Nucleocytoplasmic Shuttling of BZR1 Mediated by Phosphorylation Is Essential in Arabidopsis Brassinosteroid Signaling

Hojin Ryu,a,1 Kangmin Kim,a,1 Hyunwoo Cho,a Joonghyuk Park,a Sunghwa Choe,b and Ildoo Hwanga,2
a Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea
b Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-747, Korea

Phytohormone brassinosteroids (BRs) play critical roles in plant growth and development. BR acts by modulating the phosphorylation status of two key transcriptional factors, BRI1 EMS SUPPRESSOR1 and BRASSINAZOLE RESISTANT1 (BZR1), through the action of BRASSINOSTEROID INSENSITIVE1/BRI1 ASSOCIATED RECEPTOR KINASE1 receptors and a GSK3 kinase, BRASSINOSTEROID INSENSITIVE2 (BIN2). It is still unknown how the perception of BR at the plasma membrane connects to the expression of BR target genes in the nucleus. We show here that BZR1 functions as a nucleocytoplasmic shuttling protein and GSK3-like kinases induce the nuclear export of BZR1 by modulating BZR1 interaction with the 14-3-3 proteins. BR-activated phosphatase mediates rapid nuclear localization of BZR1. Besides the phosphorylation domain for 14-3-3 binding, another phosphorylation domain in BZR1 is required for the BIN2-induced nuclear export of BZR1. Mutations of putative phosphorylation sites in two distinct domains enhance the nuclear retention of BZR1 and BR responses in transgenic plants. We propose that the spatial redistribution of BZR1 is critical for proper BR signaling in plant growth and development.

INTRODUCTION

Brassinosteroids (BRs) are a group of plant steroid hormones that function in diverse plant developmental and growth processes through a signaling cascade from the BR receptor to the expression of BR target genes (Mandava, 1988; Clouse et al., 1996; Vert et al., 2005). Plants that are defective in BR signaling or biosynthesis display characteristic dwarfism in the light and photomorphogenesis in the dark (Clouse et al., 1996; Li and Chory, 1997). BRs bind to the BRASSINOSTEROID INSENSITIVE1 (BRI1)/BK1 receptor complex at the plasma membrane, causing the release of BK1 (He et al., 2000; Wang et al., 2001; Kinoshita et al., 2005; Wang et al., 2006). The subsequent dimerization of BRI1 and BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1) activates a downstream signal transduction pathway that leads to BRI1 EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1), the key transcriptional factors in BR signal transduction (Li et al., 2002; Nam and Li, 2002; Wang et al., 2002; Yin et al., 2002). The phosphorylation status of BES1 and BZR1 appears to control their signaling activity (He et al., 2002; Yin et al., 2002) and is in turn determined by BIN2, a glycogen synthase kinase-3 (GSK3)-like kinase (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), and BRI1 SUPPRESSOR1 (BSU1), a plant-specific phosphatase (Mora-Garcia et al., 2004). BR signals rapidly induce the dephosphorylation of BES1 and BZR1; however, how this modulates their transcriptional activity is not fully understood. BR-induced dephosphorylation of BES1 and BZR1 and their concomitant accumulation in the nucleus are suggested to result from either nuclear translocation or protein stabilization, correlated with a shift from hyperphosphorylation to dephosphorylation (He et al., 2002; Wang et al., 2002; Yin et al., 2002).

In these characteristics and the high similarity of BIN2 to metazoan GSK3α, the BR signaling pathway has been proposed to resemble the canonical Wnt signaling pathway (He et al., 2002; Yin et al., 2002; Peng and Li, 2003). Recently, however, Vert and Chory (2006) reported that the downstream events mediated by BES1 and BIN2 occur constitutively in the nucleus and suggested that the phosphorylation status primarily regulates the DNA binding and trans-activation of BES1 rather than its stability or subcellular localization. Nevertheless, the rapid shift in BES1 and BZR1 phosphorylation status, the nucleocytoplasmic localization of BIN2, and the slightly increased nuclear accumulation of BES1 and BZR1 following BR treatment indicate that the dynamic distribution of BES1 and BZR1 could provide another layer of regulation in BR signaling. Moreover, the sequential signaling events from membrane-associated BR reception to transactivation of BR regulators in the nucleus must be mediated through a cytosolic signaling cascade; however, the location and mechanism of the regulatory events in BR signaling are still largely unknown.

We show here that BZR1 itself functions as a nucleocytoplasmic signaling mediator by shuttling between the cytoplasm and the nucleus. The localization of BZR1 is tightly correlated with its phosphorylation status. We demonstrate that BIN2-mediated phosphorylation directly induces the nuclear export and subsequent cytoplasmic retention of BZR1. We also found that the 14-3-3 proteins play a role in the cytoplasmic translocation of BZR1. Mutations of putative phosphorylation and 14-3-3 recognition sites identified in BZR1 lead to constitutive nuclear localization of BZR1 and result in a strong BR response, showing bzz1-1D–like

1 These authors contributed equally to this work.
2 Address correspondence to ihwang@postech.ac.kr.
The author responsible for distribution of materials integral to this findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ildoo Hwang (ihwang@postech.ac.kr).

OA Open Access articles can be viewed online without a subscription.
W Online version contains Web-only data.
®Online version contains Web-only data.
phenotypes. Based on these observations, we propose that the phosphorylation status of BZR1 is important for the subcellular distribution of BZR1 to modulate the sensitivity and magnitude of BR signals between the nucleus and the cytosol.

RESULTS

BZR1 Is a Nucleocytoplasmic Protein, and Its Subcellular Distribution Is Interrelated with BIN2 Activity

To understand how BIN2-mediated phosphorylation negatively regulates BZR1 activity in BR signaling, we first analyzed the expression patterns of BIN2 using ProBIN2:GUS transgenic lines (Figure 1A) that carry the β-glucuronidase (GUS) gene under the control of the BIN2 promoter. Histochemical analysis of transgenic roots revealed that the expression of BIN2 is regulated differently in different developmental zones. BIN2 was expressed largely in the epidermis of the maturation zone. By contrast, GUS activity was specifically observed in the vascular tissues but not in the epidermis of the elongation zone. Such distinct expression patterns of GUS imply that BIN2 expression and its regulatory function in the phosphorylation of BZR1 are likely regulated by upstream signals having tissue and/or developmental specificity.

