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MYB4 and HY5 integrate light and cytokinin signaling pathways during Arabidopsis seedling development

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Arabidopsis seedling growth is regulated by integration of various environmental and hormonal signals. While the interactions between light and cytokinin signaling pathways have been studied, the molecular mechanism by which their interaction regulate hypocotyl elongation remain unclear. In this study, we demonstrate that MYB4, a positive regulator of photomorphogenesis, physically interacts with HY5 and attenuates HY5-mediated regulation of *MYB4*. The expression of *MYB4* is induced by different wavelengths of light and it genetically interacts with *HY5* to regulate hypocotyl length during light-mediated seedling development. The *MYB4* and *HY5* mediated regulation of hypocotyl length is altered upon cytokinin treatment in a light intensity-dependent manner. Furthermore, on contrary to the light signals, cytokinin suppresses *MYB4* expression. MYB4 together with HY5 regulates the expression of the key genes such as *ABCG14*, *ARR4*, and *CHS* involved in cytokinin signaling pathway. Taken together, this study highlights how the HY5-MYB4 module integrates light and cytokinin signals to fine-tune Arabidopsis seedling development.

KEYWORDS

MYB4, HY5, photomorphogenesis, cytokinin, seedling development

Introduction

Plants, as photoautotrophs, are highly sensitive to their light environment. Light plays a crucial role not only as an energy source for photosynthesis but also in influencing various developmental and physiological processes throughout the plant's life cycle. These processes include seed germination, seedling photomorphogenesis, photoperiodic responses, shade avoidance, and flowering (Deng and Quail, 1999; Sullivan and Deng, 2003; Chen et al., 2004; Jiao et al., 2007; Bentsink and Koornneef, 2008; Alvarez-Buylla et al., 2010). Plants have evolved with a sophisticated sensory network that monitors key aspects of their illuminated environment, including light intensity, quality, duration, and direction (Kendrick and Kronenberg, 1994). These various light signals are detected by at least five classes of wavelength-specific photoreceptors, including phytochromes (PHYA-

PHYE), cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2), F-box-containing flavin-binding proteins (ZTL, FKF1, and LKP2), and UV-B RESISTANCE LOCUS 8 (UVR8) (Paik and Huq, 2019). These photoreceptors are biologically activated by different light signals, leading to widespread transcriptional reprogramming at the genome level (Jing and Lin, 2020).

Extensive genetic and biochemical research has shown that the ELONGATED HYPOCOTYL5 (HY5), a bZIP transcription factor, is a key regulator of light-responsive transcriptional changes. HY5 primarily binds to *ACGT*-containing cis-elements (such as the G-box and T/G-box) of numerous target genes, thereby modulating various light-regulated physiological and developmental processes in plants (Chattopadhyay et al., 1998, Lee et al., 2007; Zhang et al., 2011). Mutant seedlings lacking HY5 function exhibit significantly elongated hypocotyls under different light conditions (Oyama et al., 1997), indicating that HY5 operates downstream of multiple photoreceptors to promote photomorphogenesis. In addition to inhibiting hypocotyl elongation, HY5 also regulates various other physiological and developmental processes, including root growth, pigment biosynthesis and accumulation, responses to various hormonal signals, and adaptation to low and high temperatures (Marzi et al., 2020; Bhagat et al., 2021; Wang et al., 2021). Similar to HY5, CAM7 (Calmodulin7), also known as Z-box binding factors 3 (ZBF3) (McCormack et al.,2005; Kushwaha et al., 2008) acts as transcription factor and directly interacts with promoters of several light-inducible genes (Kushwaha et al., 2008; Kumar et al., 2016). Previous studies have demonstrated that CAM7 physically interacts with HY5 to enhance the activity of the *HY5* promoter and promote photomorphogenesis (Kushwaha et al., 2008; Abbas et al., 2014). More recently, a yeast two-hybrid screen using CAM7 as bait identified MYB4 as one of its interacting partners. *CAM7* also interacts genetically with *MYB4* to control the hypocotyl length during Arabidopsis seedling development. Additionally, it was reported that both HY5 and CAM7 bind to the promoter of *MYB4* and positively regulate its expression (Dutta et al., 2024).

The MYB protein family encompasses a large group of transcription factors that play roles in various plant-specific processes (Dubos et al., 2010). MYB4, a well-studied member of the R2R3-MYB subfamily, is known for regulating the phenylpropanoid metabolic pathway, which contributes to UV-B light resistance (Jin et al., 2000; Zhao et al., 2007; Fornalé et al., 2014; Zhou et al., 2015; Wang et al., 2019). MYB4 functions as a transcriptional repressor by directly targeting the expression of *C4H* (encodes cinnamate 4-hydroxylase, a crucial enzyme in the biosynthesis of sinapate esters), and *AtMYB7*, another transcriptional repressor of several flavonoid biosynthesis genes. *MYB4* is expressed in various organs of adult plants, including roots, stems, leaves, and flowers (Fornalé et al., 2014). Studies on light-mediated regulation of *MYB4* expression in rosette leaves have shown that it is expressed in darkness and is induced under various wavelengths of light, including white, blue, and UV light (Jin et al., 2000). MYB4 has also been found to directly influence flavonoid biosynthesis by repressing the expression of *ADT6* (*AROGENATE DEHYDRATASE 6*) and interfering with the transcriptional activity of MBW (composed of MYB, bHLH, and WD40 proteins) complexes through interaction with bHLH transcription factors (Baudry et al., 2004; Xu et al., 2014; Wang et al., 2019). More recently, it has been shown that the N-terminal MYB domains of MYB4 play a role in its stability and folding under thermal stress in Arabidopsis thaliana (Mitra et al., 2021).

The internal hormonal balance in plants plays a significant role in determining its sensitivity and response to various environmental stimuli. Light signaling components like HY5, PIF3, and PIF4 act as key integrators of light and hormonal pathways by regulating the levels of gibberellin, abscisic acid, auxin, and cytokinin in Arabidopsis (Sibout et al., 2006; Chen et al., 2008; Feng et al., 2008; de Lucas et al., 2008; Lau and Deng, 2010; Rasmussen et al., 2012; Yu et al., 2013; Gangappa and Botto, 2016; van Gelderen, 2018; Yang et al., 2018; Doroshenko et al., 2020; Bhagat et al., 2021; Duan et al., 2021; Dai et al., 2022; Chen et al., 2024; Basu et al., 2025). Among the various hormones, exogenous cytokinin treatment resulted in paler plants that exhibited stunted growth, elevated levels of anthocyanin and diminished apical dominance (Chory et al., 1994). Previously, it was also demonstrated that cytokinins enhance the *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) and *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN* (*RBCS*) levels (Fierabend and deBoer, 1978; Flores and Tobin, 1986). Additionally, the RESPONSE REGULATOR 4 (ARR4) protein, which mediates cytokinin action, directly interacts with PHYB and stabilizes its active form (Sweere et al., 2001). Despite the significant resistance of *hy5* seedlings to systemic cytokinin application in both shoot and root growth inhibition (Cluis et al., 2004), cytokinin induces the accumulation of HY5 regardless of light conditions. The MYB transcription factors in *Arabidopsis thaliana* also play a crucial role in regulating hormone signaling pathways. For instance, AtMYB59 has been shown to influence cytokinin signaling by modulating the expression of *ARR16*, a key component of the cytokinin signal transduction pathway (Mu et al., 2009). Since *CAM7* genetically interacts with *HY5* and *MYB4*, and that HY5 regulates the *MYB4* transcript abundance, we examined the genetic and molecular interrelation between HY5 and MYB4 during Arabidopsis seedling development emphasising on light and cytokinin signaling pathways.

Results

MYB4 physically interacts with HY5

A recent study has demonstrated that MYB4 physically interacts with CAM7, and both CAM7 and HY5 bind to the *MYB4* promoter to regulate its transcriptional activation (Dutta et al., 2024). To investigate whether MYB4 physically interacts with HY5, we conducted *in vitro* pull-down assays using poly-His and GST fusion proteins. GST, GST-CAM7, and GST-MYB4 were incubated with Ni-NTA beads to which HY5-6His was bound. The α-GST immunoblot of the pulled down proteins revealed that GST-MYB4 was selectively pulled down by HY5-6His similar to

GST-CAM7, used a positive control (Abbas et al., 2014), however not with GST alone (Figure 1A, Upper Panel). The same membrane was then stripped and re-probed using α-His to examine the equal loading of HY5 (Figure 1A, Lower Panel). These results suggest that MYB4 and HY5 physically interact with each other.

MYB4 has two distinct domains; the N-terminal domain (9-116 aa) comprises of two MYB domains and the C-terminal domain (117-282 aa) comprises of transcriptional activation domain (Mitra et al., 2019) (Figure 1B). To determine the HY5 interacting domain of MYB4, we conducted yeast two-hybrid assays to examine the domain wise protein-protein interaction. As shown in Figure 1C, the C terminal domain of MYB4 interacted with HY5, however not the N-terminal domain. The negative controls involving empty vectors (AD and BD) and their respective combinations with HY5 and MYB4 (full length and truncated) did not exhibit any physical interaction. These results suggest that MYB4 physically interacts with HY5 through the C terminal domain.

To validate this physical interaction *in vivo*, we performed co-immunoprecipitation assay. The rosette leaves of 30-d old *hy5* mutant (Oyama et al., 1997) and HY5 OE line (Lee et al., 2007) were infiltrated with *Agrobacterium* harbouring either GFP, GFP-CAM7 (positive control, Abbas et al., 2014), or GFP-MYB4 constructs, resulting in transient expression of these proteins. As shown in Figure 1D, HY5 was co-immunoprecipitated with GFP-CAM7 and GFP-MYB4 but not with GFP alone in HY5 OE background. However, in *hy5* mutant background, used as negative control, HY5 wasn't coimmunoprecipitated with any of the transiently expressed proteins. These results further confirm that MYB4 physically interacts with HY5.

