Running Head: GSK3 Kinase in Brassinosteroid Signaling

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BIN2 Functions Redundantly with Other Arabidopsis GSK3-Like Kinases to Regulate Brassinosteroid Signaling

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Abstract

Glycogen synthase kinase 3 (GSK3) is a highly conserved serine/threonine kinase involved in a variety of developmental signaling processes. The Arabidopsis genome encodes 10 GSK3-like kinases that are clustered into 4 groups. Forward genetic screens have so far uncovered 8 mutants, all of which carry gain-of-function mutations in BRASSINOSTEROID-INSENSITIVE 2 (BIN2), one of the three members in group II. Genetic and biochemical studies have implicated a negative regulatory role for BIN2 in brassinosteroid (BR) signaling. Here we report identification of 8 EMS-mutagenized loss-of-function bin2 alleles and one T-DNA insertional mutation each for BIN2 and its two closest homologs, BIN2-Like 1 (BIL1) and BIN2-Like 2 (BIL2). Our genetic, biochemical, and physiological assays revealed that despite functional redundancy, BIN2 plays a dominant role among the three group II members in regulating BR signaling. Surprisingly, the bin2bil1bil2 triple T-DNA insertional mutant still responds to BR and accumulates more phosphorylated form of a BIN2 substrate than the wild-type plant. Using a specific GSK3 inhibitor LiCl, we have provided strong circumstantial evidence for involvement of other Arabidopsis GSK3-like kinases in BR signaling. Interestingly, LiCl treatment was able to suppress the gain-of-function bin2-1 mutation but had a much weaker effect on a strong BR receptor mutant, suggesting the presence of a BIN2-independent regulatory step downstream of BR receptor activation.
Introduction

Glycogen synthase kinase 3 (GSK3) is a highly conserved Ser/Thr kinase that is implicated in a wide range of cellular and developmental processes (Woodgett, 2001). In mammals, GSK3 has only two isoforms, GSK3α and GSK3β. By contrast, many plant species contain a much larger set of GSK3-like kinases (Richard et al., 2005; Yoo et al., 2006). The Arabidopsis has 10 GSK3-like kinases, also known as AtSKs for Arabidopsis thaliana SHAGGY-like protein kinases, that can be classified into 4 subgroups based on phylogeny analysis (Jonak and Hirt, 2002). Genetic, transgenic, and biochemical approaches have implicated plant GSK3-like kinases in a variety of plant signaling processes, including flower development, stress/wounding responses, and hormone signaling (Jonak and Hirt, 2002). For example, transgenic Arabidopsis plants with reduced transcript levels of AtSK1-1 and AtSK1-2 contain increased numbers of floral organs and exhibit abnormal apical-basal patterning in gynoecium (Dornelas et al., 2000) whereas overexpression of a mutated AtSK3-2 displayed smaller floral organs (Claisse et al., 2007). It was also known that wounding can activate an alfalfa GSK3 kinase (Jonak et al., 2000) and that overexpression of AtSK2-2 can complement a salt-stress-sensitive mutant in yeast and results in enhanced salt resistance in Arabidopsis (Piao et al., 1999; Piao et al., 2001).

The best studied Arabidopsis GSK3-like kinase is BIN2/UCU1/DWF12/AtSK2-1 that was believed to regulate the signal transduction of brassinosteroids (BRs) (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), a unique class of plant steroid hormones essential for plant growth and development (Clouse and Sasse, 1998). Genetic and biochemical studies in the past decade have revealed a linear signaling pathway that uses protein phosphorylation to transmit BR signals from the cell surface into nucleus (Li, 2005; Li and Jin, 2007). In the absence of BR, BIN2 is constitutively active to phosphorylate BES1 and BZR1 (He et al., 2002; Yin et al., 2002; Zhao et al., 2002), two highly similar transcriptional factors (He et al., 2005; Yin et al., 2005), resulting in their degradation, cytoplasmic localization, and reduced DNA-binding activities (He et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Bai et al., 2007; Gampala et al., 2007; Ryu et al., 2007). BR binding to its cell surface receptor BRI1 triggers a rapid dissociation of BK11, a putative BRI1 substrate (Wang and Chory, 2006), as well as dimerization and transphosphorylation with BAK1, a suspected BRI1 coreceptor (Li et al., 2002; Nam and...
Li, 2002; Russinova et al., 2004; Wang et al., 2005; Wang et al., 2008). These membrane events lead to BIN2 inhibition, via protein degradation (Peng et al., 2008) and/or some yet to be discovered biochemical mechanisms, thus relieving its inhibitory effects on BES1 and BZR1 that regulate expression of BR-responsive genes (Li and Jin, 2007).

