

The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*

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Brassinosteroids (BRs) are a class of steroid hormones essential for normal growth and development in plants. BR signaling involves the cell-surface receptor BRI1, the glycogen synthase kinase-3-like kinase BIN2 as a negative regulator, and nuclear proteins BZR1 and BZR2/BES1 as positive regulators. The interactions among these components remain unclear. Here we report that BRs induce dephosphorylation and accumulation of BZR1 protein. Experiments using a proteasome inhibitor, MG132, suggest that phosphorylation of BZR1 increases its degradation by the proteasome machinery. BIN2 directly interacts with BZR1 in yeast two-hybrid assays, phosphorylates BZR1 *in vitro*, and negatively regulates BZR1 protein accumulation *in vivo*. These results strongly suggest that BIN2 phosphorylates BZR1 and targets it for degradation and that BR signaling causes BZR1 dephosphorylation and accumulation by inhibiting BIN2 activity.

Brassinosteroids (BRs) are a class of steroid hormones that play important roles in plant growth and development (1–3). Deficiency in BR biosynthesis or signaling causes dramatic growth defects that include dwarfism, reduced apical dominance and fertility, delayed flowering and senescence, and photomorphogenesis in the dark (4–6). BRs are perceived by a cell-surface receptor kinase and transduced via an undefined signal transduction pathway, leading to changes in gene expression and growth (7, 8). BR signaling thus resembles the nongenomic steroid actions observed in some animal systems (9), but differs from the well-studied genomic steroid actions mediated by the nuclear receptors in animals (10).

Extensive genetic screens for recessive BR-insensitive *Arabidopsis* mutants have identified only one gene, *brassinosteroid insensitive 1* (*BRI1*), that is essential for BR response. *BRI1* encodes a leucine-rich-repeat receptor kinase localized to the plasma membrane (11–13). Molecular biochemical studies have shown that *BRI1* functions as the BR receptor. *BRI1* perceives BRs through its extracellular domain and transduces the signal by phosphorylating downstream signaling proteins that have yet to be identified (7).

Studies of the semidominant dwarf mutant *brassinosteroid insensitive 2* (*bin2*) have led to the identification of a potential downstream component that negatively regulates BR response. *BIN2* encodes a member of the glycogen synthase kinase-3 (GSK3)-like kinases (14, 15). The increased kinase activity by the semidominant *bin2-1* mutation and the phenotypes of transgenic plants with altered *BIN2* expression levels indicate that *BIN2* is a negative regulator for BR response and cell elongation (16). In animals, GSK3-like kinases play key roles as negative regulators in a variety of signaling pathways (17). Extracellular signals, such as insulin or growth factors, inhibit GSK3 kinases, allowing dephosphorylation of the substrates and activation of downstream responses (18, 19). As a negative regulator of BR response, *BIN2* might function in a manner

similar to the animal GSK3 kinases. *BIN2* may phosphorylate and inactivate downstream positive regulators and itself be inhibited by upstream BR signaling (16).

A genetic screen using the BR-biosynthetic inhibitor brassinazole has led to the identification of two homologous nuclear proteins that positively regulate BR responses (20, 21). The dominant *brassinazole resistant1-1D* (*bzr1-1D*) mutant is insensitive to brassinazole and suppresses both the BR biosynthetic mutant *de-etiolated 2* (*det2*) and the receptor mutant *bri1*, suggesting that *BZR1* acts downstream of *BRI1* in the BR signaling pathway (20). The *bzr1-1D* mutant plants show BR hyperresponse phenotypes in the dark but a weak dwarf phenotype and increased feedback inhibition of BR biosynthesis in light. This finding suggests that *BZR1* mediates both BR-induced growth response and feedback inhibition of BR biosynthesis and that *BZR1* activity might be modulated by light. The *BZR1* protein level is high in the nucleus of elongating cells. Both BR treatment and the *bzr1-1D* mutation increase *BZR1* accumulation, and overexpression of *BZR1* suppresses a weak allele of *bri1*. These results demonstrate that *BZR1* is a positive regulator of BR response (20).

BZR1 is a member of a small plant gene family, and at least one of its five *Arabidopsis* homologs, *BZR2*, functions in BR signaling. *BZR1* and *BZR2* share 88% amino acid sequence identity, and a mutation in *BZR2* that changes the same amino acid (Pro-233 to Leu) as is altered in *bzr1-1D* (Pro-234 to Leu) is responsible for the phenotypes of the *bes1* (*bri1-EMS-suppressor 1*) mutant (20, 21). Genetic studies indicate that *BZR1* and *BES1* play overlapping roles in BR response (20, 21). Brassinolide (BL) treatment increases nuclear accumulation of both *BZR1* and *BZR2/BES1* proteins (20, 21) and affects *BZR2/BES1* phosphorylation, possibly through regulation of *BIN2* activity (21).

