COP1-Mediated Degradation of BBX22/LZF1 Optimizes Seedling Development in Arabidopsis[1][W][OA]

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Light regulates multiple aspects of growth and development in plants. Transcriptomic changes govern the expression of signaling molecules with the perception of light. Also, the 26S proteasome regulates the accumulation of positive and negative regulators for optimal growth of Arabidopsis (*Arabidopsis thaliana*) in the dark, light, or light/dark cycles. *BBX22*, whose induction is both light regulated and HY5 dependent, is a positive regulator of deetiolation in Arabidopsis. We found that during skotomorphogenesis, the expression of *BBX22* needs to be tightly regulated at both transcriptional and posttranslational levels. During photomorphogenesis, the expression of BBX22 transiently accumulates to execute its roles as a positive regulator. BBX22 protein accumulates to a higher level under short-day conditions and functions to inhibit hypocotyl elongation. The proteasome-dependent degradation of BBX22 protein is tightly controlled even in plants overexpressing *BBX22*. An analysis of BBX22 degradation kinetics shows that the protein has a short half-life under both dark and light conditions. COP1 mediates the degradation of BBX22 in the dark. Although dispensable in the dark, HY5 contributes to the degradation of BBX22 in the light. The constitutive photomorphogenic development of the *cop1* mutant is enhanced in *cop1BBX22ox* plants, which show a short hypocotyl, high anthocyanin accumulation, and expression of light-responsive genes. Exaggerated light responsiveness is also observed in *cop1BBX22ox* seedlings grown under short-day conditions. Therefore, the proper accumulation of BBX22 is crucial for plants to maintain optimal growth when grown in the dark as well as to respond to seasonal changes in daylength.

Light is one of the major environmental stimuli affecting plant growth and development. Throughout their lives, plants adopt versatile strategies to interpret the environmental light signals in their growth habitat to proceed with the most favorable growth and developmental programs, including skotomorphogenesis, photomorphogenesis, shade avoidance, circadian growth, flowering time control, and eventually senescence.

Skotomorphogenesis and photomorphogenesis are two distinct developmental processes for plants growing in the dark and in the light, respectively. Proper regulation of these two developmental stages is important for plants to optimize their growth and to ensure their success in response to environmental cues (for review, see Casal et al., 2004). Seedlings undergo a sophisticated transcriptomic adjustment during the transition from skotomorphogenesis to photomorphogenesis (for review, see Casal and Yanovsky, 2005). Both positive and negative transcriptional regulators are reported to participate in light-regulated transcriptional modulation in Arabidopsis (*Arabidopsis thaliana*). Importantly, many of these transcription factors, as well as photoreceptors and other signaling molecules, are subject to posttranslational regulation by light. For example, the 26S proteasome regulates the accumulation of proteins for light perception and signaling (for review, see Henriques et al., 2009). COP1 is a well-known E3 ligase functioning in selective degradation of proteins regulating many aspects of plant development, including photomorphogenesis (Ang et al., 1998; Osterlund et al., 2000b; Holm et al., 2002; Seo et al., 2003, 2004; Jang et al., 2005; Hong et al., 2008), photoperiodic growth (Yu et al., 2008), and flowering time control (Jang et al., 2008; Liu et al., 2008). Although the importance of COP1 in photomorphogenic growth is well documented, the repertoire of light-signaling proteins targeted by COP1 is likely incomplete.

Ample evidence exists for the coordination between light and multiple plant hormones in plant growth and development (Achard et al., 2007; Alabadí et al., 2008; Chen et al., 2008; Feng et al., 2008; Nemhauser, 2008;

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Vert et al., 2008; Song et al., 2009). Current studies suggest that HY5 is one of the key factors integrating light- and hormone-signaling pathways (Sibout et al., 2006; Chen et al., 2008; Laxmi et al., 2008). However, the molecular mechanisms underlying such cross talk are not fully understood.

BBX22, also known as *LZF1* (for light-regulated zinc finger protein 1), *STH3* (for salt tolerance homolog 3), and *DBB3* (for double B-box zinc finger 3; Chang et al., 2008; Datta et al., 2008; Kumagai et al., 2008; Khanna et al., 2009), is a newly identified signaling component acting in concert with HY5 and BBX21/STH2 to achieve a coordinated deetiolation process in Arabidopsis (Chang et al., 2008; Datta et al., 2008). *bbx22* is epistatic to *cop1* in the dark, which supports the idea that at least some of the photomorphogenic phenotypes in *cop1* are mediated by the accumulation of BBX22 (Datta et al., 2008). Previous results showed that BBX22 could be ubiquitinated in vitro by COP1, and a complex composed of BBX22, HY5, and COP1 might be formed (Datta et al., 2008). However, the expression kinetics of endogenous BBX22 protein and the biological significance of BBX22 degradation have not been carefully characterized.

In this study, we demonstrate that BBX22 is a short-lived protein and contributes to the inhibition of hypocotyl elongation under short-day (SD) conditions. As well, COP1 safeguards the destruction of BBX22 in the dark. The selective degradation of BBX22 ensures a precise skotomorphogenesis process and optimizes seedling growth under SD conditions in Arabidopsis. We also provide the molecular evidence to indicate that BBX22 regulates the expression of genes involved in light and phytohormone pathways, which may contribute to optimal seedling development in Arabidopsis.

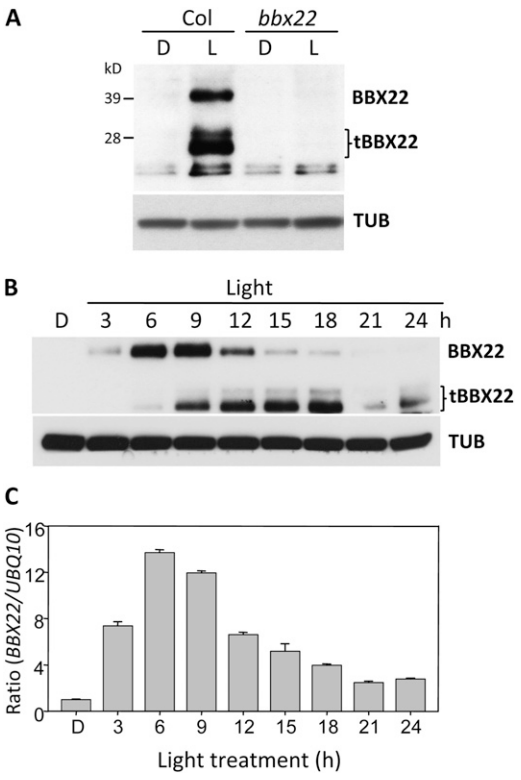

Figure 1. Both BBX22 protein and transcript are transiently accumulated. A, A BBX22-specific antibody was generated and used to determine the steady-state protein level by immunoblot analysis. Proteins were isolated from 4-d-old etiolated (dark; D) and 12-h light-treated (L) ecotype Columbia (Col) or *bbx22* seedlings. Endogenous α-tubulin was a loading control (TUB). B and C, BBX22 protein (B) and mRNA (C) are transiently accumulated during photomorphogenesis. Immunoblotting was used to detect the accumulation of BBX22 in etiolated seedlings and seedlings illuminated with light for 3 to 24 h. Real-time quantitative RT-PCR was used to monitor the expression of endogenous *BBX22* in C. The expression of *UBQ10* in each sample was used as an internal control. The *BBX22* expression in etiolated seedlings was set to 1. The expression of *BBX22* is presented as the amount of increase at each time point relative to that in etiolated seedlings and represented as "ratio." The means and SD were calculated from three replicates and plotted.

