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A Positive Feedback Loop of BBX11–BBX21–HY5 Promotes Photomorphogenic Development in *Arabidopsis*

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ABSTRACT

Light is the most important environmental factor affecting many aspects of plant development. In this study, we report that B-box protein 11 (BBX11) acts as a positive regulator of red light signaling. BBX11 loss-of-function mutant seedlings display significantly elongated hypocotyls under conditions of both red light and long day, whereas *BBX11* overexpression causes markedly shortened hypocotyls under various light states. BBX11 binds to the *HY5* promoter to activate its transcription, while both BBX21 and HY5 associate with the promoter of *BBX11* to positively regulate its expression. Taken together, our results reveal positive feedback regulation of photomorphogenesis consisting of BBX11, BBX21, and HY5, thus substantiating a transcriptional regulatory mechanism in the response of plants to light during normal development.

Keywords: BBX, HY5, COP1, photomorphogenesis, light signaling

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INTRODUCTION

Plants have evolved a fine-tuned molecular mechanism in their responsiveness to dynamically changing light conditions throughout their life span. Different wavelength-specific light signals are perceived by a variety of photoreceptors in plants. Phytochromes (phyA to phyE) perceive far-red (FR) and red (R) light (Sharrock and Quail, 1989); cryptochromes (CRY1 and CRY2) and phototropins (PHOT1 and PHOT2) sense UV-A and/or blue (B) light (Gallagher et al., 1988; Lin et al., 1995; Guo et al., 1998); and UV-B resistance locus 8 (UVR8) absorbs UV-B light (Rizzini et al., 2011). Proper light exposure converts these photoreceptors into biologically active isoforms that work synergistically with downstream components to initiate diverse molecular events and promote photomorphogenesis (Chen et al., 2014; Ma et al., 2016; Pedmale et al., 2016; Wei et al., 2020; Yadav et al., 2020; Yang and Liu, 2020; Zhai et al., 2020).

Two key regulators of light signaling, constitutively photomorphogenic 1 (COP1) and elongated hypocotyl 5 (HY5), function downstream of a variety of photoreceptors and control approximately one-third of genes in the *Arabidopsis* genome that modulate skotomorphogenic or photomorphogenic development (Ma et al., 2003; Lee et al., 2007; Zhang et al., 2011). The E3 ubiquitin ligase COP1 precisely controls the abundance of HY5, a bZIP-type transcription factor (Oyama et al., 1997; Osterlund et al., 2000). In etiolated seedlings, COP1 is enriched in the nucleus, where it directs the polyubiquitination of HY5 and promotes its degradation via the 26S proteasome. Upon light irradiation, the nuclear activity of COP1 is largely inhibited, thus promoting the accumulation of HY5 in de-etiolated seedlings. This eventually leads to changes in HY5-regulated gene expression, and thus, physiological processes in response to light in plants (Oyama et al., 1997; Ang et al., 1998; Osterlund et al., 2000). Thus, the light-regulated COP1–HY5 complex represents a key node in the transition from skotomorphogenesis to photomorphogenesis.

Light can rapidly alter the transcriptome of plants, ultimately promoting seedling development (Ma et al., 2001). A group

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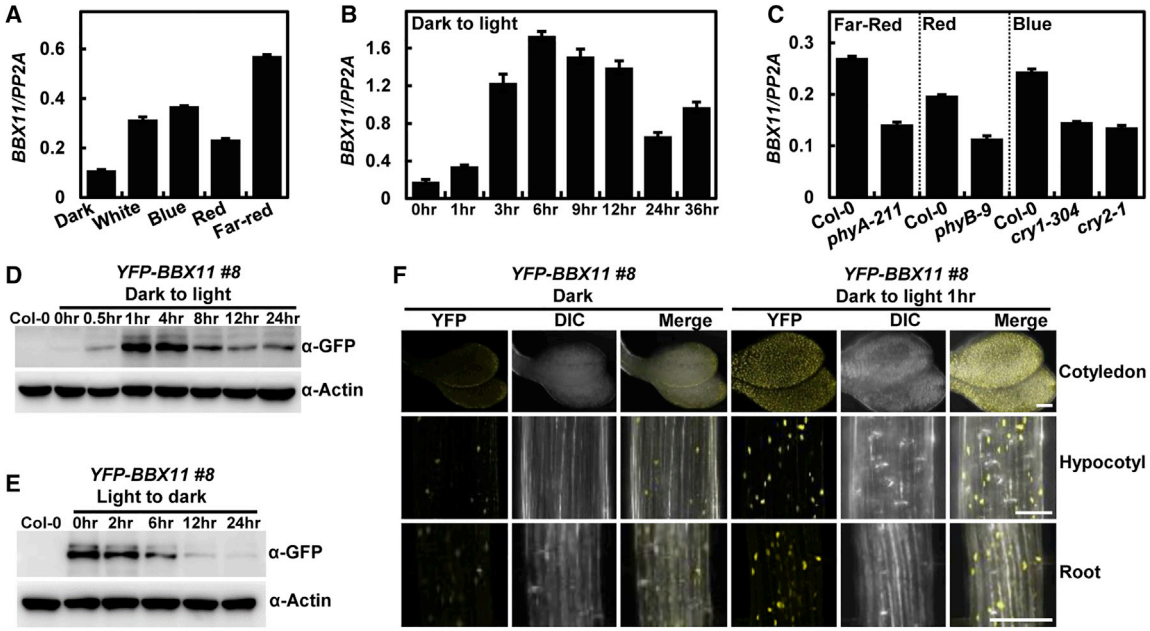

Figure 1. Transcript and Protein Levels of BBX11 Are Positively Regulated by Light.

(A) qRT–PCR analysis of the *BBX11* transcript level in 4-day-old Col-0 seedlings grown in various conditions (darkness; white, blue, red, and far-red light).

(B) qRT–PCR analysis of the *BBX11* transcript level during the dark-to-light transition. Four-day-old Col-0 seedlings grown in darkness were transferred to white light for 1–36 h as indicated.

(C) qRT–PCR analysis of the *BBX11* transcript level in different photoreceptor mutants. Col-0 and *phyA-211*, *phyB-9*, *cry1-304*, and *cry2-1* mutants were grown in far-red, red, and blue light for 4 days.

(D and E) YFP-BBX11 protein level in *YFP-BBX11 #8* transgenic seedlings during the dark-to-light **(D)** or light-to-dark **(E)** transition. Four-day-old dark-grown seedlings were transferred to white light for 0.5–24 h as indicated **(D)**. Five-day-old white-light-grown seedlings were transferred to darkness for 2–24 h as indicated **(E)**. Five-day-old white-light-grown Col-0 seedlings served as the negative control.

(F) Relative YFP fluorescence in 4-day-old *YFP-BBX11 #8* transgenic seedlings grown in darkness before and after 1 h of white light exposure. YFP, yellow fluorescent protein channel; DIC, differential interference contrast in the light microscope mode; Merge, merged images of YFP and DIC. Scale bars, 100 μm.