To further understand the molecular mechanism by which *BZR1* mediates BR responses, we examined how *BZR1* is regulated by BR and other known components of the BR signaling pathway. We demonstrate that BR induces dephosphorylation and accumulation of *BZR1* protein, whereas *BIN2* acts upstream of *BZR1* and negatively regulates *BZR1* abundance. *BIN2* interacts with *BZR1* in yeast two-hybrid assays and phosphorylates *BZR1* *in vitro*. Phosphorylated *BZR1* is stabilized by a proteasome inhibitor, suggesting that phosphorylation of *BZR1* increases its degradation by the proteasome. Our data provide strong evidence that *BIN2* kinase phosphorylates *BZR1* and targets it for degradation by the proteasome and that BR

Abbreviations: BR, brassinosteroid; *BRI1*, *brassinosteroid insensitive 1*; *bin2*, *brassinosteroid insensitive 2*; GSK3, glycogen synthase kinase-3; *bzr1-1D*, *brassinazole resistant1-1D*; *bes1*, *bri1-EMS-suppressor 1*; BL, brassinolide; CFP, cyan fluorescence protein; GFP, green fluorescent protein; GST, glutathione S-transferase; MBP, maltose binding protein.

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signaling induces dephosphorylation and accumulation of BZR1, most likely by inhibiting BIN2 activity.

Materials and Methods

Plant Growth and Treatment. Transgenic plants were generated as described (20). BZR1-cyan fluorescence protein (CFP) and mBZR1-CFP transgenic lines express wild-type and *bzr1-1D* mutant fusion proteins by using the BZR1 promoter. 35S-BZR1-CFP lines express the wild-type fusion protein driven by the cauliflower mosaic virus 35S promoter. *Arabidopsis* plants were either grown under constant light in soil or in the dark on 0.5× Murashige and Skoog medium containing 1% sucrose and indicated supplements. For hormone and MG132 treatment, dark-grown seedlings or leaves of light-grown plants were submerged in hormone or MG132 solutions for desired time. Dark-grown samples were treated under a green safe light until harvested and frozen in liquid nitrogen. The BZR1-CFP proteins were analyzed by immunoblotting.

Immunoblotting and Immunoprecipitation. Plant tissues were frozen and ground into fine powder in liquid nitrogen. Proteins were dissolved in 2× SDS sample buffer (1 μl/mg tissue), separated by either Nu-PAGE (Invitrogen) or Laemmli SDS/PAGE (22) gels, transferred to a nitro-cellulose membrane, and probed with a monoclonal anti-green fluorescent protein (GFP) antibody (CLONTECH, 1/2,000 dilution). For phosphatase and BIN2 kinase treatment of BZR1-CFP protein, extracts of BZR1-CFP transgenic plants were immunoprecipitated by using polyclonal anti-GFP antibodies (CLONTECH) at a concentration of 0.1 μl antibodies/mg plant protein. Phosphatase treatment was performed following the manufacturer's instructions (Lambda protein phosphatase, CalBiochem), and the treated proteins were analyzed by immunoblotting using the monoclonal anti-GFP antibody (CLONTECH). BIN2 kinase treatment of immunoprecipitated BZR1-CFP protein was performed as described below for *in vitro* kinase assay.

Yeast Two-Hybrid Assay. BIN2 cDNA or the BRI1 cDNA fragments encoding the intracellular kinase domain were cloned into the pAS2 bait vector (CLONTECH) in-frame with the GAL4-DNA-binding domain. The BZR1 cDNA fragments were cloned into the pGADT7 prey vector in-frame with the GAL4-activation domain. All BZR1 constructs contain deletions of the first 20 aa. Test constructs were transformed into the yeast strain AH109, and interactions were tested by His selection and β-galactosidase assays (X-Gal filter lift assay), following the instructions of the manufacturer (CLONTECH).

In Vitro Kinase Assay. Glutathione *S*-transferase (GST)-BIN2 (16), maltose binding protein (MBP), MBP-BZR1, MBP-BRI1C, and GST-BRI1K proteins were expressed in *Escherichia coli* and affinity-purified with the affinity tags. MBP-BZR1 is a MBP-BZR1 fusion protein containing amino acids 21–336 of BZR1, and MBP-mBZR1 is the corresponding fusion protein containing the *bzr1-1D* mutation. MBP-BRI1C is a MBP fusion with the C-terminal 100 aa of BRI1 (7). GST-BRI1K is a GST fusion with the kinase domain of BRI1. *In vitro* kinase reactions were performed with 10 μCi [γ-³²P]ATP (100 μM), 10 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), and 100 mM NaCl in a total volume of 20 μl. The reaction was incubated at 30°C for 1 h, or times indicated, and then stopped by adding 20 μl 2× SDS/PAGE sample buffer. After boiling for 5 min, proteins were analyzed by SDS/PAGE and autoradiography.

Results

BRs Induce Dephosphorylation and Accumulation of BZR1. We have previously shown that BL (the most active BR) induces BZR1 protein accumulation in the nucleus (20). To determine whether

BL increases total protein level or nuclear import of BZR1 and whether BL treatment affects phosphorylation of BZR1, we analyzed the BZR1-CFP proteins in transgenic plants by immunoblotting before and after BL treatment (Fig. 1). BL treatment increased the levels of both BZR1-CFP and mBZR1-CFP proteins (Fig. 1A). The increase in the BZR1-CFP protein level became obvious after 30 min of BL treatment. Because BL treatment did not increase the *BZR1* RNA level (data not shown) but increased the accumulation of BZR1-CFP protein expressed with the constitutive 35S promoter (Fig. 1A), the BL-induced BZR1-CFP accumulation must be caused by post-transcriptional regulation.

