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**COP1-mediated degradation of BBX22/LZF1 optimizes seedling development in Arabidopsis**

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Abstract

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 in the dark, light or light-dark cycles. BBX22, whose induction is both light regulated and HY5 dependent, is a positive regulator of de-etiolation in Arabidopsis. We found that during skotomorphogenesis, the expression of BBX22 needs to be tightly regulated at both transcriptional and post-translational 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 the short-day condition 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 respond to seasonal changes in day length.
Introduction

Light is one of the major environmental stimuli affecting plant growth and development. Throughout their life, 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 are 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. Importantly, many of these transcription factors, as well as photoreceptors and other signaling molecules, are subject to post-translational 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., 2000; Holm et al., 2002; Seo et al., 2003; Seo et al., 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; Alabadi et al., 2008; Chen et al., 2008; Feng et al., 2008; Nemhauser, 2008; 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 (light-regulated zinc finger protein 1), STH3 (salt tolerance homolog 3) and DBB3 (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 de-etiolation 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 demonstrated 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.
Results

Post-translational 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, including 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 kDa) and a 28-kDa truncated BBX22 (tBBX22, see below) could be detected in light-grown (L) seedlings but were barely detectable in dark-grown (D) seedlings (Figure 1A). Neither forms of BBX22 protein could be detected in the bbx22 mutant, which indicates that the antiserum is BBX22 specific (Figure 1A). The tBBX22 is unlikely a product of alternative spliced mRNA because only the full-length BBX22 transcript was observed during the de-etiolation process (Supplemental Figure 1A). When BBX22 was expressed as N- or C-terminal epitope-tagged forms in transgenic Arabidopsis, the truncated BBX22 showed a mobility shift only when expressed as an N-terminal but not 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–truncated BBX22.

The presence of the N-terminal–truncated BBX22 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 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 (Figure 1B, 1C). This finding suggests that BBX22 is under highly coordinated regulation at both the transcript and protein levels.

To further assess the impact of post-translational 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 35S promoter in this transgenic plant, the BBX22 transcripts should accumulate constitutively regardless of light or dark treatments. As expected, transcripts derived from the BBX22 transgene were expressed at high levels at all time points examined (Figure 2A). Interestingly, the accumulation kinetics of BBX22 in *bbx22BBX22ox* plants essentially mimic that in wild-type seedlings (Figure 1B, 2B). No or very low BBX22 level could be detected in dark-grown tissues, and truncated BBX22 is abundant in seedlings illuminated with 24 h of light (designated as L24 hereafter; Figure 2B). The increased level of truncated BBX22 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
A 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.

**BBX22 is a short-lived protein degraded by 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 was determined as a proportion of full-length BBX22 remaining relative to BBX22 expression at 8 h of light treatment (Figure 3). The half-lives (t_{1/2}) of BBX22 were 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 dark treatment accelerates its degradation.

The involvement of 26S proteosome 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 < 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 MG132 treatment under light or dark conditions, respectively. The effective stabilization of BBX22 protein by MG132 indicates that 26S proteasome is responsible for the degradation of BBX22 under both light and dark conditions.

**BBX22 contributes to the inhibition of hypocotyl elongation under short-day conditions**
Six-d-old *bbx22* exhibits light hyposensitivity (i.e., long hypocotyl) under SD but not long-day (LD) conditions (Datta et al., 2008). We have confirmed this phenotype for 4-d-old seedlings (Figure 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 (AUC). Results showed that BBX22 accumulates to a higher level under SD than LD conditions (Figure 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 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.

