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CONSTANS-LIKE 7 (COL7) Is Involved in Phytochrome B (phyB)-Mediated Light-Quality Regulation of Auxin Homeostasis

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\textbf{ABSTRACT} \textit{Arabidopsis} phytochrome B (phyB) is the major photoreceptor that senses the ratio of red to far-red light (R:FR) to regulate the shade-avoidance response (SAR). It has been hypothesized that altered homeostasis of phytohormones such as auxin and strigolactone is at least partially responsible for SAR, but the mechanism underlying phyB regulation of the hormonal change is not fully understood. Previously we reported that \textit{CONSTANS-LIKE 7} (COL7) enhances branching number under high R:FR but not under low R:FR, implying that COL7 may be involved in the phyB-mediated SAR. In this study, we provide evidence that COL7 reduces auxin levels in a high R:FR-dependent manner. We found that the \textit{phyB} mutation suppresses the COL7-induced branching proliferation. Moreover, COL7 promotes mRNA expression of \textit{SUPERROOT 2} (\textit{SUR2}), which encodes a suppressor of auxin biosynthesis, in high R:FR but not in low R:FR. Consistently with these results, deficiency of phyB suppresses the elevated transcription of \textit{SUR2} in \textit{COL7} overexpression plants grown in high R:FR. Taking these results together with data suggesting that photo-excited phyB is required for stabilization of the COL7 protein, we argue that COL7 is a critical factor linking light perception to changes in auxin level in \textit{Arabidopsis}.

\textbf{Key words:} \textit{phyB; COL7; SUR2; shade avoidance; auxin homeostasis.}


\textbf{INTRODUCTION}

Light is critical for plant survival because it provides both a resource for photosynthesis and useful information for attuning plant growth and development to seasonal changes or habitat conditions. Plants have evolved a battery of photoreceptors to sense ambient light and mediate transduction of light signals (Moglich et al., 2010; Yu et al., 2010). Intense research efforts have identified several classes of photoreceptors in \textit{Arabidopsis}: a UV-B photoreceptor (UVR8), seven blue light receptors, including two cryptochromes (CRY1 and CRY2), two phototropins (phot1 and phot2), three LOV/F-box/Kelch-domain proteins (ZTL, FKF, and LKP2), and five red/far-red light receptors phytochromes (phyA, phyB, phyC, phyD, and phyE). Among these photoreceptors, the phytochromes are prominent because they are able to perceive the neighboring vegetation (Franklin and Quail, 2010). Plants sense neighbor proximity by perceiving the red light (R) to far-red light (FR) ratio as nearby vegetation absorbs R and reflects or transmits FR (Ballare, 1999). The reduction of the R:FR ratio provides a signal of impending competition imposed by neighboring plants, and elicits a series of responses, called the shade-avoidance syndrome (SAS), which include increased elongation, suppression of branching, accelerated flowering, and early production of seeds (Franklin, 2008; Lorrain et al., 2008; Casal, 2012; Leivar et al., 2012). Among the five phytochromes in \textit{Arabidopsis}, phyB predominantly mediates SAS and \textit{phyB} mutants display a SAS-like phenotype that is characterized by long hypocotyls and petals, early flowering, and less branching (Finlayson et al., 2010). Like other phytochromes, phyB exists mostly

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in an active Pfr form in R or high R:FR conditions, but in an inactive Pr form in FR or low R:FR conditions. Therefore, the proportion of active phyB (Pfr/Pr) directly correlates with the R:FR ratio to which the plant is exposed (Franklin, 2008; Halliday et al., 2009; Franklin and Quail, 2010; Casal, 2012). This photochemical characteristic of phytochromes enables plants to finely adjust developmental strategies in response to neighboring light conditions.

