The Brassinosteroid-Activated BRI1 Receptor Kinase Is Switched off by Dephosphorylation Mediated by Cytoplasm-Localized PP2A B' Subunits

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ABSTRACT

Brassinosteroid (BR) binding activates the receptor kinase BRI1 by inducing heterodimerization with its co-receptor kinase BAK1; however, the mechanisms that reversibly inactivate BRI1 remain unclear. Here we show that cytoplasm-localized protein phosphatase 2A (PP2A) B' regulatory subunits interact with BRI1 to mediate its dephosphorylation and inactivation. Loss-of-function and overexpression experiments showed that a group of PP2A B' regulatory subunits, represented by B'11, negatively regulate BR signaling by decreasing BRI1 phosphorylation. BR increases the expression levels of these B' subunits, and B'11 interacts preferentially with phosphorylated BRI1, suggesting that the dynamics of BR signaling are modulated by the PP2A-mediated feedback inactivation of BRI1. Compared with PP2A B'α and B'β, which promote BR responses by dephosphorylating the downstream transcription factor BZR1, the BRI1-inactivating B' subunits showed similar binding to BRI1 and BZR1 but distinct subcellular localization. Alteration of the nuclear/cytoplasmic localization of the B' subunits revealed that cytoplasmic PP2A dephosphorylates BRI1 and inhibits the BR response, whereas nuclear PP2A dephosphorylates BZR1 and activates the BR response. Our findings not only identify the PP2A regulatory B subunits that mediate the binding and dephosphorylation of BRI1, but also demonstrate that the subcellular localization of PP2A specifies its substrate selection and distinct effects on BR signaling.

Key words: Brassinosteroid, signal transduction, receptor inactivation, BRI1 dephosphorylation, PP2A

INTRODUCTION

Receptor kinases are cellular switches of signal transduction pathways regulated by extracellular signals. Arabidopsis possesses more than 600 receptor-like kinases, among which brassinosteroid-insensitive 1 (BRI1), the receptor of brassinosteroid (BR), is the best characterized and thus serves as a paradigm for understanding the principal mechanisms of receptor kinase activation and inactivation. While many details have been elucidated about how ligand binding activates BRI1, as well as how the BR signal is transduced to regulate gene expression, little is known about how BRI1 is inactivated when the BR level drops.

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Dephosphorylation of BRI1 by PP2A B' Subunits

(A) Overexpression of different PP2A B' subunits either suppressed (B'α and B'β) or enhanced the phenotype of the bri1-5 mutant. The upper panel shows 3-week-old plants overexpressing the indicated B' subunits as YFP fusions; the lower panel shows anti-GFP immunoblots of YFP-B′ expressed in the transgenic plants shown in the upper panel. Ponceau S-stained Rubisco large subunit is shown as a loading reference.

(B) qRT-PCR analysis of the CPD and DWF4 expression levels in the seedlings shown in (A). Error bars represent SD. Wassilewskija (WS) wild type plants were used as control.

(C) The overexpression of B′γ inhibited eBL-induced BES1 dephosphorylation. Wild-type (Col) and B′γ-overexpressing Col seedlings were grown side by side on 1/2 MS medium for 1 week. The seedlings were then treated with 1 μM eBL for 5 min and harvested for immunoblotting using anti-BES1 antibodies (upper panel). The lower panel shows Ponceau S-stained Rubisco large subunit as a loading control.

BR signaling is initiated when BR binds to the ectodomain of BRI1 to create, together with the folding of a loop region, an interaction surface for the co-receptor kinase BRI1-associated receptor kinase 1 (BAK1) and its homolog SERK1, leading to their heterodimerization (Wang et al., 2001; Li et al., 2002; Nam and Li, 2002; Santiago et al., 2013; Sun et al., 2013). Transphosphorylation between BRI1 and BAK1 activates BRI1 and causes the dissociation of BRI1 kinase inhibitor (BKI1) (Wang and Chory, 2006), increasing the affinity between the kinase domains of BRI1 and BAK1 and further stabilizing the receptor/co-receptor complex (Li et al., 2002; Nam and Li, 2002; Wang et al., 2008). BR-activated BRI1 phosphorylates the membrane-localized receptor-like cytoplasmic kinases BSks and CDG1, which, in turn, activate BRI1-suppressor 1 (BSU1) family phosphatases (Tang et al., 2008; Kim et al., 2011). BSU1 dephosphorylates and inactivates the GSK3/SHAGGY-like kinase brassinosteroid-insensitive 2 (BIN2) to prevent it from phosphatolyzing brassinazole-resistant 1 (BZR1) family transcription factors (Kim et al., 2009). Upon the BR-induced inactivation of BIN2, BZR1 is dephosphorylated by protein phosphatase 2A (PP2A) (Tang et al., 2011) and accumulates in the nucleus, where it binds to DNA to regulate the expression of BR-responsive genes (He et al., 2005; Sun et al., 2010). When BR levels are low, BZR1 is phosphorylated by BIN2 and consequently loses its DNA-binding activity; it is then retained in the cytoplasm by 14-3-3 proteins (Gampala et al., 2007; Ryu et al., 2007).