In addition to the change of total protein level, BL treatment caused dephosphorylation of BZR1. Without BL treatment, BZR1-CFP protein exists in a slow migrating band with an apparent molecular mass of about 83 kDa. Within 10 min of BL treatment, BZR1-CFP protein shifted into a fast migrating band of about 65 kDa, which is similar to the molecular mass calculated from the protein sequence (64 kDa). Such mobility shift suggests a covalent modification of BZR1-CFP protein regulated by BL. When the BZR1-CFP protein from BL-untreated samples was immunoprecipitated and treated with protein phosphatase, the BZR1-CFP protein also shifted into the fast migrating band of 65 kDa (Figs. 1B and 2D), indicating that the slow migrating band in BL-untreated samples is phosphorylated, and the fast migrating band in BL-treated samples is the unphosphorylated form of BZR1-CFP. No BL-induced mobility change was observed for nonfusion CFP protein itself (data not shown), indicating that the BZR1 rather than the CFP part of the fusion protein was phosphorylated. The phosphorylation of BZR1-CFP protein was not affected by treatment with other plant hormones such as auxin, cytokinin, gibberellins, or abscisic acid (Fig. 1D), suggesting that BZR1 phosphorylation and accumulation are specifically regulated by BRs.

The majority of mBZR1-CFP protein containing the *bzr1-1D* mutation was in a band with significantly higher mobility than that of the wild-type BZR1-CFP protein in the BL-untreated samples, but had the same mobility as the wild-type protein in BL-treated samples (Fig. 1C), indicating that the *bzr1-1D* mutation specifically affected the mobility of the phosphorylated BZR1-CFP protein. Because the mobility of BZR1-CFP was decreased by phosphorylation, the increased mobility of phosphorylated mBZR1-CFP compared with wild-type protein would suggest that fewer residues of mBZR1-CFP were phosphorylated. The *bzr1-1D* mutation may reduce phosphorylation of certain residues of BZR1.

To test whether BZR1 is degraded by the proteasome and whether phosphorylation of BZR1 affects its degradation, we examined the effect of the proteasome inhibitor MG132 on BZR1 protein accumulation. We found that MG132 increased the accumulation of the phosphorylated form of BZR1-CFP (Fig. 1E) more than the unphosphorylated form, indicating that the phosphorylated BZR1 is degraded by the proteasome. The phosphorylated mBZR1-CFP protein also accumulated upon MG132 treatment (Fig. 1F), suggesting that the *bzr1-1D* mutation does not completely abolish the interaction of BZR1 with the proteasome. To further test whether the decrease of phosphorylated BZR1 upon BL treatment was because of degradation by the proteasome or conversion by a phosphatase, 5-day-old dark-grown seedlings were pretreated with MG132 before BL treatment (Fig. 1G). Compared with samples pretreated with water, samples pretreated with MG132 for 20 min (Fig. 1G) or 1 h (data not shown) showed similar kinetics of BL-induced dephosphorylation of BZR1, although the accumulation of total BZR1-CFP proteins was increased. These results suggest that a phosphatase might be involved in regulating the phosphorylation state of BZR1.

