BBX21, an *Arabidopsis* B-box protein, directly activates *HY5* and is targeted by COP1 for 26S proteasome-mediated degradation

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BBX21 (also known as SALT TOLERANCE HOMOLOG 2), a B-box (BBX)-containing protein, has been previously identified as a positive regulator of light signaling; however, the precise role of BBX21 in regulating seedling photomorphogenesis remains largely unclear. In this study, we report that CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) interacts with BBX21 in vivo and is able to ubiquitinate BBX21 in vitro. Thus, BBX21 is targeted for 26S proteasome-mediated degradation in dark-grown *Arabidopsis* seedlings in a COP1-dependent manner. Moreover, we show that BBX21 binds to the T/G-box in the ELONGATED HYPOCOTYL 5 (*HY5*) promoter and directly activates *HY5* expression in the light. Transgenic seedlings overexpressing *BBX21* exhibit dramatically shortened hypocotyls in the light, and this phenotype is dependent on a functional HY5. Taken together, our data suggest a molecular base underlying BBX21-mediated seedling photomorphogenesis, indicating that BBX21 is a pivotal component involved in the COP1-HY5 regulatory hub.

Arabidopsis | photomorphogenesis | BBX21 | COP1 | HY5

Plant seedlings undergo two distinct developmental processes dependent on the presence and absence of light, termed photomorphogenesis and skotomorphogenesis, respectively (1). CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) is a central repressor of seedling photomorphogenesis, and mutants impaired in *COP1* display constitutively photomorphogenic phenotypes in darkness (2, 3). COP1 acts as an E3 ubiquitin ligase targeting a subset of substrates for degradation in darkness, including ELONGATED HYPOCOTYL 5 (HY5), HY5 HOMOLOG (HYH), LONG AFTER FAR-RED LIGHT 1 (LAF1), LONG HYPOCOTYL IN FAR RED (HFR1), B-BOX PROTEIN 22/SALT TOLERANCE HOMOLOG 3 (BBX22/STH3), and PHYTOCHROME INTERACTING FACTOR 3-LIKE1 (PIL1) (4–11). Of these, HY5 is considered a key signal integration point from dark to light transition (10, 12, 13). Its abundance is directly correlated with the extent of seedling photomorphogenic growth, but inversely correlated with the nuclear abundance of COP1 (4, 10, 14). As a b-ZIP type transcription factor, HY5 specifically interacts with the ACGT-containing elements (ACEs) and might directly bind to approximately one-third of the *Arabidopsis* genes (12, 13). Thus, HY5 ensures proper expression of a large number of downstream regulatory genes, which in turn eventually promotes photomorphogenesis in the light. Recent studies have shown that *HY5* expression is regulated by HY5 itself, as well as by HYH and CALMODULIN7 (CAM7) (15, 16).

A total of 32 B-box (BBX)-containing proteins have been identified in the *Arabidopsis* genome (17), among which a number of BBX proteins have been shown to be involved in COP1- and HY5-mediated seedling photomorphogenesis (18). BBX4, BBX20, BBX22, BBX24, and BBX25 genetically and physically interact with COP1 and undergo COP1-mediated degradation in the dark (6, 19–24). In addition, BBX22, BBX24, and BBX25 physically interact with HY5, and, interestingly, BBX22 acts as a coactivator of HY5 action (6), whereas BBX24 and BBX25 repress the transcriptional activating activity of HY5 (22, 23). BBX21/SALT TOLERANCE HOMOLOG 2 (STH2) positively regulates anthocyanin accumulation and the inhibition of seedling hypocotyl elongation in response to light, both independently and together with HY5. Mutations in *BBX21* can partially suppress the constitutively photomorphogenic phenotypes of *cop1* in the dark (25), and BBX21 acts downstream of COP1 to mediate the shade avoidance response (26).

In this study, we explored the molecular roles of BBX21 in COP1- and HY5-mediated seedling photomorphogenesis. We found that BBX21 is ubiquitinated by COP1 in vitro, and that BBX21 abundance is controlled in a COP1-dependent manner in darkness in vivo. In addition, BBX21 directly binds to the T/G-box present in the *HY5* promoter. Accordingly, the expression of *HY5* is decreased in *bbx21* but increased in transgenic plants overexpressing *BBX21*. Collectively, our data demonstrate that BBX21 is targeted by COP1 for 26S proteasome-mediated degradation in darkness, and that BBX21 promotes photomorphogenic development in the light by activating *HY5* expression.

Results

Expression Pattern of BBX21 in Response to Light. BBX21 acts as a positive regulator of light signaling (25). To further elucidate the mechanism of action of BBX21, we sought to examine the expression pattern of BBX21 during the transition from dark to light exposure. At the transcript level, *BBX21* expression was

Significance

CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and ELONGATED HYPOCOTYL 5 (HY5) are two central regulators of photomorphogenesis. COP1 destabilizes HY5 to repress photomorphogenesis in darkness. On light illumination, accumulated HY5 regulates the expression of a large number of genes to optimize photomorphogenesis. Thus, COP1-HY5 defines a regulatory hub for light control of seedling development. In this work, our data indicate that BBX21 is a key component involved in the COP1-HY5 regulatory hub. BBX21 is controlled by COP1 via its E3 ubiquitin ligase activity in darkness. In the light, BBX21 promotes photomorphogenesis by binding to the promoter of *HY5* and activating *HY5* expression.