**Figure 1.** Nucleocytoplasmic Distribution of BZR1 Negatively Follows BIN2 Activity.

(A) Nuclear localization of BZR1-CFP is decreased in tissues in which BIN2 is actively expressed. The expression of BIN2 was monitored by the BIN2 promoter–driven GUS activity at the maturation (top panel) and elongation (bottom panel) zones of 2-week-old roots. Subcellular localization of BZR1-CFP in the epidermis and vasculature of the corresponding tissues was examined by optical sectioning using confocal laser scanning microscopy. The CFP signal at the epidermis of the root tip is also presented. Note that autofluorescence in wild-type plants (ecotype Columbia [Col-0]) was nearly undetected. Top, the top of the confocal layers; Middle, an internal layer with the vasculature. The scanning depth from the tissue surface was designated Z.

(B) BZR1 is a multiphosphorylated and nucleocytoplasmic protein. The cytoplasmic and nuclear fractions from ProBZR1:BZR1-HA plants were separated by centrifugation. W, whole leaf extract; P, protoplast extract; N, nuclear fraction; C, cytosolic fraction. Histone H2B and RHA1 (Sohn et al., 2003) were used as nuclear and cytoplasmic markers, respectively.

(C) BZR1-HA is localized in the nucleus and cytoplasm in protoplasts. The cytoplasmic and nuclear fractions from protoplasts transfected with the indicated genes were separated by centrifugation. The proteins were detected with an anti-HA antibody. N, nuclear fraction; C, cytosolic fraction. ARR2-HA and AtMPK3-HA were used as nuclear and cytosolic markers, respectively.

(D) The hyperphosphorylated BZR1 by BIN2 accumulates mainly in the cytoplasm. Protoplasts were transfected with BRI1-Myc/BZR1-HA or BIN2-Myc/BZR1-HA. The nuclear and cytosolic fractions were probed with an anti-HA antibody. ARR2-HA was cotransfected as a nuclear marker (Hwang and Sheen, 2001).
To test whether the nuclear localization of BZR1 has tissue specificity and is correlated with the spatial distribution of BIN2, we monitored the subcellular localization of BZR1-CFP (cyan fluorescent protein) (Wang et al., 2002) where BIN2 was highly expressed. We examined BZR1-CFP signals not only in the epidermis but in the vascular tissues of different developmental zones of roots by optical sectioning (Figure 1A; see Supplemental Figures 1 and 2 online). As reported previously, BZR1 was localized mainly in the nucleus around root tips (Zhao et al., 2002). Interestingly, however, we found that BZR1-CFP signals were observed in the cytosol as well as the nucleus of other developmental zones. The relative intensities of nuclear BZR1-CFP were variable among developmental zones (Figure 1A). In the maturation zone, the nuclear BZR1 was rarely detected in the epidermis, whereas strong nuclear signal was observed in the regions around vascular tissues (top panel, $Z = 27 \mu m$), where BIN2 expression appeared to be minimized. In the elongation zone, BZR1-CFP was localized mainly in the nucleus of the epidermis (bottom panel). Notably, the level of nuclear BZR1-CFP was largely decreased in the vascular tissues (bottom panel, $Z = 36 \mu m$), which have increased BIN2 expression. On the other hand, the BZR1-CFP signal in the nucleus was not detected in the epidermis of the elongation zone of Arabidopsis thaliana roots grown in a medium containing brassinazole (BRZ), a brassinosteroid biosynthesis inhibitor (see Supplemental Figure 3 online), indicating that the nuclear localization of BZR1 is positively regulated by the BR signal. Taken together, these observations suggest that BZR1 is localized in both the nucleus and the cytosol.

Subcellular localization of BZR1 interrelated with BIN2 expression implicates BIN2-catalyzed phosphorylation of BZR1 in controlling the subcellular localization of BZR1. To test this possibility, we examined the spatial distribution and the phosphorylation status of BZR1-HA (hemagglutinin) under the control of its own promoter in transgenic plants. Nuclear and cytoplasmic fractions were prepared from protoplasts of the transgenic plants, and BZR1 proteins were detected with an anti-HA antibody. BZR1 proteins were present as multiple phosphorylated bands (Figure 1B). Interestingly, hyperphosphorylated and hypophosphorylated forms exhibited distinct spatial distribution in either the cytoplasmic or the nuclear fraction (Figure 1B). The hypophosphorylated forms (the lower bands) were predominantly present in the nuclear fraction, whereas the hyperphosphorylated form (the highest band) was found exclusively in the cytoplasmic fraction, suggesting that the phosphorylation status is closely correlated with the localization. BZR1 proteins transiently expressed in protoplasts also showed a similar subcellular distribution pattern (Figure 1C). We then used a protoplast transient expression system to test whether the subcellular localization of BZR1 is modulated by BIN2-mediated phosphorylation (Figure 1D). Protoplasts were transformed with HA-tagged BZR1 and myc-tagged BIN2 or BR1. Coexpression of BIN2, a GSK3-like kinase, produced slower migrating BZR1 bands and resulted in the cytoplasmic localization of BZR1 (Figure 1D). In the presence of BR and coexpressed BR1, which favors the dephosphorylation of BZR1, BZR1 proteins were localized exclusively in the nucleus, again suggesting that the localization of BZR1 is determined by its phosphorylation status.

BZR1 Is a Nucleocytoplasmic Shuttling Protein, and Its Nuclear Export Is Largeley Regulated by BIN2

To investigate the spatial dynamics of BZR1, we fused BZR1 to green fluorescent protein (GFP) and monitored the subcellular distribution of BZR1 in the presence of BIN2 and its mutants having different catalytic efficiencies in phosphorylation events in a protoplast system. We first examined the transcription of CPD, a BR-responsive gene (Mathur et al., 1998; Noguchi et al., 1999), in protoplasts after BR treatment to determine whether BR signal is properly transmitted in Arabidopsis mesophyll protoplasts. BR treatment reduced the expression level of CPD in protoplasts as much as in seedlings (see Supplemental Figure 4A online). With a proper change in the phosphorylation status of BZR1 by BR treatment, this result suggests that the protoplast system is suitable for the elucidation of BR signaling mechanisms. Then, HA-tagged BIN2, bin2-1, or BIN2<sup>K69R</sup> was co-transfected into protoplasts with GFP-tagged BZR1 (Figure 2A). BZR1-GFP proteins were localized mainly in the nucleus but were also observed in the cytosol, as determined by the relative intensities of GFP and red fluorescent protein (RFP) fluorescent signals (Figure 2A; see Supplemental Figure 4B online). Both BIN2 and the gain-of-function bin2-1 mutant (Li and Nam, 2002) shifted the localization of BZR1 from the nucleus to the cytoplasm, whereas no shift was observed with BIN2<sup>K69R</sup>, a kinase-dead loss-of-function mutant (Zhao et al., 2002), demonstrating that the kinase activity of BIN2 correlates with both the cytoplasmic retention of BZR1 and its phosphorylation status. These results suggest that BIN2-catalyzed phosphorylation of BZR1 causes the active translocation of BZR1 proteins from the nucleus to the cytoplasm. At GSK1 and ASKd<sub>C</sub>, BIN2 homologs in Arabidopsis group II GSK3 (Jonak and Hirt, 2002; Vert and Chory, 2006) affected the localization of BZR1 in a manner similar to BIN2 and also interacted physically with BZR1 (see Supplemental Figure 5 online), indicating that the group II GSK3 kinases share the functional redundancy for the subcellular distribution of BZR1 and the negative regulation of BR signaling.