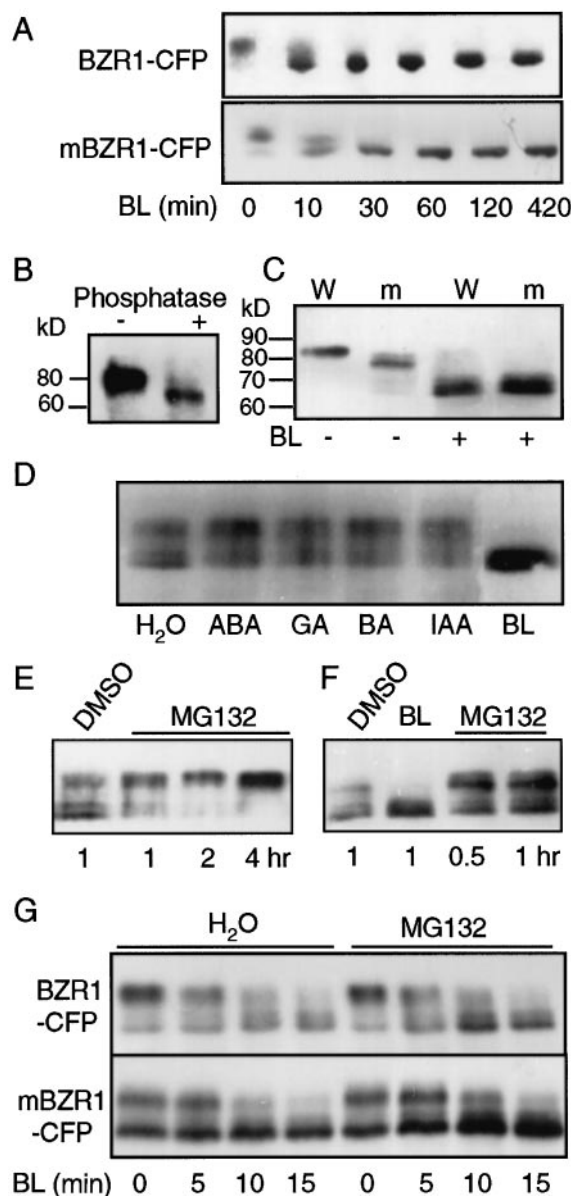


Fig. 1. Immunoblot analysis of BR-induced dephosphorylation and accumulation of BZR1 protein. (A) 35S-BZR1-CFP and mBZR1-CFP transgenic plants grown in the dark for 5 days were treated with 1 μ M BL for various times, and the BZR1-CFP proteins were analyzed by immunoblotting. (B) BZR1-CFP protein in BL-untreated sample was immunoprecipitated and treated with protein phosphatase. (C) Dark-grown BZR1-CFP (W) and mBZR1-CFP (m) plants were treated with BL or water for 1 h. (D) The 35S-BZR1-CFP plants were grown in light for 3 weeks, and the leaf tissues were treated with abscisic acid (ABA, 10 μ M), gibberellic acid 3 (GA, 10 μ M), cytokinin (BA, 10 μ M), auxin (IAA, 10 μ M), and BL (1 μ M) for 1 h. (E and F) Leaf tissues of BZR1-CFP (E) or mBZR1-CFP (F) transgenic plants grown in light for 3 weeks were treated with 10 μ M MG132, 1 μ M BL, or 0.05% DMSO (solvent) for the time shown. (G) BZR1-CFP and mBZR1-CFP seedlings were grown in the dark for 5 days and pretreated with H₂O or 10 μ M MG132 for 20 min before 1 μ M BL was added. The samples were harvested at various time points of BL treatment. Control samples (0 min) were incubated in pretreatment solution for 30 min without BL. The protein samples were analyzed on 4–8% Nu-PAGE gels (A and B) or 7.5% Laemmli SDS/PAGE gels (C–G), blotted to nitrocellulose membranes, and probed with anti-GFP antibody.

BIN2 Interacts with and Phosphorylates BZR1 *in Vitro*. The BL-induced dephosphorylation of BZR1 suggests that BZR1 is phosphorylated by a kinase such as BIN2 that negatively regu-

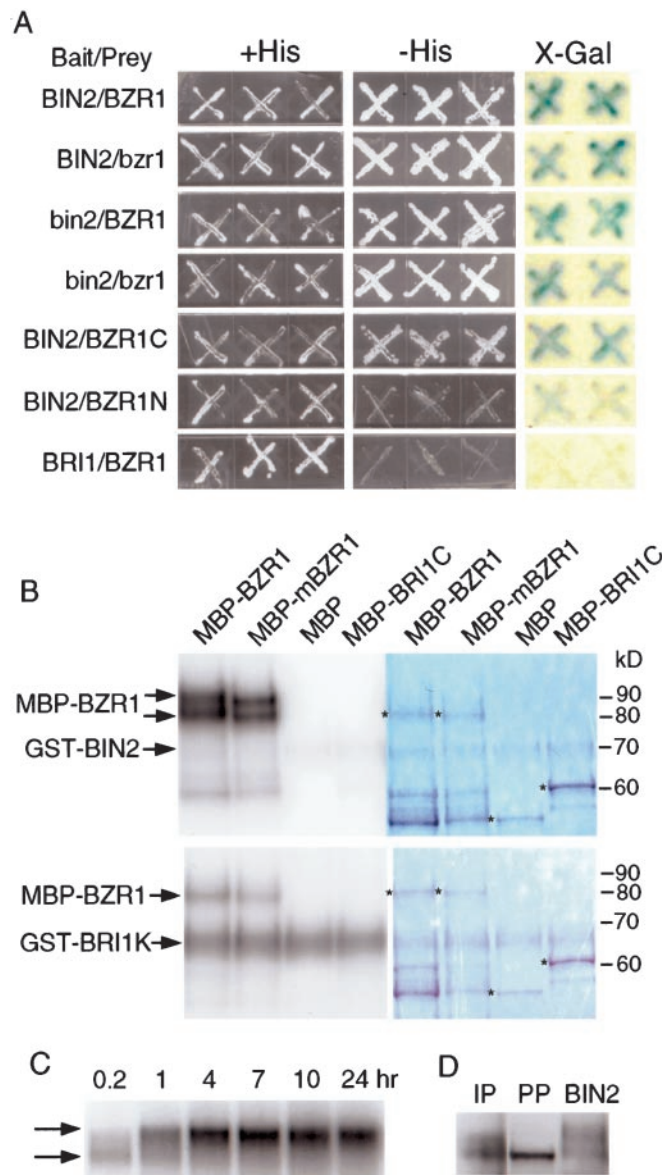


Fig. 2. BIN2 interacts with and phosphorylates BZR1. (A) BIN2 interacts with BZR1 in yeast. Yeast was transformed with a bait and a prey construct. The bait constructs contain GAL4-DNA binding domain fused with either wild-type BIN2, mutant bin2, or kinase domain of BRI1 and the prey constructs contain GAL4-activation domain fused with wild-type BZR1 or mutant bzl1 peptides (BZR1 and bzl1, amino acids 21–336; BZR1N, amino acids 21–104; BZR1C, amino acids 90–336). Interactions between each pair of test proteins were determined by selection for growth on histidine dropout (–His) medium and by 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) assay. (B) *In vitro* kinase assays using GST-BIN2 (Upper) and GST-BRI1K (Lower) kinase proteins. (Left) Autoradiographs showing protein phosphorylation; (Right) the same gels stained with Coomassie brilliant blue for total protein. Asterisks mark the substrate protein bands. Arrows show phosphorylated MBP-BZR1 and auto-phosphorylated kinases. (C) *In vitro* kinase assay using 100 ng of GST-BIN2 and 20 ng of MBP-BZR1 in each reaction incubated for various times. Arrows show the partially (Lower) and completely (Upper) phosphorylated MBP-BZR1. (D) mBZR1-CFP protein was immunoprecipitated (IP) and then treated with a protein phosphatase (PP) or GST-BIN2 kinase for 1.5 h.