MYB4 genetically interacts with *HY5* to regulate photomorphogenic growth

MYB4 and *HY5* work as positive regulators of photomorphogenesis at various wavelengths of light (Koornneef et al., 1980; Oyama et al., 1997; Ang et al., 1998; Chattopadhyay et al., 1998; Brown and Jenkins, 2008; Dutta et al., 2024). To further investigate the role of MYB4 in photomorphogenesis, we first examined whether *MYB4* expression is induced during dark-to-light transition. The semi-quantitative and quantitative PCR analyses revealed that *MYB4* expression was induced at various wavelengths of light during dark to light transition. As shown in Figures 2A–D and Supplementary Figure 2, the level of induction varied with context to time and wavelength of light tested. We then determined the physiological significance of the physical interaction between MYB4 and HY5 through genetic studies. For that, the *myb4* was genetically crossed with *hy5* mutant, and the homozygous *myb4 hy5* double mutant lines were generated Supplementary Figure 3). To determine the genetic relationship between *MYB4* and *HY5*, 6-day-old wild-type (segregated WT of F2 population), *myb4, hy5*, and *myb4 hy5* seedlings were grown in dark and at various wavelengths of light. Dark-grown seedlings exhibited hypocotyl length similar to the WT background (Figures 3A, B). The *myb4* mutant seedlings displayed significantly increased hypocotyl length as compared to WT under white light (WL), blue light (BL), and far-red light (FRL) (Figures 3C–H). No such effect was observed under red light (RL) condition (Figures 3I, J). Consistent with the previous findings, *hy5* mutant exhibited a significantly elongated hypocotyl compared to WT under all light conditions tested (Ang et al., 1998). The hypocotyl of *myb4 hy5* double mutant was found to be significantly more elongated than each of the single mutants in WL, BL, and FRL, however not in RL (Figures 3C–J). These results suggest that *MYB4* and *HY5* work in an additive manner to control the hypocotyl length under WL, BL and FRL.

MYB4 modulates HY5-mediated physiological responses

We then examined whether the genetic interaction between *MYB4* and *HY5* could influence the expression of light-inducible genes. To determine this, we assessed the transcript levels of *RBCS-1A* and *CAB1* in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings grown under constant WL (30 μmol m^{-2} s^{-1}). As previously reported, the expression of these genes was significantly decreased in *hy5* background as compared to the WT (Ang et al., 1998; Chattopadhyay et al., 1998). However, as shown in Figure 3K, the additional mutation of *MYB4* in *hy5* mutant, did not alter the expression of *RBCS-1A* and *CAB1*, resulting in the expression pattern similar to that of *hy5* in *myb4 hy5* double mutant. These results collectively suggest that additional mutation in *MYB4* in *hy5* background does not affect the expression of light inducible genes.The accumulation of chlorophyll and anthocyanin is an important physiological response controlled by light signaling pathways (Lifschitz et al., 1990; Neff and Chory, 1998; Fankhauser and Casal, 2004). It has been reported earlier that *hy5* mutant seedlings show a reduced level of chlorophyll and anthocyanin contents (Oyama et al., 1997; Shin et al., 2007). To examine the significance of the genetic interplay between *MYB4* and *HY5* in the accumulation of chlorophyll and anthocyanin, we assessed the chlorophyll and anthocyanin levels in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* double mutant seedlings grown in WL (30 $\mu molm^{-2}s^{-1}$). As shown in Figures 3L, M, while chlorophyll content between *myb4* and WT seedlings was similar (Figure 3L), an increase in anthocyanin accumulation was observed in *myb4* mutants when compared to the WT seedlings (Figure 3M). The additional mutation of *MYB4* in *hy5* mutant seedlings did not alter the level of chlorophyll accumulation (Figure 3L), suggesting that *HY5* regulates the chlorophyll accumulation independently of *MYB4*. However, as shown in Figure 3M, the anthocyanin content of *myb4 hy5* double mutant was similar to WT background, suggesting that *MYB4* and *HY5* work antagonistically to regulate the anthocyanin accumulation.

MYB4 regulates HY5 mediated activation of its own promoter

Since HY5 binds to the *MYB4* promoter and positively regulates its expression, and given that HY5 and MYB4 physically interact,

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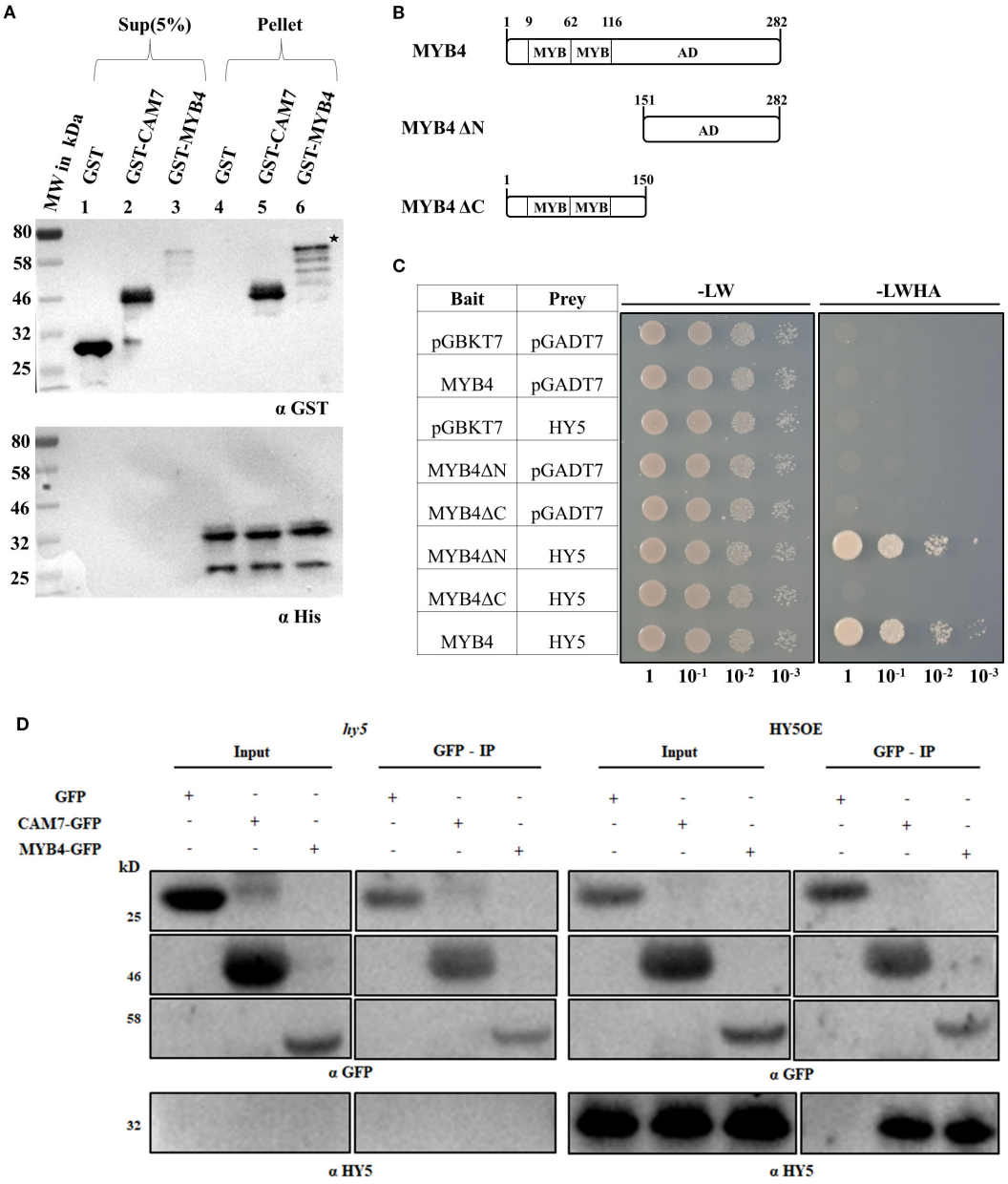

FIGURE 1
MYB4 physically interacts with HY5. **(A)** (Upper Panel). *In vitro* binding of MYB4 and HY5. Approximately 2 µg of HY5-6His was bound to Ni-NTA beads. GST, GST-CAM7 and GST-MYB4 protein was added in equimolar ratio. Supernatant (Sup5%) and pellet fractions were fractionated by SDS-PAGE, blotted, and probed with anti-GST antibodies. Lanes 2 and 5 show HY5-6His with GST-CAM7 (positive control), lanes 3 and 6 show HY5-6His with GST-MYB4, and lanes 1 and 4 show HY5-6His with GST (negative control). (Lower Panel). The membrane was stripped and re-probed with anti-His antibody (for loading control). The position of the full-length GST-MYB4 protein in elute fractions are indicated by asterisk. MW (molecular weight) is in kDa (Kilo Dalton). **(B)** Diagram illustrating different segments of MYB4, including the full-length protein and various fragments, used in yeast two-hybrid experiments. Each segment is labelled with corresponding amino acid numbers. **(C)** Co-transformed yeast cells were grown in 2D [lacking Leucine (L) and Tryptophan (W) amino acids] and 4D [lacking Leucine (L), Tryptophan (W), Adenine (A) and Histidine (H) nutrients] selective media to test the protein-protein interactions. The empty vectors (pGBKT7 and pGADT7) and their combinations with MYB4 (pGBKT7 + pGADT7-MYB4; pGBKT7 + pGADT7-MYB4ΔN; pGBKT7 + pGADT7-MYB4ΔC) and HY5 (pGBKT7-HY5 + pGADT7) were used as negative controls. Numbers below the panel indicate dilutions of the starting culture spotted on dropout media. **(D)** MYB4 interacts with HY5 *in vivo*. CAM7-GFP and MYB4-GFP were transiently co-expressed with empty vector as negative control in rosette leaves of 30-d old Arabidopsis *hy5* and HY5OE (Lee et al., 2007; Abbas et al., 2014) lines. The protein samples were immunoprecipitated with anti-GFP antibody. Both input (5%) and IP fractions were immunoblotted with anti-GFP antibody (upper panel) and the co-immunoprecipitated proteins were detected with anti-HY5 antibody (lower panel). The interaction between CAM7 and HY5 served as the positive control. The experiment was repeated three times and similar results were obtained.

