COP1 SUPPRESSOR 4 promotes seedling photomorphogenesis by repressing *CCA1* and *PIF4* expression in *Arabidopsis*

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CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and DE-ETIOLATED 1 (DET1) are founding components of two central repressor complexes of photomorphogenesis that trigger the degradation of a larger number of photomorphogenic-promoting factors in darkness. Here, we identify COP1 SUPPRESSOR 4 (CSU4) as a genetic suppressor of the *cop1-6* mutation. Mutations in *CSU4* largely rescued the constitutively photomorphogenic phenotype of *cop1-6* and *det1-1* in darkness. Loss of CSU4 function resulted in significantly longer hypocotyl in the light. Further biochemical studies revealed that CSU4 physically interacts with CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and negatively regulates its transcriptional repression activity toward its targets. CSU4 represses the expression of *CCA1* in the early morning and of *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) in the early evening. Our study suggests that CSU4 acts as a negative regulator of CCA1 via physically associating with CCA1, which in turn, likely serves to repress expression of *CCA1* and *PIF4* to promote photomorphogenesis.

photomorphogenesis | CSU4 | COP1 | CCA1 | PIF4

Light is not only the major energy source for plants but also one of the most essential environmental factors affecting various physiological and developmental processes in plants. In nature, buried and germinated seeds undergo skotomorphogenesis to penetrate upward through the soil. Upon light exposure, etiolated seedlings initiate photomorphogenesis. These two distinct developmental processes are extremely critical for a germinated seed to become a healthy seedling and, subsequently, to complete its life cycle (1, 2).

An extensive number of components, including at least four classes of wavelength-specific photoreceptors—CONSTITUTIVELY PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) and transcription factors—work synergistically to ensure the proper skotomorphogenic and photomorphogenic development and their transition (3–5). In the dark, two classes of key repressors of photomorphogenesis—COP/DET/FUS and PHYTOCHROME INTERACTING FACTORs (PIF1, PIF3, PIF4, and PIF5)—are biologically functional and highly abundant in the nucleus (6–11). COP/DET/FUS function as E3 ubiquitin ligases and target numerous photomorphogenic-promoting factors such as ELONGATED HYPOCOTYL 5 (HY5) and HY5 HOMOLOG for ubiquitination and degradation (12, 13), while the basic helix-loop-helix transcription factors—PIFs—control a large group of target genes' expression. Together, these COP/DET/FUS- and PIF-mediated molecular events inhibit photomorphogenesis and promote skotomorphogenesis. In the light, COP/DET/FUS activity is largely repressed through multiple regulatory mechanisms (8, 14–17), thus allowing the accumulation of their downstream substrates. Meanwhile, PIFs are rapidly phosphorylated, ubiquitinated, and degraded within minutes upon light illumination (9–11, 18–21). Consequently, the accumulation of COP/DET/FUS targets and the alternation of

PIF-controlled gene expression synergistically contribute to the promotion of photomorphogenesis.

Circadian rhythm has been functionally linked to photomorphogenic development in plants. Consistently, a group of circadian clock factors function in the regulation of light signaling, either positively or negatively (22–26). The key components of the morning complex of the circadian clock—CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY)—positively regulate the expression of *PIF4* to promote hypocotyl growth, thus acting as negative regulators of light-inhibited hypocotyl elongation. Consistently, loss of CCA1 or LHY function mutants display shortened hypocotyls, while *CCA1* and *LHY* transgenic plants show elongated hypocotyls (22–24). In addition to those of the CCA1-LHY morning complex, evening-expressed circadian clock proteins are also involved in photomorphogenesis. The ELF3-ELF4-LUX evening complex directly binds to the promoter of *PIF4* and represses its gene expression during early evening to gate hypocotyl elongation (26). In addition, light initiates the degradation of PIF4, and both ELF3 and TOC1 physically interact with PIF4 to repress its biochemical activity, consequently suppressing PIF4-mediated gene expression and hypocotyl elongation in the daytime (18, 27, 28). Both the PIF4 mRNA and protein levels are diurnally regulated by light and the circadian clock (26). These facts demonstrate that both light and the circadian clock converge on PIF4 to influence photomorphogenesis at both transcriptional and protein levels.

Significance

The exogenous light signal and the endogenous circadian clock are two critical factors for directing growth and development in plants. In this study, we characterized a previously unidentified positive regulator of light signaling, CSU4. CSU4 interacts with CCA1, a key component of the circadian clock, to inhibit its transcriptional repression activity. Being a transcriptional regulator, CSU4 represses *CCA1* in the early morning and *PIF4* in the early evening in gating hypocotyl elongation. Thus, CSU4 likely acts as a key regulator of light- and circadian clock-mediated hypocotyl growth in plants.
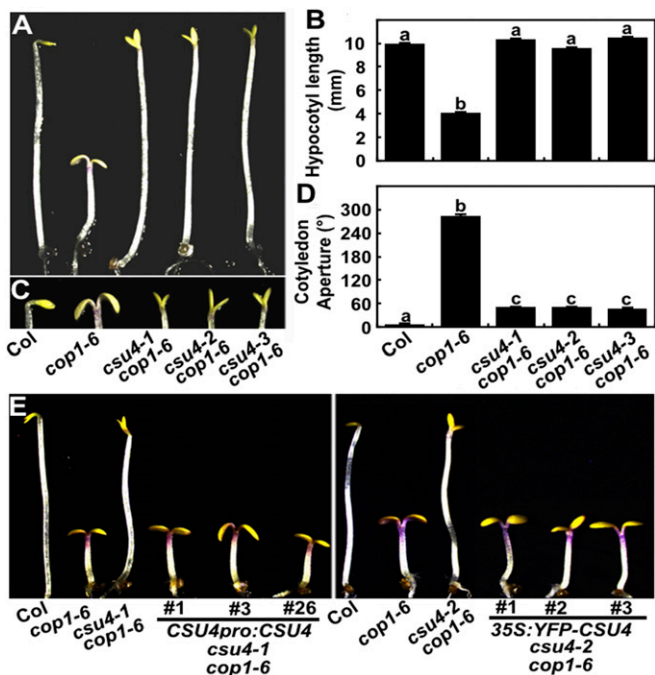

