Darkness and gulliver2/phyB mutation decrease the abundance of phosphorylated BZR1 to activate brassinosteroid signaling in Arabidopsis

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SUMMARY
Light is essential for plant survival; as such, plants flexibly adjust their growth and development to best harvest light energy. brassinosteroids (BRs), plant growth-promoting steroid hormones, are essential for this plasticity of development. However, the precise mechanisms underlying BR-mediated growth under different light conditions remain largely unknown. Here, we show that darkness increases the activity of the BR-specific transcription factor, BZR1, by decreasing the phosphorylated (inactive) form of BZR1 in a proteasome-dependent manner. We observed that COP1, a dark-activated ubiquitin ligase, captures and degrades the inactive form of BZR1. In support of this, BZR1 is abundant in the cop1-4 mutant. The removal of phosphorylated BZR1 in darkness increases the ratio of dephosphorylated to phosphorylated forms of BZR1, thus increasing the chance of active homodimers forming between dephosphorylated BZR1 proteins. Furthermore, a transcriptome analysis revealed the identity of genes that are likely to contribute to the differential growth of hypocotyls in light conditions. Transgenic misexpression of three genes under the 35S promoter in light conditions resulted in elongated petioles and hypocotyls. Our results suggest that light conditions directly control BR signaling by modulating BZR1 stability, and consequently by establishing light-dependent patterns of hypocotyl growth in Arabidopsis.

Keywords: brassinosteroids, photomorphogenesis, hypocotyl elongation, proteasome, brassinazole, Arabidopsis thaliana.

INTRODUCTION
The ability of plants to adapt their growth patterns to different light conditions underscores an important survival strategy in natural environments. When not exposed to optimal light, plants mobilize resources to grow and reach the light as part of a developmental process termed the shade avoidance syndrome (SAS) (Morelli and Ruberti, 2002; Franklin and Whitelam, 2005; Vandenbussche et al., 2005). Arabidopsis seedlings exhibiting SAS have longer hypocotyls and smaller leaf blades than their light-grown counterparts. Growth-promoting plant hormones, including auxin and gibberellins, control SAS (Pierik et al., 2004; Alabadi et al., 2008; Feng et al., 2008; de Lucas et al., 2008; Tao et al., 2008; Stavang et al., 2009). Many genes are known to be involved in SAS (Carabelli et al., 1996; Steindler et al., 1999; Kozuka et al., 2005; Bou-Torrent et al., 2008; Sorin et al., 2009; Leivar and Quail, 2011). Arabidopsis loss-of-function phytochrome B (phyB) mutants (Bae and Choi, 2008) and PHYTOCHROME INTERACTING FACTOR (PIF) overexpression lines constitutively display SAS in the light (Kim et al., 2003), suggesting that these genes control the growth pattern under various light conditions.
Brassinosteroids (BRs) are growth-promoting plant steroid hormones, and thus mutants defective in BR biosynthesis or signaling display characteristic growth-deficiency phenotypes (Clouse et al., 1996; Li et al., 1996; Szekeres et al., 1996; Choe et al., 1998, 1999a,b, 2000). Interestingly, SAS phenotypes of phyB disappear when combined with mutations defective in BR biosynthesis (Luccioni et al., 2002), suggesting that BRs are implicated in phyB-mediated hypocotyl growth. However, the mechanism by which light conditions control the degree of BR activity remains elusive. A current model depends on the interplay of the two transcription factors: PIF4 and BRASSINAZOLE RESISTANT 1 (BZR1) (Oh et al., 2012). When seedlings elongate in darkness, PIF4 and BZR1 form a heterodimer that regulates the transcription of their target genes; GERMIN-LIKE PROTEIN 1 (GER1) is repressed and PACLOBUTRAZOL RESISTANCE genes (PRE5 and PRE6) are activated (Oh et al., 2012). As PIF4 is a light-labile protein (Lorrain et al., 2007), 2012), the phosphorylation status of PIF4-3, which shows resistance to a BR biosynthetic inhibitor only in darkness (Wang et al., 2002). It is likely that the deficiency of PIF4 in light conditions limits the growth-promoting effect of bza1-D. As such, BRs seem to participate in the differential growth of hypocotyls in part through their interaction with PIF4. However, it is not known how the activity of BZR1 itself is differentially controlled in light and darkness.

