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# Predominant Actions of Cytosolic BSU1 and Nuclear BIN2 Regulate Subcellular Localization of BES1 in Brassinosteroid Signaling

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**During brassinosteroid (BR) signaling in *Arabidopsis*, BSU1 (*bri1* SUPPRESSOR1) phosphatase and BIN2 (BRASSINOSTEROID INSENSITIVE2) kinase regulate the signal intensity by determining the phosphorylation status of the transcription factors BZR1 (BRASSINAZOLE RESISTANT1) and BES1 (*bri1* EMS SUPPRESSOR1). Here, we report that BIN2 and BSU1 are nucleocytoplasmic regulators that modulate the subcellular localization of BES1, with differential activities between the nucleus and the cytosol. In our experiments, the nuclear BIN2 induced phosphorylation and nuclear export of BES1 more efficiently than cytosolic BIN2. The cytoplasmic BSU1 mediated the dephosphorylation and nuclear translocation of BES1 more efficiently than the nuclear one. BSU1 compromised the dwarf phenotype of *bri1-5*, a weak allele of BRI1 (BR-INSENSITIVE 1) receptor kinase, more effectively when localized in the cytosol than in the nucleus in transgenic plants. In conclusion, the predominance of cytosolic BSU1 and nuclear BIN2 might be required for the efficient subcellular localization of BES1 in BR signaling.**

## INTRODUCTION

Phosphorylation is the most critical post-translational modification of key molecules in a variety of signal transduction pathways in living organisms. Brassinosteroids (BRs) are a group of steroidal phytohormones that also utilize phosphorylation events to propagate their signals and modulate various developmental processes and environmental adaptations (reviewed by Bajguz and Hayat, 2009; Mandava, 1988; Vert et al., 2005). BR perception by the plasmamembrane receptor kinase BRI1 (BR-INSENSITIVE 1) triggers the sequential phosphorylation of BRI1, BSK1 (BR-SIGNALING KINASE 1), and the phosphatase BSU1 (*bri1* SUPPRESSOR1) (reviewed by Belkhadir et al., 2006; Kim et al., 2009; Tang et al., 2008; Yun et al., 2009). Then the activated BSU1 stimulates BR responses by inhibiting glycogen synthase kinase 3 (GSK3) kinases including BIN2 (BRASSINOSTEROID INSENSITIVE2)/UCU1/DWF12, a well-known negative regulator of BR signaling (Choe et al., 2002; Jonak and Hirt, 2002; Li and Nam, 2002; Perez-Perez et al., 2002). BIN2 phosphorylates and inactivates BES1 (*bri1* EMS SUPPRESSOR1) and BZR1 (BRASSINAZOLE RESISTANT1), which are key transcriptional regulators of BR responses. Thus the phosphatase activity of BSU1 on BIN2 likely plays a critical role in determining the primary signaling outputs of BRs. However, the detailed mechanisms controlling the subcellular localization and signaling events mediated by BSU1 and BIN2 remain largely unknown.

The phosphorylation statuses of BES1 and BZR1 are reliable indicators for estimating the intensity of downstream BR signaling responses, and are modulated by BIN2 and BSU1 (He et al., 2002; Vert and Chory, 2006; Wang et al., 2002; Yin et al., 2002). The phosphorylation status of these two transcription factors is believed to regulate their DNA binding, transcriptional activity, protein stabilization, and subcellular localizations. However, recent studies have provided contradictory results regarding the role of phosphorylation in the subcellular localization of BES1 and BZR1. In the hypocotyls and roots of *Arabidopsis*, the opposing actions of BIN2 and BSU1 on BZR1 and BES1 were found to occur constitutively in the nucleus (Vert and Chory, 2006; Zhao et al., 2002). On the other hand, other studies suggest that phosphorylation is important in regulating the spatial redistribution of BZR1 between the cytosol and the nucleus (Bai et al., 2007; Gampala et al., 2007; Ryu et al., 2007). In particular, the nuclear export of BZR1 was found to be mediated by its interaction with 14-3-3 proteins. Furthermore, a close positive correlation was reported between dephosphorylation and enhanced nuclear translocation of BZR1 (Ryu et al., 2007). This implies that the dephosphorylation of BZR1 by the cytosolic BSU1 phosphatase might be necessary for the efficient nuclear import of BZR1. However, this hypothesis conflicts with the constitutive nuclear localization of BSU1 reported by Mora-Garcia et al. (2004). These inconsistent results detract from our understanding of the primary roles of phosphorylation and dephosphorylation of BES1 and BZR1 in the BR signaling pathway. Moreover, the cytoplasmic signaling events that occur between BR perception at the plasma membrane and expression of BR target genes in the nucleus remain undiscovered. Therefore, we have closely examined the subcellular localizations of BSU1 and BIN2, and their physiological roles, by separately monitoring their signaling activities in the nucleus and cytosol.