Auxin is one of the core plant hormones that regulate plant growth and development (Rosquete et al., 2011; Vanstraalen and Benkova, 2012). Mounting evidence has shown that the level of auxin is closely coupled to phytochrome-mediated SAS. The Pfr form of phyB physically interacts with a subset of basic helix–loop–helix (bHLH) transcriptional factors, including PHYTOCHROME INTERACTING FACTOR 4 (PIF4), PIF5, and PIF7, and promotes their phosphorylation or degradation through the 26S proteasome when R:FR is high (Llorrain et al., 2008; Leivar and Quail, 2011; Li et al., 2012; Shin et al., 2013). In shade, phyB exists mostly in the inactive Pr form, allowing the dephosphorylation or accumulation of PIF4, PIF5, and PIF7, which promotes the expression of auxin biosynthetic genes (YUCCA2, YUCCA5, YUCCA8, and YUCCA9), auxin transporters (PIN3 and PIN4), and auxin response genes (IAA29 and GH3.3) (Li et al., 2012). As a parallel branch to YUCCA in the auxin biosynthesis pathway, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) encodes a tryptophan aminotransferase which is required for the conversion of tryptophan into IAA (Tao et al., 2008).

phyB suppresses the transcription of TAA1 and reduces the level of auxin in response to R. Consistently with this, taa1 mutants display short hypocotyls and more expanded cotyledons. In contrast, SUPERROOT 2 (SUR2) encodes the cytochrome P450 monooxygenase CYP831B, which acts as a suppressor of auxin biosynthesis (Delarue et al., 1998; Smolen and Bender, 2002). Defective SUR2 function leads to increased hypocotyl elongation and reduced cotyledon expansion in R due to the abundance of auxin, which is reminiscent of the phenotype of phyB mutants (Wagner et al., 1997). The transcriptional level of SUR2 is strongly up-regulated in seedlings growing in R compared to those growing in darkness or FR, implying that photo-activated phytochromes modulate the activation of SUR2 expression to reduce the level of auxin (Hoecker et al., 2004). Briefly, the active Pfr form of phyB decreases auxin level by concomitantly repressing auxin biosynthesis and promoting auxin homeostasis. However, the direct link integrating the light signal perceived by phyB and regulation of downstream gene expression remains elusive.

Previously, we reported that overexpression of CONSTANS-LIKE 7 (COL7) enhances branching number only when plants are grown in regular white light (high R:FR) but not in shade (low R:FR) (Wang et al., 2013). We postulated that COL7 may play a role in the phyB-mediated shade-avoidance response (SAR). In this study, we test this hypothesis and provide genetic and molecular evidence that COL7 enhances branch proliferation via the suppression of auxin level in a phyB-dependent manner. COL7 possesses transcriptional activation activity and overexpression of COL7 enhances the expression of SUR2 in high R:FR but not in low R:FR. Moreover, phyB stabilizes COL7 protein during the light period in both long-day and short-day conditions. Taken together, the results provided here suggest a possible light signal transduction pathway where COL7 links light signal perception by phyB to the modulation of auxin homeostasis by the activation of SUR2 expression.

**RESULTS**

**Promotion of Branching by COL7**

We recently reported that overexpression of COL7 driven by the constitutive 35S promoter leads to an abundant branching phenotype under long-day conditions (16h white light/8h dark) (Wang et al., 2013). In contrast, deficiency of COL7 in the T-DNA insertion mutant col7 only slightly reduces the branch number (Figures 1 and 2), implying that the branching phenotype is not significantly changed under the current growth conditions or other genes may be functionally redundant with COL7 in promoting branching. Therefore, we performed a decapitation experiment using wild-type (WT) and the col7 mutant grown in long-day conditions. Plants were decapitated at 3 d post bolting and the number of rosette branches was counted at 10 d post anthesis (DPA) (Beveridge et al., 2000; Finlayson et al., 2010). The visual phenotypes and statistical results provided in Figure 1 show that the rosette branch number of WT significantly exceeds that of the col7 mutant when both genotypes were treated by decapitation (Figure 1), further confirming that COL7 stimulates branch proliferation.