The cytoplasmic localization of BZR1 might be explained by BIN2-dependent phosphorylation of de novo synthesized proteins in the cytoplasm and then subsequent sequestration in the cytoplasm. Another possibility is that nucleus-localized BIN2 mediates the export of BZR1 from the nucleus by phosphorylating BZR1. To test these alternative hypotheses, we examined the localization of BZR1-GFP in protoplasts under tightly controlled BIN2 expression using a glucocorticoid receptor–inducible gene expression system (Figures 2B and 2C) (Yanagisawa et al., 2003). BIN2 expression was induced by dexamethasone (DEX) in a concentration- and time-dependent manner (Figure 2B). Without BIN2 induction by DEX, BZR1-GFP was localized mainly in the nucleus (Figure 2C). Following the induction of BIN2 expression, localization of BZR1 shifted from the nucleus to the cytoplasm, suggesting that preexisting nuclear BZR1 was exported to the cytoplasm after phosphorylation. To test this hypothesis further, we examined the effect of leptomycin B, a potent inhibitor of exportin-dependent nuclear export (Haasen et al., 1999), on the localization of BZR1 (Figure 2D). Treatment with leptomycin B followed by BIN2 induction with DEX caused the retention of BZR1 in the nucleus, proving that phosphorylation by BIN2 induces the nuclear export of BZR1.
**Figure 2.** BIN2 Expression Leads to BZR1 Transport from the Nucleus to the Cytosol.

(A) BIN2 increases the cytoplasmic localization of BZR1. BZR1-GFP was cotransfected into protoplasts with BIN2-HA, bin2-1-HA, or BIN2K69R-HA and examined by confocal microscopy. RFP-ARR2 was used as a nuclear marker. The fluorescent signal intensities of BZR1-GFP and RFP-ARR2 were determined along a line drawn on the confocal images using LSM Image Browser Rel. 4.0 software.

(B) BIN2-HA expression is specifically induced by DEX in a time- and concentration-dependent manner. Rubisco large subunit (RbcL) was used as a loading control.

(C) BIN2 induces the nuclear export of BZR1. Protoplasts were cotransfected with 8OP:BIN2-HA, Pro35S:VP16-GR, and BZR1-GFP. After incubation for 4 h to express GFP-tagged BZR1, BIN2 expression was induced by DEX treatment. 0 hr, time of DEX induction.

(D) Leptomycin B (LMB) blocks the BIN2-mediated nuclear export of BZR1. Protoplasts were transfected with the plasmids and incubated for 4 h as described for (C). The transformed protoplasts were treated with leptomycin B for 1 h and then further treated with DEX.
BR-Activated PP2A Phosphatases Rapidly Induce the Nuclear Translocation of BZR1

BZR1 proteins must be located in the nucleus to modulate the transcription of BR-responsive genes, meaning that BR signals must attenuate the BIN2-mediated cytosolic localization of BZR1. To test this, we coexpressed BZR1-GFP with BRI1 and BIN2 in Arabidopsis protoplasts. Without BR treatment, BZR1-GFP was located mainly in the cytoplasm (Figure 3A). However, BR increased the nuclear accumulation of BZR1 and BIN2 facilitated BR-induced nuclear localization, confirming that the BIN1-mediated BR signal inhibits the cytosolic translocation of BZR1 by BIN2. The effect of BR was due to neither the altered expression nor the stability changes of BIN2 by epi-brassinolide (BL) treatment (Figure 3B). We also monitored the phosphorylation status of HA-tagged BZR1 to examine the correlation between phosphorylation and subcellular distribution (Figure 3C). BR converted phosphorylated BZR1 proteins to the dephosphorylated state in cells coexpressing BRI1 and BIN2, suggesting that BR-induced BR1 activation potently attenuates BIN2 kinase activity.

The BIN2 action was suggested to be counteracted by BSU1, having features of both PP1- and PP2A-type phosphatase, which have several homologs in Arabidopsis (Mora-Garcia et al., 2004). To test whether the dephosphorylation and subsequent nuclear localization of BZR1 were mediated by PP2A-type phosphatases, we monitored the BR-induced nuclear localization of BZR1 in the presence of the PP1 and PP2A phosphatase-specific inhibitor, okadaic acid. When protoplasts expressing BZR1-GFP and BIN2-HA were treated with BR, BZR1 was localized to the nucleus (Figure 3D). However, 10 nM okadaic acid, a concentration that inhibits PP2A phosphatase, inhibited the translocation of BZR1 from the cytosol to the nucleus even in the presence of BR, suggesting that cytosolic PP2A-type phosphatases may mediate the BR-induced nuclear translocation of BZR1. To investigate whether BSU1 is involved in the nuclear localization of BZR1, we transiently coexpressed BZR1 with HA-tagged BIN2 and BSU1 in mesophyll protoplasts. Both BSU1-HA and BSU1-YFP facilitated the BR-induced dephosphorylation of BZR1, indicating that the tagged BSU1 proteins are functional in protoplasts (Figure 3E). BSU1 rapidly enhanced the nuclear accumulation of BZR1 upon BR treatment even in the presence of BIN2 (Figure 3F). Thus, BZR1 could be dephosphorylated in both the cytoplasm and the nucleus by BR-activated PP2A phosphatases. BSU1 would counteract the BIN2-mediated cytoplasmic translocation of BZR1 in the nucleus. Therefore, we propose that BZR1 is a nucleocytoplasmic shuttling protein whose localization is tightly regulated by its phosphorylation status, which is determined by the opposing actions of BIN2 and phosphatases, including BSU1.

