

An Essential Role for 14-3-3 Proteins in Brassinosteroid Signal Transduction in *Arabidopsis*

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SUMMARY

Brassinosteroids (BRs) are essential hormones for plant growth and development. BRs regulate gene expression by inducing dephosphorylation of two key transcription factors, BZR1 and BZR2/BES1, through a signal transduction pathway that involves cell-surface receptors (BRI1 and BAK1) and a GSK3 kinase (BIN2). How BR-regulated phosphorylation controls the activities of BZR1/BZR2 is not fully understood. Here, we show that BIN2-catalyzed phosphorylation of BZR1/BZR2 not only inhibits DNA binding, but also promotes binding to the 14-3-3 proteins. Mutations of a BIN2-phosphorylation site in BZR1 abolish 14-3-3 binding and lead to increased nuclear localization of BZR1 protein and enhanced BR responses in transgenic plants. Further, BR deficiency increases cytoplasmic localization, and BR treatment induces rapid nuclear localization of BZR1/BZR2. Thus, 14-3-3 binding is required for efficient inhibition of phosphorylated BR transcription factors, largely through cytoplasmic retention. This study demonstrates that multiple mechanisms are required for BR regulation of gene expression and plant growth.

INTRODUCTION

Steroids are used as hormones in both animals and plants. Many steroid biosynthetic enzymes are conserved, and steroids regulate many of the same physiological and developmental processes, including gene expression, cell division/expansion, reproductive development, and aging/senescence, in both kingdoms. However, plant

and animal steroid hormones appear to regulate gene expression through distinct signaling mechanisms (Thummel and Chory, 2002). In animals, steroid hormones bind the nuclear receptor family of transcription factors, which are retained in the cytoplasm by interaction with the HSP90 complex; ligand binding disrupts such interaction and leads to nuclear localization of the transcription factors and altered gene expression (Thummel and Chory, 2002). In plants, brassinosteroids (BRs) bind to a cell-surface receptor kinase BRI1, which initiates a phosphorylation cascade that regulates the activities of key transcription factors by phosphorylation/dephosphorylation (Vert et al., 2005).

BRs play essential roles in plant growth and development (Clouse and Sasse, 1998). Deficiency in BR biosynthesis or signal transduction causes severe growth defects, including dwarfism, male sterility, delayed senescence and flowering, and light-grown phenotype in the dark (Li and Chory, 1997; Li et al., 1996). BR binding to the extracellular domain of BRI1 activates its kinase activity, induces dimerization with and activation of another cell-surface receptor kinase (BAK1) (Li et al., 2002; Nam and Li, 2002), and causes disassociation of BKI1, a novel protein that represses BRI1 (Wang and Chory, 2006). BR activation of the receptor kinases is believed to inhibit BIN2, a glycogen synthase kinase 3 (GSK3)-like kinase (Li and Nam, 2002), or to activate BSU1 (Mora-Garcia et al., 2004), a phosphatase. BIN2 and BSU1 control the phosphorylation status of two homologous transcription factors, BZR1 and BZR2/BES1, which bind to specific promoter sequences to mediate BR-responsive gene expression (He et al., 2005; Yin et al., 2005). Similar to β -catenin in metazoans, which is inhibited by GSK3 β -mediated phosphorylation and activated by Wnt-induced dephosphorylation (Stadeli et al., 2006), BZR1 and BZR2/BES1 are inhibited by BIN2-mediated phosphorylation and are activated by BR-induced dephosphorylation. Whereas inhibition of GSK3 by Wnt signaling leads to nuclear translocation of dephosphorylated β -catenin, whether a similar

mechanism is important for BR regulation of BZR1 and BZR2/BES1 has been a controversial question.

BZR1 and BZR2/BES1 are key effectors of BR action. The dominant *bzr1-1D* and *bes1-D* mutations that effectively stabilize each protein suppress the phenotypes of BR-deficient or-insensitive mutants. BZR1 and BZR2/BES1 have been shown to directly bind to promoters of BR-responsive genes, but with different binding site sequence specificities and transcriptional activities. Extensive protein:DNA interaction studies have demonstrated that BZR1 has an optimal binding site of CGTG(T/C)G sequence, which was named the BR-response element (BRRE). The BRRE is conserved in the promoters of BR-repressed genes such as *CPD*, *DWF4*, *ROT3*, and *BR6OX* (He et al., 2005). BZR1 functions as a transcriptional repressor in vivo to mediate feedback inhibition of BR biosynthetic genes. In contrast, BZR2/BES1 was shown to interact with a bHLH-type transcription factor, BIM1, and together they bind to the E-box (CANNTG) elements in the promoter of *SAUR-AC1* and activate gene expression (Yin et al., 2005). Such differences between BZR1 and BZR2/BES1 are somewhat surprising given their high sequence similarity (88% identity), but these differences are consistent with the opposite cell elongation phenotypes of *bzr1-1D* and *bes1-D* mutant plants grown in the light.

How BR-regulated phosphorylation controls the activities of BZR1 and BZR2/BES1 is a key question for understanding BR action. Previous studies have yielded conflicting results about whether phosphorylation by BIN2 alters the nuclear localization of BZR1 and BZR2/BES1 (Wang et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Zhao et al., 2002). BR treatment has been shown to induce rapid dephosphorylation of BZR1-CFP and BZR2/BES1-GFP fusion proteins, which leads to an increase of the protein levels in the nuclei of hypocotyl cells (Wang et al., 2002; Yin et al., 2002). It was also shown that the *bin2-1* mutant, which encodes a hyperactive kinase, increased phosphorylation and reduced the accumulation of BZR1 and BZR2/BES1 proteins (He et al., 2002; Yin et al., 2002). These results suggested that phosphorylation of BZR1 and BZR2/BES1 reduces their protein levels in the nucleus (He et al., 2002; Yin et al., 2002). In contrast, it has been reported recently that BZR1-GFP and BZR2/BES1-GFP were constitutively localized in the nuclei (Vert and Chory, 2006; Zhao et al., 2002), while phosphorylation inhibited the DNA-binding activity of BZR2/BES1 in vitro and reduced its transcriptional activity in yeast (Vert and Chory, 2006). These observations supported a revised model that BIN2-catalyzed phosphorylation inhibits BZR2/BES1 primarily by reducing its DNA-binding and transcriptional activities rather than altering its localization or accumulation (Vert and Chory, 2006). However, all of these previous studies quantified the nuclear signals, but not the cytoplasmic signals of the fusion proteins, and thus a change of localization could not be discerned from a change of the total protein level. Besides these discrepancies, the functional importance of these possible mechanisms has not been studied in vivo. BZR1 and

BZR2/BES1 each contain 25 putative BIN2-phosphorylation sites; how each phosphorylation site affects the function of the transcription factors remains unknown.

14-3-3 proteins are phosphopeptide-binding proteins that are highly conserved in all eukaryotes. They participate in various signal transduction and regulatory processes by interacting with diverse target proteins in a sequence-specific and phosphorylation-dependent manner (Bridges and Moorhead, 2005; Muslin et al., 1996). The first plant 14-3-3 protein was identified as part of a protein-DNA complex (Lu et al., 1992), indicating a role of plant 14-3-3s in the regulation of transcription. Additional studies showed that 14-3-3s function in regulating the activities of metabolic enzymes and transcription factors (Sehnke et al., 2002). The *Arabidopsis* genome contains 15 14-3-3 genes, 12 of which are expressed (Sehnke et al., 2002). A large number of 14-3-3-target proteins have been identified in plants (Schoonheim et al., 2007a), suggesting that plant 14-3-3s play a role in a wide range of cellular processes. However, a direct role of plant 14-3-3 proteins in mediating signal transduction is yet to be illustrated.

In this study, we demonstrate an essential role for 14-3-3 proteins in BR signal transduction in *Arabidopsis*. We show that 14-3-3 proteins interact specifically with BZR1 protein that has been phosphorylated by BIN2. Mutations of a BIN2-phosphorylation site in BZR1 that abolish its binding to 14-3-3 proteins result in constitutive BR-response phenotypes similar to *bzr1-1D*. Such mutant BZR1 proteins are constitutively localized in the nucleus, but they are not affected in either protein accumulation or DNA binding. Moreover, we present convincing evidence that BR regulates nuclear localization of BZR1 and BZR2 proteins. Our results demonstrate an important role of 14-3-3 proteins in regulation of the key transcription factors by BR signal transduction.