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Fig. 1. Mutations in *CSU4* suppress *cop1-6* in the dark. (*A* and *B*) Hypocotyl phenotypes and length of 4-d-old Col, *cop1-6*, and *csu4 cop1-6* seedlings grown in the dark. Error bars represent SE (*n* ≥ 20). Letters above the bars indicate significant differences (*P* < 0.05) as determined by one-way ANOVA with Tukey's post hoc analysis. (*C* and *D*) Cotyledon phenotypes and separation angle of 4-d-old Col, *cop1-6*, and *csu4 cop1-6* seedlings grown in the dark. Error bars represent SE (*n* ≥ 30). Letters above the bars indicate significant differences (*P* < 0.05) as determined by one-way ANOVA with Tukey's post hoc analysis. (*E*) Genomic complementation test. Phenotypes of Col, *cop1-6*, *csu4-1 cop1-6*, *csu4-2 cop1-6*, three independent *CSU4pro:CSSU4 csu4-1 cop1-6*, and three independent *35S:YFP-CSU4 csu4-2 cop1-6* transgenic lines grown in darkness for 4 d. The experiments were performed three times, with similar results. The graphs depict one of three experiments.

In this study, we report the identification and characterization of a previously uncharacterized positive regulator of light signaling, COP1 SUPPRESSOR 4 (CSU4). The *csu4* gene largely suppresses the short hypocotyls, and partially rescues the opened cotyledon phenotypes of *cop1-6* in darkness. Loss-of-function mutations in the *CSU4* gene leads to significantly elongated hypocotyls in the light. CSU4 physically interacts with CCA1 and represses its transcriptional repression activity; it also negatively regulates *CCA1* expression in the early morning and *PIF4* transcript levels in the early evening. Collectively, CSU4 acts as a transcriptional coregulator in repressing *CCA1* and *PIF4* expression to inhibit hypocotyl elongation.

Results

Map-Based Cloning of *CSU4*. To explore factors involved in light signaling, we performed a forward genetic screen for mutants that suppress *cop1-6*, which is a weak allele of *cop1*, and that exhibit constitutively photomorphogenic phenotype in darkness (29). Further genetic complementation tests recovered two extragenic and recessive alleles, namely *csu4-1* and *csu4-2*, from this screen. To identify and characterize the *CSU4* gene, we combined map-based cloning and whole-genome resequencing strategies as previously described (30, 31). *CSU4* was mapped to an ~300-kb region between *5-AB009053-9339* and *5-AB007646-9467* markers on chromosome 5 (*SI Appendix*, Fig. S1*A*). The analysis of genomic sequences in this region revealed a C-to-T change at the site of amino acid 667 from the start code (*ATG*) in *At5g63440*, causing a premature stop code at the 41st codon in

csu4-1 (*SI Appendix*, Fig. S1). Next, we amplified the *At5g63440* in *csu4-2 cop1-6* using PCR. Next, the PCR products were subjected to sequencing, and a C-to-T substitution at amino acid 1147 from the *ATG* was identified, which results in a premature stop code at the 145th codon (*SI Appendix*, Fig. S1). Database searches revealed that *CSU4* is a single-copy gene encoding a domain of unknown function (DUF)167 containing protein (*SI Appendix*, Fig. S1*C*).