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## MATERIALS AND METHODS

### Plant materials

*Arabidopsis thaliana* (Col-0) seeds were germinated in media containing 0.5 × Gamborg B5 salts (Duchefa, Netherlands), 1% sucrose, and 0.8% phytoagar (pH 5.7) under long-day conditions (16 h light/8 h dark) at 22°C. After two weeks, seedlings were transferred to pots and grown under short-day conditions (10 h light/14 h dark). Generally, fully expanded leaves of three- to four-week-old seedlings were used for protoplast isolation.

### Protoplast transient expression

The cDNAs of *BIN2*, *BSU1*, *BZR1*, and *BES1* were introduced into plant expression vectors that contained hemagglutinin (HA), yellow fluorescence protein (YFP), or green fluorescence protein (GFP) tags, a nuclear export signal (NES) or a nuclear localization signal (NLS), and the 35S C4PPDK promoter (Hwang and Sheen, 2001). For transient expression assays, mesophyll protoplasts and CsCl-mediated plasmid were prepared as described in Hwang and Sheen (2001). Generally, $4 \times 10^4$ mesophyll protoplasts were transfected with 10 μg of plasmid DNA and incubated for 3 h at room temperature. Then, BL (brassinolide), okadaic acids, or cyclohexmide were treated as described in figure legends. For subcellular localization studies of BSU1 in protoplasts, *BSU1* cDNAs were introduced into vectors containing the HA or YFP-tags and the CsVMV (cassava vein mosaic virus) promoter (Verdaguer et al., 1996).

### Fluorescence microscopy

The GFP and YFP fluorescence signals from protoplasts or roots of transgenic plants were monitored using either a confocal laser scanning microscope (Carl Zeiss MicroImaging) or a fluorescence microscope (Carl Zeiss MicroImaging).

### Fractionation of subcellular organelles and immunodetection

Protoplasts were isolated from transgenic *Arabidopsis* expressing *BSU1-HA* or from wild-type plants. The protoplasts were then 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), and 0.7% Triton X-100. After centrifugation at 3000 × *g* for 10 min, the majority of the cytosolic fraction in the supernatant was removed and stored on ice until use. The nuclear pellets 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]. The proteins in each fraction were subjected to 10% SDS-PAGE, followed by immunodetection using peroxidase-conjugated high-affinity anti- HA (Roche Applied Science), anti-RHA1 (kindly gifted by Dr. Inhwan Hwang, POSTECH), and anti-Histone H2B (Upstate) antibodies. In general the immunoblotting analyses were performed using 3 to 20 μg of proteins from protoplasts or seedlings, which were separated by 10% SDS-PAGE and detected using horseradish peroxidase-conjugated anti-HA (Cell signaling).

### Physiological analysis of transgenic *bri1-5 Arabidopsis plants*

The *BSU1-HA*, *BSU1-NES*, and *BSU1-NLS* constructs were cloned into the *pCB302ES* binary vector containing an HA tag and 35S CaMV promoter (Hwang and Sheen, 2001). These were introduced into the *bri1-5 Arabidopsis* mutant using the *Agrobacterium*-mediated floral dipping method (Clough and Bent, 1998). T1 generations were used to assess the phenotypes of *bri1-5* mutants.