RESULTS

BR Induces Rapid Nuclear Localization of BZR1

To clarify whether BR regulates nuclear localization of BZR1, we analyzed the relative levels of nuclear and cytoplasmic signals of a BZR1-yellow fluorescent protein (BZR1-YFP) by using spinning-disk confocal microscopy. The results show that the ratio of nuclear to cytoplasmic BZR1-YFP signals is reduced by brassinazole (BRZ), an inhibitor of BR biosynthesis, but increased by brassinolide (BL), the most active BR (Figure 1A). BL-induced nuclear localization of BZR1-YFP was observed both when transiently expressed in tobacco leaves (Figure S1, see the Supplemental Data available with this article online) and when stably expressed in transgenic *Arabidopsis* (Figure 1A). In time course experiments, nuclear accumulation of BZR1-YFP increased rapidly, within 4 min of BL treatment, and the nuclear/cytoplasmic ratio continued to increase for 14 min (Figure 1B; Movies S1 and S2). Such kinetics of BR-induced BZR1 nuclear accumulation is consistent with the kinetics of dephosphorylation (He et al., 2002). In contrast, mock treatment did not

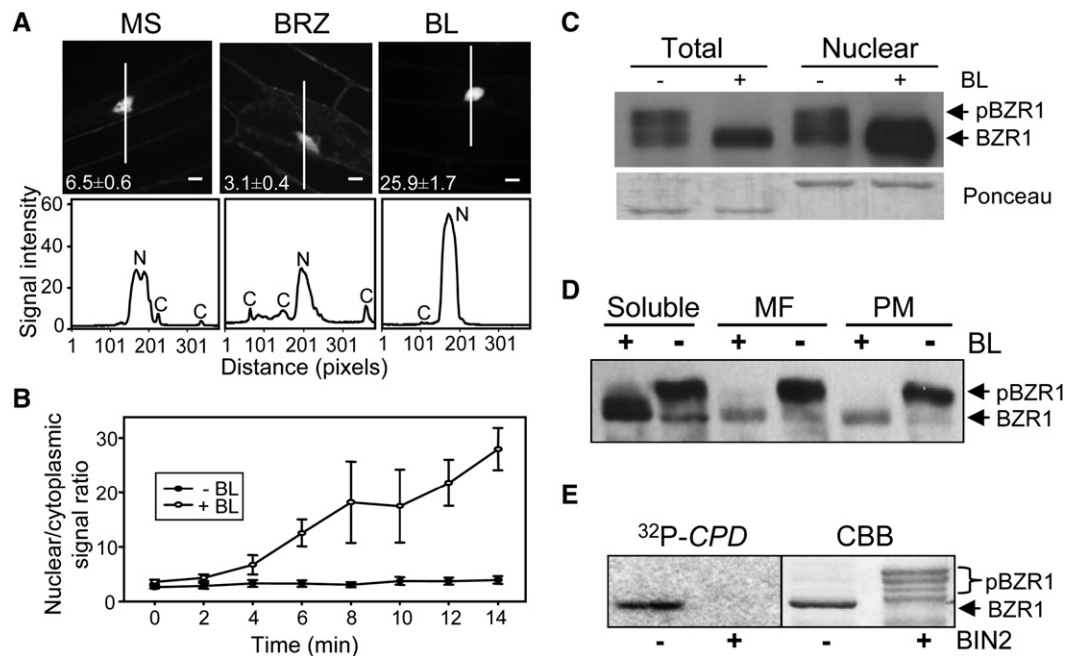


Figure 1. BR Induces Nuclear Localization of BZR1

(A) Effect of brassinazole (BRZ) and brassinolide (BL) on the subcellular localization of BZR1-YFP. Transgenic *Arabidopsis* plants expressing BZR1-YFP were grown on MS or MS plus 2 μ M BRZ medium in the dark for 4 days. Seedlings grown on BRZ medium were treated with mock or 100 nM BL solution for 1 hr, and images of the YFP signal (top) were obtained by using confocal microscopy. The numbers in each image show the average ratios between nuclear and cytoplasmic signal intensities and standard errors calculated from seven cells for each treatment. The white lines inside the images show the areas used for line scan measurements that yielded plot profiles shown in the lower panels. N, nuclear signal; C, cytoplasmic signal. Fluorescent intensity is 1000 \times , and the scale bar is 10 μ m.

(B) Kinetics of the BL-induced nuclear accumulation of BZR1-YFP. BZR1-YFP seedlings grown on 2 μ M BRZ were treated with BL or mock solution (–BL), and images were acquired at each time point to measure the nuclear/cytoplasmic ratios. Error bars are \pm SEM.

(C) Unphosphorylated BZR1 is enriched in the nuclear fraction. Immunoblot of unphosphorylated and phosphorylated BZR1-CFP proteins in total and nuclear fractions from mock- or BL-treated seedlings.

(D) Phosphorylated BZR1 is more enriched in membrane fractions than unphosphorylated BZR1. Transgenic plants expressing the BZR1-CFP protein were treated with (+BL) or without (–BL) 1 μ M BL, and the soluble, microsomal (MF), and plasma membrane (PM) fractions were analyzed by immunoblot.

(E) BIN2 phosphorylation inhibits the DNA-binding activity of BZR1. A gel blot of unphosphorylated and BIN2-phosphorylated MBP-BZR1 proteins was probed with radiolabeled CPD promoter DNA. CBB, Coomassie brilliant blue-stained gel. (C–E) Unphosphorylated BZR1 (BZR1) and phosphorylated BZR1 (pBZR1) proteins are marked by arrows.

significantly increase the nuclear/cytoplasmic signal ratio of BZR1-YFP.

We further tested the effect of BR on nuclear localization of BZR1-YFP by using subcellular fractionation experiments. These experiments demonstrated that unphosphorylated, but not the phosphorylated, BZR1-CFP was enriched in the nucleus. Furthermore, BL treatment resulted in a significant increase of the unphosphorylated BZR1 in the nuclear fraction (Figure 1C). Phosphorylated BZR1-CFP protein was also more associated with cell membranes than the unphosphorylated protein (Figure 1D). In subcellular fractionation experiments, both microsomal and plasma membrane fractions contained similar levels of phosphorylated, but much lower amounts of unphosphorylated, BZR1-CFP compared to the soluble protein fraction (Figure 1D). After BR treatment, the amount of BZR1-CFP associated with membranes was greatly reduced as BZR1 was dephosphorylated. These results collectively indicate that some

phosphorylated BZR1 protein is retained in the cytoplasm, most likely through interaction with membranes, and BR-induced dephosphorylation of BZR1 leads to its movement from cytoplasm into the nucleus.

Phosphorylation Inhibits the DNA-Binding Activity of BZR1

A recent study showed that phosphorylation inhibits DNA binding of BZR2/BES1 (Vert and Chory, 2006). We tested whether phosphorylation by BIN2 inhibits the DNA-binding activity of BZR1. A maltose-binding protein (MBP)-BZR1 fusion protein was phosphorylated by BIN2 and separated on an SDS-PAGE gel. When the gel blot was probed with radiolabeled DNA containing a BZR1-binding site (BRRE) (He et al., 2005), only the unphosphorylated, but not the phosphorylated, MBP-BZR1 showed DNA binding, indicating that phosphorylation by BIN2 inhibits BZR1's DNA-binding activity (Figure 1E).

Phosphorylation of BZR1 by BIN2 Promotes Binding to the 14-3-3 Proteins

To further understand the molecular mechanisms of BZR1 regulation, we performed a yeast two-hybrid screen for BZR1-interacting proteins. Out of 98 positive clones identified, 80 clones encode members of the 14-3-3 protein family. They represent 5 of the 12 isoforms in *Arabidopsis* (Sehnke et al., 2002), namely, 14-3-3 λ , 14-3-3 κ , 14-3-3 ϵ , 14-3-3 ϕ , and 14-3-3 ω . Additional yeast two-hybrid assays indicated that 14-3-3 λ interacts with BZR1, *bzr1-1D*, and the C termini (amino acids 90–336) of BZR1 and *bzr1-1D*, but not with BRI1, BIN2, or *bin2-1* (Figure 2A).

14-3-3s are known to bind specific sequences of target proteins in a phosphorylation-dependent manner (Muslin et al., 1996). To determine if 14-3-3 binding requires phosphorylation of BZR1 by BIN2, purified MBP-BZR1 fusion protein was phosphorylated by a GST-BIN2 protein in vitro, separated on an SDS-PAGE gel, and blotted to a nitrocellulose membrane. Incubation of the blot with GST-14-3-3 λ and anti-GST antibodies detected strong binding to the phosphorylated MBP-BZR1 and very weak binding to the unphosphorylated form (Figure 2B), indicating that phosphorylation by BIN2 facilitates BZR1 binding to 14-3-3s. The interaction in yeast is likely due to phosphorylation of BZR1 by the yeast GSK3 kinases.

