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Arabidopsis CONSTANS-LIKE3 Is a Positive Regulator of Red Light Signaling and Root Growth [W]

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CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) is an E3 ubiquitin ligase that represses photomorphogenesis in the dark. Therefore, proteins interacting with COP1 could be important regulators of light-dependent development. Here, we identify CONSTANS-LIKE3 (COL3) as a novel interaction partner of COP1. A green fluorescent protein–COL3 fusion protein colocalizes with COP1 to nuclear speckles when transiently expressed in plant cells. This localization requires the B-box domains in COL3, indicating a novel function of this domain. A loss-of-function *col3* mutant has longer hypocotyls in red light and in short days. Unlike *constans*, the *col3* mutant flowers early and shows a reduced number of lateral branches in short days. The mutant also exhibits reduced formation of lateral roots. The *col3* mutation partially suppresses the *cop1* and *deetiolated1* (*det1*) mutations in the dark, suggesting that *COL3* acts downstream of both of these repressors. However, the *col3* mutation exerts opposing effects on *cop1* and *det1* in terms of lateral roots and anthocyanin accumulation, suggesting that *COL3* also has activities that are independent of *COP1* and *DET1*. In conclusion, we have identified COL3 as a positive regulator of photomorphogenesis that acts downstream of COP1 but can promote lateral root development independently of COP1 and also function as a daylength-sensitive regulator of shoot branching.

INTRODUCTION

The perception of light participates in the gating of key developmental transitions throughout the life cycle of the plant, such as germination of the seed, photomorphogenesis or deetiolation of the seedling, and flowering. Deetiolation is arguably the most dramatic of these light-dependent transitions. Exposure of an etiolated seedling to light results in the inhibition of hypocotyl elongation, the promotion of cotyledon expansion, and the synthesis of a number of pigments, including chlorophyll and anthocyanin, and entails a dramatic transcriptional reprogramming (Ma et al., 2001).

Arabidopsis thaliana has three major classes of photoreceptors: red/far-red-light–responding phytochromes, blue light/UV-A light–responding cryptochromes, and phototropins. The cytoplasmic phototropins primarily regulate processes optimizing photosynthesis, whereas the transcriptional and developmental changes are attributed to the phytochromes and the cryptochromes. The phytochromes, encoded by the five genes *PHYA* to *PHYE*, are cytoplasmic in the dark but translocate into the nucleus in the light (Kircher et al., 2002). The active far-red-light–absorbing form of phyB was found to interact with a DNA-bound transcription factor, suggesting a rather direct signal transduction in which the photoreceptor could act in the promoter context (Martinez-Garcia et al., 2000). The two cryptochromes cry1 and cry2 are nuclear in darkness; both are phosphorylated in response to light, whereby cry1 becomes enriched in the cytoplasm and the light-labile cry2 is degraded. Cryptochromes interact genetically with multiple phytochromes (Neff et al., 2000). phyB and cry2 have been shown to tightly colocalize in vivo (Mas et al., 2000), suggesting that the different photoreceptors might act together to initiate similar developmental pathways. This is consistent with the large overlap in transcription profiles seen in microarray studies of seedlings grown in different monochromatic lights (Ma et al., 2001). Microarray studies performed in far-red light suggest that light initiates a transcriptional cascade in which a large fraction of the early affected genes are transcription factors (Tepperman et al., 2001).

In the absence of light, the seedlings become etiolated, a developmentally arrested growth mode characterized by limited root growth, an elongated hypocotyl, closed undifferentiated cotyledons, and an apical hook. The developmental arrest seen during etiolated growth is mediated by the COP/DET/FUS proteins, which act as repressors of the default photomorphogenic pathway. Mutations in any of these 10 genes result in deetiolated growth in darkness. Dark-grown *cop/det/fus* alleles have genome expression profiles closely resembling those of light-grown seedlings (Ma et al., 2003). Recent results have shown that COP/DET/FUS repression involves protein degradation. CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase, mediates ubiquitin-dependent degradation of the transcription factors HY5, HYH, LAF1, and HFR1 as well as the phyA photoreceptor (Osterlund et al., 2000a; Holm et al., 2002; Seo et al., 2003, 2004; Duek et al., 2004; Jang et al., 2005; Yang

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et al., 2005). The COP1-dependent degradation requires the activity of at least three different protein complexes: an ∼700-kD complex containing COP1 and SPA1 (Saijo et al., 2003); a 350-kD complex containing COP10, an E2 ubiquitin-conjugating enzyme variant, and DEETIOLATED1 (DET1) (Yanagawa et al., 2004); and the COP9 signalosome, a nuclear protein complex that activates cullin-containing multisubunit ubiquitin ligases (Cope and Deshaies, 2003; Wei and Deng, 2003).

The failure of plants with mutations in the *COP/DET/FUS* genes to arrest development during etiolated growth suggests that the targets of this pathway are likely to be key regulators of photomorphogenic development. To date, several photoreceptors as well as transcription factors have been shown to interact with COP1. phyB, cry1, and cry2 were found to interact with COP1 (Wang et al., 2001; Yang et al., 2001), and cry2 accumulates in *cop1-4* and *cop1-6* mutants (Shalitin et al., 2002), suggesting that COP1 might regulate cry2 abundance. Furthermore, COP1 was recently shown to interact with and ubiquitinate phyA (Seo et al., 2004). These interactions suggest that COP1 could mediate the desensitization and/or termination of signaling through the photoreceptors in the light.

By contrast, the photomorphogenic development seen in *cop/det/fus* mutants in the dark could not be mediated by photoreceptors because they are activated by light. Furthermore, genetic analysis revealed that *cop1* is epistatic to mutations disrupting phytochrome and cry1 function in darkness (Ang and Deng, 1994). The photomorphogenic development in dark-grown *cop/det/fus* seedlings, therefore, is likely caused by the loss of COP/DET/FUS repression of factors acting downstream of the photoreceptors. Four of the previously identified COP1-interacting proteins are transcription factors, and all four are positive regulators of light signaling. HY5 acts as a positive regulator in far-red, red, blue, and UV-B light conditions, HFR1 is a positive regulator in far-red and blue light, whereas LAF1 and HYH promote light-dependent development in far-red and blue light, respectively (Osterlund et al., 2000b; Ballesteros et al., 2001; Holm et al., 2002; Duek and Fankhauser, 2003; Ulm et al., 2004). Despite significant recent progress, only a few downstream regulators of light signaling have been identified, and the functional relationship between them is not well understood.

To date, mutations in only two genes have been found to suppress the phenotypes conferred by both *cop1* and *det1*. One of these is *HY5* (Ang and Deng, 1994; Pepper and Chory, 1997), the first identified target of the COP/DET/FUS pathway. The other gene, *TED3*, encodes a peroxisomal protein, and analysis of the dominant *ted3* mutation revealed that enhanced peroxisomal function partially suppresses weak *cop1* and *det1* alleles (Hu et al., 2002).

Here, we identify COL3 (for CONSTANS-LIKE3) as a COP1-interacting protein. Characterization of a *col3* mutant indicates that *COL3* positively regulates the light-dependent development and formation of lateral roots. Furthermore, *COL3* inhibits shoot elongation and promotes branching of the shoot specifically in short-day conditions. Finally, *col3* can suppress the deetiolated phenotype conferred by both *cop1* and *det1* alleles, and we characterize genetic interactions between *col3* and *hy5*, *cop1*, and *det1*.

RESULTS

COL3 Interacts with COP1 in Yeast Two-Hybrid Assays

COP1 was used as bait in a yeast two-hybrid screen in an effort to identify novel light-signaling components (Holm et al., 2001, 2002). In addition to the previously reported HYH, STH, and STO proteins, the screen identified three cDNAs encoded by the *COL3* gene, At2g24790 (Arabidopsis Genome Initiative, 2000). COL3 is one of the five CONSTANS (CO)-like proteins most closely related to CO (Robson et al., 2001). COL3, like CO, has two N-terminal tandemly repeated B-box domains, a CCT domain in the C-terminal half of the protein and a conserved motif in the C terminus (Figure 1A). The B-boxes show 59% amino acid identity (41 of 85), and the CCT domain shows 91% amino acid identity (39 of 43), between COL3 and CO, respectively. The Zn^{2+}-ligating B-box has been proposed to be a protein interaction domain, but it does not appear to be required for the interaction with COP1 in yeast because all three cDNAs identified in the screen encode a truncated COL3 protein lacking the 75 N-terminal amino acids (Figure 1A).