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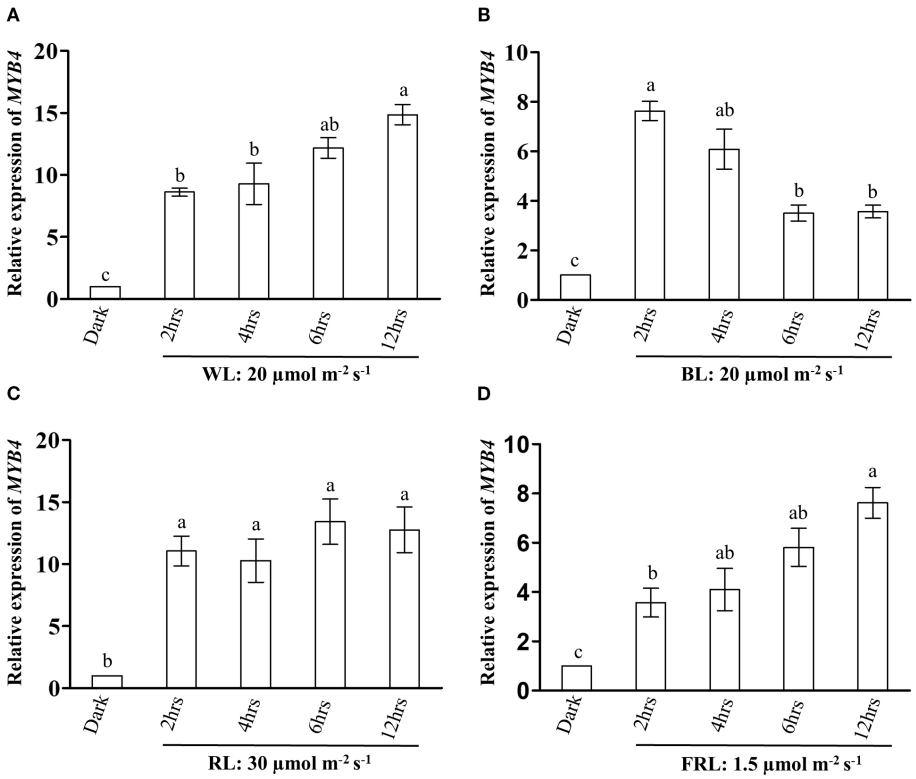

FIGURE 2
The expression of *MYB4* is induced during dark to light transitions. **(A, D)** Transcript abundance of *MYB4* in 5-day-old dark grown WT (ecotype Landsberg erecta) seedlings transferred either to white light (WL: 20 µmolm^{-2}s^{-1}) **(A)** blue light (BL: 20 µmolm^{-2}s^{-1}) **(B)** red light (RL:30 µmolm^{-2}s^{-1}) **(C)** or far-red light (FR: 1.5 µmolm^{-2}s^{-1}) **(D)** for various time points. *ACTIN2* was used as the internal control. Error bars represent ± SE of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) of *MYB4* transcript level. The data were compared by using one-way ANOVA factorial analysis followed by Tukey's HSD test.

we aimed to investigate the physiological relevance of this physical interaction in mediating the transcriptional regulation of *MYB4*. To investigate this, we analysed the DNA-protein interaction between the *MYB4* promoter and HY5 in the absence or presence of MYB4. Previously it has been reported that the *MYB4* promoter contains three E-Boxes, and HY5 specifically binds to the third E-Box (Dutta et al., 2024). We conducted electrophoretic mobility shift assays (EMSA) using purified GST-HY5 and GST-MYB4 fusion proteins, along with both wild-type and mutated versions of the *MYB4* promoter fragments (Figure 4A). As shown in Figure 4B, HY5 exhibited strong binding to the wild-type *MYB4* promoter fragment, however not to the mutated one. The binding affinity of HY5 to wild-type *MYB4* promoter fragment is decreased in the presence of MYB4 (Figure 4B, lane nos. 6 and 7). The reduction in HY5 binding affinity became more pronounced with increasing concentrations of MYB4. However, the DNA-protein complex of HY5 with the wild-type promoter fragment remained unchanged in the presence of GST alone (Figure 4B, lane no. 4). Additionally, no DNA-protein complex was formed between MYB4 and the wild-type promoter fragment (Figure 4B, lane no. 3).

To further gain insight into this regulation, we conducted transient expression assays in Arabidopsis protoplasts using a construct in which the wild-type *MYB4* promoter (DNA fragment containing HY5 but not MYB4 binding sites) was fused to the β-glucuronidase reporter gene (*Pro AtMYB4-GUS*) (Figure 4C). As shown in Figure 4D, while HY5 increased the *MYB4* promoter activity by ~2.2-fold, MYB4 alone did not alter the promoter activity. However, when both MYB4 and HY5 were introduced into the protoplast, MYB4 could drastically reduce the HY5 mediated increase in *MYB4* promoter activity (Figure 4D). Since we omitted the MYB4 binding site in the transactivation assay, the negative regulation is probably not due to direct binding to the promoter which supports our EMSA results. These results collectively suggest that the physical interaction between MYB4 and HY5 modulates HY5-mediated transcriptional activation of *MYB4*.

MYB4 and HY5 mediate the integration of light and cytokinin signaling

Previous studies have shown that HY5 protein is stabilized by light (Ang et al., 1998; Wang et al., 2021; Fang et al., 2024) and cytokinin (Vandenbussche et al., 2007). As this study revealed the genetic interplay between *HY5* and *MYB4* during photomorphogenic growth, we wanted to explore the genetic interaction between *MYB4* and *HY5* upon cytokinin treatment. To examine this, we first analyzed whether cytokinin alters *MYB4* expression. For this, we monitored