In an attempt to understand the function of 14-3-3 proteins, we obtained single and double knockout mutants of 14-3-3 λ and 14-3-3 κ , two closely homologous genes that were identified most frequently in our yeast two-hybrid screen. Neither single nor double knockout mutants displayed phenotypes or altered BR responses (data not shown), most likely due to redundancy of the gene family. 14-3-3 RNAi plants also did not show any noticeable differences in phenotypes (data not shown). Apparently, an experimental approach that specifically disrupts the BZR1:14-3-3 interaction is required to understand how 14-3-3 binding affects BZR1 function.

14-3-3 proteins are known to mostly bind two types of specific sequences: mode I, **RXXpSXP**, or mode II, **RXXXpSXP** (X = any amino acid, R = arginine, pS = phosphoserine, and P = proline), in which the S residue needs to be phosphorylated (Aitken, 2006; Muslin et al., 1996). A potential mode II-type 14-3-3-binding site sequence was identified in BZR1 at amino acids 169–175 (**RISNSCP**) (Figure 2C). Mass spectrometric analysis of BIN2-phosphorylated BZR1 protein showed that serine 173 in the putative 14-3-3-binding site of BZR1 is phosphorylated by BIN2 (Figure S2). When the binding site (RISNS, at position 169–173) was deleted (BZR1 Δ) or serine 173 was mutated to alanine (S173A) (Figure 2C), 14-3-3 binding was greatly reduced (Figure 2D). In contrast, deletion of isoleucine 170 (Δ I170) of BZR1, which changes the sequence from a mode II to a mode I 14-3-3-binding site (Figure 2C), did not abolish 14-3-3 binding (Figure 2D). These results indicate that 14-3-3 proteins bind to BZR1 through the conserved binding site that contains phosphorylated serine 173.

The in vivo interactions of wild-type and mutant BZR1s with 14-3-3 λ were studied by using a Bi-Molecular Fluorescence

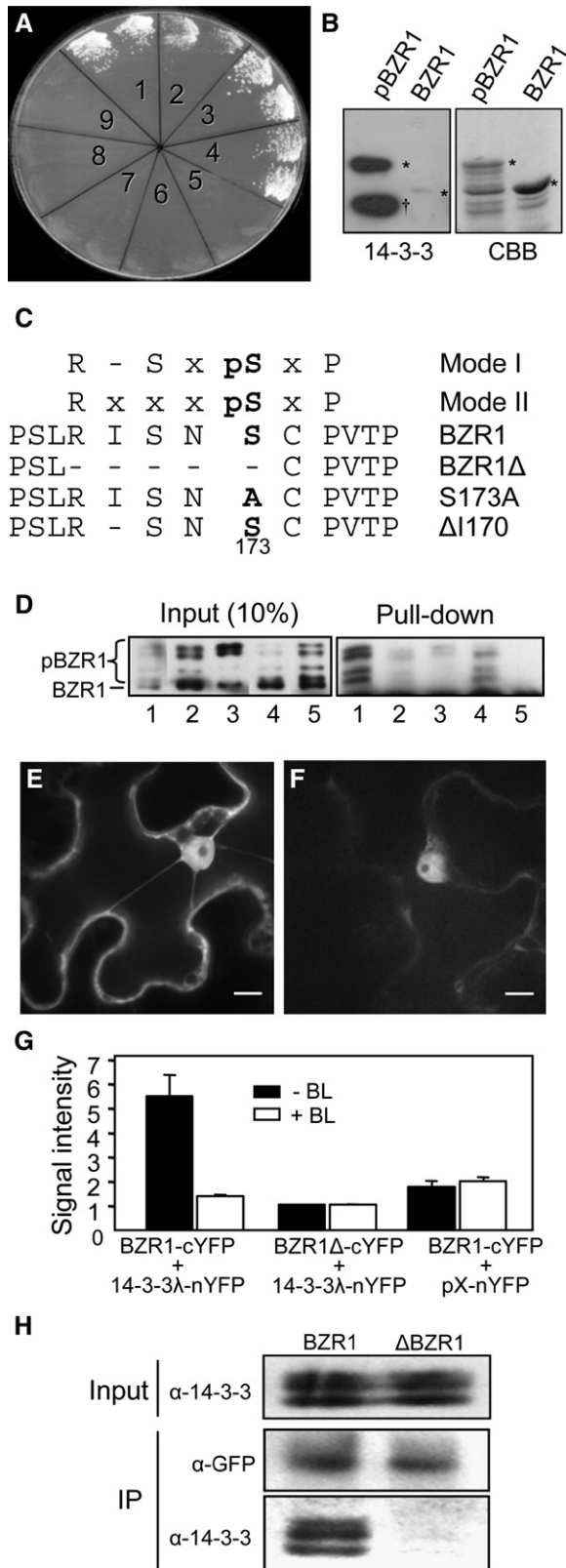
Complementation system (BiFC). BiFC is based on the principle that a functional fluorescence complex is observed when two proteins of interest fused to the N- and C-terminal halves of YFP interact in vivo. Cotransformed BZR1-cYFP and 14-3-3 λ -nYFP produced strong YFP fluorescence (Figures 2E and 2G). The fluorescence signal was reduced by BR treatment (Figures 2F and 2G), which induces BZR1 dephosphorylation. By contrast, cotransformation of BZR1 Δ -cYFP with 14-3-3 λ -nYFP or BZR1-cYFP with a non-fusion nYFP yielded a very low fluorescence signal (Figure 2G). Consistent with the BiFC data, coimmunoprecipitation experiments (Co-IP) with transgenic *Arabidopsis* plants demonstrated that wild-type BZR1 and BZR1 Δ I170, but not Δ BZR1 or BZR1S173A, interacted with 14-3-3 proteins in vivo (Figure 2H and data not shown). These results indicate that phosphorylated BZR1 interacts with 14-3-3 in vivo, and that this interaction is inhibited by BR-induced BZR1 dephosphorylation.

14-3-3 Binding Inhibits BZR1's Activity

To understand the function of the BZR1:14-3-3 interaction in BR signaling, we generated transgenic plants expressing BZR1 containing 14-3-3-binding site mutations. Interestingly, transgenic plants expressing BZR1 Δ (16/92; 17%) or BZR1S173A (14/104; 13%) showed phenotypes similar to *bzr1-1D* (Figure 3), including shorter petioles, rounder and curled leaves (Figures 3A and 3B), delayed flowering (Figure 3C), bending of the stem at the branch junction (Figure 3D), long hypocotyls when grown on BR biosynthesis inhibitor BRZ in the dark (Figure 3E), and suppression of *bri1-5* (Figure 3F). In contrast, none of the transgenic plants transformed with wild-type BZR1 (0/157) or mutant BZR1 Δ I170 (0/118) showed any *bzr1-1D*-like phenotype (Figure 3). Consistent with the morphological phenotypes, the expression of BZR1-target genes *CPD* and *DWF4* (He et al., 2005) is greatly reduced in BZR1 Δ as well as *bzr1-1D* plants (Figure 3G). Expression of BZR1S173A with the native *BZR1* promoter also caused a *bzr1-1D*-like phenotype (Figure S3). These results indicate that 14-3-3 proteins interact with BZR1 to inhibit its function, and abolition of such an interaction leads to increased BZR1 activity.

Mutations in the 14-3-3-Binding Site Do Not Affect Accumulation, Dephosphorylation, or DNA-Binding Activity of BZR1

The dominant gain-of-function mutation in the *bzr1-1D* mutant causes accumulation of BZR1 protein, leading to constitutive BR-response phenotypes (Wang et al., 2002). To determine whether the phenotypes of BZR1 Δ and BZR1S173A plants were due to altered BZR1 protein accumulation or phosphorylation, we performed immunoblot analysis. Despite causing strong phenotypes, mutations of the 14-3-3-binding site did not increase the accumulation or dephosphorylation of BZR1 protein (Figure 4A). Southwestern blot analysis showed that these mutations did not affect BZR1's ability to bind DNA, because phosphorylation by BIN2 abolished the



DNA-binding activity of the wild-type as well as the mutant BZR1 proteins (Figure 4B). Therefore, the *bzr1-1D*-like phenotypes displayed by BZR1Δ and BZR1S173A plants are unlikely due to a difference in the accumulation or DNA-binding activity of the BZR1 protein.