**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 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., 2000). 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., 2000) 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 (Figure 5A, 5B). 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 (Figure 7). 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 (Figure 5C), only residual BBX22-GFP protein was present in BBX22-GFPox plants grown in the dark (Figure 5D). In contrast, a high level of BBX22-GFP protein was detected in *cop1* mutants (Figure 5D), which suggests that COP1 is required for the selective degradation of BBX22 in the dark.
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 FRET assay (Datta et al., 2008). When co-expressed with COP1, BBX22 showed a re-distribution 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 Figure 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 pair-wise comparison, the transgene (35S:*BBX22*) was introduced into the corresponding wild-type Arabidopsis Ler ecotype by genetic crossing. As shown in Figure 6B, transcripts of the *BBX22* transgene accumulated to comparable levels in Ler and *hy5* mutants under both dark and L_24 conditions. BBX22 protein was effectively degraded in both Ler and *hy5* mutants in the dark (Figure 6C). However, in L_24, residual full-length BBX22 protein could be detected in the *hy5* mutant but not in Ler (Figure 6C). This result suggests that the efficient degradation of BBX22 protein is compromised in the light-grown *hy5* mutant. Increased level of truncated BBX22
was also observed in hy5 (L24 in Figure 6A, 6C left panel), which suggests that HY5 may contribute to the degradation of both full-length and truncated BBX22.

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 hy5hyh double mutant. The hy5 and hy5hyh mutants showed a comparable BBX22 degradation pattern (Figure 6B, 6C), 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 (Figure 2B, 5D, 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 (Figure 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 (Figure 7A) and light responsiveness under SD conditions (Figure 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% that of the wild type (Figure 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 (Figure 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 (Figure 7A and 7B).

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* [sth3*cop1* 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) < 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- (*p*-value=2 x 10^{-4}) or hormone-related (*p*-value=3.4 x 10^{-8}) pathways. Among these genes, 207 showed at
least 2-fold differential expression in cop1BBX22-GFP compared to cop1 plants at an FDR < 0.05 (Supplemental Table S1). These genes were considered to be specifically regulated by BBX22 and were selected for detailed analysis.

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 1 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 1, 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 (Figure 7). In total, 83% of BBX22-regulated genes were light-responsive genes as compared with only 27% in ATH1 (p-value=1.6 x 10^{-62}). Consistent with being a downstream gene of HY5 (Chang et al., 2008), BBX22 could regulate genes responding to various light qualities (p-value=1 x 10^{-27} to 4.6 x 10^{-62}, Table 1). Also, 42% of BBX22-regulated genes are differentially regulated by treatment with multiple plant hormones (p-value=9 x 10^{-12}, Table 1). The over-representation 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 post-translational 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 (Figures 1, 2 and 6).

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 1). Consistent with the previous observation that BBX22 regulates chloroplast development (Chang et al., 2008), genes regulated by BBX22 include \textit{ELIP1}, \textit{ELIP2}, \textit{CRY3/CRYD}, and \textit{SIG5/SIGE}, which encode chloroplast proteins (Yao et al., 2003; Tsunoyama et al., 2004; Heddad et al., 2006; Pokorny et al., 2008). \textit{ELIP1} and \textit{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 \textit{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 \textit{ELIP2} for optimal chlorophyll accumulation.

BBX22 also upregulates genes involved in flavonoid and anthocyanin biosynthesis pathways, including \textit{CHS}, \textit{CHI}, \textit{F3H} and \textit{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., 2007).
et al., 2008; Buer and Djordjevic, 2009). This observation explains the excess accumulation of anthocyanin in etiolated *cop1BBX22-GFPox* plants (Figure 7). In addition to being upregulated 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 downregulates hormone-responsive genes and genes involved in cell wall modification (Figure 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 BR (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 the SD condition. 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 (Figure 8). The short half-lives of BBX22 protein (Figure 3) allow 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 (Figure 5). Because BBX22 does not physically interact with COP1 (Datta et al., 2008), identifying protein(s) 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 (SPA1), 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 photomorphogenic 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 (Figure 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 previously proposed (Datta et al., 2008). However, BBX22 is still largely degraded in *hy5* and *hy5hyh* (Figure 6). This observation suggests that an unknown factor(s) contributes mainly to BBX22 degradation in the light. Further identification of BBX22 interacting partners by co-immunoprecipitation...
and proteomic characterization will help in 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 increased hypocotyl elongation rate in the dark phase (Figure 4). Our results indicate that *bbx22* still exhibits rhythmic growth, unlike the arrhythmic growth patterns observed in 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 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). 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 Landsberg erecta (Ler) 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 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 half-strength MS medium with 1% sucrose and 0.3% gelrite at 4°C for 4 days to synchronize the germination. Seedlings were grown at 22°C in the dark, under a 16-h/8-h or an 8-h/16-h light (100 μmol m⁻² s⁻¹)/dark photoperiod for 4 days 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 media was 1/2X MSMO with 1% sucrose; images were captured with 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 use of the ggplot2 plotting package (Wickham and Hadley, 2009).

