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Molecules and Cells

Phosphorylation Dependent Nucleocytoplasmic Shuttling of BES1 Is a Key Regulatory Event in Brassinosteroid Signaling

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Brassinosteroids (BRs) play important roles in plant growth and development. BRs modulate the phosphorylation status of two crucial transcription factors, BRI1 EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1). Here we show that BES1 functions as a nucleocytoplasmic signal transmitter, and that its subcellular localization modulates the output intensity of the BR signal. BRASSINOSTEROID INSENSITIVE2 (BIN2) and other group II GLYCOGEN SYNTHASE KINASE 3 (GSK3)-like kinases phosphorylate BES1 and induce its nuclear export by regulating its binding affinity with 14-3-3 proteins. We identified twelve putative phosphorylation residues in BES1. Two of these residues, Ser 171 and Thr 175, are critical for interaction with 14-3-3 proteins. The other putative phosphorylation sites in the N-terminal region are required for the BIN2-mediated nuclear export of BES1. Mutations of these motifs result in increased nuclear accumulation of BES1 and enhanced BR responses in transgenic plants. Taken together, our results indicate that the spatial redistribution of BES1 is important for regulation of the BR signaling output.

INTRODUCTION

Brassinosteroids (BRs) play diverse roles in plant growth and development, including photomorphogenesis, vasculature development, lateral root formation, reproductive growth, and cell elongation (Clouse et al., 1996; Mandava, 1988; Vert et al., 2005). BRs are directly perceived at the plasma membrane by the receptor kinase BRI1 (He et al., 2000; Li and Chory, 1997; Wang et al., 2001). In the absence of the BR signal, BRI1 is inactivated and is part of a complex with BRI1 KINASE INHIBITOR 1 (BKI1), a repressor of BRI1, and BR-SIGNALING KINASE 1 (BSK1), a positive regulator. BRI1 is activated by direct binding to BRs, which induce heterodimerization with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) (Li et al., 2002; Nam et al., 2002; Wang and Chory, 2006; Yun et al., 2009). Then, BSK1 and BSU1 are sequentially activated by phosphorylation. BSU1 inactivates BIN2 kinase by dephosphorylation of a phospho-tyrosine residue, and this is followed by the dephosphorylation and nuclear accumulation of BES1 and BZR1 and a rapid enhancement in their transcriptional activity (Kim et al., 2009; Mora-Garcia et al., 2004; Tang et al.,2008; Wang et al., 2002; Yin et al., 2002). In the nucleus, the dephosphorylated BES1 and BZR1 directly bind to their *cis* elements, known as E-box (CANNTG) and BR-response element (CGTG(T/C)G), and regulate the expression of diverse BR responsive genes (He et al., 2005; Yin et al., 2005).

BES1 and BZR1 were identified in genetic screens for positive regulators that function downstream of BRI1. Both *bes1-D* and *bzr1-1D*, which are gain-of-function mutants of BES1 and BZR1, display BR-activated phenotypes such as the suppression of *bri1-5* dwarfism, insensitivity to the BR biosynthetic inhibitor brassinazole (BRZ), and the up-regulation of BR-responsive genes. BES1 and BZR1 exhibit 88% amino-acid sequence identity and each contain highly conserved GSK3 phosphorylation sites, a PEST motif, and a nuclear localization signal. The phosphorylation states of BES1 and BZR1 are currently considered to be reliable molecular gauges to assess the level of the BR signal output (He et al., 2002; Wang et al., 2002; Yin et al., 2002; Zhao et al., 2002). Such phosphorylation states modulate various functions of the proteins, including transactivation activity, DNA binding affinity, protein stability, and spatial redistribution (Bai et al., 2007; Gampala et al., 2007; Ryu et al., 2007; Vert and Chory, 2006). Vert and Chory (2006) showed that the opposing actions of BIN2 and BSU1 on BES1 phosphorylation occur in the nucleus, and that these opposing actions regulate the transactivation activity of BES1. On the other hand, other studies indicate that phosphorylation induces the nuclear export of BZR1 via an interaction with 14-3-3 proteins (Bai et al., 2007; Gampala et al., 2007; Ryu et al., 2007). These differences in current opinions about the subcellular locations of phosphorylated BZR1 and BES1 imply that the BES1 and BZR1 proteins may vary in their functions, possibly depending on temporal and spatial conditions during BR signal transduction. This hypothesis is supported by the opposing phenotypes of the two gain-of-function mutants, *bes1-D* and *bzr1-1D*, under light-grown conditions. The *bes1-D* mutants exhibit constitutive BR responses, including pale green leaves and long petioles

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(Choe et al., 2001; Wang et al., 2001), whereas *bzr1-1D* mutants display semi-dwarf phenotypes and dark green leaves (Wang et al., 2002). Despite the high sequence similarity between BES1 and BZR1, they appear to have different physiological functions. This suggests that they may be regulated differently and may vary in their spatial redistribution in response to the BR signal.

Here, we show that BES1 functions as a nucleocytoplasmic shuttling protein, and that its spatial redistribution is tightly regulated by its phosphorylation status. BES1 has twelve putative phosphorylation sites at Ser/Thr residues, which are involved in its spatial redistribution. These sites are highly conserved with those of BZR1 (Ryu et al., 2007). We also demonstrate that the ectopic expression of nuclear localized BES1 leads to a BR-related phenotype and suppression of dwarfism in transgenic *bri1-5* mutants.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Col-0 and WS-2 were used as wild-type controls and as the genetic backgrounds of transgenic lines. *Arabidopsis* seeds were germinated in media (pH 5.7) containing 0.5× Gamborg B5 salts (Duchefa), 1% sucrose, and 0.8% phytoagar under long-day conditions (16 h light/8 h dark) at 22°C. Young seedlings were further grown either in medium or in soil.

Plasmid constructs and protoplast transient expression assays

The full-length cDNAs of *BRI1*, *BIN2*, *14-3-3κ*, *BES1*, and *BZR1* were cloned into plant expression vectors that contained hemagglutinin (HA), myc, or GFP tags and the 35S C4PPDK promoter (Hwang and Sheen, 2001). The *ARR2* cDNA was fused to a monomeric red fluorescent protein (mRFP) tag in a vector that contained the cassava vein mosaic virus promoter (Verdaguer et al., 1996). All point mutations in *BES1* were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) following the manufacturers' instructions. For transient expression assays, typically, 4×10^4 mesophyll protoplasts were isolated from four-week-old seedlings, and transfected with 20 to 40 μg of plasmid DNA purified using CsCl gradient ultracentrifugation. Transfected protoplasts were incubated under constant light at 22°C (Hwang and Sheen, 2001). The dexamethasone (DEX) inducible expression of *BIN2-HA* and the nuclear export/import assays of BES1 were performed as described previously (Ryu et al., 2007). For the nuclear export inhibition study, protoplasts were pretreated with 30 nM leptomycin B (Sigma) or 10 μM AICAR (Sigma) for 30 min before DEX treatment. All transient expression experiments were repeated at least three times.

Fluorescence microscopy

GFP and RFP fluorescence signals were observed either with a confocal laser scanning microscope (LSM 510 Meta system; Carl Zeiss MicroImaging, Inc.) or with a fluorescence microscope (Axioplan; Carl Zeiss MicroImaging, Inc.). The confocal laser scanning microscope was used to monitor BES1-GFP localization in the root tips and in the elongation and maturation zones of two-week-old *35S::BES1-GFP* transgenic plants (Vert and Chory, 2006) that were grown on 0.5× Gamborg B5 medium.

Protein-protein interaction assays

To perform the co-immunoprecipitation assays, HA-tagged *BES1*, *bes1-D*, *BES1*^{T175A}, *BZR1*, *bzr1-1D*, or *BZR1*^{T177A} were introduced into protoplasts together with myc-tagged *14-3-3κ*. Protoplasts were then incubated for 6 h to allow the expression of the transgenes. Total proteins were extracted from the transfected protoplasts using a buffer containing 50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 × protease inhibitor cocktail (Roche Applied Science), and 1% Triton X-100. Protein complexes that bound to a monoclonal anti-c-myc antibody (Cell Signaling) were co-precipitated using protein A/G plus-agarose beads (Calbiochem). Precipitated proteins were detected using a peroxidase-conjugated high-affinity anti-HA antibody (Roche Applied Science).

Fractionation of subcellular organelles and immunodetection

The nuclear and cytoplasmic fractions of protoplasts were separated as described previously (Ryu et al., 2007). The protoplasts were lysed with a buffer containing 20 mM Tris-HCl (pH 7.0), 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 30 mM β-mercaptoethanol, 1× protease inhibitor cocktail (Roche Applied Science, Germany), and 0.7% Triton X-100, and then centrifuged at 3,000 × *g*. The supernatant (i.e., the cytosolic fraction) was removed and stored on ice until use. The pellets (i.e., the nuclear fraction) were further washed with a resuspension buffer [20 mM Tris-HCl (pH 7.0), 25% glycerol, 2.5 mM $MgCl_2$, and 30 mM β-mercaptoethanol] and then reconstituted as nuclear fraction. The proteins in each fraction were separated by 10% SDS-PAGE, transferred to a PVDF membrane, and probed using peroxidase-conjugated high-affinity anti-HA (Roche Applied Science), anti-RHA1 (gifted from Dr. Inhwan Hwang, POSTECH), and anti-Histone H2B (Upstate) antibodies. For other immunoblotting analyses, 3 to 20 μg of proteins from protoplasts or seedlings were separated by 10% SDS-PAGE, blotted, and probed using the horseradish peroxidase-conjugated anti-HA (Roche Applied Science), anti-myc (Cell Signaling), anti-actin (MP Biomedicals), or anti-GFP (Clontech) antibodies.

Physiological analysis of transgenic *Arabidopsis*

Transgenic plants overexpressing the various *BES1* constructs were generated by cloning the coding sequences into the pCB302ES binary vector containing the 35S promoter and the HA tag (Hwang and Sheen, 2001). These were used to transform *Arabidopsis* by the *Agrobacterium*-mediated floral dipping method (Clough and Bent, 1998). Transgene expression was verified by immunoblotting using an anti-HA antibody. For hypocotyl elongation assays, T3 homozygous seeds were sown and grown for 4 days in the dark on 0.5× Gamborg B5 medium containing 1% sucrose and 1 μM BRZ (Sekimata et al., 2001). To determine the expression levels of the *CPD* and *DWF4* genes, total RNA was isolated from each transgenic line and reverse transcribed using oligo dT primers. Quantitative real-time PCR was performed using gene-specific primers, SYBR Premix Ex Taq (Takara), and the LightCycler 2.0 system (Roche), according to the manufacturers' instructions.

RESULTS

BES1 is a nucleocytoplasmic protein and its localization is determined by its BIN2-mediated phosphorylation status

Our previous study showed that the cytoplasmic localization of BZR1 was highly correlated with the *BIN2* expression pattern in different developmental zones of roots (Ryu et al., 2007). To determine whether BIN2 also affects BES1 localization, we monitored the BES1-GFP subcellular locations in different developmental zones of roots expressing *BIN2* at high levels (Ryu

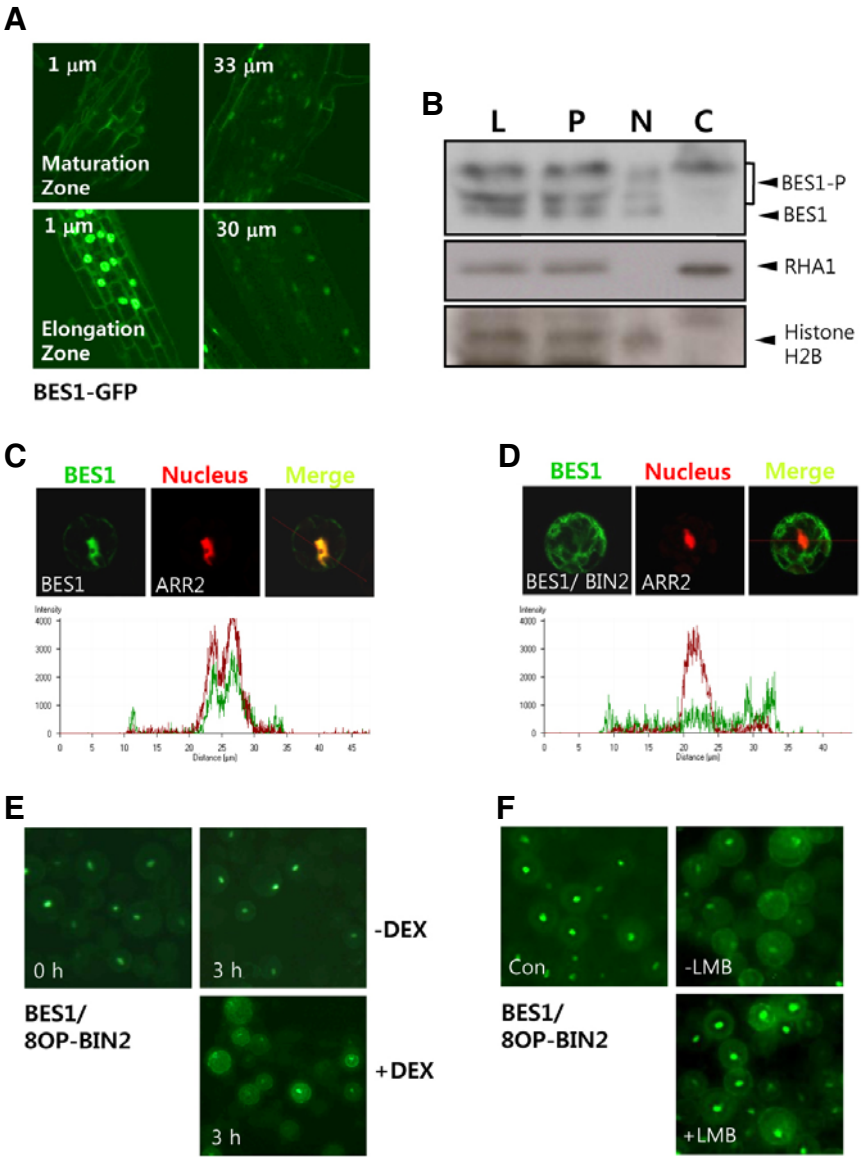

Fig. 1. BIN2-mediated phosphorylation induces the cytoplasmic localization of BES1. (A) BES1 is differently distributed in subcellular compartments depending on the tissue type. The subcellular locations of BES1-GFP were examined in the epidermis and vasculature of the maturation and elongation zones of two-week-old roots by optical sectioning using confocal microscopy. (B) Phosphorylated BES1 (BES1-P) is mainly localized in the cytoplasm. Proteins isolated from leaves (L), protoplasts (P), nuclear fractions of protoplasts (N), and cytosolic fractions of protoplasts (C) from *35S:: BES1-HA* transgenic plants were analyzed by immunoblotting with the anti-HA antibody. Histone H2B and RHA1 were used as nuclear and cytoplasmic markers, respectively. (C) BES1 is a nucleocytoplasmic protein. *BES1-GFP* and *ARR2-mRFP* were introduced into protoplasts and their subcellular locations were examined using fluorescence microscopy (upper panel). The intensities of the fluorescent signals along the line drawn on the merged image are shown in the lower panel. ARR2-mRFP was used as a nuclear marker. (D) BIN2 facilitates the cytoplasmic localization of BES1. Protoplasts were transfected with *BES1-GFP*, *BIN2-HA*, and *ARR2-mRFP* and examined by fluorescence microscopy (upper panel). The intensities of the fluorescent signals along the line drawn on the merged image are shown in the lower panel. (E) BES1 is exported from the nucleus by BIN2. Protoplasts were co-transfected with *8OP:: BIN2-HA*, *35S::VP16-GR*, and *BES1-GFP*. After incubation for 4 h, the cells were treated without DEX (0 h and 3 h) or with DEX for 3 h to induce BIN2 expression. (F) Leptomycin B (LMB) blocked the BIN2-mediated cytoplasmic translocation of BES1. Protoplasts were transfected with the genes described in (E), incubated for 4 h, treated with or without LMB for 1 h, and then treated with DEX.

et al., 2007; Yin et al., 2002) (Fig. 1A, Supplementary Figs. S1 and S2). The subcellular location of BES1 was closely correlated with the abundance of the *BIN2* transcript. Nuclear-localized BES1 was rarely detected in the epidermis of the maturation zone, where *BIN2* is highly expressed, whereas a strong BES1-GFP nuclear signal was observed in vascular regions of the maturation zone, where *BIN2* expression is low (Fig. 1A, upper panel, and Supplementary Fig. S1). However, in the elongation zone, BES1-GFP was localized mainly in the nuclei in the epidermis and in the cytosols in vascular tissues, where *BIN2* expression is low and high, respectively (Fig. 1A, lower panel, and Supplementary Fig. S2) (Ryu et al., 2007). We further confirmed the subcellular location of BES1 using biochemical fractionation (Fig. 1B). The hypophosphorylated forms of BES1 in the *BES1-HA* overexpressing plants were predominantly observed in the nuclear fraction, whereas the hyperphosphorylated forms were exclusively detected in the cytoplasmic fraction. To clarify the role of BIN2 in the subcellular localization of BES1, we monitored BES1-GFP signals in protoplasts, in the absence and presence of BIN2-HA, using confocal laser microscopy. In the absence of BIN2-HA, the fluorescent BES1-GFP signal was detected in both the nucleus and the cytoplasm (Fig. 1C). However, the coexpression of BIN2-HA and BES1-GFP increased the cytoplasmic accumulation of BES1-GFP (Fig. 1D). BIN2 is a group II GSK3 kinase (Jonak and Hirt, 2002; Ryu et al., 2007). Therefore, we also investigated whether other *Arabidopsis* group II GSK3 kinases also regulated the phosphorylation and subcellular localization of BES1. Other group II GSK3 kinases physically interacted with BES1 in yeast cells and induced the phosphorylation and nuclear export of BES1 (Supplementary Fig. S3). This implies that there is functional redundancy among these kinases in the regulation of BES1. Taken together, our results suggest that BES1 is a nucleocytoplasmic protein and that its subcellular

localization is regulated via BIN2-mediated phosphorylation.

The cytoplasmic localization of BES1 can be explained by either dynamic translocation from the nucleus or by inhibition of nuclear import. To investigate this further, we monitored the effect of BIN2 on the localization pattern of BES1 by tightly controlling *BIN2* expression using the dexamethasone (DEX)-activated BIN2-glucocorticoid receptor system in protoplasts (Ryu et al., 2007). BES1 was mainly localized in the nucleus before DEX treatment; however, the induction of *BIN2* expression by DEX treatment led to a decrease in the level of BES1-GFP in the nucleus (Fig. 1E). This suggested that the phosphorylated BES1 is translocated into the cytoplasm. In eukaryotes, many NES-containing proteins are translocated into the cytoplasm via CRM1/XPO1 (Exportin 1) (Fornerod et al., 1997; Fukuda et al., 1997). If BES1 is exported from the nucleus by the same mechanism, then its cytoplasmic translocation would be inhibited specifically by leptomycin B (LMB), which is a CRM1/XPO1 inhibitor (Haasen et al., 1999). LMB effectively blocked the BIN2-induced nuclear export of BES1, indicating that the export of phosphorylated BES1 to the cytoplasm was mediated by CRM1/XPO1 (Fig. 1F). We then investigated the primary structure of BES1 to identify an NES, which is characterized by a series of hydrophobic amino acids (e.g., leucine) (Bogerd et al., 1996). BES1 carries a putative NES, consisting of a series of closely spaced large hydrophobic amino acids, which is similar to those observed in TF III-type transcription factors (Supplementary Fig. S4A). To test whether this putative NES is functional, we generated a BES1 mutant in which the NES was disrupted. Interestingly, the mutant protein was translocated from the nucleus (Supplementary Fig. S4B), which implies that the nuclear export of BES1 is not mediated by this putative NES.

BRs promote the nuclear translocation of BES1

BES1 is required in the nucleus for the transcriptional regulation of various BR-related genes (Vert and Chory, 2006; Yin et al., 2002; 2005). This implies that BRs induce the dephosphorylation and nuclear translocation of BES1. To test this idea, we examined whether BRs promote the nuclear import of BES1. GFP-tagged *BES1* was coexpressed with *BIN2,* or with *BRI1* and *BIN2*, in protoplasts. BES1-GFP localization was monitored in the presence or absence of BR treatment. Before BR treatment, BES1-GFP was mainly located in the cytoplasm and coexpression with *BRI1* did not affect this localization (Fig. 2A). However, the application of an exogenous BR increased the nuclear translocation of BES1 (Fig. 2A, upper panel), and this was further facilitated in the presence of BRI1 (Fig. 2A, lower panel). The level of expression of *BIN2* was similar in the presence or absence of exogenous BR (Fig. 2B), indicating that the BR signal may override the phosphorylation potential of BIN2. BR treatment induced the dephosphorylation of BES1 in the presence of BRI1 and BIN2 (Fig. 2C). These results suggest that the canonical pathway initiated by BRI1 suppressed the BIN2 kinase activity.

Putative phosphorylation residues in the N-terminus of BES1 are important for BIN2-mediated nuclear export

To identify the regulatory residues that mediate BES1 phosphorylation events, we selected 25 potential phosphorylation sites at Ser/Thr residues, which were predicted in a previous study (Wang et al., 2002). We replaced these residues with alanine by site-directed mutagenesis and identified twelve mutants that failed to exhibit BIN2-induced cytoplasmic translocation (Figs. 3A and 3B; Supplementary Fig. S5). These residues were distributed in two distinct domains, which were designated as the first and second domains in this study. The second domain comprises S171 and T175, and residues corresponding to these are known to be critical in interactions between BZR1 and the 14-3-3 binding sites (Ryu et al., 2007). To clarify the

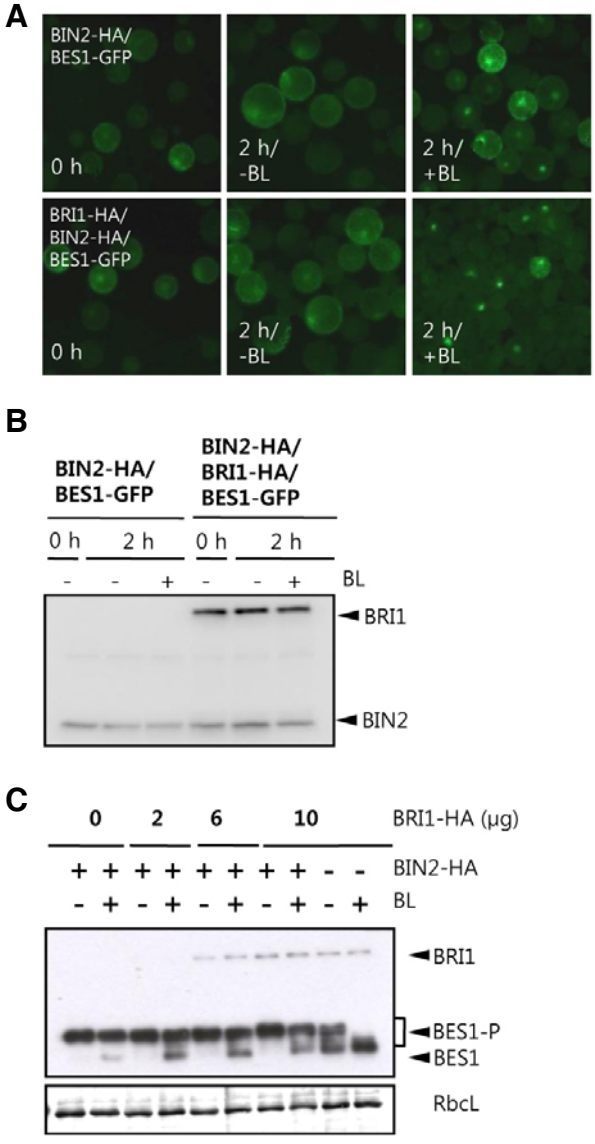

Fig. 2. BR induces the nuclear accumulation of BES1 through the canonical pathway initiated by BRI1. (A) BRI1 facilitated the BR-mediated nuclear translocation of BES1 in protoplasts. *BES1-GFP* and *BIN2-HA* were introduced into protoplasts in the presence (lower panel) or absence (upper panel) of *BRI1-HA*. After 5 h of incubation, the protoplasts were pretreated with cycloheximide for 30 min and were then incubated in the absence (BL-) or presence (BL+) of *epi*-BL for 2 h. (B) The expression of BRI1-HA and BIN2-HA was confirmed using immunoblot assays. (C) BRI1 accelerates the BR-mediated de-phosphorylation of BES1 in protoplasts. HA-tagged *BES1* was cotransfected with the indicated amounts of *BRI1-HA* and 4 μg of *BIN2-HA*. After 4 h, the transfected protoplasts were incubated with cycloheximide for 30 min before treatment with BL for 1 h. Proteins were extracted from the protoplasts and examined by immunoblotting with the anti-HA antibody. RbcL was used as a loading control.

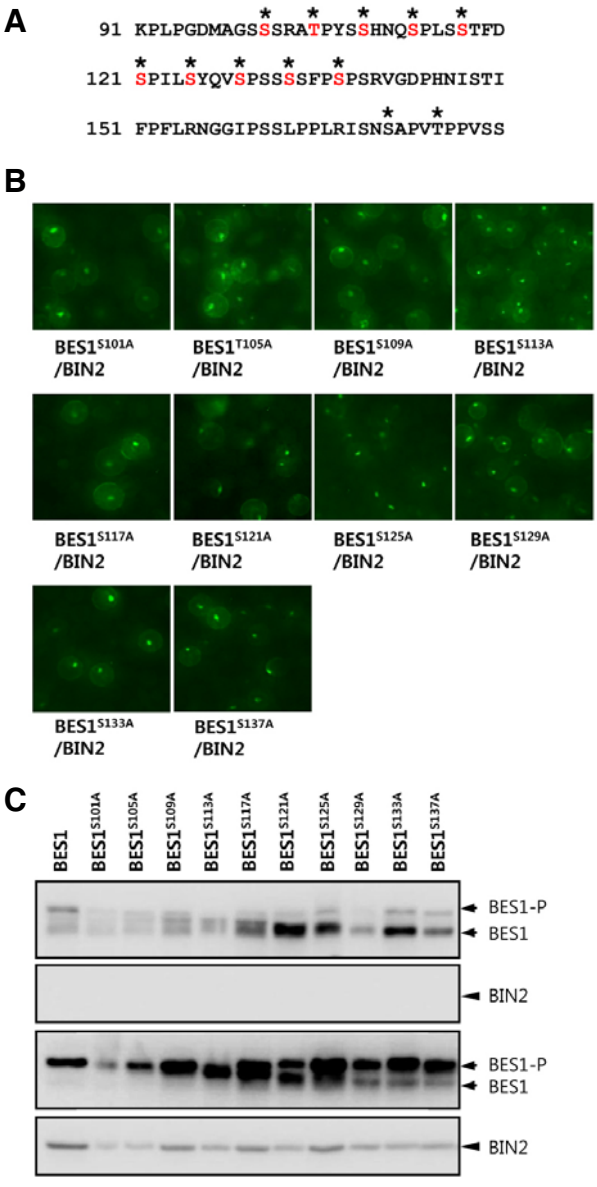

Fig. 3. Identification of putative phosphorylation sites required for the nuclear export of BES1. (A) Twelve amino acid residues of BES1 are predicted to be phosphorylated by BIN2. The putative phosphorylation sites (marked by asterisks) occur in two domains and were identified based on conserved amino acids that are known to be phosphorylated by GSKs. The first domain contains Ser 101 to Ser 137, and the second domain contains Ser 171 and Thr 175. (B) Mutations of the Ser/Thr residues in the first putative phosphorylation domain blocked the BIN2-mediated nuclear export of BES1. *BIN2-HA* was introduced into protoplasts with mutated *BES1-GFPs*, as indicated. (C) Mutations at some of the putative phosphorylation sites in the first domain partly abolished BIN2-mediated phosphorylation. GFP-tagged wild-type or mutant *BES1* was expressed in protoplasts in the presence (upper two panels) or absence (lower two panels) of *BIN2-HA*. The BIN2 and BES1 proteins were analyzed using an anti-HA antibody and an anti-GFP antibody, respectively.

functional role of the first domain, we investigated the phosphorylation status of each mutant in the presence and absence of BIN2-HA. Interestingly, in the absence of BIN2-HA, these mutants exhibited differences in phosphorylation status when compared with wild-type BES1 (Fig. 3C, upper panels). The wild-type BES1 protein was mostly phosphorylated, whereas $BES1^{S113A}$, $BES1^{S117A}$, $BES1^{S121A}$, $BES1^{S125A}$, $BES1^{S129A}$, $BES1^{S133A}$, and $BES1^{S137A}$ remained largely dephosphorylated, although the degree of dephosphorylation varied among these mutant proteins. Furthermore, in the presence of BIN2-HA, these mutants also showed varying levels of dephosphorylation (Fig. 3C, lower panels). These results suggest either that these residues are potential targets of BIN2 or that they play regulatory roles in the BIN2-mediated phosphorylation of BES1.

The Ser 171 and Thr 175 residues of BES1 regulate its interaction with 14-3-3 proteins for nuclear export

In recent studies, the guidance role of 14-3-3 proteins has been defined in the nuclear export of BZR1 (Gampala et al., 2007; Ryu et al., 2007). BES1 contains a putative 14-3-3 binding domain, which suggests that BES1 is translocated into the cytosol via the action of 14-3-3 proteins, as is BZR1 (Fig. 4A). We found that BES1 interacts with 14-3-3κ in a co-immunoprecipitation assay (Fig. 4B). It is notable that the hyperphosphorylated forms of BES1 and BZR1 appeared to co-precipitate more readily with the 14-3-3κ protein than the hypophosphorylated forms. We then disrupted the putative 14-3-3 binding sites, Thr 177 of BZR1 and Thr 175 of BES1, and assessed the *in vivo* interactions between the BZR1 and BES1 mutants and the 14-3-3 protein. The $BZR1^{T177A}$ and $BES1^{T175A}$ mutations hampered their interactions with 14-3-3κ (Fig. 4B). Interestingly, the bes1-D and bzr1-1D mutant proteins did not interact with 14-3-3κ (Fig. 4B), although their protein stability was similar to that of the wild-type proteins. We next examined the localization of the $BES1^{S171A}$ and $BES1^{T175A}$ mutants. The GFP-tagged $BES1^{S171A}$ and $BES1^{T175A}$ proteins were mainly localized in the nucleus, regardless of whether they were coexpressed with *BIN2-HA* (Fig. 4C). Treatment with AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), which is an inhibitor of 14-3-3 interactions (Gampala et al., 2007; Paul et al., 2005), blocked the BIN2-induced nuclear export of BES1 (Fig. 4D). These results imply that the Ser 171 and Thr 175 residues of BES1 are required for its interaction with 14-3-3 proteins, which act as molecular chaperones in the BIN2-mediated nuclear export of BES1.

Nuclear-localized BES1 confers enhanced BR responses in *Arabidopsis*

We analyzed *Arabidopsis* transgenic lines overexpressing BES1 mutants that were constitutively localized in the nucleus. The protein levels of BES1 and its mutants in transgenic plants were verified using immunoblot assays (Fig. 5A). The same lines were subjected to hypocotyl elongation assays in the presence or absence of BRZ (Fig. 5B). Hypocotyl lengths were similar in dark-grown wild-type seedlings and in transgenic seedlings harboring $BES1^{S129/133A}$, $BES1^{S171A}$, and $BES1^{T175A}$. However, in the presence of BRZ, the transgenic lines carrying the BIN2-insensitive BES1 mutants exhibited longer hypocotyls, with lengths that were similar to those of the *35S::bes1-D* line (Fig. 5B). Three-week-old $BES1^{S129/133A}$, $BES1^{S171A}$, and $BES1^{T175A}$ transgenic plants also had curled rosette leaves, which is a typical phenotype of the enhanced BR response mutants, *35S::bes1-D* and *bes1-D* (Wang et al., 2002; Yin et al., 2002) (Fig. 5C). These results suggest that BES1 mutants that are constitutively localized to the nucleus may enhance BR responses in plants. We then investigated the expression patterns of the BR biosynthetic genes *CPD* and *DWF4*, which are negatively regulated by BR-

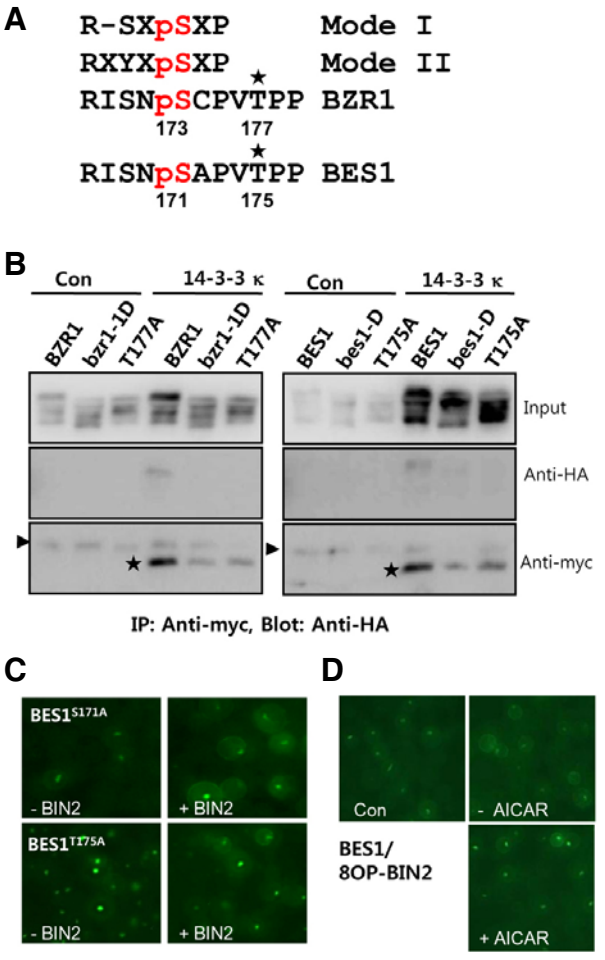

Fig. 4. 14-3-3 proteins mediate the cytoplasmic translocation of phosphorylated BES1. (A) The putative 14-3-3 binding sites of BES1 and BZR1. Two conserved 14-3-3 binding site sequences (Mode I and Mode II) were aligned with the BES1 and BZR1 sequences. pS, phospho-serine. (B) Mutations at Thr 175 of BES1 or Thr 177 of BZR1 exert critical effects on the interactions of these molecules with 14-3-3 proteins. HA-tagged *BZR1*, *bzr1-1D*, *BZR1*^{T177A}, *BES1*, *bes1-D*, or *BES1*^{T175A} were introduced into protoplasts with or without (Con; control) myc-tagged *14-3-3κ*. The 14-3-3κ protein was immunoprecipitated using an anti-myc monoclonal antibody. The co-immunopre-cipitated BZR1 and BES1 proteins were detected using an anti-HA antibody. The arrows and asterisks indicate non-specific bands and 14-3-3κ-myc, respectively. (C) Mutations at the 14-3-3 binding sites abolished the BIN2-mediated nuclear export of BES1. Protoplasts transfected with either *BES1*^{S171A}*-GFP* or *BES1*^{T175A}*-GFP* and *BIN2-HA* were examined using fluorescence microscopy. (D) AICAR, which is a specific 14-3-3 inhibitor, blocked the BIN2-mediated nuclear export of BES1. The expression of BIN2 was induced by DEX, as described for Fig. 1E.

activated BES1 and BZR1, in the transgenic lines. *CPD* and *DWF4* are subject to negative feedback regulation under prolonged BR stimulated conditions (He et al., 2005; Vert and Chory, 2006). The expression of *CPD* and *DWF4* was reduced in the transgenic lines expressing *BES1*$^{S129/133A}$, *BES1*^{S171A}, or *BES1*^{T175A}, to a level similar to that observed in the *35S::bes1-D* lines. This suggested that the increased nuclear localization of BES1 enhances the BR signaling output (Fig. 5D). We also tested whether the nuclear-localized BES1 and BZR1 mutants suppressed *bri1-5*, which is a weak allele of *bri1* (Noguchi et al., 1999). Inflorescence stem lengths were similar in the *bri1-5* lines and transgenic lines that expressed wild-type *BES1* (Figs. 5E and 5F). However, *bri1-5* plants expressing the mutant versions of BES1 showed longer inflorescence stems (Figs. 5E and 5F). Collectively, our results suggest that the phosphorylation status of BES1 regulates its spatial distribution, and that the correct spatial distribution of BES1 is required for the proper BR regulation of gene expression and plant development.

DISCUSSION

We have previously reported that the phosphorylation-mediated spatial redistribution of BZR1 is essential for proper BR signal transduction and that 14-3-3 proteins act as molecular chaperones during the nuclear export of BZR1 (Ryu et al., 2007). In this study, we provided evidence that BES1 is also a nucleocytoplasmic shuttling protein, and that its subcellular localization is modulated by its phosphorylation status. The nuclear accumulation and nuclear export of BES1 are promoted by BSU1-induced dephosphorylation and BIN2-mediated phosphorylation, respectively. Here, we propose a revised model of BR signaling that emphasizes the importance of the subcellular locations of the key transcription factors BES1 and BZR1 in the BR signal transduction cascade (Supplementary Fig. S6). The activation of BRI1 by BRs induces the dissociation of the negative regulator BKI1 and the subsequent dimerization between BRI1 and BAK1. The BRI1/BAK1 complex then phosphorylates BSK1 to stimulate downstream signaling pathways. These signals activate cytosolic BSU1 phosphatase, which inactivates the BIN2 kinase by dephosphorylating it at Tyr 200 (Kim et al., 2009). Under these conditions, the BES1 and BZR1 transcription factors accumulate in the nucleus, activate the expression of various BR-responsive genes, and suppress BR biosynthetic genes, thus producing a negative feedback loop. In the absence of the BR signal, the active nuclear BIN2 induces the phosphorylation of BES1 and BZR1. The 14-3-3 proteins mediate the nuclear export of the phosphorylated transcription factors to prevent BR signaling. This spatial redistribution of the two key transcription factors in the BR signaling pathway may be broadly used in the plant kingdom. A recent study in rice showed that the Os14-3-3 proteins similarly regulate the BR signaling pathway (Bai et al., 2007). In addition, the phosphorylation domain (positioned between residues S101 and S137) and the 14-3-3 binding domain (residues S171 and T175) of BES1 are conserved in its tomato and rice homologs (Wang et al., 2002). These observations suggest that the spatial redistribution of BR-related transcription factors is a general mechanism for the tight control of BR signaling in the plant kingdom.

The specific mechanism that controls the phosphorylation and transcriptional activity of BES1 and BZR1 remains a controversial but essential subject in our understanding of the BR signaling pathway. We found that phosphorylated BES1 accumulated in the cytoplasm (Fig. 1), whereas the BR signal caused the dephosphorylation and subsequent nuclear translocation of BES1 (Fig. 2). Interestingly, various BES1 mutants (BES1^{S101A}, BES1^{T105A}, BES1^{S109A}, BES1^{S171A}, and BES1^{T175A}), which appeared to be fully phosphorylated by BIN2, displayed constitutive nuclear localization and conferred hyper-responses to BR in transgenic plants (including curled leaves, resistance to BRZ, and suppression of the *bri1-5* dwarfism) to levels that were similar to those of other less phosphorylated mutants (Figs. 3, 4, and 5). On the other hand, it was suggested that *bes1-D* and *bzr1-1D* enhanced BR-responses as a result of high protein accumula-

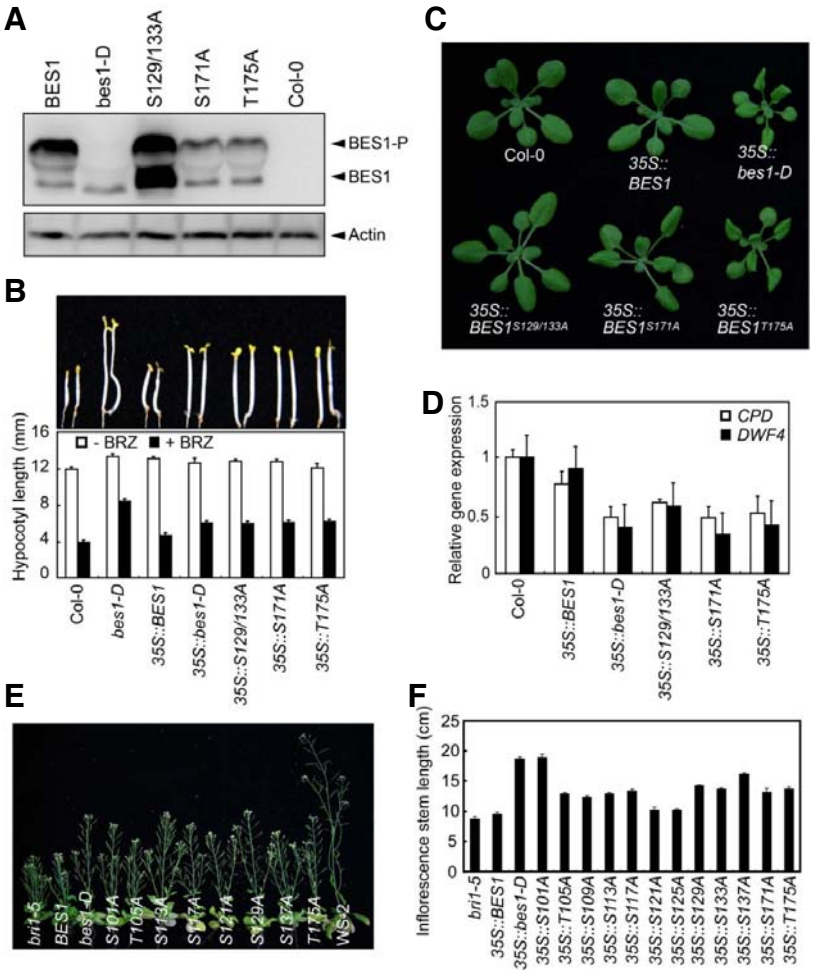

Fig. 5. Nuclear localized BES1 confers constitutive BR responses in transgenic plants. (A) The levels of accumulation of proteins encoded by *BES1-HA* and its various mutant forms. Protein levels and their phosphorylation status were determined by immunoblotting using an anti-HA antibody. Actin was used as a loading control. (B) Mutation of the putative phosphorylation sites of BES1 suppressed the dwarfism and short hypocotyl phenotypes typically caused by BRZ treatment. Seedlings were grown in the presence or absence of 1 μM BRZ (upper panel) for 4 days in the dark, and the hypocotyl lengths were measured using the Scion Image software. Error bars indicate standard errors (n = 10). (C) Phenotypes of plants expressing mutated versions of *BES1*, after growth under long-day conditions for 3 weeks in soil. (D) The expression of BR biosynthetic genes was reduced in transgenic lines that expressed nuclear BES1 constitutively. The levels of the *CPD* and *DWF4* transcripts were investigated using quantitative real-time PCR. Error bars indicate standard errors (n = 5). (E, F) The inflorescence stems of transgenic *bri1-5* plants expressing the indicated BES1 mutants were photographed (E), and the lengths of the inflorescence stems were measured (F). Error bars indicate standard errors (n = 4).

tion due to increased protein stability (He et al., 2002; Wang et al., 2002; Yin et al., 2002). However, we found that their levels were similar to those of the wild-type proteins, but that the *bes1-D* and *bzr1-1D* mutation disrupted interaction with 14-3-3 proteins (Fig. 4B). These results suggest that the positive effects of *bes1-D* and *bzr1-1D* on various BR responses could be ascribed to the constitutive nuclear localization of the mutant proteins, caused by a disruption in their 14-3-3-mediated export, rather than their protein stability. Our data collectively indicate that decreases in the DNA-binding affinity, transactivation activity, and stability of BES1, via BIN2-mediated phosphorylation, are insufficient to deactivate BES1 in the nucleus. In other words, the nuclear export of BES1 and BZR1 may be necessary for their complete inactivation in BR signaling.

The discrepancies between the localization of BES1 and BZR1 found in this study compared with others (Vert and Chory, 2006; Zhao et al., 2002) may be explained by temporal and spatial differences in expression patterns of BR biosynthetic or signaling components. *BES1*, *BZR1*, *BRI1*, and *BSU1* are ubiquitously expressed in *Arabidopsis* seedlings (Mora-Garcia et al., 2004; Wang et al., 2002; Yin et al., 2002). However, *DWF4* and *CYP85*, which encode rate-limiting BR biosynthesis enzymes that are negatively regulated by BR signaling, are mainly expressed in root tips and lateral root primordia (Castle et al., 2005; Kim et al., 2006). This expression pattern indicates that the BR signaling pathway is constitutively activated in these regions, and could explain why BES1 and BZR1 appear to be mainly localized in the nuclei in root tips (Vert and Chory, 2006; Zhao et al., 2002). In addition, we also found that the distributions of BES1-GFP and BZR1-CFP in the root tips are not altered by exogenous BR treatment (our unpublished data and Vert and Chory, 2006).

Furthermore, the temporal and spatial regulation of *BIN2* expression seems to be tightly regulated by light signals (our unpublished data), and the homeostasis of BR levels is diurnally regulated (Bancos et al., 2006) and highly correlated with BIN2 kinase activity (Choe et al., 2002). It is possible that the light- and BR signal-dependent regulation of BIN2 kinase activity, especially through transcriptional regulation, plays a critical role in BR-mediated plant growth and development. The identification of transcriptional controls for *BIN2* expression and an elucidation of the developmental specificities of the group II GSK3 kinases are necessary to our understanding of BR signaling in plant development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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