**COL7 Is Not Directly Involved in the Strigolactone Pathway**

Branching plasticity is a common development trait determined by the integrated regulation of external and internal factors (Franklin, 2008). The plant hormones strigolactone and auxin are the core endogenous suppressors in the regulation of shoot branching. Deficiency in either the biosynthesis or signal transduction of strigolactone or auxin leads to an extensive branching phenotype. To characterize the function of COL7 in branching control, we first tested the role of COL7 in strigolactone responses under low or high R:FR conditions. We examined the effect of GR24, a synthetic analog of strigolactones, on the hypocotyl elongation of WT, col7, and 35S:COL7 #10 seedlings grown on the surface of 0.5 MS agar medium containing increasing amounts of GR24. The results demonstrate that,
from WT were not able to rescue the extensive branching phenotype of 35S:COL7 #10 scions (Figure 3D, 35S:COL7 #10 & WT), and 35S:COL7 #10 rootstocks were also not able to enhance branching of WT scions (Figure 3D, WT & 35S:COL7 #10). This result contrasts with the reported observation that rootstocks from WT can restore WT branching to the scions of strigolactone-deficient mutants, such as max3 (Booker et al., 2005). Taking the reciprocal graft results together with the fact that strigolactone is mainly synthesized in the root, we argue that COL7 is not directly involved in strigolactone biosynthesis.

COL7 Down-Regulates Auxin Content in High R:FR

Because auxin is produced in shoots, the grafting experiment in Figure 3D suggests that COL7 may reduce auxin production. To test this possibility, we performed a root excision experiment, which investigates the auxin level by measuring the formation of auxin-induced adventitious roots (Sukumar et al., 2013). WT, col7, 35S:COL7 #10, and 35S:COL7 #11 seedlings were grown in low white light (3 μE m⁻² s⁻¹) for 5 d, then transferred to high-intensity light condition (100 μE m⁻² s⁻¹) with or without excision of the basal half of the hypocotyls (Figure 4A). The growth of excision-induced adventitious roots above the site of excision was recorded continuously for 8 d (Figure 4B and 4C). The number of adventitious roots between each genotype was detectable on the fifth day. Deficiency of COL7 in the col7 mutant induces more adventitious roots (P < 0.01, Student’s t-test, n > 10, day 6), whereas overexpression of COL7 in 35S:COL7 #10 and 35S:COL7 #11 leads to fewer adventitious roots (P < 0.001, Student’s t-test, n > 10, day 7) in comparison with the WT control (Figure 4C), suggesting that COL7 acts as a negative regulator of auxin level.

Taking advantage of the DR5rev:GFP reporter, we further investigated the auxin content in the hypocotyls of various genotypes acquired by crossing the col7 mutant or the 35S:MYC–COL7 line with the DR5rev:GFP reporter line (Benková et al., 2003). Confocal microscopy images and statistical analysis of GFP fluorescence intensities indicate that the GFP signal in the hypocotyl of DR5rev:GFP/35S:MYC–COL7 was significantly lower (P < 0.001, Student’s t-test) than that of DR5rev:GFP/NT or DR5rev:GFP/col7 when the seedlings were grown in high R:FR (Figure 5A and 5B), supporting the hypothesis that COL7 acts as a suppressor of auxin production. Furthermore, we observed that COL7 suppression of auxin is conditionally dependent on the R:FR ratio because, when the seedlings were grown in low R:FR, the GFP signal in DR5rev:GFP/35S:MYC–COL7 was restored to a similar level to that in DR5rev:GFP/NT or DR5rev:GFP/col7 (Figure 5C and 5D).
PhyB Mutation Suppresses COL7-Induced Branching Proliferation