RESULTS

Posttranslational Regulation Is Responsible for the Transient Accumulation of BBX22 Protein

BBX22 functions to convey light signals from HY5 by activating three major photomorphogenic growth features in Arabidopsis: inhibition of hypocotyl elongation, anthocyanin biogenesis, and chloroplast development (Chang et al., 2008; Datta et al., 2008). To further elucidate the action mechanism of BBX22, we sought to characterize the expression pattern of BBX22 protein during the transition from dark to light environments. A BBX22-specific antiserum was generated to monitor the expression of BBX22 protein under both skotomorphogenic and photomorphogenic growth. In wild-type seedlings, full-length (40-kD) BBX22 and a 28-kD truncated BBX22 (tBBX22; see below) could be detected in light-grown seedlings but were barely detectable in dark-grown seedlings (Fig. 1A). Neither form of BBX22 protein could be detected in the *bbx22* mutant, which indicates that the antiserum is BBX22 specific (Fig. 1A). The tBBX22 is unlikely to be a product of alternative spliced mRNA because only the full-length *BBX22* transcript was observed during the deetiolation process (Supplemental Fig. S1A). When BBX22 was expressed as an N- or C-terminal epitope-tagged form in transgenic Arabidopsis, the tBBX22 showed a mobility shift only when expressed as an N-terminal but not a C-terminal tagged form (data not shown). This finding suggests that, rather than being a product of alternative translational initiation, tBBX22 represents the N-terminal tBBX22.

The presence of the N-terminal tBBX22 implies that BBX22 abundance might be regulated by selective degradation. This suggestion prompted us to perform a more detailed examination of BBX22 expression kinetics. Total protein isolated from 4-d-old etiolated

seedlings illuminated with 3 to 24 h of light was subjected to immunoblot analysis. As shown in Figure 1B, full-length BBX22 protein peaked at 6 to 9 h after light illumination, then was rapidly degraded. The lag in appearance and the eventual degradation of tBBX22 during the time course examined indicated that the truncated form is an intermediate form temporally accumulated during BBX22 degradation.

Real-time quantitative reverse transcription (RT)-PCR analysis of *BBX22* transcripts in samples from these time-course experiments revealed nearly synchronized expression kinetics between *BBX22* transcripts and the full-length protein (Fig. 1, B and C). This finding suggests that BBX22 is under highly coordinated regulation at both the transcript and protein levels.

To further assess the impact of posttranslational regulation on BBX22 protein accumulation, we analyzed the BBX22 protein expression pattern in *bbx22BBX22ox* (*lzf1LZF1ox* in Chang et al., 2008), which has phenotypes indistinguishable from those of wild-type Arabidopsis (Chang et al., 2008). Because the expression of *BBX22* is under the control of the 35S promoter in this transgenic plant, the *BBX22* transcripts should accumulate constitutively regardless of light or dark treatment. As expected, transcripts derived from the *BBX22* transgene were expressed at high levels at all time points examined (Fig. 2A). Interestingly, the accumulation kinetics of BBX22 in *bbx22BBX22ox* plants essentially mimic that in wild-type seedlings (Figs. 1B and 2B). No BBX22 or a very low BBX22 level could be detected in dark-grown tissues, and tBBX22 was abundant in seedlings illuminated with 24 h of light (designated as L_{24} hereafter; Fig. 2B). The increased level of tBBX22 likely reflects the higher level of BBX22 produced from the transgene. These results imply that even when BBX22 is overproduced, its accumulation is under rigid control in both dark-grown and light-illuminated Arabidopsis seedlings. As well, an efficient degradation system exists in Arabidopsis seedlings for the removal of BBX22 protein in a time- and light-dependent manner, which allows for the accumulation of BBX22 only in a restricted window after light illumination.

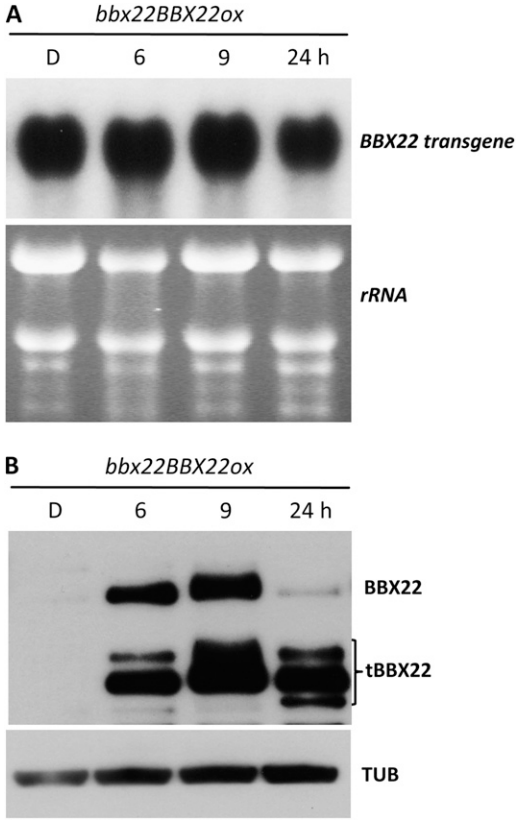

Figure 2. Transient accumulation of BBX22 is regulated at the protein level. The stability of BBX22 was determined in 4-d-old *bbx22* expressing *35S:BBX22* (*bbx22BBX22ox*) under the conditions indicated. A, Northern blot showing the overexpression of *BBX22* in etiolated (dark; D) *bbx22BBX22ox* seedlings or etiolated seedlings illuminated with light for 6, 9, or 24 h. The ethidium bromide-stained image was used to show equal loading of the total RNA samples. B, Immunoblot showing the transient accumulation of BBX22 in plant samples used in A. Endogenous α-tubulin was a loading control (TUB).