Two Putative Phosphorylation Domains Are Important for the Nuclear Export of BZR1

We next analyzed the primary structure of BZR1 for putative phosphorylation sites and used site-directed mutagenesis to test the roles of the Ser/Thr residues in regulating subcellular distribution. We substituted each of 25 Ser/Thr residues, which were previously proposed as possible phosphorylation sites (Wang et al., 2002), with Ala and screened for mutants that have altered subcellular distribution by BIN2. We identified 10 residues in BZR1 that affect the BIN2-induced nuclear export of BZR1 in protoplasts and grouped them into two distinct domains by their phosphorylation status (Figure 4A; see Supplemental Figure 6 online). We followed two Ser mutants, BZR1S130A and BZR1S134A, as representatives of the first domain. BZR1S130A and BZR1S134A mutants appeared to be insensitive to BIN2, remaining localized in the nucleus (Figure 4B). The dephosphorylated proteins were significantly enriched in these mutants (Figure 4C). We then examined the effect of BIN2 on the phosphorylation status of the selected mutants. GFP-tagged wild-type BZR1 proteins exhibited both hyperphosphorylated and hypophosphorylated forms, but the cotransfection of BIN2 largely induced the hyperphosphorylation of wild-type BZR1 proteins (Figure 4C). However, substantial amounts of BZR1S130A and BZR1S134A still remained in the dephosphorylated state even in the presence of BIN2 (Figure 4C). The double BZR1S130/134A mutation further enhanced the dephosphorylation of BZR1 and the insensitivity to BIN2. These data suggest that the putative phosphorylation residues of the first domain, including Ser-130 and Ser-134, could be direct targets of BIN2 required for the nuclear export of BZR1.

Mutations at Ser-173 and Thr-177 in BZR1 also disrupted the cytoplasmic translocation of BZR1 induced by BIN2 (Figure 4B). However, BZR1S173A and BZR1T177A mutants were still hyperphosphorylated by BIN2 at a similar level to wild-type BZR1 (Figure 4C). These data indicate that Ser-173 and Thr-177 residues may not be direct targets of BIN2 but are still important as regulatory sites for the BIN2-mediated nuclear export of BZR1 by an unknown mechanism. Alternatively, it is also possible that these residues are phosphorylated by BIN2, although phosphorylation was not detected in our gel-shift assays.

Ser-173 and Thr-177 Are Critical for Interaction with the 14-3-3 Proteins in the Nuclear Export of BZR1

We previously generated BZR1 mutants disrupting a predicted nuclear export signal, but no alteration in subcellular localization was observed, implying that the nuclear export of BZR1 might require a guidance protein (data not shown). 14-3-3 proteins are known to play diverse regulatory roles as molecular chaperones in signaling networks and have been shown to mediate the nuclear export of Repression of Shoot Growth (RSG) protein in Arabidopsis GA signaling (Igarashi et al., 2001). Interestingly, the second putative phosphorylation domain of BZR1 encompassing Ser-173 contains a potential mode II type 14-3-3 binding site (RXXXpSXP), and phosphorylation of a conserved Ser residue in signaling networks and have been shown to mediate the nuclear export of Repression of Shoot Growth (RSG) protein in Arabidopsis GA signaling (Igarashi et al., 2001). Interestingly, the second putative phosphorylation domain of BZR1 encompassing Ser-173 contains a potential mode II type 14-3-3 binding site from the 169th to the 175th residues, RISNSCP (Figure 5A). We thus examined the physical interaction between BZR1 and 14-3-3 proteins in yeast two-hybrid assays (Figure 5B). We found that 14-3-3s and 14-3-3c: proteins interacted specifically with BZR1. 14-3-3 proteins are known to interact specifically with the conserved motif designated mode II (RXXpXpXpX), and phosphorylation of a conserved Ser residue is required for this interaction (Muslin et al., 1996; Aitken, 2006). Ser-173 in the putative 14-3-3 binding motif of BZR1 is likely to be phosphorylated for 14-3-3 binding (Figure 5A). We also...
Figure 3. Nuclear Translocation of BZR1 Is Induced by BR-Activated PP2A Phosphatase.

(A) BR induces the nuclear translocation of BZR1. BZR1-GFP was cotransfected with BRI1-HA and BIN2-HA into protoplasts and expressed for 5 h. The protoplasts were then treated with epi-BL for 2 h. 0 hr, time of BR treatment. Note that BRI1 expression facilitates the BR-induced nuclear localization of BZR1.

(B) The expression levels of BRI1-HA and BIN2-HA were similar in transformed cells.

(C) BRI1 facilitates the BR-mediated dephosphorylation of BZR1 in protoplasts. HA-tagged BES1 and BZR1 were cotransfected into protoplasts with the indicated amounts of BRI1-HA and 4 μg of BIN2-HA. After 4 h of transfection, the protoplasts were incubated with cycloheximide for 30 min before treatment with BR for 1 h.

(D) Okadaic acid, a PP2A phosphatase inhibitor, blocks BR-induced nuclear translocation of BZR1. Protoplasts were transfected with BIN2-HA and BZR1-GFP and incubated for 5 h to express the effectors. The transfected protoplasts were treated with 1 μM epi-BL alone and 10 or 30 nM okadaic acid (OA) together for 3 h.

(E) BSU1 accelerates the BR-induced dephosphorylation of BZR1. After incubation for 4 h, protoplasts transfected with BSU1-HA or BSU1-YFP and BZR1-HA were treated with or without epi-BL for 1 h in the presence of cycloheximide. BSU-HA and BZR1-HA proteins were detected using an anti-HA antibody.

(F) BSU1 enhances nuclear localization. Four hours after transfection, protoplasts expressing BSU1-HA, BIN2-HA, and BZR1-GFP were incubated without or with epi-BL for 1 h in the presence of cycloheximide. Subcellular localization of BZR1-GFP was followed by fluorescence microscopy.
predicted that the adjacent Thr-177 residue might have a similar role. However, mutations of these residues did not affect the interaction of BZR1 with 14-3-3 proteins in the yeast two-hybrid assay (data not shown). We thus examined the in vivo interaction of BZR1 and 14-3-3 proteins using coimmunoprecipitation studies. HA-tagged BZR1, BZR1S173A, or BZR1T177A was cotransfected with myc-tagged 14-3-3k or 14-3-3e into Arabidopsis protoplasts. When the whole protoplast lysate was immunoprecipitated with an anti-myc antibody, HA-tagged BZR1 but not BZR1S173A or BZR1T177A was pulled down with myc-tagged 14-3-3 proteins (Figure 5C). We also found that BZR1S130/134A coimmunoprecipitated with 14-3-3 proteins (data not shown). These data indicate that 14-3-3 proteins bind to BZR1 through the second phosphorylation domain containing Ser-173 and Thr-177 and modulate the subcellular distribution of BZR1. We here suggest that at least two putative phosphorylation domains of BZR1 regulate the nucleocytoplasmic shuttling of BZR1 via distinct regulatory mechanisms.