It has been shown that BRI1 is negatively regulated by several mechanisms. First, BRI1 activity is inhibited by the unphosphorylated form of its C terminus and by association with BK1 (Wang et al., 2005; Wang and Chory, 2006). Both mechanisms appear to help maintain a low level of BRI1 activity before BR activation, rather than the inactivation of BR-activated receptor molecules, because these inhibitory effects are released by BR-induced phosphorylation. A recent genetic study suggested that the abundance of BRI1 in the plasma membrane is regulated by PP2A. BR induces the expression of a leucine carboxyl methyltransferase known as SBI1, which methylates the PP2A C subunits and causes PP2A association with the plasma membrane, possibly leading to BRI1 dephosphorylation and degradation (Wu et al., 2011). However, there is no direct evidence showing the interaction of BRI1 with PP2A or the dephosphorylation of BRI1 by PP2A in Arabidopsis. Furthermore, how the positive and negative effects of PP2A on BR signaling via BZR1 and BRI1, respectively, are specified and balanced remains an outstanding question.

The PP2A holoenzyme is a heterotrimeric complex composed of a catalytic C subunit, a regulatory B subunit that determines the substrate specificity, and a scaffolding A subunit that brings the B and C subunits together (Janssens and Goris, 2001). In Arabidopsis, the B-subunit proteins can be further grouped into three subfamilies: B, B', and B'' (Farkas et al., 2007). Previously, we demonstrated that B′α and B′β subunits interact directly with BZR1 and target BZR1 for dephosphorylation, thereby positively regulating BR signaling (Tang et al., 2011). In this study, we provide both genetic and biochemical evidence to show that the PP2A B' subfamily members B′γ, B′η, B′θ, B′ζ, B′δ, and B′κ inhibit BR signaling, at least for B′η and B′γ, through a direct interaction with and the dephosphorylation of activated BRI1. Our study not only identifies the B′ subunits that mediate the dephosphorylation of BRI1 by PP2A in Arabidopsis, it also demonstrates that the positive (through BZR1) and negative (through BRI1) effects of PP2A on BR signaling are determined by its subcellular localization.

RESULTS

BR Signaling Is Negatively Regulated by a Set of PP2A B' Subunits

It was reported previously that PP2A B′α and B′β positively regulate BR signaling by targeting BZR1 for dephosphorylation (Tang et al., 2011). There are nine genes encoding B'-subfamily members in the Arabidopsis genome (Supplemental Figure 1). Yeast two-hybrid assays have shown that four PP2A B' subunits interact strongly with BZR1 while three interact weakly (Tang et al., 2011). To understand the functions of these B' subunits, we overexpressed nine B'-subfamily members in weak bri1 mutant background. Surprisingly, the overexpression of B′η, B′γ, B′ζ, B′δ, B′κ, and B′θ enhanced the semi-dwarf phenotype.
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**Figure 2. PP2A B′, B′γ, B′ζ, and B′θ Are Negative Regulators of BR Signaling.**

(A) Diagrams show the T-DNA insertion sites in the pp2a b′η, b′γ, b′ζ, and b′θ mutants.

(B) RT–PCR analysis of the expression of B′η, B′γ, B′ζ, and B′θ in the B′γζη quadruple mutant.

(C and D) Quantitative analysis of hypocotyl length in B′ single mutant, quadruple mutant (b′q), and B′-overexpressing (γox and ζox) seedlings grown in the presence of PCZ (C) and eBL (D). Seedlings were grown on normal 1/2 MS medium with or without 0.1 or 0.2 μM PCZ in darkness for 5 days, or on a medium with or without 0.1 or 1 μM eBL under dim light (30 μmol/m² s) for 1 week. Relative growth presents the average ratio between PCZ or eBL treatment and untreated control of at least 45 individual seedlings from three biological repeats. Error bars represent SE. One-way ANOVA was performed. Statistically significant differences are indicated by different lowercase letters (P < 0.05).

(E) The b′γζη quadruple mutant partially suppressed the det2 mutant phenotype. The upper panel shows 3-week-old pp2a b′γζη/det2 pentuple and det2 mutant plants. The lower panel shows leaves detached from the 3-week-old plants.

of bri1-5 or bri1-301 (Figure 1A and Supplemental Figures 2 and 3), whereas the overexpression of B′α and B′β suppressed the dwarf phenotype of bri1-5, as reported previously (Figure 1A and Supplemental Figure 2). The overexpression of B′, which belongs to the same phylogenetic clade as B′α and B′β (Supplemental Figure 1), had no obvious effect on the growth of bri1-5 (Figure 1A and Supplemental Figure 2). Consistent with these phenotypes, real-time quantitative PCR (qRT-PCR) analysis showed that the expression levels of the BR biosynthetic genes CPD or DWF4, which are feedback-inhibited by BR signaling, were decreased in bri1-5 mutant plants overexpressing B′α and B′β, but increased in bri1-5 plants overexpressing B′η, B′γ, B′κ, B′θ, B′ζ, and B′ (Figure 1B); or bri1-301 overexpressing B′η and B′γ (Supplemental Figure 3).

Furthermore, BR-induced BES1 dephosphorylation was delayed by the overexpression of B′ζ in a wild-type background (Figure 1C). These results suggest that PP2A B′η, B′γ, B′κ, B′θ, B′ζ, and B′θ negatively regulate BR signaling, whereas PP2A B′α and B′β positively regulate BR signaling.

