

A HY5-COL3-COL13 regulatory chain for controlling hypocotyl elongation in Arabidopsis

Bin Liu¹, Hong Long², Jing Yan², Lili Ye², Qin Zhang², Hongmei Chen², Sujuan Gao², Yaqin Wang², Xiaojing Wang², and Shulan Sun²

¹Shanghai Jiao Tong University - Minhang Campus

²South China Normal University

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Abstract

CONSTANS-LIKE (COL) family members are commonly implicated in light signal transduction during early photomorphogenesis. However, some of their functions remain unclear. Here, we propose a role for COL13 in hypocotyl elongation in *Arabidopsis thaliana*. We found that COL13 RNA accumulates at high levels in hypocotyls and that a disruption in the COL13 function via a T-DNA insertion or RNAi led to the formation of longer hypocotyls of *Arabidopsis* seedlings under red light. On the contrary, overexpression of COL13 resulted in the formation of shorter hypocotyls. Using various genetic, genomic, and biochemical assays, we proved that another COL protein, COL3, directly binds to the promoter of COL13, and the promoter region of COL3 was targeted by the transcription factor LONG HYPOCOTYL 5 (HY5), to form an HY5-COL3-COL13 regulatory chain for regulating hypocotyl elongation under red light. Additionally, further study demonstrated that COL13 interacts with COL3, and COL13 promotes the interaction between COL3 and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), suggesting a possible COP1-dependent COL3-COL13 feedback pathway. Our results provide new information regarding the gene network in mediating hypocotyl elongation.

Introduction

Light is one of the most important environmental cues influencing the early stages of post-germination plant development (Kami, Lorrain, Hornitschek, & Fankhauser, 2010; Olle & Viršile, 2013; Wu, Cameron, Ljung, & Spalding, 2010). Light-grown seedlings exhibit a developmental response termed photomorphogenesis, resulting in short hypocotyls and expanded green cotyledons. In contrast, dark-grown seedlings are characterized by long hypocotyls and unexpanded etiolated cotyledons; this process is called skotomorphogenesis (Josse & Halliday, 2008; McNellis & Deng, 1995; Smith, 2000).

As a central light signal repressor, the RING finger protein CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) is involved in many light-regulated responses and is responsible for the ubiquitination and degradation of several positive transcription factors, including B-BOX CONTAINING PROTEINs (BBXs) and ELONGATED HYPOCOTYL 5 (HY5) (Dornan et al., 2004; Duek, Elmer, van Oosten, & Fankhauser, 2004; Lau & Deng, 2012; Osterlund, Hardtke, Wei, & Deng, 2000; Seo, Watanabe, Tokutomi, Nagatani, & Chua, 2004; Seo et al., 2003).

BBXs family has 32 members (Kumagai et al., 2008), which are divided into five groups based on whether their respective proteins contain one or two BBX motifs and whether or not they possess a CCT domain (Khanna et al., 2009). BBX family members, some of which have been characterized and implicated in light signal transduction during early photomorphogenesis (Cheng & Wang, 2005; Graeff et al., 2016; Li et al., 2014; Park et al., 2011; Preuss et al., 2012; Wang, Guthrie, Sarmast, & Dehesh, 2014; Xu, Jiang, Li, Holm, & Deng, 2018; Xu et al., 2016; Yang et al., 2014). The first BBX protein identified in *Arabidopsis thaliana* was

CONSTANS (CO) (Putterill, Robson, Lee, Simon, & Coupland, 1995). In addition to CO, 16 other CO-Like (COL) proteins have been identified, which contain one or two B-box domains at the N-terminus and a CCT domain at the C terminus (Cheng & Wang, 2005). However, most of their functions remain unclear. COP1 can interact with BBXs proteins. For example, COP1 interact with COL3, which acts as a positive regulator under red light and localizes to nuclear speckles. The *col3* mutant partially suppresses the *cop1* mutation, suggesting that COL3 acts genetically downstream of COP1 (Datta, Hettiarachchi, Deng, & Holm, 2006). The loss-of-function *col3* mutant has longer hypocotyls and flowers early and exhibits a reduced number of lateral branches (Datta et al., 2006). COL3 also directly interacts with BBX32, which is regulated by the circadian clock to mediate flowering (Tripathi, Carvallo, Hamilton, Preuss, & Kay, 2017). Interestingly, both COL3 and BBX32 belong to the BBXs family.

HY5 is a positive regulator under far-red, red, blue, and UV-B light conditions (Ang et al., 1998; Delker et al., 2014; Hardtke et al., 2000). It mediates about one-third of genes expression throughout the *Arabidopsis* genome, including *BBX* s (Lee et al., 2007). For example, HY5 binds to the promoters of *BBX30* and *BBX31*, both of which negatively regulate light response (Heng et al., 2019). Meanwhile, the expression of *HY5* is regulated by BBXs. For instance, BBX21, BBX22, and BBX23 directly binds to the *HY5* promoter to activate its transcription and promote photomorphogenesis (Datta et al., 2008; Zhang et al., 2017; Job et al., 2018), whereas BBX24, BBX25 and BBX28 repress *HY5* action (Gangappa et al., 2013; Job et al., 2018; Lin et al., 2018). Therefore, BBXs and *HY5* constitute a light signal regulatory network that was essential for promoting photomorphogenesis.