The COP1 protein used as bait contains three protein-interacting domains: a RING finger, a coiled-coil domain, and a WD40 repeat domain. To further examine the interaction between COP1 and COL3, we used Gal4 DNA binding domain fusions of COP1 proteins identified in three *cop1* alleles, *cop1-4*, *cop1-8*, and *cop1-9*. COP1-4 lacks the WD40 domain, whereas COP1-8 and COP1-9 contain a deletion and an amino acid substitution in the WD40 domain, respectively (Figure 1B) (McNellis et al., 1994). We found that COL3 is unable to interact with either of the COP1 proteins containing deletions or mutations in the WD40 domain, suggesting that the WD40 domain in COP1 is required for the interaction with COL3 (Figure 1C). Previous studies have identified Val–Pro (VP) pairs in the HY5, HYH, STH, and STO proteins that are critical for their interaction with COP1 (Holm et al., 2001, 2002). COL3 contains five VP pairs, and we substituted three of these with Ala (VP91AA, VP204AA, and VP291AA) to examine whether they were involved in the interaction with COP1. The COL3 proteins were all expressed at similar levels in yeast (Figure 1D). As shown in Figure 1C, both the VP91AA and VP204AA COL3 proteins interact with COP1, but the VP291AA substitution renders COL3 unable to interact with COP1, suggesting that, as in the B-box–containing proteins STH and STO, a VP pair in the C terminus is required for the interaction with COP1.

The COL3 Protein Colocalizes with COP1 When Transiently Expressed in Plant Cells

The COP1 protein localizes to nuclear speckles in the dark, and the nuclear abundance of COP1 decreases in the light (von Arnim and Deng, 1994). Furthermore, COP1 has been found to colocalize with several interaction partners, such as HY5, LAF1, HYH, HFR, and the CCT domain of CRY1 (CCT1), in nuclear speckles when expressed in onion (*Allium cepa*) epidermal cells (Ang et al., 1998; Wang et al., 2001; Holm et al., 2002; Seo et al., 2003). Both LAF1 and HFR localize to nuclear speckles when expressed

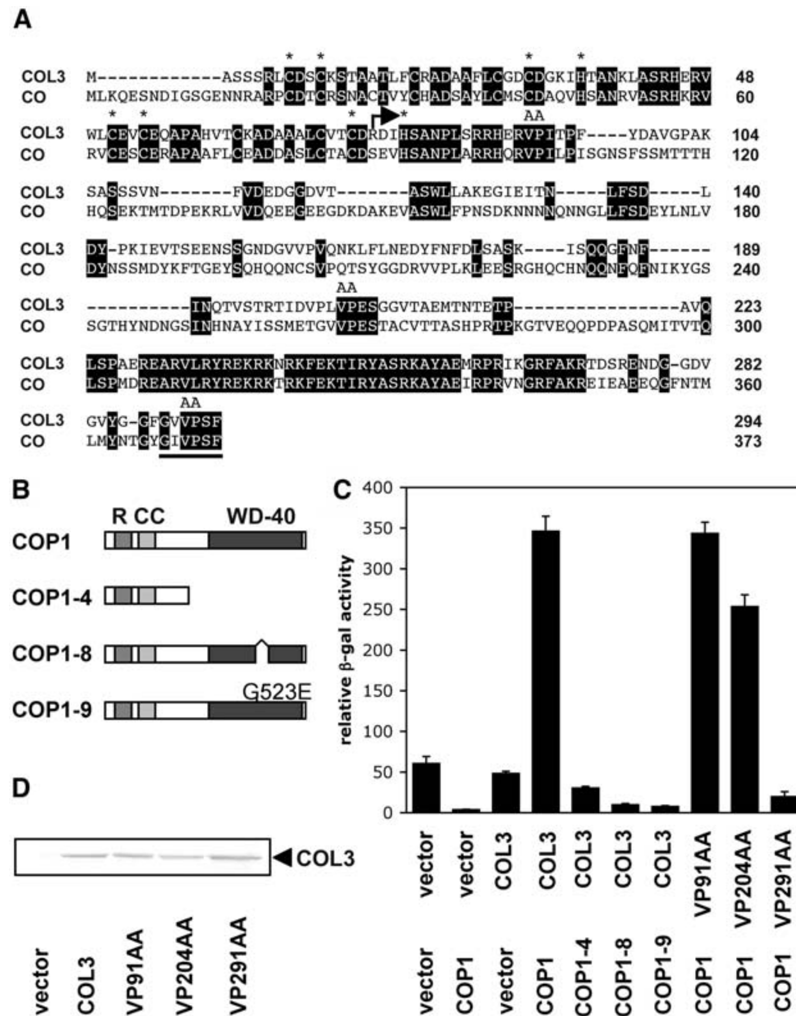

Figure 1. COL3 Interacts with COP1 in Yeast, and a VP Pair in the C Terminus Is Critical for the Interaction.

(A) Alignment of COL3 and CO. Identical amino acids are shaded in black. The arrow indicates the 5′ end of the cDNA identified in the screen, the asterisk indicates the Cys and His residues binding Zn^{2+}, and the underlined amino acids represent a motif conserved among the CO and COL1 to COL5 proteins.

(B) Scheme of the domain structure of COP1 and the mutated COP1 proteins. R, CC, and WD-40 indicate the RING finger, coiled-coil, and WD40 repeat domains present in COP1.

(C) Yeast two-hybrid interactions between the indicated COP1 and COL3 proteins. The Gal4 DNA binding domain–fused COP1 proteins are all expressed at similar levels in yeast (Holm et al., 2002). VP-n-AA indicates the Ala substitution of the three VP pairs at positions 91, 204, and 291, as indicated in **(A)**. Error bars indicate SE ($n = 5$).

(D) Immunoblot showing the similar expression levels of COL3 and substituted COL3 proteins in yeast.

in onion cells, but HY5, HYH, and CCT1 give a diffuse nuclear fluorescence when expressed alone and require coexpression of COP1 for speckle localization. We made two green fluorescent protein (GFP)–COL3 fusion constructs to examine the subcellular localization of COL3: GFP-COL3, containing the entire coding sequence of COL3; and GFP-COL3ΔB, lacking amino acids 1 to 75 encoding the B-box domains (Figure 2A). The GFP-COL3 protein is exclusively nuclear when expressed in onion cells, and it localizes to nuclear speckles both in the dark and in the light (Figure 2B). The speckles were consistently smaller and more numerous in cells incubated in the dark compared with cells incubated in the light (Figure 2B). By contrast, no speckles were observed for GFP-COL3ΔB. The truncated COL3ΔB protein was predominantly nuclear and gave a diffuse nuclear fluorescence both in the dark and in the light (Figure 2C).

Thus, the N-terminal B-boxes in COL3 are required for COL3 to localize to speckles, whereas the C terminus is required for interaction with COP1 in yeast. GFP fusions of HY5, HYH, and CCT1 give diffuse nuclear fluorescence when expressed alone but localize to speckles when coexpressed with COP1. To test whether overexpression of COP1 also can localize GFP-COL3ΔB to speckles, we coexpressed the two proteins and

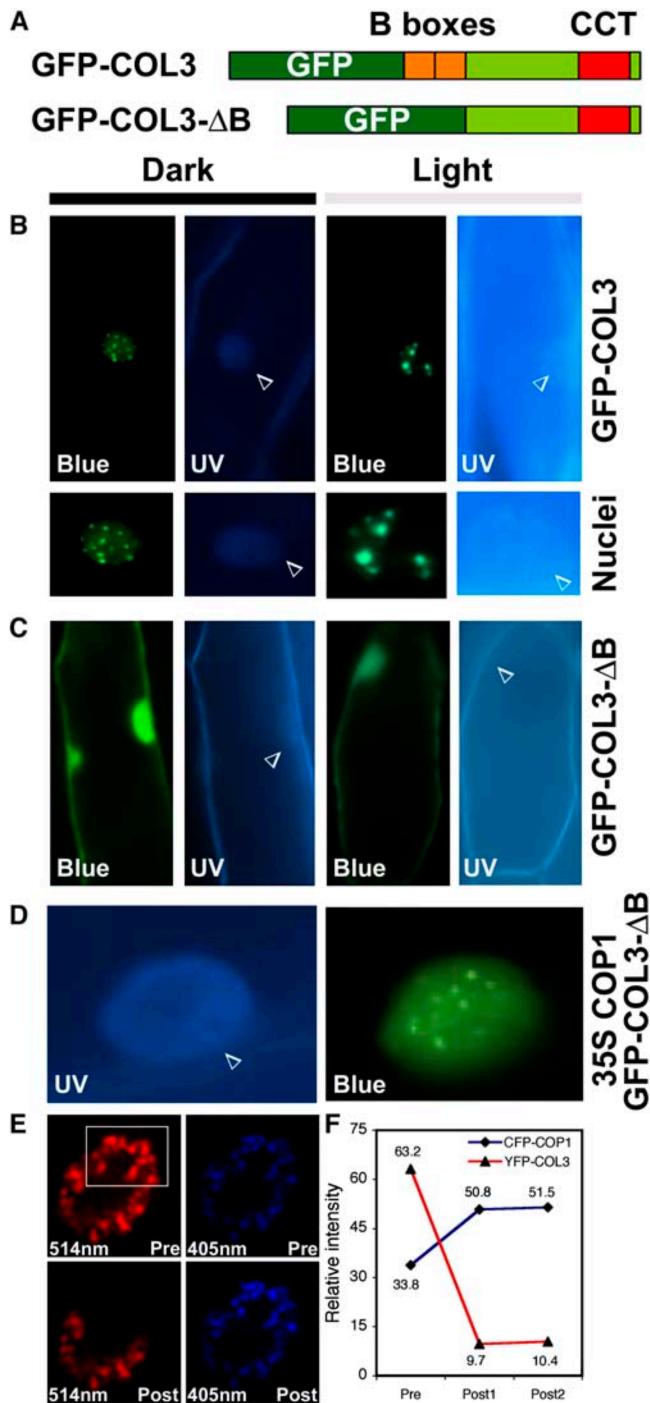

Figure 2. COL3 Localizes to Nuclear Speckles and Colocalizes with COP1 in Onion Cells.