lates BR response. The presence of 25 putative phosphorylation sites for GSK3-kinases in BZR1 (20) further suggests that BZR1 might be a substrate of BIN2. We analyzed the interaction between BIN2 and BZR1 by using yeast two-hybrid and *in vitro* kinase assays. Full-length BIN2 kinase fused to the GAL4-DNA

binding domain interacted with BZR1 fused to the GAL4-activation domain in the yeast two-hybrid assays, leading to expression of the *HIS3* and *LacZ* reporter genes (Fig. 2A). The *bin2-1* and *bzr1-1D* mutations did not have an obvious effect on the interaction. The carboxyl-terminal fragment of BZR1 (amino acids 90–336), but not the N-terminal fragment (amino acids 21–104), interacted with BIN2. By contrast, BZR1 did not interact with the kinase domain of BRI1 (Fig. 2A).

In vitro kinase assays were performed to determine whether BIN2 kinase phosphorylates BZR1. GST-BIN2 and MBP-BZR1 fusion proteins were expressed and purified from *E. coli* and incubated together with radiolabeled ATP. GST-BIN2 protein did not phosphorylate MBP itself or the MBP-fused with the C-terminal 100 aa of BRI1 (MBP-BRI1C), but it did phosphorylate the MBP-BZR1 fusion proteins. GST-BIN2 phosphorylated MBP-BZR1 much more effectively than GST-BIN2 phosphorylated itself (Fig. 2B), indicating that BZR1 protein is a good substrate of BIN2 kinase.

Phosphorylation of MBP-BZR1 was detected as two bands in autoradiographs, one with an apparent molecular mass of 82 kDa, similar to that predicted from the protein sequence, and the other ≈ 94 kDa. Comparing the autoradiograph and the protein staining, the slower band had a similar amount of radiolabeling as the faster band but a much smaller amount of protein, suggesting that the slower migrating band was more heavily phosphorylated. Increasing the incubation time of the kinase assay increased the slow migrating band but decreased the fast migrating band (Fig. 2C), indicating that the former was completely phosphorylated and the latter was a partially phosphorylated intermediate. Thus, phosphorylation of MBP-BZR1 by BIN2 *in vitro* caused a mobility shift that was similar to that observed for the phosphorylated BZR1-CFP protein in BL-untreated plants (Fig. 1). By contrast to BIN2, BRI1 kinase only weakly phosphorylated BZR1 and did not cause an obvious shift of mobility, while it autophosphorylated effectively (Fig. 2B).

Mutant MBP-mBZR1 protein containing the *bzr1-1D* mutation was also phosphorylated by GST-BIN2. Consistent with the observation of faster mobility of mBZR1-CFP proteins in immunoblot analysis of transgenic plants, *bzr1-1D* mutation appeared to increase the mobility of the *in vitro*-phosphorylated MBP-mBZR1 protein but had little effect on the mobility of the unphosphorylated BZR1 fusion protein (Fig. 2B), suggesting that the *bzr1-1D* mutation blocks BIN2 phosphorylation of certain BZR1 residues. Recombinant GST-BIN2 also phosphorylated the BZR1-CFP fusion protein expressed in transgenic plants, causing an increase of the phosphorylated form (slower band) and a decrease of the unphosphorylated form of BZR1-CFP (faster band, Fig. 2D). More detailed analyses are required to determine precisely the residues phosphorylated by BIN2 and the effect of the *bzr1-1D* mutation on BZR1 phosphorylation and accumulation.

BIN2 Negatively Regulates BZR1 Dephosphorylation and Accumulation *in Vivo*

To determine whether BIN2 is involved in BR regulation of BZR1 phosphorylation and accumulation *in vivo*, we first analyzed the genetic interaction between *bzr1-1D* and *bin2*. The *bin2-1*, *bzr1-1D/bin2-1*, *bzr1-1D* doubly homozygous mutant plants showed phenotypes of *bzr1-1D* in both dark and light (Fig. 3A), indicating that *bzr1-1D* is epistatic to *bin2* and BZR1 acts downstream of BIN2. Overexpression of wild-type BZR1-CFP in *bin2+/-* background partially suppressed its dwarf phenotype (Fig. 3B), suggesting that the reduced BZR1 level is responsible for the *bin2* phenotypes and that increased BZR1 expression can offset the negative regulation by BIN2. We then analyzed the BZR1-CFP protein accumulation in the *bin2-1* genetic background, which presumably has increased BIN2 activity (16). Immunoblot analysis showed that the *bin2+/-* [BZR1-CFP] plants had greatly reduced BZR1-CFP protein accumulation