A previous study showed that COL3 played multiple roles in plant development (e.g., flowering, hypocotyl elongation, and lateral root formation) (Datta et al., 2006). Although COL3 is known to interact with BBX32 to regulate flowering (Tripathi et al., 2017), there has been little research on how COL3 regulates hypocotyl elongation and the respective downstream pathways are uncharacterized. In the present study, we proposed a role for COL13 and an *HY5*-COL3-COL13 regulatory chain for controlling hypocotyl growth in *A. thaliana*.

Materials and methods

Plant materials and growth conditions

The *A. thaliana* mutant *col13* (GK657F04-023194) in the Columbia background (Col-0) was purchased from Germany (GABI-Kat, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany) (Rosso et al., 2003), whereas *col3* was generously provided by Professor Magnus Holm (Datta et al., 2006). Col-0, Ws, and their F1 hybrids were used as wild-type controls. Seed sterilization and growth were performed as previously described (Liu et al., 2016).

Hypocotyl Experiments

The light-response assays were performed as previously described with some changes (Datta et al., 2006). Plates were treated at 4 °C for 72 h and then moved to white light for 10 h to induce uniform germination. After that, the plates were transferred to different light conditions (dark, white, red, and blue light) and incubated at 22 °C for 3–6 d for hypocotyl measurement. Red and blue lights were generated by light emission diodes at 670 and 470 nm, respectively, (model E-30LED; Percival Scientific). The light intensity was approximately 100 $\mu\text{mol}/\text{m}^2 \text{ s}$ ppfd. The hypocotyl lengths of seedlings were measured and counted using ImageJ software.

Plasmid construction

Constructs for overexpression and RNAi assays: To construct the *COL13* overexpression construct, the predicted full-length *COL13* cDNA was cloned and inserted into the pCambia1390 vector between the *Sal* I and *Eco* RI restriction sites. To generate the *COL13* -RNAi transgenic plants, two fragments of the *COL13* coding sequence were amplified by PCR using primers containing *Pst* I (5' end) and *Mlu* I (3' end) restriction sites, and *Hin* dIII (5' end) and *Bam* HI (3' end) restriction sites. The two fragments were inserted into the pRNAi-0 vector in reverse orientation.

Constructs for GUS-staining assays: To construct the p*COL13* -GUS-2000 construct, a region comprising the 2000-bp promoter sequence of *COL13* was cloned and inserted into the pBI121 vector between the *Hin* dIII and *Bam* HI sites. To construct the p*COL13* -GUS-2812 construct, a region comprising the 2812-bp promoter sequence of *COL13* was cloned and inserted into the “1301 vector” between the *Sac* I and *Sal* I sites. To construct the p*COL3* -GUS construct, a region comprising the 967-bp promoter sequence of *COL3* was cloned and inserted into the pBI101 vector between the *Hind* III and *Xba* I sites.

Constructs for yeast assays: To make the *COL3* -pGBKT7, *COP1* -pGBKT7, *COL13* -pGBKT7, *COL3* -pGADT7, *COP1* -pGADT7, and *COL13* -pGADT7 constructs, the *COL3*, *COP1*, and *COL13* fragments were subcloned into the pGBKT7 vector (Gal4 DNA binding domain, Cat. No. 630489, Clontech) and pGADT7 (Gal4 activation domain, Cat. No. 630442, Clontech), as appropriate. To construct the *COL3*-pBbidge and *COL3*-*COL13*-pBbidge constructs, the *COL3* and *COL13* fragments were subcloned into the pBbidgeTM vector (Cat. No. 630404, Clontech), as appropriate. To construct the GAD-HY5 or GAD-*COL3* fusion protein in yeast, the HY5 or *COL3* coding sequence was subcloned into pJG4-5 with the *Eco*RI and *Xho*I sites. To construct the *COL3*-LacZ reporter gene, the 1119-bp *COL3* promoter region was amplified from genomic DNA and cloned into the pLacZi2u vector with the *Hind*III and *Xho*I sites (Lin et al., 2007). Other LacZ reporter gene plasmids containing various truncated *COL13* promoters were similarly constructed using the primers listed in Supplementary Table S1.

Constructs for GFP, CFP, and YFP assays: To construct the *COL13* -GFP construct, the full-length *COL13* coding region was cloned and inserted into the pBEGFP vector between the *Xba* I and *Kpn* I restriction sites. To construct the *COL3*-CFP, *COP1*-CFP, *COL13*-CFP, *COL3*-YFP, *COP1*-YFP, and *COL13*-YFP constructs, the full-length coding regions of *COL3*, *COP1*, and *COL13* were cloned and inserted into the pBluescript II Phagemid vector (Y. Liu et al., 2016), as appropriate.

Constructs for Co-IP assays: To construct the 35S:*COL3*- HA construct, the full-length *COL3* cDNA was cloned and inserted into the pCAMBIA1390-HA vector (Fang et al., 2019).

Constructs for dual-luciferase assays: Fragments of the *COL3* or *COL13* promoter were cloned into pGREEN0800-LUC to generate reporter vectors. A modified pBluescript vector (pBS) was used as an effector (Han et al., 2017).

The primers used are listed in Supplementary Table 1.

Plant transformation

Constructs in binary vectors were introduced into the *Agrobacterium tumefaciens* strain LBA4404 and transformed into *Arabidopsis* wild type (WT) or mutant plants by the floral-dip method (Clough & Bent, 1998). Approximately 30 T1 transgenic plants for each transgene were screened on MS medium supplemented with the appropriate antibiotics, and phenotypic analyses were performed on T2 or later generations.