(A) Schemes of the GFP-COL3 and GFP-COL3ΔB constructs.

(B) Onion epidermal cells expressing GFP-COL3 incubated in the dark and light and excited with blue light to induce GFP fluorescence and with UV light to show the positions of nuclei in 4′,6-diamidino-2-phenylindole–stained cells. Enlarged images of the nuclei, showing the size and number of speckles, are shown below. White arrowheads indicate the positions of the nuclei.

(C) GFP expression of GFP-COL3ΔB (COL3 with the B-box deleted), showing diffused fluorescence in the nuclei under the same conditions as in **(B)**.

(D) Nucleus of a cell coexpressing 35S:COP1 (untagged) and GFP-COL3ΔB, excited with UV and blue light.

(E) and **(F)** FRET between CFP-COP1 and YFP-COL3 analyzed by acceptor bleaching in nuclei ($n = 10$). The top panels in **(E)** show a representative prebleach nucleus coexpressing YFP-COL3 and CFP-COP1 excited with either a 514- or a 405-nm laser, resulting in emission from YFP (red) or CFP (blue), respectively. The white square indicates the region of interest that will be bleached with the 514-nm laser. The bottom panels in **(E)** show the same nucleus after bleaching excited with a 514- or a 405-nm laser. The relative intensities of both YFP and CFP in the region of interest were measured once before and twice after the bleaching, as indicated in **(F)**. An increase in donor fluorescence (blue) is seen only if a protein–protein interaction occurs.

found that in these cells GFP-COL3ΔB localized to speckles (Figure 2D).

Because both full-length COL3 and COP1 localize to speckles, we set out to examine whether the two proteins are found in the same subnuclear structures using the fluorescence resonance energy transfer (FRET) technique. To this end, we coexpressed cyan fluorescent protein (CFP)–fused COP1 with yellow fluorescent protein (YFP)–fused COL3 and analyzed FRET by acceptor photobleaching using a confocal microscope. As shown in the top panels of Figure 2E, a nucleus coexpressing YFP-COL3 and CFP-COP1 excited with 514- and 405-nm lasers resulted in the emission of YFP and CFP, respectively, before the 514-nm bleach of the region of interest. After the bleach, emission from YFP-COL3 in the region of interest was reduced dramatically, whereas we saw a clear increase in the emission of CFP-COP1 in the region of interest (Figure 2E, bottom panels), indicating that FRET had occurred. The relative intensities of emissions from CFP-COP1 and YFP-COL3 in the region of interest, before and after bleach, are shown in Figure 2F.

Identification of a T-DNA Insertion Mutation in the *COL3* Locus

To further characterize the role of *COL3* in plants, we screened the *Arabidopsis* knockout collection at Madison, Wisconsin, for T-DNA insertions in the *COL3* gene (Sussman et al., 2000). The collection was screened with primers annealing to sequences 5′ and 3′ of *COL3*, and we identified a T-DNA insertion within the gene (Figure 3A). The same T-DNA insertion was identified with both 5′ and 3′ primers, suggesting that the insertion consists of at least two T-DNAs inserted in a head-to-head orientation. Sequencing of the flanking regions revealed that the T-DNA was inserted in the first exon at nucleotide position 455 from the translational start site. The T-DNA results in the insertion of codons for the amino acids KSTCPAE followed by a stop codon after Glu-151 in COL3.

RNA gel blot hybridization revealed that a truncated mRNA is expressed at wild-type levels in the *col3* mutant (Figure 3B). The truncated mRNA in *col3* was amplified with RT-PCR, and sequencing confirmed that an mRNA fusion between *COL3* and the T-DNA was transcribed (data not shown).

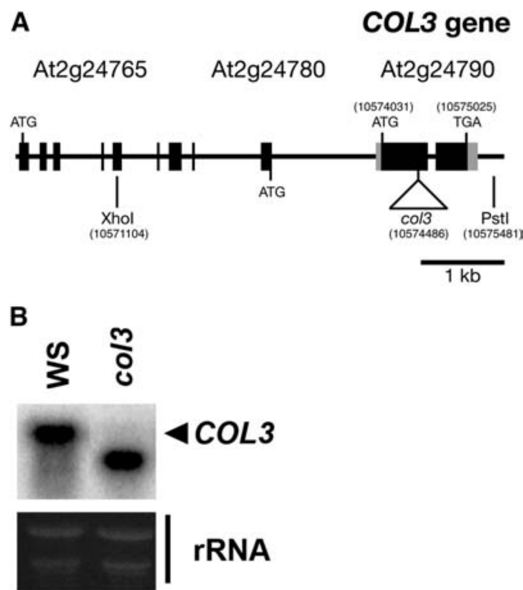

Figure 3. Identification of a T-DNA Insertion in the *COL3* Gene.

(A) Scheme of the *Arabidopsis COL3* gene (At2g24790). The T-DNA insert in *col3* and the *Xho*I and *Pst*I restriction sites used to create the pFP100-*COL3* complementation vector are indicated at positions 10,574,486, 10,571,104, and 10,575,481, respectively, on chromosome II.

(B) RNA gel blot showing *col3* transcript accumulation in wild-type Wassilewskija (Ws) and *col3* seedlings 6 d after germination in continuous white light. rRNA bands are shown to serve as a loading control.

The T-DNA line was backcrossed into the wild type (Ws) and crossed into *hy5-ks50*, *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* alleles. Analyses of these crosses revealed a single T-DNA locus cosegregating with the phenotype conferred by *col3*. To confirm that any observed phenotypes were indeed caused by disruption of the *COL3* gene, we introduced a 4377-bp genomic construct containing the *COL3* gene and 2927-bp 5′ and 456-bp 3′ sequences into the *col3* mutant as well as into each of the *col3* double mutants. For these genomic complementation experiments, we used the pFP100 vector, which allowed analysis in the T1 generation (Bensmihen et al., 2004).

COL3 Is a Positive Regulator of Light Signaling

To examine whether *COL3* is involved in light responses, *col3* seedlings were germinated in different fluences of blue, red, and far-red light. The *col3* seedlings did not differ significantly from wild-type seedlings in blue or far-red light (see Supplemental Figure 1 online) but had longer hypocotyls in high-fluence red light (Figures 4A and 4B). The finding that *col3* is specifically hyposensitive to high-fluence red light suggests that *COL3* acts as a positive regulator of the phytochrome-mediated inhibition of hypocotyl elongation. T1 transgenic *col3* seedlings transformed with pFP100-*COL3* (*col3COL3*) displayed hypocotyl lengths similar to wild-type plants (Figure 4B), indicating that a functional *COL3* gene could complement the phenotype conferred by *col3* in red light. Analysis of a segregating *col3* population revealed that the *col3* mutation is recessive. The complementation experiments and the recessive nature of the *col3* mutation are consistent with *col3* being a loss-of-function mutation.

We then examined *col3* seedlings grown in white light under different daylength conditions. We found no significant difference between wild-type and *col3* seedlings in constant light or under long-day conditions (16 h of light/8 h of dark) (see Supplemental Figure 2 online), but *col3* showed reduced inhibition of hypocotyl elongation in short-day conditions (8 h of light/16 h of dark) (Figure 4C). Also, this phenotype was complemented in T1 transgenic *col3* seedlings transformed with pFP100-*COL3* (Figures 4C and 4D).

The *hy5* mutation resulted in reduced inhibition of hypocotyl elongation in all light conditions. We generated a *col3 hy5* double mutant and examined the hypocotyl length in different light conditions. In all conditions tested, *col3 hy5* behaved like the *hy5* mutation (Figures 4B and 4C; see Supplemental Figure 2 online).

col3 Plants Flower Early in Both Long and Short Days

CO, the founding member of the CO-like family, was identified as a factor promoting flowering in long days (Putterill et al., 1995). To examine whether *COL3* affects flowering time, we compared *col3* with *co-2* grown in short days (8 h of light/16 h of dark) and long days (16 h of light/8 h of dark), respectively. We found that *col3* plants flower earlier than wild-type plants in both short and long days (Figure 5). The early flowering seen in long-day-grown *col3* plants is opposite that seen in *co* (Figure 5B) but similar to the early flowering seen in mutations in the genes encoding two COP1-interacting proteins, *hy5* and *hyh* (Holm et al., 2002), whereas neither *laf1* nor *hfr1* affects flowering time (Fankhauser and Chory, 2000; Ballesteros et al., 2001).