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slightly decreased on 1 h of light exposure, reached its lowest level after 3 h of light exposure, and then gradually increased after 6, 12, 24, and 48 h of light exposure (Fig. S1*A*).

We next analyzed the dynamic changes of BBX21 in *YFP-BBX21 bbx21-1* (*#12*) (Fig. S2) seedlings grown in darkness for 4 d and then exposed to white light for 1–48 h. Interestingly, the BBX21 protein level was significantly increased after 1 h of light exposure and peaked after 3 h of light exposure, then gradually attenuated on prolonged light exposure (6, 12, and 24 h). After 48 h of light exposure, the BBX21 level declined to a level comparable to that seen in dark-grown seedlings (Fig. S1*B*). These findings indicate that light tightly controls BBX21 in a time-dependent manner at both the transcript and protein levels.

BBX21 Interacts with COP1 and Is Ubiquitinated by COP1. It was previously shown that BBX21 is recruited to the nuclear speckles by COP1 (25). Therefore, we asked whether BBX21 physically interacts with COP1 in *Arabidopsis*. To this end, we performed coimmunoprecipitation (co-IP) assays using homozygous *myc-BBX21 bbx21-1* (*#17*) transgenic plants (Fig. S2). As shown in Fig. 1*A*,

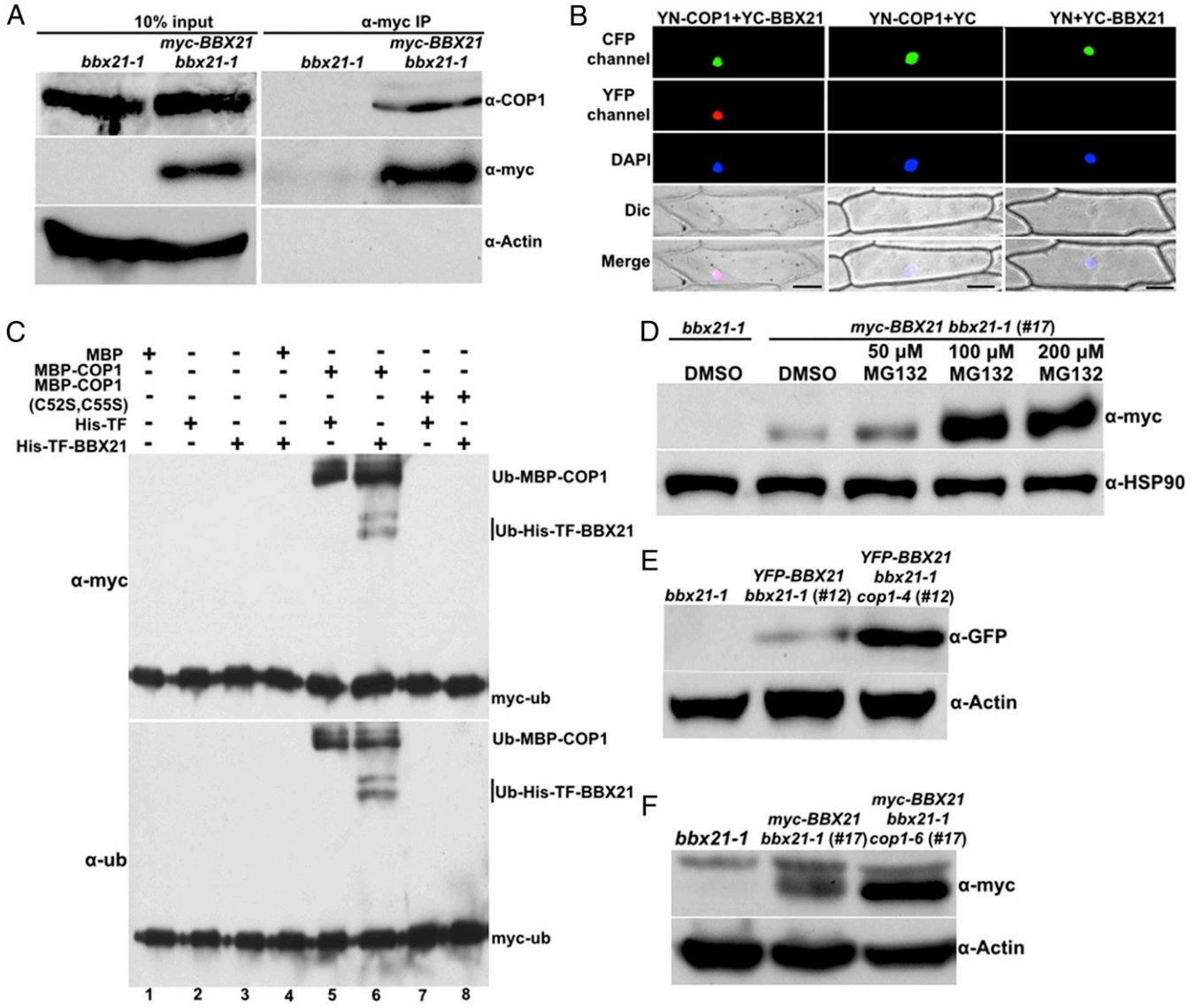

Fig. 1. BBX21 is controlled by COP1 in dark-grown seedlings. (*A*) Co-IP analysis showing that BBX21 interacts with COP1 in vivo. Four-day-old white light-grown *bbx21-1* and *myc-BBX21 bbx21-1* (*#17*) seedlings were transferred to darkness for 16 h and then subjected to a co-IP assay using anti-myc antibodies, with the immunoprecipitates detected using anti-COP1 and anti-myc antibodies, respectively. Actin served as a negative control. (*B*) BiFC assay showing the interaction of BBX21 with COP1 in onion epidemal cells. Full-length COP1 and BBX21 were fused to the split N- or C-terminal fragments of YFP (YN-COP1 or YC-BBX21). Nuclear localized CFP-CSU1 served as a marker for successful transfection. Unfused YFP N-terminal (YN) or C-terminal (YC) fragments served as negative controls, as indicated. DAPI staining marked the positions of nuclei. Dic, differential interference contrast in light microscope mode; Merge, merged images of YFP channel, DAPI, and Dic. (Scale bar: 100 μm.) (*C*) COP1 ubiquitinates BBX21 in vitro. Ubiquitination assays were performed in a reaction mix containing UBE1 (E1), rice 6×His-Rad6 (E2), and myc-tagged ubiquitin (myc-Ub). Ubiquitinated MBP-COP1 and 6×His-TF-BBX21 were detected by anti-ubiquitin and anti-myc monoclonal antibodies, respectively. The "+" and "−" indicate presence and absence, respectively. (*D*) Immunoblot analysis of myc-BBX21 protein levels in dark-grown *myc-BBX21 bbx21-1* (*#17*) transgenic seedlings treated with DMSO or various concentrations of MG132 (50, 100, or 200 μM) for 3 h. (*E*) Immunoblot analysis of YFP-BBX21 protein levels in *YFP-BBX21 bbx21-1* (*#12*) and *YFP-BBX21 bbx21-1 cop1-4* (*#12*) transgenic seedlings grown in the dark for 4 d. (*F*) Immunoblot analysis of myc-BBX21 protein levels in *myc-BBX21 bbx21-1* (*#17*) and *myc-BBX21 bbx21-1 cop1-6* (*#17*) transgenic seedlings grown in the dark for 4 d. In *D–F*, *bbx21-1* served as a negative control, and anti-HSP90 or anti-actin served as a loading control.