Most of our knowledge about BIN2 function came mostly from gain-of-function results. Genetic screens in Arabidopsis for BR-insensitive dwarf mutants or leaf development mutants resulted in isolation of 8 gain-of-function bin2 alleles (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), 7 of which carry mutations in the highly conserved TREE (Thr261Arg262Glu263Glu264) motif within the BIN2 catalytic domain (Peng and Li, 2003). In contrast, similar genetic screens identified 30 loss-of-function alleles of BRI1 (Clouse et al., 1996; Li and Chory, 1997; Noguchi et al., 1999; Friedrichsen et al., 2000; Bouquin et al., 2001; Tanaka et al., 2005; Xu et al., 2008). As a matter of fact, up to now no single loss-of-function allele of any GSK3-like kinase has been discovered in any forward genetic screen in Arabidopsis, implying that the Arabidopsis GSK3-like kinases most likely function redundantly to regulate plant development. Expression profiling results seem to support this hypothesis. Real-time PCR analysis showed that 8 of the 10 AtSK genes displayed a “pseudo-constitutive” expression patterns under several different growth conditions (Charrier et al., 2002). A recent study using T-DNA insertional mutants of BIN2 and its two closest homologs concluded that the three group II AtSKs function redundantly in BR signaling (Vert and Chory, 2006). However, it remains unknown why no single gain-of-function mutation in the two BIN2 homologs was discovered in the forward genetic screens and if other Arabidopsis GSK3-like kinases might also participate in BR signaling.

In this study, we conducted a genetic screen looking for second site mutations that suppress the weak dwarf phenotype of the Arabidopsis ucu1-3 mutant and identified 8 new loss-of-function bin2 alleles. Though genetic and pharmacological experiments using a well-known GSK3 inhibitor Li⁺ (Klein and Melton, 1996; Stambolic et al., 1996), we discovered that BIN2 plays a major role among the group II AtSKs in regulating BR signaling and that other AtSKs can also phosphorylate BIN2 substrates. Our Li⁺ experiments also suggested existence of a BIN2-independent regulatory mechanism in the BR signaling pathway.
Results

Isolation of bin2 Loss-of-Function Mutants

Unlike BRI1 that was discovered through recessive loss-of-function mutants, all the bin2 mutants uncovered from forward genetic screens carry semi-dominant gain-of-function alleles (Peng and Li, 2003). To test if the wild type BIN2 plays a role in BR signaling, we carried out a suppressor screen using a weak bin2 mutant, ucu1-3 (Fig. 1A), which can produce many seeds suitable for a large-scale genetic screen. Although ucu1-3 was previously described as a weak recessive mutant (Perez-Perez et al., 2002), transformation of a genomic BIN2 construct carrying the ucu1-3 (P284S) mutation into wild type Arabidopsis plants resulted in many dwarf plants similar to the bin2-1 mutant (Fig. 1B), indicating that ucu1-3 carries a weak gain-of-function bin2 allele. We reasoned that a suppressor screen in the ucu1-3 background could lead to identification of not only extragenic suppressors but also intragenic loss-of-function mutations that abolish the BIN2 kinase activity.

Screening a total of 200,000 M2 seeds derived from 20,000 EMS-mutagenized M1 plants resulted in identification of 50 putative suppressors. Genetic studies and PCR-based mapping of 12 putative suppressors revealed that at least 8 of them are intragenic suppressors (Fig. 1D), which were confirmed by sequencing analysis of their entire BIN2 genes, each containing an additional single nucleotide change besides the ucu1-3 mutation. We therefore name them bin2-4 to bin2-11. As shown in Figure 1E, bin2-5, bin2-6, and bin2-8 each contain a nonsense mutation, while bin2-9 and bin2-10 carry a single nucleotide mutation at exon/intron and intron/exon junctions of the 9th intron, respectively, most likely leading to defective RNA splicing and premature translational termination. The remaining three mutants, bin2-4, bin2-7, and bin2-11, have a missense mutation at G49, C183, and R312, respectively. G49 and R312 are absolutely conserved in the catalytic domain of all known Ser/Thr kinases while C183 is immediately adjacent to the absolutely conserved DFG motif of the Ser/Thr kinase activation fragment (Hanks et al., 1988). Thus these mutations most likely lead to complete loss of the BIN2 kinase activity. All these intragenic suppressors were morphologically indistinguishable from the corresponding wild type plants [ecotype Landsberg erecta (Ler)] (Fig. 1D), suggesting that BIN2 functions redundantly with one or more AtSKs in regulating BR
signaling. We also obtained a T-DNA insertional mutant in ecotype Wassilewskija (Ws), FLAG_593C09, from the FLAGdb/FST collection (Samson et al., 2002), which was previously reported as bin2-3 (Vert and Chory, 2006). Similar to all the bin2 loss-of-function mutants of ecotype Ler, bin2-3 exhibits no obvious morphological defect compared with the corresponding Ws wild type plant (Fig. 1C).