BZR1 is a substrate of a glycosyl synthase kinase 3β-like kinase, BR INSENSITIVE 2 (BIN2; Choe et al., 2002; Li and Nam, 2002; Li et al., 2001; Perez-Perez et al., 2002). Upon phosphorylation, BZR1 is captured by 14-3-3 proteins and sequestered in the cytoplasm (Gampala et al., 2007; Ryu et al., 2007), or its DNA binding affinity is reduced as part of an inactivation mechanism (Vert and Chory, 2006). Thus, the ratio of dephosphorylated to phosphorylated BZR1 serves as an index to estimate the signaling status of the plant (Wang et al., 2002). The phosphorylation status of BZR1 does not seem to change under different light conditions (He et al., 2002; Wang et al., 2002), however, and the hypocotyls of the quadruple mutant of pif1-4 (pifq) still elongate in response to darkness (Shin et al., 2009; Leivar and Quail, 2011), suggesting that a mechanism that directly regulates BZR1 activity exists and remains to be identified.

During our quest to discover the mechanisms that govern BZR1 activity, we found that the gulliver2/phyB-77 mutant possesses a reduced level of phosphorylated BZR1 in light. Surprisingly, we found that when plants are transferred from light to darkness, the protein levels of BZR1, mostly the phosphorylated forms, are gradually degraded in a proteasome-dependent manner. In vivo pull-down analyses revealed that CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ligase, binds to phosphorylated forms of BZR1. In addition, transcriptome analyses of phyB-77, bri1-5 and the phyB-77 bri1-5 double mutant revealed the unique set of genes that is likely to be regulated by BZR1 under the direction of both BR and light signaling. We show that light and darkness directly control components of the BR signaling pathway to confer optimal growth patterns according to the specific light conditions.

RESULTS AND DISCUSSION

Upregulation of feedback downregulation signaling in the phytochrome B-77 mutant

To understand the molecular mechanisms underlying the interaction between BRs and light, we isolated gulliver 2 (gul2) mutants by screening Arabidopsis mutant populations for elongated hypocotyls in the presence of both a BR biosynthetic inhibitor, brassinazole (Brz), and light. We isolated two alleles: gul2-1 and gul2-2. The gul2-1 mutant was in the Wassilewskija-2 (Ws-2) background and was mutagenized by ethyl methanesulfonate (EMS), whereas gul2-2 was obtained from a T-DNA activation-tagged mutant population in the Columbia-0 (Col-0) ecotype (Kim et al., 2014). Through the combined methods of map-based cloning and candidate gene sequencing, we revealed that both gul2-1 and gul2-2 mutants have defects in PHYTOCHROME B (PhyB), so they were renamed as phyB-77 and phyB-78, respectively. The phyB-77/gul2-1 mutant had a single base-pair substitution that changed Gln at the 438th residue to a premature stop codon. The untagged phyB-78/gul2-2 allele had a deletion mutation at 582–613 bp from the start codon, which caused a frameshift mutation, eventually introducing a premature stop codon at amino acid 196.

To learn how BR signaling interacts with this gul2/phyB, we first examined the epistatic interaction between phyB and a weak brassinosteroid insensitive 1 mutant bri1-5 (Noguchi et al., 1999). The phenotype of a double mutant of the strong allele bri1-1 and phyB-78 was indistinguishable from that of the bri1-1 single mutant, whereas the weak allele bri1-5 was dramatically suppressed by phyB-77 mutation (Figure 1a,b), suggesting that BR signaling is essential for light-dependent growth. The elongated phenotype of the phyB-77 single or phyB-77 bri1-5 double mutant could stem from increased BR biosynthesis or signaling, or both. Therefore, we quantified endogenous BRs and noticed that the levels of the most bioactive BR, brassinolide (BL), were significantly lower in the phyB-77 bri1-5 double mutant than in the bri1-1 single mutant (Figure 1c). As a reduced level of BL is considered a consequence of increased BR signaling activity, mediated by BZR1 (Choe et al., 2001; Wang et al., 2002; Chung et al., 2011, 2012; Kim et al., 2013), the reduction in bioactive BR levels in phyB-78 and phyB-77 bri1-5 mutants implies that feedback downregulation signaling is activated by phyB.
Consistently, the transcript levels of BR biosynthetic genes were low in phyB-77 bri1-5 mutant seedlings (Figure 1d). The transcript levels of CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD), DWARF 4 (DWF4) and CYP85A2 were high in bri1-5, but were suppressed in the double mutant, suggesting that feedback downregulation mechanisms are functional in the double mutant because of phyB-77.