BBX22 Is a Short-Lived Protein Degraded by the 26S Proteasome

We next examined the capacity for Arabidopsis to degrade BBX22 protein. We used 4-d-old etiolated seedlings treated with 8 h of light for optimal accumulation of BBX22 protein. The seedlings were then incubated with the translation inhibitor cycloheximide and kept under light or transferred to dark treatment for the times indicated. The degradation kinetics of BBX22 protein were determined as a proportion of full-length BBX22 remaining relative to BBX22 expression at 8 h of light treatment (Fig. 3). The half-life of BBX22 was calculated to be 20 min in the dark and 60 min in the light. These results indicate that BBX22 is a short-lived protein and that dark treatment accelerates its degradation.

The involvement of the 26S proteasome in the fast degradation of BBX22 was also examined in parallel by adding the 26S proteasome inhibitor MG132. As shown in Figure 3, as compared with less than 10% of BBX22 detected in the absence of MG132, in the presence of MG132, BBX22 was largely stabilized, as represented by 54.9% or 66.7% residual BBX22 after 4 h of MG1132 treatment under light or dark conditions, respectively. The effective stabilization of BBX22 protein by MG132 indicates that the 26S proteasome is responsible for the degradation of BBX22 under both light and dark conditions.

BBX22 Contributes to the Inhibition of Hypocotyl Elongation under SD Conditions

Six-day-old *bbx22* exhibits light hyposensitivity (i.e. long hypocotyl) under SD conditions but not long-day (LD) conditions (Datta et al., 2008). We have confirmed

this phenotype for 4-d-old seedlings (Fig. 4A). The strict expression regulation of BBX22 prompted us to examine a possible correlation between the BBX22 protein levels and the differential light responsiveness in seedlings grown under SD or LD conditions. As shown in Figure 4B, BBX22 protein showed an expression peak at 8 h after dawn under both SD and LD conditions. After dusk, BBX22 protein level was quickly degraded in both SD- and LD-grown seedlings. Total BBX22 protein in both light and dark periods under SD and LD conditions was estimated, separately, by calculating the areas under the SD or LD protein curves. Results showed that BBX22 accumulates to a higher level under SD than LD conditions (Fig. 4B, top right).

Previous studies indicated that the hypocotyl elongation of Arabidopsis seedlings follows a rhythmic pattern (Dowson-Day and Millar, 1999; Nozue et al., 2007). We thus characterized, under SD conditions, whether mutation of *BBX22* influences a specific growth phase of hypocotyl elongation by real-time imaging of hypocotyl growth under SD conditions, as described previously (Nozue et al., 2007). As shown in Figure 4C, *bbx22* exhibited an increased growth rate during the fast-elongating phase in the dark period. The most noticeable difference was observed on day 4, consistent with the maximal growth capability seen in Arabidopsis seedlings (Gendreau et al., 1997; Nozue et al., 2007).

BBX22 is a positive regulator of the inhibition of hypocotyl elongation during photomorphogenesis (Chang et al., 2008; Datta et al., 2008). The higher level of BBX22 protein under SD than LD conditions may explain its more prominent contribution in the inhibition of hypocotyl elongation under SD conditions. The increased growth rate observed in *bbx22* suggests that BBX22 could positively transmit the light signal for attenuated hypocotyl elongation during the night.

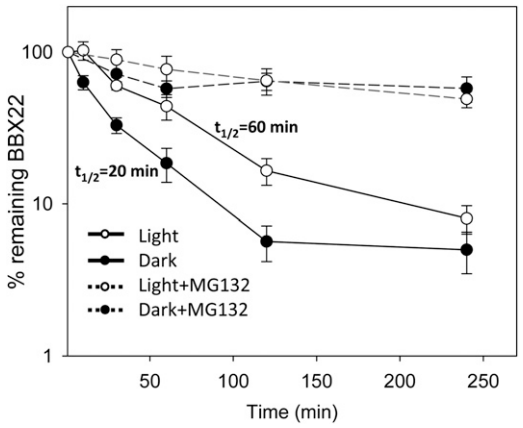

Figure 3. BBX22 is a short-lived protein degraded by the 26S proteasome. The degradation kinetics of BBX22 protein were determined as a percentage of full-length BBX22 remaining relative to BBX22 at 8 h of light treatment in the presence of cycloheximide. Half-life ($t_{1/2}$) was calculated by regression analysis. The half-life of BBX22 is 60 min in the light (white circles, solid lines) and 20 min in the dark (black circles, solid lines). The degradation of BBX22 was blocked by treatment with MG132 under both light (white circles, dashed lines) and dark (black circles, dashed lines) conditions. $n = 3$.

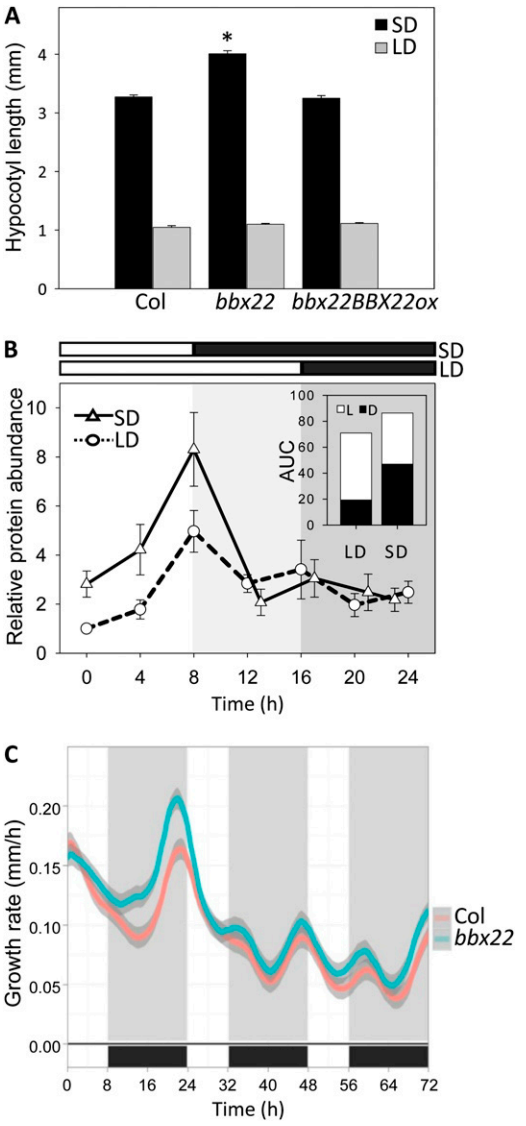

Figure 4. BBX22 protein functions in the inhibition of hypocotyl elongation under SD conditions. A, The *bbx22* mutant is hyposensitive to light only under SD conditions. Hypocotyl length was measured for 4-d-old seedlings grown under SD or LD conditions. * $P < 0.01$, Student's *t* test; $n = 38$ to 76. B, BBX22 protein accumulates to a higher level in SD-grown Col plants (SD; white triangles, solid lines) compared with that in plants under LD conditions (white circles, dashed lines). BBX22 protein levels at different times of the day were determined by immunoblot analysis. Results are presented as values relative to that at dawn for 4-d-old seedlings grown under LD conditions. White bars indicate light periods and black bars indicate dark periods. $n = 4$. AUC, Areas under the SD or LD protein curves. C, *bbx22* shows an increased growth rate during the dark period of SD conditions, with the most noticeable difference at day 4 after germination. Time 0 indicates dawn of day 4 after germination. Shaded and black areas indicate night (darkness), and white areas indicate day (lights on). Shaded areas around each growth trace (blue and red) show SE. $n = 29$.