To further assess the function of these B′-subunit isoforms, we obtained T-DNA insertion mutants for B′γ, B′ζ, B′η, and B′θ (Figure 2A and 2B), and analyzed their growth response to exogenously applied epi-brassinolide (eBL) and the BR biosynthesis inhibitor propiconazole (PCZ). These mutants showed a reduced hypocotyl response to PCZ in the dark (Figure 2C) and an increased response to eBL in terms of hypocotyl elongation under dim light (Figure 2D), demonstrating a role for these B′-isoforms as negative regulators of BR responses. In contrast to B′α and B′β (Tang et al., 2011), the overexpression of B′γ or B′η caused hypersensitivity to PCZ (Figure 2C). We also generated a b′γζη/θ quadruple mutant by genetic crossing. Compared with the single-insertion mutants, the quadruple mutant showed a larger increase in hypocotyl length in response to 0.1 μM eBL, indicating that the quadruple mutant was more sensitive to BR than the single mutants (Figure 2D). Furthermore, the b′γζη quadruple mutations partially suppressed the dwarf phenotype of det2-1, as the b′γζη/θ det2 pentuple mutant had larger leaves and occasionally longer leaf petioles (around 13%) than det2 (Figure 2E). The expression level of CPD was decreased in the pentuple mutant compared with that in det2, consistent with increased BR signaling (Supplemental Figure 4). These results demonstrate that, unlike B′α and B′β, the B′γ, B′ζ, B′η, and B′θ members of the B′ family negatively regulate BR signaling in Arabidopsis.

**PP2A B′γ and B′η Inhibit BR Signaling in a BIN2-Dependent Manner**

To determine whether the B′ subunits that inhibit BR signaling act upstream or downstream of BIN2, we grew transgenic bri1-5 mutant plants overexpressing B′α, B′β, B′γ, or B′η on Murashige and Skoog (MS) medium containing bikinin, a chemical inhibitor of BIN2, and several other GSK3 kinases involved in BR signaling. In the absence of bikinin, the hypocotyls of the bri1-5 mutants overexpressing B′α and B′β were longer than those of the nontransgenic bri1-5 control plants, while those of plants overexpressing B′γ and B′η were shorter (Figure 3). Hypocotyl elongation was significantly increased by bikinin in bri1-5 due to the inhibition of BIN2 and GSK3s. In comparison, bikinin only slightly increased hypocotyl elongation in bri1-5 overexpressing B′α and B′β, but almost fully recovered the short-hypocotyl phenotypes of bri1-5 overexpressing B′γ and B′η (Figure 3). These results suggest that B′γ and B′η inhibit hypocotyl elongation in a BIN2-dependent manner, possibly by inhibiting BR signaling upstream of BIN2.

**PP2A Negatively Regulates BR Signaling by Dephosphorylating BRI1**

To test whether BR-induced BRI1 phosphorylation is affected by cellular PP2A B′ subunits, we overexpressed B′α, B′β, B′γ, and B′η in det2 mutant plants transformed with a pBRI1:BR1-GFP construct and also introduced pBRI1:BR1-GFP into b′γζη/θ det2 pentuple mutant by genetic crossing. The expression of BR1-GFP partially rescued the dwarf phenotype of det2. Similar to our observation in bri1-5, the overexpression of B′γ and B′η caused dwarfism in the pBRI1:BR1-GFP/det2 plants, while the overexpression of B′α and B′β partially suppressed the semi-dwarf phenotype of the pBRI1:BR1-GFP/det2 plants (Supplemental Figure 5). To test whether BR1 phosphorylation
was affected, we immunoprecipitated BRI1-GFP using anti-GFP antibodies and analyzed the immunoblots using anti-phosphothreonine (pThr) antibodies. Our results show that eBL treatment induced obvious BRI1 phosphorylation in pBRI1: BRI1-GFP/det2 seedlings. This eBL-induced BRI1 phosphorylation was weaker in plants overexpressing B\(\alpha\), B\(\beta\), B\(\eta\), or B\(\gamma\) in a pBRI1:BRI1-GFP/det2 background (A and B) or the pBRI1:BRI1-GFP-expressing b\(\gamma\)I\(\gamma\)/det2 pentuple mutant (C) were treated with 1 \(\mu\)M eBL or mock solution for 30 min (A and B) or 5 min (C). BRI1-GFP was immunopurified using anti-GFP antibodies, blotted onto a nitrocellulose membrane, and probed with anti-pThr antibodies followed by anti-GFP antibodies. Numbers represent the average ratios (±SE) of pThr/GFP signals from three independent experiments.

To test whether PP2A can directly dephosphorylate BRI1, we immunopurified a PP2A enzyme complex containing PP2A A1 (named RCN1), from pRCN1:YFP-RCN transgenic plants using anti-GFP antibodies and incubated it with \(^{32}\)P-labeled glutathione S-transferase (GST)-tagged BRI1 kinase domain (GST-BRI1). This PP2A complex significantly reduced the radiographic signal of \(^{32}\)P-labeled GST-BRI1 and shifted the GST-BRI1 protein to a lower position on the gel (Figure 5A). In contrast, PP2A had no effect on \(^{32}\)P-labeled GST-BIN2, indicating that PP2A specifically dephosphorylates BRI1 but not BIN2. In addition, B\(\alpha\)\(\gamma\) and B\(\eta\)\(\gamma\)-containing PP2A complexes were immunopurified from pB\(\alpha\)\(\gamma\):B\(\alpha\)\(\gamma\)-YFP or pB\(\eta\)\(\gamma\):B\(\eta\)\(\gamma\)-YFP transgenic plants using anti-GFP antibodies. Incubation of these PP2A complexes with autophosphorylated BRI1 kinase domain caused the dephosphorylation of BRI1 and significantly reduced the ability of BRI1 to phosphorylate its substrate, BAK1 (Figure 5B).