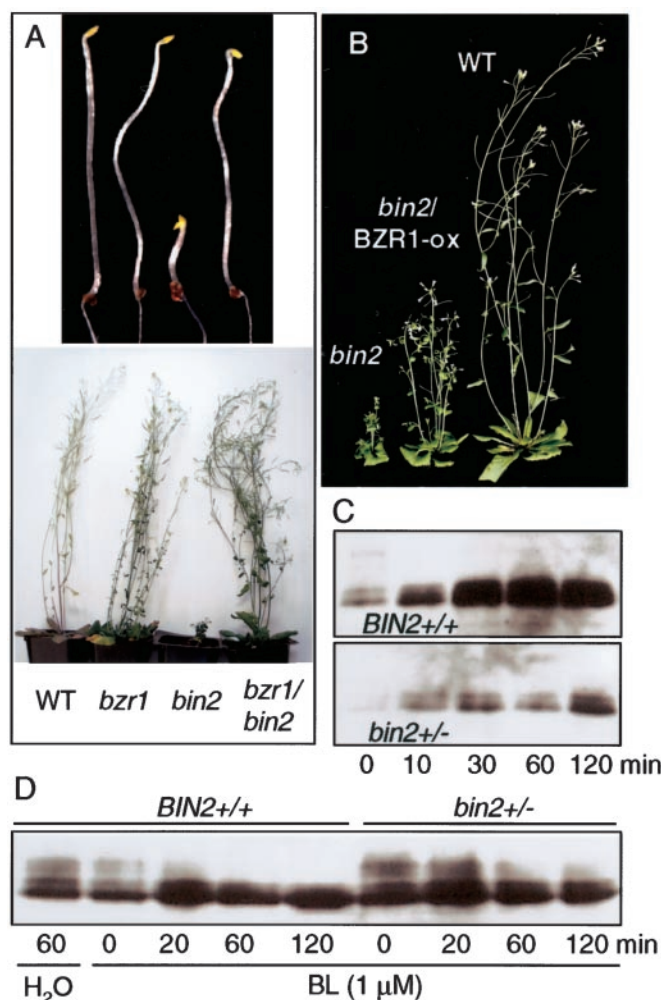


Fig. 3. BIN2 negatively regulates BZR1 accumulation. (A) *bzr1-1D* is epistatic to *bin2*. Six-day-old dark-grown seedlings (Upper) and 6-week-old light-grown plants of wild-type, *bzr1-1D*, *bin2*, and *bzr1-1D/bin2* double mutants. (B) Heterozygous *bin2+/-* mutant, heterozygous *bin2+/-* mutant containing the 35S-BZR1-CFP transgene (*bin2/BZR1-ox*), and wild-type (WT) plants were grown in light for 5 weeks. (C and D) Heterozygous *bin2+/-* plants were crossed with a homozygous 35S-BZR1-CFP line (C) or an mBZR1-CFP line (D). The wild-type BIN2^{+/+} and heterozygous *bin2+/-* F₁ plants were treated with 1 μM BL for the time shown and analyzed by immunoblotting using anti-GFP antibody.

compared with the BIN2^{+/+}[BZR1-CFP] plants (Fig. 3C). Both the phosphorylated and unphosphorylated forms of the mutant mBZR1-CFP protein accumulated at similar levels in *bin2+/-* as in BIN2^{+/+} background (Fig. 3D), indicating that *bzr1-1D* mutation disrupts the negative regulation of BZR1 accumulation by BIN2. BL treatment effectively induced dephosphorylation of mBZR1-CFP in the BIN2^{+/+} background but not in the *bin2+/-* background (Fig. 3D), suggesting that the *bin2* mutation attenuates the dephosphorylation of BZR1 induced by BR. These results indicate that BIN2 negatively regulates BZR1 accumulation *in vivo* by increasing BZR1 phosphorylation.

Discussion

Previous molecular genetic studies identified BIN2 as a negative regulator (16) and BZR1 and its homolog BZR2/BES1 as positive regulators of BR response in *Arabidopsis* (20, 21). We have shown that BR-induced BZR1 accumulation in the nucleus

is an important signaling event that leads to activation of growth response and feedback inhibition of BR biosynthesis (20). In this article, we demonstrate that BR induces BZR1 dephosphorylation and accumulation. We also provide strong evidence that BIN2 phosphorylates BZR1 and targets it for degradation by the proteasome. These results support our conclusion that BR signaling mediated by the cell surface receptor BRI1 leads to dephosphorylation and accumulation of BZR1 in the nucleus, most likely by inhibiting the BIN2 activity.

BRs Induce BZR1 Protein Dephosphorylation and Accumulation. Regulation of BZR1 protein accumulation in the nucleus appears to be critical for BR signaling. Increased accumulation of BZR1 protein by the *bzr1-1D* mutation and the 35S-BZR1-CFP transgene suppresses the *bri1* and *bin2* mutants, and the reduced BZR1 level in the *bin2* mutant is apparently responsible for its BR-insensitive phenotype. The correlation between BZR1 accumulation and BR response, as demonstrated in this and a previous study (20), suggests that BR-induced BZR1 accumulation leads to downstream growth response and feedback inhibition of BR biosynthesis.

Our data suggest that BL-induced BZR1 accumulation is caused by increased protein stability as a result of dephosphorylation. Because BL treatment does not increase BZR1 RNA levels and BL induces accumulation of BZR1-CFP expressed from the constitutive 35S promoter, BL must regulate BZR1 accumulation posttranscriptionally. Furthermore, BZR1 is dephosphorylated within 10 min of BL treatment, preceding the obvious increase of protein abundance. Finally, the proteasome inhibitor MG132 increases the accumulation of the phosphorylated BZR1-CFP, indicating that the phosphorylation of BZR1 increases its degradation by the proteasome, and dephosphorylation of BZR1 should reduce the degradation and increase the accumulation of BZR1. This finding implies that BZR1 is phosphorylated by a kinase that negatively regulates BR responses.