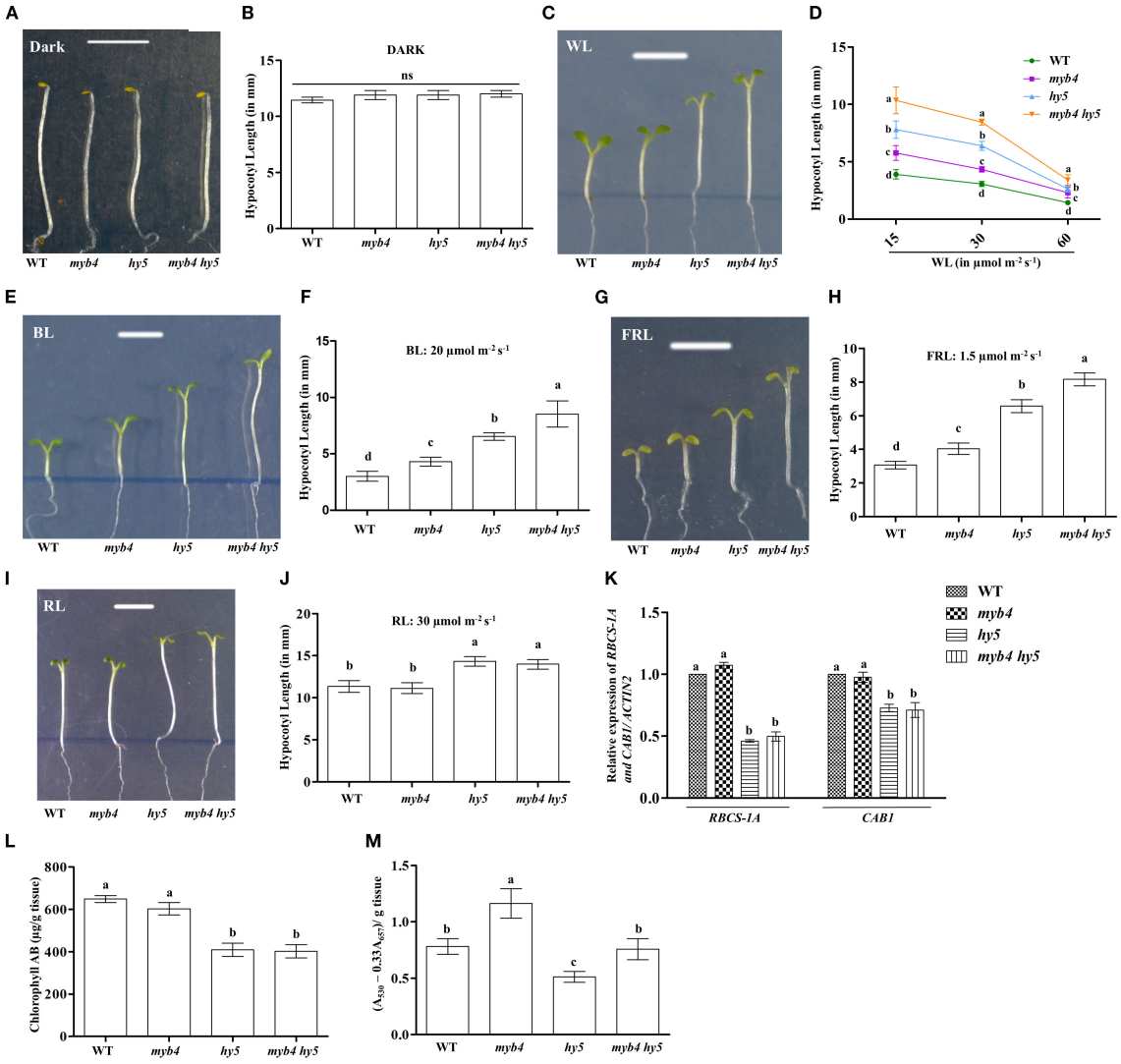

FIGURE 3
MYB4 works additively with *HY5* to regulate the hypocotyl elongation. **(A, B)** Visible Phenotype **(A)** and Quantification of hypocotyl length **(B)** of 6-day-old wild type (Segregated WT, Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant dark. Scale bar = 5 mm. The error bars indicate ± SD (n=20). **(C, D)** Visible Phenotype **(C)** of 6-day-old wild type (Segregated WT, Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant WL (15 µmolm^{-2}s^{-1}). Scale bar = 5 mm. Quantification **(D)** of hypocotyl length of WT (Segregated WT Ws-Ler), *myb4*, *hy5* and *myb4 hy5* double mutant seedlings grown in various fluence of WL. The error bars indicate ± SD (n=20). **(E, F)** Visible Phenotype **(E)** and Quantification of hypocotyl length **(F)** of 6-day-old wild type (Segregated WT, Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant BL (20 µmolm^{-2}s^{-1}). Scale bar = 5 mm. The error bars indicate ± SD (n=20). **(G, H)** Visible Phenotype **(G)** and Quantification of hypocotyl length **(H)** of 6-day-old wild type (Segregated WT, Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant FRL (1.5 µmolm^{-2}s^{-1}). Scale bar = 5 mm. The error bars indicate ± SD (n=20). **(I, J)** Visible Phenotype **(I)** and Quantification of hypocotyl length **(J)** of 6-day-old wild type (Segregated WT, Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant RL (30 µmolm^{-2}s^{-1}). Scale bar = 5 mm. The error bars indicate ± SD (n=20). **(K)** Real-time PCR analyses of *RBCS-1A* and *CAB1* in 6-day-old WT (Segregated WT Ws-Ler), *myb4*, *hy5* and *myb4 hy5* seedlings grown under constant white light (30 µmolm^{-2}s^{-1}). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. *ACTIN2* was used as endogenous control. **(L)** Quantification of accumulation of chlorophyll in 6-day-old WT (Segregated WT Ws-Ler), *myb4*, *hy5* and *myb4 hy5* double mutant seedlings grown under constant white light (30 µmolm^{-2}s^{-1}). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. **(M)** Quantification of accumulation of anthocyanin in 6-day-old WT (Segregated WT Ws-Ler), *myb4*, *hy5* and *myb4 hy5* double mutant seedlings grown under constant white light (30 µmolm^{-2}s^{-1}). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test.

MYB4 transcript levels in WT seedlings grown on MS plates, with or without 2 µM and 4 µM trans-zeatin. It was observed that at lower light intensity (15 µmol m^{-2}s^{-1}) *MYB4* expression was significantly reduced at 4 µM trans-zeatin, however not at 2 µM, suggesting that higher concentrations of cytokinin represses *MYB4* expression (Supplementary Figure 4). Next, we examined the hypocotyl length of 6-day-old WT, *myb4*, *hy5*, *myb4 hy5* mutants in both cytokinin-treated and untreated seedlings grown in the darkness. It was observed that the dark-grown seedlings of each genotype studied exhibited similar hypocotyl length in absence or presence of different

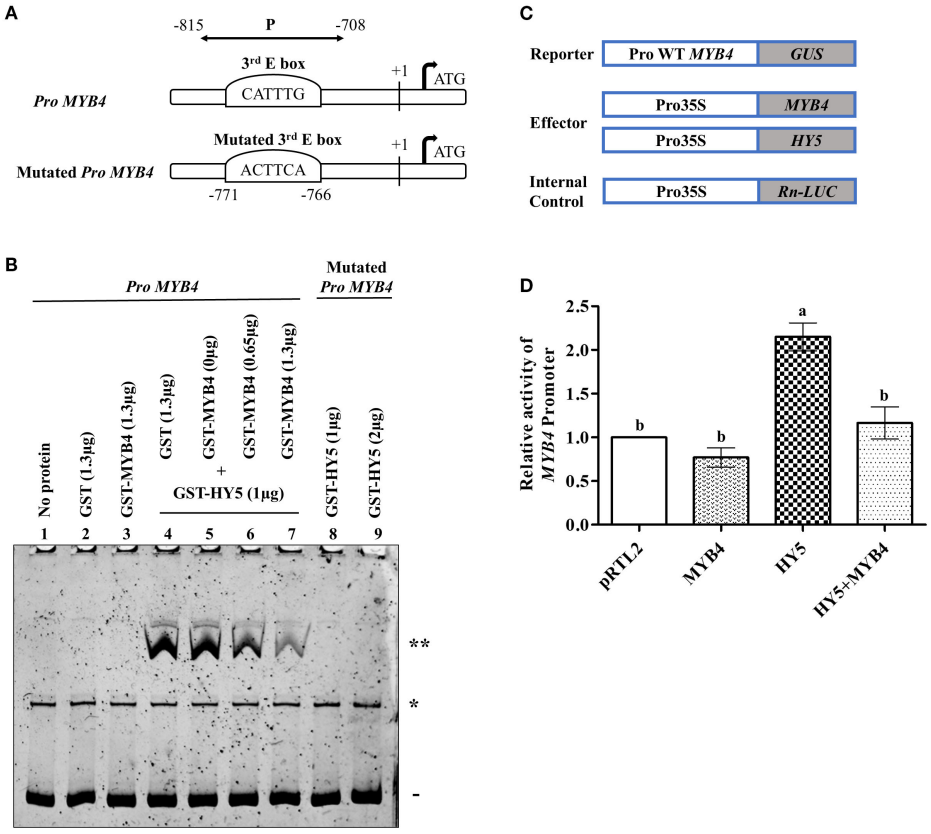

FIGURE 4
MYB4 autoregulates the HY5 mediated regulation of its own promoter. **(A)** A visual depiction of the *MYB4* promoter showing the wild type and mutated sequence of the 3rd E-Box along with their positions. The (+1) notation denotes the transcription initiation site. The double-headed line labeled as "P" denotes the location of the DNA fragment (from -708 to -815) used in DNA-protein interaction analyses during the competitive EMSA experiment. **(B)** Electrophoretic mobility shift assay (EMSA) analysis of HY5 protein binding to *MYB4* promoter in presence or absence of MYB4 protein. Lane 1 had no protein added, lane 2 contained 1.3 µg of GST and lane 3 had 1.3 µg of GST-MYB4 as control. In lane 4-7, 1 µg of GST-HY5 was added with 100 ng of wild type *MYB4* promoter fragments (107 bp long: -708 to -815 bp) along with GST or GST-MYB4 in increasing concentrations. In lane 8-9, 1 µg and 2 µg of GST-HY5 were respectively added with 100 ng of mutated MYB4 promoter fragments as control. The DNA-protein complexes were separated using an 8% native polyacrylamide gel and visualized using SYBR® Green EMSA staining. The presence of a double asterisk (**) signifies the DNA-protein complex, a solid line (-) indicates the free probe, and a single asterisk (*) denotes a spurious band consistently observed across all lanes. **(C)** Constructs used in the protoplast experiment in different combination to measure the *MYB4* promoter activity. **(D)** The relative *MYB4* promoter activity values in *Arabidopsis* protoplasts transiently transformed with the indicated effectors and reporters constructs. The experiments were repeated three times with similar results, and a representative result has been shown. The error bars indicate ± SD. Different alphabets denote statistically significant differences ($p < 0.05$) of *MYB4* promoter activity. The data were compared by using one-way ANOVA factorial analysis followed by Tukey's HSD test.

concentrations of cytokinin. (Supplementary Figure 5). These results suggest that mutation in *MYB4* doesn't alter the cytokinin mediated inhibition of hypocotyl elongation in dark.

To determine the effect of light and cytokinin on hypocotyl growth, we examined hypocotyl length of 6-day-old WT, *myb4*, *hy5*, and *myb4 hy5* double mutant seedlings grown under lower and higher WL fluences on MS plates, with or without 1 µM, 2 µM, and 4 µM trans-zeatin. As shown in Figures 5A, C, under lower intensity of WL (15 µmol m^{-2} s^{-1}), among the various cytokinin concentrations tested, only the highest concentration (4 µM) was able to rescue the etiolated growth of the *myb4* mutant. However, at higher WL intensity (60 µmol m^{-2} s^{-1}), both 2 µM and 4 µM cytokinin were able to suppress hypocotyl elongation, resulting in hypocotyl length in the *myb4* mutant similar to that of the WT (Figures 5B, D). Consistent with earlier reports (Vandenbussche et al., 2007), we observed that similar to the control condition, *hy5* mutants exhibited elongated hypocotyl as compared to the WT upon cytokinin treatment. Notably, the hypocotyl length of the *myb4 hy5* double mutant was significantly higher than that of *hy5* mutant under all the conditions tested (Figures 5A–D). These results suggest that the additional loss of MYB4 function alters the *hy5* phenotype upon cytokinin treatment. At lower intensity of WL and lower cytokinin concentration, *MYB4* and *HY5* function additively to regulate hypocotyl length which is similar to the effect of WL alone. However, at the same light intensity but higher cytokinin concentration this genetic interaction is altered and *MYB4* and *HY5* exhibit synergistic mode of interaction. At higher light intensity even lower cytokinin concentration is sufficient for the cytokinin mediated synergistic interaction between *MYB4* and *HY5*. Thus, the genetic interaction between *MYB4* and *HY5* is modulated by both light intensity and cytokinin levels.