We also attempted to identify the PP2A dephosphorylation sites on BRI1 using a quantitative proteomic approach, whereby peptides from PP2A-treated and -untreated GST-BRI1 protein were labeled with different TMT 6-plex isobaric tags, combined and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS); see Methods. Mass spectrometry unambiguously identified 14 and 10 BRI1 autophosphorylation sites in two independent experiments, respectively. Of the phosphopeptides identified, the abundance of seven phosphopeptides was significantly decreased in PP2A-treated BRI1, suggesting that these are primary targeting sites of PP2A (Table 1). A functional analysis showed that four of the seven PP2A targeting phosphopeptides are located at the C-terminal autoinhibitory domain of BRI1, which has been shown to bind the kinase domain of BRI1 and inhibit BRI1 kinase activity when it is dephosphorylated. Taken together, these \textit{in vivo} and \textit{in vitro} experiments demonstrate that PP2A negatively regulates BR signaling by dephosphorylating and inactivating BRI1.

Figure 3. The Inactivation of BIN2/GSK3s Suppresses the Phenotypes Caused by the Overexpression of PP2A B\(\gamma\) and B\(\eta\).

(A) Etiolated seedlings of the brl1-5 mutant and brl1-5 overexpressing different PP2A B\(\gamma\) subunits. The seedlings were grown on regular 1/2 MS medium with or without 15 \(\mu\)M bikinin in the dark for 5 days. (B) Quantification of the hypocotyl lengths of the plants shown in (A). Error bars represent the SE (\(n \geq 40\) plants). One-way ANOVA was performed. Statistically significant differences are indicated by different lowercase letters (\(P < 0.05\)).

Figure 4. PP2A Negatively Regulates BR Signaling by Counteracting BR-Stimulated BRI1 Phosphorylation.

Seven-day-old transgenic plants overexpressing PP2A B\(\alpha\), B\(\beta\), B\(\eta\), or B\(\gamma\) in a pBRI1:BRI1-GFP/det2 background (A and B) or the pBRI1:BRI1-GFP-expressing b\(\gamma\)I\(\gamma\)/det2 pentuple mutant (C) were treated with 1 \(\mu\)M eBL or mock solution for 30 min (A and B) or 5 min (C). BRI1-GFP was immunopurified using anti-GFP antibodies, blotted onto a nitrocellulose membrane, and probed with anti-pThr antibodies followed by anti-GFP antibodies. Numbers represent the average ratios (±SE) of pThr/GFP signals from three independent experiments.
The same membrane was probed further with anti-MBP antibody or with anti-pThr antibodies to examine BRI1 and mBAK1 phosphorylation.Rated by SDS–PAGE, blotted onto a nitrocellulose membrane, and probed cantharidin (a PP2A inhibitor) for 30 or 60 min. The proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes (Supplemental Figure 7B). Both the upper and lower bands were co-immunoprecipitated with BRI1–FLAG (Figure 6C). Both the upper and lower bands were co-immunoprecipitated with BRI1–FLAG (Figure 6C). Based on the ratio between the anti-B′ζ and anti-FLAG immunoblot signals in three independent experiments, eBL treatment increased the interaction between BRI1–FLAG and PP2A B′ζ by 2.14 ± 0.56 fold, demonstrating the BR-stimulated interaction of BRI1 with B′ζ in vivo.

Subcellular Localization Determines the Function of PP2A in BR Signal Transduction

The interaction of BRI1 with B′α, B′β, B′γ, and B′ζ, which oppositely regulate BR signaling, raises the question of how the specificity of PP2A B′ function in BR signaling is determined. We noticed that different PP2A B′ subunits showed different subcellular localization patterns. Using either a C- or N-terminal YFP fusion, the BR-activating B′α and B′β subunits were localized in both the nucleus and the cytoplasm, while all of the BR-inhibiting B′ζ subunits were localized in the cytoplasm and cell periphery but were excluded from the nucleus (Supplemental Figures 8–10).

To investigate whether nuclear localization discriminates the role of different B′ subunits in BR signal transduction, we generated transgenic plants overexpressing B′η fused with a heterologous nuclear localization signal (B′η-nNLS) and B′ζ fused to a nuclear exporting signal (B′ζ-NES). Fusions with a non-functional NLS (B′η-nNLS) or NES (B′ζ-nNES) were used as negative controls. As expected, confocal microscopy showed that YFP-B′η-nNLS and YFP-B′ζ-NES were localized exclusively in the cytoplasm while YFP-B′ζ-NLS was mostly localized in the nucleus. Similar results were found using B′ζ-NES, YFP-B′ζ-nNLS was detected in both the nucleus and cytoplasm (Figure 7A and Supplemental Figure 10). When overexpressed in bri1-5, the nuclear-localized B′ proteins (B′ζ-nNES and B′η-NLS) rescued the dwarf phenotype of the mutant, whereas the cytoplasm-localized B′ proteins (B′ζ-NES and B′η-nNLS) enhanced the dwarf phenotype of the mutant (Figure 7B and 7C, and Supplemental Figure 11). Consistent with this phenotype, the expression levels of CPD and DWF4 were increased in plants overexpressing B′ζ-NES and B′η-nNLS, but reduced in plants overexpressing B′ζ-nNES and B′η-NLS, compared with non-transgenic bri1-5 plants (Figure 7D). As such, relocating PP2A B′ζ from the nucleus to the cytoplasm converted it from a positive regulator to a negative regulator of BR signaling, whereas relocating PP2A B′ζ from the cytoplasm to the nucleus converted it from a negative regulator to a positive regulator. These results demonstrate that the subcellular localization of PP2A B′ subunits is a key determinant of their dual role in BR signal transduction.