14-3-3 Binding Increases Cytoplasmic Localization of BZR1

14-3-3 proteins have been shown to negatively regulate nuclear localization of target proteins in both metazoans and plants (Igarashi et al., 2001; Muslin and Xing, 2000). Considering that BR induces nuclear localization of BZR1, we examined whether BZR1Δ- and BZR1S173A-YFP have altered subcellular localization (Figure 5). Indeed, BZR1Δ- and BZR1S173A-YFP proteins showed significantly increased nuclear localization in *Arabidopsis*

(A) BZR1 interacts with 14-3-3λ in yeast two-hybrid assays. Each yeast clone contains the pGAD-14-3-3λ prey construct and one of the following genes in the pGBKT7 vector: 1, BZR1; 2, *bzr1-1D*; 3, BZR1C; 4, *bzr1-1DC*; 5, BIN2; 6, *bin2-1*; 7, BRR1-KD; 8, *p53*; 9, no gene insert. Growth of yeast cells indicates interaction between the test protein and 14-3-3λ.

(B) 14-3-3λ specifically interacts with phosphorylated BZR1. Recombinant MBP-BZR1 protein was phosphorylated by GST-BIN2, gel blotted, and probed with GST-14-3-3λ and anti-GST antibody. The right panel shows the Coomassie brilliant blue (CBB)-stained gel. Asterisks mark the unphosphorylated (BZR1) and phosphorylated (pBZR1) MBP-BZR1 bands. The dagger sign (†) marks the GST-BIN2 band.

(C) BZR1 contains a putative 14-3-3-binding site. Two known 14-3-3-binding site sequences (Mode I and Mode II) are aligned against the BZR1 sequence. “x” represents any given amino acid. Amino acids are presented as single letters, namely: R, arginine; I, isoleucine; pS, phosphoserine; N, asparagine; C, cysteine; P, proline. A conserved serine residue at the -3 position that is crucial for 14-3-3 binding was numbered and marked in bold. Various mutations created in the 14-3-3-binding sites are also shown. The hyphen (-) represents the deletion of an amino acid residue.

(D) Effects of mutations of the 14-3-3-binding site of BZR1 on the binding of 14-3-3λ. Wild-type and mutant MBP-BZR1 proteins were phosphorylated by GST-BIN2 and then mixed with unphosphorylated proteins before a pull-down assay (right panel) with glutathione agarose beads containing GST-14-3-3λ (lanes 1–4) or GST (lane 5). Lanes 1–5: MBP-BZR1, MBP-BZR1Δ, MBP-BZR1S173A, MBP-BZR1Δ170, and MBP-BZR1, respectively.

(E) In vivo interaction between BZR1 and 14-3-3λ detected by BiFC. The N-terminal and C-terminal halves of YFP were fused to 14-3-3λ and BZR1, respectively, the constructs were cotransformed into tobacco leaf cells, and fluorescence images were obtained by using confocal microscopy. The scale bar is 10 μm.

(F) The BZR1-cYFP and the 14-3-3λ-nYFP constructs were cotransformed into tobacco cells with 1 μM BL. YFP fluorescence images were obtained by using a confocal microscope. Scale bar is 10 μm.

(G) Quantification of BiFC signals. Each pair of indicated constructs was cotransformed into tobacco cells with (+BL) or without (-BL) 1 μM BL. Fluorescence signal intensity (1000x) is an average of the measurements from at least seven transformed cells after background subtraction. Error bars are ± SEM.

(H) Mutations in the 14-3-3-binding site abolish in vivo interaction of BZR1 with 14-3-3s. Coimmunoprecipitation (Co-IP) was carried by using an anti-GFP antibody coupled to protein A Sepharose beads with tissue material from either BZR1-YFP or ΔBZR1-YFP transgenic plants. Western blot was probed with α-GFP and α-14-3-3 antibodies.

Figure 2. BIN2-Phosphorylated BZR1 Interacts with 14-3-3 Proteins

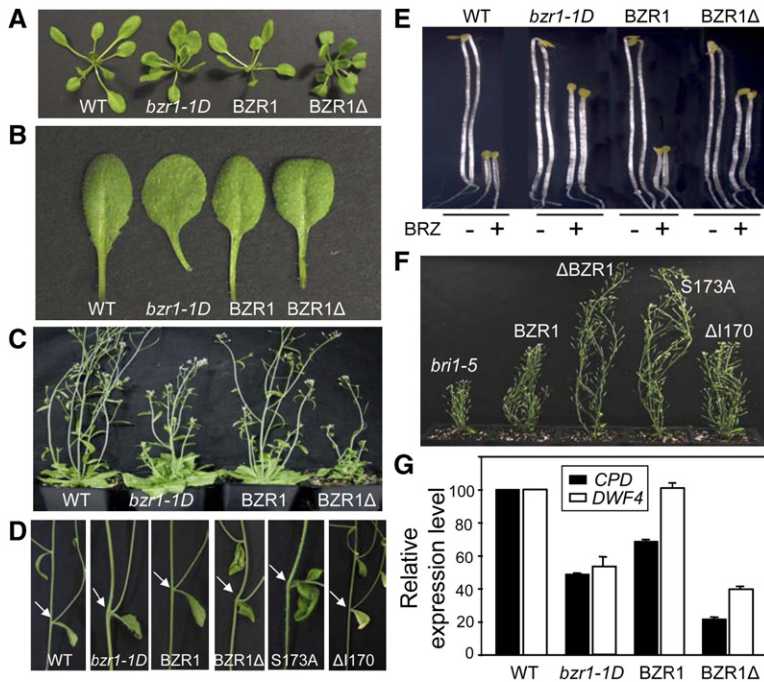


Figure 3. 14-3-3 Binding Inhibits BZR1 Activity

(A–D) Phenotypes of BZR1Δ and BZR1S173A transgenic plants compared with wild-type Columbia (WT) and *bZR1-1D*. Three-week-old (A) plants and (B) leaves, 5-week-old (C) plants and (D) bending of the stems at the branch junction (pointed by arrows).

(E) BZR1Δ and BZR1S173A mutations suppress BR-deficiency phenotypes. Seedlings were grown in the dark on MS medium with or without 2 μM BRZ for 4 days. Two representative seedlings for each treatment are shown. (F) Mutations in BZR1 that reduce 14-3-3 binding suppress the *bri1-5* phenotype. Plants shown from left to right are *bri1-5*, *bri1-5/BZR1-YFP*, *bri1-5/BZR1Δ-YFP*, *bri1-5/BZR1S173A-YFP*, and *bri1-5/BZR1ΔI170-YFP*.

(G) BR biosynthesis genes are downregulated in BZR1Δ plants. Expression levels of *CPD* and *DWF4* genes were measured by quantitative real-time PCR. The data were normalized first to *UBC* and then to Col. Error bars are ± SEM.

and tobacco cells (Figures 5A, and 5B; Figure S4), whereas BZR1ΔI170-YFP was localized in both the cytoplasm and nucleus, similar to wild-type BZR1-YFP (Figures 5A; Figure S4). Quantification of the nuclear/cytoplasmic signals confirmed these microscopic observations (Figure 5B). BRZ increased the cytoplasmic signal of wild-type BZR1-YFP, but had little effect on BZR1Δ-YFP (Figure 1A; Figure S5). When transiently coexpressed in tobacco leaf cells, both BZR1-CFP and *bZR1-1D*-CFP proteins showed identical localization patterns with that of cotransformed BZR1-YFP, indicating that the *bZR1-1D*

mutation, which increases the protein accumulation, does not affect the subcellular localization of the protein (Figure 5C). In contrast, BZR1Δ-CFP showed much reduced levels of cytoplasmic signal compared to BZR1-YFP (Figure 5C). This difference in localization disappeared when cells were treated with BR, presumably due to BR-induced nuclear localization of BZR1-YFP (Figure 5C; Figure S1). Consistent with microscopic observations, subcellular fractionation showed that BZR1S173A-YFP protein levels, compared to those of BZR1ΔI170-YFP, are reduced in the cytosolic fraction (Figure S6). These results demonstrate that mutation of the 14-3-3-binding site increases the nuclear localization of BZR1.

Previous studies have shown that 14-3-3 interactions with the target proteins can be disrupted by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (Paul et al., 2005). We observed that AICAR treatment increased the nuclear/cytoplasmic ratio of BZR1 and reduced the expression of BZR1-target genes *CPD* and *DWF4* (Figures 5D and 5E), further supporting the thought that disrupting 14-3-3 binding increases BZR1's nuclear localization and activates its function. Together, these results strongly support a model in which phosphorylated BZR1 is retained in the cytoplasm by binding to 14-3-3 proteins, and in which BR-induced dephosphorylation abolishes 14-3-3 binding to allow nuclear localization and transcriptional regulation of BZR1.