To further examine the contribution of BZR1 in phyB signaling, we generated the phyB-78 35Spro:BZR1 line and determined the ratio of dephosphorylated BZR1 (dBZR1) to phosphorylated BZR1 (pBZR1; Figure 2a), a read-out of the BR signaling status (Wang et al., 2002). The phyB-78 35Spro:BZR1 line exhibited a long hypocotyl phenotype relative to 35Spro:BZR1 transgenic seedlings (Figure 2a). Furthermore, the phosphorylated form of BZR1 was reduced in the phyB-78 background (Figure 2b). Next, we examined the expression levels of genes known to be regulated by BZR1 in a light-dependent manner (Oh et al., 2012): GER1 was suppressed, and PRE5 and PRE6 were upregulated, in phyB mutants relative to the wild type (Figure 2c), suggesting that the elongated phenotype of phyB depends on the activation of BZR1 signaling.

phyB-77 activates BR signaling by reducing the abundance of BZR1 proteins

To identify the precise role of BZR1 in phyB-77, we sought to establish how light signaling modulates BR signaling pathways. As phyB mutants exhibit the same growth patterns as those of plants grown under shade, we performed the following experiments in total darkness or continuous white light (Figure 2d). After subjecting BZR1pro:BZR1-HA

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plants to various light conditions (L, light-grown control; LD, transferred to darkness for 6 h; LDL, transferred to darkness for 6 h and subsequently to light for 6 h), we found that most of the phosphorylated forms of BZR1 (pBZR1) disappeared in LD (Figure 2d), similar to the case under BL treatment (lane BL in Figure 2d), whereas the amount of protein in the dephosphorylated form (dBZR1) was similar for both LD and L (Figure 2d). The ratio of dBZR1 to pBZR1 increased upon both dark and BL treatment, indicating that BR signaling was activated by dark treatment. Conversely, when the dark-treated samples were returned to the light, pBZR1 became the dominant form (Figure 2d). Furthermore, a time-course analysis of a 3Spro:BZR1-HA line confirmed the darkness-dependent removal of BZR1 (Figure 2e). BZR1 levels gradually decreased and the dBZR1/pBZR1 ratio increased from as early as 3 h after dark treatment (Figure 2e). In support of the effect of darkness on BZR1 activity, the expression of marker genes known to be activated by BZR1, such as ACC SYNTHASE 5 (ACS5), PRE5, PRE6 and CPD (Oh et al., 2012), was upregulated in the LD12 sample (light-grown seedlings transferred to darkness for 12 h before RNA isolation), and reverted to control levels upon return to light in the sample designated as ‘LD12 → L12’ (Figure 2f). In addition, genes known to be repressed by darkness were downregulated in LD (Figure 2f). This dark-specific removal of BZR1, preferentially the phosphorylated form, and the simultaneous regulation of known marker genes suggest that darkness activates BR signaling and hypocotyl growth by changing the abundance of BZR1.
Regulation of BZR1 stability by COP1