**DISCUSSION**

During growth and development, many receptor kinases are reversibly activated by ligand binding and inactivated upon ligand association of B′η with BRI1 was further confirmed in vivo by a co-immunoprecipitation assay using an antibody against native B′η. This antibody recognizes recombinant B′η, B′ζ, and B′ζ (Supplemental Figure 7A), and detects a strong upper gel band corresponding to B′η (it disappeared in the B′η knockout mutant), as well as a weak lower band that is likely B′ζ or B′ζ (Supplemental Figure 7B). Both the upper and lower bands were co-immunoprecipitated with BRI1–FLAG (Figure 6C). Based on the ratio between the anti-B′η and anti-FLAG immunoblot signals in three independent experiments, eBL treatment increased the interaction between BRI1–FLAG and PP2A B′ζ by 2.14 ± 0.56 fold, demonstrating the BR-stimulated interaction of BRI1 with B′ζ in vivo.

**PP2A B′ Subunits Interact Directly with Active BRI1**

We tested whether PP2A B′ subunits inhibit BR-stimulated BRI1 activation by directly interacting with BRI1. In bimolecular fluorescence complementation (BiFC) assays, tobacco epidermal cells co-transformed with either B′ζ or B′η fused to the N-terminal half of yellow fluorescent protein (YFPs) or BRI1 fused to the C-terminal half of cyan fluorescent protein (BRI1-cCFP) showed strong fluorescence signals on the plasma membrane, demonstrating an interaction with BRI1. In contrast, tobacco cells co-transformed with B′α-nYFP and BRI1-cCFP showed no fluorescence signal (Figure 6A). Interestingly, B′α and B′ζ, which positively activate BR signaling, also interacted with BRI1 in BiFC assays. In vitro gel blot overlay assays confirmed the direct interaction of B′α and B′ζ with BRI1, and further showed that B′ζ bound more strongly to phosphorylated BRI1 than to hypophosphorylated BRI1 (Figure 6B), suggesting the recruitment of PP2A B′ζ by activated BRI1 and the disassociation of PP2A B′ζ from dephosphorylated BRI1. The
Dephosphorylation of BRI1 by PP2A B’ Subunits

Our genetic and biochemical data demonstrate that a group of PP2A B’ subunits negatively regulates BR signaling by targeting activated BRI1 for dephosphorylation. Overexpression experiments suggested that the PP2A B’-subunit isoforms B’η, B’γ, B’κ, B’δ, B’ζ, and B’θ act as negative regulators of BR signaling. These functions were further confirmed by creating loss-of-function mutations in B’η, B’γ, B’ζ, and B’θ. The inhibition of growth and BR-responsive gene expression caused by the overexpression of B’η and B’γ was correlated with reduced levels of BR-induced BRI1 phosphorylation, whereas the quadruple B’γζκζη mutation increased hypocotyl elongation and BR-induced BRI1 phosphorylation. Immunopurified PP2A specifically dephosphorylated BRI1 but not BIN2, and reduced BRI1 phosphorylation of its co-receptor BAK1. In vivo and in vitro assays demonstrated that B’η interacts preferentially with phosphorylated and BR-activated BRI1. Together, our results provide strong evidence showing that a set of PP2A B’ subunits (B’η, B’γ, B’κ, B’δ, B’ζ, and B’θ) negatively regulate BR signaling by facilitating the dephosphorylation of BR-activated BRI1.

Several lines of evidence support the notion that subcellular localization contributes to the specific roles of different B’ subunits in regulating BR signaling. First, the PP2A B’ subunits that inhibit BR signaling were all localized to the cytoplasm; only B’%; and B’δ, which positively regulate BR signaling, were localized to both the cytoplasm and the nucleus. Second, fusing B’δ with an NES removed B’δ from the nucleus and transformed it into a negative regulator of BR signaling. Conversely, fusing B’η with an NLS brought B’η into the nucleus and transformed it into a positive regulator of BR signaling. These results demonstrate that B’δ and B’η are both able to mediate the dephosphorylation of either BRI1 or BZR1 when co-localized. However, in wild-type plants, cytoplasm-localized B’ subunits mostly mediate the dephosphorylation of BRI1, whereas PP2A B’% and B’δ are localized in both the nucleus and cytoplasm, and thus contribute to the dephosphorylation of both BZR1 and BRI1. Genetic evidence indicates that B’% and B’δ are positive regulators of BR signaling, suggesting that their effects on BZR1 activation are dominant over their effects on BRI1 inactivation.