In **(A)** to **(C)**, the *BBX11* expression level was normalized to that of *PP2A*. Data are presented as means ± SD (n = 3).

of transcription factors mediates the light-controlled reprogramming of a variety of transcripts. Of these, HY5 is a key component that directly binds to the promoters of light-regulated genes to control their expression (Lee et al., 2007; Zhang et al., 2011; Burko et al., 2020). HY5 and B-box proteins (BBXs) are components of a delicate regulatory network, in which light optimally controls the timely expression of a variety of genes (Gangappa and Botto, 2014; Xu, 2019; Song et al., 2020). BBX21 and BBX22 promote HY5 activity by forming heterodimers (Datta et al., 2007, 2008), whereas BBX24, BBX25, and BBX28 inhibit its transcriptional activity through a similar molecular mechanism (Gangappa et al., 2013; Lin et al., 2018). In addition, BBX21 directly binds to the *T/G-box cis*-element present in the *HY5* promoter through its second B-box domain to activate its expression (Xu et al., 2016, 2018), whereas HY5 positively controls *BBX22* and represses *BBX30* and *BBX31* at the transcriptional level (Chang et al., 2008; Heng et al., 2019a; Yadav et al., 2019). BBX23 and HY5 associate with each other to regulate the expression of downstream targets that promote photomorphogenesis (Zhang et al., 2017). Thus, HY5 and specific BBXs constitute a critical regulatory network, whose function is to gain absolute control over the expression of thousands of genes to ensure normal plant growth and development (Xu, 2019; Song et al., 2020).

In this study, we characterized a previously unidentified positive regulator of R light signaling, BBX11, which contains two tandem conserved B-box domains in the N-terminal region. *BBX11* loss-of-function mutants show elongated hypocotyls under conditions of both R light and long day (LD; 16 h light/8 h dark), whereas *BBX11* overexpression results in shortened hypocotyls under white (W), B, R, and FR light. BBX11 associates with *HY5* chromatin regions and promotes its expression, whereas both BBX21 and HY5 bind to the *BBX11* promoter and activate its transcription, suggesting that BBX11, BBX21, and HY5 form a positive feedback loop at the transcriptional level. These results demonstrate that BBX11, BBX21, and HY5 promote photomorphogenesis, and this positive feedback regulation is critical for light-mediated seedling development.

RESULTS

Light Induces BBX11 at Both Transcriptional and Protein Levels

It has been shown that multiple BBX proteins are involved in light-regulated seedling development (Gangappa and Botto, 2014; Xu, 2019; Song et al., 2020). In an effort to identify the previously uncharacterized BBX member(s) acting in light signaling, we examined the transcript levels of a group of *BBX*s in wild-type *Arabidopsis* (Columbia-0 [Col-0] ecotype) grown under various

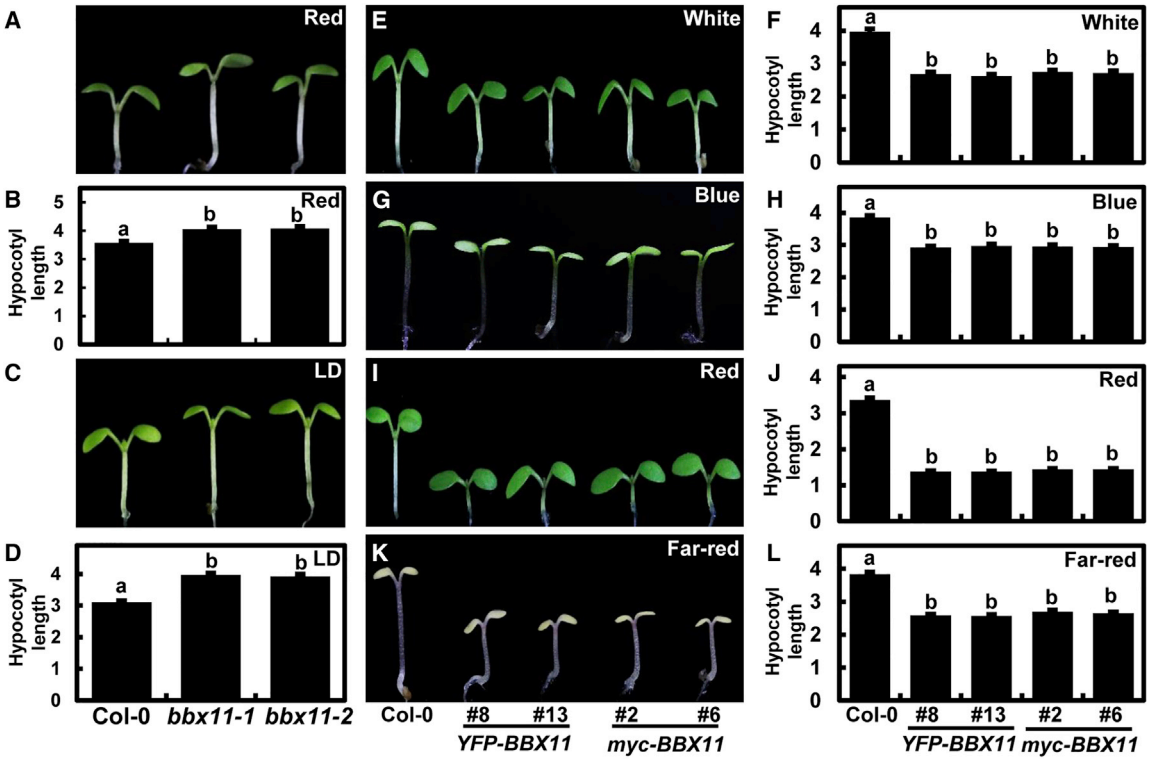

Figure 2. *Bbx11* Mutant Seedlings Grown in Red Light Show Elongated Hypocotyls.

Hypocotyl phenotype and length of 5-day-old Col-0 and two independent *bbx11* single-mutant seedlings grown in red light (129 μmol/m^2/s) **(A and B)** and LD conditions (16 h light/8 h dark) **(C and D)**. Hypocotyl phenotype and length of 5-day-old Col-0 and *BBX11* transgenic seedlings grown in white (14.74 μmol/m^2/s) **(E and F)**, blue (10.5 μmol/m^2/s) **(G and H)**, red (129 μmol/m^2/s) **(I and J)**, and far-red (4.5 μmol/m^2/s) **(K and L)** light. The unit of hypocotyl length is millimeters. Data are presented as the means ± SE ($n \geq 60$) 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.

light conditions (darkness; W, B, R, and FR light). The transcript level of *BBX11* in light-grown Col-0 seedlings was much higher than that in dark-grown seedlings (Figure 1A), indicating that *BBX11* is induced by light, and thus, a candidate for functioning in light signaling. We also found that the expression level of *BBX11* significantly increased when dark-grown seedlings were transferred to W light for various time points as indicated in Figure 1B. These results indicate that light can induce the expression of *BBX11*. As different light signals are perceived by different photoreceptors, we investigated whether phyA, phyB, CRY1, and CRY2 could affect *BBX11* at the transcriptional level. The expression of *BBX11* in FR light-grown *phyA-211*, R light-grown *phyB-9*, and B light-grown *cry1-304* and *cry2-1* mutant seedlings was significantly decreased compared with that in Col-0 seedlings grown in the corresponding wavelength-specific light conditions (Figure 1C), suggesting that photoreceptors phyA, phyB, CRY1, and CRY2 positively regulate *BBX11* expression in response to light, respectively.

