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BZS1, a B-box Protein, Promotes Photomorphogenesis Downstream of Both Brassinosteroid and Light Signaling Pathways

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ABSTRACT Photomorphogenesis is controlled by multiple signaling pathways, including the light and brassinosteroid (BR) pathways. BR signaling activates the BZR1 transcription factor, which is required for suppressing photomorphogenesis in the dark. We identified a suppressor of the BR hypersensitive mutant *bzr1–1D* and named it *bzr1–1D* suppressor1-*D*ominant (*bzs1–D*). The *bzs1–D* mutation was caused by overexpression of a B-box zinc finger protein BZS1, which is transcriptionally repressed by BZR1. Overexpression of *BZS1* causes de-etiolation in the dark, short hypocotyls in the light, reduced sensitivity to BR treatment, and repression of many BR-activated genes. Knockdown of *BZS1* by co-suppression partly suppressed the short hypocotyl phenotypes of BR-deficient or insensitive mutants. These results support that BZS1 is a negative regulator of BR response. BZS1 overexpressors are hypersensitive to different wavelengths of light and loss of function of BZS1 reduces plant sensitivity to light and partly suppresses the *constitutive photomorphogenesis 1* (*cop1*) mutant in the dark, suggesting a positive role in light response. BZS1 protein accumulates at an increased level after light treatment of dark-grown *BZS1–OX* plants and in the *cop1* mutants, and BZS1 interacts with COP1 *in vitro*, suggesting that light regulates BZS1 through COP1-mediated ubiquitination and proteasomal degradation. These results demonstrate that BZS1 mediates the crosstalk between BR and light pathways.

Key words: photomorphogenesis; light signaling; Brassinosteroid; *BZS1*; *Arabidopsis*.

INTRODUCTION

Wild-type *Arabidopsis* seedlings grown in the dark have long hypocotyls and closed cotyledons with undifferentiated chloroplast, a phenomenon termed etiolation or skotomorphogenesis. When exposed to light, seedlings undergo de-etiolation (also called photomorphogenesis) and thus display short hypocotyls, open cotyledons, and chloroplast differentiation (Wei and Deng, 1996). This light-induced developmental switch is controlled by the photoreceptor-mediated signaling transduction pathways, which act in part by inactivating the COP1 E3 ubiquitin ligase and stabilizing positive transcription factors. In addition to light signaling, brassinosteroid (BR) is another key signal that controls the switch between skotomorphogenesis and photomorphogenesis, as BR-deficient and insensitive mutants show de-etiolation phenotypes in the dark (Chory et al., 1991; Li et al., 1996; Szekeres et al., 1996; Song et al., 2009). Light and BR act antagonistically in photomorphogenesis. However, the molecular mechanisms of this antagonism are not fully understood.

BR signals are perceived by BRI1 receptor kinase and transduced through a well-defined signal transduction pathway to activate members of the BZR family transcription factors (Kim et al., 2009; Kim and Wang, 2010; Clouse, 2011). BZR1 and BZR2/BES1 transcription factors play central roles in BR regulation of plant growth and gene expression (Wang et al.,

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2002; Yin et al., 2002; He et al., 2005; Yin et al., 2005). The dominant *bzr1–1D* mutation causes constitutive BZR1 activation due to enhanced dephosphorylation by PP2A (Tang et al., 2011), and *bzr1–1D* fully suppresses the photomorphogenesis phenotype of *bri1-116* and reverses the expression changes of about 80% of the genes affected in *bri1-116* (Sun et al., 2010). A large number of light-induced genes are activated in the BR-deficient or insensitive mutants in the dark (Chory et al., 1991; Li et al., 1996; Szekeres et al., 1996; Song et al., 2009), suggesting that light and BR may regulate common transcription factors. Indeed, a recent study showed that a member of the GATA family transcription factors (GATA2) is transcriptionally repressed by BZR1 but posttranslationally activated by light signaling (Luo et al., 2010). GATA2 controls a subset of genes co-regulated by light and BR to promote photomorphogenesis. Many other transcription factors mediate light-regulated gene expression (Lau and Deng, 2010), but whether any of them are also regulated by BR remains unknown.

B-box proteins are a group of transcription factors containing one or more B-box domains that are stabilized by binding to zinc ions (Klug and Schwabe, 1995). There are 32 B-box containing proteins with diverse functions in *Arabidopsis* (Khanna et al., 2009). For example, CONSTANS (CO) is an important regulator of photoperiodic flowering (Putterill et al., 1995; Onouchi et al., 2000). The CO-like genes *COL1* and *COL2* are involved in regulating the circadian clock (Ledger et al., 2001). *STH2*, *STH3*, and *COL3* are positive regulators of light responses (Datta et al., 2006, 2007, 2008), whereas *STO* and *STH1* are negative regulators of photomorphogenesis (Khanna et al., 2006; Indorf et al., 2007).

In this study, we identified an activation-tagged *bzr1–1D* suppressor (*bzs1–D*), which showed dwarfism and suppressed *bzr1–1D*'s phenotypes of stem kink and insensitivity to BR biosynthesis inhibitor BRZ. The phenotypes of *bzs1–D* are caused by overexpression of a B-box protein we named BZS1 (also named BBX20), which is repressed at the transcription level by BR. Overexpression and loss-of-function experiments demonstrate that BZS1 plays a negative role in BR responses but positively regulates light responses. BZS1 protein level is increased upon light treatment of dark-grown seedlings and in the *cop1* mutant. BZS1 interacts with COP1 *in vitro*, suggesting that light may regulate BZS1 accumulation through COP1. These data demonstrate that BZS1 acts downstream of both BR and light pathways. This study supports a mechanism of BR-light crosstalk through transcriptional repression and posttranslational activation of photomorphogenesis-promoting transcription factors.