**Analysis of bin2-3 Mutants Confirms a Regulatory Role of BIN2 in BR Signaling**

Sensitized genetic backgrounds have been used widely to detect redundant gene function. To test if BIN2 plays a role in BR signaling, we used a sensitized genetic background of bri1-5 mutation that causes ER retention of a functionally competent BR receptor (Hong et al., 2008). Because bri1-5 is in ecotype Ws, we used bin2-3 to generate the bin2-3bri1-5 double mutant to avoid any ecotype effect on individual F2 plants. As shown in Figure 2A, the bin2-3bri1-5 double mutant has a bigger rosette with visible petioles and flatter leaves than the bri1-5 mutant. The partial suppression of bri1-5 by loss of BIN2 activity confirmed that BIN2 plays a negative role in BR signaling.

Consistent with our genetic result, bin2-3 mutants exhibit enhanced BR sensitivity measured by BR-induced root growth inhibition and resistance to brassinazole or Brz, a specific inhibitor of BR biosynthesis (Asami et al., 2000). As shown in Figure 2B, the root growth of bin2-3 mutants was slightly more inhibited than that of wild type seedlings by brassinolide (BL), the most active member of the BR family, giving rise to a slightly down-shifted dose-response curve. Figures 2C and 2D revealed that hypocotyl elongation of dark-grown bin2-3 seedlings was less prohibited by Brz compared to wild type seedlings, producing a slightly up-shifted dose-response curve.

Enhanced BR sensitivity of bin2-3 was also observed at both biochemical and molecular levels. Previous studies have shown that BR treatment leads to rapid dephosphorylation of BES1, a presumed BIN2 substrate (Mora-Garcia et al., 2004). As revealed by Figure 2E, wild type plants predominantly accumulate the hyperphosphorylated form of BES1, and BL treatment results in rapid disappearance of the hyperphosphorylated BES1 accompanied by accumulation of hypophosphorylated BES1 that has a faster electrophoretic mobility on western blot. Interestingly, without BR treatment, bin2-3 mutants accumulate an easily detectable amount of hypophosphorylated BES1 compared to wild type control grown under the same growth condition (Fig. 2E),
suggesting that loss of BIN2 results in enhanced BR signaling. We also measured the transcript levels of two BZR1 target genes, *CPD* and *DWF4* (He et al., 2005), and a BES1 target gene, *SAUR-AC1* (Yin et al., 2005) by northern blot and RT-PCR analyses. Consistent with previous reports (Mathur et al., 1998; Goda et al., 2002), 1 hour BL treatment stimulated expression of *SAUR-AC1* but inhibited accumulation of transcripts of both *CPD* and *DWF4* genes (Fig. 2F). Interestingly, without BR treatment, *bin2-3* mutants accumulated more *SAUR-AC1* transcripts but lower levels of *DWF4* and *CPD* mRNAs than wild type plants. Taken together, our results demonstrated that the *bin2-3* knockout mutant exhibits measurable physiological and molecular phenotypes despite lack of any visible morphological defect in a *BRI1* background, confirming that BIN2 is an important negative regulator of the BR signaling pathway.

**The Two Closest Homologs of BIN2 Are Also Involved in BR Signaling**

Although our genetic, physiological, and biochemical assays demonstrated that BIN2 is a negative regulator of the BR signaling pathway, the lack of morphological defects of all isolated *bin2* loss-of-function mutants is in sharp contrast to the severe BR-insensitive dwarf phenotype of most previously characterized *bin2* gain-of-function mutants. This is likely caused by functional redundancy between BIN2 and one or more Arabidopsis GSK3-like kinases. Based on sequence similarity, BIN2 and its two closest homologs, AtSK2-2 and AtSK2-3 (named hereinafter BIL1 and BIL2 for BIN2-Like 1 and 2, respectively), belong to the group II of the Arabidopsis AtSKs (Jonak and Hirt, 2002). Amino acid sequence alignment revealed that the two highly similar BILs share extensive sequence identity with BIN2, despite having extra amino acids at their N-terminal ends and different C-terminal tails (Fig. 3A), suggesting that both BIL1 and BIL2 might also be involved in BR signaling.