To examine whether light can regulate the abundance of BBX11, we generated YFP-tagged *BBX11* (*YFP-BBX11*) transgenic plants overexpressing *BBX11* (Supplemental Figure 1) and characterized the amount of YFP-BBX11 during the transition from dark to light. YFP-BBX11 accumulated and peaked at 1–4 h after light illumination and then gradually decreased (Figure 1D). In addition, YFP-BBX11 gradually decreased when light-grown *YFP-BBX11* overexpressing plants were transferred to dark conditions for various time points (Figure 1E). Consistent with these observations, YFP signals were only slightly detectable in the cotyledons and hypocotyls of dark-grown *YFP-BBX11* seedlings; however, they became significantly evident at 4 h after light illumination (Figure 1F). Taken collectively, these data indicate that BBX11 degrades in the dark but accumulates in the light.

BBX11 Acts as a Positive Regulator of Red Light Signaling

To characterize the role of BBX11 in light signaling, we generated two independent *bbx11* loss-of-function mutants, namely *bbx11-1* and *bbx11-2*, using the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 technique (Wang et al., 2015) (Supplemental Figure 2). The *bbx11* mutants showed a phenotype similar to that of Col-0 when grown in the dark and W, B, and FR light (Supplemental Figures 3 and 4), whereas the two independent *bbx11* mutants displayed significantly elongated hypocotyls when grown in R light (Figure 2A and 2B), suggesting that BBX11 promotes photomorphogenic development in R light. Next, we investigated the expression pattern of *BBX11* under LD conditions. The transcript level of *BBX11* was under diurnal control with peak expression in the morning (zeitgeber time 8) (Supplemental Figure 5), indicating that the *BBX11* mRNA level is regulated by the circadian clock. Consistently, the

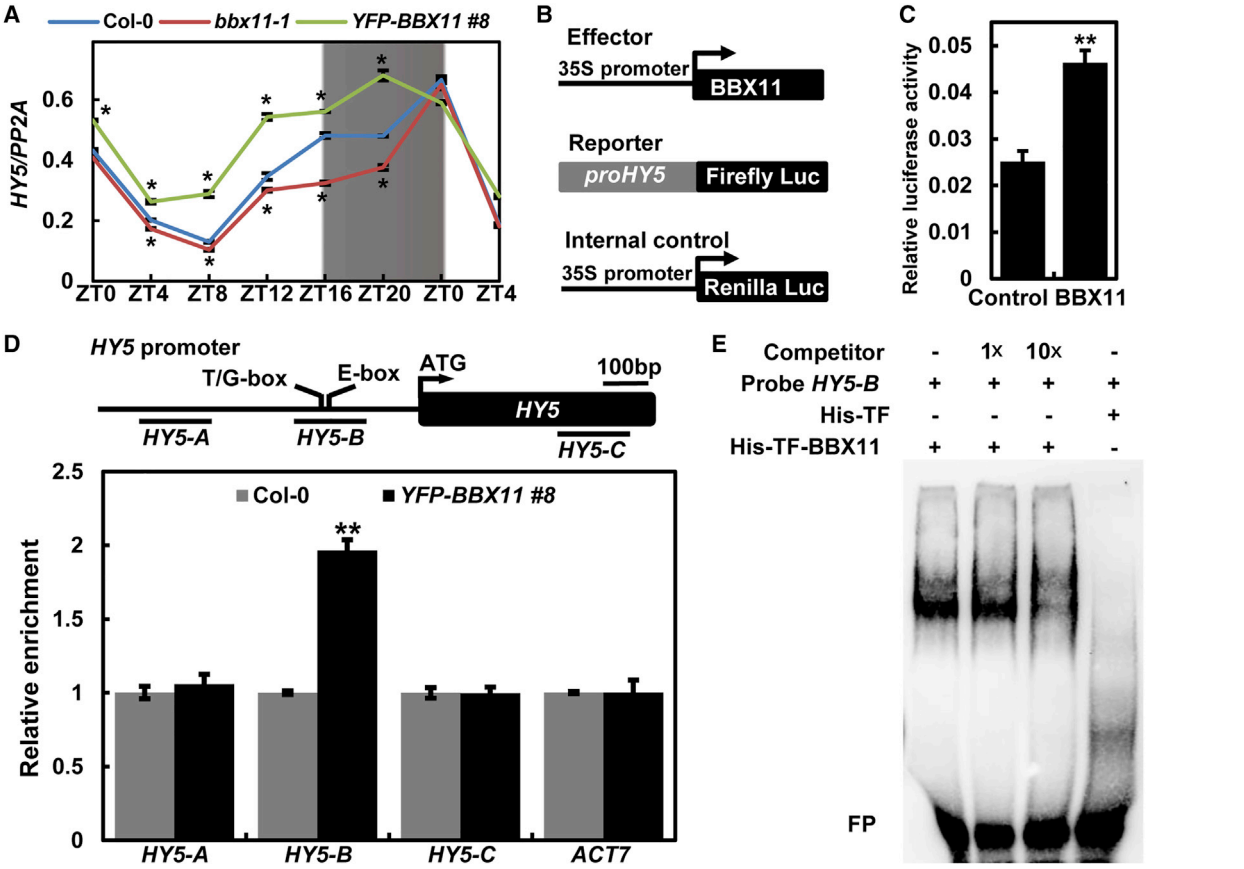

Figure 3. BBX11 Associates with the *HY5* Promoter and Upregulates Its Transcription.

(A) qRT–PCR analysis of the *HY5* transcript level in Col-0, *bbx11-1*, and *YFP-BBX11 #8* seedlings grown in LD conditions (16 h light/8 h dark). ZT indicates the zeitgeber time. Data are presented as means ± SD ($n = 3$). *$P < 0.05$, *compared with Col-0, as determined by the* two-tailed Student's *t*-test. The *HY5* expression level was normalized to that of *PP2A*.

(B) Schematic representation of various constructs used in transient transfection assays in *Arabidopsis* protoplasts. Arrows after the *35S* promoter indicate the transcriptional start site. The *HY5* promoter was fused to firefly luciferase to create the reporter constructs.

(C) Relative luciferase activity of the *proHY5:LUC* reporter construct cotransformed with BBX11 or the control into protoplasts. Data are presented as means ± SD ($n = 3$). **$P < 0.01$, as determined by the two-tailed Student's *t*-test.

(D) Results of a representative ChIP–qPCR assay showing that BBX11 associates with the *HY5* promoter *in vivo*. ChIP was performed with an anti-GFP monoclonal antibody. Data are presented as means ± SD ($n = 3$). **$P < 0.01$, as determined by the two-tailed Student's *t*-test.