RESULTS

Isolation and Characterization of the *bzr1–1D* Suppressor Mutant

The activation tagging strategy has been successfully used in identification of novel BR signaling components, including BRS1, BAK1, BRL1, BSU1, BEN1, ATBS1, and TCP1 (Li et al., 2001, 2002; Mora-Garcia et al., 2004; Zhou et al., 2004; Yuan et al., 2007; Wang et al., 2009; Guo et al., 2010; Kang et al., 2010). To better understand the molecular mechanism of BZR1 function and identify additional components of the BR pathway, we previously carried out a large-scale activation tagging screen in *bzr1–1D* background (Cao et al., 2008). One of the mutant lines partially suppressed phenotypes of *bzr1–1D*; therefore, we named it *bzr1–1D suppressor1-Dominant* (*bzs1–D*). The *bzr1–1D;bzs1–D* mutant showed stronger BR-deficient phenotypes, such as dwarfism and extended lifecycle, compared to *bzr1–1D* itself (Figure 1A). The *bzr1–1D;bzs1–D* plants showed no stem kink, which is one of the most obvious phenotypes of light-grown *bzr1–1D* (Figure 1B), and a reduced degree of silique kink compared to *bzr1–1D* (Figure 1C).

The *bzr1–1D* mutant is insensitive to brassinazole (BRZ), a BR biosynthesis inhibitor (Wang et al., 2002). BRZ inhibits hypocotyl elongation of wild-type in the dark but not of *bzr1–1D* (Figure 1D and 1E). While *bzr1–1D;bzs1–D* plants had similar hypocotyl length compared to wild-type and *bzr1–1D* when grown on medium without BRZ, their hypocotyls were shorter than *bzr1–1D* on the medium containing

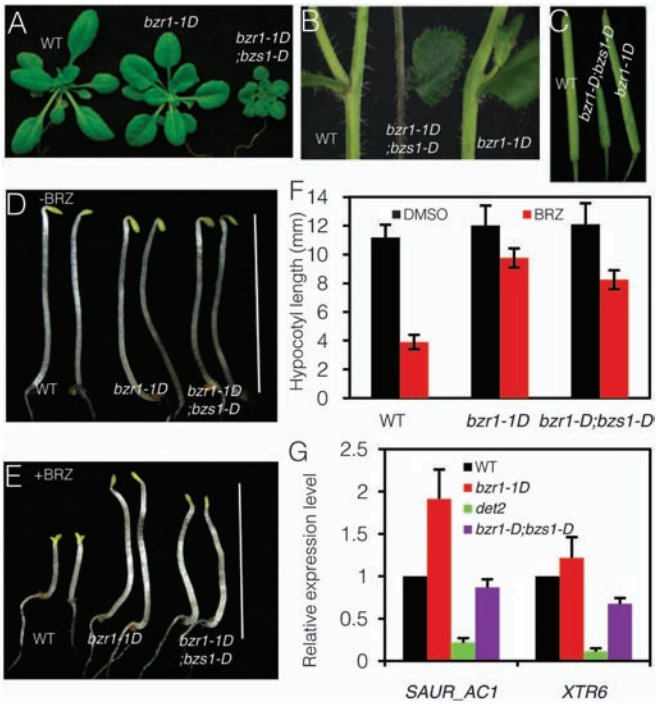

Figure 1. The *bzs1–D* Mutation Partly Suppresses *bzr1–1D*'s Phenotypes.

(A) Phenotypes of soil-grown 3-week-old plants.
(B) The stem kink phenotypes.
(C) The silique kink phenotype.
(D, E) Selective dark-grown seedlings on regular half-strength MS medium (D) or half-strength MS medium containing 2 μM BRZ (E).
(F) The average hypocotyl length of seedlings grown on medium with or without 2 μM BRZ. Error bars indicate SD (n = 40).
(G) Quantitative RT–PCR analysis of the expression levels of *SAUR_AC1* and *XTR6*. *UBC30* was used as internal control.

2 μM BRZ, suggesting *bzs1–D* partially suppressed *bzr1–1D*'s BRZ-insensitive phenotype (Figure 1E and 1F).

To test whether *bzs1–D* affected the expression of BR-regulated genes, the expression levels of two BR-responsive genes, *SAUR-AC1* and *XTR6*, were analyzed by quantitative reverse transcription (RT)–PCR. The expression levels of these two genes increased in *bzr1–1D* but decreased in the BR-deficient mutant *det2*. In *bzr1–1D;bzs1–D*, the expression levels of these two genes were reduced dramatically compared to *bzr1–1D* (Figure 1G). The results indicate that *bzs1–D* partially suppresses multiple developmental, physiological, and molecular phenotypes of *bzr1–1D*.

The *bzr1–1D* mutation suppresses the phenotypes of BR-deficient mutant *det2* and BR-insensitive mutant *bin2-1* (Wang et al., 2002). When *bzs1–D* was crossed into *det2* and *bin2-1*, and the double mutants *bzs1–D;det2* and *bzs1–D;bin2-1(+/–)* had shorter hypocotyls in the dark and more dramatic dwarf phenotypes in the light compared with *det2* single mutant and *bin2-1* heterozygote, respectively, while the segregated *bzs1–D* itself was phenotypically indistinguishable from wild-type (Figure 2A–2D). These genetic results suggest that *bzs1–D* has a negative effect on the BR pathway.

bzs1–D Overexpresses a B-box Protein

The population of segregated heterozygous *bzr1–1D;bzs1–D* lines showed a 3:1 (Basta+:Basta–) ratio, and the basta resistance co-segregated with the mutant phenotypes, indicating a single T-DNA insertion is responsible for the phenotypes of *bzr1–1D;bzs1–D*. Using thermal asymmetric interlaced PCR (TAIL–PCR), a T-DNA flanking sequence was identified at 1084 bp upstream of the translational start codon of gene *At4g39070* (Figure 3A). As activation tagging usually activates the genes in the vicinity of the insert (Weigel et al., 2000), we investigated the expression levels of the genes near the T-DNA insertion site. Quantitative RT–PCR result showed that *At4g39070* was overexpressed in mutant *bzr1–1D;bzs1–D*, but the other two flanking genes *At4g39080* and *At4g39090* were not significantly affected (Figure 3B). To confirm that the mutant phenotype is caused by overexpression of *At4g39070*, we overexpressed *At4g39070* driven by the CaMV35S promoter in the *bzr1–1D* background. From 16 independent transgenic lines, 10 lines recapitulated *bzr1–1D;bzs1–D* mutant phenotype and the level of recapitulation was correlated to the transcript level (Figure 3C and 3D). Overexpression of *At4g39070* in *bzr1–1D* also partly restored the sensitivity of *bzr1–1D* to BRZ (Supplemental Figure 1). RNA interference (RNAi) was also used to knock down the expression of *At4g39070* in *bzr1–1D;bzs1–D* mutant. From 11 transgenic plants, 10 plants restored *bzr1–1D* phenotype, and the phenotypes correlated with reduced expression levels of *At4g39070* (Figure 3E and 3F). Both overexpression and knockdown experiments confirmed that the phenotype of *bzr1–1D;bzs1–D* was due to overexpression of *At4g39070*, so we defined this gene as *BZS1*.