We also measured the primary root length in WT, *myb4*, *hy5*, and *myb4 hy5* double mutant seedlings grown under cytokinin

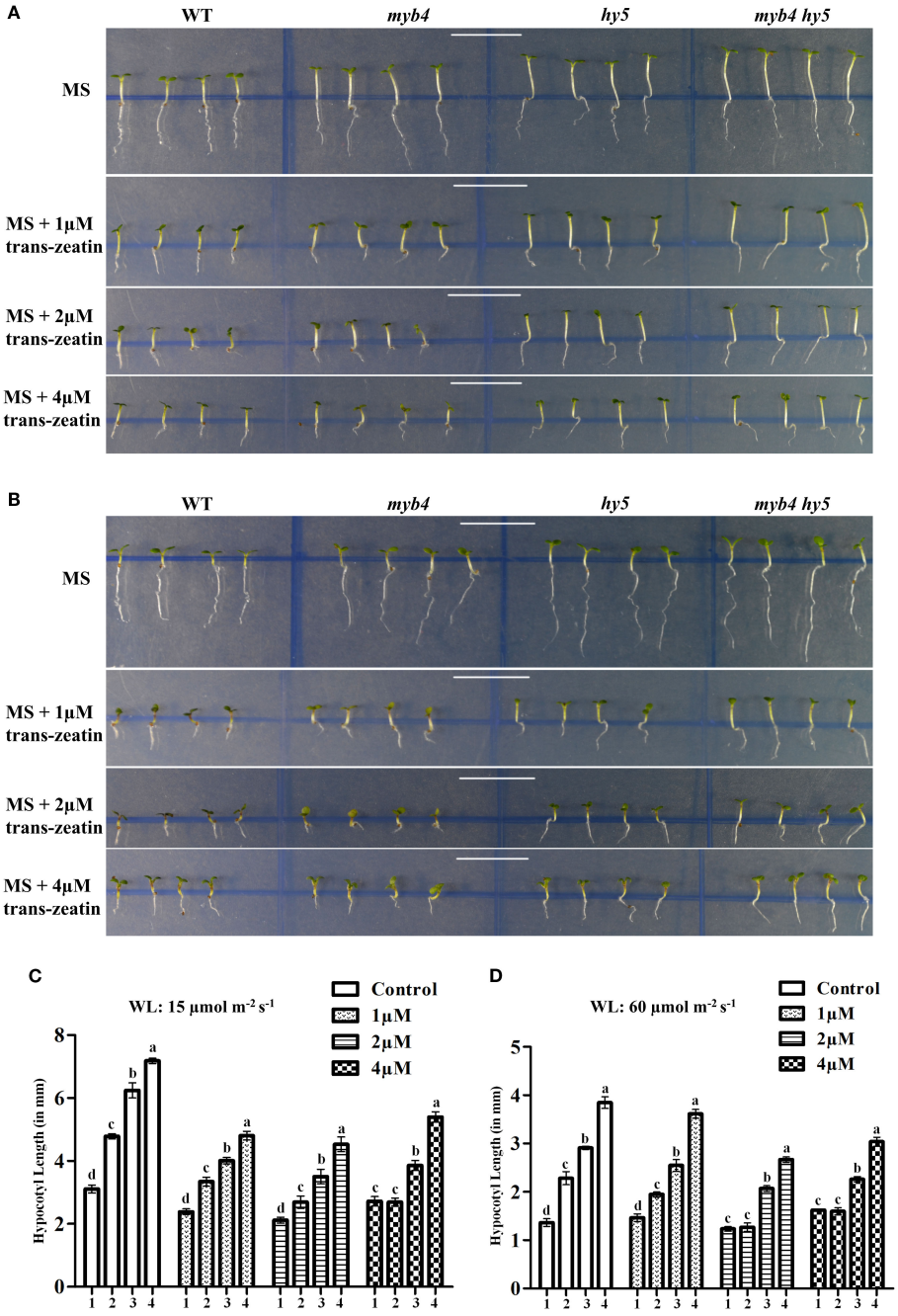

FIGURE 5
MYB4 and *HY5* mediates the integration of light and cytokinin signaling. **(A, B)** Visible phenotype of 6-day-old WT (Segregated WT Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant WL (15 µmolm^{-2}s^{-1}) **(A)** or in constant WL (60 µmolm^{-2}s^{-1}) **(B)** in 0 µM, 1 µM, 2 µM or 4 µM (Top to bottom) trans-Zeatin concentration. Error bars represent ± SD (n=10). Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. 1, 2, 3, 4 under the bar represents WT, *myb4, hy5* and *myb4 hy5* double mutant respectively. Scale bar = 10 mm. **(C, D)** Quantification of hypocotyl length of 6-day-old WT (Segregated WT Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant WL (15 µmolm^{-2}s^{-1}) **(C)** or in constant WL (60 µmolm^{-2}s^{-1}) **(D)** in 0 µM, 1 µM, 2 µM or 4 µM (Top to bottom) trans-Zeatin concentration. Error bars represent ± SD (n=10). Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. 1, 2, 3, 4 under the bar represents WT, *myb4*, *hy5* and *myb4 hy5* double mutant respectively. Scale bar = 10 mm.

treatment in darkness and under various intensities of white light. While cytokinin suppresses primary root length in WT in both dark and WL (Liu et al., 2022), our results showed no significant differences in inhibition ratios between WT and *myb4* mutant seedlings across all conditions (Supplementary Figures 6A–C). Consistent with previous findings by Cluis et al. (2004), the *hy5* mutant exhibited resistance to cytokinin treatment under both the intensities of the white light. The *myb4 hy5* double mutant

displayed primary root inhibition patterns similar to the *hy5* mutant in white light, indicating that *HY5* works independent of *MYB4* in regulating primary root length upon cytokinin treatment (Supplementary Figures 6B, C).

MYB4 and HY5 differentially regulate the expression of cytokinin responsive genes

Our study shows that while cytokinin rescues the etiolated growth of *myb4* seedlings, the primary root length of myb4 remains similar to WT in both presence and absence of cytokinin. The *ATP-binding cassette (ABC)* transporter subfamily *G14* (*ABCG14*) in Arabidopsis is primarily active in roots and is crucial for transporting cytokinin to the shoots. When *ABCG14* expression is disrupted, it leads to significant stunted growth in the shoots, which can be reversed by applying trans-zeatin externally (Ko et al., 2014; Zhang et al., 2014). To explore whether altered *ABCG14* levels is responsible for the suppression of the hypocotyl length of *myb4*, we assessed the transcript levels of *ABCG14* in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings grown under low (15 µmol m^{-2} s^{-1}) and high (60 µmol m^{-2} s^{-1}) white light in MS or in MS with 2 µM and 4 µM trans-zeatin (Figures 6A, B). Under control conditions (without cytokinin), at both low (15 µmol m^{-2} s^{-1}) and high intensities (60 µmol m^{-2} s^{-1}) of WL, the expression of *ABCG14* in the *myb4* mutant is similar to that in the WT, whereas the *hy5* mutants exhibit a significant upregulation (~2.2-fold increase) of *ABCG14* expression compared to WT (Figures 6A, B). An additional mutation in *MYB4* could suppress the elevated expression of *ABCG14* in the *hy5* mutant, resulting in expression levels similar to WT in the *myb4 hy5* double mutant. Earlier it was reported that the expression of *ABCG14* was induced by cytokinin treatment (Ko et al., 2014). When we assessed the transcript levels of *ABCG14* in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings grown under constant low white light (15 µmol m^{-2} s^{-1}) and under constant high white light (60 µmol m^{-2} s^{-1}) in MS with 2 µM and 4 µM trans-zeatin, we observed that in low light intensity and low cytokinin concentrations, the expression of *ABCG14* is significantly reduced in *myb4* mutant than that of WT (Figures 6A, B). The expression level pattern of *ABCG14* remains same like the untreated for both *myb4* and *hy5* mutant in all other conditions. Interestingly, unlike the untreated conditions, in *myb4 hy5* double mutant, the expression of *ABCG14* is similar to that of *hy5* mutants. This suggests that upon 2 µM cytokinin treatment at low intensity of WL, *HY5* works downstream to *MYB4* to regulate the expression of *ABCG14* (Figure 6A).

Sweere et al. (2001) suggested a potential mechanism through which cytokinin and light signaling pathways interact to control hypocotyl elongation through RESPONSE REGULATOR 4 (ARR4). To determine if ARR4 plays a role in the altered response of *myb4* mutant seedlings, we measured *ARR4* transcript levels in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings grown under continuous low white light (15 µmol m^{-2} s^{-1}) and high white light (60 µmol m^{-2} s^{-1}) conditions, both in untreated MS and in MS supplemented with 2 µM and 4 µM trans-zeatin (Figures 6C, D). The results show that *ARR4* expression was significantly increased in the *myb4* mutant but decreased in the *hy5* mutant. In the *myb4 hy5* double mutant, *ARR4* expression was similar to that in the *hy5* mutant, indicating that *HY5* functions downstream of *MYB4* in regulating *ARR4* expression. As it was previously reported that the *ARR4* gene expression is also induced by cytokinin (Sweere et al., 2001), we checked the transcript levels of *ARR4* in 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings grown under constant low white light (15 µmol m^{-2} s^{-1}) and under constant high white light (60 µmol m^{-2} s^{-1}) in MS with 2 µM and 4 µM trans-zeatin. In low light, the pattern of expression of *ARR4* is similar to that of the untreated for both *myb4* and *hy5* mutants. Interestingly, the expression of *ARR4* in *myb4 hy5* double mutant is similar to that of *myb4* mutants, suggesting that under cytokinin treatment in low light, *MYB4* works downstream to *HY5* to regulate the expression of *ARR4*. However, in high light, there were no significant differences in the *ARR4* transcript in any cytokinin concentrations tested (Figure 6D). This suggests that the role of *MYB4* and *HY5* in the regulation of cytokinin induced *ARR4* expression is specific to low intensity of WL.