(E) Results of a representative EMSA showing that BBX11 binds to the *HY5* promoter region. "−" and "+" represent the absence or presence, respectively, of the corresponding probe or protein. For probe *HY5-B*, "+" indicates 5 pmol. For His-TF, "+" indicates 5.5 pmol. For His-TF-BBX11, "+" indicates 6.5 pmol. FP indicates the free probe.

hypocotyl length of *bbx11* mutant seedlings was significantly longer than that of Col-0 when grown in LD conditions (Figure 2C and 2D). These results suggest that BBX11 inhibits hypocotyl growth under LD conditions.

To substantiate these observations, we analyzed the phenotypes of *YFP-BBX11* and *myc-BBX11* transgenic plants overexpressing *BBX11* (Supplemental Figure 1). The two independent etiolated *YFP-BBX11* and *myc-BBX11* overexpressing plants exhibited a similar hypocotyl length compared with Col-0; however, they developed a significantly larger apical hook angle compared with Col-0 in the dark (Supplemental Figure 6). All *BBX11* transgenic lines showed markedly shortened hypocotyls when grown in various light conditions (W, B, R, and FR) (Figure 2E–2L), suggesting that the overexpression of *BBX11* confers hypersensitivity in the response to various wavelength-specific light signals in *Arabidopsis*.

BBX11 Activates the Transcription of *HY5*

We have previously reported that multiple BBX proteins converge on HY5 to regulate photomorphogenesis (Xu et al., 2016, 2018; Lin et al., 2018; Heng et al., 2019a). Thus, we examined whether BBX11 affects *HY5* at the transcriptional level. As shown in Figure 3A, the *HY5* transcript level was decreased in *bbx11-1* but increased in *YFP-BBX11* at various time points under LD conditions, suggesting that BBX11 positively regulates *HY5* expression. Next, we transiently co-expressed *35S:BBX11* and *proHY5:LUC* in *Arabidopsis* protoplasts and found that BBX11 indeed activated the *proHY5:LUC* reporter in plant cells (Figure 3B and 3C), further confirming the activation of *HY5* by BBX11. To explore whether BBX11 binds to the promoter of *HY5*, we employed chromatin immunoprecipitation (ChIP)–qPCR to examine this possibility. As expected, BBX11 specifically bound to the *HY5* promoter region B (−350 to −252 bp), which contains an *E-box* and a *T/G-box cis*-element

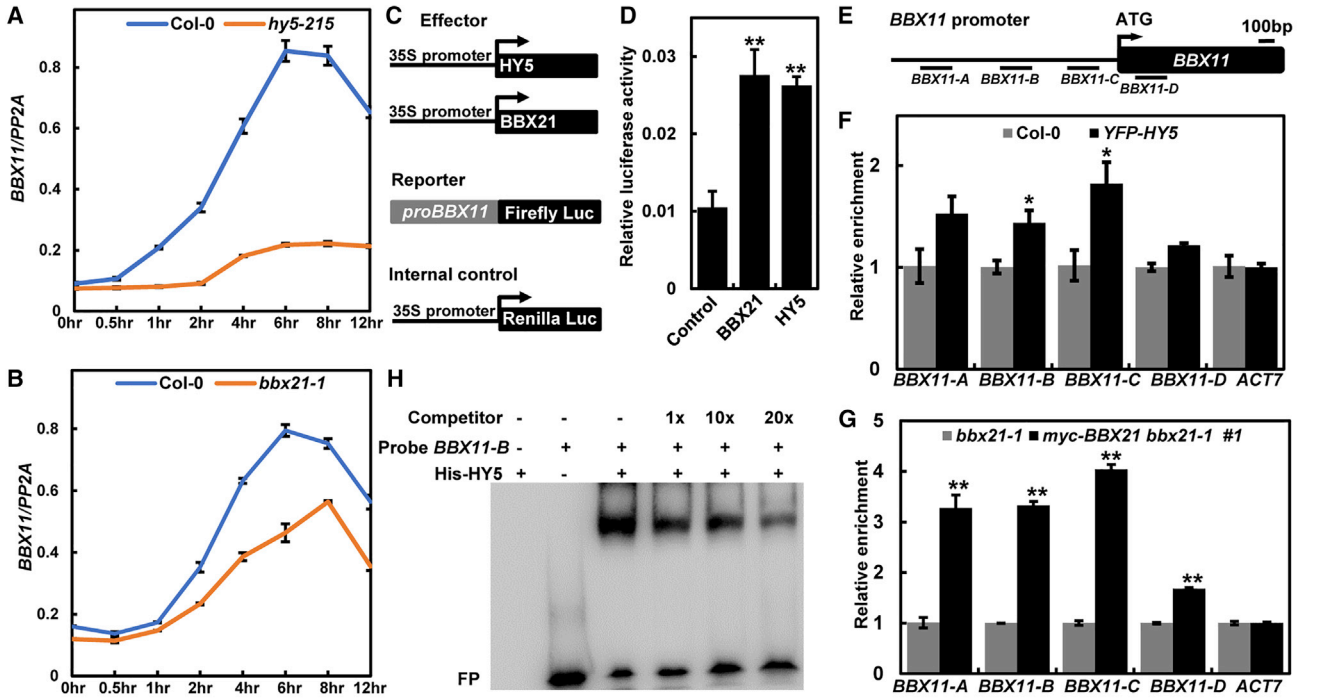

Figure 4. HY5 and BBX21 Associate with the *BBX11* Promoter and Upregulate Its Expression.

(A and B) qRT–PCR analysis of the *BBX11* transcript level during the dark-to-light transition. Four-day-old dark-grown Col-0, *hy5-215* **(A),** and *bbx21-1* **(B)** seedlings were exposed to white light (14.74 μmol/m^2/s) for up to 12 h at the indicated time points. Data are presented as means ± SD (n = 3). The *BBX11* expression level was normalized to that of *PP2A*.

(C) Schematic representation of various constructs used in transient transfection assays in *Arabidopsis* protoplasts. Arrows after the *35S* promoter indicate the transcriptional start site. The *BBX11* promoter was fused to firefly luciferase to create the reporter constructs.

(D) Relative luciferase activity of the *proBBX11:LUC* reporter construct cotransformed with *BBX11*, *HY5*, or the control into protoplasts. Data are presented as means ± SD (n = 3). $^{**}P < 0.01$, as determined by the two-tailed Student's t-test.

(E) Schematic representation of the regions examined in ChIP–qPCR assays.

(F and G) Results of a representative ChIP–qPCR assay showing that HY5 and BBX21 associate with the *BBX11* promoter *in vivo*. ChIP was performed with anti-GFP **(F)** and anti-Myc **(G)** monoclonal antibodies. Data are presented as means ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$, as determined by the two-tailed Student's t-test.