Semi-quantitative reverse transcriptase (RT)-PCR and quantitative reverse transcriptase (qRT)-PCR

Semi-quantitative RT-PCR and qRT-PCR analyses were performed as previously described (Zhang, Liu, et al., 2014). RNA was extracted from 5-d-old seedlings. Three biological and three technical repetitions were performed for each combination of cDNA samples and primer pairs. The primers used are listed in Supplementary Table 1.

Dual-luciferase assay

Protoplasts were isolated, and the dual-luciferase assay was performed as previously described (Han et al., 2017). Transformed protoplasts were incubated at room temperature (~22) for 20-22 h and luciferase activities were measured using the dual-luciferase assay system (Dual-Luciferase[®] Reporter Assay, Promega, United States) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Three biological replicates were performed for all experiments.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, United States) (Han et al., 2017). The dual-luciferase assay mapped the COL3 binding site to a 1059 bp region of the *COL13* promoter, located between 676 and 1675 bp upstream of the transcription start site (ATG). This promoter region was used as a 5' end biotin-labeled probe and the same fragment, but unlabeled, was used as a competitor. To investigate the core-binding motif of the 1059 bp region, a series of EMSAs involving deletions of this region were performed. We divided the 1059 bp promoter sequence into five overlapping regions: -1675 to -1391 bp (probe 1), -1421 to -1184 bp (probe 2), -1201 to -1040 bp (probe 3), -1060 to -868 bp (probe 4), and -898 to -616 bp (probe 5). The sequence of probes is listed in Supplementary Table 2.

Yeast assays

Yeast one-hybrid assays were performed as described by Lin et al. (2007). The yeast two-hybrid and three-hybrid assays were performed using a Clontech kit (PT3024-1 (PR973283)). For yeast two-hybrid assays, the bait vectors (pGBKT7 plus candidate genes) and prey vectors (pGADT7 plus candidate genes) were transformed into the gold and Y187 yeast strains, respectively, and then each colony was picked for mating, and the mating solution was sprayed on SD/-Trp/-Leu/X- α -Gal/AbA (DDO/X/A) agar plates, and positive results were confirmed by growing them on SD/-Ade/-His/-Trp/-Leu/X- α -Gal/AbA (QDO/X/A) agar plates. For the yeast three-hybrid assay, COP1-pGADT7 and COL3-COL13-pBbridge constructs were co-transformed into the gold yeast strain. Each colony was picked and grown in SD/-Leu/-Met/-Trp and SD/-Leu/-Trp solutions, respectively. Normalized Miller units were calculated as a ratio of α -galactosidase activity in yeast. For all yeast assays, we used empty vectors as controls.

Co-immunoprecipitation (Co-IP)

Co-IP was performed as previously described with some changes (Fiil, Qiu, Petersen, Petersen, & Mundy, 2008). 35S:COL3-HA and 35S:COL13-GFP constructs were transformed into EHA105 *Agrobacterium* cells and then used to generate 35S:COL3-HA and 35S:COL3-HA::COL13-GFP transgenic plants. Proteins were extracted from 18-d-old seedlings. Anti-GFP used in this assay was bought from Abcam (ab290), Anti-HA used in this assay was bought from Sigma (H6908).

A 0.5 g sample of *Arabidopsis* seedlings were ground in liquid nitrogen, 1 ml (2 volumes) of lysis buffer was added (50 mM Tris pH 8.0, 150 mM NaCl, and 1 mM EDTA, containing 0.01 volume of 1 \times Protease Inhibitor Cocktail [Sigma]) or protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin). It was spun at 16,000 *g* for 20 min at 4 °C, and the supernatant was transferred to a new microcentrifuge tube. Protein concentration was determined using the Bradford reagent (Bio-Rad) and bovine serum albumin was used as a standard. For immunoprecipitation (IP) reactions, 1 mg of total protein was incubated with 5 μ l of polyanitiserum (or pre-immune serum) in a total volume of 1 ml of lysis buffer and incubated for 1 h to overnight at 4 °C, and the sample was mixed by inversion. During incubation, a 40 μ l wash per reaction of Protein-A-Agarose beads with 1 ml of cold lysis buffer or phosphate-buffered saline (PBS) was used by gentle vortexing and spinning in a microcentrifuge at 12,000 *g* for 30 s. The supernatant was carefully removed by aspiration. This process was repeated twice. A 50 μ l sample of fresh lysis buffer or PBS was added. The beads were ready to be used. To precipitate the immune complexes, 50 μ l of Protein-A-Sepharose (Amersham Pharmacia Biotech) slurry was added and incubated for 2 h to overnight at 4 °C, mixing the sample head-over-tail. The beads were washed in the microcentrifuge tube by resuspension in 700 μ l of lysis buffer. It was then centrifuged at 12,000 *g* for 15–30 s at 4 °C. The effluent was discarded. Steps 7 and 8 were repeated three more times. Next, 40 μ l of 2 \times SDS sample buffer was added to the beads. The beads were gently mixed (no vortexing) to avoid spreading the beads on the column walls. The sample was heated at 95 °C for 5 min to ensure that the Protein-A-Sepharose complex was within the heating well. The sample was then centrifuged at 12,000 *g* for 30 s. A sample of 10 μ l of the eluted immunoprecipitate was loaded on an SDS-PAGE gel (Bio-Rad). For standard western blotting analysis, 50 μ g of total protein was loaded. Proteins were electroblotted onto PVDF membranes (Amersham), blocked for at least 1 h at room

temperature in PBS-Tween containing 5% (wt/vol) non-fat dried milk. Primary antibodies were added to PBS-Tween containing 5% (wt/vol) non-fat dried milk and incubated for 1 h. Blots were developed using an ECL Kit from Amersham Pharmacia Biotech.

