploid sporophyte or the female gametophyte did not show such drastic phenotypes (Reichheld et al., 2007). In a similar way, the *ntra ntrb gr1* triple mutant produced a lethal phenotype in pollen, while the female embryo sac was unaffected (Marty et al., 2009). This strong difference is probably associated with the exceptional burst metabolism occurring during pollen germination, that produces important redox imbalances and would justify the exceptional requirement of both thiol-dependent redox GR/GRX and NTR/TRX systems. This is in agreement with the results compiled in our present review, showing how the h-type TRXs or the GRXs are specifically found in pollen grain, pollen tube or pollen coat.

Within this context, it must be noted the importance of GSH and auxins for pollen functionality. Zechmann et al. (2011) have shown that GSH availability is essential for pollen germination and early elongation steps of the pollen tube, since its depletion triggers disturbances in the auxin metabolism, which led to inhibition of pollen germination. Considering that NTR/TRX and GSH pathways are involved in auxin homeostasis (Bashandy et al., 2010, 2011), we suggest that both redox systems can act somehow linking the GSH cellular status and auxin downstream signals during and/or after pollen germination.

Redoxins in female reproductive tissues

TRXs h have been shown to be specifically expressed in the stigmatic papillae of different plants (**Figure 3B**). The PsTRXh1 from *P. sativum* has been immunolocalized in the receptive stigmatic papillae and the mature pollen grain of this plant (Traverso et al., 2007). THL-1 from *B. rapa*, a TRXs h from the same subgroup, which has been involved in SI (Cabrillac et al., 2001) also shows a similar pattern of expression in flower tissues than PsTRXh1. Considering that *P. sativum* is a self-compatible species, the role associated with this dual localization in pea flower is unlikely implicated in SI. Besides, an h-type TRX from subgroup I was included within the 50 most highly expressed genes characterized in a transcriptomic analysis from saffron stigmas (D'Agostino et al., 2007), and another TRX, h-type *Arabidopsis* h1 was distinctively detected in the style (Reichheld et al., 2002).

Classical chloroplastidial TRXs or TRX-related proteins have also been associated with pistils (**Figure 3C**). For example, expression of Pea TRXs m and f types was specifically detected in pollen grains, tapetum, style and ovules (de Dios Barajas-López et al., 2007). Other plastidial TRX-related proteins like the NTRC or CP12 redox proteins are also present in the style (**Figure 3C**). CP12 are small, dithiol-based redox-sensitive proteins which together with the plastidial TRX f, regulate the activity of the Calvin cycle in response to rapid light changes (Howard et al., 2008). The redox state of CP12 has been shown to be regulated by TRXs (Marri et al., 2009). The *Arabidopsis* genome contains three genes encoding CP12 proteins (CP12-1, 2 and 3), which could be involved in plant reproduction since CP12-1 and 2 are specifically expressed in the style (Singh et al., 2008). No clear roles are associated with these expressions, although CP12-antisense lines of tobacco and *Arabidopsis* display a complex phenotype including reduced fertility (Singh et al., 2008). AtNTRC is also a redox plastidial protein characterized as the principal reducer of 2-Cys PRXs (Kirchsteiger et al., 2009; Pulido et al., 2010). This protein allows the use of NADPH to maintain the redox status of the chloroplast (Spínola et al., 2008), as well as acting as a general molecular switch able to convert NADPH into redox signal in non-photosynthetic plastids (Kirchsteiger et al., 2012). Remarkably, this protein also shows a high level of expression in the style (Kirchsteiger et al., 2012).

Pollen tube growth throughout the style depends on nutrients supported by female tissues (Lind et al., 1996; Taylor and Hepler, 1997; Wu et al., 2001). The presence of photosynthesis regulatory proteins like those described here might be derived from the necessity of extra nutrients from the stylar photosynthetic cell to feed pollen tubes. However, we cannot exclude other possibilities like their involvement in the control of the redox imbalance derived from the burst metabolism of the growing pollen tube. Under this context, the redox state of two key enzymes of the Benson-Calvin cycle (fructose-1,6-bisphosphatase and phosphoribulokinase) could be regulated by S-nitrosylation, since they have been previously identified as S-nitrosylated proteins (Lindermayr et al., 2005; Begara-Morales et al., 2013).

SELF-INCOMPATIBILITY AND h-type TRXs

Self-incompatibility (SI) is a mechanism adopted by flowering plants to prevent self-fertilization as well as to avoid crossings with genetically-related plants, promoting outcrossing and subsequently, genetic diversity in the offspring (Iwano and Takayama, 2012). Therefore, this mechanism has been critically relevant on the dominant position reached by flowering plants in the biosphere during evolution (Gaude and Cabrillac, 2001). Genes involved in SI display high allelic variability, and are referred as haplotypes in the literature. SI systems are based on the discrimination between male-specificity and female-specificity determinants (S-determinants) localized in the surface of pollen grains and the stigmatic papillae respectively, both encoded at the S-locus. Therefore, SI depends on a specific interaction between male and female S-determinants derived from the same or different S-haplotypes (depending on the plant family. For a review see Iwano and Takayama, 2012). Although these determinants differ among plant species, the redox activity of the TRXs h has been described as critically involved within most of the proposed SI molecular models.