The above findings, that COL7 suppresses auxin production under high R:FR but not under low R:FR, are consistent with our previous observations that the extensive branching phenotype of the COL7 overexpression line appears under high R:FR but not under low R:FR (Wang et al., 2013). Together, these results imply that red light receptors may modulate the function of COL7 in promoting branching. To test this possibility, we crossed the 35S:COL7 #10 line with the phyB mutant and acquired 35S:COL7 #10/phyB homozygotes, the genotype of which was confirmed by both RT–PCR (Supplemental
Figure 3 Hypocotyl Elongation Assay and Grafting Assay.
(A) Representative hypocotyl images of each genotype grown on 0.5 Murashige and Skoog salts supplemented with 0 μM (mock), 5 μM, or 10 μM GR24 in low R:FR (R:FR = 0.3) (left panel) or in high R:FR (white light, R:FR = 1.2) (right panel).
(B, C) Statistical analysis of hypocotyl lengths in seedlings of each genotype grown in low R:FR (B) or high R:FR (C) as shown in (A). Similar results were obtained from three independent biological replicates, and representative results are shown with standard deviations (n≥20 plants).
(D) Visual images of grafting experiments carried out with the indicated genotypes. Graft combinations are annotated as ‘scion & rootstock’. The average numbers of rosette branches with standard deviations (n≥10) are indicated at the bottom of each genotype or graft combination.

Figure 4 Root-Excision Assay to Analyze Auxin Level in Each Genotype.
(A) Schematic representation of the root-excision assay according to the method described by Sukumar et al. (2013).
(B) Representative images of root formation in intact or root-excised seedlings of WT, col7, 35S:COL7#10, and 35S:COL7#11.
(C) Statistical analysis of the number of adventitious roots determined in intact or root-excised hypocotyls of each genotype as shown in (B). Standard errors are shown (n>10).

of flowering (Figure 2A–2C), when grown in long-day conditions. We noticed that the rosette leaf numbers of the examined genotypes are different (Figure 2C). Because the leaf and branch numbers are generally correlated, simple comparisons of branch numbers may not adequately reflect the difference of branching phenotype between genotypes with different numbers of leaves (Finlayson et al., 2010). Therefore, we calculated the standard branch numbers of each genotype at the observed mean leaf values for the phyB mutant according to a described method (Finlayson et al., 2010). The result shows that the standard branch number of the 35S:COL7 #10 line is much higher than that of the 35S:COL7 #10/phyB line (Figure 2D), demonstrating that deficiency of phyB can efficiently suppress COL7-induced promotion of branching.
COL7 Possesses Transcriptional Activation Activity

COL7 belongs to a family of transcriptional regulators containing one or two N-terminal B-box zinc finger domains and a C-terminal CCT (CONSTANS, CONSTANS-like, and TOC1) motif (Khanna et al., 2009). To test whether COL7 acts as a transcriptional activator like CONSTANS (Tiwari et al., 2010), we investigated the transcriptional activation potentials of intact COL7 or a series of truncated fragments in yeast cells (Figure 6A). We fused the LexA DNA-binding domain with the full-length COL7 (LexA–COL7) or with a partial form with a deletion of the N-terminal 149 amino acids containing the B-box domain (LexA–COL7 △1–149), and found that both fusion proteins could activate the expression of reporter genes (Figure 6B). This suggested that the transactivation domain of COL7 is located in the

Figure 5 Investigation of Auxin Levels Using the DR5rev:GFP Reporter.

(A) Expression analysis of DR5rev:GFP in WT, col7, and 35S:MYC–COL7 backgrounds grown in white light (R:FR = 1.2) for 4 d. Hypocotyls were fixed in 4% formaldehyde, and the GFP signal was investigated by confocal microscopy. The experiments were repeated three times, yielding similar results, and representative images are shown.
(B) Statistical analysis of GFP signals in the experiment described in (A). Relative expression units (REU) of GFP are shown with standard deviations (n ≥ 10). The average GFP fluorescence intensity was measured by Image J and REU was calculated by the formula [REU = (average fluorescence intensity in the hypocotyl) − (average fluorescence intensity in the background)] * *P < 0.01
(C) Expression analysis of DR5rev:GFP in WT, col7, and 35S:COL7–MYC backgrounds grown in low R:FR (R:FR = 0.3) for 4 d.
(D) Statistical analysis of GFP signals in the experiment described in (C).

Figure 6 Assay of COL7 Transcription Activation Potential in Yeast Cells and Tobacco Leaf Cells.