The dramatic removal of BZR1 upon dark treatment led us to speculate that BZR1 degradation is associated with a 26S proteasome. When light-grown seedlings were treated with both darkness and MG132, a 26S proteasome inhibitor, BZR1 noticeably accumulated (Figure 3a), suggesting that the 26S proteasome is involved in the removal of BZR1. Therefore, we next sought to identify an E3 ligase that binds to BZR1. We tested COP1, because it is known to be active in darkness (Osterlund et al., 2000; Seo et al., 2004). Compared with the Col-0 wild type (Figure 3b, lane 2), the cop1-4 mutant accumulated BZR1 even after dark treatment (lane 5), suggesting that COP1 is involved in regulating BZR1 stability. An interaction between COP1 and BZR1 was further assessed through in vivo co-immunoprecipitation (CoIP) analyses (Figure 3c). Seedlings of double transgenic lines, 35Spro:BZR1-HA 35Spro:COP1-GFP, were grown under long-day conditions, transferred to darkness and incubated for two more days. The total proteins were prepared and immunoprecipitated using anti-GFP antibody. When the precipitated proteins were separated and immunoblotted with anti-HA antibody, BZR1-HA proteins were detected (Figure 3c). CoIP data indicate that COP1 interacted with BZR1, especially pBZR1 (Figure 3c). A possible interaction between COP1 and BZR1 was further assessed through yeast two-hybrid analysis (Figure 3d). Similar to a positive control that involved the binding of BIN2 to BZR1 (He et al., 2002), COP1 bound to BZR1 (Figure 3d). Both CoIP and the yeast two-hybrid test suggest that BZR1 and COP1 bind together as part of biological signal processing mechanisms.

Figure 3. BZR1 stability is regulated by COP1 in a 26S proteasome-dependent manner.
(a) Immunoblot analysis of BZR1 proteins. Seven-day-old light-grown seedlings expressing BZR1pro:BZR1-HA were treated or not with MG132 in the dark for 6 h. Actin was used as a loading control.
(b) Immunoblot analysis results of the 10-day-old seedlings grown under continuous light and subsequently subjected to LD (light to dark for 12 h) or LDL (light to dark for 12 h and back to light for 12 h) growth conditions. The total protein extracted from 10-day-old seedlings was immunoblotted with anti-BZR1. Actin was used as a loading control.
(c) Coimmunoprecipitation assay to test for the interaction between BZR1 and COP1 in 35Spro:BZR1-HA 35Spro:COP1-GFP Arabidopsis seedlings. Seedlings were grown under long-day conditions (with a photon fluence rate of 103 μmol m−2 s−1), and then transferred to the dark for 2 days. Total proteins were first immunoprecipitated with anti-GFP, and were subsequently immunoblotted with anti-HA and anti-GFP.
(d) Yeast two hybrid analysis. −LW indicates growth media lacking leucine (L) and tryptophan (W). DBD and AD are the vectors harboring the DNA binding domain and the DNA activation domain, respectively. Vector indicates the empty vector.
(e) qRT-PCR analyses of ACS5, GER1, PRE5 and PRE6 in Col-0 and cop1-4 treated with the same growth and light conditions as described in (b).
We then reasoned that the expression patterns of the marker genes would be affected in the cop1-4 mutant background. We monitored the expression of various marker genes in both wild-type and cop1-4 seedlings grown in three different conditions: continuous light (cL); continuous light-grown seedlings transferred to darkness for 12 h (LD12); and LD12 seedlings transferred back to the light for 12 h (LD12 → L12). Compared with the wild-type control, the induction of ACS5, PRE5 and PRE6 by darkness was greatly suppressed in cop1-4 mutants (Figure 3e). However, the darkness-dependent induction of these genes was canceled in the LD12 → L12 samples of both wild-type and cop1-4 backgrounds, suggesting that light-dependent repression regularly occurs in cop1-4 (Figure 3e). In support of this, we found that BZR1 accumulated in a similarly treated LDL sample of cop1-4 mutant seedlings (Figure 3b).

An oppositely regulated gene, GER1, was also misregulated in cop1-4 (Figure 3e). Our CoIP results, coupled with the findings that BZR1 accumulates in cop1-4 seedlings and that gene expression patterns are altered in cop1-4, suggest that binding of COP1 to BZR1 mediates proteasome-dependent degradation, and that the uncoupling of BZR1 degradation from darkness-triggered signaling caused mis-expression of the BZR1-regulated marker genes.