COP1 Is Required for Selective Degradation of BBX22 in the Dark

COP1 possesses the ability to ubiquitinate BBX22 in vitro and was proposed to be responsible for the regulation of BBX22 protein (Datta et al., 2008). However, the role of COP1 in BBX22 degradation in vivo has not been examined. The nuclear localization of BBX22 (Datta et al., 2008) prompted us to test whether BBX22 protein could indeed accumulate in *cop1* mutants by examining BBX22 abundance in the dark when COP1 is also present in the nucleus (von Arnim et al., 1997; Osterlund et al., 2000a). The direct measurement of BBX22 in the *cop1* mutant could be hindered by the accumulation of HY5. HY5 is a direct target of COP1 E3 ligase activity (Osterlund et al., 2000a) and also a direct transcriptional activator of *BBX22* expression (Chang et al., 2008). Indeed, both our northern and immunoblot analyses showed an increased accumulation of both *BBX22* transcripts and protein in *cop1* mutants (Fig. 5, A and B). To bypass the regulation of *BBX22* by HY5, we introduced the *35S*:*BBX22-GFP* transgene from a wild-type background (*BBX22-GFPox*) into the *cop1* mutant to directly compare BBX22-GFP protein abundance in wild-type plants and the *cop1* mutants *cop1-4* and *cop1-6*. Similar to *bbx22BBX22ox* plants, *BBX22-GFPox* plants also showed wild-type phenotypes (see Fig. 7 below). Therefore, the accumulation of BBX22-GFP is likely within the wild-type threshold, even though its expression was driven by a 35S promoter. As expected, although *BBX22-GFP* transcripts accumulated to high levels (Fig. 5C), only residual BBX22-GFP protein was present in *BBX22-GFPox* plants grown in the dark (Fig. 5D). In contrast, a high level of BBX22-GFP protein was detected in *cop1* mutants (Fig. 5D), which suggests that COP1 is required for the selective degradation of BBX22 in the dark.

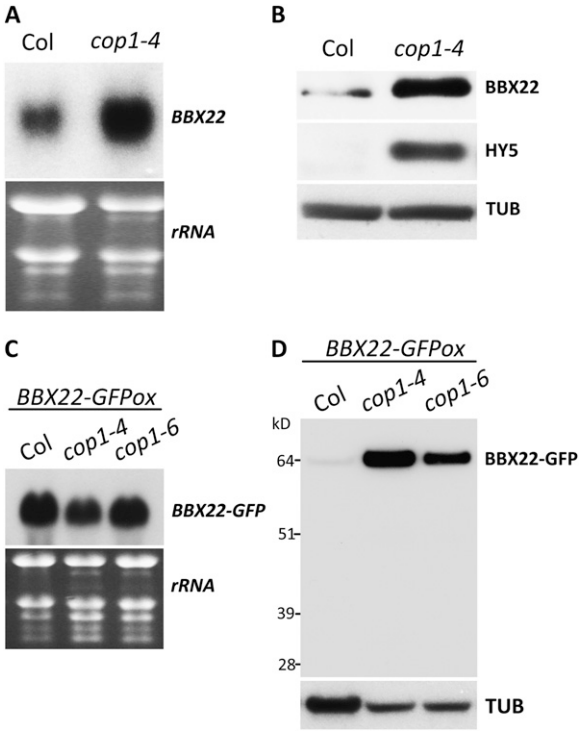

Figure 5. COP1 is required for selective degradation of BBX22 in the dark. A and B, The expression of both *BBX22* transcript (A) and BBX22 protein (B) is higher in dark-grown *cop1-4* plants than in wild-type (Col) plants. C, Northern-blot analysis was used to confirm the comparable *BBX22-GFP* transgene expression in these lines. The ethidium bromide-stained image was used to show equal loading of the total RNA samples. D, BBX22-GFP protein accumulates to high levels in *cop1* mutants. Endogenous α-tubulin was a loading control (TUB).

HY5 Contributes to Light-Mediated Degradation of BBX22

Although COP1 could mediate a low level of BBX22 ubiquitination in vitro, no direct interaction of COP1 and BBX22 could be detected with yeast two-hybrid or fluorescence resonance energy transfer assay (Datta et al., 2008). When coexpressed with COP1, BBX22 showed a redistribution to nuclear speckles, which implies an indirect interaction between COP1 and BBX22 (Datta et al., 2008). Because HY5 can interact with both COP1 and BBX22 (Datta et al., 2008), COP1, HY5, and BBX22 may form a large complex in Arabidopsis seedlings (Datta et al., 2008). Investigating whether the integrity of this protein complex is essential for the COP1-dependent degradation of BBX22 is of interest.

One way to test this hypothesis is to examine the BBX22 degradation patterns in the *hy5* mutant, although the results are likely to be compromised because of a HY5-dependent transcriptional activation of *BBX22* (Chang et al., 2008). Indeed, our immunoblot analyses revealed decreased BBX22 protein level in *hy5* mutants (L_8 in Fig. 6A). To circumvent this limitation, we manipulated the overproduction of *BBX22* transcripts in the *hy5-1* mutant (*hy5BBX22ox* represents *hy5LZF1ox* in Chang et al., 2008). For pairwise comparison, the transgene (*35S*:*BBX22*) was introduced into the corresponding wild-type Arabidopsis Landsberg *erecta* (L*er*) ecotype by genetic crossing. As shown in Figure 6B, transcripts of the *BBX22* transgene accumulated to comparable levels in L*er* and *hy5* mutants under both dark and L_{24} conditions. BBX22 protein was effectively degraded in both L*er* and *hy5* mutants in the dark (Fig. 6C). However, in L_{24}, residual full-length BBX22 protein could be detected in the *hy5* mutant but not in L*er* (Fig. 6C). This result suggests that the efficient degradation of BBX22 protein is compromised in the light-grown *hy5* mutant. An increased level of tBBX22 was also observed in *hy5* (L_{24} in Fig. 6, A and C, left panel), which suggests that HY5 may contribute to the degradation of both full-length BBX22 and tBBX22.