immunoprecipitation of myc-BBX21 pulled down COP1 in *myc-BBX21 bbx21-1* transgenic seedlings, but not in *bbx21-1* seedlings, demonstrating that BBX21 associates with COP1 in vivo.

We next performed bimolecular fluorescence complementation assays (BiFCs) to further confirm the BBX21–COP1 interaction. When YN-COP1 (COP1 fused with the N-terminal of YFP) was coexpressed with YC-BBX21 (BBX21 fused with the C-terminal of YFP) in onion (*Allium cepa*) epidermal cells, strong YFP fluorescence signals were observed in the nucleus; however, no YFP fluorescence signals were detected when YN-COP1 was coexpressed with YC, or when YC-BBX21 was coexpressed with YN, supporting the conclusion that BBX21 physically interacts with COP1 (Fig. 1*B*).

To test whether COP1 is able to ubiquitinate BBX21, we performed the in vitro ubiquitination assays. MBP-COP1 itself exhibited self-ubiquitination activity (Fig. 1*C*, lane 5), consistent with a previous report (11), and did not ubiquitinate 6×His-tagged Trigger Factor (TF), a soluble tag (Fig. 1*C*, lane 5). However, when MBP-COP1 was added together with 6×His-TF-BBX21 in the reaction, both ubiquitinated MBP-COP1 and 6×His-TF-BBX21 were detected (Fig. 1*C*, lane 6). Moreover, mutant forms of COP1 (C52S and C55S) with impaired E3 ubiquitin ligase activity (11) could not ubiquitinate COP1 itself and 6×His-TF-BBX21 (Fig. 1*C*, lanes 7 and 8). Taken together, these results suggest that COP1 is able to ubiquitinate BBX21 in vitro.

BBX21 Undergoes Degradation in Darkness in a COP1-Dependent Manner. To test whether the stability of BBX21 is controlled by COP1, we first examined whether BBX21 is degraded via the 26S proteasome system. For this, *myc-BBX21 bbx21-1* (*#17*) transgenic seedlings were first grown in darkness for 4 d and then treated with DMSO or various concentrations of MG132, a proteasome inhibitor, for 3 h. As shown in Fig. 1*D*, BBX21 protein levels were increased when the transgenic seedlings were treated with 50 μM MG132, and this increase became more evident when the concentration of MG132 was increased to 100 or 200 μM. Moreover, the YFP fluorescence signal increased by ∼20-fold when dark-grown *YFP-BBX21 bbx21-1* (*#12*) seedlings were treated with 100 μM MG132 (Fig. S3 *A* and *B*). Consistently, markedly more BBX21 accumulated in *YFP-BBX21 bbx21-1* (*#12*) transgenic seedlings after treatment with 100 μM MG132 (Fig. S3*C*). Taken together, these data demonstrate that MG132 treatment significantly blocked the degradation of BBX21, suggesting that BBX21 is subjected to 26S proteasome-mediated degradation in darkness.

We next introduced the *cop1* mutation into *YFP-BBX21 bbx21-1* (*#12*) and *myc-BBX21 bbx21-1* (*#17*) by genetic crossing. Significantly higher YFP-BBX21 or myc-BBX21 protein levels were found in *bbx21-1 cop1* compared with *bbx21-1* background in darkness (Fig. 1 *E* and *F*). These findings are also consistent with the observations of YFP fluorescence in *YFP-BBX21 bbx21* transgenic seedlings with or without functional COP1 (Fig. S3 *D* and *E*). Taken together, these data demonstrate that COP1 promotes the degradation of BBX21 in darkness.

Overexpression of *BBX21* Leads to Hyperphotomorphogenic Growth in the Light. To further examine the physiological role of BBX21, we examined the light responsiveness of BBX21 overexpressors (i.e., *YFP-BBX21 bbx21-1* and *myc-BBX21 bbx21-1* transgenic plants in different light conditions (Fig. S2). When grown in the dark, all of the transgenic lines were indistinguishable from the wild type (WT) or *bbx21-1* mutant seedlings (Fig. S4); however, both *YFP-BBX21 bbx21-1* and *myc-BBX21 bbx21-1* transgenic lines exhibited significantly shorter hypocotyls compared with WT when grown in various fluence rates of different light [white (W), blue (B), red (R), and far-red (FR)] conditions (Fig. 2). These data suggest that both YFP-BBX21 and myc-BBX21 transgenes are biologically functional in vivo, and that overexpression of BBX21 could confer hypersensitivity to different light, which is consistent with the positive role of BBX21 in light signaling (25).

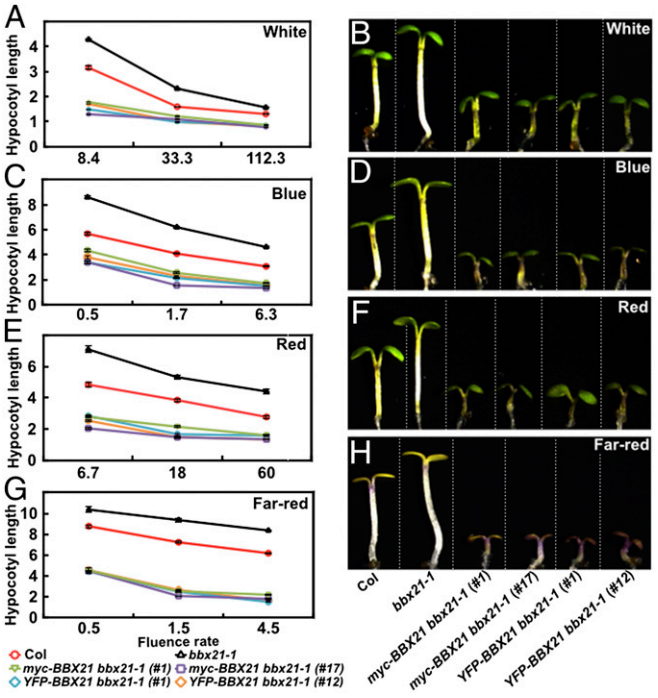

Fig. 2. *BBX21* transgenic seedlings are hypersensitive to light. (*A*, *C*, *E*, and *G*) Fluence rate response curves of 4-d-old Col, *bbx21-1*, and *BBX21* transgenic seedlings grown under white light (*A*), blue light (*C*), red light (*E*), and far-red light (*G*) conditions. Hypocotyl lengths are in millimeters. Data are mean ± SE; $n \geq 20$. (*B*, *D*, *F*, and *H*) Hypocotyl phenotypes of 4-d-old Col, *bbx21-1*, and *BBX21* transgenic seedlings grown under white light (33.3 μmol/m^2/s) (*B*), blue light (6.37 μmol/m^2/s) (*D*), red light (59.5 μmol/m^2/s) (*F*), and far-red light (1.46 μmol/m^2/s) (*H*) conditions. In *B*, *D*, *F*, and *H*, Col, *bbx21-1*, and various transgenic seedlings are separated by dotted lines. The experiments were performed three times, with similar results. The graphs depict one of these experiments.

We also asked whether overexpression of *BBX21* affects anthocyanin accumulation of seedlings. To this end, we measured the anthocyanin levels in WT, *bbx21-1*, *YFP-BBX21 bbx21-1*, and *myc-BBX21 bbx21-1* transgenic seedlings grown in different light conditions (W, B, R, and FR) for 4 d. We observed that whereas *bbx21-1* mutant seedlings accumulated less anthocyanin, consistent with a previous study (25), all of the transgenic seedlings overexpressing *BBX21* accumulated dramatically more anthocyanin than WT when grown in the different light conditions (Fig. S5). These findings suggest that BBX21 positively regulates anthocyanin accumulation in the light.