Identification of genes co-regulated by both light and BR signaling
To define the list of genes that are co-regulated by BRs and light, we performed a genome-wide transcriptome analysis using total RNAs prepared from the seedlings of four genotypes, i.e. Ws-2 wild type, phyB-77, bri1-5 and phyB-77 bri1-5 (Figure 4). We constructed a heat map based on 624 genes obtained after filtering with the criteria of \( P < 0.01 \) and fold change > 1.5 in the following three comparisons: phyB-77 versus Ws-2; bri1-5 versus Ws-2; and phyB-77 bri1-5 versus Ws-2 (Figure 4a,b; Tables S1–S5). We used these comparisons because they enabled us to identify epistatic interactions. According to the expression patterns summarized in Figure 4(b), bri1-5 is epistatic to phyB-77 for both ‘bd’ (i.e.genes upregulated in phyB-77 but downregulated in the bri1-5 single and phyB-77 bri1-5 double mutant, relative to the wild type) and ‘bu’ (i.e. upregulated in the bri1-5 single and phyB-77 bri1-5 double mutant), and phyB-77 is epistatic to bri1-5 for both the ‘pu’ (i.e. upregulated in phyB-77 and the double mutant) and ‘pd’ (i.e. downregulated in phyB-77 and the double mutant, but upregulated in bri1-5) types. To validate the microarray data, we subjected the genes marked by arrowheads (Figure 4b) to quantitative RT-PCR analysis. The two genes, At5g53870 and At1g17810, encode early nodulin-like protein 1 (ENODL1) and a major intrinsic protein family water channel protein, respectively, and were upregulated in phyB-77 but were repressed in bri1-5. In contrast, At3g30180 and At5g57530 encode CYP85A2 (BR6ox2) and xyloglucan endotransglycosylase 12 (XTH12), respectively, and were found to be repressed in phyB-77 but induced in bri1-5 in both microarray and qRT-PCR analyses (Figure 4c). In addition, a Venn diagram analysis revealed 123 genes at the intersection of phyB-77, bri1-5, phyB-77 bri1-5 and the genes defined as BR-regulated BZR1 targets (BRBTs; Figure 4d; Oh et al., 2012). We postulate that the growth pattern regulated by light and BRs depends on complex interactions between these genes (Table S6).

Functional validation of the identified genes
To understand the effects of these genes on growth, the 35Spro:At5g53870-HA construct was introduced into the bri1-5 mutant background (Figure 5a,b). We found that the dwarf phenotype of bri1-5 was suppressed, such that petioles elongated and leaves expanded (Figure 5a). The expression of At5g53870 was confirmed by protein gel blot analysis (Figure 5b). Furthermore, we expressed two PEROXIDASEs (PRX2 and PRX73) that were downregulated in phyB-77 but were upregulated in bri1-5 in the antisense orientation; both PRX2 RNAi and PRX73 RNAi transgenic plants exhibited elongated hypocotyls (Figure 5c,d), and the expression of the target genes was greatly reduced (Figure 5e).

Formation of dimers between phosphorylated and dephosphorylated forms of BZR1
Next, we sought to explain the darkness-dependent degragation of pBZR in terms of BR signaling. We hypothesized that BZR1 proteins with different levels of phosphorylation may bind with each other as part of a regulatory mechanism. To demonstrate binding between BZR1 proteins, we transiently expressed the epitope-tagged BZR1 constructs in protoplasts and carried out a CoIP analysis (Figure 6a,b). In our transient expression system, 35Spro:BZR1-HA and 35Spro:BZR1-cMyc constructs produced phosphorylated forms of BZR1 (Figure 6a, lane 1). The 35Spro:mBZR1-HA and 35Spro:mBZR1-cMyc constructs were previously reported to have mutations of S130A and S130A/S134A, respectively, in BZR1 that reduced the levels of phosphorylation (Ryu et al., 2007). We were able to clearly discern the proteins derived from these constructs as phosphorylated (shifted toward the top of the gel; Figure 6a, lanes 1 and 4) or dephosphorylated (Figure 6b, lanes 1 and 4) forms.