(H) Results of a representative EMSA showing that HY5 binds to the *BBX11-B* promoter region. "−" and "+" represent the absence or presence, respectively, of the corresponding probe or protein. For probe *BBX11-B*, "+" indicates 4 pmol. For His-HY5, "+" indicates 10 pmol. FP indicates the free probe.

(Figure 3D). In addition, the results of an *in vitro* electrophoretic mobility shift assay (EMSA) showed that His-Trigger Factor-BBX11 (His-TF-BBX11) could directly bind to the *HY5* promoter region B (−300 to −237 bp). As the amount of competitor (cold probe) increased in the reactions, the His-TF-BBX11 binding of *HY5* promoter clearly decreased. By contrast, the negative control His-TF could not bind to the same *HY5* promoter fragment (Figure 3E). Taken collectively, these results suggest that BBX11 directly binds to the *HY5* promoter to activate its transcription.

BBX21 and HY5 Activate the Transcription of *BBX11*

The results of a genome-wide ChIP-chip study indicate that HY5 can associate with the *BBX11* promoter (Lee et al., 2007). Moreover, BBX21 cannot only enhance the activity of HY5 but also activate its expression (Datta et al., 2007; Xu et al., 2016, 2018). Thus, we examined whether HY5 and BBX21 could regulate the transcription of *BBX11*. The expression of *BBX11* was markedly decreased in *hy5-215* and *bbx21-1* compared with Col-0 during the transition from dark to light at various time points as indicated in Figure 4A and 4B. In addition, the *BBX11* transcript level was decreased in both *bbx21-1* and *hy5-215* mutants but increased in *myc-BBX21 bbx21-1* and *YFP-HY5 hy5-215* overexpressing plants grown in continuous W light (Supplemental Figure 7). In the transient activation assay, both HY5 and BBX21 activated the *proBBX11:LUC* reporter (Figure 4C and 4D). ChIP–qPCR analysis also showed that both HY5 and BBX21 associated with the *BBX11* promoter *in vivo* (Figure 4E–4G). Next, we performed EMSAs to determine whether HY5 and BBX21 could directly bind to the promoter of *BBX11 in vitro*. His-HY5 directly bound to the subfragments of the *BBX11* promoter (−628 to −569 bp), which contains a typical *G-box cis*-element (CATGCG). As the amount of competitor increased, the affinity of His-HY5 binding to the *BBX11* promoter subfragments decreased (Figure 4H). However, BBX21 could not bind to the same DNA subfragments under the same experimental conditions (Supplemental Figure 8), indicating that BBX21 may indirectly associate with the *BBX11* promoter or that the binding site(s) for BBX21 may reside in other regions within the *BBX11* promoter. Taken collectively, these data suggest that both

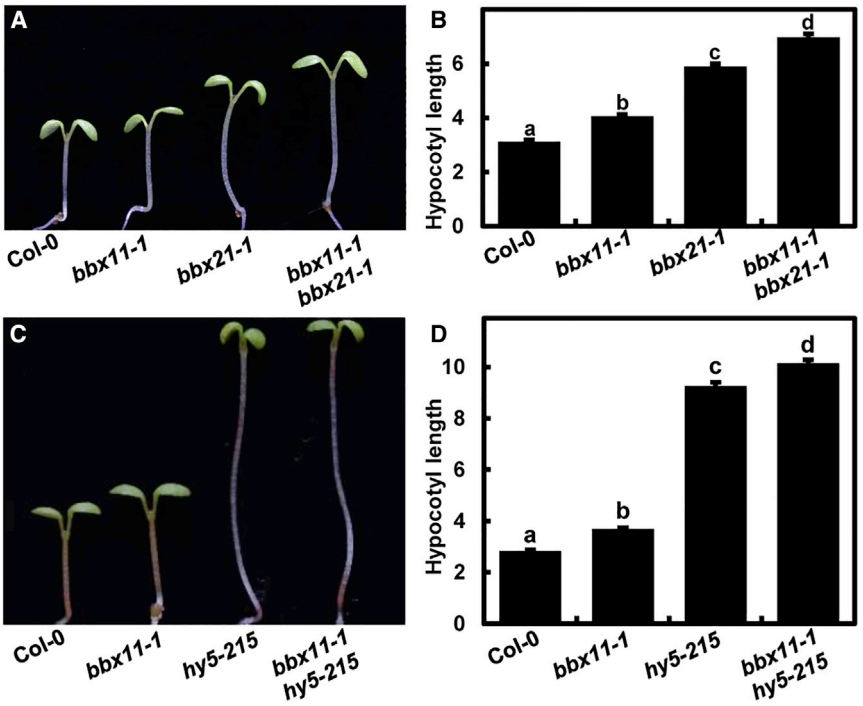

Figure 5. Hypocotyl Phenotype and Length of *bbx11-1*, *bbx21-1*, and *bbx11-1 hy5-215* Seedlings Grown in Red Light.

(A and B) Hypocotyl phenotype and length of 5-day-old Col-0, *bbx11-1*, *bbx21-1*, and *bbx11-1 bbx21-1* seedlings grown in red light (129 μmol/m^2/s).

(C and D) Hypocotyl phenotype and length of 5-day-old Col-0, *bbx11-1*, *hy5-215*, and *bbx11-1 hy5-215* seedlings grown in red light (129 μmol/m^2/s). The unit of hypocotyl length is millimeters. Data are presented as means ± SE ($n \geq 60$) 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.

BBX21 and HY5 bind to the promoter of *BBX11* to activate its expression.

Genetic Interaction between *BBX11*, *BBX21*, and *HY5*

To genetically examine functional interactions between *BBX11*, *BBX21*, and *HY5*, we generated *bbx11-1 bbx21-1* and *bbx11-1 hy5-215* double mutants by genetic crossing. Consistently, both *bbx11-1* and *bbx21-1* displayed elongated hypocotyls in R light, and the hypocotyl length of *bbx11-1 bbx21-1* was significantly longer than those of Col-0, *bbx11-1*, and *bbx21-1* when grown in R light (Figure 5A and 5B), suggesting that BBX11 and BBX21 may function additively in the regulation of R light-mediated hypocotyl growth. Furthermore, the hypocotyl length of *bbx11-1 hy5-215* was significantly longer than that of *hy5-215* when grown in R light (Figure 5C and 5D). Consistently, *myc-BBX11* #2 transgenic seedlings displayed shortened hypocotyls in W, B, R, and FR light, and *hy5-215 myc-BBX11* #2 seedlings were shorter than *hy5-215*, but longer than Col-0 and *myc-BBX11* #2 seedlings when grown in W and B light (Supplemental Figure 9). The hypocotyl length of *hy5-215 myc-BBX11* #2 was indistinguishable from that of *hy5-215* when grown in R and FR light. Myc-BBX11 accumulated at comparable levels in *myc-BBX11* #2 and *hy5-215 myc-BBX11* #2 transgenic seedlings grown in various light conditions (W, B, R, and FR) (Supplemental Figure 10), suggesting that HY5 may not affect the abundance of BBX11 in the light. These data suggest that BBX11 may act independently of HY5 in W and B light, while it is likely dependent on functional HY5 in R and FR light.