BBX21 Directly Up-Regulates *HY5* Expression. We next examined the expression of several key regulators of photomorphogenesis in the *bbx21-1* mutant and *BBX21* overexpression lines. Our data show that the transcript levels of *COP1*, *HYH*, *PIF1*, and *PIF3* were not altered regardless of either a loss or gain of BBX21 function in *Arabidopsis* seedlings (Fig. S6); however, *HY5* expression was dramatically decreased (by ∼5-fold) in *bbx21-1*, but increased (by ∼19- to 21-fold) in transgenic seedlings overexpressing *BBX21* (Fig. 3*A*). In addition, the expression of six direct target genes of HY5 (i.e., *CHS*, *CHI*, *F3′H*, *F3H*, *LDOX*, and *DOF*) involved in the anthocyanin biosynthesis pathway (27) was accordingly reduced in *bbx21-1* but drastically elevated in *BBX21* overexpression seedlings (Fig. S7). These data suggest that BBX21 controls *HY5* and *HY5*-regulated genes in *Arabidopsis* seedlings.

To test whether BBX21 can directly bind to the *HY5* promoter and regulate the expression of *HY5*, we first performed chromatin immunoprecipitation (ChIP) experiments using anti-myc monoclonal antibodies and *myc-BBX21 bbx21-1* (*#17*) transgenic plants. We

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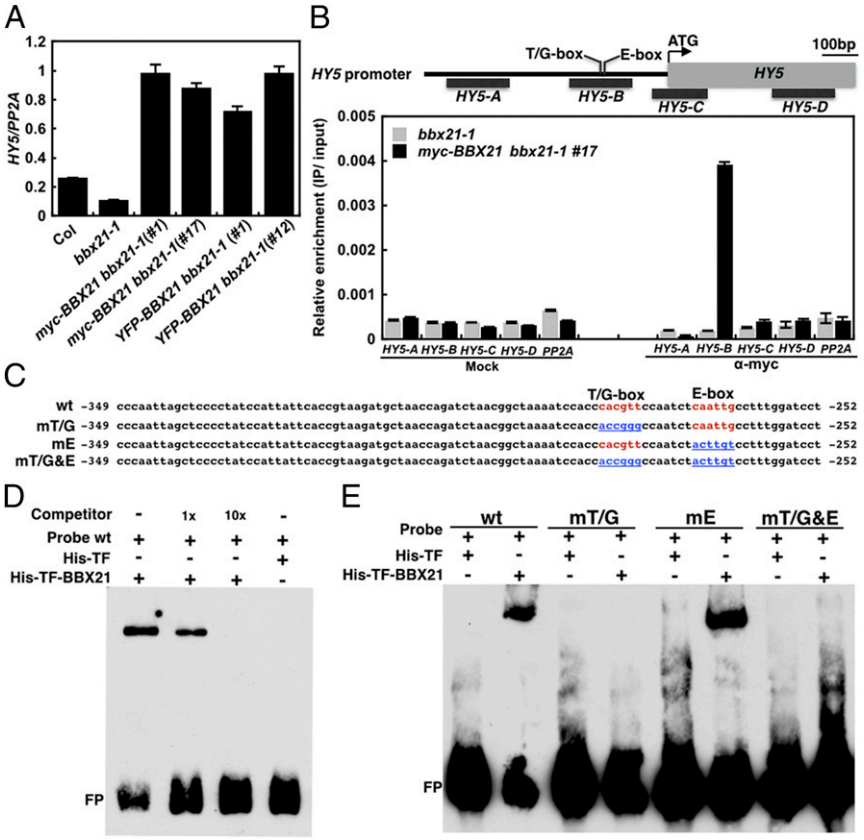

Fig. 3. BBX21 directly up-regulates the expression of *HY5*. (*A*) Real-time qPCR analyses of *HY5* transcript levels in Col, *bbx21-1*, and *BBX21* overexpression seedlings grown under white light for 4 d. (*B*) ChIP assays showing that BBX21 associates with the *HY5* promoter in vivo. ChIP was performed with anti-myc monoclonal antibodies, and the ChIP DNA was analyzed by real-time qPCR. Error bars represent SD of three technical replicates. (*C*) Diagram of the WT and various mutated versions of the *HY5* promoter subfragments used in the EMSA assays. The WT T/G-box and E-box elements are shown in red, and nucleotide substitutions in the mutant subfragments are shown in blue and underscored. (*D* and *E*) EMSA assays using 6×His-TF-BBX21 and the WT (*D*) or various mutated versions (*E*) of the *HY5* promoter subfragments as the probes. 6×His-TF protein served as the negative control. The "+" and "−" indicate presence and absence, respectively. FP, free probe.

found that BBX21 specifically associated with the *B* promoter fragment of *HY5* (−414 to −215 bp) (Fig. 3*B*), but not with any examined promoter fragments of *HYH* (Fig. S8). These data indicate that BBX21 directly associates with the *HY5* promoter in vivo.