Since phosphorylated BZR1 is mostly localized to the cytoplasm by 14-3-3 proteins (Gampala et al., 2007; Ryu et al., 2007), it is surprising that the cytoplasm-localized B’ subunits do not increase the dephosphorylation of BZR1 while the

### Table 1. PP2A-Dephosphorylated BRI1 Peptides Identified by Mass Spectrometry.

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<th>Peptide</th>
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Methionine sulfoxide is denoted as M*. Identified phospho-seryl and phospho-threonyl residues are underlined. The charge (z), mass (m/z), mass accuracy (ppm), searching score, and expected value of the identified phosphopeptides are shown. Ratio represents the normalized abundance of the peptide identified from PP2A-treated BRI1 protein divided by the normalized abundance of the same peptide identified from PP2A-untreated BRI1 protein.
Dephosphorylation of BRI1 by PP2A B’ Subunits

that inhibit BR signaling (Supplemental Figure 13) may contribute to the additional aspect of negative feedback regulation of BR signaling.

It was recently reported that PP2A negatively regulates plant innate immunity by controlling BAK1 activation (Segonzac et al., 2014); however, a direct interaction between PP2A subunits and BAK1 has not been shown. It will be interesting to investigate whether PP2A interacts directly with additional BR signaling components, and to examine whether BRI1 phosphorlates and regulates PP2A.

BRI1 is negatively regulated by several molecular mechanisms. Previous studies have shown that BRI1 activity is inhibited by BRI1’s C-terminal autoregulatory domain and by BKI1. However, these mechanisms appear to play important roles in maintaining low-level BRI1 activity in the absence of BR, rather than the inactivation of active BRI1, because these inhibitory activities are released upon BR-induced phosphorylation (Wang et al., 2005; Wang and Chory, 2006). In contrast, PP2A appears to be recruited to inactivate BRI1 following its activation by BR. PP2A B’11 binds more strongly with phosphorylated BRI1 than with unphosphorylated BRI1, and BR treatment, which induces BRI1 phosphorylation, increased the interaction between BRI1 and PP2A B’11. A previous study suggested that membrane-localized PP2A causes the irreversible removal of BRI1 by endocytosis and degradation (Wu et al., 2011). Our results show that PP2A decreases BRI1 phosphorylation without significantly altering BRI1 accumulation; thus, dephosphorylated BRI1 can presumably be reactivated by ligand-induced transphosphorylation between BRI1 and BAK1. Such rapid, activation-induced, and reversible inactivation of BRI1 may ensure real-time monitoring of dynamic changes in BR levels during plant development and in the face of environmental change. Direct evidence for such cycles of BR-induced activation and PP2A-mediated inactivation of BRI1, however, will likely require real-time single-molecule analyses of BRI1 activity in future studies.

METHODS

Plant Materials and Growth Conditions

The br1-5 mutant is in the Wassilewskija ecotype background; all other BR-related mutants are in the Columbia ecotype background. For hypocotyl and root growth assays, seedlings were grown on vertical agar plates containing half-strength MS medium supplemented with 1% (w/v) sucrose and the indicated concentration of hormone and inhibitors. Growth images were acquired, and the hypocotyls and root lengths were measured using ImageJ. For short-term hormone treatments, 1-week-old seedlings (n ≥ 40) grown on agar plates were submerged in hormone or mock solution for the indicated time. Each experiment was performed at least three times with similar results; representative data from one repetition are shown in the figures.

Molecular Cloning and Generation of Transgenic Arabidopsis

Full-length cDNA sequences of the PP2A B’ subunits without a stop codon were amplified by PCR, cloned into pENTR/SD/D-TOPO vector (Invitrogen, Carlsbad, CA), and subcloned into the Gateway-compatible binary vectors pEarleyGate 104 or pEarleyGate 101 to generate N- and C-terminal fusions with YFP using LR Clonase (Invitrogen). For the subcellular localization studies, full-length genomic sequences (without the stop codon or 3’ untranslated region) encoding PP2A B’α, B’β, and B’γ, including 1285 bp (B’α), 2006 bp (B’β), and 1217 bp (B’γ) of the promoter sequence relative to the translation start site, were amplified by PCR.
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Figure 7. The Subcellular Localization of PP2A B’ Subunits Determines Their Roles in the BR Signaling Pathway.

(A) Confocal images showing the subcellular localization of YFP-B’\(\beta\) fused with an NES (YFP-B’\(\beta\)-NES) or a non-functional NES (YFP-B’\(\beta\)-nNES), and YFP-B’\(\eta\) fused with an NLS (YFP-B’\(\eta\)-NLS) or a non-functional NLS (YFP-B’\(\eta\)-nNLS) in the primary root of 1-week-old transgenic seedlings grown on normal \(\frac{1}{2}\) MS medium in the light. Scale bar represent 20 \(\mu\)m. (B) Upper panel shows 3-week-old bri1-5 mutant plants and bri1-5 plants overexpressing YFP-B’\(\beta\)-NES or YFP-B’\(\beta\)-nNES. Middle panel shows immunoblotting for YFP-B’\(\beta\)-NES and YFP-B’\(\beta\)-nNES in transgenic plants using anti-GFP antibodies. Lower panel shows Ponceau S staining of the Rubisco large subunit on the nitrocellulose membrane. (C) Upper panel shows 3-week-old bri1-5 plants and bri1-5 plants overexpressing YFP-B’\(\eta\)-NLS or YFP-B’\(\eta\)-nNLS. Middle panel shows immunoblotting for YFP-B’\(\eta\)-NLS and YFP-B’\(\eta\)-nNLS in the transgenic plants using anti-GFP antibodies. Lower panel shows Ponceau S staining of the Rubisco large subunit on the nitrocellulose membrane. (D) qRT-PCR analysis of the CPD and DWF4 expression levels in the seedlings shown in (B) and (C). Error bars represent the SE. One-way ANOVA was performed. Statistically significant differences are indicated by different lowercase letters (\(P < 0.05\)). Wassilewskija (WS) wild type plants were used as control.