Histochemical GUS staining, GFP, and fluorescence resonance energy transfer (FRET) experiments

Histochemical GUS staining, GFP microscopy, and FRET were performed as previously described with some modifications (Datta et al., 2006; Hou, Wu, & Gan, 2013; Zhang, Zhang, et al., 2014). For the GUS-staining assay, the young seedlings (4 d after germination) were fixed and incubated in GUS-staining solution for 24 h at 37 °C. The stained samples were then cleaned with 75% ethanol and observed under a dissecting microscope.

For the GFP assay, the fusion *COL13* -GFP constructs were transformed into protoplasts for transient expression as previously described (Wu et al., 2009). Ten stable transgenic plants with *COL13* -GFP were obtained using the floral-dip method. Photographs of GFP were taken using a confocal microscope Olympus.

For the FRET assay, images were acquired using an Olympus confocal microscope, and protoplasts were visualized 16 h after transformation. The CFP was excited by a laser diode 405 laser and YFP, by an argon-ion laser. The target regions were bleached with 100 iterations using an argon-ion laser at 100% power.

Statistical analysis

Experimental data were analyzed using an ANOVA, and the statistical significance of any differences between treatments was tested using Duncan's test or t-tests. All analyses were conducted using SPSS for Windows.

Results

COL13 RNA accumulates at high levels in hypocotyls

By searching the gene expression information in the Arabidopsis Information Resource (TAIR) database (Klepikova, Kasianov, Gerasimov, Logacheva, & Penin, 2016), we found that *COL13*(*AT2G47890*) is highly expressed in the hypocotyl. qRT-PCR analysis confirmed that *COL13* was expressed in most plant organs, with higher expression in the hypocotyl and stem (Fig. 1a). To determine the spatial patterns of *COL13* expression in more detail, transgenic lines expressing GUS driven by the 2812 bp *COL13* promoter fragment were generated. As shown in Fig. 1b, GUS expression was predominantly in the hypocotyl.

COL13 regulates hypocotyl elongation under red-light conditions

To characterize the role of *COL13* in plants, we obtained the corresponding *Arabidopsis* T-DNA insertion mutant (GK-657F04-023194, termed *col13* in the following; Fig. S1a) from GABI-Kat, Max Planck Institute for Plant Breeding Research (Rosso et al., 2003). The mutation was verified by PCR (Fig. S1b), which amplified the *Sulphonamide*(*sul*) gene by using the primers listed in Supplementary Table 1. To confirm that the phenotype of the *col13* mutant was indeed caused by disruption of the *COL13* gene, we generated *COL13* overexpression (OX) (Fig. 2a) and *COL13* RNAi transgenic lines (Fig. 2b) for comparison.

To examine whether *COL13* was involved in light responses, the WT, *COL13* RNAi, and *col13* seedlings were germinated and grown under different light wavelengths (white, red, blue), as well as under dark conditions. As shown in Fig. S1c, under white or red light, the *COL13* RNAi and *col13* seedlings had longer hypocotyls than that of the WT, whereas in blue light or dark conditions, the hypocotyl length of all seedlings was not significantly different. Therefore, our research focused on red light. For further study, *COL13* OX, *COL13* RNAi, *col13*, and WT seedlings were germinated and grown under red light. We found that the *COL13* OX seedlings had shorter hypocotyls than the WT seedlings under red light (Fig. 2c, d). In contrast, the *COL13* RNAi and *col13* seedlings had longer hypocotyls than the WT seedlings under the same conditions (Fig. 2c, e). These findings suggested that *COL13* acts as a positive regulator of red-light-mediated inhibition of hypocotyl elongation.

Genetic interaction and physiological characterization of hypocotyl elongation

Given that the *phyB*, *hy5*, *col3*, and *cop1* mutations can affect hypocotyl elongation under red-light conditions (Datta et al., 2006; Lee et al., 2007; Peter H Quail, 2002; von Arnim & Deng, 1994), we investigated the expression of *COL13* in the absence of *PHYB*, *COL3*, *HY5*, and *COP1*. Semi-quantitative RT-PCR and qRT-PCR analysis revealed that the expression of *COL13* in *phyB*, *col3*, or *hy5* knockout plants was significantly reduced compared with that of the WT, whereas the expression of *COL13* in the *cop1* mutants was increased (Fig. 3a, b). As the expression of *COL13* decreased the most in the *col3* mutant, we generated transgenic lines expressing GUS under the control of the *COL13* promoter with the *col3* mutant background. Interestingly, although the *COL13* promoter was active in the hypocotyls and cotyledons in the WT seedlings, GUS expression was not detected in the hypocotyl in the *col3* mutant background (Fig. 3c).