Interestingly, the *B* promoter fragment of *HY5* contains a T/G-box and an E-box, which are known to be bound by HY5/HYH and CAM7, respectively (15, 16). To investigate whether BBX21 could bind to the *HY5* promoter directly, we performed electrophoretic mobility shift assays (EMSAs) using recombinant 6×His-TF-BBX21 proteins and a 98-bp *HY5* promoter subfragment (−349 to −252 bp) containing the T/G-box and E-box (Fig. 3*C*). As shown in Fig. 3*D*, 6×His-TF alone showed no binding activity, whereas 6×His-TF-BBX21 was able to bind to the *HY5* promoter subfragment with high affinity. Moreover, increasing amounts of unlabeled subfragments obviously decreased BBX21 binding to the biotin-labeled probes (Fig. 3*D*), indicating that BBX21 directly binds to the *HY5* promoter subfragment in vitro.

We next performed EMSAs to delineate the exact binding *cis*-element of BBX21 in the *HY5* promoter. We used the 98-bp WT and mutant probes in which T/G- and E-boxes were mutated individually or together (Fig. 3*C*). We found that BBX21 could bind to the WT and mE subfragments that contain the WT T/G-box; however, mutations of the T/G-box (in either mT/G or mT/G&E) efficiently abolished BBX21 binding to the promoter subfragment (Fig. 3*E*), indicating that the T/G-box is the binding site for BBX21 in the *HY5* promoter. Taken together, our data demonstrate that BBX21 up-regulates *HY5* expression by directly binding to the T/G-box present in the *HY5* promoter.

Mutation of *HY5* Restores the Hyperphotomorphogenic Phenotypes of *BBX21* Overexpressors in the Light. To obtain genetic evidence supporting the role of HY5 as a pivotal target of BBX21, we introduced the *hy5-215* mutation into the *YFP-BBX21 bbx21-1* transgenic plants by genetic crossing. We observed that the *YFP-BBX21 bbx21-1 hy5-215* seedlings were indistinguishable from the WT and *YFP-BBX21 bbx21-1* seedlings when grown in the dark (Fig. 4 *A* and *B*); however, when grown in the light (W, R, B, and FR), the *YFP-BBX21 bbx21-1 hy5-215* seedlings exhibited significantly longer hypocotyls than the WT and *YFP-BBX21 bbx21-1* seedlings (Fig. 4 *C*–*J*). Notably, the hypocotyl lengths of *YFP-BBX21 bbx21-1 hy5-215* seedlings were similar to those of *bbx21-1* seedlings in FR light; however, in W, R, and B light conditions, they were longer than those of *bbx21-1* but shorter than those of *hy5-215* and *bbx21-1 hy5-215* (Fig. 4 *C*–*J*). These data indicate that disruption of HY5 in *BBX21* overexpressors efficiently restored their hyperphotomorphogenic phenotypes, supporting the notion that BBX21 functions in photomorphogenesis by directly activating *HY5* expression.

Discussion

As an E3 ubiquitin ligase, COP1 is able to target multiple substrates for ubiquitination and promote their protein turnover via the 26S proteasome system. A series of COP1 interacting proteins have been identified and characterized as COP1 direct targets, including several BBX proteins (28, 29). CO/BBX1, BBX20, BBX22, BBX24, and BBX25 interact with COP1 and are regulated in a COP1-dependent manner (5, 6, 20–22, 30, 31).

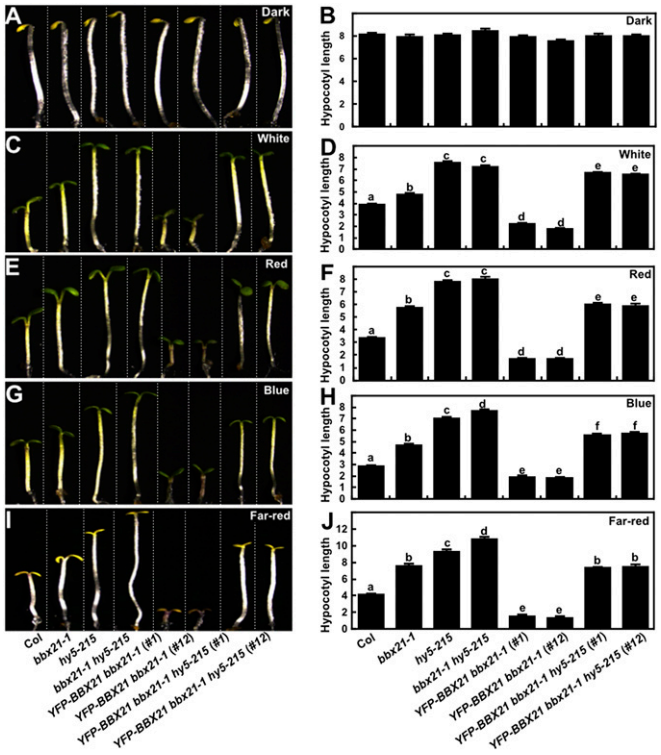

Fig. 4. Mutation of *HY5* restores the hyperphotomorphogenic phenotypes of *BBX21* overexpressors in the light. Hypocotyl phenotypes and lengths of 4-d-old Col, various mutants, and transgenic seedlings grown in darkness (*A* and *B*), white light (33.3 μmol/m^2/s) (*C* and *D*), red light (59.5 μmol/m^2/s) (*E* and *F*), blue light (6.37 μmol/m^2/s) (*G* and *H*), and far-red light (1.46 μmol/m^2/s) (*I* and *J*) conditions. Hypocotyl lengths are in millimeters. Data are means ± 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. In *A*, *C*, *E*, *G*, and *I*, Col, various mutant and transgenic seedlings are separated by dotted lines. The experiments were performed three times, with similar results. The graphs depict one of these experiments.

Previouslypublished results show that BBX21 colocalizes with COP1 in the nuclear speckles, and that *bbx21* partially suppresses the constitutively photomorphogenic phenotypes of *cop1* in darkness, implying that BBX21 is a potential downstream target of COP1 (25, 26). In this study, we provide evidence for this hypothesis based on several lines of evidence: BBX21-COP1 interaction in vivo, ubiquitination of BBX21 by COP1 in vitro, stabilization of BBX21 by MG132 treatment, and COP1-mediated degradation of BBX21 in vivo. Our data suggest that BBX21 acts downstream of COP1 and is targeted by COP1 via its E3 ubiquitin ligase activity in darkness (Fig. 5).

HY5 is a key regulator not only in seedling photomorphogenesis, but also in root development, various hormone signaling pathways, and other processes (32–34). In the dark, HY5 is ubiquitinated by COP1 and subsequently degraded via the 26S proteasome system (28, 29). In the light, HY5 is required for proper expression of approximately one-third of *Arabidopsis* genes to promote various developmental processes (12, 13). At the posttranslational level, light triggers the inactivation of COP1, which ultimately allows the accumulation of HY5; however, at the transcriptional level, a complicated regulatory network converges on the *HY5* promoter to precisely modulate its expression in response to light. It has been shown that HY5, HYH, and CAM7 can directly bind to the *HY5* promoter to activate its expression. Specifically, HY5 and HYH associate with the T/G-box, and CAM7 binds to the E-box in the *HY5* promoter (15, 16). In this study, we show that BBX21 directly binds to the T/G-box in the *HY5* promoter as well (Fig. 3*E*). These findings suggest that multiple transcription factors converge on the *HY5* promoter to ensure fine-tuned control of *HY5* expression (Fig. 5); however, how BBX21, HY5, HYH, and CAM7 work in concert to precisely control *HY5* expression in response to dynamic light changes awaits further investigation.

Given that BBX21, HY5, HYH, and CAM7 individually play positive roles in light signaling (7, 25, 34, 35), it is possible that these factors may modulate a common set of downstream target genes, and that they have partially overlapping functions in light-mediated development. In addition, BBX21 interacts with both HY5 and HYH in yeast and living plant cells (25), and HY5 associates and forms heterodimers with HYH to bind to the G-box *cis*-element (7). Thus, BBX21, HY5, and HYH may form heterodimers to bind to the T/G-box in the *HY5* promoter to mediate its expression. It has been proposed that a potential as-yet unknown transcription factor binding to ACG-box in the *HY5* promoter may be responsible for repression of *HY5* in response to light (16). Therefore, it appears that BBX21, HY5, HYH, and CAM7, together with additional unidentified factors, are coordinately involved in the transcriptional regulation of *HY5* in the light.

Collectively, our findings support a model in which BBX21 acts as a direct downstream target of COP1 and a direct activator of *HY5* (Fig. 5). Our elucidation of the molecular roles of BBX21 in the COP1-HY5 signaling hub provides previously unidentified insight into the complicated but delicate regulatory network that controls plants' responses to their dynamic light environment.

Materials and Methods

Plant Materials and Growth Conditions. The *bbx21-1* (25), *hy5-215* (34), *cop1-4*, and *cop1-6* (36) mutants are in the Columbia-0 (Col-0) ecotype. Seeds were surface-sterilized with 30% (vol/vol) commercial Clorox bleach and 0.02% Triton X-100 for 10 min, washed three times with sterile water, and then sown on 1× Murashige and Skoog (MS) medium supplemented with 0.4% Difco Bacto agar (BD Diagnostic Systems) and 1% (wt/wt) sucrose. The seeds were stratified in darkness for 3 d at 4 °C and then transferred to light chambers maintained at 22 °C. For generating transgenic plants overexpressing *BBX21*, the full-length *BBX21* ORF was first cloned into the *pDONR-221* vector (Invitrogen), and then introduced into the plant binary vector *pEarley Gateway 104* or *pEarley Gateway 203* (37) using Gateway LR Clonase Enzyme Mix (Invitrogen). These constructs were then introduced into *Arabidopsis bbx21-1* mutant plants via the floral dip method (38).

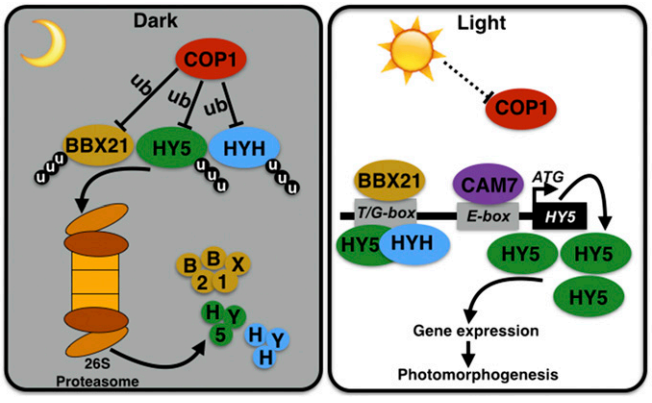

Fig. 5. A working model depicting how BBX21 is involved in the COP1-HY5 signaling hub. In darkness, activated COP1 directly targets downstream substrates including BBX21, HY5, and HYH for ubiquitination, which subsequently triggers their proteolysis via the 26S proteasome system. On light illumination, the activity of COP1 is inhibited, resulting in the accumulation of BBX21, HY5, and HYH. Accumulated BBX21, HY5, and HYH directly bind to the T/G-box, and CAM7 associates with the E-box in the *HY5* promoter. Together, these factors induce *HY5* expression, leading to a high abundance of HY5, which regulates a large number of genes involved in promoting photomorphogenesis. ub, ubiquitination; u, ubiquitin.

In Vitro Ubiquitination Assays. The *pMAL-c2X-MBP-COP1* and *pMAL-c2X-MBP-COP1* (*C52S* and *C55S*) vectors (11) were described previously. To generate *pCold-6×His-TF-BBX21*, the full-length *BBX21* coding sequence was amplified by PCR and then cloned into the NdeI/XbaI sites of the *pCold-6×His-TF* vector (Takara). In vitro ubiquitination assays were performed as described previously (39) with minor modifications. Ubiquitination reaction mixtures (60 μL) contained 30 ng of UBE1 (E1; Boston Biochem) and rice His-Rad6 E2 (6), and 500 ng of myc-tagged ubiquitin (myc-Ub; Boston Biochem) in a reaction buffer containing 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, and 0.5 mM DTT. In addition, 500 ng of MBP or MBP-COP1 (previously incubated with 20 μM zinc cloride), and 500 ng of 6×His-TF-BBX21 or 6×His-TF were applied in the reactions as indicated. After a 2-h incubation at 30 °C, the reactions were stopped by adding 10 μL of 5× sample loading buffer. One-half of each mixture (~35 μL) was then separated onto 8% SDS/PAGE gels. Ubiquitinated MBP-COP1 and TF-BBX21 were detected using monoclonal anti-ubiquitin (Santa Cruz Biotechnology) and anti-myc (Sigma-Aldrich) antibodies, respectively.

ChIP. The ChIP assays were performed as described previously (12). Chromatin isolation was performed using *bbx21-1* or *myc-BBX21 bbx21-1* transgenic seedlings grown under constant white light for 4 d. The resuspended chromatin was sonicated at 4 °C to ~250- to 500-bp fragments. The sheared chromatin was immunoprecipitated, washed, reverse cross-linked, and finally amplified. Approximately 10% of sonicated but nonimmunoprecipitated chromatin was reverse cross-linked and used as an input DNA control. Both immunoprecipitated DNA and input DNA were analyzed by real-time quantitative PCR (qPCR; Applied Biosystems). Monoclonal anti-myc antibody (Sigma-Aldrich) was used. The primers used for this assay are listed in Table S1.

EMSA. EMSAs were performed using biotin-labeled probes and the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific) as described previously (40). The 98-bp promoter subfragment of *HY5* upstream of ATG (-349 to -252 bp) was cloned into the *pLacZi2μ* vector. Various mutated versions of *HY5* promoter subfragments were produced by primer-based site-directed mutagenesis using the WT promoter subfragment (in the *pLacZi2μ* vector) as the template. The WT and various mutated versions of *HY5* promoter subfragments were PCR-amplified, mixed with biotin, and kept in UV light for 30 min for biotin labeling. Then 500 ng of purified 6×His-TF-BBX21 or 6×His-TF protein was incubated together with 40 fmol of biotin-labeled probes in a 20-μL reaction mixture containing 10 mM Tris·HCl pH 7.5, 0.05% Nonidet P-40, 10 mM $MgCl_2$, 5% (vol/vol) glycerol, and 0.1 μg/mL poly (dI•dC).

Reactions were incubated at 25 °C for 20 min and separated on 6% (vol/vol) native polyacrylamide gels in 0.5×TBE buffer. The gels were electroblotted to Hybond N+ nylon membranes (Millipore) in 0.5×TBE for 40 min, after which the labeled probes were detected according to the manufacturer's protocol provided with the EMSA kit.

Detailed descriptions of co-IP analysis, immunoblot analysis, BiFC assays, anthocyanin measurements, hypocotyl length measurements, and real-time qPCR assays are provided in *SI Materials and Methods*.

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