COP1 and DET1 Stabilize BBX11

As the E3 ubiquitin ligase COP1 promotes the degradation of BBX21 and HY5 (Osterlund et al., 2000; Xu et al., 2016), we examined whether COP1 could affect the stability of BBX11 and introduced a *cop1-4* mutation by genetic crossing into *YFP-BBX11* transgenic plants. Dark-grown *YFP-BBX11* (line #8) accumulated more YFP-BBX11 compared with that of *YFP-BBX11 cop1-4* (Figure 6A). Moreover, the YFP-BBX11 protein level in *YFP-BBX11 #8* was higher than that in *YFP-BBX11 cop1-4* transgenic seedlings after the transition from the dark to light for 0.5 h and 1 h, respectively (Figure 6B). Consistently, YFP fluorescence signals were clearly observed in the hypocotyls and roots of dark-grown *YFP-BBX11* seedlings; however, YFP signals were barely detectable in *YFP-BBX11 cop1-4* or *YFP-BBX11 det1-1* hypocotyls and root cells (Figure 6C–6E). The *BBX11* transcript level in *YFP-BBX11 cop1-4* was comparable to that in *YFP-BBX11* (Supplemental Figure 11), implying that COP1 may have little effect on the transcript level of *BBX11*. Taken collectively, these data suggest that both COP1 and DET1 stabilize BBX11 at the protein level in planta.

DISCUSSION

Skotomorphogenesis and photomorphogenesis are two contrasting developmental patterns of a germinated seed under conditions of dark or light. These two developmental processes are tightly controlled by light, which can rapidly change the transcriptome of young seedlings. A group of transcription factors modulates the expression of light-regulated genes in response to light (Jiao et al., 2007; Shi et al., 2018; Xu, 2019; Song et al., 2020). Here, we identify a B-box containing protein, BBX11, which promotes photomorphogenesis. BBX11, together with BBX21 and HY5, forms a positive feedback loop at the transcriptional level to maintain normal seedling development.

Previous studies have shown that many positive regulators of light signaling are induced by light at transcriptional and/or protein levels (Osterlund et al., 2000; Xu et al., 2016; Heng et al., 2019a, b). The expression of *BBX11* peaks at 3–9 h after light exposure and then gradually decreases (Figure 1B). The induction of *HY5* peaks even earlier than that of *BBX11, specifically at* 1 h after light exposure (Osterlund et al., 2000). The levels of BBX11, BBX21, and HY5 are barely detectable in

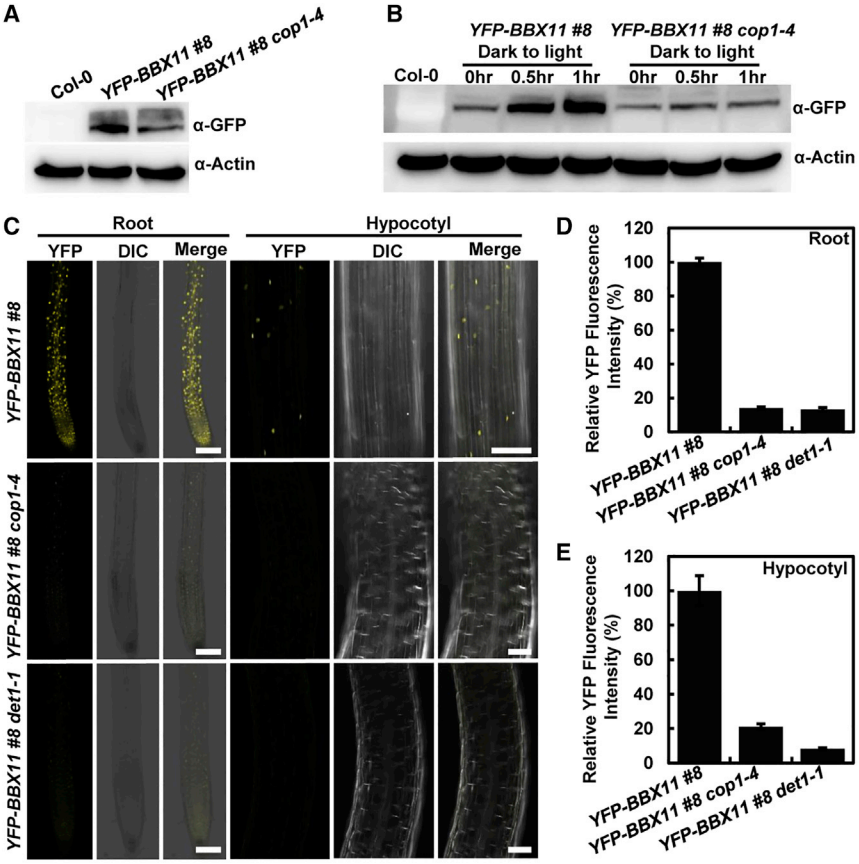

Figure 6. Abundance of BBX11 Is Stabilized by COP1 and DET1.

(A) YFP-BBX11 protein level in *YFP-BBX11 #8* and *YFP-BBX11 #8 cop1-4* seedlings grown in darkness for 4 days. Four-day-old dark-grown Col-0 seedlings served as the negative control.

(B) Immunoblot analysis showing the YFP-BBX11 protein level in *YFP-BBX11 #8* and *YFP-BBX11 #8 cop1-4* seedlings. Four-day-old dark-grown seedlings were transferred to white light for 0, 0.5, and 1 h as indicated. Four-day-old dark-grown Col-0 seedlings transferred to white light for 1 h served as the negative control.

(C) Relative YFP fluorescence in *YFP-BBX11 #8*, *YFP-BBX11 #8 cop1-4*, and *YFP-BBX11 #8 det1-1* seedlings grown in darkness for 4 days. Scale bars, 100 μm.

(D and E) Relative YFP fluorescence in roots **(D)** and hypocotyls **(E)** of *YFP-BBX11 #8*, *YFP-BBX11 #8 cop1-4*, and *YFP-BBX11 #8 det1-1* seedlings grown in darkness for 4 days. Fluorescence intensity was measured using ImageJ software. Data are presented as means ± SD ($n \geq 10$).

etiolated seedlings. However, the levels of BBX11 and BBX21 are highest at 1–4 h and 3 h after light treatment, respectively (Figure 1D; Xu et al., 2016), and that of HY5 peaks at 1 h after light exposure (Osterlund et al., 2000). These findings indicate that light can upregulate BBX11, BBX21, and HY5, which is critical for changes in gene expression and photomorphogenesis at an early stage.