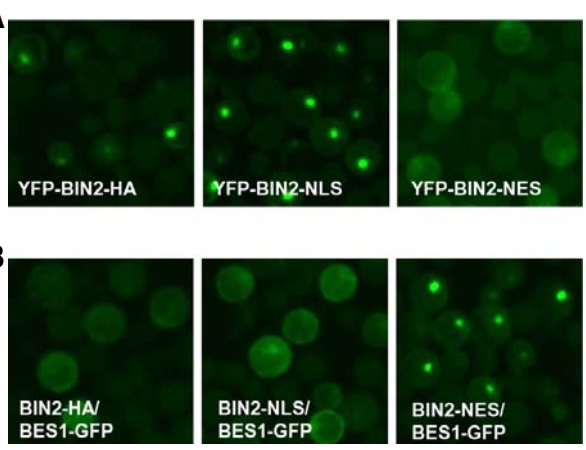


**Fig. 1.** The cytosolic localization of BES1 is mediated mainly by BIN2 in the nucleus. (A) The localization of YFP-BIN2 with no localization signal (YFP-BIN2-HA; left) or fused with a NLS (YFP-BIN2-NLS; middle) or a NES (YFP-BIN2-NES; right). (B) Nuclear BIN2 actively induces the cytoplasmic accumulation of BES1-GFP. *BES1-GFP* was co-expressed in protoplasts with *BIN2-HA, BIN2-NLS*, or *BIN2-NES*. The YFP (in A) and GFP (in B) signals were observed using fluorescence microscopy.

## RESULTS

### The cytosolic localization of BES1 is mediated mainly by nuclear BIN2

BIN2 is localized in the nucleus and cell periphery, but its negative effect on BR signaling occurs predominantly in the nucleus (Vert and Chory, 2006). To identify the subcellular location of BIN2 where it functions in regulating the nuclear export of BES1, we separately monitored the effects of cytosolic and nuclear BIN2 on the localization of BES1. Since BIN2 lacks both a conserved nuclear localization signal (NLS) and a nuclear export signal (NES), we produced chimeric fusion constructs, *YFP-BIN2-HA*, *YFP-BIN2-NLS*, and *YFP-BIN2-NES*, and transiently expressed them in *Arabidopsis* mesophyll protoplasts. The subcellular localization of each fusion protein was verified by monitoring the fluorescent signals emitted by YFP (Fig. 1A). The YFP-BIN2-HA signals were detected both in the nucleus and the cytosol, indicating that BIN2 is a nucleocytoplasmic protein, as reported previously (Vert and Chory, 2006). YFP-BIN2-NLS was preferentially detected in the nucleus, whereas YFP-BIN2-NES was found in the cytosol. We then co-expressed *BES1-GFP* with the three *BIN2* constructs in the protoplasts and monitored the localization of BES1 using the fluorescent signals from the GFP tag. BES1-GFP was mostly detected in the cytosol in the presence of *BIN2-HA* or *BIN2-NLS* (Fig. 1B), suggesting that BES1 was actively exported from the nucleus. Remarkably, however, BIN2-NES did not alter the nuclear localization of BES1-GFP, as indicated by the strong green fluorescence in the nucleus (Fig. 1B). These results suggest that the presence of BIN2 in the nucleus enhances the export of BES1 from the nucleus, and this is probably an important mechanism controlling the desensitization to BR signaling by BIN2.

### The BR-induced nuclear localization of BES1 is mediated by phosphatases

Under BR-stimulated conditions, BES1 must be dephosphorylated and transferred into the nucleus. In addition, the cytoplsamic BES1 exported by BIN2 likely re-enters the nucleus

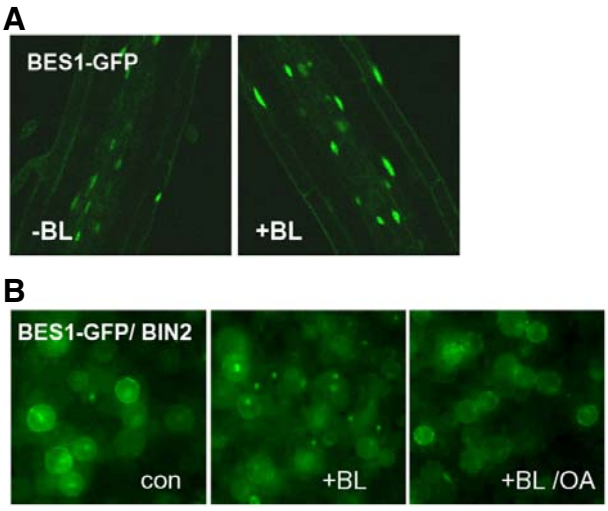


**Fig. 2.** The BR-stimulated nuclear localization of BES1 is mediated by phosphatases. (A) BRs enhance the nuclear accumulation of BES1 in plants. Transgenic *Arabidopsis* plants harboring *35S::BES1-GFP* were incubated in the presence or absence of 1 μM *epi*-BL for 1 h. The subcellular localization of BES1-GFP in the vasculature of the maturation zone was examined using confocal microscopy. (B) The phosphatase 2A inhibitor okadaic acid (OA) prevented the BR-induced nuclear accumulation of BES1. Protoplasts were co-transfected with *BES1-GFP* and *BIN2-HA*. After 5 h of incubation, the protoplasts were treated with 100 μM CHX for 30 min and then treated with or without (con) 1 μM *epi*-BL, in the presence or absence of OA (30 nM), for 3 h.