In the plant *Phalaris coerulescens*, the SI model system is based on two multiallelic not-linked loci, called S and Z (Hayman and Richter, 1992; Li et al., 1997; Klaas et al., 2011). Locus S encodes a monocot-conserved protein exclusively expressed in mature pollen. Its N-terminal domain is highly variable and contains the allelic information, whereas the C-terminal end contains a conserved TRX h-type motif responsible of the catalytic activity and its disruption yields the lost of the SI (Li et al., 1994, 1995). Although no enough data are available for assembling a clear model, the redox activity of the TRX domain has been shown to be essential for the SI response in *P. coerulescens* (Li et al., 1996).

In the classical model of *Brassica*, several genes are involved in SI, all of them linked to the S-locus. Two of these genes encode for the female and male determinants, the S-locus Receptor Kinase (SRK) expressed in the stigma-papilla cells and its ligand, the S-locus Cys rich protein (SCR) localized in the pollen coat (Schopfer et al., 1999; Takasaki et al., 2000). Pollen rejection is achieved when SRK recognizes SCR coming from the same S-allele. SRK is a transmembrane protein with a serine threonine kinase activity in the cytoplasmic region, which triggers the downstream biochemical pathways of the SI response (Giranton et al., 2000). In a classical model suggested by Cabrillac et al. (2001), this kinase activity is reversibly blocked by the direct interaction with a h-type TRX (THL-1) in the subcortical cytoplasmic side of stigma papillae cells, and only the allele-specific interaction between SCR and SRK produces the release of the TRX, therefore releasing the kinase activity (Cabrillac et al., 2001). However, according to Ivanov and Gaude (2009a), SRK is mostly intracellular, and the scarce part present in different domains of the plasma membrane can interact with SCR. After ligand recognition, the receptor-ligand complex is internalized in endosomes enriched in the negative regulator TRX h (Ivanov and Gaude, 2009a). In fact, these authors proposed a new model, the SI "domain" model, where the role of the TRX is mainly relegated to the endomembrane system (Ivanov and Gaude, 2009b). h-type TRXs are the biggest cluster of plant TRXs, encoded by almost eleven TRXs in the genome of *A. thaliana*, which have been usually described as soluble proteins due to the lack of transit peptides (Florencio et al., 1988). However, some TRXs h have recently been described to be modified by lipidation (myristoylation and palmitoylation) at their N-terminal extensions. These modifications result in their localization to the endomembrane system or to the plasma membrane (Traverso et al., 2008, 2013) and are in agreement with the endomembrane localizations of the TRXs h shown by Ivanov and Gaude (2009a). Independently from the models suggested, the role of the TRX h in *Brassica* SI mechanism was demonstrated, since antisense transgenic lines for THL-1 displayed a limited level of SI only (Haffani et al., 2004). Furthermore, it has been shown that this role depends on both the redox activity of the TRX, and the occurrence of a specific sequence in their active centers -WCPPC- (Mazzurco et al., 2001).

The SI model mechanism described for *Solanaceae, Rosaceae, and Plantaginaceae* families is based on the activity of secreted S-RNases. In all three families, compatibility is also controlled by a polymorphic S-locus containing at least two genes. S-RNases determine the specificity of pollen rejection in the pistil, and S-locus F-box proteins fulfill this function in pollen. In *N. alata*, Juárez-Díaz et al. (2006) suggested the participation of a h-type TRX (NaTRXh), which interacts with the S-RNase in the extracellular matrix of the stylar transmitting tract (**Figure 3C**). Although these authors suggested that NaTRXh was secreted due to some information present in its N-terminal extension, the secretion mechanism remained unexplained. Remarkably, NaTRXh has also been characterized as a N-myristoylated membrane-associated protein. Therefore, this characteristic could be involved in its secretion (**Figure 3C**; Traverso et al., 2013).

ROS and RNS have also been shown to be involved in SI mechanisms. In the *Papaver* model of SI (where no TRXs have been directly implicated), SI response seems to be mediated by ROS/NO redox signaling. Allele-specific interaction was shown to induce a rapid and transitory increase of ROS and NO in *Papaver rhoeas* pollen tubes. In this model, SI is triggered by an increase of intracellular Ca^{2+} in the pollen tube, which ultimately originates actin reorganization and programmed cell death resulting in the destruction of the self-pollen. ROS/NO seem to act mediating the signal between calcium and PCD (Wilkins et al., 2011). In the S-RNase-based model of SI response, the S-RNase specifically disrupts tip-localized ROS of incompatible pollen tubes via arresting ROS formation in mitochondria and cell walls of *Pyrus pyrifolia* (Wang et al., 2010a,b).

All these SI mechanisms usually originate the rejection of the allele-incompatible pollen grains by affecting the pollen tube growth throughout the stigma and the style. Although independent SI molecular mechanisms are known, which indicates that SI has clearly evolved independently several times during plant evolution (Takayama and Isogai, 2005), the involvement of the TRXs (and probably other redoxins) as well as the signaling mediated by ROS or NO have been evidenced in different SI models proposed. Further experiments are needed to clarify the role of redoxins in all these models within the general context of redox regulation and signaling, which includes reactive species-mediated signaling.

CONCLUDING REMARKS

In this review, we have listed and discussed the most remarkable evidences suggesting that molecular and cellular events involved in sexual plant reproduction are critically influenced by plant-conserved and specific thiol-based redox mechanisms, which include ROS and NO together with several isoforms of TRXs, GRXs and other redox-related proteins. These plant reproductive-specific redox mechanisms likely appeared as a consequence of the high number of isoforms of redoxins available, which emerged during plant evolution and somehow resulted in beneficial characteristic for plant physiology. However, and comparatively with non-reproductive plant tissues and organs, much is still unknown about the nature, presence, localization and molecular features of these proteins.

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