COL3 Regulates Lateral Organ Formation

When grown in short-day conditions, *col3* plants were taller and their primary shoots had fewer lateral branches than those of wild-type plants (Figures 6A and 6B). Neither the wild type nor *col3* produced secondary shoots under our short-day growth conditions. The elongated shoot and reduced branching phenotypes were observed only in short-day conditions: no significant difference in either height or branching was seen between *col3* and the wild type under long-day conditions (see Supplemental Figure 3 online). These results suggest that *COL3* promotes the formation of branches and inhibits the growth of the primary shoot specifically during short days.

The observation that the *col3* mutation affects the growth of the shoot prompted us to examine whether *col3* has any effect on root growth. To this end, we germinated *col3* and wild-type seeds on vertical plates in constant white light and measured the growth of the primary root. As shown in Figure 6C, *col3* seedlings had shorter primary roots than wild-type seedlings. The difference in root length was most pronounced at day 7 after germination and decreased at later time points. Interestingly, we found that *col3* seedlings produced fewer lateral roots than wild-type seedlings (Figure 6D). T1 *col3* seedlings transformed with pFP100-*COL3* displayed wild-type primary root length and number of lateral roots (Figures 6C and 6D), indicating that the

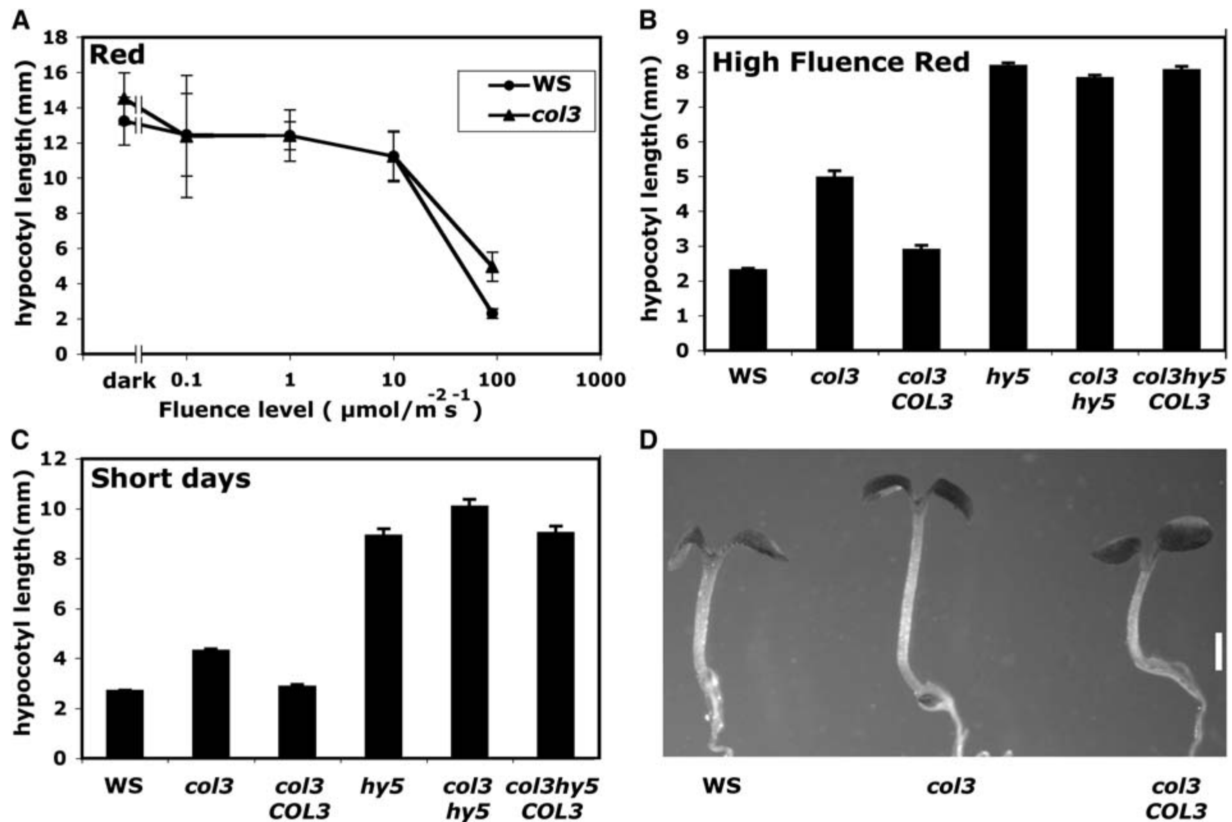

Figure 4. *col3* Seedlings Have Longer Hypocotyls When Grown under High-Fluence Red Light or Short Days.

(A) Fluence response curve of wild-type (Ws) and *col3* seedlings grown under continuous monochromatic red light. The experiment was performed twice with similar results. The graph depicts one of these experiments. Error bars represent SE ($n = 30$).

(B) Bar graph showing the difference in hypocotyl length between Ws, *col3*, *col3COL3*, *hy5*, *col3 hy5*, and *col3 hy5COL3* seedlings grown under high-fluence red light (90 μmol·m^{-2}·s^{-1}). *col3COL3* and *col3 hy5COL3* represent T1 *col3* and *col3 hy5* seedlings transformed with pFP100-*COL3*. Error bars represent SE ($n = 18$).

(C) Hypocotyl lengths of the indicated seedlings grown on plates under short-day conditions. Error bars represent SE ($n = 18$).

(D) Representative Ws, *col3*, and *col3COL3* seedlings grown on plates under short-day conditions.

COL3 gene complemented both phenotypes. Because the reduction of lateral branches in the shoot was seen in short-day conditions only, we examined lateral root formation in both short and long days, but similar results were obtained in all three light conditions, indicating that the lateral root phenotype, unlike the branching phenotype, is independent of daylength.

Both COP1 and the COP1-regulated transcription factor HY5 affect lateral root formation. The *cop1* mutation reduces the number of lateral roots, whereas the *hy5* mutation enhances both the initiation and elongation of lateral roots (Oyama et al., 1997; Ang et al., 1998). In addition to the lateral root phenotype, *hy5* seedlings show altered gravitropic and touching responses, enhanced cell elongation in root hairs, and reduced greening and secondary thickening of the root. We examined whether *col3* affects any of these processes, but we found no difference in gravitropic responses, greening, secondary thickening, or root hair elongation between *col3* and the wild type (data not shown).

To examine the genetic relationship between *col3* and *hy5* on lateral root formation, *col3 hy5-ks50* double mutant seedlings were analyzed. The double mutants were indistinguishable from *hy5* (Figure 6D), suggesting that *hy5* is epistatic to *col3* with respect to lateral root formation.

col3 Acts as a Suppressor of Both *cop1* and *det1*

We generated double mutants between *col3* and the *cop1* alleles *cop1-1*, *cop1-4*, and *cop1-6* as well as with *det1-1* to examine the genetic relationships between these genes. *col3* seedlings germinated in the dark were indistinguishable from wild-type plants (Figures 7A and 7B). However, when the *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, and *col3 det1-1* double mutants were germinated in the dark, we found that the double mutants had longer hypocotyls than either the *cop1* or *det1* single mutant, indicating that *col3* can partially suppress the hypocotyl phenotype of *cop1* and *det1* in the dark (Figures 7A and 7B). T1 *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, and *col3 det1-1* seedlings transformed with pFP100-*COL3* displayed hypocotyl lengths similar to those of the *cop1* and *det1* single mutants (Figure 7B),

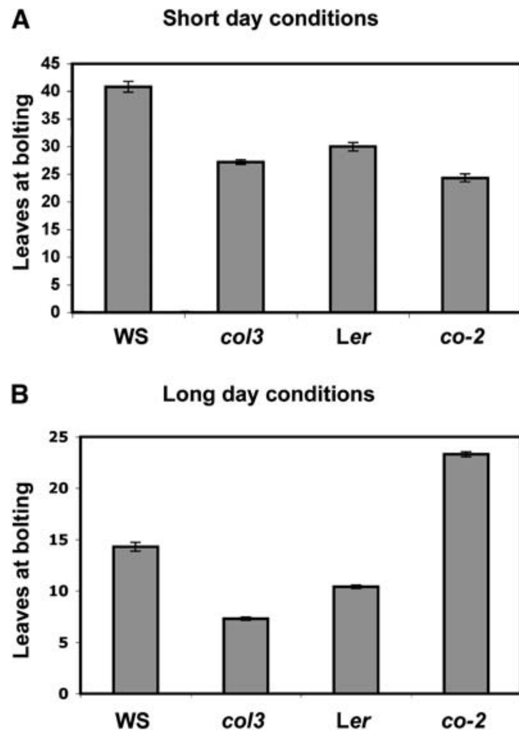

Figure 5. Unlike *co*, *col3* Flowers Early in Both Long and Short Days.