**Nuclear Retention of BZR1 Mutants Leads to Constitutive BR Responses in Arabidopsis, Independently of the Phosphorylation Status**

To investigate the physiological role of BZR1 subcellular localization, we generated transgenic plants that express BZR1, bzr1-1D, BZR1S130/134A, BZR1S173A, or BZR1T177A under the control of the 35S promoter. All of the dark-grown transgenic seedlings showed similar hypocotyl elongation to the wild type (Figure 6A). However, the bzr1-1D, BZR1S130/134A, BZR1S173A, and BZR1T177A transgenic lines were relatively resistant to the BR biosynthetic inhibitor BRZ. These lines had longer hypocotyls than wild-type and transgenic lines that overexpress BZR1, similar to the bzr1-1D mutant. Hypocotyl and root elongation of these transgenic lines grown in long-day conditions was more sensitive to exogenous BL (Figure 6B; see Supplemental Figure 7A online). Notably, the BZR1T177A line under the control of the 35S promoter responded to BL similarly to the ProBRI:BRI1-GFP transgenic lines (Nam and Li, 2002), which showed typical BR hypersensitivity, whereas the bri1-5 mutant was not affected by BL. Three-week-old BZR1S130/134A, BZR1S173A, and BZR1T177A transgenic lines also showed curled leaves and dwarfism, which was typically observed in the bzr1-1D transgenic plants (Figure 6C). The expression pattern of the transgene was verified by RT-PCR and immunoblotting with an anti-HA antibody (see Supplemental Figures 7B and 7C online). As shown by transient expression in protoplasts (Figure 4B), BZR1S130/134A was mainly dephosphorylated, whereas BZR1S173A and BZR1T177A were highly phosphorylated in the transgenic plants. The protein level of BZR1T177A was similar to that of BZR1.

We then examined the localization of BZR1T177A in the transgenic lines by subcellular fractionation (Figure 6D). Consistent with the nucleocytoplasmic localization of BZR1 under its own promoter shown in Figure 1B, overexpressed wild-type BZR1 proteins also localized to the nucleus and cytosol (Figure 6D), with an apparent distribution of the phosphorylated forms in the cytosolic fraction. By contrast, the phosphorylated form of BZR1T177A was localized mainly in the nucleus, further supporting the role of Thr-177 in the nuclear export of BZR1 in planta.
These results also indicate that the nuclear retention of BZR1\textsuperscript{T177A} may increase the binding capacity of BZR1 to its target genes and confer strong BR responses on the transgenic lines. To test this idea, we performed the chromatin immunoprecipitation (ChIP) assay on the CPD promoter (Figure 6E). The HA-tagged BZR1 and BZR1\textsuperscript{T177A} proteins in transgenic lines were immunoprecipitated with an anti-HA monoclonal antibody. The CPD target sequences that were communoprecipitated with BZR1-HA or BZR1\textsuperscript{T177A}-HA were amplified by standard PCR. The amount of the CPD promoter precipitated from the BZR1\textsuperscript{T177A} lines was slightly higher than that precipitated from the BZR1 lines, suggesting that BZR1 T177A represses CPD expression by direct binding to regulatory cis elements. Consistent with strong BR response phenotypes, the expression of the BZR1 target genes CPD and DWF4 was strongly reduced in the transgenic lines that ectopically express the mutated BZR1 transgenes (Figure 6F). It is notable that DWF4 transcription was greatly suppressed in the Pro35S:BZR1T177A lines, similar to the level in the bzr1-1D lines (Figure 6F) (Mora-Garcia et al., 2004; He et al., 2005). These results together indicate that increased nuclear retention of BZR1 by either its dephosphorylation or its inhibition of 14-3-3 interaction leads to BZR1 activation and strong BR responses in Arabidopsis.

**DISCUSSION**

BZR1 is a highly phosphorylatable protein and is rapidly dephosphorylated by brassinosteroid treatment. Various in vitro/in vivo studies have suggested that the phosphorylation status of BZR1 was regulated by BIN2 and BL. The role of the phosphorylation of BZR1 or BZR2/BES1 has been proposed to regulate a variety of molecular events: protein stability, cytoplasmic retention, multimerization, and inhibition of the transactivation/repression of target genes via direct interaction with cis elements (Vert et al., 2005; Vert and Chory, 2006). Our study showed that BIN2-mediated phosphorylation also caused the nuclear export of BZR1 (Figure 2), whereas BR induced the nuclear translocation of the protein. This result was correlated with previous observations that the nuclear localization of BZR1 and BZR2/BES1 was enhanced by exogenous BR treatment (Wang et al., 2002; Yin et al., 2002). However, other studies suggested that BZR1 and BZR2/BES1 are constitutively localized in the nucleus (Zhao et al., 2002; Vert and Chory, 2006). The discrepancy in BZR1 localization might be explained by differences in the analyzed tissues, developmental stages of the plants, and experimental conditions. The nuclear export of BZR1 appears to be minimal due to a strong input of BR signals in expanding tissues like hypocotyls and root tips of young seedlings, resulting in either constitutive or dominant nuclear retention of BZR1. However, dynamic redistribution of the nuclear BZR1 could have physiological importance in many cells under regulatory conditions in which the BR signal is needed to be desensitized or BIN2 activity is increased. Under such conditions, BIN2 could mediate the cytoplasmic translocation of BZR1 from the nucleus and thus minimize BR signaling activity in cells. Phosphorylation-mediated nucleocytoplasmic shuttling of BZR1 allows plants to respond swiftly to environmental or developmental stimuli without the de novo synthesis of BZR1. Multiple regulatory mechanisms for
Figure 6. Mutations of Putative Phosphorylation Residues Enhance BR Responses in Transgenic Plants.

(A) BZR1S130/134A, BZR1S173A, and BZR1T177A mutations suppress the BR-deficiency phenotype. Transgenic seedlings harboring HA-tagged bnr1-1D,
BZR1 and BZR2/BES1 likely contribute to tight regulation of the magnitude and sensitivity of BR signals in plant growth and development.