Whether HYH, a HY5 homolog (Holm et al., 2002), substitutes the functions of HY5 in the *hy5* mutant was tested by examining BBX22 protein abundance in the

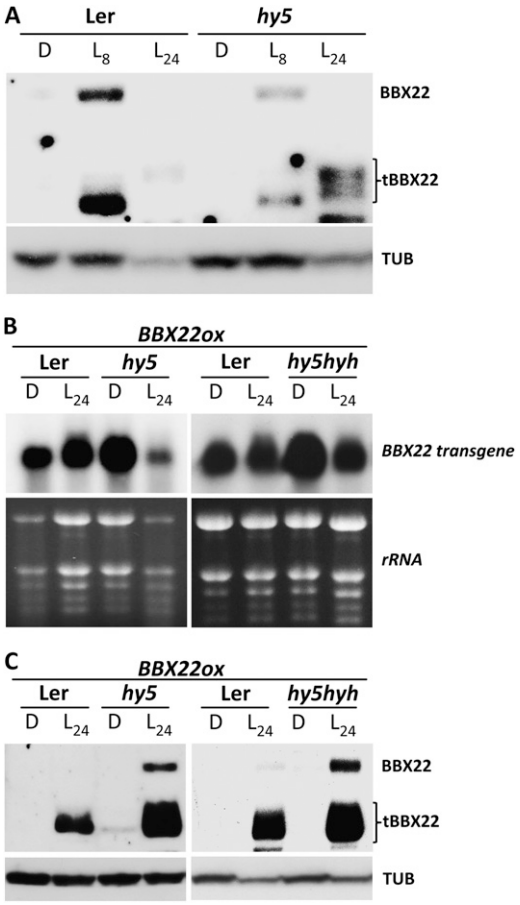

Figure 6. BBX22 degradation depends in part on HY5 in the light but is independent of HY5 and HYH in the dark. A, BBX22 decreases in *hy5* due to a lack of HY5-dependent transcriptional activation of *BBX22*. B, Northern-blot analysis was used to confirm the comparable expression of *BBX22* transgene in L*er*, *hy5*, and *hy5hyh* plants expressing *35S*:*BBX22* and grown in the dark (D) for 4 d or in 4-old etiolated seedlings illuminated with light for 24 h (L_{24}). The ethidium bromide-stained image was used to show the amount of total RNA samples loaded in each lane. C, Immunoblotting was used to detect BBX22 protein in the plant samples used in B. The detection of endogenous α-tubulin was performed as a loading control (TUB).

hy5hyh double mutant. The *hy5* and *hy5hyh* mutants showed comparable BBX22 degradation patterns (Fig. 6, B and C), which indicates that HYH does not play a decisive role in the degradation of BBX22. Therefore, both HY5 and HYH are dispensable for the degradation of BBX22 in the dark but may contribute to the efficiency of BBX22 degradation in light-illuminated seedlings.

The Selective Degradation of BBX22 Ensures Proper Seedling Development

The results shown above indicate that BBX22 could be effectively degraded even if it is overexpressed, especially in the dark (Figs. 2B, 5D, and 6C). Arabidopsis seedlings have a strong capacity for the removal of BBX22 even at an excess amount. Therefore, BBX22 might have a detrimental role if its level or time of accumulation is not strictly controlled. Our data indicate that the exaggerated accumulation of BBX22 could be achieved only in the *cop1* mutant (Fig. 5). To assess whether the overproduction of BBX22 would negatively affect Arabidopsis seedling development, we analyzed the phenotypes of *cop1BBX22-GFPox* plants under both dark and SD conditions. The parameters measured included the inhibition of hypocotyl elongation and anthocyanin accumulation, both characteristics reported for *cop1* mutants (McNellis et al., 1994). Overexpression of *BBX22-GFP* enhanced the *cop* phenotype during skotomorphogenesis (Fig. 7A) and light responsiveness under SD conditions (Fig. 7B), including the exaggerated hypocotyl shortening and the excess accumulation of anthocyanin. During skotomorphogenesis, the hypocotyl length of *cop1* is about 50% that of the wild type. Overexpression of *BBX22-GFP* further reduced the hypocotyl length to 13% of that of the wild type (Fig. 7A). Excess anthocyanin accumulation could also be seen in both cotyledons and hypocotyls, as indicated by arrowheads in Figure 7A. Similar results were observed in *cop1BBX22-GFPox* plants grown under SD conditions (Fig. 7B). In contrast, overexpression of *BBX22-GFP* in a wild-type background did not result in these phenotypic alterations, which indicates that the presence of COP1 is sufficient to mediate the degradation of excess *BBX22-GFP* and that the wild-type level of biologically active BBX22-GFP could be properly maintained (Fig. 7, A and B).

The phenotypes of *cop1BBX22-GFPox* are consistent with the previous observation that *bbx22* could partially suppress the short hypocotyl and high anthocyanin level of dark-grown *cop1* (*sth3cop1* in Datta et al., 2008). These results also emphasize the importance of the COP1-mediated protein surveillance of positive regulators such as BBX22 in optimizing seedling growth under both dark and SD conditions.

BBX22 Influences Genes on Light Signaling and Hormone Responses

Target genes responsible for the exaggerated light phenotype in *cop1BBX22-GFPox* plants were revealed by comparing transcriptomes among dark-grown wild-type, *cop1*, and *cop1BBX22-GFPox* plants. In total, 1,494 genes showed at least 2-fold difference in expression between *cop1BBX22-GFPox* and wild-type plants at a false discovery rate (FDR) of less than 0.05. These genes were further interrogated for their association with specific biological pathways by use of Gene Ontology analysis. These genes were associated with light-related ($P = 2 \times 10^{-4}$) or hormone-related ($P = 3.4 \times 10^{-8}$) pathways. Among these genes, 207 showed at least 2-fold differential expression in *cop1BBX22-GFP* compared with *cop1* plants at an FDR of less than 0.05 (Supplemental Table S1). These genes were considered to be specifically regulated by BBX22 and were selected for detailed analysis.