Isolation of Arabidopsis Mutants

The T-DNA knockout mutants used in this study were SALK_039172 (\(b’\gamma\)), SALK_057440 (\(b’\eta\)), SAIL-300-B01 (\(b’\delta\)), and SALK_107944c (\(b’\zeta\)), which were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). The confirmed single mutants were crossed with each other to generate the \(b’\gamma\)\(\eta\)\(\zeta\eta\) quadruple mutant, which was crossed with the det2 mutant to generate the \(b’\gamma\)\(\eta\)\(\zeta\eta\)det2 pentuple mutant. F3 segregated homozygous lines were used for growth assays. The gene-specific primers used to genotype the mutants were: 5’-tgtaaattgagttggactg-3’ and 5’-aattacagaa actctataatcttataatcc-3’ for SALK_039172; 5’-atggaggacagagatctagtaag ctct-3’ and 5’-actgttggactgttacggtctcctctcc-3’ for SALK_057440; 5’-aac gaagatggtagcggttaatctgtt-3’ and 5’-tctattaaggtgcatcgcagatgtaa-3’ for SALK_107944; and 5’-tggattattatccattcagg-3’ and 5’-gaacatcttg caagctctctc-3’. ss 5’-gtctgttctttgtttcctaa-3’. C. The expression level of CPD or DWF4 was normalized by UBQ5 (At3g62250). The relative expression levels of CPD and DWF4 are presented as the ratio to the expression level in non-transgenic control plants. The average ratio and SD were calculated based on values from at least three biological repeats. The primers used were: 5’-ttgctcaactcaaggaa gaggttgaatc-3’ and 5’-gcaaattacttatatccttggactcgtg-3’. pBRI1:BRI1-GFP/det2 mutant was then crossed with the \(b’\gamma\)\(\eta\)\(\zeta\eta\) quadruple mutant. F3 segregated pBRI1:BRI1-GFP-expressing double and sextuple homozygous mutants were used for the immunoprecipitation assays.

qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). The first-strand cDNA was synthesized using Reverse Transcriptase M-MLV (Takara Bio Inc., Otsu, Japan) according to the manufacturer’s instructions. qRT-PCR was performed according to a standard protocol using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA). The expression level of CPD or DWF4 was normalized by UBQ5 (At3g62250). The relative expression levels of CPD and DWF4 are presented as the ratio to the expression level in non-transgenic control plants. The average ratio and SD were calculated based on values from at least three biological repeats. The primers used were: 5’-ttgctcaactcaaggaa gaggttgaatc-3’ and 5’-gcaaattacttatatccttggactcgtg-3’.

BiFC Assays

BiFC assays were performed as reported previously (Gampa et al., 2007). The coding sequences of full-length AtBRI1 and different B’ subunits were cloned in frame into the Gateway-compatible BiFC binary vectors pX-CCFP and pX-nYFP, in which gene expression is under the control of the 3S promoter, using LR Clonase (Invitrogen). The vectors were transformed into A. tumefaciens strain GV3101 and co-transformed into abaxial epidermal cells of 4-week-old tobacco leaves. YFP fluorescence was detected 48 h after infiltration.
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Protein Purification and In Vitro Gel Blot Overlay Assays

The procedure was carried out as described previously (Tang et al., 2011). GST-tagged PP2A B′-subunit proteins, BR1, BIN2, mBAK1, and 14-3-3 proteins, as well as malsee-binding protein (MBP)-tagged BR1 and BZR1, were expressed in and purified from Escherichia coli by standard procedures using Glutathione Separose 4B (GE Healthcare, Little Chalfont, UK) or amylase agarose beads (New England Biolabs, Ipswich, MA). To prepare phosphorylated and hypophosphorylated MBP-BR1, 2 μg of MBP-tagged BR1 was incubated in the presence and absence of 200 μM ATP for 16 h at 30°C to allow BR1 autophosphorylation. To prepare phosphorylated and hypophosphorylated BZR1, 2 μg of MBP-BZR1 on amylase agarose beads was incubated with 1 μg of GST-BIN2 in kinase buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl2, 100 mM NaCl, 1 mM DTT, and 200 μM ATP) at 30°C for 16 h. BIN2-phosphorylated BZR1 was incubated with immunopurified PP2A complex at 30°C with constant agitation for 2 h in the presence of 30 μM bixinikin to inhibit BIN2 kinase activity. Phosphorylated and hypophosphorylated BR1 or BZR1 were then separated by SDS-PAGE, blotted on a nitrocellulose membrane, and incubated in blocking solution (5% non-fat milk in PBS buffer) overnight at 4°C. The membrane was then incubated with 2 μg/ml of GST-tagged B′ subunits or GST-14-3-3-3 in blocking solution for 2 h at room temperature. After washing three times (10 min each) with PBS containing 0.1% Tween 20, the membrane was incubated at room temperature with horseradish peroxidase-labeled anti-GST antibodies (Sigma, St. Louis, MO) in blocking solution for 1 h. The overlay signal was detected using SuperSignal West Dura chemiluminescence reagent (Pierce, Rockford, IL).