5 Immunoblot analysis and degradation kinetics

To generate a BBX22-specific antibody, we used C-terminal 143 amino acid (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, Madison, NJ) for generating rat anti-BBX22 polyclonal antibody. Total protein was isolated as described (Al-Sady et al., 2006) with minor modification. In brief, seedlings underwent extraction with boiled extraction buffer [4M urea, 5% SDS, 15% glycerol, 100 mM Tris-HCl, pH 8, with freshly added 10 mM 2-mercaptoethanol, 2 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 3 μg/ml leupeptin and 1X complete protease inhibitor (Roche, Penzberg, Germany)]. In total, 30 to 50 μg protein was separated on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA), and blots were probed with the anti-BBX22, then horseradish peroxidase (HRP)-conjugated anti-rat antiserum (Sigma, St. Louis, MO) as a secondary antibody. Immunoblotting with Anti-α-tubulin (Sigma) and alkaline phosphatase-conjugated anti-mouse antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) was used to measure endogenous α-tubulin as a loading control. Chemiluminescence HPR substrate (Millipore, Billerica, MA) 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 UVP
Biospectrum 600 Imaging System (UVP, Cambridge, UK). Protein half-lives ($t_{1/2}$) were calculated by regression analysis. For determining the proteasome dependent-degradation of BBX22, seedlings were pre-incubated with 50 μM MG132 (Biomol/Enzo, Farmingdale, NY) for 1 h before the addition of cycloheximide.

For determining BBX22 protein levels at different times of a 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 vs. time curves (AUC) were obtained by use of the Area below Curves macro (trapezoidal rule) in SigmaPlot 9 (Systat Software, Inc., San Jose, CA).

**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 RNA was separated by 1% formaldehyde-agarose gel and transferred to Hybond N+ membrane (GE Healthcare, Buckinghamshire, UK). 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 Arabidopsis ATH1 Genome Array (ATH1-121501, Affymetrix Inc., San Jose, CA) for gene expression analysis as described (Chang et al., 2008). MicroArray Suite 5.0 (Affymetrix Inc.) and GeneSpring 7.3 (Agilent
Technologies, Santa Clara, CA) 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 pair-wise expression comparison for each probe set. Only genes with FDR < 5% [SAM, (Tusher et al., 2001)] in triplicate biological repeats were selected for further analyses. Genes with ≥2 or ≤0.5 fold change in expression levels between cop1BBX22-GFPox and the wild type were analyzed for association with use of Gene Ontology (GO) and GeneSpring GX 10. Among those genes with > 2-fold change in level between cop1BBX22-GFPox and cop1-4 were considered BBX22 regulated. The datasets have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through the GEO Series accession number GSE22983.
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Figure legends

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) Col or *bbx22* seedlings. Endogenous α-tubulin was a loading control (TUB). (B) BBX22 protein and (C) mRNA are transiently accumulated during photomorphogenesis. Immunoblot was used to detect the accumulation of BBX22 in etiolated (dark; D) 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 (dark; D) 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 standard deviations were calculated from 3 replicates and plotted. tBBX22: truncated BBX22.