To directly test this possibility, we first expressed both BIL1 and BIL2 as fusion proteins with maltose-binding protein (MBP) and measured their *in vitro* kinase activities towards BES1 expressed also in *E. coli* as a fusion protein with glutathione-S-transferase (GST). As shown in Figure 3B, both MBP-BIL1 and MBP-BIL2 displayed similar GST-BES1 phosphorylation activity as the MBP-BIN2 fusion kinase. Since the three MBP-fused kinases failed to phosphorylate the GST tag itself (data not shown), our results demonstrated that two BILs are biochemically capable of phosphorylating known BIN2 substrates *in vitro.*
We also took a gain-of-function approach and overexpressed either a mutated \textit{gbil1}(E295K) or \textit{gbil2}(E293K) gene driven by their native promoters in wild type Arabidopsis plants. Both E295K and E293K mutations correspond to the gain-of-function E263K mutation responsible for the \textit{bin2-1} dwarf phenotype (Li and Nam, 2002) (Fig. 3A). As shown in Figure 3C, some of the \textit{gbil1}(E295K) and \textit{gbil2}(E293K) transgenic plants are morphologically similar to the \textit{bin2-1} mutant or \textit{gbin2-1} transgenic plants overexpressing a mutated \textit{BIN2} gene that carries the \textit{bin2-1} mutation. It should be interesting to note that while almost 100% of the \textit{gbin2-1} plants exhibited the \textit{bin2-1} phenotypes, only 35% of the \textit{gbil1}(E295K) transgenic seedlings and 28% of the \textit{gbil2}(E293K) transgenic plants (out of >100 transgenic lines for each transgene) were \textit{bin2}-like (Fig. 3C). These results suggested that both BIL1 and BIL2 are capable of blocking BR signaling albeit with lower efficiency when the conserved acidic residue (E295 in BIL1 and E293 in BIL2) is changed to a basic residue.

To confirm their involvement in BR signaling, we screened and obtained one T-DNA insertional mutant in the Ws ecotype for either \textit{BIL} gene from the Arabidopsis Knockout Facility at the University of Wisconsin-Madison (Fig. 4A). RT-PCR analysis revealed that \textit{bil1} and \textit{bil2} mutants contained no detectable levels of \textit{BIL1} and \textit{BIL2} transcripts, respectively, suggesting that both \textit{bil1} and \textit{bil2} are likely null mutants (Fig. 4B). Similar to all the \textit{bin2} loss-of-function mutants, neither single nor double mutants of \textit{bil1} and \textit{bil2} displayed any visible morphological/growth defect (Fig. 4C, the 3\textsuperscript{rd}, 4\textsuperscript{th}, and 5\textsuperscript{th} plants). We also generated double and triple mutants of \textit{bil1}, \textit{bil2}, and \textit{bri1-5}. Unlike \textit{bin2-3bri1-5} that exhibited a partial suppression phenotype, \textit{bil1bri1-5} and \textit{bil2bri1-5} double or \textit{bil1bil2bri1-5} triple mutants are morphologically indistinguishable from \textit{bri1-5} (Fig. 4D, the 3\textsuperscript{rd}, 4\textsuperscript{th}, and 5\textsuperscript{th} plants).

Despite lack of any morphological effect, root growth inhibition assay and etiolated hypocotyl elongation assay revealed that simultaneous elimination of BIL1 and BIL2 had a detectable effect on BR sensitivity. Figures 5A and 5B showed that dose-responsive curves of BL-induced root growth inhibition and Brz-inhibited hypocotyl elongation of the \textit{bil1bil2} double mutant sit between those of the wild type control and the \textit{bin2-3} mutant. The effect of the \textit{bil1bil2} double mutation on BR signaling was also examined by the BES1 phosphorylation assay and the \textit{SAUR-AC1} and \textit{CPD} gene expression analyses.
Although no detectable amount of non-phosphorylated BES1 was detected in the bil1bil2 mutant seedlings by anti-BES1 immunoblotting (Fig. 5C), RT-PCR and northern blot analyses did show changes in transcript levels of SAUR-AC1 and CPD, respectively. The bil1bil2 mutation increases the SAUR-ACS1 transcript level by approximately two fold (Fig. 5D) but reduces the CPD mRNA level by ~ 30% (Fig. 5E).

We also constructed triple mutants with bil1 or bil2 single mutant with the bin2-3bri1-5 double mutant. As shown in Figure 4D (the 6th and 7th plants), bin2-3bil1bri1-5 and bin2-3bil2bri1-5 mutants are larger than the parental bin2-3bri1-5 double mutant or the bil1bil2bri1-5 triple mutant with much longer petioles, revealing that suppression of the bri1-5 phenotype by bin2-3 can be significantly enhanced by elimination of either BIL1 or BIL2. Taken together, our results suggest that BIN2 functions redundantly with BIL1 or BIL2 in regulating BR signaling with BIN2 playing a dominant role.