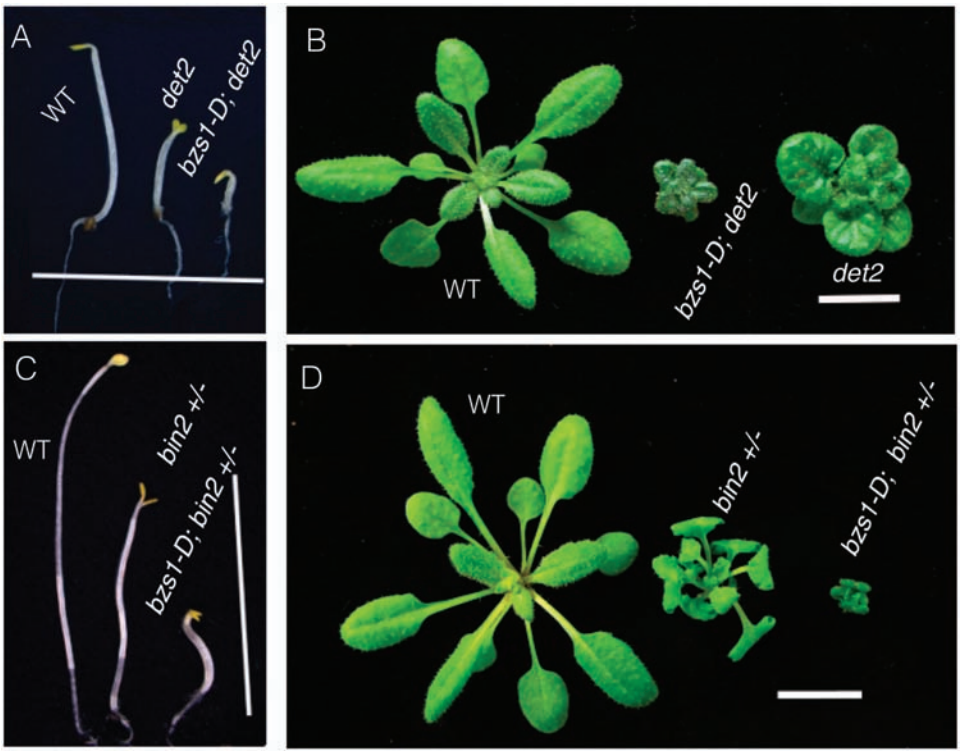

Figure 2. The *bzs1–D* Mutation Aggravates Phenotypes of BR-Deficient Mutant *det2* and Insensitive Mutant *bin2-1*.
(A, C) Five-day-old etiolated seedlings.
(B, D) Three-week-old soil-grown plants.

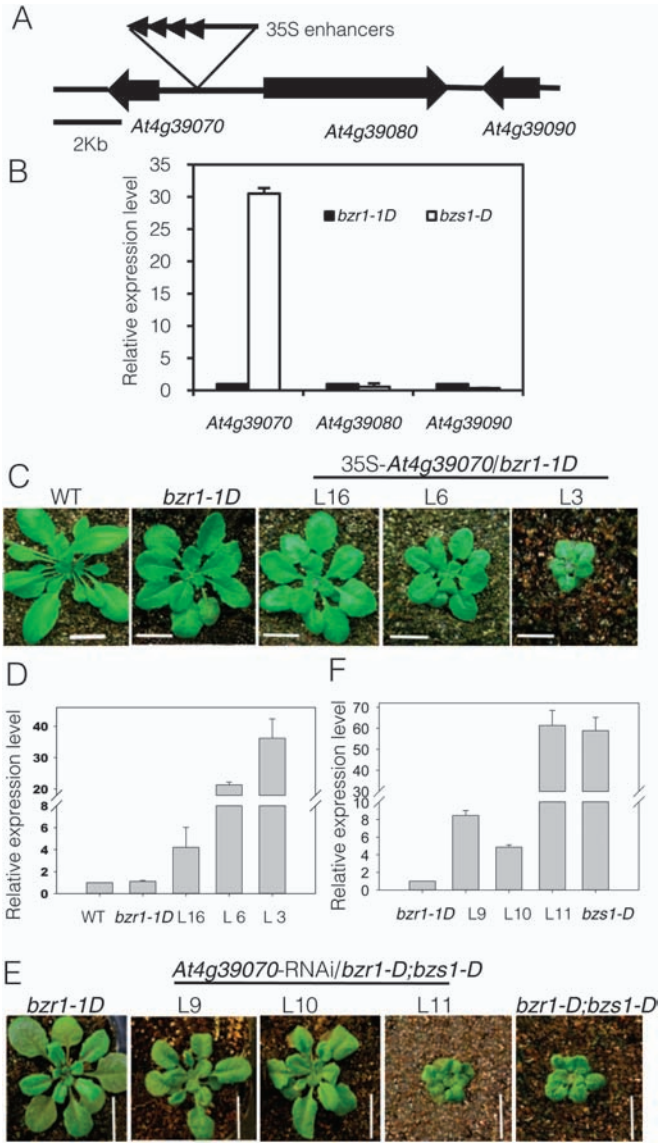

Figure 3. *BZS1/BBX20* Is Responsible for the *bzs1–D* Phenotypes.

(A) A diagram of the genomic region flanking the T-DNA insertion site in *bzr1–1D;bzs1–1D*.

(B) Quantitative RT–PCR analysis of the genes flanking the T-DNA insertion.

(C) Overexpression of *BZS1* in *bzr1–1D* (two independent lines: L6 and L3) recapitulates phenotype of *bzr1–1D;bzs1–D*.

(D) Quantitative RT–PCR analysis of the expression level of *BZS1* in three independent overexpression lines.

(E) Suppressing the expression of *BZS1* using RNAi in *bzr1–1D;bzs1–D* (two independent lines: L9 and L10) restores *bzr1–1D* phenotype.

(F) Quantitative RT–PCR analysis of the expression level of *BZS1* in *BZS1–RNAi* plants. For all the qRT–PCR analysis, *UBC30* was used as internal control.