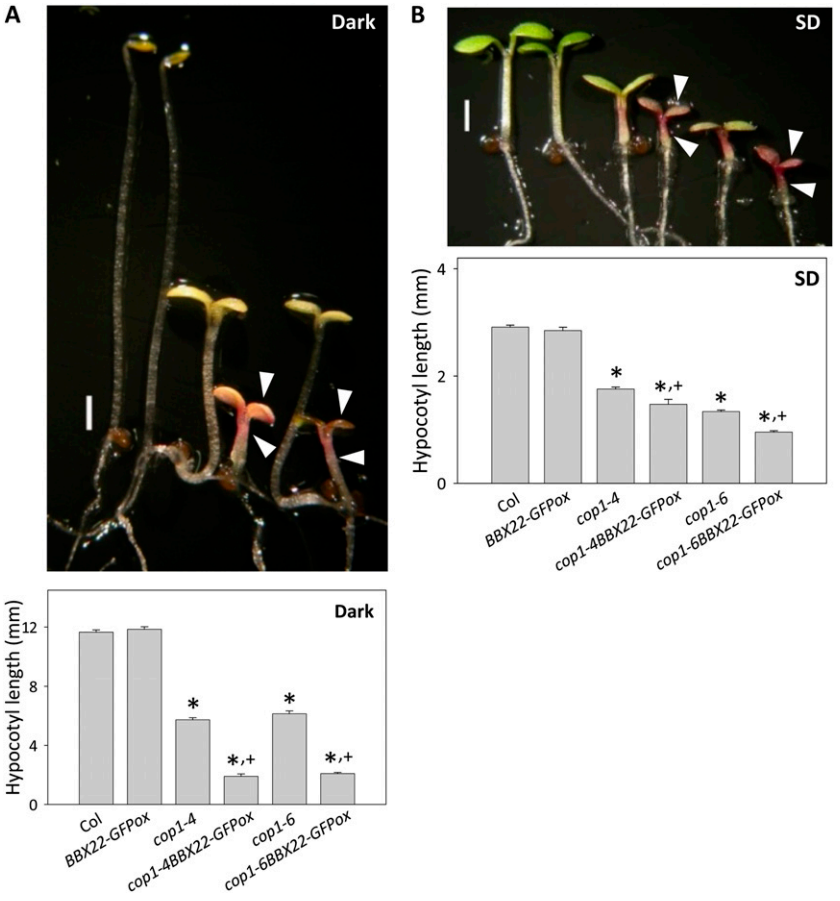

Figure 7. *cop1BBX22-GFPox* has exaggerated light responsiveness. Exaggerated *cop* phenotypes (top panels), the extra inhibition of hypocotyl growth and excess anthocyanin accumulating both in cotyledons and hypocotyls (marked by arrowheads), were seen when BBX22-GFP was overexpressed under both dark (A) and SD (B) conditions. Hypocotyl length (bottom panels) was measured in 4-d-old seedlings grown under dark (A) and SD (B) conditions. $^{*,+}$ Significantly different from Col and the corresponding *cop1* allele, respectively ($P < 0.01$, Student's *t* test; $n = 20–32$). Bars = 1 mm.

Whether BBX22 preferentially participates in specific light or hormone pathways was examined by comparing the 207 BBX22-regulated genes with genes regulated by a specific quality of light or plant hormones during the seedling stage. Table I lists genes retrieved from various experimental conditions treated with light or plant hormones as well as genes associated with the *cop1*-like phenotype. For each gene list, the percentage representation of the genes in the Arabidopsis genome (ATH1) and in the 207 BBX22-regulated genes was calculated. Fisher's exact test (http://www.matforsk.no/ola/fisher.htm; Agresti, 1992) was used to evaluate whether BBX22-regulated genes are significantly enriched with any given treatment or genetic material.

As shown in Table I, 70% of BBX22-regulated genes were differentially expressed in plants with *cop*-like phenotypes, which is consistent with the exaggerated phenotype observed for *cop1BBX22-GFPox* plants (Fig. 7). In total, 83% of BBX22-regulated genes were light-responsive genes as compared with only 27% in ATH1 ($P = 1.6 \times 10^{-62}$). Consistent with being a downstream gene of HY5 (Chang et al., 2008), BBX22 could regulate genes responding to various light qualities ($P = 1 \times 10^{-27}$ to 4.6×10^{-62}; Table I). Also, 42% of BBX22-regulated genes are differentially regulated by treatment with multiple plant hormones ($P = 9 \times 10^{-12}$; Table I). The overrepresentation of BBX22-regulated genes in response to light and plant hormones suggested a possible role of BBX22 in regulating light signaling and hormone responses.

DISCUSSION

Selective Degradation of BBX22 Is Important for Proper Transcriptomic Responses in Developing Arabidopsis Seedlings

The optimal responsiveness of plants to the light/dark environment could be achieved by combinations of rapid transcriptional adjustments and posttranslational degradation of both positive or negative factors in light-sensing and -signaling pathways (Casal and Yanovsky, 2005; Henriques et al., 2009). Our results demonstrated that both the transcription of *BBX22* and the accumulation of BBX22 protein are strictly controlled (Figs. 1 and 2).

The COP1- and 26S proteasome-mediated selective degradation of BBX22 is crucial to avoid unfavorable seedling development under dark or SD conditions. When overproduced, BBX22 preferentially alters the expression of genes in response to light and multiple plant hormones (Table I). Consistent with the previous

Table I. *BBX22 regulates light- and hormone-responsive genes*

A total of 207 BBX22-regulated genes were compared with genes regulated by light or plant hormones. Fisher's exact test was used to evaluate significant enrichment under each given treatment. *PHYB*^{Y276H}, Tyr-to-His mutant allele of *phyB*; *pifq, pif1345* quadruple mutant; *35S:MIF1, MINI ZINC FINGER* overexpressor; Wc, continuous white; Rc, continuous red; FRc, continuous far-red; Bc, continuous blue; IAA, indole-3-acetic acid; MJ, methyl jasmonate; ABA, abscisic acid; ACC, 1-amino-cyclopropane-1-carboxylic acid; CK, cytokinin.

Treatment	ATH1	BBX22 Regulated	*P*	Reference
	%			
cop1-like	14.7	70.0	7.2×10^{-71}	
PHYB^{Y276H}	13.1	63.8	2.7×10^{-63}	Hu et al. (2009)
pifq	4.5	30.0	1.1×10^{-32}	Leivar et al. (2009)
35S:MIF1	0.5	3.9	1.4×10^{-5}	Hu and Ma (2006)
Light	27.2	83.1	1.6×10^{-62}	
Wc	15.7	61.8	2.3×10^{-50}	Jiao et al. (2005); Chang et al. (2008)
Rc	17.2	70.0	4.6×10^{-62}	Jiao et al. (2005); Leivar et al. (2009)
FRc	8.2	42.0	5.6×10^{-39}	Jiao et al. (2005)
Bc	11.8	42.0	1.0×10^{-27}	Jiao et al. (2005)
High light	6.4	36.7	1.3×10^{-36}	Kleine et al. (2007)
Hormone	20.5	41.5	9.0×10^{-12}	
IAA	3.5	14.0	3.3×10^{-10}	Nemhauser et al. (2006)
BR	4.0	13.0	1.2×10^{-7}	Lisso et al. (2005); Nemhauser et al. (2006); Song et al. (2009)
GA	0.5	1.4	0.11	Nemhauser et al. (2006)
MJ	6.7	18.4	1.7×10^{-08}	Nemhauser et al. (2006)
ABA	12.9	24.6	5.6×10^{-6}	Nemhauser et al. (2006)
ACC	2.4	14.0	4.6×10^{-14}	Nemhauser et al. (2006)
CK	2.2	4.8	0.027	Nemhauser et al. (2006)

observation that BBX22 regulates chloroplast development (Chang et al., 2008), genes regulated by BBX22 include *ELIP1*, *ELIP2*, *CRY3/CRYD*, and *SIG5/SIGE*, which encode chloroplast proteins (Yao et al., 2003; Tsunoyama et al., 2004; Heddad et al., 2006; Pokorny et al., 2008). *ELIP1* and *ELIP2*, transiently induced by different qualities of light, encode thylakoid proteins functioning in chlorophyll biosynthesis (Casazza et al., 2005; Rossini et al., 2006). Plants overproducing ELIP2 have reduced chlorophyll content (Tzvetkova-Chevolleau et al., 2007). Our results suggest that the selective degradation of BBX22 might fine-tune the expression of *ELIP2* for optimal chlorophyll accumulation.