Graphs showing the number of rosette leaves at bolting for wild-type Ws, *col3*, wild-type Landsberg *erecta* (L*er*), and *co* plants grown under short-day conditions (8 h of light and 16 h of dark) **(A)** and long-day conditions (16 h of light and 8 h of dark) **(B)**. Error bars represent SE ($n = 15$).

indicating that a functional *COL3* gene could reverse the *col3*-dependant suppression.

Deetiolated *cop1* seedlings that have been germinated in darkness are sensitive to high-fluence light, and most of them are unable to green and will die upon transfer to white light (Ang and Deng, 1994). This COP1-dependent block-of-greening phenotype follows an allelic series and becomes more pronounced the longer the seedlings have been grown in the dark. We found that a higher percentage of *col3 cop1-4* (85%) and *col3 cop1-6* (57%) seedlings were able to green when germinated in the dark for 6 d and then transferred to light for 6 d compared with the *cop1* single mutant (19 and 19%, respectively) (Figure 7C). The difference was smaller between *cop1-1* and *col3 cop1-1* (54% compared with 75%), suggesting that *col3* acts as an allele-specific suppressor of this *cop1* phenotype (Figure 7C). We found a reduced competence to green also in *det1-1* (33% were able to green), the *col3 det1-1* double mutant displayed slightly improved greening (50%), and the *col3* suppression of *det1-1* was similar in magnitude to the suppression of *cop1-1*.

In conclusion, the *col3* mutation, like the *hy5* mutation, can suppress the hypocotyl phenotypes of both *cop1* and *det1* in the dark. Furthermore, similar to *hy5* and *hyh*, *col3* acts as an allele-specific suppressor of the COP1-dependent block-of-greening phenotype.

col3 Exerts Opposing Effects on *cop1* Alleles and *det1-1* in Terms of Emerged Lateral Roots under High-Fluence Red Light

Although the *hy5* mutation enhances the formation of lateral roots, both *col3* and *cop1* show reduced numbers of lateral roots. We consistently found a higher number of lateral roots formed in red light. To facilitate the analysis of the emergence of lateral roots in the double mutants, we performed the experiments in red light. Similar results, albeit with fewer lateral roots, were seen in white light.

In red light, *col3*, *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* all showed reduced numbers of emerged lateral roots (Figure 8). When analyzing the double mutants, we found that *col3* enhanced the phenotypes of *cop1-1* and *cop1-6*, whereas no significant difference was seen between *col3*, *cop1-4*, and *col3 cop1-4* (Figure 8), suggesting that *col3* acts as an allele-specific enhancer of the lateral root phenotype in *cop1*. Surprisingly, *col3* suppresses the lateral root phenotype of *det1-1* (Figure 8). The reduced number of lateral roots in *col3*, the *col3* enhancement of *cop1-1* and *cop1-6* lateral root phenotypes, and the suppression of *det1-1* lateral root phenotypes were complemented in T1 seedlings carrying a functional *COL3* gene (Figure 8), indicating that the phenotypes were caused by the *col3* mutation.

Thus, although *col3* partially suppresses the dark phenotype of weak *cop1* and *det1* alleles alike, we found very different genetic interactions in lateral root formation. Although the *col3* mutation partially suppresses the reduced formation of lateral roots in the *det1-1* mutant, it acts as an allele-specific enhancer of *cop1-1* and *cop1-6*.

col3 Has Reduced Levels of Anthocyanin and Has Opposite Effects on Anthocyanin Accumulation in *cop1* and *det1*

We found that the *col3* seedlings were slightly paler than wild-type seedlings during the first days after emergence from the seed coat. We assayed chlorophyll and anthocyanin contents of the seedlings to ascertain whether *col3* affects the accumulation of either of these pigments. We found no significant difference in chlorophyll content; however, *col3* seedlings had approximately half the amount of anthocyanin compared with the wild type in both red and white light (40 and 46%, respectively) (Figures 9A and 9B). The difference was more pronounced at day 4 after germination and had decreased to 9% at day 7. The reduction in anthocyanin levels in 4-d-old red light–grown *col3* seedlings was complemented in T1 seedlings transformed with pFP100-*COL3*, indicating that the phenotype was caused by the loss of the *COL3* gene (Figure 9A).

The *hy5* mutation causes reduction in both chlorophyll and anthocyanin levels during deetiolation (Holm et al., 2002). Because *hy5* is epistatic to *col3* with respect to lateral root formation, we analyzed anthocyanin levels in the *col3 hy5* double mutant. However, we found that the effect of the *col3* and *hy5* mutations was additive in red light, whereas the anthocyanin levels of *col3 hy5* were intermediate between those of the *col3* and *hy5* mutants in white light, suggesting that *col3* and *hy5* regulate anthocyanin accumulation independently (Figures 9A and 9B).

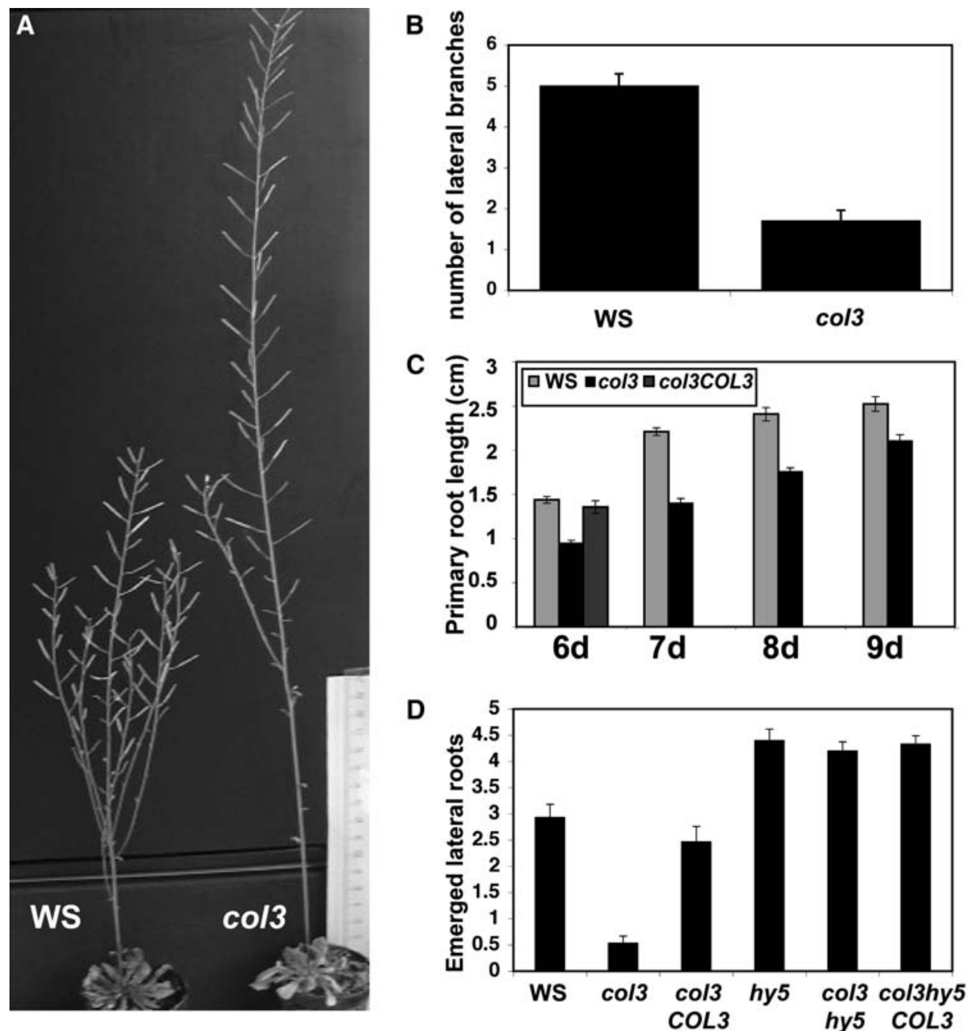

Figure 6. *COL3* Regulates Lateral Organ Formation as the Mutant Exhibits Reduced Branching in Both the Shoot and the Root.

(A) Representative Ws and *col3* plants showing lateral branching in the shoot grown under short-day conditions for 70 d.

(B) Number of lateral branches arising from the main shoot in 70-d-old Ws and *col3* plants grown under short-day conditions. Error bars represent SE ($n = 15$).

(C) Primary root length in Ws, *col3*, and *col3COL3* plants grown on vertical plates in continuous white light for the indicated number of days. Error bars represent SE ($n = 22$).

(D) Number of emerged lateral roots in Ws, *col3*, *col3COL3*, *hy5*, *col3 hy5*, and *col3 hy5COL3* seedlings grown under continuous white light for 10 d. Error bars represent SE ($n = 15$).