Immunoprecipitation Assay

To immunoprecipitate BR1, 10–20 g of 10-day-old liquid-grown seedlings were ground in an extraction buffer containing 20 mM Tris-HCl (pH 8.8), 150 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 50 mM microcystin, 1 mM PMSF, and 1 μM protease inhibitor cocktail (Roche, Basel, Switzerland). The homogenate was spun first at 15,000 g for 15 min to remove organelles and cell debris, followed by a 1-h spin at 100,000 g to pellet the microsomal fraction. Membrane protein was resuspended in 1 ml of resuspension buffer (10 mM Tris-HCl [pH 7.3], 150 mM NaCl, 1 mM EDTA, and 10% glycerol) by pipetting. Triton X-100 was added to a final concentration of 0.5% to solubilize membrane protein. The mixture was preincubated with 50 μl (50% slurry) of Protein A Sepharose CL-4B beads (Sigma, St. Louis, MO) to block any non-specifically bound proteins. After a quick spin at 500 g for 2 min, 1 μg of monoclonal anti-FLAG antibody (Sigma) or monoclonal anti-GFP antibody (Roche) was added to the supernatant and incubated with agitation for another hour. A fresh preparation of Protein A Sepharose CL-4B beads (50 μl) or monoclonal anti-GFP antibody (Roche) or anti-pThr antibodies (Cell Signaling Technology Inc., Danvers, MA) for critical reading of the manuscript. No conflict of interest declared.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Molecular Plant Online.

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AUTHOR CONTRIBUTIONS

W.T. and Z.-Y.W. conceived the project and designed the experiments. R.W. contributed data in Figures 1B, 2B, 3B, 5A, 7A–7D, and Supplemental Figures 1, 4, 9, 11, and 13; M.L. contributed data in Figures 1A, 2C–2E, 4A–4C, 5B, and Supplemental Figures 2, 3, 5, 6, 10, and 12; M.Y. contributed data in Figures 1A, 1C, 6A–6C, and Supplemental Figures 7 and 8. J.A.O. and A.L.B. contributed data in Supplemental Information, or anti-pThr antibodies (Cell Signaling Technology Inc., Danvers, MA) to detect the in vivo interaction between BR1 and B′-1 or BR1-induced phosphorylation of BR1.

In Vitro BR1 Dephosphorylation Assay

YFP-tagged PP2A A1 (RCN1), B′(1), or B′(1) was immunopurified from 1-week-old liquid-grown pRCN1:YFP-RCN1, pBR′B:β′YFP, or pBR′B:β′YFP transgenic Arabidopsis seeds using anti-GFP antibodies as described above. After 4 h of autophosphorylation, GST-BR1, MBP-BR1, or GST-BIN2 was purified using Glutathione Sepharose 4B (GE Healthcare) or amylase agarose beads (New England Biolabs), eluted, and incubated with immunopurified PP2A complex on Protein A beads at 30°C for 2 h with constant agitation. The reaction was stopped by adding an equal volume of 2 x SDS sample buffer, or further incubated with GST-tagged kinase-dead mutant form of the BAK1 kinase domain (GST-mBAK1) in the presence of 200 μM ATP and 5 μM cantharidin (a PP2A inhibitor) for 30 or 60 min. The protein was separated by SDS-PAGE. The phosphorylation levels of BR1, BIN2, and mBAK1 were determined by autoradiography or using anti-pThr antibodies.

To identify the PP2A dephosphorylation sites on BR1, autophosphorylated BR1 kinase domain was incubated with or without PP2A complex immunopurified from pBR′B:pβ′YFP transgenic Arabidopsis seeds using anti-GFP antibodies at 30°C for 6 h. The proteins were separated by SDS-PAGE, and the Coomassie blue-stained MBP-BR1KD band was cut into 2–3-mm pieces and in-gel digested. The extracted peptides were cleaned up using ZipTip pipette tips containing C18 (Millipore, Billerica, MA) and labeled with TMT sixplex isobaric labeling reagents (Life Technologies) according to the manufacturer’s instructions. Peptides from MBP-BR1KD treated with or without PP2A were labeled with different isobaric tags, combined, and analyzed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) coupled online to a NanoAcquity UPLC system (Waters Corp., Milford, MA) in data-dependent mode. The peptides were separated by reverse-phase LC with an Easy-Spray PepMap column (75 μm × 15 cm; Thermo Fisher Scientific Inc.). The mobile phases for LC contained buffer A (2% acetoniol and 0.1% formic acid) and buffer B (98% acetoniol and 0.1% formic acid). A linear gradient of buffer B from 2% to 50% for 120 min was used for separation. Phosphopeptide identification and quantitation were analyzed in high-energy collision dissociation mode with the collision energy set to 35%. The analytical peak lists were generated from the raw data using PAVA software (Guan et al., 2011). The MS/MS data were searched against the Arabidopsis proteome in the Swiss-Prot database using the Protein Prospector search engine (http://prospector.ucsf.edu/prospector/mshome.htm). Phosphopeptide quantitation was performed according to Blanc et al. (2012).

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