A group of transcription factors converges on HY5 or the *HY5* promoter to modulate its activity and/or transcription. BBX21, CAM7, WRKY36, HYH, and HY5 itself can bind to the *HY5* promoter to activate its expression (Abbas et al., 2014; Binkert et al., 2014; Xu et al., 2016; Yang et al., 2018). In addition, BBX21 and BBX22 form heterodimers to enhance its activity (Datta et al., 2007, 2008), whereas BBX24, BBX25, and BBX28 repress its transcriptional activity through a similar molecular mechanism (Gangappa et al., 2013; Lin et al., 2018). This study revealed that BBX11 could associate with the *HY5* promoter and upregulate its transcription (Figure 3), indicating that BBX11 also acts as an activator of *HY5*. Light induces BBX11 and HY5 at both transcriptional and protein levels (Figure 1; Osterlund et al., 2000), implying that light may enhance the binding of BBX11 to the *HY5* promoter, thereby at least partially activating *HY5* transcription. Interestingly, *BBX11* is under the transcriptional control of BBX21 and HY5. Both BBX21 and HY5 bind to the *BBX11* promoter and positively regulate its expression (Figure 4), suggesting that BBX21 and HY5 are positive regulators of *BBX11*. Furthermore, both BBX21 and HY5 can bind to the *T/G-box* present in the *HY5* promoter to activate its expression (Abbas et al., 2014; Binkert et al., 2014; Xu et al., 2016). Thus, these findings suggest that BBX11, BBX21, and HY5 form a positive feedback loop for the precise control of downstream target genes. The phenotypic analysis demonstrates that BBX11, BBX21, and HY5 are positive regulators of light signaling, as *bbx11*, *bbx21*, and *hy5* mutants display elongated hypocotyls, whereas transgenic seedlings overexpressing *BBX11*, *BBX21*, or *HY5* show shortened hypocotyls in light (Figure 2; Oyama et al., 1997; Datta et al., 2007; Xu et al., 2016). These genetic observations indicate that the BBX11–BBX21–HY5-mediated transcriptional cascade promotes photomorphogenic development. These three key proteins accumulate in light, which is consistent with their respective modes of action for promoting photomorphogenesis in response to light. Taken collectively, these facts support the contention that HY5 represents a regulatory node in light-controlled transcriptional reprogramming, and multiple transcription factors regulate the expression of downstream genes at least in part by controlling the *HY5* transcript level. The BBX11–BBX21–HY5 positive feedback loop likely orchestrates a transcriptional cascade that regulates light-mediated development in plants.

BBX11, BBX21, and HY5 degrade in darkness and accumulate in light (Figure 1D–1F; Osterlund et al., 2000; Xu et al., 2016). Both BBX21 and HY5 are ubiquitinated by COP1 and subsequently degraded by the 26S proteasome system in darkness (Osterlund et al., 2000; Xu et al., 2016). However, COP1 stabilized BBX11 rather than promoting its degradation (Figure 6). This fact suggests that a yet unidentified component(s) might promote the degradation of BBX11 in etiolated seedlings. Recent studies have shown that COP1 promotes the degradation of EBF1 and EBF2, which target EIN3 and PIF3 for ubiquitination and degradation, and inhibits

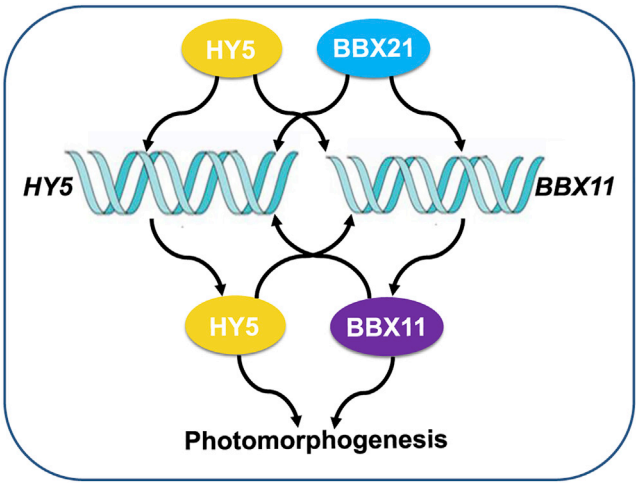

Figure 7. Proposed Working Model of the BBX11–BBX21–HY5 Positive Feedback Loop in Light Signaling.
Light promotes the accumulation of BBX11, BBX21, and HY5, which bind to the promoter of *HY5* and activate its expression. BBX21 and HY5 associate with the promoter of *BBX11* and upregulate its expression. Consequently, the BBX11–BBX21–HY5 feedback loop controls the expression of a variety of genes to promote photomorphogenic development in plants.

BIN2-mediated PIF3 phosphorylation and degradation, thereby resulting in the stabilization of EIN3 and PIF3 (Shi et al., 2016; Dong et al., 2017; Ling et al., 2017). It appears that COP1 likely promotes the degradation of a yet unknown component(s) targeting BBX11 for destabilization. Interestingly, two COP1 and two SPAs can form a stable core E3 ubiquitin ligase complex and work synergistically to control the stability of substrates in etiolated *Arabidopsis* seedlings (Zhu et al., 2008; Ordoñez-Herrera et al., 2015). Therefore, COP1 and SPAs may have similar effects on the accumulation of BBX11.

Based on previous studies and our current study, three transcription factors, BBX11, BBX21, and HY5, associate with the *HY5* promoter to activate its transcription. BBX21 and HY5 bind to the *BBX11* promoter to upregulate its expression (Figure 7). Thus, this positive feedback loop serves to orchestrate light-mediated transcriptional cascades to precisely control photomorphogenic development in plants.

METHODS

Plant Materials and Growth Conditions

Hy5-215 (Oyama et al., 1997), *phyA-211* (Reed et al., 1994), *phyB-9* (Reed et al., 1994), *bbx21-1* (Datta et al., 2007), *cry1-304* (Mockler et al., 1999), *cry2-1* (Mockler et al., 1999), *cop1-4* (McNellis et al., 1994), and *det1-1* (Chory and Peto, 1990) mutants and *myc-BBX21 bbx21-1* (Xu et al., 2016) transgenic lines were derived from the *Arabidopsis thaliana* Col-0 ecotype. *Bbx11* mutants and transgenic lines overexpressing *BBX11* were generated in this study. Multiple mutants were generated by genetic crossing and genotyped with PCR or antibiotic screening methods. Seeds were surface sterilized and sown on 1× Murashige and Skoog (MS) medium containing 1% (w/v) sucrose and 0.8% (w/v) agar. Seeds were stratified in darkness for 3 days at 4°C and then transferred to white light for 8–12 h at 22°C to induce uniform germination. To prepare seeds for phenotypic analysis, they were transferred to W light (14.74 μmol/m^2/s), B light (10.5 μmol/m^2/s), R light (129 μmol/m^2/s), FR light (4.5 μmol/m^2/s), or LD conditions (16 h light/8 h dark, W light 14.74 μmol/m^2/s) at 22°C.