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). tBBX22: truncated BBX22.
Figure 3. **BBX22 is a short-lived protein degraded by 26S proteasome.**

The degradation kinetics of BBX22 protein were determined as 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 (open circle, solid line) and 20 min in the dark (closed circle, solid line). The degradation of BBX22 was blocked by treatment with MG132 under both light (open circle, dash line) and dark (closed circle, dash line) conditions. $n = 3$.

Figure 4. **BBX22 protein functions in the inhibition of hypocotyl elongation under short-day conditions.**

(A) *bbx22* mutant is hyposensitive to light only under the short-day (SD) condition. Hypocotyl length was measured for 4-d-old seedlings grown under SD or long-day (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; open triangle, solid line) compared to that in plants under LD conditions (open circle, dash line). 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. Open bar indicates light periods and solid bar indicates dark periods. $n = 4$. (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), white areas indicate day (lights on). Shaded areas around each growth trace (blue and red) show standard error. $n = 29$. 


Figure 5. **COP1 is required for selective degradation of BBX22 in the dark.**

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).

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 Ler, *hy5* and *hy5hyh* plants expressing 35S:*BBX22* and grown in the dark (D) for 4 d or in 4-d-old etiolated seedlings illuminated with light for 24 h (L24). The ethidium bromide-stained image was used to show the amount of total RNA samples loaded in each lane. (C) Immunoblot was used to detect BBX22 protein in plant samples used in (B). The detection of endogenous α-tubulin was performed as a loading control (TUB). tBBX22: truncated BBX22.

Figure 7. **cop1BBX22-GFPox has exaggerated light responsiveness.**

Exaggerated *cop* phenotypes, the extra-inhibition of hypocotyl growth and excess anthocyanin accumulating both in cotyledons and hypocotyl (marked by arrowheads) were seen when BBX22-GFP was overexpressed under both (A) dark and (B) SD conditions. Hypocotyl length was measured in 4-d-old seedlings grown under (A) dark or (B) SD conditions. * and #: significantly different from Col and corresponding *cop1*
allele, respectively. (p < 0.01, Student’s t test; n = 20 to 32). Bar size 1 mm.

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. Over-accumulated BBX22 in cop1 gives rise to an exaggerated light responsiveness by altering the expression of genes responsive to light and hormone signals.

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

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

Northern blot analysis was used to examine BBX22 transcripts in etiolated (dark; D) seedlings and seedlings illuminated with light for 1 to 24 h. BBX22 transcripts of a single size (~1.3 kb) were detected. The full-length coding region of BBX22 was used as a probe. The methylene blue-stained image was used to show the amount of total RNA samples loaded in each lane.
Table 1. BBX22 regulates light- and hormone-responsive genes.

| Treatment       | ATH1 (%) | BBX22-regulated (%) | p-value     | References                  |
|-----------------|----------|----------------------|-------------|-----------------------------|
| cop1-like       | 14.7     | 70.0                 | $7.2 \times 10^{-71}$ | (Hu et al., 2009)           |
| Y276H-phyB      | 13.1     | 63.8                 | $2.7 \times 10^{-63}$ | (Hu et al., 2009)           |
| pifq            | 4.5      | 30.0                 | $1.1 \times 10^{-32}$ | (Leivar et al., 2009)       |
| MIF1ox          | 0.5      | 3.9                  | $1.4 \times 10^{-5}$  | (Hu and Ma, 2006)           |
| light           | 27.2     | 83.1                 | $1.6 \times 10^{-62}$ |                             |
| Wc              | 15.7     | 61.8                 | $2.3 \times 10^{-50}$ | (Jiao et al., 2005; Chang et al., 2008) |
| Rc              | 17.2     | 70.0                 | $4.6 \times 10^{-62}$ | (Jiao et al., 2005; Leivar et al., 2009) |
| FRc             | 8.2      | 42.0                 | $5.6 \times 10^{-39}$ | (Jiao et al., 2005)         |
| Be              | 11.8     | 42.0                 | $1.0 \times 10^{-27}$ | (Jiao et al., 2005)         |
| High Light      | 6.4      | 36.7                 | $1.3 \times 10^{-36}$ | (Kleine et al., 2007)       |
| hormone         | 20.5     | 41.5                 | $9.0 \times 10^{-12}$|                             |
| IAA             | 3.5      | 14.0                 | $3.3 \times 10^{-10}$ | (Nemhauser et al., 2006)    |
| BR              | 4.0      | 13.0                 | $1.2 \times 10^{-7}$  | (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                 | $1.7 \times 10^{-08}$ | (Nemhauser et al., 2006)    |
| ABA             | 12.9     | 24.6                 | $5.6 \times 10^{-6}$  | (Nemhauser et al., 2006)    |
| ACC             | 2.4      | 14.0                 | $4.6 \times 10^{-14}$ | (Nemhauser et al., 2006)    |
| CK              | 2.2      | 4.8                  | 0.027        | (Nemhauser et al., 2006)    |