BZS1 encodes a 242-amino-acid B-box transcription factor that is a member of the BBX family and has been named BBX20 (Khanna et al., 2009). The expression pattern of *BZS1* was examined using *BZS1* promoter fused with GUS reporter gene. GUS stain results showed that *BZS1* is expressed through the entire life circle, from etiolated seedlings, young seedlings, margins of adult leaves, flower buds, flowers, and siliques (Supplemental Figure 2A–2H). Consistently with *BZS1* being repressed by BZR1 (Sun *et al*., 2010), the *BZS1::GUS* expression was reduced by BR treatment (Supplemental Figure 2B and 2D). In addition, the subcellular localization of BZS1 was examined using *35S::BZS1–YFP* transgenic plants. Confocal microscope imaging results showed that BZS1 localized to both nucleolus and cytoplasm (Supplemental Figure 2I–2K).

BZS1 Is a Negative Regulator in BR Response

To further analyze the functions of BZS1, a *BZS1* overexpression construct (*35S::BZS1*) was transformed into wild-type plants. Among 23 transgenic plants, 19 plants were smaller than wild-type control. RT–PCR analysis showed that the dwarf phenotype correlated with overexpression of *BZS1*, and two transgenic lines (*BZS1–OX8* and *BZS1–OX21*) showing medium and strong phenotypes were selected for further analysis (Figure 4A and 4B). Two of the four transgenic lines showing no obvious phenotype when grown in soil (*BZS1–CS1* and *BZS1–CS2*) showed lower expression levels of *BZS1* than wild-type control, apparently due to co-suppression (Figure 4A and 4B).

When grown on half-strength Murashige-Skoog (MS) medium in the light, the *BZS1–OX* lines showed shorter hypocotyls, and the *BZS1–CS* lines showed significantly longer hypocotyls than wild-type (Figure 4C). While average hypocotyl lengths of the *BZS1–CS* lines were only slightly longer than wild-type, the tallest *BZS1–CS* individuals were obviously taller than the tallest wild-type. It seems that not all *BZS1–CS* seedlings maintain co-suppression. The *BZS1–OX* plants had shorter roots than wild-type when grown on medium without BR. Increasing concentration of BR inhibits root growth of wild-type plants but the *BZS1–OX* plants showed reduced response (Figure 4D), suggesting that accumulation of BZS1 reduces BR sensitivity. By contrast, two *BZS1–CS* lines had slightly longer roots than wild-type on medium without BR and had slightly shorter roots on medium with high concentration of BR, although the differences are not statistically significant. These results suggest that BZS1 reduces BR sensitivity (Figure 4D).

Quantitative RT–PCR results revealed that the expression levels of three BR-induced genes *DREPP*, *At5g44670*, and *SAUR-AC1* were decreased in the *BZS1–OX* plants compared to wild-type (Figure 4E). To further analyze the possible role of BZS1 in BR-regulated responses, the *BZS1–CS* lines (*CS1* and *CS2*) were crossed into BR mutants *det2* and *bin2-1*. The double mutants *BZS1–CS1;det2* and *BZS1–CS2;det2* had longer hypocotyls than *det2* in the dark (Figure 4F and 4G). Similarly, *BZS1–CS1* and *CS2* also increased the hypocotyl elongation of *bin2+/–* in the dark (Figure 4H and 4I). These results together demonstrate that BZS1 is a negative regulator of BR response.

BZS1 Promotes Photomorphogenesis

When grown in the dark, *BZS1–OX* lines showed de-etiolation phenotypes with short hypocotyls and open cotyledons

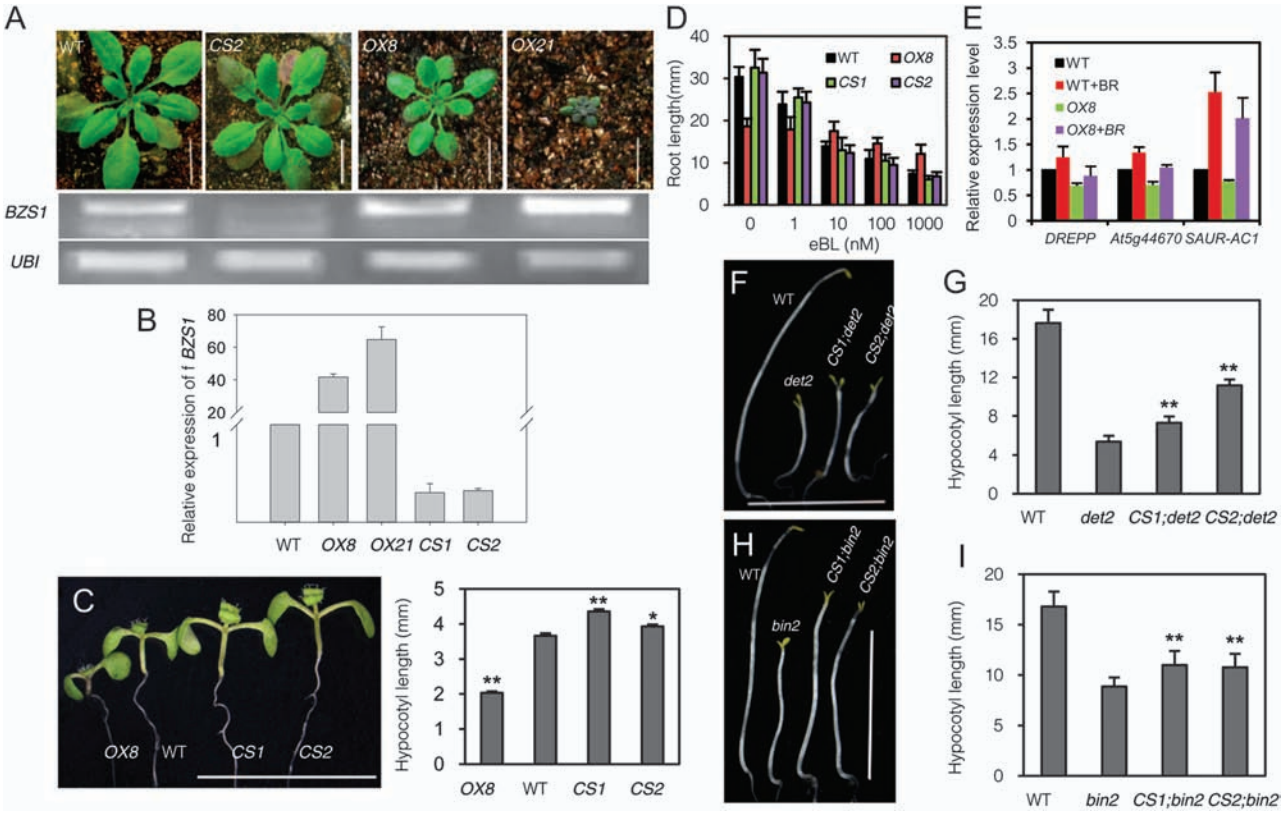

Figure 4. Overexpression of *BZS1* Partly Inhibits BR Signaling.