Elimination of BIN2, BIL1, and BIL2 Constitutively Activates BR Signaling

To further test functional redundancy between BIN2 and two BILs, we generated bin2-3bil1bil2 triple mutant. Unlike bin2-3 or the bil1bil2 double mutant, the triple mutant has elongated and wavy petioles with narrow and twisted rosette leaves (Fig. 4C, the 6th and 7th plants), resembling the previously characterized bes1-D or bzr1-D mutants and the BES1(P233L):GFP or BZR1(P234L):GFP transgenic plants (Wang et al., 2002; Yin et al., 2002; Zhao et al., 2002) (Fig. 6A). The P233/234L mutation is known to be responsible for a BR hypersensitive phenotype of the bes1-D and bzr1-D mutants that accumulates high levels of both hyperphosphorylated and hypophosphorylated forms of BES1 and BZR1, respectively (He et al., 2002; Wang et al., 2002; Yin et al., 2002). The phenotypic similarity between bin2-3bil1bil2 and known mutants or transgenic plants with enhanced BR signaling outputs further support our conclusion that BIN2 functions redundantly with BIL1 and BIL2 in regulating the BR signal transduction pathway.

To test if the phenotype of the bin2-3bil1bil2 triple mutant depends on the presence of BRI1 or active BRs, we first crossed the triple mutations into bri1-5. The resulting quadruple mutant exhibited a more or less similar morphological phenotype as the bin2-3bil1bil2 triple mutant (Fig. 4D, the 8th plant). We also germinated and grew the triple mutant in the dark on Brz-containing medium. As shown in Figures 5B, while hypocotyl elongation of wild type, bin2-3, or bil1bil2 mutants were inhibited 85%, 73% and 83%,
respectively, by 2 μM Brz, the triple mutant was still etiolated when grown on the same concentration of Brz with a mere 17% reduction in hypocotyl elongation.

Consistent with our genetic and physiological results, western blot analysis of the BES1 phosphorylation status revealed that the bin2-3bil1bil2 triple mutant accumulated higher levels of both hypophosphorylated and hyperphosphorylated forms of BES1 than wild type, bin2-3, or the bil1bil2 double mutant (Fig. 5C, lane 7). As a result, SAUR-AC1 expression was significantly enhanced in the bin2-3bil1bil3 triple mutant with a near 7 fold increase compared to a ~ 3 fold BR-elicited induction in wild type seedlings (Fig. 5D). By contrast, the transcript level of CPD was reduced by ~80% in the triple mutant compared with a ~40% reduction in BR treated wild type plants (Fig. 5E). Taken together, these results conclude that elimination of BIN2 and its two closest homologs leads to constitutive activation of BR signaling. Additional support for our conclusion came from our observations that both the dark- and light-grown seedlings of the triple mutants are morphologically similar to the dark- and light-grown wild type seedlings grown in the presence of 1 μM BL, respectively (Fig. 6B and 6C).

The bin2-3bil1bil2 Triple Mutant Still Responds to BR
The fact that the Arabidopsis genome encodes 7 other GSK3-like kinases raises an interesting question as whether or not the BR responsiveness of the bin2-3bil1bil2 triple mutant is saturated. To answer this question, we used the BR-induced root growth inhibition assay to test if the triple mutant still exhibits a BR response. As shown in Figure 5A, roots of the triple mutant are much shorter than those of wild type, bin2-3, or bil1bil2 mutants without BR treatment but could be further inhibited by increasing concentrations of BL, suggesting that other GSK3 kinases might also be involved in BR signaling. Alternatively, the BR-elicited further inhibition of root growth of the bin2-3bil1bil2 triple mutant might be mediated by a GSK3-independent BR signaling pathway.

As mentioned before, our immunoblotting analysis revealed that the triple mutant accumulated not only the hypophosphorylated form but also the hyperphosphorylated form of BES1 (Fig. 5C). This observation contradicts our prediction that elimination of BIN2, BIL1, and BIL2 should significantly reduce rather than increase BES1 phosphorylation. This apparent contradiction implies that other GSK3-like kinases are able to compensate for the loss of BIN2, BIL1, and BIL2 in the triple mutant by
phosphorylating BES1 in a manner that does not lead to degradation of the phosphorylated BES1. Alternatively, the increased amount of phosphorylated BES1 could be due to increased production of the BES1 protein coupled with constant rate of phosphorylation.

In agreement with the root growth inhibition result, hyperphosphorylated BES1 disappeared while the amount of hypophosphorylated BES1 was further increased when the triple mutant was treated with BL (Fig. 5C, lanes 7 and 8). This result indicated that the kinase(s) that phosphorylate BES1 in the bin2-3bil1bil2 triple mutant could also be inhibited by a BR-activated BIN2 regulatory mechanism, thus supporting our hypothesis that BES1 in the triple mutant is likely phosphorylated by other AtSKs.

**Li⁺ Treatment Leads to Complete Dephosphorylation of BES1**

To further test whether the BES1-phosphorylation activity in the triple mutant is contributed by other Arabidopsis GSK3-like kinases, we conducted a series of pharmacological experiments using a well-studied GSK3 kinase inhibitor, lithium (Klein and Melton, 1996; Stambolic et al., 1996), which was capable of inhibiting both *in vitro* and *in vivo* phosphorylation of BES1 and BZR1 by BIN2 (Zhao et al., 2002; Peng et al., 2008). Growth of bin2-1 mutants on synthetic medium containing 10 mM LiCl rescued their short hypocotyl phenotype in the dark and short petiole defect in the light, despite its failure to suppress their leaf phenotype (Fig. 7A and 7B). Consistently, treatment with 100 mM LiCl even for 10 min resulted in complete disappearance of hyperphosphorylated BES1 accompanied by accumulation of hypophosphorylated BES1 (Fig. 7C) and significant inhibition of the CPD gene expression (Fig. 7E), whereas a similar treatment with 100 mM KCl had little effect on the BES1 phosphorylation status (Fig. 7D).

To eliminate the possibility that the inhibitory effect of Li⁺ on the BES1 phosphorylation activity is mediated by another well-known Li⁺ target, inositol monophosphatase (Phiel and Klein, 2001), we treated Ws wild-type seedlings with a widely used inositol monophosphatase inhibitor L690330 (Atack et al., 1993). As revealed by Figure 7F, L690330 had no measurable effect on the *in vivo* BES1 phosphorylation activity. We thus concluded that Li⁺ is quite effective to inhibit GSK3 kinases and constitutively activates BR signaling in Arabidopsis. As shown in Figure 7G, treatment of the bin2-
3bil1bil2 triple mutant with either 1 μM BL or 100 mM LiCl for 1 hr completely eliminated hyperphosphorylated BES1 with a significant increase in the abundance of hypophosphorylated BES1. Taken together, these results strongly suggested that the BES1 protein in the bin2-3bil1bil2 triple mutant is phosphorylated by other GSK3 kinases that can also be inhibited by a BR-activated regulatory mechanism.

**Li⁺ treatment failed to suppress a strong bri1 mutation**

It was previously thought that BIN2 inhibition is the sole signaling output of the BR-activated BRI1 receptor complex (Vert and Chory, 2006). Our discovery that Li⁺ treatment was able to completely block the BES1-phosphorylation activity provided us a great opportunity to directly test this idea. We thus germinated and grew bri1-101 seedlings, carrying a mutated BR receptor with an inactive serine/threonine kinase activity (Nam and Li, 2004), on synthetic medium containing 10 mM LiCl under both dark and light growth condition. To our surprise, the Li⁺ treatment only slightly enlarged the rosettes of light-grown bri1-101 seedlings (Fig. 8A) and slightly elongated the hypocotyls of dark-grown bri1-101 seedlings (Fig. 8B). The two most obvious morphological alterations, elongated petioles in the light and twisted hypocotyls in the dark that are associated with constitutive activation of BR signaling (Fig. 6B and 6C), were not observed on Li⁺-treated bri1-101 seedlings. The different morphological responses of bin2-1 and bri1-101 mutants to the Li⁺ treatment thus suggested that there is a BIN2-independent regulatory mechanism downstream of BRI1 activation that is crucial for BR-regulated plant growth.

**Discussion**

**Functional Redundancy between BIN2 and Its Two Closest Homolog with BIN2 Playing a Major Role in BR Signaling**

In this study, we have identified several loss-of-function alleles of the Arabidopsis BIN2 gene, including a T-DNA insertional allele and 8 intragenic suppressors of the previously characterized weak ucu1-3 mutation (Perez-Perez et al., 2002). The lack of observable morphological phenotypes in any loss-of-function bin2 mutants suggested functional redundancy between BIN2 and other AtSKs. Using T-DNA insertional mutants of BIN2 and its two closest homologs in the same Ws ecotype, we showed that simultaneous elimination of all three group II AtSKs constitutively activate BR signaling, as revealed by
morphological alterations, changes in \textit{CPD} and \textit{SAUR-AC1} expression, and accumulation of hypophosphorylated BES1. These results support the earlier claim that \textit{BIN2} functions redundantly with its two closest homologs in regulating BR signaling (Vert and Chory, 2006).

Our careful genetic, biochemical and molecular analysis of several BR signaling outputs allowed us to conclude that \textit{BIN2} plays a dominant role among the three group II \textit{GSK3}-like kinases despite their functional redundancy. First, while 8 gain-of-function \textit{bin2} mutants were discovered as BR-insensitive dwarf mutants (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), no single gain-of-function mutant for \textit{BIL1} or \textit{BIL2} or any other AtSK has been isolated so far that exhibits the BR-insensitive dwarf phenotype. Second, loss of \textit{BIN2} function alone was able to partially suppress the \textit{bri1-5} mutation and results in detectable decrease in BES1 phosphorylation, whereas single or even double mutations of \textit{bil1} and \textit{bil2} had little effect on the weak \textit{bri1-5} mutant or BES1 phosphorylation. Third, almost all transgenic plants overexpressing \textit{gbin2-1(E263K)} exhibit the strong \textit{bin2-1}-like phenotype whereas only approximately one third of \textit{gbil1(E295K)} or \textit{gBIL2(E293K)} transgenic plants are morphologically similar to \textit{bin2-1} mutants. Similarly, ~10% of transgenic plants overexpressing the wild-type \textit{BIN2} transgene exhibit \textit{bin2}-like phenotype (Li and Nam, 2002), no single transgenic plant expressing wild type \textit{BIL1} or \textit{BIL2} gene out of >150 independent transgenic T1 lines (per transgene) was morphologically similar to \textit{bin2} mutant. The EK mutation in the highly conserved TREE motif is thought to prevent the BR-mediated \textit{BIN2} inhibition (Peng and Li, 2003), resulting in increased \textit{BIN2} stability and/or its nuclear accumulation (Vert and Chory, 2006; Peng et al., 2008), whereas overaccumulated \textit{BIN2} can saturate the BR-activated \textit{BIN2} regulatory machinery, leading to accumulation of constitutively-active \textit{GSK3} kinases in plant cells that inhibit BR signaling. One would predict that the corresponding EK mutation in either \textit{BIL1} or \textit{BIL2} or overexpression of the two wild type \textit{BIL} genes would produce a similar morphological effect if both \textit{BILs} play a significant role in regulating BR signaling. The failure to identify a single \textit{bin2}-like \textit{gBIL1} or \textit{gBIL2} transgenic plant could be due to different expression profiles of the three group II AtSKs. However, a recent gene expression profiling study (Charrier et al., 2002) and a web-based analysis of existing microarray data (Supplemental Fig. S1) showed similar expression profiles between \textit{BIN2} and the two \textit{BILs}. One may argue that differential protein abundance of the three related \textit{GSK3s} might be responsible for their differential
effect on BR signaling. Consistent with this argument, two recent studies demonstrated that both BIN2 and a *Medicago* GSK3 kinase are regulated by proteasome-mediated protein degradation (Wrzaczek et al., 2007; Peng et al., 2008). Alternatively, the differential effect of the three group II AtSKs could be attributed to their primary sequence. Both BIL1 and BIL2 contain longer N-terminal ends (Fig. 5) that could affect their subcellular localizations, thus affecting their interactions with yet to be identified BIN2 regulatory proteins.

**Involvement of Other GSK3-Like Kinase in Regulating BR Signaling**

Our discovery that simultaneous elimination of BIN2, BIL1, and BIL2 did not completely eliminate but instead increased the phosphorylated BES1 suggested that BES1 can be phosphorylated *in vivo* by kinases other than the three group II AtSKs. In this study, we provided strong circumstantial evidence to suggest that the BES1-phosphorylating activity in the triple mutant is likely due to other AtSKs. Our conclusion was based on the following reasoning. First, the BES1-phosphorylation activity in the triple mutant could be completely eliminated by treatment of BL, suggesting that the BR-activated BIN2 regulatory machinery can inhibit other BES1-phosphorylating kinases. Second, the TREE motif and the Pro284 residue critical for BIN2 regulation are almost absolutely conserved among the 10 AtSKs except AtSK3-1 that carries an Ala residue at the position of Thr (Supplemental Fig. S2), suggesting that six other AtSKs could interact with the BIN2 regulatory mechanism. Third, despite its resemblance to BR mutants or transgenic plants with constitutively-activated BR signaling, the *bin2-3bil1bil2* triple mutant still responds to BR in a dose-dependent manner, arguing strongly for functional redundancy between the group II AtSKs and other AtSKs. Forth, we discovered that the BES1-phosphorylation activity in the triple mutants could be completely inhibited by treatment with Li+ known to inhibit *in vitro* and *in vivo* BIN2-catalyzed phosphorylation of BES1/BZR1 (Zhao et al., 2002; Peng et al., 2008). In addition, LiCl treatment resulted in phenotypic suppression of the *bin2-1* mutant. Although we can not rule out the possibility that some non-GSK3-like kinases, which can be inhibited by both BL and Li+, are responsible for phosphorylating BES1 in the *bin2-3bil1bil2* triple mutant, the data presented here strongly suggested involvement of other AtSKs in BR signaling.