Seedlings treated with exogenous cytokinins accumulate anthocyanin pigments, with increased transcript levels of *CHALCONE SYNTHASE (CHS)* gene, a key player in the anthocyanin biosynthesis pathway (Deikman et al., 1995). HY5 enhances anthocyanin biosynthesis by directly binding to *CHS* promoter resulting in its transcriptional activation (Ang et al., 1998; Chattopadhyay et al., 1998). Conversely, *MYB4*, a known negative regulator of anthocyanin biosynthesis, indirectly repress *CHS* expression (Wang et al., 2020). Given these observations, we asked how loss of MYB4 and HY5 functions influence *CHS* regulation under cytokinin treatment. To answer this, we grew 6-day-old WT, *myb4, hy5*, and *myb4 hy5* seedlings under constant low white light (15 µmol m^{-2} s^{-1}) and high white light (60 µmol m^{-2} s^{-1}) on MS media without trans-zeatin and with 2 µM and 4 µM trans-zeatin (Figures 6E, F). Consistent with previous findings, we observed that in untreated conditions, *CHS* transcript level was significantly elevated in the *myb4* mutant whereas it was significantly reduced in the *hy5* mutant. In the *myb4 hy5* double mutant, *CHS* expression was similar to the *hy5* mutant, indicating that *HY5* acts downstream of *MYB4* in regulating *CHS* expression under control condition (Figures 6E, F). However, under both low and high cytokinin treatment across both light intensities, *CHS* expression was significantly reduced (~2-3 fold) in the *myb4* mutant. In contrast, *CHS* expression patterns were similar in *hy5* and *myb4 hy5* double mutants across both treated and untreated conditions. These findings suggest that MYB4 plays opposing roles in the regulation of *CHS* expression depending on the presence or absence of cytokinin and *HY5* works downstream to *MYB4* in this regulation.

Discussion

A signal transduction pathway relies on its interconnected transcriptional regulatory network for proper functional

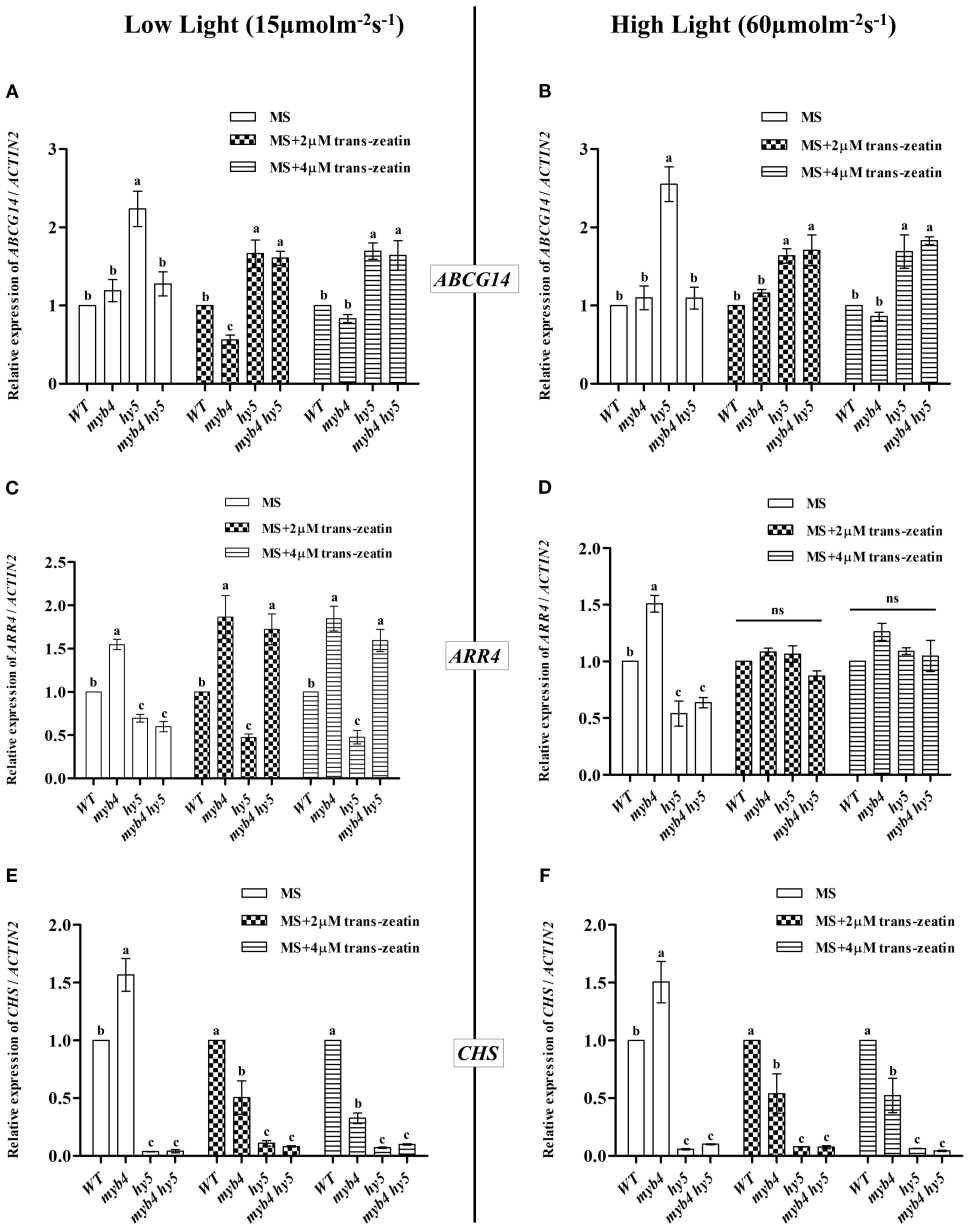

FIGURE 6
Differential regulation of cytokinin responsive genes by *MYB4* and *HY5*. **(A, B)** Real-time PCR analyses of *ABCG14* in 6-day-old wild type (Segregated wild type Ws-Ler), *myb4*, *hy5* and *myb4 hy5* seedlings grown under constant white light (15 µmolm^{-2}s^{-1}) **(A)** and constant white light (60 µmolm^{-2}s^{-1}) **(A)** in normal MS media (Control) or in MS media supplemented with 2 µM and 4 µM trans-zeatin (Treated). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. *ACTIN2* was used as endogenous control. **(C, D)** Real-time PCR analyses of *ARR4* in 6-day-old wild type (Segregated wild type Ws-Ler), *myb4*, *hy5* and *myb4 hy5* seedlings grown under constant white light (15 µmolm^{-2}s^{-1}) **(C)** and constant white light (60 µmolm^{-2}s^{-1}) **(D)** in normal MS media (Control) or in MS media supplemented with 2 µM and 4 µM trans-zeatin (Treated). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. *ACTIN2* was used as endogenous control. **(E, F)** Real-time PCR analyses of *CHS* in 6-day-old wild type (Segregated wild type Ws-Ler), *myb4*, *hy5* and *myb4 hy5* seedlings grown under constant white light (15 µmolm^{-2}s^{-1}) **(E)** and constant white light (60 µmolm^{-2}s^{-1}) **(F)** in normal MS media (Control) or in MS media supplemented with 2 µM and 4 µM trans-zeatin (Treated). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. *ACTIN2* was used as endogenous control.

execution. In this study, we show that MYB4 is functionally connected with HY5 to integrate light and cytokinin responses. Furthermore, our results suggest that MYB4, through its physical interaction, attenuates HY5 from binding to the *MYB4* promoter. Multiple lines of experimental evidences, including *in vitro* and *in vivo* assays, demonstrate a physical interaction between MYB4 and HY5 proteins. Domain-specific interaction studies further reveal that HY5 interacts with the C-terminal domain (transcriptional

activation domain, Mitra et al., 2019) of MYB4. Depending on the growth conditions, two or more substrates can compete for binding to the same domain of a protein. For example, upon blue light exposure, photoactivated CRY2 competes with PAP2 for binding to the WD40 domain of COP1, thereby stabilizing PAP2 and resulting in anthocyanin biosynthesis (Ponnu et al., 2019). Previous studies have shown that CAM7 interacts with HY5 (Abbas et al., 2014), and recent findings indicate that CAM7 interacts with the C-terminal domain of MYB4 as well (Dutta et al., 2024). This raises intriguing questions about whether CAM7, HY5 and MYB4 compete for binding with each other or form a transient or obligatory multiprotein complex to regulate photomorphogenesis. One plausible functional significance of MYB4-HY5 physical interaction could be that MYB4 modulates HY5-mediated regulation of *MYB4* expression in order to maintain the balance between light and cytokinin mediated hypocotyl elongation. The EMSA results show that the presence of MYB4 significantly reduces HY5-binding to the *MYB4* promoter which is further supported by trans-activation experiments in Arabidopsis protoplasts.

Genetic analysis of the *myb4 hy5* double mutant shows that long hypocotyl phenotype of *hy5* is further enhanced in *myb4 hy5* double mutant, resulting in an exaggerated tall phenotype under WL, BL, and FRL, but not in RL. Thus, *MYB4* and *HY5* function additively under those wavelengths of light. While light-inducible genes like *RBCS-1A* and *CAB1* display expression levels similar to WT in *myb4* mutants, their expression in *myb4 hy5* mutant is similar to *hy5* mutant. Additionally, while *HY5* regulate chlorophyll accumulation independent of *MYB4*, they work antagonistically to regulate anthocyanin levels. Therefore, *MYB4* and *HY5* coordinate both independently and dependently to regulate photomorphogenic growth.