(A) Soil-grown 3-week-old plants of wild-type and transgenic plants (upper panel); RT–PCR analysis of *BZS1* expression (lower panel).
(B) Quantitative RT–PCR analysis of *BZS1*expression level in WT and *BZS1–OX* transgenic lines (*OX8* and *OX21*) and co-suppressed lines (*CS1* and *CS2*). *UBC30* was used as internal control.
(C) Phenotypes of 7-day-old light-grown seedlings of wild-type, *OX8*, *CS1*, and *CS2* (left panel) and the measured hypocotyl lengths (right panel). Representatives of the tallest seedlings of each population were chosen for photographs. Error bars indicate SD (n = 30), and significant differences from wild-type are marked (** $P < 0.01$, * $P < 0.05$).
(D) Quantification of the root length of seedlings grown on half-strength MS containing various amounts of BR.
(E) qRT–PCR analysis of downstream genes in wild-type and *BZS1–OX8* plants with or without 100 nM eBL treatment (3 h). *UBC30* was used as internal control.
(F, H) Dark-grown phenotypes of *det2* (F) or *bin2-1* (H) crossed with *BZS1–CS* lines (*CS1* and *CS2*).
(G, I) Hypocotyl lengths of dark-grown seedlings in (F) and (H), respectively. Error bars indicate SD (n = 20), and significant differences from wild-type are marked (** $P < 0.01$, * $P < 0.05$).

(Figure 5A). The phenotypes of de-etiolation in the dark and hypersensitive to light of *BZS1–OX* plants indicated that BZS1 promotes photomorphogenesis and may be involved in light signaling. To investigate whether BZS1 is downstream of a specific photoreceptor, *BZS1–OX* plants and the two *BZS1–CS* lines were grown in different light conditions. *BZS1–OX* had shorter hypocotyls, while *CS1* and *CS2* showed slightly but significantly longer hypocotyls compared to wild-type in blue and red light (Figure 5B–5E and Supplemental Figure 3). Similarly, *bzr1–1D;bzs1–D* was hypersensitive to both red and blue light (Supplemental Figure 4). These results suggest that BZS1 is a common factor downstream of multiple photoreceptors and promotes photomorphogenesis.

STH2, the closest homolog of BZS1, has been shown to play a positive role in light signaling. The loss-of-function mutant *sth2-1* is hyposensitive to blue, red, and far-red light (Datta et al., 2007). We generated a *BZS1* co-suppression line in *sth2-1* background. Quantitative RT–PCR analysis showed the expression of *BZS1* was reduced by about 30% in *BZS1–CS3;sth2-1* plants (Figure 6B). Fluence response test showed that *BZS1–CS3;sth2-1* plants had longer hypocotyls under various fluence rates of different lights than *sth2-1* single mutant (Figure 6A, 6C, and 6D). These data suggest that *BZS1* and *STH2* play overlapping or redundant roles as positive regulators of light responses.

BZS1 Affects Genes Responsive to Both Light and BR

To further understand the function of BZS1 in BR and light signaling pathways, we compared the transcriptomic changes caused by *BZS1* overexpression, light treatment, and *bri1* (Luo et al., 2010; Sun et al., 2010). Seven-day-old light-grown seedlings of *BZS1–OX* and wild-type (WT) were analyzed by

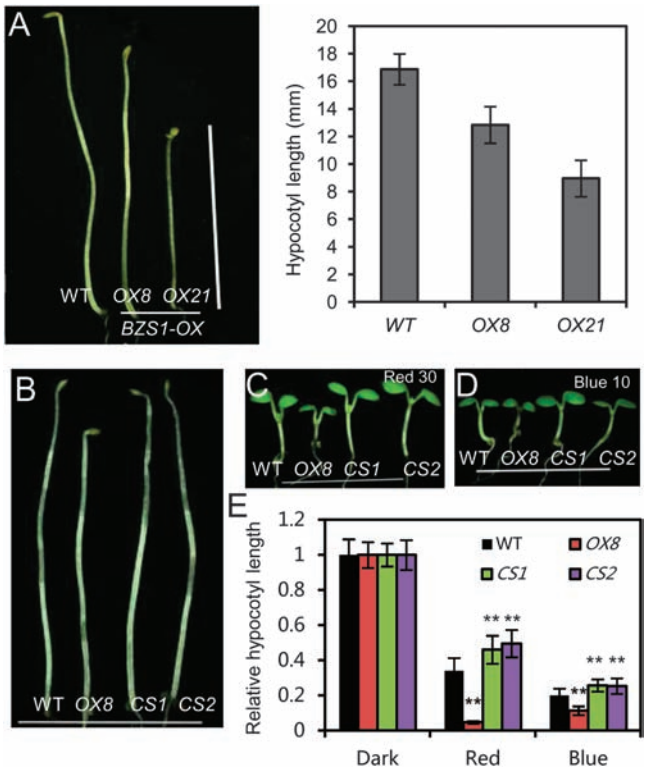

Figure 5. BZS1 Promotes Photomorphogenesis.

(A) Phenotypes of wild-type (WT) and two *BZS1–OX* transgenic lines (*OX8* and *OX21*) grown in the dark. The hypocotyl length measurement of seedlings shown in (A) is in the right panel. Error bars indicate SD (n = 30).

(B–D) Phenotypes of *BZS1–OX* and *BZS1–CS* lines grown in the dark (B), red light (30 μmol m^{-2} s^{-1}) (C), and blue light (10 μmol m^{-2} s^{-1}) (D), for 7 d. Representatives of the tallest seedlings of each population were chosen for photographs.