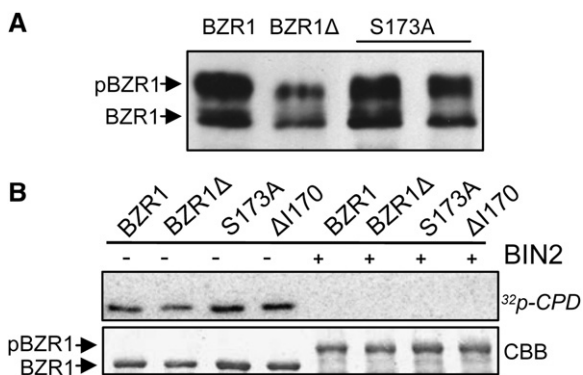


Figure 4. Mutations in the 14-3-3-Binding Site Do Not Affect Stability or the DNA-Binding Activity of BZR1

(A) Immunoblot of BZR1-YFP, BZR1Δ-YFP, and BZR1S173A-YFP (two independent lines) proteins in the transgenic plants. BZR1-YFP was detected with an α-GFP antibody.

(B) Mutations in the 14-3-3-binding site do not alter the DNA-binding activity of BZR1. The gel blot of unphosphorylated and BIN2-phosphorylated MBP-BZR1 proteins was probed with radiolabeled *CPD* promoter DNA. CBB, Coomassie brilliant blue-stained gel.

14-3-3 Binding Negatively Regulates BZR2/BES1 Function

A yeast two-hybrid screen also showed interaction between BZR2/BES1 and 14-3-3 proteins (Figure 6A). To determine if subcellular localization of BZR2/BES1 is also

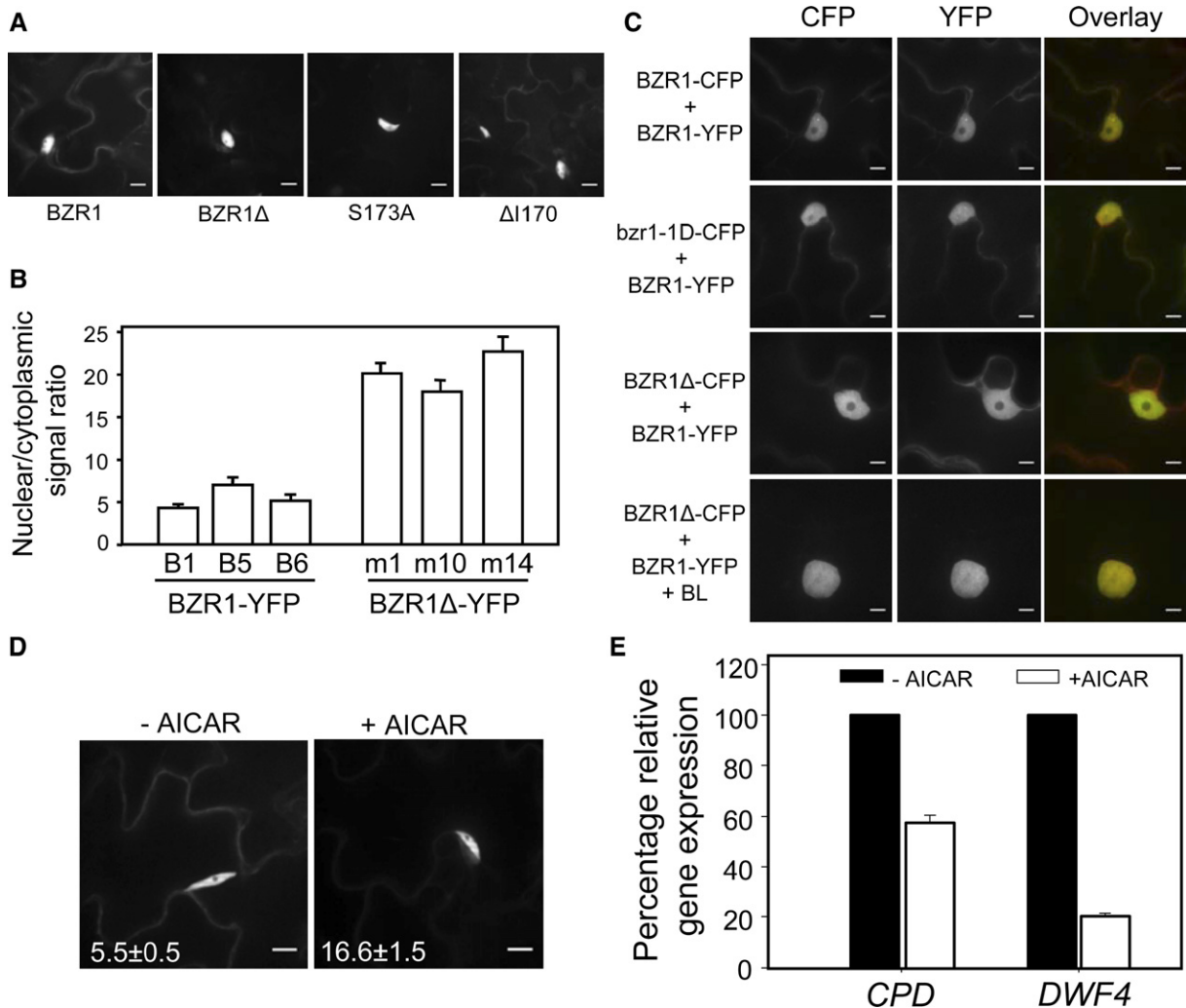


Figure 5. 14-3-3 Binding Reduces Nuclear Localization of BZR1

(A and B) Subcellular localization of BZR1-, BZR1Δ-, BZR1S173A-, and BZR1Δ1170-YFP proteins in the leaves of transgenic *Arabidopsis* plants. Representative images of (A) YFP fluorescence and (B) quantification of nuclear/cytoplasmic signal ratios of three independent lines from BZR1-YFP and BZR1Δ-YFP are shown. Error bars are \pm SEM.

(C) BZR1Δ-CFP protein predominantly localizes in the nucleus. Pairs of indicated YFP and CFP fusion constructs were cotransformed into tobacco leaves with or without 1 μ M BL. Images in CFP and YFP channels were false-colored as green and red, respectively, in overlay. The Scale bar is 10 μ m.

(D) AICAR treatment decreases cytoplasmic localization of BZR1. Transgenic plants expressing BZR1-YFP were treated with either mock solution or 20 mM AICAR for 1 hr, and images were obtained with a confocal microscope. The scale bar is 10 μ m. Numbers in each image show the average ratios between nuclear and cytoplasmic signal intensities and standard errors calculated from seven cells for each treatment.

(E) AICAR treatment downregulates BZR1-target gene expression. Transgenic plants expressing BZR1-YFP were treated with either mock solution or 20 mM AICAR for 3 hr, and expression of *CPD* and *DWF4* was measured by using qRT-PCR. Error bars are \pm SEM.

regulated by BR and 14-3-3 proteins, we measured the nuclear/cytoplasmic signal ratio of BZR2/BES1-GFP in transgenic *Arabidopsis* plants by using confocal microscopy. We observed that, similar to BZR1-YFP (Figures 1A and 1B), BZR2/BES1-GFP localization in the nucleus was decreased by BRZ treatment and increased by BL treatment (Figure 6B). BZR2/BES1 contains a putative 14-3-3-binding site (Figure S7), and mutation of S171A in BZR2/BES1 (equivalent to S173A in BZR1) increased the nuclear localization of the protein (Figure 6C; Figure S7). These results collectively indicate that 14-3-3

proteins function in BR signal transduction by regulating the subcellular localization and activity of both BZR1 and BZR2/BES1.