Integration of light and hormone signaling pathway in Arabidopsis is well studied (Feierabend and de Boer, 1978; Tian and Reed, 1999; Moore et al., 2003; Xiong et al., 2023). Among the various phytohormones, cytokinin mimics the effect of light signals, resulting in the de-etiolation of dark-grown seedlings and the inhibition of hypocotyl elongation at lower intensities of light (Chory et al., 1994; Deikman and Hammer, 1995; Vandenbussche et al., 2007). Our study demonstrates that upon cytokinin treatment, the elongated hypocotyl phenotype of *myb4* in WL, is suppressed. At higher light intensities, where the effect of cytokinin is otherwise diminished, exogenous cytokinin could still suppress the hypocotyl elongation of *myb4* mutants. One possible explanation for this observation is that, due to impaired light responses in the *myb4* mutant, the inhibitory effect of light on cytokinin-mediated suppression of hypocotyl elongation is rescued. Additionally, cytokinin application downregulates *MYB4* expression. The genetic interaction between *HY5* and *MYB4*, typically additive, becomes synergistic under cytokinin treatment in WL. These altered morphologies are probably due to changes in the dynamics of physical interactions and transcriptional regulation under different growth conditions. The shift in genetic interaction following treatment with exogenous signals or environmental stimuli, which alters the regulatory dynamics between genes, is not unprecedented. Sandhu et al. (2012) explores the intricate interactions between photoreceptors and brassinosteroid-inactivating P450 enzymes, demonstrating how light treatments influence flowering and gene expression through various genetic pathways. Additionally, the genetic interaction between *HY5* and *GBF1* also varies in a light intensity- and wavelength-dependent manner (Singh et al., 2012). Apart from regulating light-responsive genes, MYB4 and HY5 regulate the expression of genes involved in cytokinin signaling pathway. While the expression of *ABCG14*, which is involved in the transport of cytokinin from root to shoot, is significantly downregulated in the *myb4* mutant, the expression of *ARR4*, an A-type response regulator, is significantly upregulated as compared to the corresponding cytokinin-treated WT seedlings. Furthermore, HY5 and MYB4 work in the same pathway to regulate the expression of *ABCG14* and *ARR4*. The reduced expression of *ABCG14* in *myb4* might be responsible for altered cytokinin export to shoot resulting in the observed stunted growth. Whereas in light signaling MYB4 negatively regulates *CHS* expression, it positively regulates *CHS* expression upon cytokinin treatment. Since plants are continuously exposed to changing environmental cues, understanding how they integrate and respond to fluctuating light signals and internal hormone levels provides insights into their intricate mechanisms of adaptability and survival.

In summary, HY5 binds to the *MYB4* promoter, and positively regulates its expression (Dutta et al., 2024). Conversely, MYB4 physically interacts with HY5, inhibiting HY5 to bind to its promoter, thus autoregulating its own expression (Figure 7). Furthermore, under higher light intensity, *myb4* mutants show hypersensitivity to lower cytokinin concentrations, whereas under lower light intensity, higher cytokinin levels are required to observe this effect. This stoichiometric balances between HY5 and MYB4 provide maintain homeostasis and accordingly control hypocotyl elongation in response to light and cytokinin.

Methods

Plant materials and growth conditions

Arabidopsis thaliana plants used in this study were homozygous *myb4* mutant (CS26404), a Ds transposon tagged mutant in Ler background (Dutta et al., 2024) and homozygous *hy5-ks50* mutant, a T-DNA insertion mutant in Ws background (Oyama et al., 1997). The *myb4 hy5* double mutants were created through genetic crosses using standard methods outlined by Yadav et al. (2005) by crossing homozygous *myb4* mutant with *hy5* homozygous mutant lines. The homozygosity of both the mutation in T2 generation were confirmed by the inability of gene-specific primer sets, which spanned the full length of *MYB4* (MYB4FP and MYB4RP primers) and *HY5* (HY5FP and HY5RP) to amplify the *MYB4* and *HY5* gene respectively from genomic DNA isolated from the plants. One homozygous *myb4 hy5* double mutant plant, used for different experiments was additionally confirmed by assessing the transcript levels of both *MYB4* and *HY5* using RT-PCR analyses. To analyse double mutants from different ecotype backgrounds, a segregated WT line of Ws-Ler from the T2 generation was

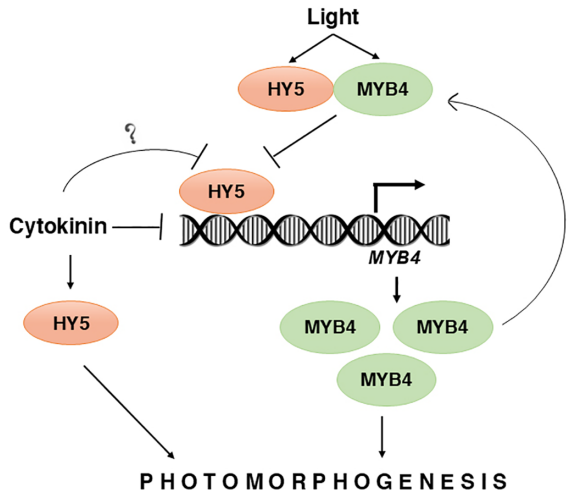

FIGURE 7
Simplified Model depicting the role of MYB4 and HY5 in light and cytokinin signaling pathway. Light stimulates both *HY5* and *MYB4* expression, whereas cytokinin stimulates *HY5* but suppresses *MYB4*. HY5 interacts with the 3rd E box on the *MYB4* promoter (Dutta et al., 2024) to enhance *MYB4* expression. As a feedback response, MYB4 interacts with HY5, preventing its binding to *MYB4* promoter, thus finely regulating its expression to balance light and cytokinin signals in controlling hypocotyl growth during *Arabidopsis* seedling development.

marked as WT and used as a control to compare phenotypic and molecular differences.

Arabidopsis seeds were surface-sterilized and sown on Murashige and Skoog (MS) plates, stratified in the dark at 4°C to break dormancy before being transferred to growth chambers. They were then grown under specific wavelengths of light at designated light intensities at 22 °C. For different phenotypic studies, seedlings were photographed, and their hypocotyl lengths were measured using ImageJ software. For cytokinin responsive experiments, seeds were sown on MS plates with different concentration of trans-Zeatin.

In vitro pull-down assay

The full-length coding sequences (CDS) of CAM7 and MYB4 were cloned into the pGEX-4T2 vector to produce fusion proteins with Glutathione S-transferase (GST). The GST (negative control), GST-CAM7 (positive control) and GST-MYB4 were overexpressed and purified from E. coli BL21 (DE3) using Glutathione Sepharose 4B beads (Amersham Biosciences). The full-length CDS of HY5 was cloned into the pET-20b (+) vector, adding a 6× Histidine tag to the C-terminus. The HY5-His protein was overexpressed in E. coli BL21 (DE3) and purified with Ni-NTA Agarose beads (Qiagen). The *in vitro* binding assay was performed following the protocol from Senapati et al. (2019). For the assay, 2µg of HY5-His was added to microcentrifuge tubes containing Ni-NTA Agarose beads and incubated with *in vitro* binding buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.2% glycerol, 0.1% Triton-X100, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40, and protease inhibitor cocktail; Sigma, Calcutta, India) for 2 hours at 4 °C. After washing off the unbounded proteins, GST, GST-CAM7 or GST-MYB4 was added in equimolar amounts and incubated overnight at 4 °C. Following incubation, the supernatant was collected by centrifugation, and the beads were washed three times with binding buffer. The beads were then resuspended in 5X sodium dodecyl sulfate (SDS) loading buffer and boiled for 10 minutes. Both the supernatant (5%) and pellet fractions were analyzed using 12% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti-GST antibodies to detect protein interactions. The same membrane was then stripped and probed using α-His to examine the equal loading of HY5-His.

Yeast two-hybrid assay

For the domain-wise interaction study of MYB4 with HY5, full-length CDS and truncated version of MYB4 was combined with the GAL4 DNA-binding domain (BD-MYB4- bait) and the full-length CDS of HY5 was combined with the GAL4 activation domain (AD-HY5- prey). The fusion constructs were then introduced into yeast *AH109* strain using polyethylene glycol/lithium acetate transformation (Clontech), and the growth were assessed of the co-transformed yeast cells on 2D plates (lacking leucine and tryptophan) and 4D plates (lacking leucine, tryptophan, adenine, and histidine). As previously demonstrated by Ang et al., 1998, COP1 and HY5 were used as a positive control for this study.

Co-immunoprecipitation assays

Co-IP assay was performed as described previously (Senapati et al., 2019) with slight modifications. Full length CDS of MYB4 was cloned into pCAMBIA 1303 to yield a GFP fusion protein. Total protein was extracted from the leaves of 30-d old *hy5* and HY5OE plants transiently expressing GFP, CAM7-GFP (Senapati et al., 2019) or MYB4-GFP using Co-IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 10% glycerol and protease inhibitor cocktail). 500 µg of total protein for each sample was incubated with 15 µl of anti-GFP monoclonal antibody (Sigma) for 6h at 4 °C. To this 30 µl of pre blocked protein A-agarose beads (Sigma) was added and incubated for 2 hr at 4 °C. Then the beads were washed three times with Co-IP buffer, and after the last wash the beads were boiled in 5X protein loading dye for 10 min to elute the protein and along with that 5% input was run in SDS-PAGE. Immunoblots were developed using anti-GFP antibody (Sigma, dilution- 1:2500) and co-immunoprecipitated protein was detected by stripping and re-probing with anti-HY5 antibody (Srivastava et al., 2015; Sigma, dilution- 1:5000).

Gene expression analysis

Total plant RNA was isolated from the 6-day-old *Arabidopsis thaliana* seedlings grown under required light conditions using

TABLE 1 Primers used in various experiments.