Mutations in *CSU4* Suppress *cop1-6*. The *cop1-6* mutant seedlings display constitutively photomorphogenic phenotype in darkness (29). Each of the *csu4* mutant alleles (*csu4-1* and *csu4-2*) largely suppressed the *cop1-6* in the dark (Fig. 1). Two independent *csu4 cop1-6* double mutants displayed similar hypocotyl length compared with the wild type (WT), and drastically longer length compared with *cop1-6* (Fig. 1 *A* and *B*). Although the cotyledons of *csu4 cop1-6* mutant seedlings were obviously expanded, the opening angles were significantly smaller than that of *cop1-6* in darkness (Fig. 1 *C* and *D*). The hypocotyl length of *csu4 cop1-6* was longer than *cop1-6* but shorter than WT when grown in various light conditions tested [white (W), blue (B), red (R), and far-red (FR)] (*SI Appendix*, Fig. S2 *A–H*). In addition, the *csu4 cop1-6* adult plants grown in long-day (LD) conditions (16 h light/8 h dark) showed intermediated phenotype compared with WT and *cop1-6* (*SI Appendix*, Fig. S2*I*). Together, these results indicate that *csu4* largely suppressed the *cop1-6* in the dark and partially suppressed *cop1-6* in the light. Further, we isolated and characterized a T-DNA insertion *csu4* mutant, namely *csu4-3*
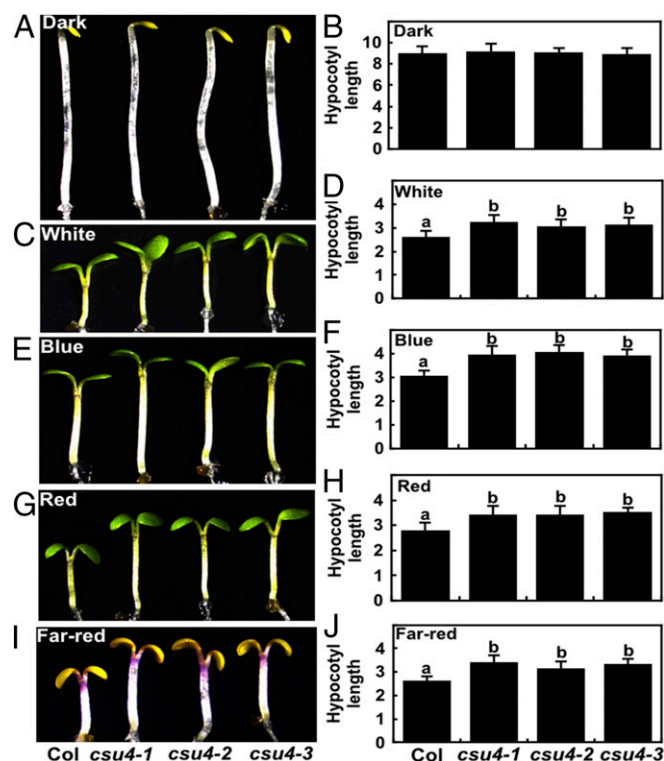

Fig. 2. The *csu4* seedlings are hyposensitive to light. (*A–J*) Hypocotyl phenotypes and length of 5-d-old Col and three independent *csu4* single-mutant seedlings grown in darkness (*A* and *B*) and in W (18.1 μmol·m⁻²·s⁻¹) (*C* and *D*), B (3.88 μmol·m⁻²·s⁻¹) (*E* and *F*), R (75.1 μmol·m⁻²·s⁻¹) (*G* and *H*), and FR (2.05 μmol·m⁻²·s⁻¹) (*I* and *J*) light conditions. The unit of hypocotyl length is millimeters. Error bars represent SE (*n* ≥ 20). Letters above the bars indicate significant differences (*P* < 0.05) as determined by one-way ANOVA with Tukey's post hoc analysis. The experiments were performed three times, with similar results. The graphs depict one of three experiments.

(SALK_070923C) from the Arabidopsis Biological Resource Center, in which *CSU4* gene expression was dramatically reduced (*SI Appendix*, Fig. S3). As expected, the constructed *csu4-3 cop1-6* double-mutant plants displayed similar phenotype with *csu4-1 cop1-6* and *csu4-2 cop1-6* double mutants, both in the dark and in light conditions (Fig. 1 and *SI Appendix*, Fig. S2). Furthermore, *csu4-1 cop1-6* transformed with genomic *CSU4* driven by its own promoter (*CSU4pro:CSU4 csu4-1 cop1-6*) and *csu4-2 cop1-6* transformed with *CSU4* coding sequences fused with yellow fluorescent protein (YFP) at the N terminus driven by the cauliflower mosaic virus 35S promoter (*35S:YFP-CSU4 csu4-2 cop1-6*) both exhibited *cop1-6* phenotype in darkness (Fig. 1E). These results demonstrate that disruption of CSU4 indeed rescues constitutively photomorphogenic phenotype of *cop1-6* and that a functional CSU4 complements the suppressor phenotype conferred by *csu4* in the *cop1-6* background.

In addition to COP1, DET1 also acts as a central repressor of photomorphogenesis (32). Our genetic evidence revealed that *csu4 det1-1* showed significantly longer hypocotyls compared with *det1-1*, suggesting that mutations in *CSU4* not only suppress *cop1-6* but also largely restore *det1-1* phenotype in darkness (*SI Appendix*, Fig. S4).

At the 3′ end of the fourth intron, *cop1-6* possesses a point mutation, "AG" to "GG", leading to multiple cryptically spliced products at intron 4 (29, 31). Thus, we examined the splicing pattern of *COP1-6* in *csu4 cop1-6* double mutants, and found that mutations in *CSU4* barely affect the splicing pattern and the transcript levels of *COP1-6* mRNA (*SI Appendix*, Fig. S5).