(E) Relative hypocotyl length from (B), (C), and (D). Error bars indicate SD (n = 20), and significant differences from wild-type are marked (** $P < 0.01$, * $P < 0.05$).

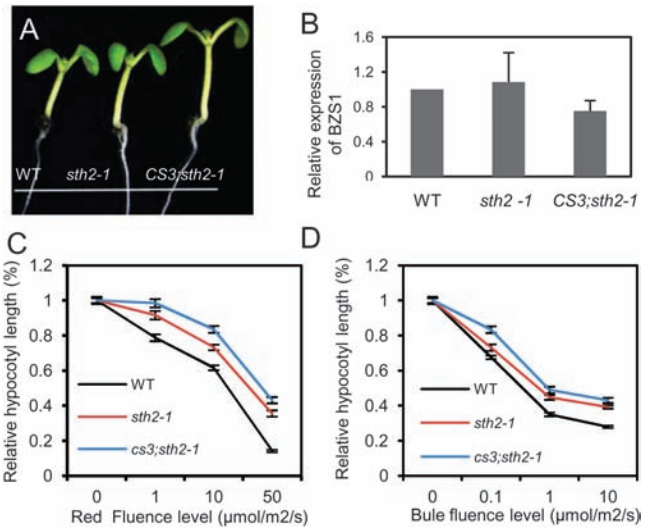

Figure 6. BZS1 Functions Redundantly with STH2.

(A) Phenotypes of 7-day-old seedlings of wild-type, *sth2-1*, and *BZS1–CS3;sth2-1* grown in blue light (10 μmol m^{-2} s^{-1}).

(B) Expression level of *BZS1* analyzed by quantitative RT–PCR.

(C, D) Fluence response of wild-type, *sth2-1*, and *BZS1–CS3;sth2-1* measured as relative hypocotyl lengths under red light (C) or blue light (D). Error bars present standard error (n = 20).

microarray using the *Arabidopsis* ATH1 array (Affymetrix). The results showed that expression levels of 285 genes were altered in *BZS1–OX* plants, with 145 genes up-regulated and 140 down-regulated (fold ⩾ 1.5 and q ⩽ 0.05; Supplemental Table 1). About 66% (189 of the 285) of these genes were affected by light in at least one of the microarray analyses of light-responsive genes (Luo et al., 2010). Among these, about 89% (168) were affected in the same way by *BZS1–OX* and light treatment (Supplemental Figure 5 and Supplemental Table 2). confirming BZS1 as a positive regulator in the light signaling pathway. About 65 genes (23% of the 285) differentially expressed in *BZS1–OX* were also affected by *bri1-116* mutation, and 83% of these 65 genes were also regulated by light (Supplemental Figure 5 and Supplemental Table 3). Among the genes co-regulated by BZS1 and light or *bri1-116* mutation, about 89% or 77% were affected in the same way by *BZS1–OX* and light treatment or by *bri1-116* mutation, respectively. The gene expression results indicate a role of BZS1 in mediating the antagonistic effects of BR and light on gene expression and photomorphogenesis.

Light Regulates BZS1 Protein Accumulation Through COP1

Quantitative RT–PCR result showed that the transcript level of *BZS1* was decreased in the light, but induced in the dark (Figure 7A and 7B). Similarly, *BZS1* expression was inhibited in the *cop1* mutants, suggesting light inhibits the transcription of *BZS1* (Figure 7C). Such RNA expression patterns are opposite to the positive function of BZS1 in promoting photomorphogenesis. We thus examined the BZS1 protein level. Interestingly, the BZS1 protein level was increased in *BZS1–OX* plants after light treatment and in the *cop1* mutant compared to WT (Figure 7D and 7E). The opposite changes of BZS1 RNA and protein by both light and *cop1* mutation is likely due to negative feedback inhibition of BZS1 transcription by BZS1 protein or independent regulation at transcriptional and post-translational levels. Suppression of *BZS1* in *cop1-4* and *cop1-6* partly suppressed the photomorphogenic phenotypes of *cop1* in the dark, confirming that accumulation of BZS1 contributes to the de-etiolation phenotype of *cop1* mutant (Figure 7F and 7G).

COP1 is an E3 ubiquitin ligase that inhibits photomorphogenesis by promoting ubiquitination and degradation of positive regulators of light signal transduction pathways, such as HY5 and STH3 (Osterlund et al., 2000; Datta et al., 2008). The accumulation of BZS1 protein in *cop1* mutants suggests that BZS1 might be a substrate of COP1. *In vitro* pull-down assay showed that BZS1 interacted physically with COP1 (Figure 7H). These results suggest that BZS1 is another

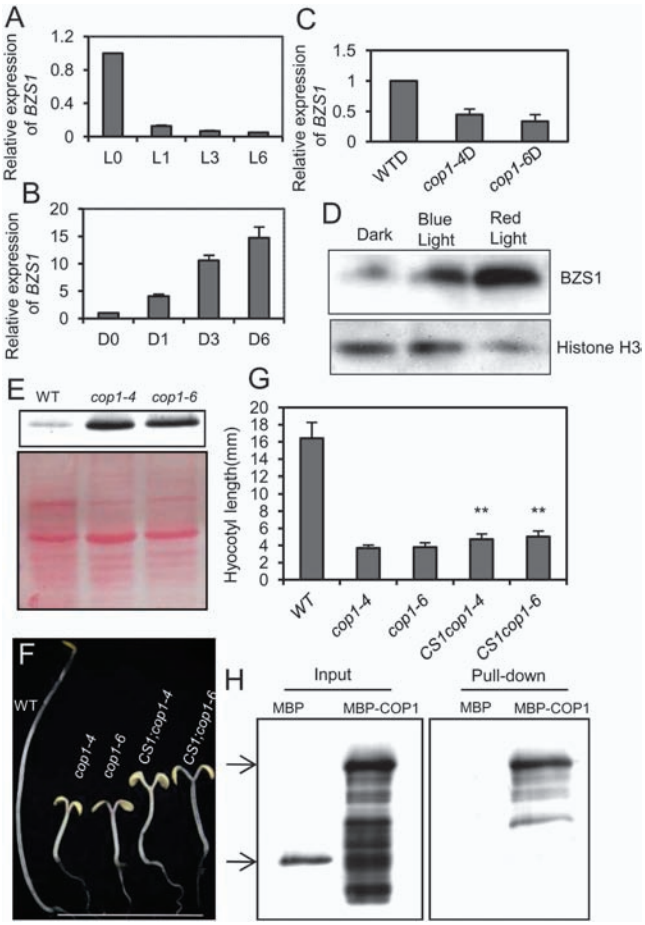

Figure 7. Light Regulates BZS1 Accumulation through COP1.