DISCUSSION

In the present study, we illustrate a critical role of 14-3-3 proteins in BR signal transduction. Our data demonstrate that BIN2-catalyzed phosphorylation inhibits the function of the BZR1 and BZR2/BES1 proteins not only by inhibiting DNA binding, but also by promoting binding to the

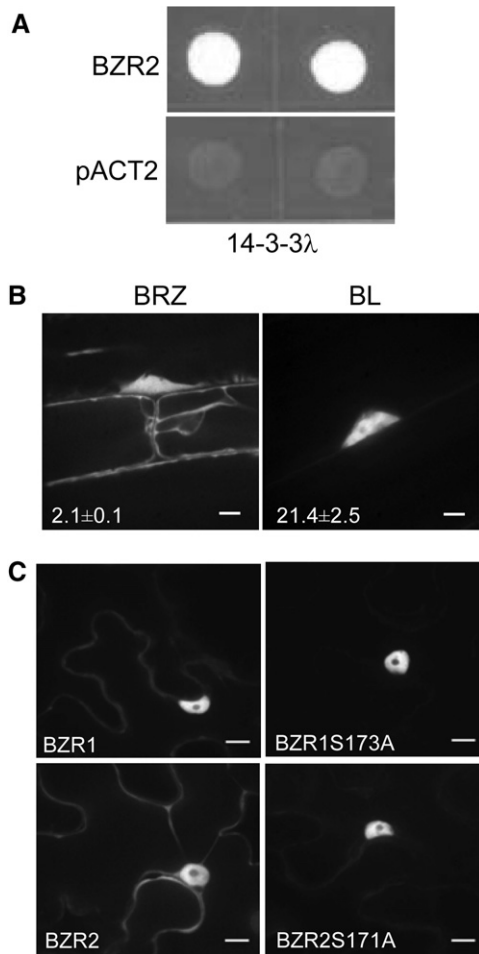


Figure 6. BR Treatment and Mutation of the 14-3-3-Binding Site Increase Nuclear Localization of BZR2/BES1

(A) 14-3-3 λ interacts with BZR2 in yeast two-hybrid assays. pACT2 is the empty prey vector as a negative control.

(B) BL treatment increases the nuclear localization of BZR2. BZR2-GFP transgenic seedlings were grown on media containing 2 μ M BRZ or 10 nM BL in the dark for 4 days, and BZR2-GFP subcellular localization was visualized by using confocal microscopy. Numbers in each image show the average ratios between nuclear and cytoplasmic signal intensities and standard errors calculated from seven cells for each treatment. Error bars are \pm SEM.

(C) YFP fusion constructs of BZR1, BZR1S173A, BZR2, or BZR2S171A were transiently expressed in tobacco leaves, and subcellular localization of YFP was observed. The scale bar is 10 μ m.

14-3-3 proteins, which increases cytoplasmic retention of BZR1 and BZR2/BES1. Together with previous studies, we have shown that phosphorylation can inhibit BZR1 and BZR2/BES1 through at least four mechanisms: increasing proteolysis, inhibiting DNA binding, promoting 14-3-3 binding, and cytoplasmic retention (Figure 7). These multiple mechanisms of regulation are consistent with the presence of large numbers of putative BIN2-phosphorylation sites in BZR1 and BZR2/BES1, and they likely act together to confer efficient and precise regulation of key transcription factors required for normal BR response and plant growth.

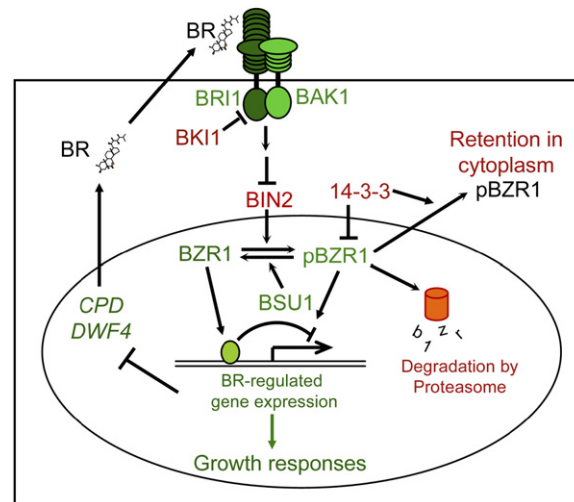


Figure 7. A Model for the BR Signaling Pathway in Arabidopsis

BR signaling involves a cell-surface receptor complex (BRI1/BAK1), a GSK3 kinase (BIN2), a phosphatase (BSU1), and two homologous transcription factors (BZR1 and BZR2/BES1). Green and red colors represent positive and negative functional roles in BR signaling, respectively. Arrows and bars represent the actions of promotion and inhibition, respectively. In the absence of BR, BKI1 suppresses BRI1, and BIN2 phosphorylates and inhibits BZR1 and BZR2/BES1 (not shown). BR binds to the extracellular domain of BRI1 to activate its kinase, which leads to disassociation of BKI1 from BRI1 and dimerization with and activation of BAK1. Then, through an unknown mechanism, the activated receptor kinases inhibit BIN2 or activate BSU1, yielding dephosphorylated BZR1 and BZR2/BES1, which directly regulate BR-responsive gene expression. BIN2 phosphorylation negatively regulates BZR1 by increasing proteasomal degradation and BZR2/BES1 by inhibiting DNA binding. The current study shows that BR promotes nuclear localization of BZR1 and BZR2/BES1. BIN2 phosphorylation of BZR1 not only inhibits its DNA binding, but also promotes its binding with 14-3-3 proteins, which is required for cytoplasmic retention and efficient inhibition of phosphorylated BZR1 in vivo. 14-3-3 proteins also regulate subcellular localization of BZR2/BES1. We propose that the large numbers of BIN2-phosphorylation sites in BZR1 and BZR2/BES1 allow BIN2 to inhibit BZR1/BZR2 through multiple mechanisms, conferring an efficient control of gene expression and plant growth by BR signaling.

Multiple mechanisms of regulation of key transcription factors have been observed in signaling pathways in animals. For example, the Ci/Gli transcription factor of the Hedgehog (Hh) pathway is controlled by multilayered regulatory mechanisms. It is believed that tissue- and developmental stage-specific expression of some of the additional regulators in vertebrates provide a mechanism for ensuring precision in spatial and temporal control of Hh signaling (Jiang, 2006). Similarly, multiple mechanisms of BZR1 regulation may provide higher levels of efficiency and precision than a single mechanism in BR signaling. One might expect that inhibiting DNA binding should be sufficient to turn off the transcription factors. However, BZR1 Δ -YFP and BZR1S173A-YFP, but not the wild-type BZR1-YFP, cause *bzr1-1D*-like phenotypes in the transgenic plants, despite the fact that phosphorylation inhibits their DNA-binding activity in vitro. Apparently

phosphorylation per se is not sufficient, and binding to the 14-3-3 proteins is required, for efficient inhibition of BZR1 activity in vivo. This is not surprising since genome-wide surveys of in vivo DNA binding by transcription factors have revealed discrepancies between in vitro and in vivo DNA-binding specificities of transcription factors (Biggin, 2001). In vivo DNA binding by transcription factors is not only influenced by their intrinsic sequence-specific recognition properties, but it is also affected by other factors, such as chromatin structure or cooperative interactions with other DNA-binding proteins. It is conceivable that when not bound by 14-3-3s, phosphorylated BZR1 and BZR2/BES1 are localized in the nucleus and can regulate target genes by interacting with partner DNA-binding proteins, such as BIM1 (Yin et al., 2005).

Multiple mechanisms would also allow for fine-tuning of BR sensitivity by other environmental or developmental signals. For example, sugars globally affect 14-3-3 binding to target proteins (Cotelle et al., 2000), and stresses and abscisic acid (ABA) increase expression of plant 14-3-3 proteins (Chen et al., 2006; Schoonheim et al., 2007b), which could potentially contribute to the antagonistic interaction between BR and ABA (Friedrichsen et al., 2002). Such regulation of 14-3-3s by environmental and developmental cues might explain the previous conflicting observations of constitutive nuclear accumulation of BZR1 and BZR2/BES1 (Vert and Chory, 2006; Zhao et al., 2002). In fact, we observed a strong nuclear-localization response in young growing tissues, but weak response in hypocotyls of older seedlings (Figure S8) and no obvious response in fully developed mature leaves that have finished cell elongation (Figure S8). Therefore, 14-3-3s provide a potential point of crosstalk for other pathways to modulate BR response, which is important for optimizing growth according to physiological and environmental conditions. Nevertheless, the strong *bzr1-1D*-like phenotypes shown by the BZR1 Δ and BZR1S173A transgenic plants grown in both light and dark support a ubiquitous and critical role for 14-3-3 proteins in regulating BZR1.

14-3-3 proteins increase the cytoplasmic retention of phosphorylated BZR1. Disrupting 14-3-3 binding, either by mutating the binding site in BZR1 or treatment with AICAR, leads to increased nuclear localization of BZR1. BR-induced nuclear localization correlates well with BZR1 dephosphorylation and inhibition of 14-3-3 binding. These results strongly support an important role of 14-3-3s in BR regulation of subcellular localization of BZR1. BIN2 phosphorylation promotes cytoplasmic retention of BZR1 by promoting 14-3-3 binding, and BR-induced dephosphorylation increases nuclear localization of BZR1 by abolishing 14-3-3 binding.

The importance of cytoplasmic retention for inhibition of phosphorylated BZR1 is supported by the strong *bzr1-1D*-like phenotypes and altered expression of BZR1-target genes that are associated with increased BZR1 nuclear localization. It is possible, however, that 14-3-3 proteins may also inhibit other aspects of BZR1 function, such as BZR1's transcriptional activity or its interaction with other

partner proteins. However, such additional effects on transcriptional activity would be difficult to discern in the presence of altered nuclear localization. It should be noted that the nuclear BiFC signal of the BZR1:14-3-3 λ interaction suggests that 14-3-3s might inhibit the phosphorylated BZR1 in the nucleus, although it is possible that BZR1 cytoplasmic retention is mediated by other members of the 14-3-3 family rather than 14-3-3 λ in vivo. Four additional 14-3-3 isoforms interacted with BZR1 in our yeast two-hybrid screens, and at least three isoforms coimmunoprecipitated with BZR1-YFP. In fact, 14-3-3 λ is predominantly localized in the nucleus, while 14-3-3 ω , which interacts with BZR1 in yeast, 14-3-3 ϕ , and 14-3-3 ν all show wide distribution in the cytoplasm, at least in certain cell types (Paul et al., 2005; Sehnke et al., 2002). Therefore, it is unclear whether a particular 14-3-3 isoform(s) mediates cytoplasmic retention of BZR1 and BZR2/BES1 in vivo. Although cytoplasmic retention may not be the only mechanism of 14-3-3 inhibition of BZR1, it certainly contributes to the inhibition.

Similar functions of 14-3-3 proteins in regulating protein nuclear localization have been observed in several other systems. For example, in tobacco, 14-3-3s mediate gibberellin (GA) regulation of nuclear localization of transcriptional activator REPRESSION OF SHOOT GROWTH (RSG) (Ishida et al., 2004), in a manner similar to the regulation of BZR1. However, it remains unclear how GA signaling, which is mediated by a soluble receptor that controls degradation of a nuclear component, regulates the RSG:14-3-3 interaction (Ishida et al., 2004). In yeast, phosphorylation of Cdc25 by Chk1 kinase and MSN2 by TOR kinase creates binding sites in the transcription factors for 14-3-3s, which promote nuclear export of Cdc25 and MSN2, leading to DNA-damage checkpoint and inhibition of carbon-source-regulated genes, respectively (Beck and Hall, 1999; Lopez-Girona et al., 1999). Interestingly, 14-3-3 family proteins have also been shown to differentially regulate the glucocorticoid receptor (GR). 14-3-3 η is known to bind to GR and function as a positive regulator of GR by blocking its degradation (Kim et al., 2005), whereas 14-3-3 σ binds to GR and function as a negative regulator by retaining GR in the cytoplasm (Kino et al., 2003). A more striking parallel to the BR pathway is the regulation of heat-shock transcription 1 (HSF1) by GSK3 phosphorylation and 14-3-3 binding in humans (Wang et al., 2003, 2004). It remains unclear whether the BR signaling pathway is evolutionarily related to any animal signaling pathways, such as the heat-shock pathway, the Wnt signaling pathway, or cell-surface receptor-mediated steroid signaling pathways. The use of evolutionarily conserved signaling proteins such as GSK3 and 14-3-3s suggests that the BR signal transduction pathway is an ancient steroid signaling pathway.

In summary, this study illustrates a molecular mechanism by which 14-3-3 proteins mediate phosphorylation-dependent control of the BZR1 and BZR2/BES1 transcription factors in the BR signaling pathway of *Arabidopsis*. A similar mechanism of 14-3-3 regulation of the rice BZR1 homolog has also been observed (M.-Y.B. and Z.-Y.-W.,

unpublished data), suggesting that the regulation of localization and activity of BZR1 by 14-3-3s is a conserved mechanism for steroid regulation of gene expression in higher plants. 14-3-3 proteins may also regulate other components of the BR signaling pathway. Recent studies have shown that 14-3-3s copurify with BRI1 (Karlova et al., 2006) and interact directly with BAK1's *Arabidopsis* homolog, SERK1 (Rienties et al., 2004), and barley homolog, HvBAK1 (Schoonheim et al., 2007a). How 14-3-3s regulate the receptor kinases remains to be elucidated, and the physiological implications of 14-3-3 regulation of BR signaling is yet to be fully appreciated by future studies.

EXPERIMENTAL PROCEDURES

Plant Material

Arabidopsis thaliana ecotype Columbia-0, *bzr1-1D*, *bri1-5*, transgenic *Arabidopsis* plants harboring either full-length YFP fusions or Bi-Molecular Fluorescence Complementation (BiFC) constructs, and *Nicotiana benthamiana* (tobacco) plants were all grown in green houses under 16 hr light-long day conditions.

Dark-Grown Phenotypic Analysis

Arabidopsis seeds were sterilized with bleach and germinated on half-strength Murashige and Skoog (MS) medium supplemented with or without 2 μ M BRZ. Seeds were stratified for 3 days at 4°C and were kept under white light for 6 hr before being moved into the dark. Hypocotyl lengths from 4-day-old vertically grown seedlings were measured by using ImageJ software, available at the NIH website ([Abramoff et al., 2004]; <http://rsb.info.nih.gov/ij/>).

Isolation of the Nuclear Fraction

Transgenic seedlings expressing BZR1-CFP were grown in liquid medium containing half-strength MS and 1% sucrose for 9 days under continuous light. They were treated with 100 nM BL or mock solution for 1 hr, and samples were harvested. Nuclei protein fractions (Figure 1C) were extracted according to a published protocol (Bae et al., 2003).

For the data shown in Figure S6, nuclei fractions were isolated by using the following protocol. Plant material was ground in NEB buffer (20 mM HEPES [pH 7.5], 40 mM KCl, 10 mM MgCl₂, 1% Triton X-100, 1 mM EDTA, 10% glycerol) and filtered through Miracloth (Calbiochem, San Diego, CA) to obtain the total extract. It was then centrifuged at 5000 \times g for 2 min to separate the cytosolic fraction (supernatant) from the nuclei fraction (pellet). SDS gel loading buffer was added to each fraction, heated at 65°C for 5 min, centrifuged at 20,000 \times g for 5 min, and analyzed on a 4%–12% gradient SDS-PAGE gel (Invitrogen).

Plasma Membrane Fractionation

One-week-old liquid-grown seedlings treated with either mock or 100 nM BL solution for 2 hr were processed for plasma membrane (PM) preparation (Figure 1D). Seedlings were ground in 2 vol. Buffer H (100 mM HEPES-KOH [pH 7.5], 330 mM sucrose, 10% [v/v] glycerol, 5 mM EDTA, 5 mM ascorbic acid, 0.6% [w/v] PVP 40, 5 mM DTT, 25 mM NaF, 2 mM imidazole, 1 mM sodium molybdate, and protease inhibitors) with a Powergen 700D homogenizer at 5,000 rpm for 3 min. Homogenate was filtered through a single layer of Miracloth and was centrifuged at 10,000 \times g for 10 min to remove cell debris and organelles. The microsomal fraction was collected by centrifuging at 60,000 \times g for 30 min. An aliquot of supernatant containing the soluble protein was snap frozen in liquid nitrogen. The microsomal pellet was resuspended in Buffer R (5 mM potassium phosphate [pH 7.8], 330 mM sucrose, 3 mM KCl, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors). Similarly, an aliquot of the microsomal fraction was loaded onto a two-phase partitioning system containing 6% polymers and 8 mM KCl, and

partitioning was done according to Larsson et al. (1994). U3 upper phase was diluted with 10 vol. Buffer R and was centrifuged at 200,000 \times g for 1 hr to pellet PM vesicles. After the final centrifugation, PM vesicles were resuspended in a buffer containing 5 mM potassium phosphate (pH 7.8), 250 mM sucrose, 3 mM KCl, 0.1 mM EDTA. All protein samples were quantified by using the Bradford assay (Bio-Rad, Hercules, CA), and equal amounts of proteins were loaded on the SDS-PAGE gels for immunoblots, which were probed with a polyclonal anti-GFP antibody (Wang et al., 2002).

Southwestern Blot

Phosphorylation of MBP-BZR1 by GST-BIN2 was carried out in the presence of 100 μ M ATP as described previously (He et al., 2002). Unphosphorylated and phosphorylated MBP-BZR1 proteins were separated on SDS-PAGE gels and blotted on nitrocellulose membrane. The blot was incubated with a CPD probe that is chemically synthesized (5'-AAAAGGTCCTACTTTATGCAGAAACCCCGTGTGCCCACTCTCCCTTC-3') and labeled with ³²P-dTTP.

Overlay Western Blot

A gel blot containing unphosphorylated and phosphorylated MBP-BZR1 proteins was incubated with 20 μ g recombinant GST-14-3-3 λ protein, washed, and then probed with anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Mass Spectrometry

MBP-BZR1 protein phosphorylated by GST-BIN2 in vitro was subjected to standard in-solution alkylation/tryptic digestion. Briefly, 1000 \times molar excess DTT over total proteins in 25 mM ammonium bicarbonate was added, and the reduction reaction was carried out for 1 hr at 37°C. Then, 5.5 \times molar excess iodoacetamide over DTT was added in 25 mM ammonium bicarbonate, and the alkylation reaction was carried out at 25°C in the dark for 1 hr. Low-molecular weight compounds in the reaction mixture were reduced by using Amicon Ultra-4 10K (Millipore, Billerica, MA), and proteins were in a buffer containing 25 mM ammonium bicarbonate and 20% acetonitrile. Tryptic digestion was carried out overnight by 1% (w/w) modified trypsin (Promega, Madison, WI) and was stopped by adding formic acid to a final concentration of 1% in the solution.

The digestion mixture was diluted in water to a concentration of 100 fmole/ μ l, and 1 μ l of the aliquot was injected into an Ultimate Capillary LC system via a FAMOS Autosampler (LC Packings, Sunnyvale, CA). Peptides were separated on a 75 μ m \times 15 cm C₁₈ reverse-phase capillary column at a flow rate of 300 nl/min while running a 3%–32% acetonitrile gradient with 0.1% formic acid. The HPLC eluent was connected directly to an LTQ/FT instrument (Thermo Finnigan, Bremen, Germany). The LC-MS/MS method consisted of one survey scan in FT mode with a resolution of 25,000, followed by three CID scans in LTQ. The raw LC-MS/MS data were converted to ASCII peak list by Mascot Distiller software (Matrix Science, Boston, MA), with carbamidomethylation as a fixed modification and phosphorylation and methionine oxidation as variable modifications.

In Vitro Pull-Down Assay

Unphosphorylated and phosphorylated MBP-BZR1 proteins were mixed together and were incubated with beads containing either GST-14-3-3 λ or GST alone in binding buffer (50 mM Tris-Cl [pH 7.5], 5 mM MgCl₂). After incubation for 1 hr, beads were washed with binding buffer, and 14-3-3-binding proteins were eluted with elution buffer (50 mM Tris-Cl [pH 7.5], 5 mM glutathione) and detected by using a polyclonal anti-MBP antibody in western blot.

Transient Transformation

Agrobacterium cells containing either full-length or BiFC expression vectors were washed and resuspended in the induction medium (10 mM MES buffer [pH 5.6], 10 mM MgCl₂ and 150 μ M acetosyringone) and were infiltrated into young leaves of 4-week-old tobacco plants. A total of 36 hours after infiltration, expression of various fluorescent

proteins was analyzed by using confocal microscopy. The data generated by using this system were also confirmed in stable transgenic *Arabidopsis* plants.

Coimmunoprecipitation, IP

Plant material was ground in NEB buffer (20 mM HEPES [pH 7.5], 40 mM KCl, 1 mM EDTA, 1% Triton X-100), filtered, and centrifuged at 20,000 × g for 10 min. Supernatant (1 ml) was incubated with anti-GFP antibody coupled to protein A Sepharose beads for 20 min. Beads were washed four times with wash buffer (20 mM HEPES [pH 7.5], 40 mM KCl, 0.1% Triton X-100), and bound proteins were eluted with buffer containing 2% SDS.

Plasmids

The BiFC system was engineered based on the 172 N-terminal amino acids (aa 1–172) of enhanced Yellow Fluorescent Protein (eYFP) and the 85 C-terminal amino acids (aa 154–239) of enhanced Cyan Fluorescent Protein (eCFP), which were subcloned into pPZP312 driven by the CaMV 35S promoter and a CaMV terminator derived from pRT100. These two halves were named nYFP and cYFP, respectively (the cYFP differs from cCFP by two amino acids, namely, V164A and Y204T). All vectors were converted into gateway-compatible destination vectors by PCR cloning the Gateway cassette from Gateway Vector Conversion System (Invitrogen, Carlsbad, CA) by using the BglII and HindIII restriction sites. Entry clones containing various interacting proteins were recombined with the Gateway-destination vectors by using the LR reaction kit (Invitrogen). The full-length coding sequence of 14-3-3 λ was cloned directly into binary vectors by using XbaI sites engineered into pGREEN0229 and pPZP312 vector backbones. Full-length CFP and YFP fusions of wild-type and mutant BZR1 (containing mutations in the 14-3-3-binding site) were made in pEarleyGate (pEG) vectors (Earley et al., 2006) driven by the CaMV 35S promoter. The BZR1:BZR1S173A-CFP construct was created by using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the wild-type construct as template (Wang et al., 2002). The primers used for site-directed mutagenesis PCR are listed in Supplemental Experimental Procedures. All binary vectors were introduced into *Agrobacterium* strain GV3101 by electroporation and then transformed into *Arabidopsis* by using the floral dip method. Basta was used for selecting the transgenic plants.

RNA and Protein Analysis

RNA samples were isolated from 2-week-old transgenic plants by using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA by using M-MuLV Reverse Transcriptase (MBI Fermentas, Hanover, MD) and oligo dT. Quantitative real-time PCR was performed by using iQ SYBR Green reagent (Bio-Rad, Hercules, CA) to amplify *BZR1-YFP*, *UBC*, *CPD*, and *DWF4* gene-specific regions. Primer sequences used for amplification are listed in Supplemental Experimental Procedures. Transgenic lines were compared for transcript abundance and were subsequently analyzed by western blots. BZR1-YFP protein was extracted in 2× SDS-PAGE buffer and was detected by using a polyclonal antibody against GFP (Wang et al., 2002).

Spinning-Disk Confocal Microscopy

Fluorescence of CFP, YFP, and BiFC (YFP) was visualized by using a spinning-disk confocal microscope (Leica Microsystems, Heerbrugg, Germany) (Paredes et al., 2006). In brief, CFP was excited by a HeCd laser at 442 nm, YFP was excited by an argon laser at either 488 or 514 nm, and emission filtering was achieved by using band pass filters 480/40, 525/50, 570/65 (Chroma Technology Corp., Rockingham, VT). Both glycerin (n.a. = 1.3) and oil-immersion (n.a. = 1.4) 63× objectives were used for confocal microscopy, and all images were acquired with a 512b Roper Cascade EMCCD camera with a gain of 3800. Two-frame averaging of image acquisition improved the signal-to-noise ratio. All images were acquired by using MetaMorph software (Molecular Devices, Sunnyvale, CA). Images of BZR1-YFP and mutant BZR1-YFPs were obtained with identical

image-acquisition settings; YFP was excited at 488 nm, and images were obtained with a 500 ms exposure time. For quantifying the effect of BRZ and BL on BZR1-YFP localization, images were obtained with a 200 ms exposure time (Figure 1A). For the time course experiment (Figure 1B), seedlings were grown on 2 μ M BRZ in the dark for 4 days, then mounted on a slide in 100 nM BL, and imaged immediately to obtain the image at time zero. The same field of view was imaged subsequently for the duration of the time course described in Figure 1B. A similar change in nuclear localization was observed when seedlings already under the microscope in liquid MS medium were profused with BL solution. Time course experiments were repeated three times, and representative data are shown in Figure 1B. Images of YFP fluorescence in BiFC assays were obtained by using 488 nm excitation with a 500 ms exposure time (Figure 2).

Quantification of Fluorescent Protein Signal

All images were analyzed in ImageJ (<http://rsb.info.nih.gov/ij/>). To measure the ratio between nuclear and cytoplasmic signals of BZR1-YFP for each cell, a small area of fixed size (21 pixels) was drawn, and measurements of integrated densities were taken from representative areas within the nucleus, cytoplasm, and background (central vacuole) of each cell. Three repeat measurements were performed for each cell, and the average of background values was then subtracted from the average values for the nuclear and cytoplasmic signals. The nuclear to cytoplasmic signal ratio was then calculated for each cell. The average nuclear/cytoplasmic signal ratio and standard error for independent T1 transgenic lines were calculated from measurements of at least seven cells from each plant (Figure 4B). Line scan measurements spanning both the nucleus and cytoplasm were also carried out, and representative plot profiles of sample measurements are presented in Figure 1A. For quantifying the BiFC signal, an outline was drawn around each transformed cell, and the signal intensity (integrated density) of the whole cell was calculated after subtracting the mean background density measured from an adjacent untransformed cell. Signal intensities from at least seven transformed cells were then averaged (Figure 2G).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, eight figures, and two movies and are available at <http://www.developmentalcell.com/cgi/content/full/13/2/177/DC1/>.

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