unless it has been degraded. This would increase the nuclear localization of BES1 in the presence of BRs. To examine this idea, we monitored the changes in the nuclear accumulation of BES1 in transgenic *Arabidopsis* lines overexpressing *BES1-GFP* in the absence or presence of brassinolide (BL). The plants were also treated with cycloheximide (CHX) to minimize the *de novo* synthesis of BES1-GFP. In the absence of exogenous BL, weak green fluorescent signals were detected in both the nucleus and peripheral cytoplasmic regions of transgenic root cells (Fig. 2A). However, fluorescence signals became intensified in the nucleus following the treatment with exogenous BL, indicating that BRs induce the nuclear import of BES1.

Based on the close correlation between the dephosphorylation state and the nuclear localization of BES1, it is likely that the BSU1 phosphatase and its functional homologs are required for the nuclear accumulation of BES1. To test this, we monitored BR-induced nuclear localization of BES1 in the presence of the phosphatase 2A inhibitor okadaic acid (Fig. 2B). First, we transfected *Arabidopsis* mesophyll protoplasts with *BES1-GFP* and *BIN2-HA*. In the presence of the overexpressed *BIN2*, most of the BES1 protein was expelled from the nucleus (Fig. 2B, left panel). BL treatment caused the rapid nuclear localization of BES1 in the absence of okadaic acid (Fig. 2B, middle panel). However, the nuclear localization of BES1 was inhibited by okadaic acid treatment even with BL treatment (Fig. 2B, right panel), indicating that the BR-induced nuclear localization of BES1 requires cytosolic phosphatase activity. In addition, since the *de novo* synthesis of BES1 was inhibited by CHX, the increased nuclear accumulation of BES1 was likely due to the import to the nucleus of cytosolic BES1 that had been exported by BIN2. Interestingly, the nuclear exporting activity of the overexpressed BIN2 was apparently overridden by BL treatment, implying that the endogenous BR-activated dephosphorylation activity was greater than the activity of the overexpressed BIN2 kinase. It is possible that the endogenous BR-activated component might inhibit BIN2 activity via high catalytic turnover rates. This is consistent with a recent finding that BR-activated BSU1 directly inhibits BIN2 function (Kim et al., 2009). Taken together, our results suggest that BSU1 and its homologs may functionally activate the BR signaling pathway by mediating the nuclear translocation of BES1.

**BSU1 is a nucleocytoplasmic protein**

It was challenging to directly trace the locations of the (de)phosphorylation events of BES1 which are highly dynamic processes and correlated with the nuclear translocation. Alternatively, to gain insight into the BES1 nuclear import mechanisms, we closely examined the spatial distribution of the BSU1 phosphatase, which is thought to be responsible for the dephosphorylation of BES1 (Kim et al., 2009; Mora-Garcia et al., 2004). We created transgenic *Arabidopsis* plants overexpressing *BSU1-HA* and isolated protoplasts from 4-week-old seedlings. The nuclei and cytoplasmic components were fractionated and the BSU1-HA protein was detected using an anti-HA antibody. Overexpression of *BSU1-HA* caused curled leaves (Fig. 3A, line #25), which resembled the phenotypes of gain-of-function mutants in BR signaling (Wang et al., 2002; Yin et al., 2002). This result indicated that the BSU1-HA protein is functional. Noticeably, transgenic line #8 (a low accumulator) exhibited less leaf curling than line #25 (a high accumulator), implying that the morphological phenotypes were correlated with the BSU1-HA protein levels (Figs. 3A and 3B). Interestingly the BSU1-HA protein was detected in both the nucleus and the cytoplasm in two independent transgenic lines (Fig. 3B), and this result conflicts with the exclusively nuclear pattern reported in a previous study (Mora-Garcia et al., 2004). It is possible that the high abundance of the BSU1 protein, due to the use of the strong 35S CaMV promoter, caused the nucleocytoplasmic pattern found in this study. However, transgenic lines accumulating BSU1-HA to a minimum level (Fig. 3B #8), with similar morphologies to those of the wild-type Col-0 plants, also exhibited the nucleocytoplasmic localization. Further, we re-examined the subcellular localization of BSU1, fused to either the YFP or the HA tag, using the protoplast transient expression system. In mesophyll protoplasts, the tagged BSU1 proteins were localized in both the cytoplasm and the nucleus, as determined by a fractionation study and by fluorescence microscopy (Figs. 3C and 3D). Regardless of the signal intensities in individual cells, YFP-BSU1 was always detected both in the cytoplasm and the nucleus (data not shown). Again, this suggested that the localization of the protein was not due to overexpression of the *YFP-BSU1* construct. Remarkably, in transgenic plants, the BSU1 protein was more abundant in the cytosol than the nucleus (Fig. 3B). Taken together, our study clearly indicated that BSU1 is a nucleocytoplasmic phosphatase. Although where BES1 is dephosphorylated is still not clear, the prevalent accumulation of BSU1 in the cytosol indirectly suggests that cytosolic BES1 is more likely to be dephosphorylated than nuclear BES1.

**The nuclear translocation of BES1 is mainly mediated by BSU1 localized in the cytoplasm**

To identify the subcellular location of BSU1 where it contributes to the rapid activation of the BR response, we monitored the activities of various BSU1 constructs on the dephosphorylation and nuclear translocation of BES1. YFP-BSU1 was tagged with HA, the NES, or the NLS, and the NES- and NLS-tagged proteins were predominantly localized in the cytoplasm and the

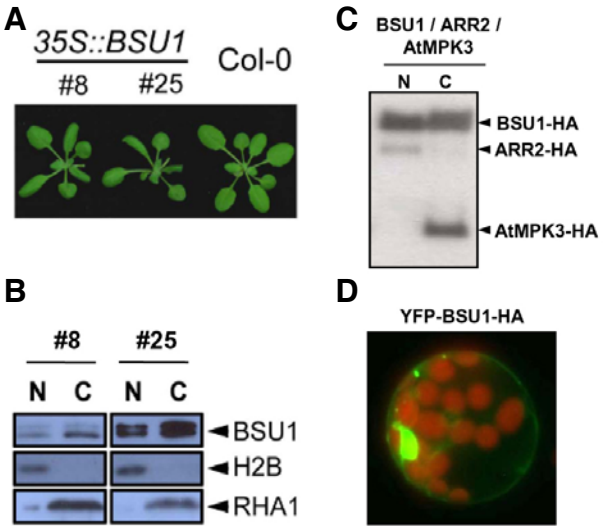


**Fig. 3.** BSU1 localizes both in the nucleus and cytosol. (A) Transgenic *Arabidopsis* harboring *35S::BSU1-HA* (plant lines #8 and #25) exhibited curled leaf in young seedlings, when compared with the wild-type (Col-0). (B) The subcellular localization of BSU1 protein. Protoplasts were isolated from transgenic plants expressing *35S::BSU1-HA*, and the nuclear (N) and cytoplasmic (C) fractions were separated. The equal volumes of protein extracts were loaded to SDS-PAGE. BSU1-HA was detected using an anti-HA antibody. Histone H2B and RHA1 were used as nuclear and cytoplasmic markers, respectively. (C, D) The localization of BSU1 was re-examined in *Arabidopsis* mesophyll protoplasts transiently expressing BSU1 fused to either YFP or HA. (C) Detection of BSU-HA in nuclear and cytoplasmic fractions. ARR2-HA and AtMPK3-HA were used as a nuclear and a cytoplasmic marker, respectively. (D) Detection of YFP-BSU1 by fluorescence microscopy. The YFP fluorescence signals and autofluoresence signals from chloroplasts were pseudo-colored as green and red, respectively.

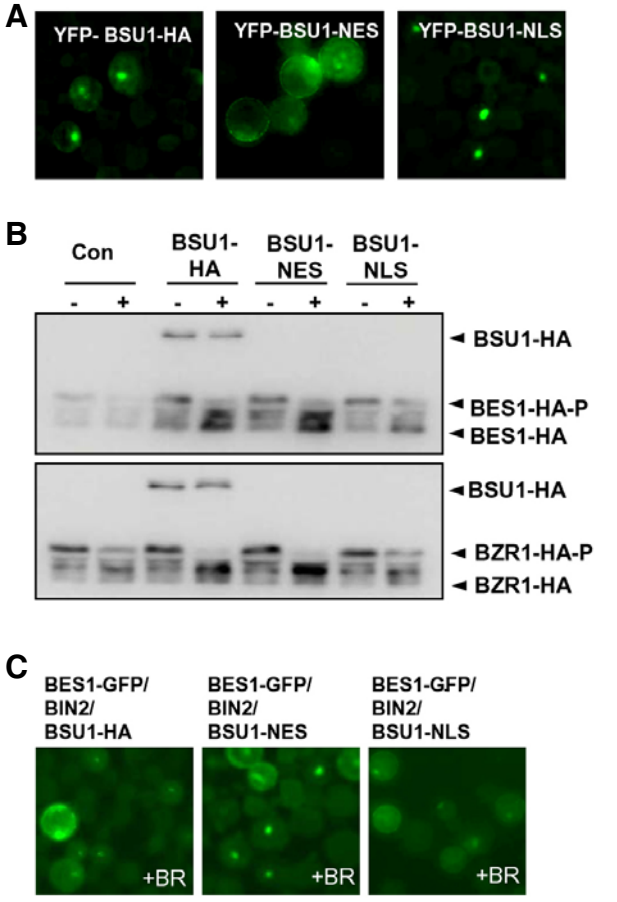


**Fig. 4.** Cytoplasmic BSU1 activates the BR signaling pathway more effectively than the nuclear proteins. (A) The subcellular localization of YFP-BSU1 with no localization signal (YFP-BSU1-HA; left) or fused with a NES (YFP-BSU1-NES; middle) or a NLS (YFP-BSU1-NLS; right). YFP fluorescence was monitored in transfected protoplasts by fluorescence microscopy. (B) The cytoplasmic-localized BSU1 promoted the BR-induced dephosphorylation of BES1 and BZR1. Protoplasts were transfected with *BSU1-HA*, *BSU1-NES*, or *BSU1-NLS* together with *BES1-HA* (top panel) or *BZR1-HA* (bottom panel). The protoplasts were incubated with or without 1 μM *epi*-BL (BR) for 1 h in the presence of CHX (100 μM). The phosphorylation statuses of BES1-HA and BZR1-HA were determined on the basis of their electrophoretic mobility on SDS-PAGE, followed by immunodetection with an anti-HA antibody. (C) The BR-mediated nuclear accumulation of BES1 was facilitated by cytosolic BSU1. Protoplasts were co-transfected with *BES1-GFP*, *BIN2-HA*, and *BSU1-HA*, *BSU1-NES*, or *BSU1-NLS*. After 5 h of incubation, the protoplasts were treated with *epi*-BL for 30 min. The localization of BES1-GFP was determined using fluoresence microscopy.

nucleus, respectively (Fig. 4A). Next, the BSU1-mediated dephosphorylation of BES1 and BZR1 was monitored using electrophoretic mobility shift assays. The cytoplasmic BSU1-NES facilitated the BR-induced dephosphorylation of both BES1 and BZR1 to similar extents as BSU1-HA. In contrast, the extent of BES1 and BZR1 dephosphorylation was much lower in the presence of the BSU1-NLS protein (Fig. 4B). Then, we transiently co-expressed *BES1-GFP* and *BIN2-HA* with *BSU1-HA, BSU1-NES,* or *BSU1-NLS* in the protoplasts. In the absence of exogenous BR, BES1-GFP was detected most strongly in the cytosol due to the BR-independent action of BIN2 (data not shown). However, when the protoplasts were treated with exogenous BR, BSU1-NES was more effective in the nuclear translocation of BES1-GFP than BSU1-HA or BSU1-NLS (Fig. 4C). Taken together, our data strongly indicate that the dephosphorylation and nuclear translocation of BES1-GFP is affected by BSU1 in the cytosol.

### Cytosolic BSU1 facilitates BR responses in planta

To examine the differential actions of nuclear and cytoplasmic BSU1 *in vivo*, we assessed the phenotypes of transgenic *bri1-5* plants expressing *BSU1-HA*, *BSU1-NLS*, or *BSU1-NES* (Fig. 5). The *bri1-5* mutant showed a severe dwarfed phenotype and less sensitivity to exogenous BR than wild-type *Arabidopsis* (Noguchi et al., 1999). Since *bri1-5* is a weak allele of *BRI1* that is believed to transmit the BR signal at a minimal level, we speculated that overexpression of BSU1 might improve the BR signaling output of *bri1-5*. The overexpression of BSU1-HA overcame the dwarf phenotype in 15 of 52 T1 plants (29%). Notably, the recovered wild-type phenotypes, which included longer inflorescence stems, were observed more frequently in transgenic lines expressing *BSU1-NES* (31/86; 36%) than in lines expressing *BSU1-NLS* (10/70; 14%). Taken together with the results presented in Fig. 4, these observations suggest that BSU1 works more efficiently for BR signaling when localized in the cytoplasm than in the nucleus.


| | Dwarf | Recovered | Total |
|---|---|---|---|
| *BSU1-HA* | 37 | 15 (29%) | 52 |
| *BSU1-NES* | 55 | 31 (36%) | 86 |
| *BSU1-NLS* | 60 | 10 (14%) | 70 |

**Fig. 5.** Cytosolic BSU1 facilitates BR responses *in planta*. Overexpression of *BSU1-HA* suppressed the dwarfism phenotype of *bri1-5* (upper panel). The phenotypes of transgenic *bri1-5* lines (T1 generations) ectopically expressing *BSU1-HA*, *BSU1-NES*, or *BSU1-NLS* were categorized as either "Dwarf" or "Recovered" (suppressed dwarf). The transgenic lines that were taller than *bri1-5* lines were defined as "Recovered" and are presented as the percentage of the total number of individual T1 plants (lower panel).

## DISCUSSION

In BR signaling, the BSU1 phosphatase and BIN2 kinase are major modulators of the transcriptional activities of BES1 and BZR1, by determining their phosphorylation status and subcellular localization. In BR-stimulated conditions, consecutive phosphorylation events sequentially activate the BRI receptor kinase, BSK1, and BSU1 (Belkhadir et al., 2006; Kim et al., 2009; Tang et al., 2008). The activated BSU1 in turn inactivates the BIN2 kinase by direct dephosphorylation (Kim et al., 2009). These findings disclosed a complete signaling pathway from the membrane receptor kinase to the transcription factors. In this study, BSU1 and BIN2 were shown to occur both in the nucleus and the cytosol. However, BSU1 was initially identified as a nuclear phosphatase (Mora-Garcia et al., 2004). Considering its critical role in BR signaling, the location where BSU1 exerts its action is an important issue to be investigated. Our study, using fluorescence microscopy and biochemical fractionation assays, ultimately clarifies an ambiguity in current opinions on the spatial distribution of BSU1. We also propose that BSU1 and BIN2 modulate the subcellular location of BES1, via differential activities between the nucleus and the cytosol. In our study, BSU1 proteins appear to be more abundant in the cytosol than the nucleus (Fig. 3). In contrast, the activated mutant form of BIN2, *bin2-1*, accumulated predominantly in the nucleus (Vert and Chory, 2006). The differential subcellular activities of BSU1 and BIN2 observed in our study could be due to stoichiometric differences in these two enzymes when located in the nucleus and the cytosol.

Other BSU1 homologs, known as BSU1-like phosphatases (BSLs), are known to function redundantly with BSU1 and to inactivate group II GSK3 kinases including BIN2 (Kim et al., 2009; Mora-Garcia et al., 2004). Thus, the activities of endogenous homologs also need to be considered in estimating the effects of BSU1 and BIN2 tagged with the NLS or NES in this study. We found that, despite the overexpression of BIN2-HA, BL treatment increased the nuclear localization of BES1 (Fig. 2B, middle panel), suggesting that endogenous BSU1 and its homologs overcame the negative action of BIN2. However, BES1 was rarely detected in the nuclei of cells co-expressing BSU-NLS and BIN2-HA (Fig. 4C, right panel), indicating that when it is located in the nucleus, BSU1 stays inactivated and loses its ability to directly inhibit the activity of BIN2-HA. Therefore, the excessive amount of inactive BSU1-NLS could compete with endogenous BSU1 and BSLs for interaction with BIN2 and the group II GSK3 kinases which as a result are kept in the activated state. Probably, cytosolic activation by BSK1, followed by nuclear import, is a prerequisite process before BSU1 can dephosphorylate the nuclear BIN2. Interestingly, in BSU1-NES expressing cells, the endogenous BSU1 and its homologs were apparently fully activated by BL treatment and efficiently inhibited the BIN2-HA activity. This would explain why BES1 was mainly located in the nucleus even though the overexpressed BSU1 was retained in the cytosol by the NES-tag (Fig. 4C, middle panel). On the other hand, nuclear BIN2 (BIN2-NLS) dominantly induced the cytosolic accumulation of BES1, whereas cytosolic BIN2 (BIN2-NES) did not (Fig. 1B, middle and right panel). This supports the idea that BIN2 acts on BES1 in the nucleus (Vert and Chory, 2006). Altogether, our results indicate that the BR-induced nuclear accumulation of BES1 likely results from the nuclear import of BSU1 after activation in the cytosol, and the concomitant inactivation of nuclear BIN2 (see Model in Supplementary Fig. 1 online).

Clarification of the mechanisms controlling the nuclear import of BSU1 is another important issue in our understanding of the nuclear accumulation of BES1 and BZR1. A recent report showed that BSU1 dephosphorylated BIN2 kinase *via* direct interactions, strongly suggesting that the BR-stimulated dephosphorylation of BES1 and BZR1 results from the inactivation of BIN2 by BSU1 (Kim et al., 2009). On the other hand, BSU1 failed to dephosphorylate BES1 directly *in vitro* (Mora-Garcia et al., 2004). However, it is still an open question whether BSU1 or its homologs directly dephosphorylate BES1 *in planta*. Possibly, BSU1 might require interactions with other components for the full scale dephosphorylation of BES1 *in planta*. Given the localization of BSU1 and BIN2 in the nucleus even without the NLS, BSU1 and BIN2 might form a highly ordered complex with other components containing nuclear localization signal. The kelch domain in BSU1 is suggested to interact with other proteins. Therefore, BSU1 might interact with its potential substrates and cargo proteins, resulting in nucleocytoplasmic shuttling. In vertebrate Wnt signaling, which is similar to plant BR signaling, Axin acts as a cargo protein in the nucleocytoplasmic shuttling of relevant components including β-catenin, GSKs, and group 2A phosphatases (Hsu et al., 1999; Itoh et al., 1998; Yang et al., 2003). Even though the roles of the group 2A phosphatases in Wnt signaling are highly diversified and include both negative and positive regulators, it may be that the spatial distribution of BSU1 is mediated by Axin-like cargo proteins. Interestingly, a recent proteomic screening of *Arabidopsis* identified BSU1 and its homolog BSL1 as strong interactors with 14-3-3 proteins (Chang et al., 2009). Based on the suggested roles of 14-3-3 proteins in regulating the subcellular partitioning of a variety of signaling

molecules, including BZR1 (Gampala et al., 2007; Ryu et al., 2007), it is possible that the nucleocytoplasmic shuttling of BSU1 might be regulated *via* 14-3-3 proteins.

We propose that BSU1 and BIN2 modulate the nuclear localization of BES1 *via* differential subcellular activities. Probably, these types of regulation provide swift mechanisms for the nuclear import and export of BES1 in response to BRs. In the cytoplasms of BR-stimulated cells, BSK1-activated BSU1 would primarily inhibit BIN2 activity. Cytosolic BES1 would be excluded from BIN2-mediated phosphorylation and become more available for dephosphorylation. The phosphorylated BES1 and BZR1 proteins exported from the nucleus probably undergo similar tight regulation by BSU1, unless degraded by cytosolic proteasomes (He et al., 2002). The dephosphorylated forms of BES1 and BZR1 are highly enriched in the nucleus, suggesting that they are immediately imported into the nucleus after dephosphorylation (Ryu et al., 2007). Conversely, in the absence of BR, the nuclear BIN2 proteins dominantly phosphorylate and export BES1 and BZR1 from the nucleus (see Model in Supplementary Fig. 1 online).

Our study was aimed at elucidating the differential stoichiometries of BSU1 and BIN2 between the cytosol and the nucleus. These proteins orchestrate the subcellular localizations of BES1 and BZR1 by modulating their phosphorylation statuses. The predominance of cytosolic BSU1 might be required to rapidly transmit cytosolic BR signals to the nucleus. Further investigations of the mechanisms controlling the shuttling of BSU1 and BIN2 between the nucleus and cytosol will provide deeper insights into BR signaling. Such dynamic translocations of key regulators and transcription factors enable the cell to rapidly respond to the BR signal. Plants may benefit from these regulatory mechanisms by swiftly responding to environmental or developmental stimuli.

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

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