To understand the functional relationship and genetic interaction between *COL13* and *COL3* and their role in the regulation of hypocotyl growth, we generated a *col13 col3* double mutant and examined hypocotyl length under red-light conditions. Given that *col13* was in Col-0 and *col3* was in the WS background, crossing lines from different backgrounds would likely affect hypocotyl length. To reduce the effect of the background, we used the F1 hybrid of Col-0 × WS as the WT. We found that, although hypocotyl length in the double-mutant *col13 col3* was longer than in the WT seedlings, it was not significantly different from hypocotyl length in the single mutants, *col13* or *col3*. (Fig. 3d). To confirm this result, we created the RNAi lines of *COL13* in the *col3* mutant background (Fig. 3e), and we obtained the same result as in Fig. 3d. Additionally, we also generated a *COL13*-OX line in the *col3* mutant background and showed that the hypocotyl length in this strain was similar to that of the WS and significantly shorter than that of the *col3* mutant (Fig. 3e). In other words, *COL13* overexpression rescued the phenotype exhibited by the *col3* mutant. Taken together, our results suggest that *COL13* might be downstream of *COL3* in the red-light-mediated signaling pathway.

HY5-COL3-COL13 regulatory chain

Based on the genetic data, the *col3 hy5* double mutant behaved like the *hy5* mutation (Datta et al., 2006), and *COL13* might be downstream of *COL3* in regulating hypocotyl elongation. In addition, it has been well established that HY5 binds to “CACG” and “GACGTG” in the promoters of light-responsive genes (Lee et al., 2007; Nawkar et al., 2017). *COL3* is a light-responsive gene, and we examined the promoter sequence of the *COL3* gene and found that it contains two “CACG” and one “GACGTG”. Thus, we hypothesized that there would be an HY5-COL3-COL13 regulatory chain for controlling hypocotyl growth. To test this hypothesis, the HY5 and *COL3* coding sequences, as well as a deletion series of the *COL13* promoter, were cloned into the dual-luciferase system (Fig. 4a). As shown in Figure 4b, these dual-luciferase experiments and yeast-one hybrid assays confirmed the ability of HY5 to bind to the *COL3* promoter and *COL3* to bind to the *COL13* promoter. Additionally, these experiments also mapped the *COL3* target regions (1059 bp) to between -1675 bp and -616 bp of the *COL13* promoter (Fig. 4b). To investigate the core-binding motif of the 1059 bp region, a series of EMSAs involving deletions of this region were performed. We divided the 1059 bp promoter sequence into five overlapping regions (Fig. 4c): -1675 to -1391 bp (probe 1), -1421 to -1184 bp (probe 2), -1201 to -1040 bp (probe 3), -1060 to -868 bp (probe 4), and -898 to -616 bp (probe 5), and showed that probe 2 (-1421 to -1184 bp) was essential for binding of *COL3* to the *COL13* promoter (Fig. 4d). The *in vivo* interaction of *COL3* with probe 2 was further confirmed by EMSA competition experiments that were conducted by adding excess amounts of the competitor (5-, 10-, and 25-fold higher amounts) (Fig. 4e). Next, we analyzed the sequence of probe 2, and found that in the 238 bp fragment, there were three light responsive elements (ATCT-motif, G-Box and TCT-motif) and one core promoter element for transcription start (TATA-box) (Fig. 4f). Then we wondered if these promoter elements were required for binding with *COL3*. To address this question, yeast one-hybrid assays were performed and the results showed that a 49-bp region of *COL13* promoter containing G-Box and TCT-motif were the binding requirement (Fig. 4g).

COL13 is located in the nucleus

Transformation of *Arabidopsis* protoplasts with a construct expressing COL13-CFP indicated that COL13

is located in the nucleus (Fig. 5a), and a similar result was obtained when the root apical cells of stable COL13-GFP transgenic plants were examined (Fig. 5b).

COL13 interacts with COL3, but not COP1

According to previous reports, both COL3 and COL13 are CONSTANS (CO)-like proteins, which are related to CO (Robson et al., 2001), and as shown for COL13 above, COL3 also positively regulates red-light-mediated inhibition of hypocotyl elongation in *Arabidopsis* (Datta et al., 2006). We also demonstrated that COL13 shares the same subcellular localization as COL3 (Fig. 5a, b). Given that COL3 can interact with BBX32 and that COL13 also belongs to the BBX zinc finger TF family, we hypothesized that COL3 might interact with COL13. This idea was supported by a two-hybrid assay revealing that COL3 interacts with COL13 protein in yeast (Fig. 6a). Next, we examined the interaction in transgenic plants expressing both COL3 and COL13 and showed that COL13 was co-immunoprecipitated with COL3 from seedling tissues (Fig. 6b). The phenotypes of 35S:COL3-HA and 35S:COL13-GFP transgenic plants were the same as 35S:COL3 and 35S:COL13 transgenic plants, respectively, which produced shorter hypocotyl than WT grown in the presence of red light (Fig. S2). The interaction between COL13 and COL3 was also demonstrated in plant cells in a FRET assay (Fig. 6c-f). As shown in Fig. 6c, both cyan fluorescent protein (CFP)-fused COL3 and yellow fluorescent protein (YFP)-fused COL13 were observed in the nucleus after excitation with a 405 nm or 514 nm laser, respectively. After bleaching an area of interest with the 514 nm laser, YFP-COL13 fluorescence was reduced dramatically, whereas there was a clear increase in CFP-COL3 emission in the same area (Fig. 6d), indicating that FRET had occurred. The relative intensities of emissions from CFP-COL3 and YFP-COL13 in the area of interest, before and after bleaching, are shown in Fig. 6e, f.