BBX22 also up-regulates genes involved in flavonoid and anthocyanin biosynthesis pathways, including *CHS*, *CHI*, *F3H*, and *4CL3* (Li et al., 1993; Shirley et al., 1995; Ehlting et al., 1999; Raes et al., 2003; Solfanelli et al., 2006; Poustka et al., 2007; Owens et al., 2008; Buer and Djordjevic, 2009). This observation explains the excess accumulation of anthocyanin in etiolated *cop1BBX22-GFPox* plants (Fig. 7). In addition to being up-regulated by BBX22, the genes *CHS* and *F3H* are direct targets of HY5 (Shirley et al., 1995; Lee et al., 2007). The interaction of BBX22 with HY5 (Datta et al., 2008) raises the possibility that BBX22 and HY5 function in a complex for the activation of these two genes.

HY5 and PIF3/4 function to link the light- and hormone-signaling pathways (de Lucas et al., 2008; Lau and Deng, 2010). The enrichment of hormone-responsive genes upon the overexpression of BBX22 implies that BBX22 functions downstream of HY5 in both light and hormone signaling pathways. The overproduction of BBX22 down-regulates hormone-responsive genes and genes involved in cell wall modification (Fig. 8). For example, BBX22 represses *EXP3*, which, when overexpressed, promotes plant growth (Kwon et al., 2008), and *EXP8*, which is known be repressed by blue light through *cry1* (Kleine et al., 2007). *EXP8* is also induced by brassinosteroid (BR;

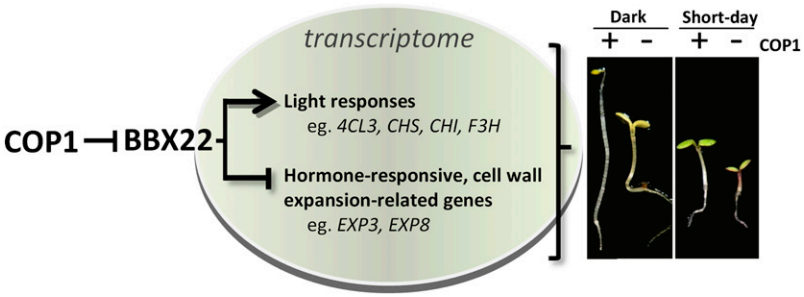

Figure 8. A model illustrating the biological impact of BBX22 degradation on seedling development. Protein abundance of the positive regulator BBX22 is tightly controlled by COP1 to regulate the development of Arabidopsis grown in the dark and under SD conditions. Overaccumulated BBX22 in *cop1* gives rise to an exaggerated light responsiveness by altering the expression of genes responsive to light and hormone signals.

Yin et al., 2002) and auxin through ARF7 (Esmon et al., 2006). Whether BBX22 functions to suppress either auxin- or BR-mediated expression of *EXP8* remains to be examined.

Collectively, the efficient degradation of BBX22 in wild-type seedlings ensures adequate skotomorphogenesis of elongated hypocotyl, with minimal pigments accumulating in the dark. Also, degradation of BBX22 could fine-tune hypocotyl length and anthocyanin accumulation under SD conditions. However, the unwarranted accumulation of BBX22 in *cop1* or *cop1BBX22-GFPox* plants under both conditions leads to the exaggerated inhibition of hypocotyl elongation and hyperaccumulation of anthocyanin by activating light-responsive genes and repressing hormone-responsive and cell wall expansion genes (Fig. 8). The short half-life of BBX22 protein (Fig. 3) allows Arabidopsis seedlings to adjust downstream gene expression in a timely manner in response to environmental light changes.

Degradation Mechanism of BBX22 Protein

Our data provide evidence to support that, in the dark, COP1 is required for the selective degradation of BBX22 (Fig. 5). Because BBX22 does not physically interact with COP1 (Datta et al., 2008), identifying any proteins assisting in the targeted degradation of BBX22 by COP1 is of great interest. Possible candidates are early flowering 3 (ELF3) and suppressor of phyA-105, which are COP1-interacting partners and function to facilitate COP1-mediated degradation of GIGANTEA and HY5/CONSTANS, respectively, in the dark (Saijo et al., 2003; Laubinger et al., 2006; Yu et al., 2008). Also, reduced expression of CULLIN4 enhanced the *cop* phenotype in *cop1-4* (Chen et al., 2010), *cop10*, and *det1* (Chen et al., 2006). This finding suggests that, in addition to COP1, CULLIN4 is needed to suppress photomophogenic growth in the dark. Because CULLIN4 is proposed to assist COP1-mediated HY5 degradation (Chen et al., 2006), whether CULLIN4 also accelerates COP1-mediated BBX22 degradation in the dark could be examined.

Although COP1 exists predominantly in the cytoplasm in the light (von Arnim and Deng, 1994), a recent study reported that the nuclear phyB degradation under red light is mediated by COP1 (Jang et al., 2010). BBX22 is also a nuclear protein, and its degradation in the light depends in part on HY5 (Fig. 6). This finding suggests that a small portion of BBX22 protein could be degraded in a protein complex composed of BBX22, HY5, and COP1, as was proposed previously (Datta et al., 2008). However, BBX22 is still largely degraded in *hy5* and *hy5hyh* (Fig. 6). This observation suggests that an unknown factor(s) contributes mainly to BBX22 degradation in the light. Further identification of BBX22-interacting partners by coimmunoprecipitation and proteomic characterization will help in the study of the time-dependent degradation of BBX22 in the light. This mechanism is likely common for the timely elimination of positive regulators in light signal transduction pathways.