Lane 3 in Figure 6a shows the pBZR1-cMyc protein derived from a complex of pBZR1-cMyc and pBZR1-HA, and lane 6 indicates dBZR1-cMyc derived from the pBZR1-HA and dBZR1-cMyc complex (asterisk). This CoIP result suggests that BZR1 proteins with different levels of phosphorylation can form dimers with each other. To avoid a possible bias derived from the epitopes, and to determine the affinity of dBZR1 for dBZR1, we repeated the pull-down assay (Figure 6b). The pBZR1-HA derived from the pBZR1-HA and dBZR1-cMyc complex (lane 3), and the dBZR1-HA...
proteins from the dBZR-HA and dBZR1-cMyc complex (lane 6), were clearly detected. Interestingly, it seemed that pBZR-HA and dBZR1-cMyc (lane 3) had a greater affinity for each other than did dBZR-HA and dBZR1-cMyc (lane 6). The results of our pull-down assays suggest that BZR1 proteins can form dimers, and that pBZR1-pBZR1, pBZR1-dBZR1 and dBZR1-dBZR1 pairings are possible.

In conclusion, we propose a model that explains the roles of BZR1 in light and dark conditions. In the light (Figure 6c, left panel), the majority of BZR1 is phosphorylated by BIN2 and remains inactive in the cytoplasm (Ryu et al., 2007). Our results show that pBZR1 can trap dBZR1 to form a dimer between pBZR1 and dBZR1. In addition, PIF4 is labile in the light and less likely to form an active heterodimer with dBZR1. Because COP1 is not active in the nucleus in the light, the pools of pBZR1 and dBZR1 are maintained; however, in darkness (Figure 6c, right panel), COP1 becomes active and preferentially targets BZR1 for degradation, because pBZR1 is the major form present. The dBZR1 proteins, being freed from the complex of pBZR1

Figure 4. Identification of genes regulated by both light and brassinosteroids (BRs). (a, b) Heat maps of the 624 hierarchically clustered genes for which expression levels changed by more than 1.5-fold in phyB-77, bri1-5 and phyB-77 bri1-5, relative to the Ws-2 wild type. Seedlings were grown in red light for 5 days with a photon fluence rate of 30 μmol m−2 s−1 at 22°C. Genes labeled as 'bd' at the left of the heat map were upregulated in phyB-77 but downregulated in the bri1-5 single and phyB-77 bri1-5 double mutant, relative to the wild type. Genes downregulated in phyB-77, but upregulated in the bri1-5 single and phyB-77 bri1-5 double mutant were labeled 'bu'. Genes designated as 'pu' were upregulated in phyB-77 and the double mutant, but downregulated in bri1-5, and those named 'pd' were downregulated in phyB-77 and the double mutant, but upregulated in bri1-5. (b) Definition of the gene clusters, and magnified view of the groups marked with vertical bars. (c) Validation of the microarray data using qRT-PCR. Error bars are the standard deviations (n = 3). (d) Venn diagram of the co-regulated genes in phyB-77, bri1-5 and phyB-77 bri1-5, relative to Ws-2, and the set of genes defined as BZR1 binding target (BRBT) genes (Oh et al., 2012).
and dBZR1, can now form dimers with either dBZR1 or PIF4 to actively control downstream genes. It was previously shown that PIF4 can form a dimer with BZR1 and that the dimer controls the target genes that are regulated by both light and BRs (Oh et al., 2012).

In addition, GA is known to modulate BR signaling through controlling the stability of DELLA proteins (Bai et al., 2012). In darkness, the endogenous GA level goes up (Bae and Choi, 2008), resulting in the clearance of DELLA proteins (Itoh et al., 2003; Wang and Deng, 2011). Signaled by this increased GA level, both BZR1 and PIF proteins are released from DELLA, which enables these two proteins to form an active heterodimer and regulate the genes involved in hypocotyl growth (Feng et al., 2008; de Lucas et al., 2008; Bai et al., 2012; Jaillais and Vert, 2012). Our results fit well with this model. COP1-mediated degradation of pBZR1 should increase the possibility of dBZR1 to meet either dBZR1 or PIF4. The concerted action of GA and BR signals explains the mechanisms of hypocotyl growth under different light conditions. It is expected that future studies will reveal whether DELLA preferentially binds to pBZR1, and whether this binding affects the stability of pBZR1 protein in light.

Figure 5. Knock-in of ENODL1 (At5g53870) and knock-down of peroxidases stimulates growth. (a, b) Phenotypes and protein levels of bri1-5 plants and two transgenic lines overexpressing At5g53870 in the bri1-5 background. (a) Comparison of 4-week-old plants exhibiting the partial suppression of bri1-5. (b) Immunoblot analysis showing At5g53870 expression in transgenic lines, compared with the bri105 control. Proteins were extracted from the aerial tissues of adult plants. (c) 35Spro:PRX2 RNAi and 35Spro:PRX73 RNAi seedlings display elongated hypocotyls. (d) Measurement of hypocotyl and root lengths. Seedlings were grown on MS medium for 7 days under long-day conditions (16-h light/8-h dark, with a photon fluence rate of 80 μmol m⁻² s⁻¹ at 22°C. Error bars are standard deviations (n = 20; P < 0.001). Different lowercase letters above each bar indicate statistically significant differences. (e) Relative RNA levels of nascent PRX2 and PRX73 in wild-type and transgenic lines expressing PRX RNAi. Error bars are standard deviations (n = 3; P < 0.001).
Interaction of light and BRs in Arabidopsis

We thus revealed a mechanism by which Arabidopsis seedlings can grow faster in darkness without a simultaneous increase in the level of bioactive BRs. Light conditions regulate the stability of BZR1 via COP1 E3 ligase to control the formation of active dimers of dBZR1–dBZR1 or dBZR1–PIF4.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis seeds were surface sterilized and plated on agar-solidified MS media. After stratification at 4°C for 3 days, seedlings were grown under long-day (16-h light/8-h dark), continuous red light or continuous white light conditions at 22°C.

To introduce 35Spro:ENODL1 (At5g53870) into bri1-5, and 35Spro:COP1-GFP, 35Spro:BZR1-HA and 35Spro:PRX RNAi constructs into wild-type plants, coding sequences of each gene were cloned into pENTR/SD/D_TTOPO (Invitrogen, now Life Technologies, http://www.lifetechnologies.com), and then combined with the destination vector pEarley101, pMDC83 and pB7GWIWGII in frame with epitopes, respectively. The constructs were transformed into Arabidopsis using conventional techniques.

Quantitative analysis of endogenous BRs and sterols by gas chromatography–mass spectrometry (GC-MS)

To determine the endogenous levels of both sterols and BRs, the aerial parts of 5-week-old Arabidopsis plants were harvested and used to yield approximately 30 g of fresh tissues, and then lyophilized at –80°C. The tissues were extracted twice with 300 ml of methanol. Deuterium-labeled internal standards were added to the extracts. Purification and quantification of sterols and BRs were performed according to a method described previously (Fujikawa et al., 2002).

Coimmunoprecipitation

Previously, it was shown that site-directed substitution of Ser130 alone, or of both Ser130 and Ser134, with Ala in BZR1, designated as mBZR1-HA<sup>S130A</sup> and mBZR1-cMyc<sup>S130,134A</sup>, respectively, exhibited lower levels of phosphorylation than a wild-type form (Ryu et al., 2007). These mutated versions were used to represent dephosphorylated forms. The plasmid DNA constructs were transformed into yeast mesophyll-derived protoplasts that were freshly isolated from 3-week-old wild-type plants as previously described (Hwang and Sheen, 2001). Briefly, approximately 2 × 10⁶ protoplasts were transfected with 30 μg BZR1 and mBZR1 plasmid DNA. Transfected protoplasts were incubated for 6 h at room temperature and homogenized in immunoprecipitation buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM DTT, 0.1% Triton X-100, and full strength (1X) protease inhibitor cocktail (GenDEPOT, http://www.gendepot.com). The supernatant was incubated for 3 h at 4°C with agarose-conjugated anti-HA (Roche, http://www.roche.com) or anti-HA (Roche) diluted at 1/2000. Then, 30 μl of denatured proteins was subjected to SDS-PAGE on a 10% gel. After transfer to polyvinylidene difluoride membranes, the blot was immunoblotted with anti-HA (Roche) or anti-HA (Roche) diluted at 1/2000. The immunoblot was washed with phosphate-buffered saline buffer and incubated with the corresponding anti-horseradish peroxidase (HRP) antibodies for visualization of the protein bands (GenDEPOT).