Both *cop1* and *det1* have increased expression of chalcone synthase, the first committed enzyme in the anthocyanin biosynthetic pathway (Chory and Peto, 1990; Deng et al., 1991). To further characterize the genetic relationship between *col3*, *cop1*, and *det1*, we analyzed anthocyanin accumulation in *col3 cop1-6* and *col3 det1-1*. As seen in Figures 9C and 9D, *cop1-6* and *det1-1* have increased levels of anthocyanin in 4-d-old seedlings germinated in red or white light. The *cop1* mutation has 12.1- and 4.5-fold increases in anthocyanin content compared with the wild type in red and white light, respectively. The *det1-1* mutation results in 8.0- and 1.7-fold higher anthocyanin content than the wild type in red and white light, respectively. Double mutant analysis revealed that *col3* has opposite effects on anthocyanin accumulation in the *cop1* and *det1* seedlings. In the case of *col3 cop1-6*, the anthocyanin content was reduced to 53 and 44.7% of *cop1-6* levels in red and white light, respectively (Figures 9C and 9D). By contrast, *col3 det1-1* had higher anthocyanin content than the *det1-1* single mutant, 9.6- and 3.2-fold above wild-type levels in red and white light, respectively (Figures 9C and 9D). These results suggest that although the *col3* mutation can suppress the accumulation of anthocyanin in both red light– and white light–grown *cop1* seedlings, it enhances the anthocyanin accumulation in *det1-1*, particularly in white light.

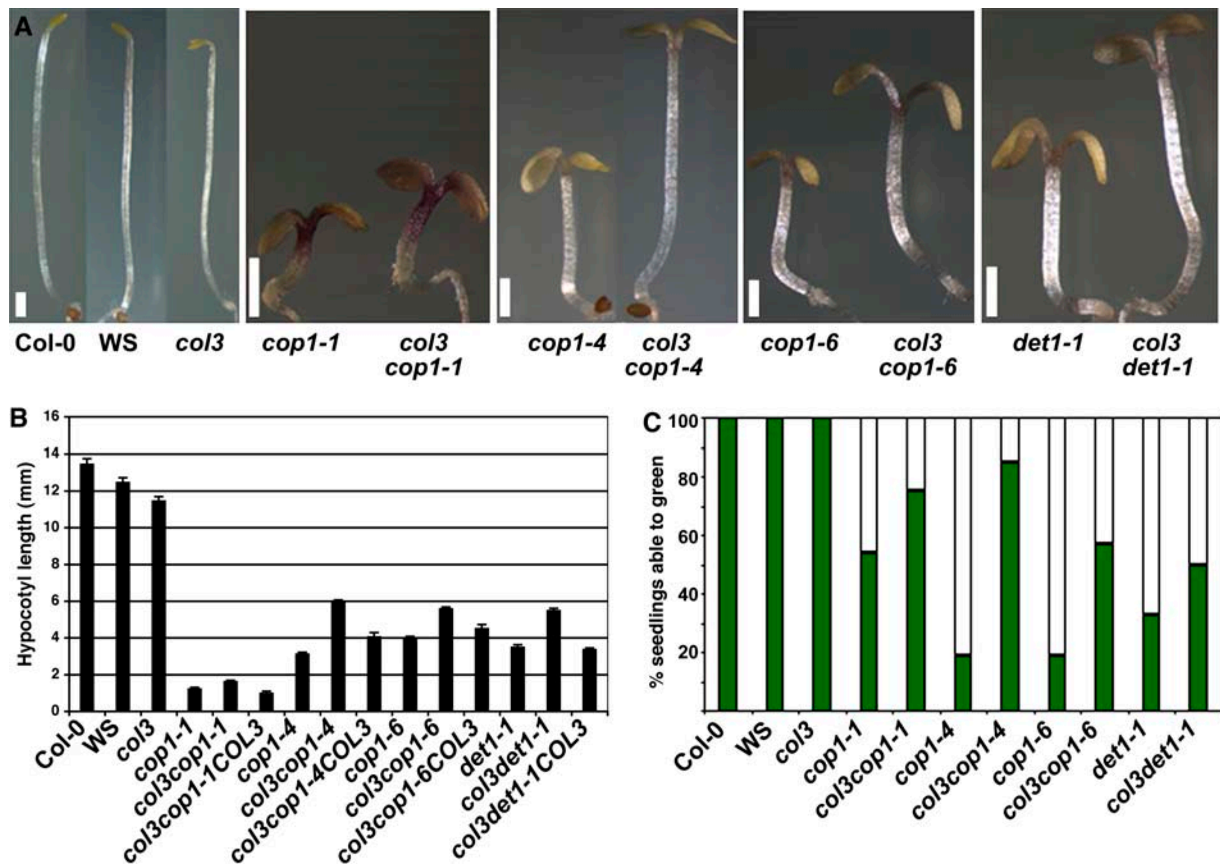

Figure 7. *col3* Suppresses the Phenotypes Conferred by *cop* and *det* in Darkness and in the Light.

(A) Wild-type and mutant seedlings (as labeled) grown in the dark for 6 d. Bars = 1 mm.

(B) Hypocotyl lengths of the indicated seedlings grown in the dark for 6 d. Error bars represent SE ($n \geq 8$).

(C) The *col3* mutation partially suppresses the light-dependent block-of-greening phenotype of the *cop1* and *det1* alleles. Seedlings were germinated in the dark for 6 d and then transferred to constant white light for 6 d. Seedlings with green cotyledons and/or true leaves were scored as able to green, and those with bleached cotyledons and/or true leaves were scored as unable to green ($n = 50$). The number of seedlings able to green is expressed as a percentage of the total number of seedlings.

DISCUSSION

Here, we report the identification of COL3 as a COP1-interacting protein and the characterization of a *col3* loss-of-function mutant. The interaction between COP1 and COL3 that was identified in yeast two-hybrid assays is supported by colocalization and positive FRET signals between the proteins in onion epidermal cells. A functional interaction between *COP1* and *COL3* is further supported by phenotypic and genetic analyses of the *col3* mutant.

Functional Domains in COL3

Analysis of the 16 CO-like proteins in *Arabidopsis* has revealed that the family is divided into three broad groups (Robson et al., 2001; Griffiths et al., 2003). COL3 is included together with CO and COL1 to COL5 in a group that has two B-boxes, a CCT domain, and a conserved six–amino acid motif in the C terminus. In animals, B-boxes are usually found in proteins that also have RING finger and coiled-coil domains. These proteins are often referred to as the RBCC (for RING, B-box, coiled-coil) or tripartite motif family. The RBCC family includes a large number of genes involved in functions such as axial patterning, growth control, differentiation, and transcriptional regulation (Torok and Etkin, 2001). The tumor suppressor Promyelocytic Leukemia (PML) gene, identified at the chromosomal breakpoint in t(15;17)-associated acute promyelocytic leukemia (de The et al., 1991; Kakizuka et al., 1991), encodes what is arguably the best-characterized RBCC protein. PML localizes to the PML nuclear body (NB), a subnuclear structure to which at least 30 proteins have been found to colocalize (Salomoni and Pandolfi, 2002). PML appears to be required for NB formation, because all NB components tested to date acquire an aberrant nuclear localization pattern in $PML^{-/-}$ primary cells, and their normal localization patterns can be restored by expression of PML (Zhong et al., 2000; Lallemand-Breitenbach et al., 2001). The localization of PML to NB requires functional RING finger and B-box domains, because substitutions of Zn^{2+} ligating residues in

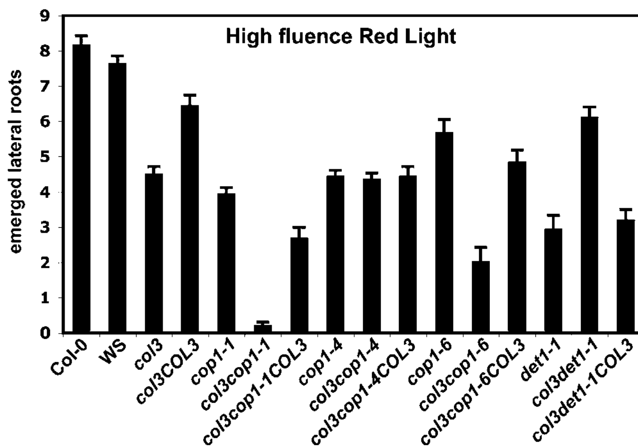

Figure 8. *col3* Exerts Opposing Effects on *cop1* Alleles and *det1-1* in Terms of Emerged Lateral Roots.

Number of emerged lateral roots on the indicated seedlings grown on vertical plates for 8 d under high-fluence red light (90 μmol·m^{-2}·s^{-1}). Error bars represent SE ($n \geq 11$ except for *col3 cop1-1COL3*, for which $n = 3$).

either of these domains disrupt PML NB formation (Borden et al., 1996). In addition, PML NB formation requires the sumoylation of Lys-160 in the first of the two B-boxes of PML, further indicating the role of the B-box domain in NB formation (Zhong et al., 2000; Lallemand-Breitenbach et al., 2001). The B-box is generally considered to mediate protein–protein interactions either directly or indirectly (Torok and Etkin, 2001), and the B-boxes in PML have been shown to interact with the GATA-2 transcription factor (Tsuzuki et al., 2000). The finding that deletion of the B-boxes in COL3 results in uniform nuclear fluorescence suggests that the COL3 B-boxes, like the PML B-boxes, are involved in speckle formation (Figure 2).