The Role of BBX22 in Arabidopsis Rhythmic Growth

bbx22 has a light-hyposensitive phenotype under SD conditions because of an increased hypocotyl elongation rate in the dark phase (Fig. 4). Our results indicate that *bbx22* still exhibits rhythmic growth, unlike the arrhythmic growth patterns observed in the light perception and signaling mutants *hy2* and *hy5* (Nozue et al., 2007). Consistent with BBX22 carrying a branch of HY5-mediated light-signaling outputs, the light-mediated inhibition of hypocotyl elongation during the light phase is intact in *bbx22* but absent in *hy5*. Interestingly, in addition to an increased growth rate at dawn, *bbx22* also has an accelerated growth rate in the first half of the dark phase, as was observed in the circadian clock mutants *CCA1ox*, *elf3*, and *elf4* (Nozue et al., 2007). A previous study indicated that the expression of *BBX22* is regulated by the circadian clock (Kumagai et al., 2008). Whether BBX22 conveys the circadian information for the regulation of rhythmic growth is also of interest.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

bbx22, *bbx22BBX22ox*, and *hy5BBX22ox* plants were described in our previous study, and *LZF1* was the original name (Chang et al., 2008). *hy5BBX22ox* was backcrossed to L*er* to generate *BBX22ox* plants. *hy5BBX22ox* was crossed to *hy5-ks50hyh* (Holm et al., 2002) to generate *hy5hyhBBX22ox* plants. For generating *BBX22-GFPox* transgenic plants, the *BBX22* coding region was amplified and fused to *GFP* in frame in a vector of *35S:GFP* (Lee et al., 2001). The fragment of *p35S:BBX22-GFP* was subcloned into pCAMBIA1390 for generating transgenic Arabidopsis (*Arabidopsis thaliana*) overexpressing *BBX22-GFP*. Transgenic Arabidopsis expressing *35S:BBX22-GFP* was used for crossing with two weak *cop1* alleles, *cop1-4* and *cop1-6* (McNellis et al., 1994).

Plants were grown on one-half-strength Murashige and Skoog medium with 1% Suc and 0.3% gelrite at 4°C for 4 d to synchronize the germination. Seedlings were grown at 22°C under a 16-h/8-h or an 8-h/16-h light (100 μmol m^{-2} s^{-1})/dark photoperiod for 4 d for phenotype observation under LD versus SD conditions. For data shown in Figures 1, 2, and 6, 4-d-old etiolated seedlings were illuminated with 100 μmol m^{-2} s^{-1} light for the times indicated before RNA and protein extraction.

Growth Rate Analysis

Plants were grown and analyzed essentially as described (Nozue et al., 2007) except that the growth medium was one-half-strength Murashige and Skoog medium with minimal organics with 1% Suc; images were captured with the use of a PixeLINK PL-A781 camera driven by LabView (National Instruments). For growth rate calculations, we did not use a rolling average; instead, change in growth was calculated for each 30-min time, local polynomial regression fitting (Loess) smoothing with smoothing parameter = 0.15 was used in R (R Development Core Team, 2009), and results were plotted with the use of the ggplot2 plotting package (Wickham, 2009).

Immunoblot Analysis and Degradation Kinetics

To generate a BBX22-specific antibody, we used the C-terminal 143 amino acids (C-143) as antigen to reduce cross-recognition of antiserum of BBX22 and other BBX proteins (Khanna et al., 2009). C-143 of BBX22 was constructed into pET28a(+) and expressed in Rosetta2 (DE3) cells (Novagen) for generating rat anti-BBX22 polyclonal antibody. Total protein was isolated as described

(Al-Sady et al., 2006) with minor modifications. In brief, seedlings underwent extraction with boiled extraction buffer (4 M urea, 5% SDS, 15% glycerol, 100 mM Tris-HCl, pH 8, with freshly added 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 2 μg mL^{-1} apopectin, 1 μg mL^{-1} pepstatin, 3 μg mL^{-1} leupeptin, and 1× complete protease inhibitor [Roche]). In total, 30 to 50 μg of protein was separated on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen), and blots were probed with the anti-BBX22 antiserum and then horseradish peroxidase-conjugated anti-rat antiserum (Sigma) as a secondary antibody. Immunoblotting with anti-α-tubulin antiserum (Sigma) and alkaline phosphatase-conjugated anti-mouse antiserum (Santa Cruz Biotechnology) was used to measure endogenous α-tubulin as a loading control. Chemiluminescence horseradish peroxidase substrate (Millipore) was used for signal detection.

For determination of degradation kinetics, seedlings were immersed in 100 μM cycloheximide and incubated under light or dark conditions for the times indicated. The quantity of protein was analyzed by immunoblotting and quantified by the Biospectrum 600 Imaging System (UVP). Protein half-lives were calculated by regression analysis. For determining the proteasome-dependent degradation of BBX22, seedlings were preincubated with 50 μM MG132 (Biomol/Enzo) for 1 h before the addition of cycloheximide.

For determining BBX22 protein levels at different times of day, 3-d-old seedlings grown under LD or SD conditions were harvested at the times indicated starting at dawn of day 4 for protein extraction and immunoblot analysis. BBX22 protein levels were shown as relative values to BBX22 at 0 h under LD conditions. Areas under the BBX22 protein levels versus time curves were obtained by use of the Area below Curves macro (trapezoidal rule) in SigmaPlot 9 (Systat Software).

Real-Time Quantitative RT-PCR and Northern-Blot Analysis

Total RNA was isolated and analyzed by real-time quantitative RT-PCR as described (Chang et al., 2008). A total of 3 μg of RNA was separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N$^+$ membrane (GE Healthcare). The full-length coding region of *BBX22* was used to generate a gene-specific probe by incorporating DIG-11-UTP (DIG RNA labeling mix; Roche) by PCR. Hybridization and signal detection were performed as suggested by the manufacturer.

Affymetrix ATH1 Genome Array Hybridization and Data Analyses

Total RNA from 4-d-old etiolated wild-type, *cop1-4*, and *cop1-4BBX22-GFPox* seedlings was isolated and applied to the Arabidopsis ATH1 Genome Array (ATH1-121501; Affymetrix) for gene expression analysis as described (Chang et al., 2008). MicroArray Suite 5.0 (Affymetrix) and GeneSpring 7.3 (Agilent Technologies) were used for chip quantification, normalization, and further analysis. In brief, the intensity of all probe sets of each chip was scaled up to 500 for equivalent chip-to-chip comparison. The wild type, *cop1-4*, and *cop1BBX22-GFPox* underwent pairwise expression comparison for each probe set. Only genes with FDR of less than 5% (significance analysis of microarrays; Tusher et al., 2001) in triplicate biological repeats were selected for further analyses. Genes with 2-fold or greater or 0.5-fold or less change in expression levels between *cop1BBX22-GFPox* and the wild type were analyzed for association with the use of Gene Ontology and GeneSpring GX 10. Among those, genes with greater than 2-fold change in level between *cop1BBX22-GFPox* and *cop1-4* were considered BBX22 regulated.

The data sets have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through the Gene Expression Omnibus series accession number GSE22983.

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library with the following locus identifiers: *BBX22* (At1g78600), *COP1* (At2g32950), *HY5* (At5g11260), and *HYH* (At3g17609).

Supplemental Data

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

Supplemental Figure S1. *BBX22* expresses as a single-sized transcript.

Supplemental Table S1. A list of 207 BBX22-regulated genes.

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