Yeast two-hybrid assays

Full-length coding sequences of BZR1, BIN2 and COP1 were cloned into the low-copy yeast expression vectors pDEST22 (Gal4 AD) and pDEST32 (Gal4 BD). To test protein interactions, the corresponding plasmids were co-transformed into the yeast MaV203 strain. Successfully transformed colonies were identified on yeast synthetic drop-out media that lacked Leu and Trp (Clontech, http://www.cellsignal.com) or anti-HA (Roche) diluted at 1/2000. The immunoblot was washed with phosphate-buffered saline buffer and incubated with the corresponding anti-horseradish peroxidase (HRP) antibodies for visualization of the protein bands (GenDEPOT).
Protein extraction and immunoblot analysis

Total proteins were extracted from the tissues of 7-day-old seedlings grown on agar-solidified media using protein extraction buffer [50 mM Tris–Cl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1X protease inhibitor cocktail (GenDEPOT)]. Proteins were equally loaded on an 8 or 10% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The antibodies used (at 1:3000) were anti-αTUB (MP Biomedicals, http://www.mpbio.com) and anti-BZR1 (generated in our lab). Following three washings with Tris-buffered saline Tween-20 (TBST) buffer, membranes were incubated with HRP-linked secondary antibodies (GenDEPOT). The membranes were then washed again with TBST, and antibodies were visualized with a chemiluminescence method (Amer sham Bioscience, now GE Healthcare, http://www.gelifesciences.com). Protein gel blot analyses were performed multiple times, and the data shown are representative blots.

Microarray and quantitative RT-PCR analysis

Microarray experiments were performed using an Affymetrix ATH1 GeneChip system. Seedlings of four genotypes, Ws-2 wild type, bri1-5, phyB-77 and bri1-5 phyB-77, were grown for 7 days on 1X MS agar-solidified medium under continuous red light. Biological samples were analyzed in triplicate and on three chips per genotype. The microarray analysis generated 12 different CEL files. All data generated during this experiment were submitted to the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE46456. Statistical analyses of the data were performed according to methods described previously (Chung et al., 2011).

For validation of the microarray data, 7-day-old light-grown seedlings were ground to a fine powder in liquid nitrogen. Total RNA was prepared using Trizol (Sigma-Aldrich). Two micrograms of total RNA were reverse-transcribed (Fermentas, now Thermo Scientific, http://www.thermoscientific.com/fermentas), and equal quantities of reverse-transcribed products were used for the PCR reactions. Each template RNA was normalized using the UBO10 (AT4G05320) or TUB2 (AT5G62600) genes as loading controls. Quantitative RT-PCR was performed in 96-well plates (Applied Biosystems, http://www.appliedbiosystems.com) using a commercial qPCR kit (KapaBiosystems, http://www.kapabiosystems.com) in a volume of 20 μl. The reactions were performed in triplicate. Samples were quantified by generating standard curves using the obtained results with serially diluted control DNA. The relative mRNA levels represent fold change over the control. Each cDNA was normalized using UBO10 or TUB2. The oligonucleotide sequences used in the qRT-PCR are listed in Table S7.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Complete data of the microarray analysis using the RNAs prepared from Ws-2 wild type, phyB-77, bri1-5 and phyB-77 bri1-5 seedlings grown for 7 days under continuous red light.

Table S2. Expression data for 3201 genes, with a comparison of phyB-77 versus Ws-2 (P < 0.01).

Table S3. Expression data for 3157 genes, with a comparison of bri1-5 versus Ws-2 (P < 0.01).

Table S4. Expression data for 4453 genes, with a comparison of phyB-77 bri1-5 versus Ws-2 (P < 0.01).

Table S5. Expression data for 624 genes, with P < 0.01 at all three comparisons of phyB-77 versus Ws-2, bri1-5 versus Ws-2 and phyB-77 bri1-5 versus Ws-2.

Table S6. Expression data for the genes at each intersection in the Venn diagram.

Table S7. List of primers used in quantitative RT-PCR and cloning.

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