Construction of Plasmids

The full-length *BBX11* coding sequence (CDS) was cloned into the *pDONR223* vector using the Gateway BP Clonase Enzyme Mix (Invitrogen). CDSs were introduced into the *pEarly Gate-104* or *pEarly Gate-203* plant binary vector using the Gateway LR Clonase Enzyme Mix (Invitrogen) to generate *35S:YFP-BBX11* and *35S:myc-BBX11* constructs, respectively (Earley et al., 2006). To generate constructs for transient luciferase transfection assays, *BBX11*, *HY5*, and *BBX21* CDSs were cloned into the *Eco*RI/*Xho*I sites of the *pGreenII 62-SK* vector (Hellens et al., 2005). The 2540-bp *BBX11* promoter upstream of *ATG* was cloned into the *Hin*dIII/*Nco*I sites of the *pGreen II 0800-LUC* vector. The generation of *pGreen II 0800-HY5pro-LUC* (Lin et al., 2018), *pET28a-HY5* (Heng et al., 2019a), and *pCold-TF-BBX21* (Xu et al., 2016) has been previously described. To produce the construct for prokaryotic expression, the *BBX11* CDS was cloned into the *Eco*RI/*Hin*dIII sites of the *pCold-TF* vector (Takara). Primers used for plasmid construction are listed in Supplemental Table 1.

Generation of *bbx11* Mutants Using CRISPR/Cas9

Bbx11 mutants were generated using the CRISPR/Cas9 system described by Wang et al. (2015). In brief, CRISPR-GE (http://skl.scau.edu.cn/) was used to identify 23-bp target sites (5′-$N_{20NGG-3'}$) (Xie et al., 2017). Primers were synthesized, and the products were subcloned into the *pHEE401E* vector. After transforming into the *Agrobacterium tumefaciens* GV3101 strain by the freeze–thaw method, binary constructs were introduced into Col-0 using the floral-dip method. T_1 seeds were sown on MS plates containing 50 mg/l hygromycin, and the resistant seedlings (T_1) were transferred to soil. Genomic DNA was extracted and used to amplify the *BBX11* gene. PCR products were sequenced to identify mutations. Homozygous mutants were crossed with Col-0 to remove the T-DNA insertion. Seedlings carrying mutations in *BBX11* and without hygromycin resistance were selected for further studies.

Transgenic Plants

pEarly Gate-35S:YFP-BBX11 and *pEarly Gate-35S:myc-BBX11* constructs were transformed into the *A. tumefaciens* GV3101 strain by the freeze–thaw method. The floral-dip method was used to generate transgenic plants (Clough and Bent, 1998). Transgenic plants were selected on MS medium containing 20 mg/l BASTA. Homozygous lines were used for genetic and biochemical studies.

Measurement of Hypocotyl Length

Seeds were surface sterilized, sown on MS plates, stratified in darkness for 3 days at 4°C and then transferred to white light for 8 h to induce uniform germination. Thereafter, seeds were exposed to darkness or different light conditions and cultivated at 22°C. Seedling hypocotyls were scanned with a scanner, and the hypocotyl length was measured using ImageJ software (Schneider et al., 2012).

Total RNA Isolation and Quantitative RT–PCR

Total RNA was isolated from 5-day-old *Arabidopsis* seedlings using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized using the 5× All-In-One RT MasterMix (Applied Biological Materials) according to the manufacturer's instructions. cDNA templates and primer pairs were mixed with Hieff qPCR SYBR Green Master Mix (Yeasen), and quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems). Each experiment was performed at least three independent times with similar results, and each sample was assayed three times within each experiment. The expression level of each target gene was normalized to that of a housekeeping gene, *PP2A*. Primers used for qRT–PCR are listed in Supplemental Table 1.

Immunoblot Analysis

Arabidopsis seedlings were homogenized in protein extraction buffer containing 100 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0), 200 mM NaCl, 8 M urea, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor cocktail (Roche). Primary antibodies used in this study were anti-GFP (Abmart, cat. #M20004M), anti-Myc (Sigma-Aldrich, cat. #M4439), and anti-Actin (Sigma-Aldrich, cat. #A0480).

Electrophoretic Mobility Shift Assays

Probe oligos used for EMSA are presented in Supplemental Table 1. Oligos were diluted and mixed with EZ-Link Psoralen-PEG3-Biotin (Thermo Scientific). After 30 min of UV exposure, biotin-labeled probes were precipitated with potassium acetate (pH 5.2) in ethanol, air-dried, and dissolved in water, and the concentration was determined. For prokaryotic expression, the *pCold-TF-BBX11* construct was transformed into the *Escherichia coli* BL21 (DE3) strain, and His-TF-BBX11 protein was purified according to the manufacturer's instructions. The Light Shift Chemiluminescent EMSA Kit (Thermo Scientific) was used. In brief, purified proteins were incubated with biotin-labeled probes in 20-μl reaction mixtures containing 10 mM Tris–HCl (pH 7.5), 0.05% (v/v) Nonidet P-40, 10 mM $MgCl_2$, 5% (v/v) glycerol, and 0.1 μg/ml poly(dI-dC) at room temperature for 20 min. Thereafter, 6% (w/v) native polyacrylamide gels were used to separate the labeled probes, which were then electroblotted onto Hybond N^+ (Millipore) nylon membranes in 0.5× Tris–Borate–EDTA buffer for 40 min. Labeled probes were detected according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assays

ChIP assays were performed as previously described (Xu et al., 2016). In brief, 7-day-old seedlings grown in LD conditions (16 h light/8 h dark) were collected and treated with formaldehyde to crosslink protein–DNA complexes. After washing three times, the excess liquid was removed. Samples were frozen in liquid nitrogen and stored at -80°C or processed further. In brief, samples were ground to a fine powder with a pestle and mortar in liquid nitrogen. After isolation and sonication of chromatin, samples were centrifuged at 16 000 *g* for 5 min, and the supernatants were collected. Anti-GFP and anti-Myc antibodies were used for immunoprecipitation. The serum served as the control. Ten percent of each supernatant served as the input. Primers used for real-time qPCR are listed in Supplemental Table 1.

Transient Luciferase Expression Assays

Arabidopsis plants grown in LD conditions (16 h light/8 h dark) were used for the isolation of protoplasts. Leaves were minced and digested as described by Yoo et al. (2007). Reporter and effector constructs were transformed into protoplasts. After 20 h of incubation in darkness, the protoplasts were pelleted. Firefly luciferase (LUC) and Renillia luciferase (Ren) were assayed using the Dual-Luciferase Reporter Assay System (Promega). The Ren gene driven by the cauliflower mosaic virus 35S promoter was used as the control. The relative activity was expressed as a ratio of LUC/Ren.

Statistical Analysis

Statistical analyses were performed using Microsoft Excel, GraphPad Prism 5.0, or an online program (http://astatsa.com/OneWay_Anova_with_TukeyHSD/).

ACCESSION NUMBERS

Sequence data from this article can be found in GenBank and EMBL libraries under the following accession numbers: BBX11 (TAIR: AT2G47890), BBX21 (TAIR: AT1G75540), HY5 (TAIR: AT5G11260), and COP1 (TAIR: AT2G32950).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

X.Z., Y.H., X.W., and D.X. conducted the experiments. D.X. and X.W.D. designed the experiments, analyzed the data, and wrote the article.

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