The COP1-SPA Complexes and COP10-DDB1-DET1 Do Not Affect CSU4. The COP1-SPA complexes and the COP10-DDB1-DET1 complex act as E3 ubiquitin ligases and promote degradation of a number of downstream targets (3, 4). We tested whether those types of E3 complexes play roles in regulating the CSU4 abundance in *Arabidopsis* seedlings. CSU4 protein levels were not significantly altered in *cop1-4*, *cop1-6*, multiple *spa* triple mutants, and *det1-1* compared with WT in the dark and light (*SI Appendix*, Fig. S6A), indicating that the COP1-SPA complexes and the COP10-DDB1-DET1 complex do not affect CSU4 abundance. In addition, CSU4 protein accumulated at comparable levels in seedlings grown in various light conditions tested (continuous darkness, W, B, R, and FR) (*SI Appendix*, Fig. S6B). Seedlings grown in different intensities of W light also accumulated similar CSU4 protein levels (*SI Appendix*, Fig. S6B). Together, these data suggest that light signals might have little effect on CSU4 in plants.

In addition, the COP1 protein level in Columbia (Col) WT seedlings was indistinguishable from that in *csu4* seedlings grown in the dark or in W light (*SI Appendix*, Fig. S6C), suggesting that CSU4 may not control COP1 abundance. COP1 exclusively localized in the nucleus, but not in the cytosol, in both etiolated Col and *cus4* seedlings (*SI Appendix*, Fig. S6D), indicating that CSU4 may not affect the nuclear subcellular localization of COP1 in the dark.

csu4 Mutant Seedlings Are Hyposensitive to Light. To substantiate CSU4's role in light signaling, we analyzed the hypocotyl phenotypes of three independent *csu4* (*csu4-1* to *csu4-3*) single mutants (without the *cop1-6* mutation) grown in various light conditions. All *csu4* mutant seedlings showed indistinguishable phenotype with WT when grown in darkness for 4 d (Fig. 2 A and B). In all of the light conditions tested (W, B, R, and FR), all individual *csu4* single-mutant seedlings displayed significantly longer hypocotyls than WT (Fig. 2 C–J), indicating that CSU4 promotes light-inhibited hypocotyl elongation and plays a positive role in light signaling.

CSU4 Physically Interacts with CCA1 and Represses Its Transcriptional Repression Activity. To understand the molecular base for CSU4 in promoting photomorphogenesis, we performed a yeast two-hybrid screen and revealed that CSU4 interacts with CCA1, a

key component of the circadian clock and a promoter of hypocotyl growth (23) (Fig. 3 A and B). Further analysis showed that the N terminus of CSU4 (amino acids 1 to 141), but not the C terminus of CSU4 (amino acids 142 to 232) containing a DUF, interacted with CCA1 (Fig. 3 A and B). The data suggest that the N terminus of CSU4 is sufficient and necessary for its interaction with CCA1. To verify the interaction between CSU4 and CCA1, we performed a bimolecular fluorescence complementation (BiFC) assay. Strong YFP signals were clearly detected when CSU4, fused with the N terminus of YFP (YFP^N-CSU4), and CCA1, fused with the C terminus of YFP (YFP^C-CCA1), were transiently cotransfected into *Nicotiana benthamiana* leaves. However, no detectable YFP signals were produced when transiently coexpressing the controls—YFP^N-CSU4 and YFP^C or YFP^N and YFP^C-CCA1 (Fig. 3C). Next, we further employed a coimmunoprecipitation (co-IP) assay using *N. benthamiana* leaves transiently expressing 35S:myc-CSU4 and 35S:Flag-CCA1. As shown in Fig. 3D, myc-CSU4 coimmunoprecipitated with Flag-CCA1. Together, these data demonstrate that CSU4 physically interacts with CCA1 in planta.

CCA1, a Myb-domain transcriptional factor, can bind to its numerous targets and is considered a bona fide transcriptional repressor (23). Thus, we examined whether CSU4 affects the binding of CCA1 to its targets using electrophoretic mobility shift assays (EMSAs). His-trigger factor (TF)-CCA1, but not His-TF (negative control), could directly bind to *ELF4* or *PRR9* promoters (two targets of CCA1) (33, 34) (Fig. 3 E and F). In the presence of His-CSU4, the binding of His-TF-CCA1 to *ELF4* or *PRR9* promoter DNA subfragments was obviously reduced. In addition, as the amount of His-CSU4 increased in the reactions, the His-TF-CCA1 binding affinity to *ELF4* and *PRR9* promoters was markedly decreased (Fig. 3 E and F), indicating that CSU4 may interfere with the binding of CCA1 to its target promoters. In yeast cells, activating domain (AD)-fused CCA1 could activate *proELF4:LacZ* reporter (Fig. 3G), indicating that CCA1 can directly bind to the *ELF4* promoter, thus allowing AD to activate *LacZ* reporter in yeast cells. AD-CSU4 alone did not have a significant effect on *proELF4:LacZ* reporter; however, when cotransformed with AD-CCA1, activation of AD-CCA1 on *proELF4:LacZ* was dramatically reduced in yeast cells, as indicated by β-galactosidase activity (Fig. 3G). These results imply that CSU4 may interfere with CCA1's ability to regulate promoter activity of *ELF4*. Similar to the previous finding (34), CCA1 alone could repress the transcription of *proELF4:LUC* or *proPRR9:LUC* when it was transiently expressed in *Arabidopsis* protoplasts (Fig. 3 H–J). CSU4 alone did not have any effect on these reporters; however, the repression of CCA1 on *proELF4:LUC* or *proPRR9:LUC* was significantly reduced when it was transiently coexpressed with CSU4 (Fig. 3 H–J). These results collectively suggest that CSU4 can reduce the transcriptional repression activity of CCA1 possibly through interfering with CCA1's ability to bind its target promoters.

CSU4 Represses *CCA1* in the Early Morning and *PIF4* in the Early Evening. CCA1 is a central component of the circadian clock, which represses its own gene expression and phenotypically promotes hypocotyl elongation (23). Given that CSU4 inhibits hypocotyl elongation, interacts with CCA1, and negatively regulates *CCA1* transcriptional repression activity (Figs. 2 and 3), we tested whether CSU4 affects the expression of *CCA1*. As shown in Fig. 4A, *CCA1* transcript levels were clearly increased in *csu4* mutants in the early morning [zeitgeber time (ZT)0 and ZT4], suggesting that CSU4 negatively regulates *CCA1* at dawn.

CCA1 promotes hypocotyl elongation predominantly through the activation of *PIF4* expression (35). In agreement, *PIF4* expression was markedly elevated in all three independent *csu4* mutant seedlings at ZT12 (Fig. 4B), and *IAA19* and *IAA29* (PIF4-controlled genes) (36) were also increased in the *csu4*
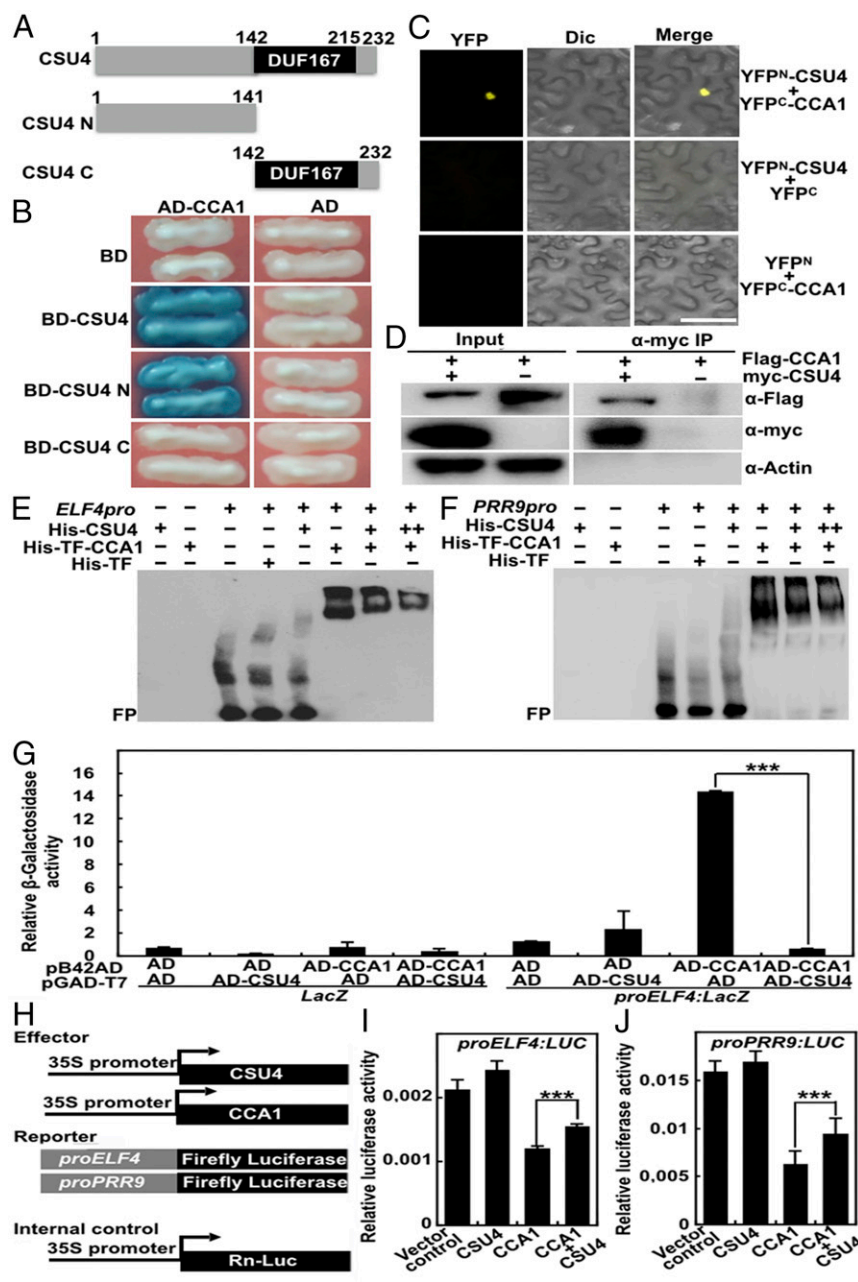
Fig. 3. CSU4 physically interacts with CCA1 and represses its transcriptional activity. (*A*) Schemes of the domain structure of CSU4 and the truncated CSU4 proteins. Numbers indicate the amino acid positions in CSU4. (*B*) Yeast two-hybrid interactions between the indicated CSU4 and CCA1. BD, binding domain. (*C*) BiFC assay showing the interaction of CSU4 with CCA1. Full-length CSU4 and CCA1 were fused to the split N- or C-terminal fragments of YFP (YFP^N or YFP^C, respectively). Unfused YFP^N and YFP^C fragments were used as negative controls. Merge, merged YFP channel and differential interference contrast (Dic) brightfield images. (Scale bar = 40 μm). (*D*) Co-IP analysis showing that myc-CSU4 interacts with Flag-CCA1. Total protein was extracted from tobacco leaves transiently expressing 35S:myc-CSU4 alone or together with 35S:Flag-CCA1; the immunoprecipitates were detected using anti-myc and anti-Flag antibodies, respectively. Actin served as a negative control. (*E* and *F*) EMSA showing that CSU4 interferes with the binding of CCA1 to *ELF4* (*E*) or *PRR9* (*F*) promoters. A (−) symbol indicates the absence of corresponding of probes or proteins. For His-CSU4, (+) and (++) indicate that 18 and 36 pmol is present, respectively. For His-TF, (+) indicates that 9.2 pmol is present. For His-TF-CSU4, (+) indicates that 8 pmol is present. TF (trigger factor), a prokaryotic ribosome 3-associated chaperone protein. FP indicates free probes. (*G*) Yeast one-hybrid analysis showing that CSU4 interferes with CCA1's ability to bind to *ELF4* promoter. Error bars represent the SD of four independent yeast cultures. ***$P < 0.001$ as determined by Student's *t* test. The experiments were performed three times, with similar results. (*H*) Schematic representation of various constructs used in the transient transfection assay in *Arabidopsis* protoplasts. Arrows after the 35S promoters indicate the transcriptional start site. *ELF4* or *PRR9* promoter was fused to firefly luciferase to create the reporter constructs. (*I* and *J*) Bar graphs showing CSU4 represses the repression of CCA1 on the *proELF4:LUC* (*I*) and *proPRR9:LUC* (*J*) reporters. Error bars represent the SD of three independent transient transfections in *Arabidopsis* protoplasts. ***$P < 0.001$ as determined by Student's *t* test. The experiments were performed three times, with similar results.

mutants (Fig. 4 *C* and *D*), indicating that CSU4 represses the expression of *PIF4* specifically in the early evening.

Considering that CSU4 affected the temporal regulation of *CCA1* and *PIF4* expression (Fig. 4 *A* and *B*), we examined whether CSU4 regulates the expression of other circadian clock genes. Expression levels of *TOC1*, *GI*, *ELF3*, *ELF4*, *LUX*, *PRR5*, *PRR7*, and *PRR9* were changed at one or multiple time points in a 24-h period in the *csu4* mutants compared with those in the WT (*SI Appendix*, Fig. S7), suggesting that CSU4 is a common regulator for the expression of these circadian clock genes. Although CSU4 affects the transcription of these clock genes, CSU4 itself was not regulated by the circadian clock at either the transcriptional or protein level (*SI Appendix*, Fig. S8).

Overexpression of Either *CCA1* or *PIF4* Partially Suppresses *cop1* or *det1*. Mutations in *CSU4* genetically suppressed *cop1-6* or *det1-1* (Fig. 1 and *SI Appendix*, Fig. S4), and CSU4 repressed the *CCA1*

and *PIF4* transcript levels (Fig. 4 *A* and *B*). These results indicate that suppression of *cop1-6* or *det1-1* by *csu4* is likely, at least in part, caused by elevation of *CCA1* and *PIF4*. To test this, we generated *CCA1-OX cop1-4*, *CCA1-OX cop1-6*, *CCA1-OX det1-1*, *PIF4-OX cop1-4*, and *PIF4-OX cop1-6* transgenic lines by genetic crossing. Overexpression of either *CCA1* or *PIF4* significantly suppressed the short hypocotyl phenotype of *cop1-4, cop1-6*, or *det1-1* in the dark (Fig. 5). A previous study has shown that overexpression of *PIF4* partially suppresses *det1-1* (37). Together, these findings support the notion that elevation of *CCA1* and *PIF4* transcript levels due to *csu4* mutations partially contributes to the suppression of *cop1-6* or *det1-1*.

Next, we examined the genetic interactions between *CSU4* and *CCA1* or *PIF4*. Consistent with previous studies, the *pif4-2* mutant showed shorter hypocotyls as compared with WT in W and R light (38). The hypocotyl length of *csu4-1 pif4-2* was similar to *pif4-2* in W and R light (Fig. 6 *A–D*), suggesting that *PIF4* acts

genetically downstream of *CSU4*. Loss of CCA1 function resulted in shortened hypocotyls under light, which was consistent with results from previous studies (23, 39, 40). The *csu4-1 cca1-22* double mutant showed indistinguishable hypocotyl length with *csu4-1*, which was significantly longer than WT and *cca1-22* in LD conditions (Fig. 6 *E* and *F*). In short-day (SD) conditions (i.e., continuous W, B, R, and FR light conditions), the hypocotyl length of *csu4-1 cca1-22* was shorter than that of *csu4-1* but longer than those of WT and *cca1-22* (Fig. 6 *G* and *H* and *SI Appendix*, Fig. S9). These data suggest that while the short hypocotyl phenotype of *cca1-22* is dependent on a functional CSU4 in LD conditions, it is independent of CSU4 in SD W, B, R, and FR light conditions.