One important finding in our study is that, in a BR-stimulated growth state, cytosolic BZR1 itself may be an efficient transmitter of BR signals from receptor kinases at the plasma membrane to influence the expression of BR-responsive genes in the nucleus. This process must be delicately regulated by a BR-activated toggle switch composed of a kinase BIN2 and phosphatases like BSU1. Dissociation of BRI1 from BKI1, a negative regulator of BRI1 signaling, upon BR perception leads to the activation of an as yet unidentified cytosolic phosphatase or modification of BSU1, which might shift the equilibrium between BIN2 and BSU1 toward dephosphorylation and translocation of BZR1 to the nucleus. Simultaneously, BIN2 might be subjected to negative regulation, such as protein degradation, to facilitate the nuclear translocation of BZR1. It is also possible that the temporal and spatial regulation of BIN2 or BSU1 expression modulates BZR1 function as a cytoplasmic signal transmitter. This is supported by the interrelation between the subcellular distribution of BZR1 and the tissue and developmental specificity of BIN2 expression (Figure 4). Furthermore, BIN2 expression was not detected in the elongating region of dark-grown hypocotyls (data not shown); by contrast, BSU1 was reported to be highly expressed in expanding hypocotyls and completely silenced in fully expanded stems and leaves (Mora-Garcia et al., 2004). Such opposite expression patterns between BIN2 and BSU1 could explain the constitutive localization of BZR1 in the nucleus of rapidly elongating tissues. In other tissues, different degrees of subcellular distribution of BZR1 and possibly BIN2/BES1 might be determined by the differential activity of BIN2 and BSU1, which could contribute to the optimization of BR signaling during plant development.

In this study, we identified at least two domains carrying putative phosphorylation sites that modulated the subcellular localization of BZR1 (Figure 4). Positioned in the first domain, Ser-130 and Ser-134 residues are critical for the nuclear export of BZR1. Mutations of these residues enriched the dephosphorylation form of BZR1 and abolished BIN2-mediated phosphorylation, which is well correlated with increased nuclear retention of BZR1. As the putative phosphorylation residues, including Ser-130 and Ser-134, in the first domain are positioned in the conserved S/TxxxS/T GSK3 phosphorylation motifs, they are likely to be direct targets for BIN2 kinase. It is possible that the phosphorylation degree in this domain may directly determine the DNA binding affinity of BZR1 on cis elements of the BR-responsive genes. The second domain containing Ser-173 and Thr-177 is also important for the nuclear localization of BZR1. However, as opposed to the mutants in the first domain, S173A and T177A mutants were still sensitive to BIN2-mediated phosphorylation. The hyperphosphorylated state of these mutants is probably due to phosphorylation in the first domain. This result indicated that phosphorylation of the first domain alone may not be sufficient to mediate the nuclear export and that phosphorylation of the second domain may also be required, implying that the second domain is essential for the nuclear export of BZR1.

The second putative phosphorylation domain of BZR1 has a short stretch of amino acid sequence that is similar to the conserved 14-3-3 recognition motif, designated mode II RXYXpSXP residues (Aitken, 2006) (Figure 5). It is well known that phosphorylation of the fifth Ser residue is required for proper interaction of the 14-3-3 proteins with interacting partners. Interestingly, BZR1 appears to interact with 14-3-3v and 14-3-3c proteins, and Ser-173 and Thr-177 residues in the putative 14-3-3 binding domain of BZR1 are essential for this interaction (Figure 5). We also found that two additional isoforms, 14-3-3w and 14-3-3b, also interact with BZR1 in the yeast two-hybrid system (data not shown). Besides mutation at Ser-173 of the putative binding domain, mutation at Thr-177 also abolished interaction with the 14-3-3 proteins and resulted in the constitutive nuclear localization of BZR1. These results strongly suggest that Ser-173 or Thr-177 of BZR1 is directly targeted by phosphorylation for binding to the 14-3-3 proteins. However, it remains unknown whether Ser-173 is directly phosphorylated by BIN2 or a yet unidentified kinase in

Figure 6. (continued).

BZR1S130/134A, BZR1S173A, or BZR1T177A driven by the 3SS promoter display a BRZ-resistant phenotype similar to that of the bzr1-1D mutant. Seedlings were grown without or with BRZ (top panel) for 5 d in the dark, and the hypocotyl length of each seedling was measured. Error bars indicate SD (n = 10).

(B) The BZR1 mutations result in increased BR sensitivity in hypocotyl elongation. Transgenic lines that overexpress BZR1S130/134A-HA, BZR1S173A-HA, or BZR1T177A-HA show BR hypersensitivity similar to the Pro3SS:bzr1-1D and ProBRI1:BRI1-GFP plants. The brie-1 mutant is insensitive to BR. Seedlings were grown on Murashige and Skoog medium containing various concentration of epi-BL for 6 d, and the hypocotyl length of each seedling was measured. Error bars indicate SD (n = 10).

(C) Phenotypes of mutated BZR1 transgenic leaves (top panel) and plants (bottom panel) grown for 3 weeks under long-day conditions in soil. The mutants show BR-response phenotypes similar to that of the bzr1-1D mutant.

(D) The BZR1T177A mutation abolishes the cytoplasmic localization of hyperphosphorylated BZR1. Subcellular fractionation was performed with protoplasts isolated from Pro3SS:BZR1-HA and Pro3SS:BZR1T177A-HA transgenic lines. Note that the ectopic expression of BZR1 does not affect its subcellular distribution in the transgenic line. W, whole leaf extract; P, protoplast extract; N, nuclear fraction; C, cytosolic fraction. Histone H2B and RHA1 were used as the nuclear and cytoplasmic markers, respectively.

(E) The BZR1T177A mutation in the 14-3-3 binding site does not affect the DNA binding capacity of BZR1. ChIP assays of the CPD promoter were performed with BZR1-HA or BZR1T177A-HA transgenic plants using anti-HA monoclonal antibody. The specific primer set (He et al., 2005) was used to analyze the CPD promoter bound to the proteins.

(F) Expression of BR biosynthesis genes is suppressed in BZR1S130/134A-HA, BZR1S173A-HA, and BZR1T177A-HA transgenic lines. The transcript levels of CPD and DWF4 were quantified using quantitative real-time PCR. UBQ10 was used for normalization of the data. Error bars indicate SD (n = 3).
Role of BZR1 as a Nucleocytoplasmic BR Signaling Transmitter

BR signaling. Although the BR-induced nuclear retention of BZR1 and the constitutive nuclear localization of BZR1S173A and BZR1T177A mutants in protoplasts and transgenic plants are well correlated with a critical role of 14-3-3 proteins in the subcellular distribution of BZR1, it was difficult to test whether 14-3-3 interaction directly mediates the export of BZR1 from the nucleus, in part due to functional redundancy and high levels of 14-3-3 proteins. However, several studies have suggested that 14-3-3 proteins mediate the subcellular localization of interacting protein partners (Igarashi et al., 2001). Furthermore, BZR1 appeared to be exported from the nucleus to the cytosol via CRM1/exportin1 receptors (Figure 2G) (Fornèrd et al., 1997).