However, although RBCC proteins are found in several eukaryotes, they seem to be absent in *Arabidopsis* (Kosarev et al., 2002). In light of this, the interactions between the RING finger and coiled-coil domain containing COP1 protein and the B-box containing COL3, STH, and STO proteins (Holm et al., 2001) are interesting because this could bring the three domains together through protein–protein interaction. The interactions require the WD40 domain in COP1 and the C termini of COL3, STH, and STO, which would leave the RING, coiled-coil, and B-box domains available to interact with other proteins.

The CCT (for CONSTANS, CO-like, and TOC1) domain is a highly conserved basic module of ~43 amino acids often found in association with other domains, such as B-boxes, the response regulatory domain, the ZIM motif, or the DNA binding GATA-type zinc finger. Alleles with mutations in the CCT domain have been identified in both *TOC1* and *CO* (Strayer et al., 2000; Robson et al., 2001), suggesting that the domain is functionally important. The CCT domain contains a putative nuclear localization signal within the second half of the CCT motif and has been shown to be involved in nuclear localization (Robson et al., 2001). The CCT domain probably also has a role in protein–protein interaction, and the CCT domains of CO and TOC1 (also called ABI3-interacting protein1 or APRR1) were found to interact with the *Arabidopsis* transcription factor ABI3 in yeast cells (Kurup et al., 2000). Furthermore, the C-terminal portion of TOC1, including the CCT domain, was found to interact with several basic helix-loop-helix (bHLH) transcription factors, including PIF3 (Yamashino et al., 2003).

Thus, both the B-boxes and the CCT domain appear to mediate protein–protein interactions, and although the domains are found together in the CO-like proteins, each domain is also found in proteins with no other defined domains, suggesting that they can function independently.

In addition to B-boxes and the CCT domain, the CO and COL1 to COL5 proteins contain a conserved six–amino acid motif with the consensus sequence G-I/V-V-P-S/T-F in their C termini. The motif is separated from the CCT domain by 16 to 22 amino acids. The finding that the VP pair in the COL3 motif is required for the interaction with COP1 in yeast suggests a functional role for this motif. The conservation of the motif might indicate that other group members could be COP1-interacting partners and perhaps targets of COP1-mediated degradation. Interestingly, the CO protein is stabilized by light in the evening but degraded by the proteasome in the morning and in darkness (Valverde et al., 2004). However, studies of the COP1-interacting motifs in HY5, HYH, STH, and STO indicate that although the VP pair at the core of the motif is critical, residues before the core contribute to the interaction (Holm et al., 2001), and these residues show little conservation between the CO and COL1 to COL5 proteins. Further studies are needed to determine whether the COP1 interaction with the COL3 motif regulates COL3 protein stability, but the identification of the conserved C-terminal motif in COL3 as a COP1 interaction motif could facilitate the characterization of the motif in the CO and COL1 to COL5 proteins.

COL3 Is a Positive Regulator of Light Signals and Affects Lateral Organ Formation

The T-DNA insertion in the first exon of *COL3* results in a truncated mRNA that, if translated, would produce a protein consisting of the N-terminal amino acids 1 to 151 in COL3 followed by the amino acids KSTCPAE and a translational stop codon. This protein would contain the B-box domains but lack the C-terminal half of the COL3 protein, including the CCT domain.

However, the fact that we could complement all tested *col3* phenotypes by introducing the *COL3* gene (note that we have not tested complementation of the flowering-time phenotypes), together with the recessive nature of the *col3* mutation, indicates that *col3* is a loss-of-function mutation.

Because COL3 was identified as a COP1-interacting protein, we were interested in examining whether the *col3* mutation is defective in any known *COP1*-regulated process(es). Our analysis of the *col3* mutation reveled that this is indeed the case. The *col3* mutation resulted in reduced inhibition of hypocotyl elongation in short-day conditions and in high-fluence red light and in early flowering in both long-day- and short-day-grown plants (Figures 4 and 5). Furthermore, we observed reduced branching of the shoot in short-day-grown plants and found that *col3* seedlings form fewer lateral roots and show reduced accumulation of anthocyanin. These results suggest that *COL3* is

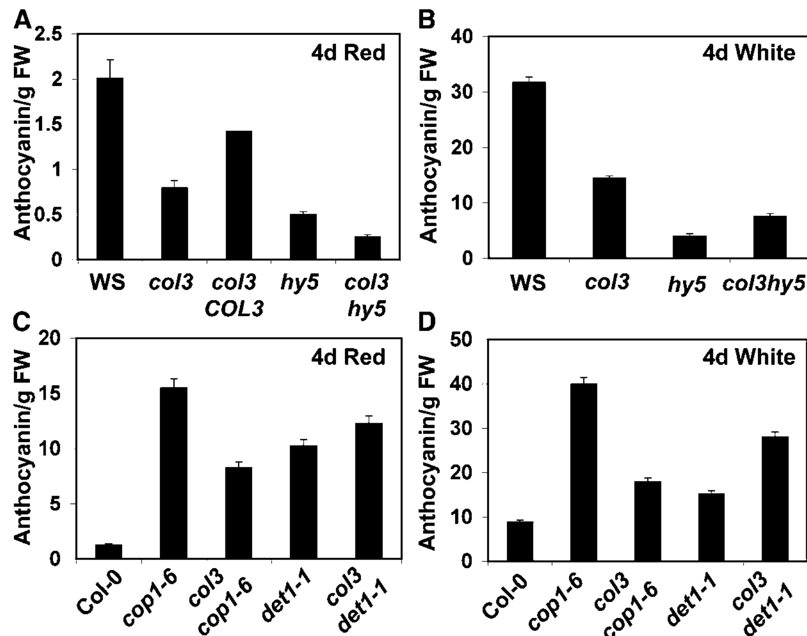

Figure 9. *col3* Has Reduced Levels of Anthocyanin and Has Opposite Effects on Anthocyanin Accumulation in *cop1* and *det1* Mutants.

(A) and **(B)** Anthocyanin content of the indicated seedlings grown for 4 d under continuous red and white light, respectively. Error bars represent SE ($n = 4$ except for *col3COL3*, for which 50 GFP-positive T1 seedlings were compared with 50 GFP-negative siblings in one experiment). FW, fresh weight.
(C) and **(D)** Anthocyanin accumulation in 4-d-old Columbia, *cop1-6*, *col3 cop1-6*, *det1-1*, and *col3 det1-1* seedlings grown under continuous red and white light, respectively. Error bars represent SE ($n = 3$).

a positive regulator of light signaling involved in a subset of the pathways regulated by *COP1*. The fact that COL3 contains the B-box and CCT domains, both of which have been found in other proteins to interact with transcription factors, suggests that COL3 acts as a downstream regulator, possibly in a promoter context.

COP1 has been shown previously to interact with and promote the degradation of the transcription factors HY5, LAF1, HYH, and HFR1 in the dark. By contrast, COP1 positively regulates PIF3 accumulation in darkness (Bauer et al., 2004). All of the transcription factors degraded by COP1 act as positive regulators of light signals of single or multiple wavelengths, whereas the phytochrome-interacting bHLH proteins PIF3, PIF1, PIF4, and PIF5 act mainly as negative regulators of phytochrome signaling (Huq and Quail, 2002; Kim et al., 2003; Fujimori et al., 2004; Huq et al., 2004). However, PIF3 might differentially affect distinct branches of red light signaling, because it acts as a positive factor in anthocyanin and chlorophyll accumulation (Kim et al., 2003; Monte et al., 2004).

To further define and characterize *COL3*, we analyzed genetic interactions between *col3* and *hy5*. In addition to the hypocotyl phenotype, *hy5* seedlings show enhanced initiation and elongation of lateral roots, altered gravitropic and touching responses, enhanced cell elongation in root hairs, reduced greening and secondary thickening of the root, and reduced chalcone synthase expression (Oyama et al., 1997; Ang et al., 1998). Of the spectrum of phenotypes seen in *hy5*, the more subtle phenotypes of *col3* are restricted to reduced inhibition of hypocotyl elongation in short days and red light, reduced anthocyanin accumulation, and lateral root formation. However, surprisingly and in contrast with *hy5*, *col3* mutants have reduced formation of lateral roots. Analysis of *hy5 col3* double mutants revealed that *hy5* is epistatic to *col3* with respect to lateral roots (Figure 6), whereas *HY5* and *COL3* appear to act as independent positive regulators of anthocyanin accumulation (Figure 9).