COL13 promotes interaction between COL3 and COP1

Interestingly, although COL13 and COL3 have similar structures, containing two N-terminal tandemly repeated B-box domains and a CCT domain in the C-terminal, only COL3 can interact with COP1, and COL13 does not bind to COP1 (Fig. 6a). These results were also demonstrated by the FRET assay (Fig. S3). To investigate whether COL13 influences the interaction between COP1 and COL3, we performed a yeast three-hybrid assay. In this yeast system, the COL3-COL13-pBridge construct allowed expression of *COL3-BD* /bait and *COL13* in yeast, and *COL13* was expressed only in the absence of methionine (Met). As shown in Fig. 7a, the growth of yeast carrying indicated constructs on selective medium (+Met or -Met) along with an α -galactosidase assay that showed that COP1 and COL3 had a stronger binding activity with the expression of COL13. Based on a previous report, COP1 interacted with COL3 and inhibited the production of COL3 (Datta et al., 2006). By combining our results above, we propose a possible COP1-dependent COL3-COL13 feedback pathway (Fig. 7b), which is involved in the regulation of hypocotyl elongation.

Discussion

Light regulates photomorphogenesis in plants. A large number of genes that are involved in such photomorphogenesis processes have been identified as light receptors (Datta et al., 2006; Kircher et al., 2002; Peter H. Quail, 2002), signal transduction factors (Gangappa et al., 2013; Osterlund et al., 2000) or degradation proteins (Crocco, Holm, Yanovsky, & Botto, 2010; Crocco et al., 2015; Delker et al., 2014). One of the immediate questions is how these genes act in a network to mediate various light-related phenotypes. It has been shown that multiple pathways are interlinked to form a gene network of photomorphogenesis (Lau & Deng, 2012; Lee, Park, Ha, Baldwin, & Park, 2017). Among these pathways, it is worth mentioning the ones formed by a subset of family genes termed the COL genes (Cheng & Wang, 2005). These family of genes plays multiple roles in plant development (Datta et al., 2006; Graeff et al., 2016; Muntha et al., 2018; Tripathi et al., 2017; Wang et al., 2014). As an effort toward COL networking, we investigated the relationship between *COL3* and *COL13* and provided evidence that these two COLs and HY5 were connected together to form an HY5-COL3-COL13 regulatory chain that controls hypocotyl elongation in *Arabidopsis* (Fig. 7b). Hypocotyl elongation is a genetically well-controlled process that responds to light. In *Arabidopsis*, several key genes are required for hypocotyl growth. Among these, COP1 is a negative regulator (McNellis, von Arnim, & Deng, 1994), whereas HY5 and COL3 are considered to be positive (Datta et al., 2006; Hardtke

et al., 2000). A previous study showed that COL3 plays a role in flowering and hypocotyl elongation (Datta et al., 2006), and COL3 is known to interact with B-BOX32 to regulate flowering (Tripathi et al., 2017). However, there has been no research on how COL3 regulates hypocotyl elongation. In this study, we demonstrated that *COL13*, whose RNA accumulated to a high level in the hypocotyl (Fig. 1), was one more positive regulator in the regulation of hypocotyl elongation under red-light conditions. For example, overexpression of *COL13* or knockdown of its transcript resulted in a shorter or longer hypocotyl, respectively, (Fig. 2).

As a positive regulator under far-red, red, blue, and UV-B light conditions (Ang et al., 1998; Delker et al., 2014; Hardtke et al., 2000), HY5 mediates about one-third of genes expression throughout the *Arabidopsis* genome (Lee et al., 2007). This study revealed that HY5 bound to the promoter of *COL3* and upregulate its transcription (Fig. 4b), indicating that *COL3* also acted as a downstream target of HY5. Interestingly, COL3 bind to the *COL13* promoter and positively regulate its expression (Fig. 4b-g), suggesting that COL3 are positive regulators of COL13. Thus, these findings suggest that HY5, COL3, and COL13 constitute a hypocotyl regulatory pathway.

BBX family members are commonly involved in photomorphogenesis and that they can interact with other BBX proteins to regulate plant growth (Tripathi et al., 2017; Wang et al., 2014). For example, COL3 belong to the BBX family and COL3 interact with BBX32 to regulate flowering (Tripathi et al., 2017). In this study, we demonstrated that COL13 could interact with COL3 (Fig. 6). Furthermore, we found that the expression of COL13 promoted the interaction between COP1 and COL3 (Fig. 7a). To our knowledge, COP1 is responsible for the degradation of several positive TFs, such as COL3, in the dark (Datta et al., 2006; Dornan et al., 2004; Duek et al., 2004; Lau & Deng, 2012; Osterlund et al., 2000; Seo et al., 2004; Seo et al., 2003). Increasing the binding activity of COP1 and COL3 would lead to the degradation of COL3. As a result, there would be less COL3 to activate the expression of COL13 (Fig. 7b). Therefore, we proposed a possible COP1-dependent COL3-COL13 feedback pathway as a hypothesis to optimize the HY5-COL3-COL13 regulatory pathway (Fig. 7b). This feedback pathway could enrich the regulation network in hypocotyl elongation.

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Author contributions

S.S. and W.X. proposed the project. S.S., W.X., and L.B. designed the experiments. L.B., L.H., Y.J., Y.L., Z.Q., C.H., and G.S. performed the experiments. L.B. and S.S. analyzed the data. L.B. wrote the manuscript, with the participation of S.S. and W.Y.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure legends

Fig. 1 *COL13* RNA accumulates at high levels in the hypocotyl. (a) Quantitative real-time PCR analysis of *COL13* transcript abundance in different tissues. R=Root; S=Stem; L=Leaf; SAM=Shoot apical meristem; H=Hypocotyl; F=Flower. (b) Activity of the *COL13* promoter revealed by β -glucuronidase (GUS) staining in *Arabidopsis* seedlings. Bar = 100 μ m.