(A) Schematic representations of intact or truncated fragments of COL7 fused to the LexA DNA-binding domain.
(B) β-Galactosidase assay in yeast cells. Plate assays (middle panel) and quantitative liquid assays (right panel) define domains of COL7 that are essential for transcription activation activity.
(C) Schematic illustration of the dual-luciferase reporter construct which contains two reporter genes: REN, driven by the 35S promoter, and, LUC, driven by the minimal 35S promoter fused with four CCAAT-boxes.
(D) Dual-luciferase assay to test the binding of COL7 to the CCAAT-box in N. benthamiana. Leaves were infiltrated with an Agrobacterium strain harboring the reporter plasmid described in (C), in the presence (+) or absence (−) of the co-transfecting Agrobacterium strain harboring an effector plasmid expressing COL7. The images were obtained 72 h after infiltration.
(E) Quantitation of relative reporter activities in the samples described in (D). LUC activities, normalized to REN activity, are defined as relative expression units (REU). Results of three independent experiments are shown with standard deviations.
C-terminal part of the protein containing the CCT motif. Moreover, deletion of an N-terminal fragment (Δ1–219) or the CCT motif (Δ320–392) completely abolished reporter gene expression, indicating that amino acids 149–219 and the CCT motif are both required for the transcriptional activation activity of COL7 (Figure 6B).

We then examined whether COL7 might act as a transcriptional regulator in plant cells, using a dual-luciferase in planta assay similar to the one we previously reported (Meng et al., 2013). Because CONSTANS-like family members are likely to form a large transcription regulator complex in plant cells by interacting physically with Nuclear Factor-Y (NF-Y), which recognizes and binds a unique CCAAT-box DNA motif (Ben-Naim et al., 2006; Wenkel et al., 2006; Song et al., 2008), the potential transcriptional regulatory activity of COL7 was tested by its effect on the firefly luciferase (LUC) reporter gene driven by a hybrid promoter that contains the minimal 35S promoter and four copies of the CCAAT-box sequence (Figure 5C). In this experiment, tobacco leaves were co-transformed with Agrobacterium tumefaciens strains harboring a plasmid expressing a dual-luciferase reporter and/or a plasmid expressing COL7. Expression of the Renilla reniformis luciferase (REN) driven by the standard 35S promoter was used as the internal control. The result of this ex vivo experiment shows that the recombinant promoter drove expression of the reporter genes in a COL7-dependent manner (P < 0.001, Student’s t-test) (Figure 6D and 6E). These results suggest that COL7 is a component of the CCAAT-box-specific DNA-binding complex and may act as a transcriptional activator in plant cells.

**COL7 Activates the Transcription of SUR2**

To further investigate the mechanism of COL7-mediated branching regulation in Arabidopsis, we examined whether altering the expression of COL7 or the ratio of R:FR affected mRNA expression of the genes involved in auxin or strigolactone biosynthesis or signal transduction. We surveyed the mRNA expression of seven genes and found that most of them are down-regulated in COL7 overexpression plants grown in high R:FR (Supplemental Figure 2). As an exception, the transcription of SUR2 increased in the COL7 overexpression line but decreased in the col7 mutant when compared to the WT control grown in high R:FR (Figure 7A). Given that COL7 shows transcriptional activation activity in both yeast and tobacco cells, and that SUR2 expression is up-regulated in the COL7 overexpression line, we surmise that SUR2 is a putative target gene of COL7 and COL7 may decrease auxin levels by activating the transcription of SUR2, which is a suppressor of auxin biosynthesis. Consistently with this hypothesis, overexpression of COL7 leads to an extensive branching phenotype similar to that caused by overexpression of SUR2 (Bak et al., 2001; Wang et al., 2013). Moreover, SUR2 mRNA in the COL7 overexpression line was restored to the WT level when the plants were in low R:FR, indicating that the activity of COL7 on SUR2 transcription is sensitive to changes in the R:FR ratio (Figure 7A). We further tested the mRNA levels of SUR2 in WT, 35S:COL7 #10, 35S:COL7 #10/phyB, and phyB grown in high R:FR. The quantitative PCR result demonstrates that high expression of SUR2 activated by overexpression of COL7 can be efficiently suppressed by phyB deficiency in the phyB mutant (Figure 7B). The above molecular results are consistent with the observation that overexpression of COL7 results in the extensive branching phenotype when the plants are grown in high R:FR or in the presence of functional phyB, but not when the plants are grown in low R:FR or with a deficiency of phyB.