(A) Light represses *BZS1* transcription levels. Etiolated wild-type seedlings were treated with white light for the indicated time and mRNA levels of *BZS1* were analyzed by qRT–PCR.

(B) Dark induces *BZS1* transcription levels. Light-grown wild-type seedlings were moved into dark for the indicated time and mRNA levels of *BZS1* were analyzed by qRT–PCR.

(C) Quantitative RT–PCR analysis of *BZS1* expression in the dark-grown wild-type and *cop1* mutants.

(D) Immunoblot analysis of BZS1 protein in 3-day-old dark-grown *OX8* line seedlings treated with blue and red light for 1 h.

(E) Immunoblot analysis of BZS1 protein levels in dark-grown wild-type and *cop1* mutants.

(F) Dark-grown phenotypes of *cop1* mutants crossed with *BZS1–CS1*.

(G) Hypocotyl lengths of wild-type, *cop1* mutants, *CS1*;*cop1-4*, and *CS1*;*cop1-6*. Representatives of the tallest seedlings of each population were chosen for photographs. Error bars represent SD ($n = 30$) and significant differences from *cop1* mutants are marked (** $P < 0.01$, * $P < 0.05$).

(H) *In vitro* pull-down assay showing the interaction between BZS1 and COP1.

positive transcription factor of photomorphogenesis that is directly regulated by COP1. Light-induced inactivation of COP1 leads to accumulation of BZS1 protein, which promotes photomorphogenesis, whereas BR inhibition of BZS1 transcription contributes to BR's repression effects on photomorphogenesis.

DISCUSSION

Interactions between light and brassinosteroid signals are important for photomorphogenic development in higher plants. The underlying molecular network is not fully understood. In this study, we show that the B-box protein BZS1/BBX20 is another component of the network integrating BR and light signaling on gene expression and photomorphogenic development. BR represses the transcriptional level of BZS1/BBX20 through BZR1, whereas light promotes BZS1/BBX20 protein accumulation through inhibiting COP1's activity. Our results provide further evidence for a general mode of BR-light crosstalk whereby photomorphogenesis-promoting transcription factors are activated by light-induced stabilization, through inhibition of the COP1-mediated ubiquitination/degradation, but are inhibited by BR signaling through BZR1-mediated transcriptional repression (Luo et al., 2010).

Genetic interactions with BR mutants support a negative role of BZS1/BBX20 in BR response. Overexpression of *BZS1* suppressed the BR-activation phenotypes of *bzr1–1D*, but enhanced the dwarf phenotypes of BR-deficient *det2-1* and BR-insensitive *bin2-1*. In contrast, knockdown of *BZS1* expression partly suppressed the phenotypes of *det2-1* and *bin2-1*. These results strongly support that BZS1/BBX20 plays a role in repressing BR-regulated response. BZS1/BBX20 has been shown in Chromatin-immunoprecipitation experiments to be a BZR1 target gene, and its expression is repressed by BR, suggesting that BR represses *BZS1* expression through BZR1 (Sun et al., 2010). Consistently with BZS1/BBX20 function downstream of BZR1, *bzs1–D* had no effect on BZR1 accumulation and phosphorylation (Supplemental Figure 6). *BZS1–OX* suppressed multiple phenotypes of *bzr1–1D*, including BRZ-insensitivity and stem kink, suggesting that BZS1/BBX20 is a major component that mediates multiple BZR1-regulated developmental processes.

The phenotypes of *BZS1–OX* and *BZS1–CS* plants grown under different light conditions indicate a role of BZS1 in photomorphogenesis. The *BZS1–OX* plants showed constitutive photomorphogenesis phenotypes in the dark, and hypersensitivity to blue, red, and white light. In contrast, *BZS1–CS* plants showed increased hypocotyl elongation under all light conditions. Furthermore, *BZS1–CS* partly suppresses the photomorphogenic *cop1* mutant. These results support that BZS1 is a positive regulator of light response. The weak phenotype of the *BZS1–CS* plants is likely due to partial suppression of *BZS1* and redundant function with homologous genes. The close homologs of BZS1/BBX20, namely STH2/BBX21, STH3/BBX22, and STO/BBX24, have been shown to positively regulate photomorphogenesis (Datta et al., 2007; Indorf et al., 2007). BZS1/BBX20 shares 71.82%, 54.13%, and 56.48% of amino-acid identity in the B-box domain to STH2/BBX21, STH3/BBX22, and STO/BBX24, respectively (Supplemental Figure 7). While *sth2-1* and *BZS1–CS* plants each show weak long-hypocotyl phenotypes, the *BZS1–CS/sth2-1* plants showed

stronger phenotypes than each parental line, consistently with their redundant functions in promoting photomorphogenesis.

To mediate light-regulated development, BZS1/BBX20 protein level is increased by light signaling. Light regulation of BZS1/BBX20 protein is likely mediated by the COP1 ubiquitin ligase, as BZS1/BBX20 accumulates in the *cop1* mutants and interacts with COP1 *in vitro*. Similarly, other members of the BBX family, including CO, COL3, STO, STH1, STH2, and STH3, have been shown to interact with COP1 (Holm et al., 2001; Datta et al., 2006, 2007, 2008; Liu et al., 2008). The triple mutant *sth2-1 sth3 cop1* showed a stronger cop1-suppression phenotype compared to the *sth3cop1* double mutant, suggesting that STH2 might also be the target of COP1 degradation (Datta et al., 2008). As such, interaction with COP1 seems a conserved feature of the B-box proteins consistently with their roles in light-regulated development.

Our microarray data of *BZS1–OX* plants provide further evidence for a positive role in regulating light-responsive gene expression. Although only a small set of genes are affected by *BZS1* overexpression, the majority of these genes are light-responsive and the expression changes caused by *BZS1–OX* are similar to the effects of light. How BZS1 regulates gene expression remains unknown, but its homolog STH2/BBX21 has been shown to interact with HY5 (Datta et al., 2007; Holtan et al., 2011), a COP1-targeted b-ZIP transcription factor that positively regulates photomorphogenesis. It is likely that BZS1/BBX20 also interacts with HY5 or other light signaling transcription factors to control a subset of light-responsive genes.