Fig. 2 *COL13* regulates hypocotyl elongation under red-light conditions. (a) Relative expression of *COL13* in Col-0 and overexpression (OX) lines. (b) Relative expression of *COL13* in Col-0, T-DNA mutant (*col13*) and RNAi lines (R1-1 etc.). Asterisks indicate that the expression of *COL13* in OX9, OX11, OX20, *col13*, and *COL13* RNAi lines are significantly different with Col-0 under red light ($P < 0.05$). (c)-(e) Phenotypic analysis seedlings of the indicated genotypes were grown in the presence of red light. Images of representative seedlings are shown in (c), white bar=0.5cm. The hypocotyl lengths of the indicated genotypes were measured at the 5th or 3rd day, and are shown in (d) and (e), respectively. Error bars indicate SD ($n > 15$). Asterisks indicate that hypocotyl lengths in OX9, OX11, OX20, *col13*, and *COL13* RNAi lines are significantly different with Col-0 under red light ($P < 0.05$).

Fig. 3 Genetic interaction and physiological characterization of hypocotyl elongation. (a) Semi-quantitative reverse transcriptase (RT)-PCR analyses of *COL13* expression in *phyB*, *col3*, *hy5* and *cop1* mutants. (b) qRT-PCR analyses of *COL13* expression in *phyB*, *col3*, *hy5* and *cop1* mutants. (c) Activity of the *COL13* promoter revealed by β -glucuronidase (GUS) staining in WT and *col3* mutant backgrounds. (d) Hypocotyl length in WT, single- and double-mutant plants. Here we use the F₁ hybrid of Col-0 \times WS as WT. (e) Hypocotyl length in WT and *col3* plants compared to transgenic plants with *COL13* RNAi or *COL13* overexpression (OX) in the *col3* background. Here we use WS as WT. Error bars indicate SD ($n > 15$). Lower-case letters indicate significantly different data groups (hypocotyl length) of the indicated seedlings grown in red light.

Fig. 4 Analysis of the binding of HY5 to the COL3 promoter and COL3 to COL13 promoter truncations. (a) Diagram of constructs used in this study. For luciferase system, the AD-HY5 or AD-COL3 fusion proteins driven by the 35S promoter produces a potential effector protein, while the AD protein alone represents a negative control for basal activity of *COL3* promoter or each *COL13* promoter truncation. The *LUC* gene driven by the series of *COL3* promoter or *COL13* promoter truncations tests the ability of the

AD-HY5 or AD-COL3 fusion protein to bind to each promoter truncation. For yeast-one hybrid system, the GAD-HY5 or GAD-COL3 fusion proteins driven by the *GAL1* (P_{GAL1}) promoter serves as effectors. The GAD protein served as negative control to see if there exists the self activity of *COL3* or *COL13* promoters. The *LacZ* gene driven by *COL3* or *COL13* promoter truncations served as the reporter to test the binding activity of the GAD-HY5 or GAD-COL3 fusion protein to individual promoter truncations. (b) For luciferase assay, the fusion protein AD-HY5 can up-regulate *LUC* expression from the *COL3* promoter, but not from *COL13* promoter; and the fusion protein AD-COL3, but not AD alone, can up-regulate *LUC* expression from some of the *COL13* promoter truncations. For Yeast-one hybrid assay, the fusion protein GAD-HY5 can strongly bind to the promoter of *COL3*, but not *COL13* to direct *LacZ* expression in yeast cells that turns 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside to blue compound; and the fusion protein AD-COL3, but not AD alone, can bind to some of the *COL13* promoter truncations. (c) The 1059-bp promoter (-1675 bp to -616 bp) of *COL13* was divided into five overlapping regions: -1675 to -1391 bp (probe 1), -1421 to -1184 bp (probe 2), -1201 to -1040 bp (probe 3), -1060 to -868 bp (probe 4), and -898 to -616 bp (probe 5). (d) Electrophoretic mobility shift assay (EMSA) analysis showing the binding of COL3 on the -1421 to -1184 bp promoter of *COL13* (probe 2) in vitro. The + and - represent the positive and negative control, respectively. (e) Electrophoretic mobility shift assay (EMSA) analysis showing the binding of COL3 on the -1421 to -1184 bp promoter of *COL13* in vitro. The black arrow indicates binding of COL3 to the biotin-labeled *COL13* promoter. The + and - represent the presence and absence of corresponding components, respectively. Competition experiments were carried out by adding 5-, 10- and 25-fold excessive competitor. (f) In probe 2, there were three light responsive elements (ATCT-motif, G-Box and TCT-motif) and one core promoter element for transcription start (TATA-box). (g) The fusion protein GAD-COL3, but not GAD alone, can strongly bind to a promoter truncation of *COL13* to direct *LacZ* expression in yeast cells. The 238-bp promoter region (-1421 bp to -1184 bp) of *COL13* was divided into four fragments: -1421 to -1356 bp, -1355 to -1307 bp, -1306 to -1242 bp, and -1241 to -1184 bp. The colour rectangles represented indicated promoter elements in (f).

Fig. 5 Subcellular localization of COL13 (a) COL13-CFP localizes to the nucleus in protoplasts. (d) COL13-GFP localizes to the nucleus in root tip cells.