The flowering-time phenotype seen in *col3* is opposite to the long-day late-flowering phenotype of *co* and similar to the early-flowering phenotype seen in *hy5* and *hyh. CO* promotes flowering in response to long days; flowering is induced when *CO* mRNA expression coincides with the exposure of plants to light. Recent results suggest that the daily rhythm of *CO* transcription is refined by photoreceptor-dependent regulation of CO protein levels (Valverde et al., 2004). Light stabilizes the CO protein in the evening, whereas CO is degraded by the proteasome in the morning or in darkness.

The early flowering in *col3* mutants suggests that *COL3* does not act as a promoter of flowering. However, the reduced branching of the shoot seen only in short days suggests that *COL3* has a positive role in this process and that *COL3* might decode a subset of daylength-sensitive outputs.

All six *CO* and *COL1* to *COL5* genes are represented on the ATH1 array, and review of the diurnal experiments performed by Smith et al. (2004) using the genevestigator website revealed that the expression of all six genes shows diurnal changes. The expression of *COL3* peaks at dawn, and the expression profile of *COL3* most closely resembles that of *COL4*.

The ability to translate daylength into physiological responses requires crosstalk between light signals and the circadian clock (Hayama and Coupland, 2003). Interestingly, overexpression of *COL1* in plants shortened the period of two distinct circadian rhythms in a fluence rate–dependent manner, suggesting an effect on a light-input pathway (Ledger et al., 2001). The light-dependent regulation of CO protein levels, together with the diurnal expression and conserved domain structure of all six CO and COL1 to COL5 proteins, make it tempting to speculate that these proteins act at the crossroads of light signals and the circadian clock. However, further genetic and biochemical studies are needed to address this issue.

The finding that *col3* partially suppresses the hypocotyl phenotype of dark-grown *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* alleles firmly establishes *COL3* as a gene affecting *COP/DET/FUS*-regulated processes. Surprisingly, although *col3* suppresses both *cop1* and *det1* in darkness, we found that *col3* has different and sometimes opposing effects on *cop1* and *det1* in light-grown seedlings. In the root, where *col3*, *cop1*, and *det1* all show reduced numbers of emerged lateral roots, *col3* enhances the effect of *cop1-1* and *cop1-6* and suppresses the phenotype conferred by *det1-1*. Furthermore, although *col3* suppresses the enhanced anthocyanin accumulation in *cop1-6*, *col3 det1* seedlings have higher anthocyanin content than the *det1-1* mutant.

The different effects seen in the dark and light could be attributable to the reduced nuclear abundance of COP1 in light-grown seedlings. However, it is also possible that although COP1 represses COL3 in the dark, COL3 might be regulated independently of COP1 in the light.

Several lines of evidence suggest that the mechanisms whereby *col3* and *hy5* suppress *cop1* and *det1* in the dark are similar. Both COL3 and HY5 interact physically with COP1 in yeast, and both proteins colocalize with COP1 in onions. COL3 is a nuclear protein (Figure 2), and nuclear localization of the homologous CO protein is required for CO function (Simon et al., 1996). Both *col3* and *hy5* are recessive loss-of-function mutations. The phenotypes of the *col3* mutation suggest that *COL3* promotes photomorphogenesis. The recent mechanistic understanding of the COP/DET/FUS proteins further suggests that COL3 might be targeted for degradation in the dark.

Further biochemical analysis of the COL3 protein is needed to address the mechanism by which COL3 is regulated. Our functional and genetic studies provide a framework from which a more complete understanding of light signaling can be built.

METHODS

Plant Material, Growth Conditions, and Complementation Tests

The *Arabidopsis thaliana col3* and *hy5-ks50* (Oyama et al., 1997) alleles are in the Ws accession, the *cop9-1*, *det1-1*, *cop1-1*, *cop1-4*, and *cop1-6* alleles are in the Columbia accession, and *co-2* (Putterill et al., 1995) is in Landsberg *erecta*. Unless stated otherwise, seeds were surface-sterilized and plated on GM medium supplemented with 0.8% Bactoagar (Difco) and 1% sucrose. The plates were then cold-treated at 4°C for 3 d and transferred to light chambers maintained at 22°C with the desired light regime. For the complementation test, a 4.4-kb genomic fragment containing the full-length *COL3* gene was excised from the genomic BAC clone F27A10 and inserted into the *Xho*I and *Pst*I sites of the pBSK+ vector. The *COL3* gene was subsequently excised with *Kpn*I and *Pst*I and inserted into the pFP100 vector (Bensmihen et al., 2004), containing an At2S3:E-GFP:35S_{ter} cassette driving the expression of E-GFP in seeds, to generate pFP100-*COL3*. This construct was used to transform *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method, which was then introduced into the *col3*, *col3 cop1-1*, *col3 cop1-4*, *col3 cop1-6*, *col3 det1-1*, and *col3 hy5* mutant plants via the floral dip method (Clough and Bent, 1998). Transgenic T1 seeds were selected using the Leica MZFL III stereomicroscope equipped with a GFP filter. These transgenic seeds were used for phenotypic analyses, with untransformed siblings serving as controls.

Yeast Two-Hybrid Methods and Onion Experiments

The yeast strain Y190 (Kim et al., 1997) was used for the two-hybrid screen and for the two-hybrid assays. The conversion of the λ*ACT* cDNA expression library (ABRC number CD4-22) into a pACT library, the two-hybrid screen, and the β-galactosidase assays were performed as described by Holm et al. (2001). The pAVA321-S65TGFP-COL3 and pRTL2-mGFP-ΔCOL3 constructs, containing versions of the GFP (von Arnim et al., 1998), the pAM-PAT-35SS-CFP-COP1 and pAM-PAT-35SS-YFP-COL3, and the pRTL2-COP1 overexpression constructs were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment, incubated, and examined by epifluorescence microscopy as described previously (Holm et al., 2002).

For the FRET–acceptor photobleaching experiments, live cell images were acquired using an Axiovert 200 microscope equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss). Cells were visualized 24 h after particle bombardment using the confocal microscope through a Plan-Neofluor 40×/1.3 oil (differential interference contrast) objective. The multitracking mode was used to eliminate spill-over between fluorescence channels. The CFP was excited by a laser diode 405 laser and the YFP by an argon-ion laser, both at low intensities. Regions of interest were selected and bleached with 100 iterations using the argon-ion laser at 100%.

Expression of AD-COL3 and the three VP-substituted COL3 fusion proteins was examined by protein gel blot analysis using polyclonal rabbit antibodies raised against COL3.

RNA Gel Blotting

Total RNA was extracted from seedlings grown in continuous white light for 6 d after their germination using the RNeasy kit (Qiagen). Twenty micrograms of total RNA was loaded for RNA gel blot analysis.

Hypocotyl and Root Experiments

For all monochromatic light assays, plates were cold-treated at 4°C for 3 d and then transferred to continuous white light for 8 h to induce uniform germination. The plates were transferred to monochromatic light conditions and incubated at 22°C for 6 d in the case of hypocotyl experiments and for 6 to 12 d for the measurement of roots. Red, far-red, and blue lights were generated by light emission diodes at 670, 735, and 470 nm, respectively (model E-30LED; Percival Scientific). Fluence rates were measured with a radiometer (model LI-250; Li-Cor). Unless stated otherwise, all experiments with the roots under red light were performed using a high fluence of 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The hypocotyl lengths of seedlings, the lengths of the primary roots, and the numbers of lateral roots were measured/counted using ImageJ software.

Flowering-Time Experiments

For short-day and long-day measurements, seeds were sown on soil, cold-treated for 3 d at 4°C, transferred to a light chamber (model

AR-36; Percival Scientific) maintained at 22°C, and grown under a 16-h-light/8-h-dark photoperiod for long days and an 8-h-light/16-h-dark photoperiod for short days. Flowering time was determined by counting the number of rosette leaves at bolting.

Anthocyanin Measurements

For the anthocyanin determinations, seedlings at 4 d after germination were weighed, frozen in liquid nitrogen, and ground, and total plant pigments were extracted overnight in 0.6 mL of 1% HCl in methanol. After addition of 0.2 mL of water, anthocyanin was extracted with 0.65 mL of chloroform. The quantity of anthocyanin was determined by spectrophotometric measurements of the aqueous phase ($A_{530} - A_{657}$) and normalized to the total fresh weight of tissue used in each sample.

Block of Greening

Seedlings were germinated in the dark for 6 d and then transferred to constant white light for 6 d. Seedlings with green cotyledons and/or true leaves were scored as able to green, and those with bleached cotyledons and/or true leaves were scored as unable to green. The fluence level for white light was maintained at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Accession Numbers

The COL3, COP1, DET1, and HY5 Arabidopsis Genome Initiative locus identifiers are At2g24790, At2g32950, At4g10180, and At5g11260, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *col3* Seedlings Do Not Differ Significantly from the Wild Type in Blue or Far-Red Light.

Supplemental Figure 2. *col3* Behaves like the Wild Type and *col3 hy5* Seedlings Do Not Differ Significantly from *hy5* under Constant White Light and Long-Day Conditions.

Supplemental Figure 3. Branching in *col3* and Wild-Type Plants Is Similar under Long-Day Conditions.

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