Together, our results demonstrate that BZS1/BBX20 mediates the antagonistic interactions between light and BR signaling pathways. BZS1/BBX20 is oppositely regulated by BR and light through transcriptional and posttranscriptional mechanisms, respectively (Supplemental Figure 8). This is analogous to the GATA2 factor, which is also transcriptionally repressed by BZR1 and posttranslationally stabilized by light through a COP1-dependent mechanism (Luo et al., 2010). Therefore, it seems a general strategy of crosstalk that BR represses the expression and light increases accumulation of positive transcription factors that promote photomorphogenic gene expression and development. In addition, light inhibits BR response by increasing the expression of the Membrane Steroid Binding Protein 1 (MSBP1) (Shi et al., 2011). Furthermore, the BR signaling transcription factor BZR1 and the phytochrome-interacting factor (PIL5) co-regulate many common target genes (Sun et al., 2010). A recent study also showed that the HLH factor PAR1, which was previously identified as a negative regulator of shade-avoidance response (Bou-Torrent et al., 2008), binds to PIF4 to inhibit PIF4 DNA binding (Hao et al., 2012). The BR-activated HLH factor PRE1 interacts with and inhibits PAR1, freeing PIF4 to promote the response to shade and darkness (Hao et al., 2012). Therefore, the interactions between BR and light pathways appear to be mediated by a complex network of molecular interactions.

METHODS

Plant Material and Growth

Arabidopsis thaliana ecotype Columbia-0, various mutants, and transgenic plants obtained in this study were grown at 22°C under white light (16-h light/8-h dark cycles unless stated otherwise) either on half-strength MS medium or in the soil. *Arabidopsis* seeds were sterilized with 75% ethanol plus 0.01% Triton X-100 for 5 min, followed by three rinses with 95% ethanol, and dried in the hood. The surface-sterilized seeds were sowed on 0.7% phytoagar plates containing half-strength MS medium and 1% sucrose. The plates were kept at 4°C for 3 d and exposed to white light for 6 h before being transferred into the dark. Growth under red and blue light was carried out in an LED light chamber (E-30LEDL3, Percival) at 22°C. Seedlings were photographed and their hypocotyl lengths measured using ImageJ software (http://rsb.info.nih.gov/ij/).

Vector Construction and Transformation

A 1520-bp genomic fragment containing full-length *BZS1* open reading frame was amplified by PCR and then cloned into the *Bam*HI and *Kpn*I sites of the pSN1301 binary vector to place *BZS1* under the control of the CaMV 35S promoter. The primer sequence used was 5′-GGGGTACCCCAAGTTGGTTTGGATTCATG-3′ and 5′-CGGGATCCCGAGAGTGAGAAGAAGCAAT-3′.

To knock down *BZS1* in *bzr1–1D;bzs1–D*, a specific sequence was amplified from *bzr1–1D;bzs1–D* with primers 5′-CGAGCTCGGATCCTGGTCGGGTTTTGGCTCG-3′ and 5′-GGACTAGTGGTACCTTGCCAAGAAGACAGAGC-3′. This fragment was first digested by *Bam*HI and *Kpn*I for the reverse insert to vector pTCK309. The forward insert was generated by *Spe*I and *Sac*I digestion.

A total 2042-bp genomic fragment containing the promoter region of *BZS1* was amplified from WT genomic DNA and cloned into the vector pBI101 by enzymes *Bam*HI and *Sal*I. The primer sequence used was 5′-CGGGATCCACACCAAATCTTCATCTTTC-3′ and 5′-ACGCGTCGACGTCCAGTAGTACATCCATG-3′.

The *35S::BZS1–YFP* fusion construct was generated by inserting a full-length *BZS1* cDNA without stop codon fused to the N-terminus of the pEZR–LNY vector. Primer sequence 5′-ACGCGTCGACAGAGAAGGGTTTGTGATCC-3′ and 5′-CGGAATTCAGAGTGAGAAGAAGCAAT-3′ was used.

The *BZS1–OX*, *BZS1–RNAi*, *pBZS1::GUS*, and *35S::BZS1–YFP* binary constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101 and then introduced into *Arabidopsis thaliana* plants via floral dip method.

Total RNA Extraction and Quantitative RT–PCR Analysis

Total RNA was extracted by Trizol (Invitrogen, USA). About 500 ng RNA was reverse-transcribed by AMV reverse transcriptase (Takara Biotechnology, Ltd) following the manufacture's instruction. Quantitative RT–PCR analyses were carried out on

ABI7500 (Applied Biosystems, USA) by using SYBR Green reagent (Toyobo, Japan). Two or three biological repeats and three technical repeats were performed in each treatment. The *UBC30* gene was used as internal reference for all the qRT–PCR analysis.

Protein Expression and Antibody Preparation

The full-length *BZS1* cDNA was cloned into the pGEX–6p-1 vector to express GST–BZS1 protein in *E. coli* BL21 cells (Novagen). The recombinant fusion protein was purified using glutathione-agarose beads (GE Healthcare) and used to immunize rabbit. The anti-BZS1 antibody was purified from the immune serum using immobilize un-conserved fragment (amino acids 101–242) of BZS1 tagged with maltose binding protein (MBP) (Amino link Immobilization Kit, Pierce Biotechnology). The anti-histone H3 antibody for loading control was from Sigma (catalog number H9289).

Protein Purification and Pull-Down Assay

The GST–BZS1 protein was expressed using the pGEX–6P-1 vector in *E. coli* BL21 cells. The recombinant fusion protein was purified using glutathione-agarose beads, and the protein was mostly full-length based on it size in SDS–PAGE gel. COP1 fused to MBP was purified using amylose resin (New England Biolabs). For pull-down assay, glutathione beads containing 1 μg GST–BZS1 were incubated with MBP and MBP–COP1. The mixture was rotated in a cold room for 1 h and the beads were washed at least five times with wash buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl). The proteins were eluted from the beads by boiling in equal volume of 2 SDS buffer and loaded onto a SDS–PAGE gel. Gel blots were analyzed using anti-MBP antibody (NEB).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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