Discussion

Light and the circadian clock interact to control seedling photomorphogenic development in *Arabidopsis* seedlings. Although CSU4 is not circadian regulated at both the transcript and protein levels (*SI Appendix*, Fig. S8), it interacts with the circadian clock and controls the expression of several circadian clock genes in gating hypocotyl growth (Figs. 3 and 4), suggesting that CSU4 affects the circadian clock and its output pathway. CSU4, CCA1, and PIF4 coordinately modulate the hypocotyl under diurnal conditions. CSU4 interacted with CCA1 and reduced its DNA binding affinity, thereby repressing its transcriptional repression activity toward its targets (Fig. 3). It is possible, therefore, that CSU4 may form heterodimers with CCA1 to inhibit its ability to bind to target sites, thereby repressing CCA1 biochemical activity. Previous studies have demonstrated that CCA1 negatively regulates its own expression (23). Thus, suppression of CCA1 transcriptional repression activity by CSU4 likely leads to the elevation of its own expression, thereby inhibiting the *PIF4* and PIF4-mediated gene expression and hypocotyl elongation. CSU4 acts as a transcriptional repressor, gating rhythmic expression of *CCA1* and *PIF4* diurnally (Fig. 4). Consequently, these molecular events contribute to the light-inhibited hypocotyl growth in plants.

Mutations in *CSU4* largely rescued the *cop1-6* or *det1-1* phenotype, especially with respect to hypocotyl phenotypes in darkness (Fig. 1 and *SI Appendix*, Fig. S4). Both COP1 and DET1 function as E3 ubiquitin ligases in promoting degradation of numerous downstream targets in repressing photomorphogenesis in darkness (3, 4). CSU4 protein abundance is not controlled by COP1 and DET1 (*SI Appendix*, Fig. S6), suggesting that CSU4 is not likely a substrate of these two E3 ubiquitin

Fig. 5. Overexpression of *CCA1* or *PIF4* partially suppress *cop1* in darkness. (*A* and *B*) Hypocotyl phenotypes and length of 4-d-old Col, CCA1-OX, *cop1-4*, *cop1-6*, *det1-1*, CCA1-OX *cop1-4*, CCA1-OX *cop1-6*, and CCA1-OX *det1-1* mutant seedlings grown in darkness. (*C* and *D*) Hypocotyl phenotype and length of 4-d-old Col, PIF4-OX, *cop1-4*, *cop1-6*, PIF4-OX *cop1-4*, and PIF4-OX *cop1-6* mutant seedlings grown in darkness. Data are mean ± SE; $n \geq 20$. ***$P < 0.001$ (Student's *t* test) relative to Col. The experiments were performed three times, with similar results. The graphs depict one of three experiments.

ligases. COP1/DET/FUS contribute to the stabilization of PIF4 abundance (37), and overexpression of *PIF4* in either a *cop1* or *det1-1* single-mutant background partially suppressed their shortened hypocotyl phenotypes (Fig. 5 *C* and *D*) (37). In *csu4* mutant seedlings, both *CCA1* and *PIF4* transcript levels were significantly increased (Fig. 4 *A* and *B*). All this evidence supports a possibility
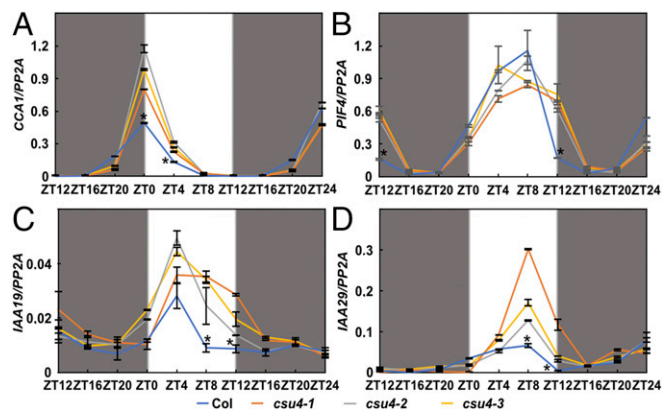