We generated a BZR1 mutant disrupting a predicted nuclear export motif, MEDLELT, but no alteration in subcellular localization was observed (data not shown). This indicates that the nuclear export of BZR1 is likely accompanied by exporting machinery complex formation, in part with binding to 14-3-3 proteins as molecular chaperones (Igarashi et al., 2001). However, it is also possible that 14-3-3 proteins mediate the cytoplasmic retention of BZR1 proteins. All of the interacting 14-3-3 proteins localize to both the nucleus and the cytosol. 14-3-3 proteins were also shown to interact with BAK1, its homolog SERK1, and the barley (Hordeum vulgare) homolog Hv BAK1 (Rientes et al., 2005; Karlova et al., 2006; Schoonheim et al., 2007), further supporting important roles of 14-3-3 proteins in BR signaling. 14-3-3 binding has recently emerged as a key regulatory mechanism in plant signaling as well as in yeast and animal systems. For example, 14-3-3 proteins negatively regulate nuclear localization of the bZIP transcriptional factor RSG to modulate gibberellin signaling in Arabidopsis (Igarashi et al., 2001; Ishida et al., 2004). A similar role of 14-3-3 proteins was also reported in DBP1 (tobacco protein phosphatase1) (Carrasco et al., 2006). Therefore, the modulation of signaling output via the subcellular redistribution of critical components by interaction with 14-3-3 proteins could be a conserved mode of regulation in plant developmental signaling.

BZR1S173A, BZR1T177A, and BZR1T177A mutations conferred increased BR responses in transgenic plants. In particular, BZR1S173A and BZR1T177A, but not wild-type BZR1, caused the strong brz1-1D–like phenotypes, although its phosphorylation status was similar to that of the wild type. We also found that BZR1T177A proteins were dephosphorylated by BL treatment (data not shown) and bound to a target CPD promoter in plants (Figure 6D). This result indicates that phosphorylation in the first domain is not sufficient to inhibit BZR1 activity, but phosphorylation in the second domain is required for full inhibition of BZR1 in vivo, even though BIN2-mediated phosphorylation inhibits DNA binding and transactivation of a BZR1 homolog, BZR2/BES1, in vitro (Vert and Chory, 2006). It is likely that enhanced nuclear localization of BZR1 increases the pool size of BSU1 substrates in the nucleus. This could enhance DNA binding capacity to its target promoters and thus induce strong BR responses in the BR-stimulated state, which would lead to BR hypersensitivity and brz1-1D–like phenotypes in the transgenic plants. It is also possible that an interaction between 14-3-3 and the phosphorylated second domain could block the DNA binding motif by either modulating the multimerization of BZR1 or regulating interaction with other proteins or exporting machinery. BZR1T177A would relieve the inhibition induced by interaction between BZR1 and 14-3-3s and thus maintain the binding activity of the DNA binding motif. In any case, the phosphorylation status of the two domains of BZR1 appears to have distinct and diverse roles in BR signaling. This further suggests that multiple regulatory switches are required to fine-tune the signaling output of BZR1 in BR signaling. The subcellular redistribution of BZR1 following phosphorylation would be essential for normal BR responses in many cells and would provide another regulatory layer to tightly control the output strength of BR signaling in various tissues and developmental stages of plants.

Further Research

While this article was under revision, Gampala et al. (2007) and Bai et al. (2007) reported that the BIN2–catalyzed phosphorylation of BZR1 inhibits the BR response through the cytosolic retention of BZR1 induced by the interaction between BZR1 and 14-3-3 proteins in Arabidopsis and rice (Oryza sativa).

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild type and genetic background for all transgenic lines. Seeds were germinated in medium containing half-strength Gamborg B5 medium (Duchefa) and 1% sucrose under fluorescent light (14-h-light/10-h-dark cycle) at 22°C. Young seedlings were further grown either in medium or in soil.

Plasmid Constructs

BZR1, BSU1, BIN2. At GSK1, ASKkd, ASKk, 14-3-3c, 14-3-3v, and BZR1 cDNAs were cloned into plant expression vectors containing HA, myc, or GFP tags driven by the 35S C4PPDK promoter (Hwang and Sheen, 2001), as indicated in the figure legends. ARR2 cDNA was fused to monomeric RFP coding sequences in a plant expression vector controlled by the cassava vein mosaic virus promoter (Verdaguer et al., 1996). All of the mutants were generated with the QuickChange site-directed mutagenesis kit (Stratagene). For DEX-inducible expression of BIN2, BIN2 cDNA was fused to eight copies of the LexA binding sites as described (Yanagisawa et al., 2003), and the produced plasmid was designated 8OP:BIN2-HA. The ProSSS:VP16-GR construct was obtained from S. Yanagisawa. For the yeast two-hybrid interaction assay, cDNAs of BIN2, bin2-1, BIN2K69R, At GSK1, ASKkd, ASKk, 14-3-3c, and 14-3-3v were cloned into the vector pGBK77, which contains the GAL4 DNA binding domain, and BZR1 was cloned into pGAD77, which contains the GAL4 activation domain (Clontech).

Protoplast Transient Expression Assay and Fluorescence Microscopy

For transient expression assays, typically, 4 × 10^4 mesophyll protoplasts were isolated from 4-week-old seedlings, transfected with 20 to 40 µg of plasmid DNA purified by CsCl gradient ultracentrifugation, and then incubated under constant light at 23°C (Hwang and Sheen, 2001). For transient induction of BIN2-HA expression, 10 µg of 8OP:BIN2-HA and 10 µg of ProSSS:VP16-GR were cotransfected into wild-type protoplasts. After 3 h of incubation, the protoplasts were treated with 0, 5, or 10 µM DEX for 1 or 3 h. To test the BIN2-mediated cytosolic translocation of BZR1, 8OP:BIN2-HA, ProSSS:VP16-GR, and BZR1-GFP were cotransfected into protoplasts. After 5 h of incubation, the protoplasts were
incubated with or without 10 µM DEX for 1 or 3 h. For the nuclear export inhibition study, protoplasts transfected with the genes described above were pretreated with 30 nM leptomycin B (Sigma-Aldrich) for 1 h before DEX treatment. Protoplasts were further incubated without or with 10 µM DEX for 5 h. For the nuclear import experiment, BZR1-GFP was cotransfected with BIN2-HA, BIN2-HA and BR11-HA, or BIN2-HA and BSU1-HA. Transfected protoplasts were incubated for 4.5 h before treatment with 100 µM cycloheximide for 30 min and then further treated with 1 µM epi-BL for 1 to 3 h. For the nuclear import inhibition study, protoplasts transfected with the genes described above were pretreated with okadaic acid (10 and 30 nM) (Sigma-Aldrich) and 100 µM cycloheximide for 30 min before TR treatment. All transient transfection experiments were repeated at least four times. GFP and RFP fluorescence was observed with either a confocal laser scanning microscope (LSM 510 Meta system; Carl Zeiss MicroImaging) or a fluorescence microscope (Axioplan; Carl Zeiss MicroImaging). The signal intensities of GFP and RFP were quantitatively determined using LSM Image Browser Rel. 4.0 software (Carl Zeiss MicroImaging). To detect BZR1 localization in plants, the root tip, elongation zone, and maturation zone of 2-week-old Pro35S: BZR1-CFP transgenic plants (Wang et al., 2002) grown on half-strength Gamborg B5 medium with or without BRZ (1 µM) were observed with a confocal laser scanning microscope.