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.
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) Col or bbx22 seedlings. Endogenous α-tubulin was a loading control (TUB). (B) BBX22 protein and (C) mRNA are transiently accumulated during photomorphogenesis. Immunoblot was used to detect the accumulation of BBX22 in etiolated (dark; D) 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 (dark; D) 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 standard deviations were calculated from 3 replicates and plotted. tBBX22: truncated BBX22.
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). tBBX22: truncated BBX22.
Figure 3. **BBX22 is a short-lived protein degraded by 26S proteasome.** The degradation kinetics of BBX22 protein were determined as 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 (open circle, solid line) and 20 min in the dark (closed circle, solid line). The degradation of BBX22 was blocked by treatment with MG132 under both light (open circle, dash line) and dark (closed circle, dash line) conditions. $n = 3$. 
Figure 4. BBX22 protein functions in the inhibition of hypocotyl elongation under short-day conditions.
(A) bbx22 mutant is hyposensitive to light only under the short-day (SD) condition. Hypocotyl length was measured for 4-d-old seedlings grown under SD or long-day (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; open triangle, solid line) compared to that in plants under LD conditions (open circle, dash line). 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. Open bar indicates light periods and solid bar indicates dark periods. n = 4. (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), white areas indicate day (lights on). Shaded areas around each growth trace (blue and red) show standard error. n = 29.
Figure 5. **COP1 is required for selective degradation of BBX22 in the dark.**
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*-trangene 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).
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 Ler, *hy5* and *hy5hyh* plants expressing 35S:*BBX22* and grown in the dark (D) for 4 d or in 4-d-old etiolated seedlings illuminated with light for 24 h (L24). The ethidium bromide-stained image was used to show the amount of total RNA samples loaded in each lane. (C) Immunoblot was used to detect BBX22 protein in plant samples used in (B). The detection of endogenous α-tubulin was performed as a loading control (TUB). tBBX22: truncated BBX22.
Figure 7. *cop1BBX22-GFPox* has exaggerated light responsiveness.
Exaggerated *cop* phenotypes, the extra-inhibition of hypocotyl growth and excess anthocyanin accumulating both in cotyledons and hypocotyl (marked by arrowheads) were seen when BBX22-GFP was overexpressed under both (A) dark and (B) SD conditions. Hypocotyl length was measured in 4-d-old seedlings grown under (A) dark or (B) SD conditions. * and +: significantly different from Col and corresponding *cop1* allele, respectively. (p < 0.01, Student’s *t* test; n = 20 to 32). Bar size 1 mm.
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. Over-accumulated BBX22 in *cop1* gives rise to an exaggerated light responsiveness by altering the expression of genes responsive to light and hormone signals.
Supplemental Figure S1. **BBX22 expresses as a single-sized transcript.**

Northern blot analysis was used to examine BBX22 transcripts in etiolated (dark; D) seedlings and seedlings illuminated with light for 1 to 24 h. BBX22 transcripts of a single size (~1.3 kb) were detected. The full-length coding region of BBX22 was used as a probe. The methylene blue-stained image was used to show the amount of total RNA samples loaded in each lane.