Sl. No.	Primer name	Sequence (5′-3′)
1	*Actin2* FP	AAAGGCTTAAAAAGCTGGGG
2	*Actin2* RP	GGGACTAAAACGCAAAACGA
3	*CAB1*-FP1	GAGGAAGACTGTTGCCAAGC
4	*CAB1*-RP1	CCCACCTGCTGTGGATAACTTC
5	*RBCS-1A*-FP1	ACCTTATCCGCAACAAGTGG
6	*RBCS-1A*-RP1	TGGGGTACTCCTTCTTGCAC
7	MYB4(GST)FP	CGGGATCCATGGGAAGGTCACCGTGC
8	MYB4(GST)RP	ATAAGAATGCGGCCGCTTATTTCATCTCCAAGCTTCG
9	HY5(His)FP	CGGAATTCGATGCAGGAACAAGCGACTACTC
10	HY5(His)RP	CCGCTCGAGAAGGCTTGCATCAGCATTAGAACC
11	FP-MYB4ΔC-Y2H	GGAATTCCATATGGGAAGGTCACCGTGC
12	RP-MYB4ΔC-Y2H	CGGAATTCGGTATTACTCGTAACTGGTTC
13	FP-MYB4ΔN-Y2H	GGAATTCCATATGATTAATATCTCATTCACTTCTGC
14	RP-MYB4ΔN-Y2H	CGGAATTCTTATTTCATCTCCAAGCTTCG
15	FP-HY5-Y2H	GGAATTCATGCAGGAACAAGCGACTAG
16	RP-HY5-Y2H	CCATCGATTCAAAGGCTTGCATCAGCATTAG
17	*MYB4* proto-WT FP	CGGGATCCGTGCTTGTCATTTGGTGAGAG
18	*MYB4* proto-WT RP	CCGCTCGAGGTTTTTTGGACAAGTGCAGGTC
19	*MYB4* pRTL2- FP	CATGCCATGGGGATGGGAAGGTCACCG
20	*MYB4* pRTL2- RP	CATGCCATGGTTATTTCATCTCCAAGCTTCG
21	*HY5* pRTL2- FP	CATGCCATGGGCATGCAGGAACAAGCGACTAGC
22	*HY5* pRTL2- RP	CATGCCATGGTCAAAGGCTTGCATCAGCATTAG
23	MYB4_qPCR_FP	AGATGAGTGCCCAGTTCAAGA
24	MYB4_qPCR_RP	AGCTGCACTTGAAACAACGT
25	HY5_qPCR_FP	AGACATATTCTGAAGAACACAACAGG
26	HY5_qPCR_RP	AGAAGAAGAAGGAGATCAAGGC
27	MYB4_R2c_FP	CGGAATTCTTACAAGTTATCCTGCCCACAC
28	MYB4_R2c_RP	CGGGATCCCTCTAATATATAGACTGCACTGG
29	MYB4_R2Am3_FP	CGGAATTCTTAGCAAGTGATTGTATTAGGG
30	MYB4_R2Am3_RP	CGGGATCCCCCTAATACAATCACTTGCTAA
31	MYB4-GFP_FP	CATGCCATGGGAAGGTCACCGTGC
32	MYB4-GFP_RP	GGACTAGTGCGGCCGCTTATTTCATCTCCAA
33	ABCG14 RT FP	TCGCCAACGGAATCCCAC
34	ABCG14 RT RP	GGTTTTTGGCAGCAGCTTTG
35	ARR4 RT FP	GAAGATTAAGGAATCGTCC
36	ARR4 RT RP	TCAAGGCATCTGTCGATTC
37	CHS FP	CTGTCCTCGTATCGCTAAGGAT
38	CHS RP	ACGTGTCGCCTCATCTTCTCTT

RNeasy plant mini kit (Qiagen). cDNA was synthesized from 1µg of total RNA using RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific). qPCR was then performed using Power SYBR® Green PCR Master Mix (Applied 509 Biosystems) in StepOnePlusTM Real-Time PCR Systems using respective qPCR gene specific primers. *ACTIN2*, a housekeeping gene was used as reference gene to normalize the transcript level of the qPCR values. To analyze the relative changes in gene expression, the common $2^{-\Delta\Delta CT}$ algorithm was used. First, ΔCt value is calculated by normalizing samples Ct to *ACTIN2* Ct values. This value for different samples was then normalized to the Ct values of the experimental control and the ΔΔCt values were obtained. Fold expression was calculated by the formula, $2^{-\Delta\Delta CT}$, which was then plotted on the graph. The primers used for qPCR analysis has been mentioned in Table 1.

Analysis of pigment accumulation

The levels of chlorophyll and anthocyanin were determined following the protocol described by Holm et al. (2002). Specifically, for chlorophyll content estimation, 6-day-old seedlings grown under continuous white light (WL) were weighed and quickly frozen in liquid nitrogen. The frozen tissues were grounded in a 1.5 ml microcentrifuge tube, and chlorophyll was extracted in the dark using 80% acetone until the pellet became colourless. Chlorophyll a and b levels were calculated using MacKinney's specific absorption coefficients: chlorophyll a = 12.7(A663) - 2.69 (A645), and chlorophyll b = 22.9(A645) - 4.48(A663). The total chlorophyll content was expressed as micrograms of chlorophyll a and b per gram of fresh tissue weight.

For anthocyanin content estimation, 6-day-old seedlings grown under continuous WL were weighed, rapidly frozen in liquid nitrogen, and ground. Total plant pigments were extracted overnight in 0.3 mL of 1% HCl in methanol. After adding 0.2 mL of water, chlorophyll was separated from anthocyanin by adding an equal volume of chloroform. The anthocyanin level was assessed by spectrophotometric measurements of the aqueous phase (A530 - A657) and normalized to the total fresh weight of the tissue. Flowering times were determined as described by Yadav et al. (2005).

Electrophoretic mobility shift assay

GST, GST-MYB4, and GST-HY5 proteins were overexpressed in *E. coli* BL21 (DE3) and affinity purified using Glutathione Sepharose 4B beads (Amersham Biosciences). For the DNA binding assays, wild type *MYB4* promoter spanning the 3rd E box and mutated 3rd E box generated through primer-based site-directed mutagenesis, were used as probes. All of these fragments were cloned into the pBluescript SK+ vector, followed by PCR amplification and purification to produce the probes. Approximately 100 ng of the DNA fragment was incubated with the purified protein in a reaction mixture containing 1X binding buffer (15 mM HEPES, pH 7.5, 35 mM KCl, 1 mM MgCl2, 1 mM EDTA, pH 8.0, 2% glycerol, and 1 mM DTT) in a total volume of 20 µl. The incubation was carried out at room temperature for 20 minutes. Following incubation, the reactions were resolved on a 7% native polyacrylamide gel, stained with SYBR® Green EMSA nucleic acid gel stain (Molecular Probe, Invitrogen), and visualized using the iBright 750 imaging system (Invitrogen) for image documentation.

Transient expression assay in Arabidopsis protoplast

Arabidopsis protoplasts were isolated and transformed following the protocol outlined by Yoo et al. (2007). Wild-type *MYB4* promoter fragments containing the three E-boxes were PCR amplified and cloned into the pGAL-UAS-GUS vector. The full-length coding sequences (CDS) of *HY5* and *MYB4* were individually cloned into the pRTL2 vector to create the effector constructs. Both the reporter and effector constructs were introduced into Col-0 protoplasts. Following transfection, the protoplasts were incubated under 20 µmol/m²/s white light for 10 hours before harvesting. The GUS activity measurement was carried out as mentioned by Yoo et al. (2007) and Chakraborty et al. (2019).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

AP: Investigation, Conceptualization, Writing – review & editing, Data curation, Writing – original draft, Validation, Methodology. RB: Writing – original draft, Investigation, Writing – review & editing. SC: Writing – review & editing, Funding acquisition, Writing – original draft, Supervision.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphgy.2025.1657264/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Full Blot of co-immunoprecipitation assay showing interaction between MYB4 and HY5.

SUPPLEMENTARY FIGURE 2
The expression of MYB4 is stimulated during dark to light transitions. **(A–D)** Semiquantitative PCR to show the transcript abundance of *MYB4* in 5-day-old dark grown WT (ecotype Landsberg erecta) seedlings transferred either to white light (WL: 20 $\mu molm^{-2}s^{-1}$) (A), blue light (BL: 20 $\mu molm^{-2}s^{-1}$) **(B)**, red light (RL:30 $\mu molm^{-2}s^{-1}$) **(C)**, or far-red light (FR: 1.5 $\mu molm\text{-}2s^{-1}$) **(D)**, at various time points. Each PCR reaction was sampled at 25 cycles to monitor the amplification process before saturation occurred. The semiquantitative PCR amplification of *ACTIN2* for the same reaction setup was used as endogenous control.

SUPPLEMENTARY FIGURE 3
Confirmation of *myb4 hy5 double* mutant. Semiquantitative PCR to confirm *myb4 hy5* double mutant plant by using gene specific primers. *ACTIN2* was used as endogenous control.

SUPPLEMENTARY FIGURE 4
Expression of *MYB4* is downregulated upon cytokinin treatment. Real-time PCR analyses of *MYB4* in 6-day-old wild type seedlings grown under constant white light (15 $\mu molm^{-2}s^{-1}$) in normal MS media (Control) or in MS media supplemented with 2 μM and 4 μM trans-zeatin (Treated). Error bars represent ± SE of the mean of three biological replicates. Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. *ACTIN2* was used as endogenous control.

SUPPLEMENTARY FIGURE 5
Response of *myb4 hy5* double mutants to external cytokinin treatment grown under dark condition. **(A, B).** Visible phenotype of 6-day-old WT (Segregated wild type Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant dark (A); Quantification of hypocotyl length **(B)** of 6-day-old WT (Segregated wild type Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under constant dark. Error bars represent ± SD (n=10). Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. 1, 2, 3, 4 under the bar represents WT, *myb4, hy5* and *myb4 hy5* double mutant respectively.

SUPPLEMENTARY FIGURE 6
HY5 works independently of *MYB4* to regulate the root growth inhibition under cytokinin treatment. **(A–C)** Quantification of primary root length of 6-day-old WT (Segregated wild type Ws-Ler), *myb4, hy5* and *myb4 hy5* double mutant seedlings grown under dark (A), constant WL (15 $\mu molm^{-2}s^{-1}$) **(B)** and WL (60 $\mu molm^{-2}s^{-1}$) **(C)** in absence or in presence of different concentrations of trans-zeatin. Error bars represent ± SD (n=8). Different alphabets denote statistically significant differences ($p < 0.05$) by One-way ANOVA followed by a Tukey-Kramer *post-hoc* test. 1, 2, 3, 4 under the bar represents WT, *myb4, hy5* and *myb4 hy5* double mutant respectively.

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