BIN2 Kinase Phosphorylates BZR1 and Negatively Regulates BZR1 Accumulation. Our *in vitro* and *in vivo* experiments strongly suggest that BIN2 is the negatively acting kinase that phosphorylates BZR1 and targets it for degradation. First, the genetic interaction between *bzr1-1D* and *bin2* suggests that BIN2 is upstream of BZR1 in the BR pathway and BIN2 negatively regulates BZR1 accumulation (Fig. 2A). Increased kinase activity of BIN2 in the *bin2-1* mutant (16) reduces the dephosphorylation and accumulation of BZR1 induced by BL, suggesting that BIN2 increases BZR1 phosphorylation and reduces its accumulation. Second, the *in vitro* phosphorylation of BZR1 by BIN2 strongly suggests that BIN2 directly phosphorylates BZR1 *in vivo* (Fig. 2). Furthermore, the *in vitro* phosphorylation of MBP-BZR1 by BIN2 causes a similar electrophoretic mobility shift in SDS/PAGE as the *in vivo* phosphorylation of BZR1-CFP in the absence of BL treatment. Phosphorylation of immunoprecipitated BZR1-CFP protein by recombinant BIN2 reversed the mobility shift caused by BL treatment (Fig. 2D). Together these results provide compelling evidence that BIN2 is the kinase that phosphorylates BZR1 and consequently targets BZR1 for degradation by the proteasome *in vivo*. It is possible, however, that additional kinases and phosphatases may be involved in regulating BZR1 accumulation and activity.

BL-induced dephosphorylation of BZR1 could be caused either by increased activity of a phosphatase that dephosphorylates BZR1 or rapid turnover of phosphorylated BZR1 in the absence of BIN2 activity. Like the GSK3 kinases in animals, BIN2 may be inhibited by upstream BR signaling. Because *bin2* mutation did not change the ratio between phosphorylated and unphosphorylated mBZR1-CFP in BL-untreated samples but attenuated BL-induced dephosphorylation of mBZR1-CFP

(Fig. 3D), it is likely that BL-induced BZR1 dephosphorylation requires inhibition of BIN2 activity. On the other hand, our observation that the proteasome inhibitor MG132 has little effect on the BR-induced decrease of the phosphorylated BZR1-CFP suggests that a phosphatase may be involved. It is thus possible that both activation of a phosphatase and inhibition of BIN2 kinase activity contribute to BR-induced BZR1 dephosphorylation.

The mechanism by which the *bzr1-1D* mutation stabilizes the BZR1 protein remains unclear. One possibility is that the mutation directly disrupts the interaction of BZR1 with the protein-degradation machinery. The proline to leucine mutation of *bzr1-1D* is within a putative PEST sequence that mediates proteolytic degradation (20, 21, 23). The *bzr1-1D* mutation may thus disrupt the function of the PEST domain and abolish the interaction of BZR1 with the protein-degradation machinery independent of BZR1's phosphorylation status (20). On the other hand, the mutant BZR1 protein appears to be incompletely phosphorylated compared with the wild-type protein (Figs. 1C and 2B). Although the *bzr1-1D* mutation is not in a putative BIN2-phosphorylation site and does not affect the BZR1-BIN2 interaction in yeast two-hybrid assays, it may nonetheless affect the phosphorylation of some residues of BZR1 by BIN2 and thereby reduce BZR1 degradation. Determination of the phosphorylation sites of wild-type and *bzr1-1D* mutant proteins may reveal the residues that are critical for the regulation of BZR1 accumulation and activity.

BR Signal Transduction Pathways. Genetic studies have suggested that BZR1 and its homolog BZR2/BES1 play overlapping yet

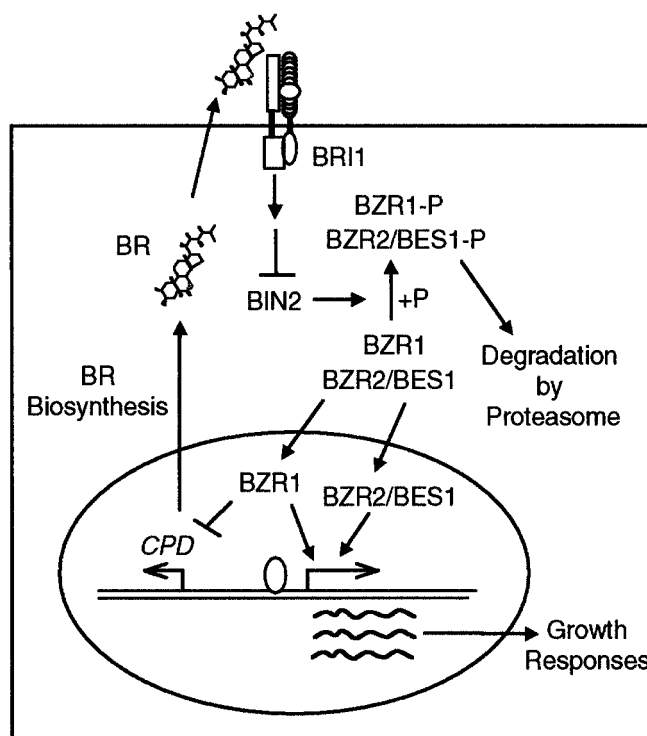


Fig. 4. A diagram of the BR signal transduction pathways. In the absence of BR, BRI1 is inactive, and BIN2 phosphorylates BZR1 and BZR2/BES1 and targets them for degradation by the proteasome. BR binding to the cell-surface receptor BRI1 activates BRI1 kinase and leads to inhibition of the BIN2 kinase, allowing dephosphorylation and accumulation of BZR1 and BZR2/BES1 proteins in the nucleus. BZR2/BES1 activates BR-regulated genes and growth responses. BZR1 mediates gene expression to confer growth response and feedback regulation of BR biosynthesis.