**Photo-Excited phyB Stabilizes the COL7 Protein**

To investigate how phyB modulates the function of COL7, we examined the mRNA or protein levels of COL7 in different genotypes with sufficient or deficient phyB. First, we compared the mRNA expression of endogenous COL7 in WT and phyB mutants during a 24-h diurnal period. The result shows that there is no obvious difference between the COL7 mRNA levels in WT and phyB mutants grown in either long-day or short-day conditions (Supplemental Figure 3). Because an anti-COL7 antibody is not available and COL7Pro:GUS demonstrates universal expression in all tissues tested (Supplemental Figure 4), we utilized the MYC–COL7 overexpression line for protein level analysis.
To compare the MYC–COL7 protein level in plants with sufficient or deficient phyB, we crossed the MYC–COL7 line with the phyB mutant to get MYC–COL7/phyB homozygotes. MYC–COL7 and MYC–COL7/phyB plants were then grown in long-day or short-day conditions, and total proteins were extracted from samples collected every 4h during a 24-h diurnal period. The Western blot images and quantification results indicate that the MYC–COL7 level in WT is substantially higher than that in the phyB mutant grown in both long-day and short-day conditions (Figure 8). Moreover, in both long-day and short-day conditions, MYC–COL7 protein accumulates during the light period but decreases in darkness. Together, the above results demonstrate that photo-excited phyB can stabilize COL7 at the protein level. It was noted that the MYC–COL7 protein still accumulated in the phyB mutant background during the light period and decreased in the dark period, indicating that other photoreceptors may be additive with phyB to inhibit the degradation of COL7 in light conditions.

**DISCUSSION**

Branching plasticity is a common developmental trait that determines the ability of the plant to compete with surrounding vegetation in terms of intercepting light and obtaining other resources (Franklin, 2008). As a core endogenous regulator, auxin is produced in the apical bud and transported basipetally to prevent the outgrowth of axillary buds—a characteristic that is referred to as ‘apical dominance’ (Thimann and Skoog, 1933). Hypothetically, nascent auxin produced in the apical bud does not enter and regulate the axillary buds directly (Morris, 1977), but interacts with other phytohormones, such as strigolactone, in the regulation of shoot branching (Vanstraelen and Benkova, 2012). A ‘secondary-messenger’ hypothesis suggests that auxin controls the levels of acropetal signals that enter the bud and directly regulate bud activity (Ongaro and Leyser, 2008). Consistently with this ‘secondary-messenger’ hypothesis, strigolactone can move acropetally into axillary buds and antagonize bud outgrowth. Auxin

![Figure 8](image-url)
positively regulates strigolactone production by stimulating the expression of MAX3 and MAX4, both of which are required for strigolactone synthesis (Sorefan et al., 2003; Foo et al., 2005; Hayward et al., 2009).

In contrast to the considerable literature describing the interplay of endogenous phytohormones in the regulation of branching architecture, the mechanism of branching plasticity achieved by light modulation of phytohormones has remained elusive. The SUR2 gene, which suppresses auxin biosynthesis, is a putative regulator of the phytochrome signal transduction pathway, but the mechanism by which phytochromes modulate SUR2 expression is still a missing piece of the puzzle. Here we provide evidence that COL7 possesses transcriptional activation potential and that overexpression of COL7 elicits SUR2 mRNA expression. Moreover, COL7 is able to enhance the expression of a LUC reporter gene controlled by a hybrid promoter that contains CCAAT-boxes (Figure 5C). Given that there are three CCAAT-boxes located in the native SUR2 promoter (Supplemental Figure 5A), these results suggest that COL7 may suppress auxin levels by direct activation of SUR2 expression. To test this hypothesis, we investigated the transcriptional activation activity of COL7 on the SUR2 promoter via the ex vivo dual-luciferase assay (Supplemental Figure 5B and 5C). The results indicate that the presence of COL7 elevates the expression of the LUC reporter gene under the control of the SUR2 promoter by about two-fold in tobacco leaves. We tested the activity of COL7 on the SUR2 promoter using the yeast one-hybrid assay and did not detect the expression of a colorimetric reporter gene (β-galactosidase). Taking these results together, we argue that COL7 alone may be unable to bind the CCAAT-box, but it may be recruited to the SUR2 promoter with the help of other partner proteins present in plant cells—a possibility which needs to be further tested using methods that detect in vivo protein–protein and protein–DNA interactions.