**Protein–Protein Interaction Assays**

For interaction among GSKs, 14-3-3, and BZR1, the yeast strain AH109 was transformed with pGBK7 vector expressing GSK1 (Yanagisawa et al., 2003). Protoplasts were lysed with a buffer (20 mM Tris-HCl, pH 7.0, 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl₂, 30 mM β-mercaptoethanol, 1× protease inhibitor cocktail, and 0.7% Triton X-100) and fractionated by centrifugation at 3000g. The supernatant was taken as the cytosolic fraction. The pellet was further washed with a resuspension buffer (20 mM Tris-HCl, pH 7.0, 25% glycerol, 2.5 mM MgCl₂, and 30 mM β-mercaptoethanol) and reconstituted as the nuclear fraction. Each fraction was resolved by SDS-PAGE, followed by immunodetection with a peroxidase-conjugated high-affinity anti-HA antibody (Roche Applied Science). Image analysis was performed using ImageJ software (Abramoff et al., 2004). Protein expression was verified by immunoblotting with an anti-HA antibody and anti-actin (MP Biomedicals), or anti-GFP (Clontech) antibody.

**ChiP Assay**

ChiP was performed as described (Gendrel et al., 2002) with a minor modification. Two-week-old seedlings (1 g) of transgenic and wild-type plants (Col-0 as a control) were cross-linked with 1% formaldehyde for 30 min before BR treatment. All transient transfection experiments were repeated at least four times. GFP and RFP fluorescence was observed with either a confocal laser scanning microscope (LSM 510 Meta system; Carl Zeiss MicroImaging) or a fluorescence microscope (Axioplan; Carl Zeiss MicroImaging). The signal intensities of GFP and RFP were quantitatively determined using LSM Image Browser Rel. 4.0 software (Carl Zeiss MicroImaging). To detect BZR1 localization in plants, the root tip, elongation zone, and maturation zone of 2-week-old Pro35S: BZR1-CFP transgenic plants (Wang et al., 2002) grown on half-strength Gamborg B5 medium with or without BRZ (1 µM) were observed with a confocal laser scanning microscope.

**Fractionation of Subcellular Organelles and Immunodetection**

Nuclear and cytoplasmic fractions in protoplasts expressing HA-tagged proteins were separated as described previously (Yanagisawa et al., 2003). Protoplasts were lysed with a buffer (20 mM Tris-HCl, pH 7.0, 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl₂, 30 mM β-mercaptoethanol, 1× protease inhibitor cocktail, and 0.7% Triton X-100) and fractionated by centrifugation at 3000g. The supernatant was taken as the cytosolic fraction. The pellet was further washed with a resuspension buffer (20 mM Tris-HCl, pH 7.0, 25% glycerol, 2.5 mM MgCl₂, and 30 mM β-mercaptoethanol) and reconstituted as the nuclear fraction. Each fraction was resolved by 10% SDS-PAGE, followed by immunodetection with a peroxidase-conjugated high-affinity anti-HA antibody, anti-RHA1, and anti-histone H2B (Upstate). For the immunoblot analyses, 3 to 20 µg of proteins from protoplasts or seedlings was analyzed by 10% SDS-PAGE and detected with a horseradish peroxidase-conjugated anti-HA, anti-myc, anti-actin (MP Biomedicals), or anti-GFP (Clontech) antibody.
clonal, the 3.4-kb Arabidopsis BIN2 promoter was cloned into the vector pCAMBIA 1303. Two-week-old T3 homozygous plants were stained with X-Gluc (Duchefa).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Subcellular Localization of BZR1-CFP in the Maturation Zone of Arabidopsis Roots.

Supplemental Figure 2. Subcellular Localization of BZR1-CFP in the Elongation Zone of Arabidopsis Roots.

Supplemental Figure 3. Subcellular Localization of BZR1-CFP in the Elongation Zone of Arabidopsis Roots Roots Grown on BRZ-Containing Medium.

Supplemental Figure 4. Mesophyll Protoplasts and Whole Plants Show a Similar Response to BR.

Supplemental Figure 5. Group II At GSKs Have Functional Redundancy in BR Signal Transduction in Arabidopsis.

Supplemental Figure 6. Identification of Putative Phosphorylation Residues Required for the Nuclear Export of BZR1 by BIN2.

Supplemental Figure 7. Transgene Expression and Physiological Analysis of Nucleus-Accumulated BZR1 Mutants.

ACKNOWLEDGMENTS

We thank Jen Sheen and Joe Kieber for comments on the manuscript and Zhiyong Wang for the Pro35S: BZR1-CFP seeds. This work was supported by grants from the Plant Diversity Research Center and the Korea Science and Engineering Foundation (Grant M10601000194-06N0100-19410), the Plant Signaling Network Research Center, and the Korea Research Foundation (Grants KRF-2005-070-C00129 and R08-2003-000-10819-0). K.K. was supported by the POSTECH Core Research Fund.

Received June 17, 2007; revised August 13, 2007; accepted August 28, 2007; published September 14, 2007.

REFERENCES


Nucleocytoplasmic Shuttling of BZR1 Mediated by Phosphorylation Is Essential in Arabidopsis
Brassinosteroid Signaling
Hojin Ryu, Kangmin Kim, Hyunwoo Cho, Joonghyuk Park, Sunghwa Choe and Ildoo Hwang

Plant Cell 2007;19;2749-2762; originally published online September 14, 2007;
DOI 10.1105/tpc.107.053728

This information is current as of July 1, 2017

Supplemental Data /content/suppl/2007/09/13/tpc.107.053728.DC1.html
References This article cites 44 articles, 17 of which can be accessed free at:
/content/19/9/2749.full.html#ref-list-1
eTOCs Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY