COLD-REGULATED GENE27 Integrates Signals from Light and the Circadian Clock to Promote Hypocotyl Growth in Arabidopsis

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Light and the circadian clock are two essential external and internal cues affecting seedling development. COLD-REGULATED GENE27 (COR27), which is regulated by cold temperatures and light signals, functions as a key regulator of the Light and the circadian clock to promote hypocotyl growth.

INTRODUCTION

In nature, a seed germinating in the soil will first undergo skotomorphogenesis (seedling development in the dark), which is characterized by an elongated hypocotyl, a closed apical hook, and small, unfolded cotyledons to provide little resistance against soil particles, until the seedling reaches the soil surface. Upon light exposure, seedlings then switch to the light-mediated developmental program known as photomorphogenesis. These two distinct developmental processes enable the proper and healthy development of a young seedling (Jiao et al., 2007; Song et al., 2020). During the transition from the dark- to the light-driven developmental process, the expression of approximately one-third of genes throughout the Arabidopsis (Arabidopsis thaliana) genome is significantly altered, indicating that light orchestrates massive transcriptomic reprogramming in plants (Ma et al., 2001).

Light signals of various wavelengths are perceived by several photoreceptor families, including phytochromes (red and far-red light), cryptochromes (blue light), phototropins (blue light), and UV-B RESISTANCE LOCUS8 (UVR8, for UV light; Gallagher et al., 1988; Sharrock and Quail, 1989; Lin et al., 1995; Guo et al., 1998; Rizzini et al., 2011; Christie et al., 2012). These photoreceptors are responsible for transducing diverse light information to downstream light signaling components to initiate a variety of cellular and physiological processes (Chen et al., 2014; Ma et al., 2016; Pedmale et al., 2016; Paik and Huq, 2019; Xu, 2019; Jing and Lin, 2020; Yadav et al., 2020; Yu and Liu, 2020). CONSTITUTIVELY PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) proteins, acting directly downstream of photoreceptors, are biologically active in the nucleus and maintain skotomorphogenesis in the dark (Lau and Deng, 2012; Hoecker, 2017; Podolec and Ulm, 2018; Han et al., 2020). Upon light exposure, light signals and light-activated photoreceptors inhibit COP/DET/FUS activity through multiple molecular regulatory mechanisms (Hoecker, 2017; Podolec and Ulm, 2018). COP/DET/FUS proteins consist of three distinct biochemical entities: the COP1-SPA complex, the COP10-DET1-DNA DAMAGE BINDING PROTEIN1 (DD81; CDD) complex, and the COP9 signalosome (CSN; Wei and Deng, 2003; Yadav et al., 2004; Zhu et al., 2008; Qin et al., 2020). The Arabidopsis genome encodes four SPA proteins (SPA1 to SPA4), and two COP1 and two SPA proteins form a stable core complex (Zhu et al., 2008). The COP1-SPA complex targets numerous photomorphogenesis-promoting factors, including the transcription...
factor ELONGATED HYPOCOTYL5 (HY5), for ubiquitination and degradation in the dark (Hoecker, 2017; Osterlund et al., 2000; Han et al., 2020). Light exposure repressed the activity of the COP1-SPA complex, leading to the accumulation of HY5, that then controls the expression of many genes, either directly or indirectly, to promote photomorphogenic development (Lee et al., 2007; Zhang et al., 2011; Burko et al., 2020).

COP1 not only represses photomorphogenesis but also mediates diverse cellular and physiological development responses, including the control of light input to the circadian clock (Huang et al., 2014). COP1 promotes the degradation of circadian clock components such as EARLY FLOWERING3 (ELF3), GIGANTEA (GI), and CONSTANS (CO) during the night at the posttranslational level (Huang et al., 2014). Similar to light, the circadian clock also mediates hypocotyl elongation. Multiple circadian clock components, such as CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION1 (TOC1), ELF3, ELF4, and LUX ARRHYTHMO (LUX) participate in the regulation of hypocotyl growth (Schaffer et al., 1998; Wang et al., 1998; Mås et al., 2003; Nusinow et al., 2011), suggesting that the circadian clock and light signaling synergistically mediate seedling development after seed germination. All these morning- or evening-phased central clock components control seedling hypocotyl growth by regulating the basic-helix-loop-helix–type transcription factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) at the transcriptional and/or protein levels under diurnal conditions (Niwa et al., 2009; Nusinow et al., 2011; Zhu et al., 2016). PIF4 promotes hypocotyl elongation mainly by regulating downstream auxin signaling, which promotes cell elongation (Sun et al., 2013). In addition to the circadian clock, multiple signaling cascades (phytochromes, cryptochromes, UV-B, low and high ambient temperature) converge on PIF4 and the PIF4-mediated auxin signal transduction pathway to control hypocotyl growth (Huq and Quail, 2002; Jung et al., 2016; Legris et al., 2016; Ma et al., 2016; Pedmale et al., 2016; Hayes et al., 2017; Dong et al., 2016; Hayes et al., 2017; Dong et al., 2016; Hayes et al., 2017). Thus, PIF4 functions as a central hub, acting downstream of multiple signaling pathways, to gate seedling growth.

COLD-REGULATED GENE27 (COR27), which is regulated by both low temperatures and light signals, acts as a nighttime repressor of the circadian clock (Li et al., 2016; Wang et al., 2017). The CCA1-LHY morning complex associates with the COR27 promoter and regulates its rhythmic expression. COR27 binds to TOC1 and PSEUDO-RESPONSE REGULATOR5 (PPIR5) chromatin to repress their transcription in the afternoon and at night (Li et al., 2016). In this study, we show that the COP1-SPA complex interacts with COR27 and promotes its proteolysis via the 26S proteasome system in the dark. COR27 negatively regulates photomorphogenic development in the light. Our biochemical studies reveal that light-induced COR27 not only negatively modulates HY5 activity but also binds to the promoter regions of PIF4 and upregulates its transcription to maintain its proper expression in the afternoon. Our study thus provides a mechanistic framework for COR27 in controlling the biochemical activity of HY5 and circadian clock–regulated PIF4 transcription in repressing seedling development.

RESULTS

COR27 Physically Interacts with the COP1-SPA1 Complex

COP1 is a central repressor of light signaling (Deng et al., 1991, 1992; Huang et al., 2014; Han et al., 2020). To identify novel
Figure 1. COR27 Physically Interacts with the COP1-SPA1 Complex.

(A) Schematic diagram of various constructs used in the yeast two-hybrid assays. Numbers indicate amino acid positions in COR27, COP1, or SPA1.
components of light signaling, we performed a yeast two-hybrid screen using COP1 as a bait. This screen identified COR27 as a COP1-interacting protein in yeast cells (Figures 1A and 1B). Further domain mapping analysis revealed that the C-terminal region (COR27-C, 121 to 246), but not the N-terminal half (COR27-N, 1 to 120), of COR27 interacted with COP1 (Figures 1A and 1B). We continued to perform yeast two-hybrid assays to identify the domain within COP1 that was responsible for interaction with COR27. None of the COP1 truncation fragments interacted with COR27, COR27-N, or COR27-C (Figures 1A and 1B). These data suggest that the overall structure of COP1 and the C-terminal portion of COR27 are required for their protein–protein interaction.

COP1 and SPA form stable core complexes in plants (Zhu et al., 2008). We thus tested whether COR27 interacts with SPA proteins (SPA1 to SPA4) by yeast two-hybrid assays. COR27 specifically interacted with SPA1, but not with SPA2, SPA3, or SPA4 (Figure 1C). COR27-N, but not COR27-C, interacted with SPA1 (Figure 1C), suggesting that the N terminus of COR27 is required for its association with SPA1. Further yeast two-hybrid assays showed that COR27 or COR27-N interacted with SPA1 (521 to 1029) and SPA1 (521 to 696), which contain an intact coiled-coil domain, but not SPA1 (1 to 520) or SPA1 (647 to 1029), which lack the coiled-coil domain (Figure 1D). These data suggest that the N terminus of COR27 and the coiled-coil domain of SPA1 mediate the COR27-SPA1 interaction in yeast cells.

To verify the interaction between COR27 and the COP1-SPA1 complex, we performed a bimolecular fluorescence complementation (BiFC) assay. COR27 was fused with a split N terminus of yellow fluorescent protein (YFPN) and COP1 or SPA1 was fused with a split C terminus of YFP (YPF). We observed strong YFP signals when we transiently coexpressed COR27-YFPN and COP1-YPFC or COR27-YPFN and SPA1-YPFC in Nicotiana benthamiana leaves. The negative controls did not produce any detectable YFP signal (Figure 1E). Next, we performed in vitro pull-down assays using recombinant His-COR27 and maltose binding protein (MBP)–COP1. His-COR27 successfully pulled down MBP–COP1, but not negative control MBP (Figure 1F), suggesting that COR27 interacts with COP1 in vitro. To verify the COR27 and COP1-SPA1 interaction in vivo, we further performed coimmunoprecipitation (Co-IP) assays using YFP-COR27 transgenic seedlings or N. benthamiana leaves transiently coexpressing 35S::YFP-COR27 and 35S::Flag-SPA1. YFP-COR27 coimmunoprecipitated with endogenous COP1 in YFP-COR27 transgenic seedlings (Figure 1G). Similarly, YFP-COR27 transiently coexpressed in N. benthamiana leaf cells, together with Flag-SPA1 or YFP-glutathione S-transferase (GST), immunoprecipitated Flag-SPA1, but not YFP-GST (negative control; Figure 1H). Together, these data firmly demonstrate that COR27 associates with the COP1-SPA1 complex in planta.

COR27 Undergoes COP1-Mediated Degradation in the Dark

COR27 is induced by blue light and accumulates during the day but decreases in the night under long-day conditions (Li et al., 2016). In agreement, YFP-COR27 transgenic seedlings grown in white light accumulated markedly more YFP-COR27 compared to those grown in the dark (Figure 2A). YFP-COR27 abundance gradually decreased in white light–grown YFP-COR27 transgenic seedlings upon transfer to darkness at the indicated time points (0, 1, 3, 6, 12, and 24 h; Figure 2B), suggesting that COR27 is subjected to degradation in the dark. As an E3 ubiquitin ligase complex, COP1-SPA1 ubiquitinates a number of downstream substrates and promotes their degradation through the 26S proteasome system in the dark (Huang et al., 2014; Hoecker, 2017). Since COP1-SPA1 interacts with COR27, which becomes degraded in the dark (Figures 1, 2A, and 2B), we thus examined whether COR27 degradation depended on the 26S proteasome system and COP1. Treating YFP-COR27 seedlings with the proteasome inhibitor MG132 clearly stabilized YFP-COR27 in dark-grown seedlings (Figure 2C), demonstrating that COR27 is indeed subject to 26S proteasome system–mediated degradation. Next, we introduced the cop1-4 and cop1-6 mutations into YFP-COR27 transgenic lines by genetic crossing. Immunoblot assays revealed that loss of COP1 function led to the

Figure 1. (continued).
accumulation of YFP-COR27 in dark-grown seedlings (Figure 2D). Consistent with these results, we detected significantly stronger YFP signal in YFP-COR27 cop1-4 and YFP-COR27 cop1-6 seedlings than in YFP-COR27 transgenic lines grown in the dark (Figures 2E and 2F). Taken together, these results suggest that COP1 promotes the degradation of COR27 via the 26S proteasome system in etiolated seedlings.

**COR27 Acts as a Negative Regulator of Light Signaling**

To explore the role of COR27 in light signaling, we identified a T-DNA insertion mutant (namely, cor27-3) with much lower COR27 expression. We also generated two additional independent cor27 mutant lines (namely, cor27-4 and cor27-5) by clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-Cas9 genome editing (Supplemental Figures 1A to 1C). These three individual cor27 mutant alleles showed a similar phenotype to the wild-type Columbia-0 (Col-0) in the dark (Supplemental Figures 2A and 2B). However, all cor27 mutant seedlings showed shorter hypocotyls relative to Col-0 when grown at different intensities of white, blue, red, and far-red light (Figures 3A to 3H).

Next, we generated transgenic plants carrying myc- or YFP-tagged COR27 driven by the cauliflower mosaic virus 35S promoter in which COR27 was overexpressed at the transcriptional level, and its protein was easily detectable using anti-myc or anti-GFP antibodies for immunoblot analysis (Supplemental Figures 3A to 3C). These etiolated COR27 transgenic seedlings were similar to Col-0 in the dark (Supplemental Figures 4A and 4B). Two independent myc-tagged and two independent YFP-tagged COR27 transgenic lines exhibited markedly elongated hypocotyls under various light conditions tested (white, blue, red, and far red; Figures 4A to 4H). These genetic and phenotypic results support the notion that COR27 promotes hypocotyl elongation and acts as a negative regulator of light signaling.

**COR27 Physically Interacts with HY5**

HY5, a central regulator of light signaling, functions directly downstream of the COP1-SPA complex (Oyama et al., 1997;
COR27 Represses HY5 Biochemical Activity

To assess the biological significance of the COR27-HY5 interaction, we tested whether COR27 affects the biochemical activity of HY5. GST-HY5, but not GST (negative control), was able to bind to the biotin-labeled FHY1 and CHS promoter subfragments in electrophoretic mobility shift assays (EMSA), which is consistent with previous studies (Li et al., 2010; Zhang et al., 2017; Lin et al., 2018). His-COR27 itself did not bind the FHY1 or CHS promoter subfragments. The presence of His-COR27 clearly reduced the binding of HY5 to these two DNA subfragments. As the amount of His-COR27 increased, the binding affinity of HY5 clearly decreased (Figures 6A and 6B). The activating domain (AD)-HY5 fusion protein activated the FHY1pro:LacZ and BBX31pro:LacZ reporters in yeast cells, as previously reported (Li et al., 2010; Lin et al., 2018; Heng et al., 2019), indicating that HY5 can bind to the DNA subfragments of FHY1 and BBX31 promoters, allowing the activation of the LacZ reporter by AD in yeast cells. AD-COR27 had no detectable effect on these two reporters;

Osterlund et al., 2000); thus, we sought to test the interaction between COR27 and HY5. As shown in Figures 5A and 5B, COR27 did interact with full-length HY5 in yeast-two hybrid assays. Further domain mapping analysis revealed that COR27-N, but not COR27-C, interacted with HY5 and the HY5D N77 (78-168) truncation lacking the N-terminal region of HY5, but not with HY5 N77 (1-77), suggesting that the N terminus of COR27 and the C-terminal region of HY5 mediate the HY5-COR27 interaction.

To verify these results, we performed a BiFC assay using *N. benthamiana* leaves. We clearly detected strong YFP signal when transiently coexpressing both COR27·YFP<sup>N</sup> and HY5·YFP<sup>C</sup> (Figure 5C). However, the negative controls (COR27·YFP<sup>N</sup> and YFP<sup>C</sup> and YFP<sup>N</sup> and HY5·YFP<sup>C</sup>) did not produce any YFP signal (Figure 5C). We further used a Co-IP assay using transgenic plants coexpressing YFP-COR27 and HA-HY5 or YFP-GST and HA-HY5. YFP-COR27, but not YFP-GST, immunoprecipitated the HA-HY5 protein in *N. benthamiana* leaves (Figure 5D). Together, these data suggest that COR27 physically interacts with HY5.

Figure 3. cor27 Seedlings Are Hypersensitive to Light.

(A), (C), (E), and (G) Hypocotyl phenotypes for 4-d-old Col-0 and three independent cor27 mutant alleles grown in white- (10 μmol/m²/s; see [A]), blue- (1 μmol/m²/s; see [C]), red- (31 μmol/m²/s; see [E]), and far-red (4.7 μmol/m²/s; see [G]) light conditions. Bar = 1 mm. (B), (D), (F), and (H) Hypocotyl length of 4-d-old Col-0 and three independent cor27 mutant alleles grown in different light intensities of white (B), blue (D), red (F), and far-red (H) light. The data represent means ± se (n = 60) of three biological replicates. Letters above the bars indicate significant differences (P < 0.05), as determined by one-way ANOVA with Tukey’s post hoc analysis.

Figure 4. COR27 Transgenic Seedlings Are Hyposensitive to Light.

(A) to (H) Hypocotyl phenotypes and length for Col-0 and four independent transgenic lines overexpressing COR27. Col-0, myc-tagged, or YFP-tagged COR27 transgenic lines were grown in white- (16 μmol/m²/s; see [A] and [B]), blue- (3.6 μmol/m²/s; see [C] and [D]), red- (110 μmol/m²/s; see [E] and [F]), and far-red (4.7 μmol/m²/s; see [G] and [H]) light conditions for 4 d. Bar = 1 mm. In (B), (D), (F), and (H), the data represent means ± se (n = 60) of three biological replicates. Letters above the bars indicate significant differences (P < 0.05), as determined by one-way ANOVA with Tukey’s post hoc analysis.
however, the activation of AD-HY5 on FHY1pro:Luc and BBX31pro:Luc reporters significantly decreased in the presence of AD-COR27 (Figures 6C and 6D). Moreover, HY5 activated the FHY1pro:LUC and CHSpro:LUC reporters in Arabidopsis protoplasts (Zhang et al., 2017; Lin et al., 2018). Although COR27 alone did not affect FHY1pro:LUC and CHSpro:LUC expression, the activation of HY5 on these two reporters markedly decreased in the same experimental system when HY5 and COR27 were transiently coexpressed (Figures 6E to 6G). The transcript and protein levels of HY5 were comparable in Col-0, cor27-3, and myc-COR27 (Supplemental Figure 5), suggesting that COR27 may not affect HY5 at either the transcriptional or protein levels in Arabidopsis. Together, these data suggest that COR27 represses the hypothetical activity toward target genes.

Next, we examined the genetic relationship between COR27 and HY5. cor27-3 displayed shorter hypocotyls, whereas hy5-215 exhibited dramatically elongated hypocotyls. The hypocotyl length of cor27-3 hy5-215 double mutant seedlings was longer than that of Col-0 and cor27-3 but was slightly shorter than that of hy5-215 (Figures 7A to 7H), suggesting that COR27 and HY5 may act independently in regulating hypocotyl growth. HY5 HOMOLOG (HYH) can largely compensate for the function of HY5 in the control of hypocotyl growth, with HYH showing comparable activity toward target genes in the control of hypocotyl growth. Moreover, the hypocotyl length of the hy5-215, hy5-215 FHY1pro:Luc, and hy5-215 CHSpro:Luc reporters was much shorter than that of Col-0 but significantly longer than that of Col-0 pif4-2 double mutant seedlings (Figures 7A to 7D). The hypocotyl length of the cor27-3 hy5-215 double mutant was indistinguishable from that of hy5-215 (Figures 9A to 9D), indicating that COR27 acts upstream of HY5 with respect to hypocotyl growth. Moreover, the hypocotyl length of GFP::PIF4 cor27-3 seedlings was similar to that of GFP::PIF4 seedlings, suggesting that the function of COR27 is dependent on PIF4 in the regulation of hypocotyl growth. Myc-COR27 pif4-2 double seedlings had significantly shorter hypocotyls than myc-COR27 seedlings, both however markedly longer than the cor27-3, pif4-2, and cor27-3 pif4-2 genotypes (Figures 9A to 9D). The long hypocotyl length of myc-COR27 and myc-COR27 pif4-2 might be caused by ectopic overexpression of myc-COR27, thereby promoting hypocotyl elongation independently of PIF4.

**DISCUSSION**

Extensive studies have documented that numerous components of light signaling and the circadian clock synergistically contribute to control hypocotyl growth, demonstrating that external light
signals and internal circadian rhythms functionally work in concert to regulate seedling development (Schaffer et al., 1998; Wang et al., 1998; Más et al., 2003; Nusinow et al., 2011). COR27 has been reported to play pleotropic roles in the circadian clock, photoperiodic flowering, and freezing tolerance (Mikkelsen and Thomashow, 2009; Li et al., 2016; Wang et al., 2017). In this study, we show that COR27 acts as a negative regulator of light signaling. COR27 is degraded in a COP1-dependent manner via the 26S proteasome system at night. Upon light exposure, COR27 is abundant in plant cells. Accumulated COR27 not only negatively regulates HY5 activity by forming heterodimers but also associates with the PIF4 promoter and upregulates its expression in the afternoon, ultimately promoting hypocotyl elongation (Figure 10).

The basic leucine zipper–type transcription factor HY5 is a positive regulator of light signaling and controls the expression of many genes involved in promoting light-inhibited hypocotyl growth (Oyama et al., 1997; Osterlund et al., 2000; Lee et al., 2007; Zhang et al., 2011; Burko et al., 2020). COR27 physically interacted with HY5 and impaired its DNA binding activity (Figures 5 and 6). Thus, COR27 promotes hypocotyl growth, at least in part, by repressing HY5 action. Previous studies have revealed that low temperatures induce the expression of COR27, which is also temporally restricted by the circadian clock (Mikkelsen and Thomashow, 2009; Li et al., 2016; Wang et al., 2017). COR27 has a negative effect in responses to low temperatures and affects the period length of various circadian outputs (Li et al., 2016). On one hand, COR27 is repressed by the morning complex CCA1-LHY in...
and PRR9 transcriptional activity through direct protein-protein interactions gate seedling growth by repressing PIFs (including PIF4) targets (Zhu et al., 2016). PRR9, PRR7, PRR5, and TOC1 sequentially repress PIF4 to repress its transcriptional activity toward its downstream targets (Nusinow et al., 2011). The evening complex ELF3-ELF4-LUX promotes hypocotyl growth (Figures 3 and 4), whereas HY5 inhibits hypocotyl elongation (Oyama et al., 1997; Osterlund et al., 2000). COR27 and HY5 play opposite roles in controlling hypocotyl growth: COR27 promotes hypocotyl growth (Figures 3 and 4), whereas HY5 inhibits hypocotyl elongation. COR27 was able to inhibit HY5 activity through a direct protein–protein interaction (Figures 5 and 6), suggesting that COR27 works in concert with HY5 to coordinate hypocotyl growth in response to light signals. The inhibition of HY5 action by COR27 appears to predominantly occur in the light, as both proteins are degraded in the dark but are abundant in the light (Figure 2; Osterlund et al., 2000; Li et al., 2016). The expression of PIF4 is under the control of the circadian clock, peaking at ZT8 under diurnal conditions (Nusinow et al., 2011). Of note, COR27 activates PIF4 expression in the afternoon, especially at ZT8 and ZT12 (Figure 8A). In agreement, our genetic studies demonstrated that COR27 acted upstream of PIF4 with respect to hypocotyl elongation (Figure 9). Together, these facts suggest that COR27 is required for the proper expression of PIF4 under diurnal conditions as well as PIF4-mediated hypocotyl growth. A recent study showed that gating of seedling growth starts in the morning and covers the entire day to dusk through a dynamically changing PRR-PIF module (Martin et al., 2018). Although COR27 is a nighttime repressor (Li et al., 2016; Wang et al., 2017), it did repress HY5 biochemical activity in the light and upregulated PIF4 expression in the afternoon (Figures 5, 6, and 8), indicating that COR27 might gate hypocotyl growth through HY5- and PIF4-mediated processes during the daytime.

Collectively, COR27 is regulated by light, the circadian clock and low temperatures. The COP1-SPA1 E3 ubiquitin ligase complex promotes the degradation of COR27 via the 26S proteasome system at night. Light-accumulated COR27, on one hand, interacts with HY5 and inhibits its action; on the other hand, it binds to the chromatin of the PIF4 locus, which subsequently leads to an increase in PIF4 transcription in the afternoon. Consequently, these molecular regulatory events serve to promote hypocotyl elongation in plants (Figure 10).

The evening complex ELF3-ELF4-LUX promotes hypocotyl growth largely through the PIF4-mediated signaling pathways. Light, the circadian clock, and low and warm temperatures affect seedling growth partly by PIF4-mediated signaling (Huq and Quail, 2002; Sun et al., 2013; Jung et al., 2016; Legris et al., 2016; Dong et al., 2020; Jiang et al., 2020), indicating that PIF4 represents a regulatory node for the gating of seedling growth downstream of multiple signaling pathways. Our study reveals that COR27 integrates light and circadian cues for the promotion of hypocotyl elongation, at least in part through the transcriptional regulation of PIF4.

COR27 is diurnally regulated and is degraded during the night but accumulates during the daytime (Li et al., 2016). COP1-SPA1 interacts with COR27 and promotes its protein turnover via the 26S proteasome system in the dark (Figures 1 and 2). Thus, destabilization of COR27 at night is mainly attributed to the enriched and bioactive COP1-SPA1 complex in the nucleus (Hoecker, 2017). Light inactivates the biochemical activity of the COP1-SPA1 complex through multiple regulatory mechanisms (Podolec and Ulm, 2018), thereby leading to the accumulation of COR27 in the daytime. The abundance of HY5 is regulated by the COP1-SPA1 complex in a similar way (Osterlund et al., 2000). COR27 and HY5 play opposite roles in controlling hypocotyl growth: COR27 promotes hypocotyl growth (Figures 3 and 4), whereas HY5 inhibits hypocotyl elongation (Oyama et al., 1997; Osterlund et al., 2000). COR27 was able to inhibit HY5 activity through a direct protein–protein interaction (Figures 5 and 6), suggesting that COR27 works in concert with HY5 to coordinate hypocotyl growth in response to light signals. The inhibition of HY5 action by COR27 appears to predominantly occur in the light, as both proteins are degraded in the dark but are abundant in the light (Figure 2; Osterlund et al., 2000; Li et al., 2016). The expression of PIF4 is under the control of the circadian clock, peaking at ZT8 under diurnal conditions (Nusinow et al., 2011). Of note, COR27 activates PIF4 expression in the afternoon, especially at ZT8 and ZT12 (Figure 8A). In agreement, our genetic studies demonstrated that COR27 acted upstream of PIF4 with respect to hypocotyl elongation (Figure 9). Together, these facts suggest that COR27 is required for the proper expression of PIF4 under diurnal conditions as well as PIF4-mediated hypocotyl growth. A recent study showed that gating of seedling growth starts in the morning and covers the entire day to dusk through a dynamically changing PRR-PIF module (Martin et al., 2018). Although COR27 is a nighttime repressor (Li et al., 2016; Wang et al., 2017), it did repress HY5 biochemical activity in the light and upregulated PIF4 expression in the afternoon (Figures 5, 6, and 8), indicating that COR27 might gate hypocotyl growth through HY5- and PIF4-mediated processes during the daytime.

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Figure 7. Genetic Relationship between COR27 and HY5.

(A) to (H) Hypocotyl phenotypes and length for Col-0, cor27-3, hy5-215, and cor27-3 hy5-215 seedlings grown in white- (10 μmol/m²/s; see [A] and [B]), blue- (3.6 μmol/m²/s; see [C] and [D]), red- (110 μmol/m²/s; see [E] and [F]), and far-red (4.7 μmol/m²/s; see [G] and [H]) light conditions for 4 d. Bar = 1 mm. In (B), (D), (F), and (H), the data represent means ± se (n = 60) of three biological replicates. Letters above the bars indicate significant differences (P < 0.05), as determined by one-way ANOVA with Tukey’s post hoc analysis.
METHODS

Plant Materials and Growth Conditions

We followed a gene editing strategy using the CRISPR/Cas9 technique as previously described by Wang et al. (1998). We searched for and identified 23-bp target sites (5'-N20NGG-3') within exons of the COR27 genomic sequence and then evaluated each candidate site for target specificity on the website of potential off-target finder (http://www.rgenome.net/cas-offinder/). We subcloned two independent single guide RNA targeting COR27 into the pHEE401E vector. We introduced the resulting vectors into Agrobacterium (Agrobacterium tumefaciens) strain GV3101 by the freeze-thaw method and then transformed Col-0 plants via the floral dip method (Clough and Bent, 1998). We selected primary transformants on Murashige and Skoog (MS) medium containing 25 mg/L hygromycin and genotyped plants using gene-specific primers by PCR amplification and sequencing. We then removed the pHEE401 T-DNA insert (including CRISPR/Cas9) in cor27-4 and cor27-5 single mutants by backcrossing to Col-0. The primers used in these experiments are listed in Supplemental Data Set 1.

Double mutants or transgenic plants were obtained by genetic crossing or constructs transduction and then verified by phenotypic inspection, antibiotic selection, PCR genotyping, and sequencing (for primers, see Supplemental Data Set 1). For germination, seeds were surface sterilized with 30% (v/v) commercial Clorox bleach for 10 min and washed three times with sterile water. Seeds were then sown on MS plates containing 1% (w/v) Suc and 0.8% (w/v) agar and incubated at 4°C in the dark for 3 d to help

Figure 8. COR27 Binds to PIF4 Promoter Regions and Upregulates Transcription of PIF4 and Its Targets.

(A) RT-qPCR analysis of rhythmic PIF4 transcript levels in Col-0 and cor27-3 mutant seedlings grown in 12-h-light/12-h-dark cycles for 5 d. Three biological replicates, each with three technical repeats, were performed. The data represent means ± SD of three biological replicates. Asterisks indicate significant differences (*P < 0.05, **P < 0.01), as determined by Student’s t test.

(B) to (E) RT-qPCR analysis of PIF4 (B) and PIF4 target genes (IAA19, C; IAA29, D; YUC8, E) expression in Col-0, cor27-3, pif4-2, cor27-3 pif4-2, myc-COR27 Col-0 #2, and myc-COR27 pif4-2 #2 seedlings. Seedlings of the indicated genotypes were grown in 12-h-light/12-h-dark cycles for 5 d. Samples were collected at ZT8 for total RNA extraction. Three biological replicates, each with three technical repeats, were performed. The data represent means ± SD of three biological repeats. Letters above the bars indicate significant differences (P < 0.05), as determined by one-way ANOVA with Tukey’s post hoc analysis.

(F) Illustration of PIF4 promoter regions with the indicated positions of primers used in ChIP-qPCR experiments.

(G) ChIP-qPCR assays showing that COR27 associates with the PIF4 promoter in vivo. ChIP-qPCR assays were performed using 5-d-old Col-0 and YFP-COR27 Col-0 #4 seedlings with anti-GFP antibodies. Plants were grown in 12-h-light/12-h-dark cycles and harvested at ZT8. The data represent means ± SD of three biological repeats. Asterisks indicate significant differences (**P < 0.01), as determined by Student’s t test.
indicated genotypes grown in white- (7.5 \mu mol/m^2/s; see [A] and [B]) or red- (110 \mu mol/m^2/s; see [C] and [D]) light conditions. Bar = 1 mm. In (B) and (D), the data represent means ± se (n ≥ 60) of three biological replicates. Letters above the bars indicate significant differences (P < 0.05), as determined by one-way ANOVA with Tukey’s post hoc analysis.

synchronize germination. Seedlings were grown in growth chambers (Percival Scientific) maintained at 22°C in constant white light (38 \mu mol/m^2/s, F17T8; Philips) conditions. For the BiFC assays, Nicotiana benthamiana plants were grown in the greenhouse at 30°C in long-day conditions (16-h light [white light, 58 \mu mol/m^2/s, TL5; Philips]-8-h dark cycle).

Plasmid Construction

We cloned the full-length COR27, COP1, SPA1, or HY5 coding sequences (CDSs) into the pDONR-221 or pDONR-223 vector (Invitrogen) using Gateway BP Clonase enzyme mix (Invitrogen) and verified all constructs by sequencing. To generate plasmids for transgenic plant production, we further introduced all CDS-containing vectors into the pEarleyGate 202 (N-terminal Flag tag), pEarleyGate 203 (N-terminal myc tag), and pEarleyGate 104 (N-terminal eYFP fusion; Earley et al., 2006) vectors using Gateway LR Clonase enzyme mix (Invitrogen) to create the plant binary vectors pEarleyGate 104 (N-terminal eYFP fusion; Earley et al., 2006) vectors using Gateway LR Clonase enzyme mix (Invitrogen) to create the plant binary vectors pEarleyGate-yeast-COR27, pEarleyGate-yeast-COR27, and pEarleyGate-Flag-SPA1. To generate constructs for BiFC assays, we cloned the full-length COR27, COP1, SPA1, or HY5 CDSs into the pSPYNE or pSPYCE vectors (Earley et al., 2006) to generate the constructs pSPYNE-35S-COR27, pSPYCE-35S-COP1, pSPYCE-35S-SPA1, and pSPYCE-35S-HY5.

For yeast-two hybrid assays, we PCR amplified the full-length COR27, COR27 (1 to 120), and COR27 (121 to 246) CDSs with their respective pairs of primers and then cloned the PCR products at the EcoRI/BamHI sites of the pGADT7 vector (Clontech) to generate the prey constructs pGADT7-COR27, pGADT7-COR27 (1 to 120) and pGADT7-COR27 (121 to 246). The bait constructs pGBK7-COP1, pGBK7-COP1 N282, pGBK7-COP1 coil, and pGBK7-COP1 WD40 were described previously by Xu et al. (2016). Furthermore, we also cloned the full-length COR27, COR27 (1 to 120) and COR27 (121 to 246) CDS at the EcoRI/BamHI sites of the pLexA vector to generate bait constructs of pLexA-COR27, pLexA-COR27 (1 to 120), and pLexA-COR27 (121 to 246). We PCR amplified the CDS for SPA1, SPA2, SPA3, SPA4, SPA1 (1 to 520), SPA1 (521 to 1029), SPA1 (521 to 698), SPA1 (647 to 1029), HY5, HY5 N77, and HY5 N77, with their respective pairs of primers and then cloned them at the EcoRI/HindIII sites of the pB42AD vector to generate prey constructs. For in vitro pull-down assay, we cloned the full-length COR27 and COP1 CDS at the BamHI/
defined (SD/-Trp-Ura dropout medium according to the Yeast Protocols Handbook (Clontech). We used a liquid assay protocol to measure yeast colony β-galactosidase activity (Thermo Fisher Scientific).

To confirm protein–protein interactions, we cotransformed the respective combinations of bait and prey constructs into yeast strain Y2H Gold (Clontech). We also cotransformed the empty pGADT7 and pGBKTK7 vectors in parallel as negative controls. After growth on SD/-Trp-Leu and SD/-Trp-Leu-His Ade dropout medium, we tested protein–protein interactions using selective SD/-Trp-Leu–His–Ade dropout medium supplied with X-a-Gal and aureobasidin A (Clontech). We checked for interaction after 3 d of incubation at 30°C. We performed all yeast transformations as described in the Yeast Protocols Handbook. To detect COR27 interactions with SPA or HIS5 proteins, we performed yeast two-hybrid assays using the Matchmaker LexA Two-Hybrid System (Clontech). We cotransformed the respective combinations of pLexA and pB42AD fusion plasmids into yeast strain EGY48 containing p8op-LacZ plasmid. We cotransformed the empty pLexA and pB42AD vectors in parallel as negative controls. We selected and grew transformants on SD/-Trp-Leu–His–Ade dropout medium at 30°C. We grew transformants on SD/-His-Trp–Ura dropout medium containing 80 mg/L X-Gal for blue color development.

**BiFC Assays**

We generated the constructs pSPYNE-3SS-COR27, pSPYCE-3SS-COP1, pSPYCE-3SS-SPA1, and pSPYCE-3SS-HIS5 as described above and introduced them into Agrobacterium strain GV3101. We grew the resulting colonies overnight in Luria–Bertani medium at 28°C, pelleted the cells by centrifugation and resuspended the pellet in infiltration buffer (10 mM MgCl₂, 150 mM acetoxyarginone, and 10 mM MES, pH 5.6) to a final cell density equivalent to OD₆₀₀ = 0.6. We infiltrated the cell suspensions containing the indicated transformant pairs into N. benthamiana leaves. We detected YFP fluorescence using a confocal laser scanning microscope (LSM510 Meta; Carl Zeiss) 24 h after infiltration.

**Co-IP Assays**

For Co-IP assays, we collected 4-d-old dark-grown Col-0 and 3SS:YFP-COR27 seedlings and then ground them to a fine powder in liquid nitrogen. We added 400 μL of protein extraction buffer (containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% [v/v] glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 × Complete Protease Inhibitor Mixture [Roche]) to extract total proteins. We incubated the mixtures at 4°C for 3 h. After four washes with binding buffer, we boiled the pellet fraction with 5 × SDS protein loading buffer. We detected input and pull-down prey proteins by immunoblot analysis using anti-His (1:5000 [v/v], H1029-2ML; Sigma-Aldrich) and anti-MBP (1:5000 [v/v], cat. no. E8031S; New England Biolabs) monoclonal antibodies.

**Immunoblot Analysis**

For immunoblot analysis, we collected the wild-type or mutant Arabidopsis seedlings and extracted total proteins using protein extraction buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 200 mM NaCl, 8 M urea, pH 8.0, 1 mM PMSF, and 1 × Complete Protease Inhibitor Cocktail [Roche]). We then separated protein samples by SDS-PAGE before transfer onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% milk and incubated with primary antibody overnight at 4°C. Primary antibodies used in this study were anti-COP1 (1:1000 [v/v], McNellis et al., 1994), anti-Myc (1:1000 [v/v], cat. no. M4438; Sigma-Aldrich), anti-GFP (1:5000 [v/v], cat. no. M20004M; Abmart), anti-Flag (1:1000 [v/v], F3165–2MG; Sigma-Aldrich), and anti-Actin (1:2000 [v/v], cat. no. A0480; Sigma-Aldrich). We then washed the membranes with protein blotting buffer and incubated them with secondary antibody (1:10,000 [v/v]) for 1 h at room temperature. After three washes with 1 × phosphate-buffered saline Tween 20 for 10 min, we detected the labeled proteins. The probes used in this study were anti-COR27, GST-HIS5, or GST proteins as indicated, together with 40 fmol of biotin-labeled probes in a 20-μL reaction mixture at 25°C for 20 min, followed by separation on 6% native polyacrylamide gels in 0.5 × Tris borate EDTA buffer. We electroblotted the resolved proteins onto Hybond N+ (Millipore) nylon membranes in 0.5 × Tris borate EDTA for 40 min and detected the labeled probes. The probes used in this study are listed in Supplemental Data Set 1.

**EMSA**

We cloned the full-length HIS5 CDS sequence into the pGEX-4T vector and introduced the resulting construct into E. coli strain BL21 (Invitrogen) to produce recombinant HIS5 protein. Next, we used biotin-labeled probes and a Light Shift Chemiluminescent EMSA kit for EMSA (Thermo Fisher Scientific) as described previously by Xu et al. (2016) and Lin et al. (2018). The promoter subfragments of FHY1 (143 bp, −280 to −138 bp) and CHS (150 bp, −547 to −398 bp) upstream of ATG were amplified by PCR. For biotin labeling, we mixed these purified PCR products with biotin and incubated them under UV light for 30 min. We then incubated purified His-COR27, GST-HIS5, or GST proteins as indicated, together with 40 fmol of biotin-labeled probes in a 20-μL reaction mixture at 25°C for 20 min, followed by separation on 6% native polyacrylamide gels in 0.5 × Tris borate EDTA buffer. We electroblotted the resolved proteins onto Hybond N+ (Millipore) nylon membranes in 0.5 × Tris borate EDTA for 40 min and detected the labeled probes. The probes used in this study are listed in Supplemental Data Set 1.

**Dual-Luciferase Reporter System**

We PCR amplified promoter subfragments for FHY1 and CHS and cloned them into the pGreenII 0800-LUC vector (Hellens et al., 2005) to drive the firefly luciferase (LUC) gene (FHY1pro:LUC and CHSpro:LUC; Heng et al., 2019). We prepared and transfected Arabidopsis mesophyll cell protoplasts as described previously by Yoo et al. (2007). pCambia1300-UBQ10:HA-HIS5 (Heng et al., 2019) and pGreenII 62:5K-COR27 were used as the effectors. We used a Dual-Luciferase kit (Promega) for transient expression analysis to detect reporter activity. The Renilla (REN) luciferase gene, driven by the cauliflower mosaic virus 35S promoter, was used as an internal control. The ratio of LUC:REN was calculated as an indicator of the final transcriptional activity.
ChIP
We conducted ChIP assays as described previously by Xu et al. (2016) and Zhao et al. (2020). We fixed 5-day-old Col-0 or 35S::YFP-COR27 transgenic seedlings grown in constant white light at room temperature in 1% formaldehyde under vacuum for 15 min. We then homogenized the fixed tissues and sonicated the resuspended chromatin in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and 0.1 mM PMSF) at 4°C to ~250- to 500-bp fragments. We immunoprecipitated, washed, reverse cross-linked, and finally amplified the sheared chromatin. The monoclonal anti-GFP antibody (Abmart) was used for immunoprecipitation. Approximately 10% of sonicated but nonimmunoprecipitated chromatin was reverse cross-linked and used as an input DNA control. We analyzed both immunoprecipitated DNA and input DNA by real-time quantitative PCR. We used 100 ng of DNA as template and mixed with SYBR Green PCR Master Mix (cat. no. DRR041A; Takara) and subjected to the Step One Plus Real-time PCR detection system (Applied Biosystems). The experiments were repeated three times with similar results. The primers used for ChIP assays are listed in Supplemental Data Set 1.

Quantitative RT-PCR and Semiquantitative PCR Analysis
We extracted total RNA from Arabidopsis samples using the RNeasy Plant Minikit (Qiagen) and synthesized first-strand cDNAs from 2 µg of total RNA using the S&X All-In-One RT Master Mix cDNA synthesis system (Applied Biological Materials) according to manufacturer’s instructions. For qPCR, we performed all reactions with SYBR Green PCR Master Mix (Takara) in a 20-µL reaction mixture and run on the StepOnePlus Real-time PCR detection system (Applied Biosystems) following the manufacturer’s instructions. We used the Arabidopsis housekeeping gene PROTEIN PHOSPHATASE2A as a reference gene. All assays for target genes were conducted three biological repeats, each with three technical repeats. The quantification of threshold cycle (CT) value analysis was achieved using the 2(–ΔΔCT) method. For semiquantitative qPCR, cDNA was combined with PCR Mix (cat. no. R040A; Takara). The PCR products were loaded onto a 1% (w/v) agarose gel for electrophoresis. The primers used in this study are listed in Supplemental Data Set 1.

Statistical Analysis
Statistical analyses were performed in Excel (Microsoft), Prism version 5.0 (GraphPad), or through an online website (http://astatsa.com/OneWay_Anova_with_TukeyHSD/). Different letters represent statistical significances determined by ANOVA (P < 0.05) for multiple comparisons, and levels that are not significantly different are indicated with the same letter. The results of all statistical analyses were provided in Supplemental Data Set 2.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative database or the GenBank/EMBL libraries under the following accession numbers: COR27 (At5g42900); COP1 (At2g32950); SPA1 (At2g46340); HY5 (At5g11260); PIF4 (At2g43010).

Supplemental Information
Supplemental Figure 1. Identification and characterization of cor27 mutants.
Supplemental Figure 2. cor27 single mutants show similar phenotype with WT in darkness.
Supplemental Figure 3. COR27 transcript and protein levels in myc- or YFP-tagged COR27 transgenic plants.

Supplemental Figure 4. Transgenic seedlings overexpressing COR27 show similar phenotypes with WT grown in darkness.

Supplemental Figure 5. COR27 did not affect the HY5 transcript and protein levels.

Supplemental Data Set 1. List of primers used in this study.

Supplemental Data Set 2. ANOVA table.

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AUTHOR CONTRIBUTIONS
W.Z., H.Z., F.L., X.Z., and D.X. performed the research. D.X. and X.W.D. designed the project, analyzed the data, and wrote the article.

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