Moreover, COL7 protein accumulates in the presence of photo-excited phyB during the light period but becomes labile in the absence of light-activated phyB in darkness or in the phyB mutant. We surmise that COL7 protein, analogous to CONSTANS, may be recognized by the E3 ubiquitin ligase and degraded through the 26S proteasome pathway (Liu et al., 2008b; Lazaro et al., 2012). Further characterization of the COL7 degradation mechanism will provide a better understanding of how phyB regulates auxin homeostasis through the modulation of COL7 stabilization in response to changes in light conditions. Nevertheless, phyB is unlikely to be the only photoreceptor that stabilizes COL7. We compared the levels of COL7 protein in WT and phyB mutant seedlings treated in the dark for 24 h and then illuminated with increasing periods of red light. Although the overall amount of COL7 protein accumulating in phyB was lower than that in WT, the level of COL7 in both WT and phyB peaked in half an hour and subsequently decreased (Supplemental Figure 6). These observations imply that phyA, which is a photo-labile light receptor, may stabilize COL7 additively with phyB during the initial period of red light irradiation.

**METHODS**

**Plant Materials and Growth Conditions**

The WT Arabidopsis thaliana plant used in this study is the Col-4 ecotype. The 35S:COL7#10, 35S:COL7#11, and 35S:MYC–COL7 transgenic lines and the col7 mutant were as previously described (Wang et al., 2013), and phyB (phyB-9) seeds were obtained from the Arabidopsis Biological Resource Center. Seedlings used for protein or mRNA analysis were grown on 0.5 MS medium (pH 5.8) in long-day (16 h light/8 h dark), half an hour and subsequently decreased (Supplemental Figure 6). These observations imply that phyA, which is a photo-labile light receptor, may stabilize COL7 additively with phyB during the initial period of red light irradiation.

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For immunoblot analysis of MYC–COL7 protein, sterilized seeds were grown on 0.5 MS medium under the indicated conditions. Seedlings were harvested, frozen, ground into powder, and suspended in 4 SDS loading buffer. Protein extractions were incubated at 98°C for 5 min, separated on 10% SDS–polyacrylamide gels, and transferred to a nitrocellulose membrane. MYC–COL7 protein was visualized using the anti-MYC antibody (Abmart). The membrane was stripped and re-probed using the anti-HSP antibody as loading control. Quantification of proteins was performed using Image J software (http://rsb.info.nih.gov/ij/).

**Dual-Luciferase Assay**

The transient transcription dual-luciferase assay was performed as previously described (Meng et al., 2013). The system includes two key constructs, the reporter vector, and the effector vector. To make the reporter vector, an artificial sequence including four repeated CCAAT-boxes (GGGCCCAATCGGGGGCCCAATCGGCGGCGCCCAATCGCA)
fused to the minimal 35S promoter was synthesized (Genewiz) and embedded into the HindIII and BamH1 sites ahead of firefly luciferase (LUC) in the reporter vector pGreen-0800-LUC. The effector vector was constructed by insertion of the COL7 coding DNA sequence (CDS) into the vector pEGAD–GFP under the control of the 35S promoter (Liu et al., 2008a). The reporter construct was co-transformed with the helper plasmid Psoup-p19 into Agrobacterium AGL0. The Agrobacterium culture containing the reporter vector was either incubated alone or as a mixture with the Agrobacterium culture containing the effector vector (at a reporter:effector ratio of 1:1), and then carefully press-infiltrated manually into the healthy leaves of 21-days-old N. benthamiana. The infiltrated plants were grown in continuous white light for 3 d, sprayed with luciferin (1 mM luciferin and 0.01% Triton X-100), and photographed with a charge-coupled device camera (Princeton Instruments). The dual-luciferase assay was performed using a commercial kit (Promega; DLR reagents).