Fig. 6 COL13 interacts with COL3. (a) Yeast two-hybrid assay between COL13 and COL3. DDO=double dropout; QDO=quadruple dropout; pGADT7=prey plasmid; pGBKT7=bait plasmid. (b) Co-immunoprecipitation (Co-IP) in *Arabidopsis* Immunoprecipitations (IPs) were performed on proteins extracted from 10 d-old *Arabidopsis* seedlings grown under long-day illumination (16L: 8D) at 22 degC. Leaf tissues were harvested 1 h after the light cycle commenced. IP was performed using an anti-HA antibody and COL13 was co-immunoprecipitated with an anti-GFP antibody. A 5% input was used. Western blots were performed on 10% (wt/vol) precast gels (Bio-Rad). (c) COL3-CFP and COL13-YFP colocalize to the nucleus in protoplasts in the light and dark. Bar=5um. (d-f) FRET between CFP-COL3 and YFP-COL13 analyzed by acceptor bleaching in the nucleus. Bar=5um. The top panels in (d) show a representative pre-bleach nucleus co-expressing YFP-COL13 and CFP-COL3 excited with either a 514 or a 405 nm laser in light and dark, resulting in emission from YFP (yellow) or CFP (blue), respectively. The bottom panels in (d) show the same nucleus post-bleaching after excitation with a 514 or a 405 nm laser. The relative intensities of both YFP and CFP were measured before and after bleaching, as indicated in (e) and (f), respectively.

Fig. 7 COL13 promotes the interaction between COL3 and COP1.

(a) Yeast three-hybrid analysis of COP1-COL3 interaction in the presence of COL13. Normalized Miller units were calculated as a ratio of α -galactosidase activity in yeast. Additionally, normalized Miller units are reported separately for yeast grown on media with or without 1 mM methionine (Met), corresponding to induction (-Met) or repression (+Met) of *Met25* promoter-driven COL13 expression, respectively. Means and standard errors of the means for three biological repetitions are shown. Lower-case letters indicate significant differences in α -galactosidase. (b) A model representing the HY5-COL3-COL13 regulatory chain and COP1-dependent COL3-COL13 feedback pathway in the regulation of hypocotyl elongation.

Supporting Information

Table S1 List of primers and their uses.

Table S2 Probes used in EMSA assay.

Fig. S1 COL13 regulates hypocotyl elongation under red-light conditions. (a) Scheme of the *Arabidopsis* COL13 gene (AT2G47890) showing the T-DNA insertion position. (b) Identification of the sulfonamide resistance gene (SUL) in the *col13* mutant by PCR. (c) The hypocotyl lengths of the wild type (WT), COL13 RNAi, and *col13* seedlings under different light conditions. Error bars indicate the standard deviations ($n > 15$). Lower-case letters indicate significant differences ($P < 0.05$).

Fig. S2 Hypocotyl lengths of the indicated genotypes were measured at the 5th day. Error bars indicate SD ($n > 15$). Asterisks indicate that hypocotyl lengths in 35S:COL-HA and 35S:COL13-GFP are significantly different with Col-0 under red light ($P < 0.05$).

Fig. S3 COP1 can interact with COL3, but not COL13. (a) COL3-CFP and COP1-YFP co-localized to the nucleus in protoplasts under both light and dark conditions. (b) FRET between CFP-COL3 and YFP-COP1 analyzed by acceptor bleaching in the nucleus. The top panels in b show a representative pre-bleach nucleus co-expressing YFP-COP1 and CFP-COL3 excited with either a 514 or a 405 nm laser in light and dark, resulting in emission from YFP (yellow) or CFP (blue), respectively. The bottom panels in (b) show the same nucleus after bleaching following excitation with a 514 or a 405 nm laser. The relative intensities of both YFP and CFP were measured once before and twice after bleaching, as indicated in (c) and (d). (e) COL13-CFP and COP1-YFP co-localized to the nucleus in protoplasts in light and dark. (f) FRET between CFP-COL13 and YFP-COP1 analyzed by acceptor bleaching in the nucleus. The top panels in f show a representative pre-bleach nucleus co-expressing YFP-COP1 and CFP-COL13 excited with either a 514 or a 405 nm laser in light and dark, resulting in emission from YFP (yellow) or CFP (blue), respectively. The bottom panels in f show the same nucleus after bleaching following excitation with a 514- or a 405-nm laser. The relative intensities of both YFP and CFP were measured once before and twice after bleaching, as indicated in (g) and (h).

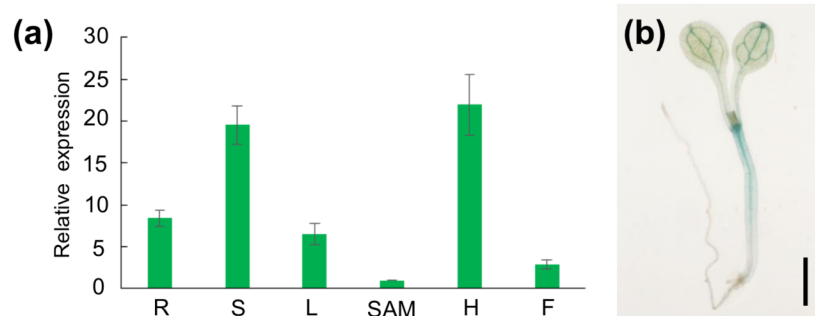


Fig.1

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