Fig. 4. CSU4 negatively regulates the expression of *CCA1* in the early morning and *PIF4* in the early evening. Real-time qPCR analysis of *CCA1* (*A*), *PIF4* (*B*), *IAA19* (*C*), and *IAA29* (*D*) transcript levels in Col and three independent *csu4* mutant seedlings grown in 12 h light/12 h dark photoperiods. Error bars represent the SD of three technical replicates. *$P < 0.05$ as determined by Student's *t* test. The experiments were performed three times, with similar results.
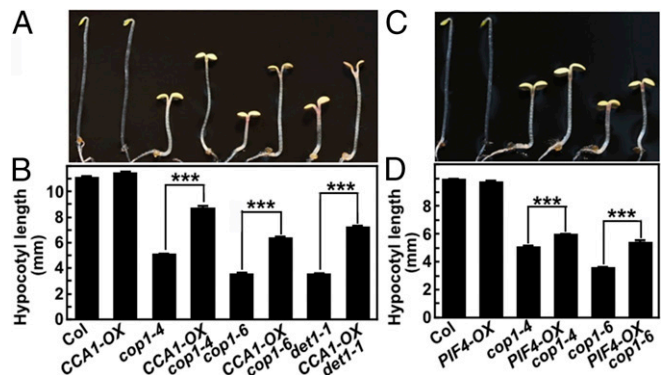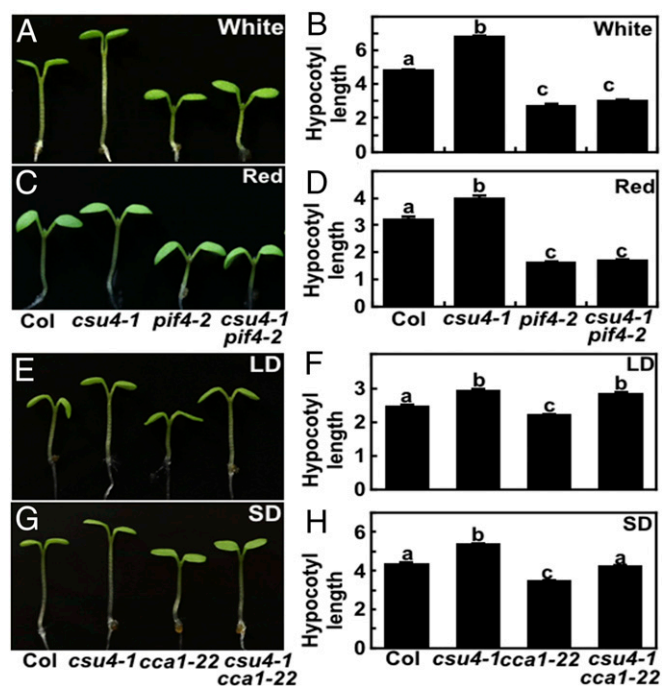

Fig. 6. CSU4 acts upstream of *PIF4*. (*A–D*) Hypocotyl phenotypes and length of 5-d-old Col, *csu4-1*, *pif4-2*, and *csu4-1 pif4-2* mutant seedlings grown in W (14.74 μmol·m^{-2}·s^{-1}) (*A* and *B*) or R (129 μmol·m^{-2}·s^{-1}) (*C* and *D*) conditions. (*E–H*) Hypocotyl phenotypes and length of 5-d-old Col, *csu4-1*, *cca1-22*, and *csu4-1 cca1-22* mutant seedlings grown in LD (*E* and *F*) or SD (*G* and *H*) conditions. The unit of hypocotyl length is millimeters. Error bars represent SE ($n \geq 20$). Letters above the bars indicate significant differences ($P < 0.05$) as determined by one-way ANOVA with Tukey's post hoc analysis. The experiments were performed three times, with similar results. The graphs depict one of three experiments.

that the suppression of *cop1-6* and *det1-1* phenotypes in the *csu4* mutants is likely, at least in part, caused by the elevation of *CCA1* and *PIF4* transcript levels. DET1 physically interacts with the morning-phase components (CCA1 and LHY) and is recruited to their evening-phase gene targets, including *TOC1* and *GI*, to repress their expression (41). Neither DET1 nor CSU4 is controlled by the circadian clock at both transcriptional and protein levels (*SI Appendix,* Fig. S8) (41). Like DET1, CSU4 associated with CCA1, and it negatively controlled *CCA1* expression at dawn (Figs. 3 and 4*A*), suggesting a direct involvement of CSU4 as a transcriptional co-repressor in mediating the circadian clock. Although the CSU4 constantly expressed under various light conditions or at different time points (*SI Appendix,* Fig. S8), it negatively controls *CCA1* specifically in the early morning and *PIF4* specifically in the early evening (Fig. 4). These facts indicate that additional or yet-unidentified factors are required for CSU4 in regulating *CCA1* or *PIF4* expression at specific time points.

PIF4 functions downstream of the circadian clock and gates circadian clock-mediated hypocotyl growth (26, 28). Both morning complex CCA1-LHY and evening complex ELF3-ELF4-LUX regulate *PIF4* expression at different time points (26, 35). On the one hand, CCA1-LHY activates *PIF4* expression to regulate hypocotyl growth during the day (35, 42) while ELF3 interacts with PIF4 independent of the evening complex to repress PIF4 biochemical activity in order to inhibit hypocotyl elongation in the daytime (28). On the other hand, the evening complex ELF3-ELF4-LUX

directly associates with the promoter of *PIF4* through transcription factor LUX to repress its gene expression, which in turn, serves to control PIF4-mediated gene expression and inhibit hypocotyl elongation in the evening (26). CSU4 repressed the *CCA1* expression in the early morning and down-regulated *PIF4* in the early night (Fig. 4 *A* and *B*), supporting that CSU4 plays an essential role in gating the timing of hypocotyl elongation probably through repression of *CCA1* and *PIF4* at dawn and dusk, respectively, to integrate photoperiod signals for growth and development.

Materials and Methods

Detailed descriptions of plant material and growth conditions, map-based cloning, sequencing by oligonucleotide ligation and detection, mutation identification, construction of plasmids, co-IP analysis, immunoblot analysis, EMSA analysis, yeast one- and two- hybrid assays, protoplast assays, BiFC assays, hypocotyl length measurements, production of specific CSU4 poly-clonal antibody, and real-time qPCR assays are provided in *SI Appendix, SI Materials and Methods*.

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