distinct roles in BR responses (20, 21). Both *bzr1-ID* and *bes1-D* are dominant mutations and increase BR response and suppress the *brl* mutation in the dark. However, light-grown *bzr1-ID* mutants have reduced BR levels and phenotypes of reduced BR response, presumably because of increased feedback inhibition of BR biosynthesis, whereas the *bes1-D* mutant plants have normal BR levels and show phenotypes of increased BR responses in light (21). Thus BZR1 and BZR2/BES1 appear to have distinct functions in BR responses, with BZR2/BES1 regulating downstream growth response and BZR1 regulating both growth response and feedback regulation of BR biosynthesis. Identification of loss-of-function alleles of *bzr1* and *bzr2* will be important for understanding their biological functions.

It has been shown that BRs induce dephosphorylation and accumulation of BZR2/BES1, and BIN2 phosphorylates BZR2/BES1 *in vitro* and negatively regulates BZR2/BES1 level *in vivo* (21). Thus, BZR1 and BZR2/BES1 seem to be regulated similarly by upstream BR signaling. It has yet to be shown whether BIN2 phosphorylates BZR2/BES1 and targets it for degradation by the proteasome *in vivo*. The *bes1-D* mutant plants show constitutive expression and hyperresponsiveness of two groups of BR-induced genes (21), suggesting that BZR1 and its related proteins regulate gene expression in the nucleus (20, 21). Identification of additional proteins that interact with BZR1 and BZR2/BES1 will shed light on the biochemical mechanism of their functions.

Based on this and previous studies, we propose a BR signal transduction pathway, in which BR activation of BRI1 kinase leads to dephosphorylation and accumulation of positive regulators BZR1 and BZR2/BES1 (Fig. 4). In the absence of BRs, BRI1 kinase is inactive and BIN2 kinase is active. BIN2 phosphorylates BZR1 and BZR2/BES1 and targets them for degradation by the proteasome. Reduced levels of BZR1 and BZR2/BES1 in the nucleus result in low expression of BR-activated genes and decreased growth responses. In the presence of BRs, activation of BRI1 kinase leads to inhibition of BIN2 activity through an unknown mechanism, increasing the levels of the unphosphorylated BZR1 and BZR2/BES1 proteins in the nucleus. BES1 activates BR-induced genes, including those encoding cell wall enzymes that contribute to cell elongation (21). BZR1 not only activates the BR-induced genes and promotes cell elongation but also suppresses BR biosynthetic genes such as *CPD*, leading to feedback inhibition of BR biosynthesis (20). Thus, BZR1 and BZR2/BES1 define two overlapping branches of the BR signal transduction pathway.

Conserved Function of GSK3 Kinases in Signal Transduction. The results reported in this and previous studies (16, 21) demonstrate

a functional conservation of GSK3 kinases as negative regulators in signal transduction pathways in plants and animals. In animals, the GSK3 kinases are involved in a wide range of signaling pathways that regulate many cell functions, including signaling by insulin, growth factor and nutrients, and cell fate specification during embryonic development (17). A common theme of GSK3 function is that it negatively regulates the activities of its substrates and that it is itself inhibited by upstream signaling (17). For example, GSK3 acts as a negative regulator in the insulin pathway by phosphorylating and inactivating glycogen synthase and the initiation factor eIF2B. In the Wnt signaling pathway, a GSK3 kinase phosphorylates β -catenin and targets it for ubiquitin-mediated degradation in the absence of upstream signaling. Wnt signaling mediated by the cell surface receptor Frizzled inhibits the GSK3 kinase, leading to accumulation of unphosphorylated β -catenin in the nucleus and activation of Wnt-regulated genes (17). There is thus a striking similarity between plant and animal GSK3 kinases in their mode of function as negative regulators in signal transduction pathways. Particularly, the phosphorylation and negative regulation of BZR1 and BZR2/BES1 accumulation by BIN2 resembles the negative regulation of β -catenin accumulation by the GSK3 kinase in the Wnt signaling pathway (18, 21, 24). Furthermore, our results using the proteasome inhibitor MG132 suggest that BZR1, like β -catenin, is targeted for proteolysis by the proteasome because of phosphorylation. It will be interesting to see whether BIN2 is inactivated by upstream BR signaling in a manner similar to that shown for the inactivation of GSK3 by insulin or Wnt signaling (16).

Although the mechanism by which GSK3 kinases function appears to be conserved in plants and animals, the rest of the BR pathway may not be conserved in animals. BIN2 is so far the only protein structurally conserved among the known components of the BR signaling pathway and the signaling pathways involving GSK3 kinases in animals. BZR1 shares no significant homology with any known animal proteins. On the other hand, steroid actions mediated by putative cell-surface receptors (nongenomic steroid actions) have been observed in some animal systems (9). Identification of signaling proteins in these pathways will reveal whether BR signaling shares a similar molecular mechanism with the nongenomic steroid actions in animals.

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