GUS Histochemical Staining
To obtain COL7Pro:GUS transgenic plants, the 2356-bp COL7 promoter region was amplified by PCR from Arabidopsis genomic DNA. The PCR fragment was purified and cloned into the pCAMBIA3301 vector through the BamH1 and NcoI sites. Homozygous COL7Pro:GUS transgenic plants were used for GUS histochemical staining. Tissues were fixed with 90% acetone for 10–30 min on ice, rinsed three times with 50 mM sodium phosphate buffer, then incubated overnight with X-Gluc solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronate, 10 mM Na2EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Triton X-100, 50 mM Na2PO4 buffer, pH 7.0) at 37°C. The treated tissues were submerged in an ascending series of ethanol solutions (50%, 70%, 90%, and 100%) for 30 min at each concentration, prior to observation and photography.

Root-Excision Assay
The procedure was performed according to a previously described method (Sukumar et al., 2013). Briefly, seeds were sterilized and grown on 0.5 MS medium supplemented with vitamins (1 μg ml−1 thiamine, 1 μg ml−1 pyridoxine HCl, and 0.5 μg ml−1 nicotinic acid), 1.5% (w/v) sucrose, and 0.05% (w/v) MES. To induce hypocotyl elongation, the seedlings were grown in constant low white light (3–5 μE m−2 s−1) for 5 d. The hypocotyl base was then excised and the upper part was grown vertically under strong white light (100 μE m−2 s−1) at 25°C. Adventitious roots were quantified daily for 7 d.

Grafting Assay
The grafting assay was performed according to the protocol reported previously (Notaguchi et al., 2009). Briefly, seeds were grown on 0.5 MS supplemented with 1% sucrose, and graft surgery was performed using 4-day-old seedlings. The grafted seedlings were kept in long-day conditions at 27°C for 5 d, and then transferred to soil and grown in long-day conditions for 3–4 weeks prior to phenotypic analysis and photography.

Transcription Activation Potential Assay in Yeast
In this study, we used the LexA system to test the transcription activation potential of COL7. Intact or truncated versions of COL7 CDS were amplified by PCR and inserted into the vector PEG202 to express the LexA DNA-binding domain fused with intact or truncated COL7. The primers used are listed in Supplemental Table 1. Yeast transformation and measurement of relative β-galactosidase activities were performed as described previously (Liu et al., 2011).

mRNA Expression Analysis
Seedlings were grown on 0.5 MS under the indicated conditions, then materials were harvested (three biological replicates per genotype/treatment) and stored in liquid nitrogen. Total RNAs were isolated using the Trizol kit (Invitrogen). cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis system (Transgene). Quantitative PCR (20 μl reaction) was performed with 0.5 μl cDNA and 0.2 mM of each gene-specific primer for 40 cycles using the Light Cycler 480 SYBR Green I Master system (Roche) according to the manufacturer’s instructions. The efficiency of the PCR reactions was taken into account when calculating the relative expression levels (Bustin et al., 2009). Three technical replicates were done for each biological treatment. Gene expression was normalized to the expression of ACTIN2. The primers used for each gene in this study are shown in Supplemental Table 1.

GFP Fluorescence Assay
The GFP fluorescence of hypocotyls was observed with a Zeiss LSM 700 laser scanning confocal microscope. The images were taken with the same exposure time of 50 milliseconds under the GFP channel excited at 488 nm and collected at emission wavelengths of 505–530 nm. The GFP fluorescence intensity was measured by Image J software.

SUPPLEMENTARY DATA
Supplementary Data are available at Molecular Plant Online.
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