**The existence of a possible BIN2-independent regulatory mechanism**
Our study made a surprising discovery that Li⁺ treatment could suppress the gain-of-function bin2-1 mutation but had little effect on a strong BR receptor mutant bri1-101. The two most obvious morphological changes, twisted hypocotyls of dark-grown seedlings and elongated petioles of light-grown seedlings, which are associated with constitutive activation of BR signaling, were not observed on Li⁺-treated bri1-101 seedlings. These observations suggested that BR perception by BRI1 activates a BIN2-independent regulatory mechanism that function together with BIN2 inhibition to regulate plant growth. Elimination of the BES1/BZR1-phosphorylation activity by Li⁺ without activation of such a BIN2-independent regulatory system is not able to fully activate BR signaling. It is quite possible that BR signaling behaves similar to the signaling process of animal steroid hormones involving both genomic and non-genomic effects. The genomic effect of BR signaling is mediated by BES1 and BZR1, which are regulated at several different levels by BIN2-catalyzed protein phosphorylation while the non-genomic effects might be mediated by known BRI1 substrates such as TTL, an Arabidopsis protein similar to vertebrate transthyretin-like proteins (Nam and Li, 2004), TRIP-1, an essential component of the translational initiation complex (Ehsan et al., 2005), or BSKs, a family of related protein kinases that are phosphorylated by BRI1 (Tang et al., 2008). It might also be possible that the activated BRI1 could regulate the proton-pumping activity of a plasma membrane or vacuolar ATPase, which is known to be critical for generating turgor pressure necessary for plant cell growth, or affect reorganization of cortical microtubules (Peng and Li, 2003). Alternatively, the BIN2-independent regulatory mechanism might also be involved in gene regulation by inhibiting a transcriptional repressor or activating a transcriptional activator that functions together with BES1/BZR1 to control gene expression. Novel genetic screens and identification of components of a BR-activated BRI1 receptor complex could lead to complete elucidation of the suspected BIN2-independent regulatory system.

Material and Methods

Plant Material and Growth Conditions
Columbia (Col-0), Landsberg erecta (Ler) and Wassilewskija (Ws-2) ecotypes were used as wild type control for phenotype comparison. Ws-2 was also used as the control in seedling measurement as well as in protein and RNA analysis. Col-0 was used to
generate all the transgenic plants. BR mutants bri1-5 (in Ws-2; Choe et al., 2000), ucu1-3 (in Ler, (Perez-Perez et al., 2002), and all other mutants and transgenic plants were germinated on half strength Murashige and Skoog’s (MS) medium, transferred into soil for continued growth at 22°C under a 16-hr-light/8-hr-dark photoperiod condition. Seed sterilization and seedling handling were carried out as previously described (Li et al., 2001).

Mutant Isolation

For isolation of loss-of-function bin2 mutants, seeds of the Arabidopsis ucu1-3 mutant (Perez-Perez et al., 2002) were treated with 0.3% ethyl methanesulfonate (Sigma-Aldrich) as described (Yin et al., 2002). About 200,000 M2 seeds derived from 20,000 M1 plants were screened on half strength MS medium. After 4-week growth in a growth chamber with a 16h-light/8h-dark photoperiod condition, putative suppressors were transferred into soil for continued growth, phenotypic analysis, and seed collection. Interesting suppressors were genotyped with the ucu13 dCAPS makers (Supplemental Table S1) to eliminate pollen/seed contaminants. The bin2-3 mutant (FLAG_593C09, Ws ecotype) was PCR screened and isolated from the FLAGdb Collection at the Versailles Genetics and Plant Biology Laboratory, Institut National de la Recherche Agronomique (http://urgv.evry.inra.fr/projects/FLAGdb++/HTML/index.shtml, (Samson et al., 2002). bil1 (At2g30980) and bil2 (At1g06390) knockout mutants were obtained from the Wisconsin Arabidopsis Gene Knockout Facility at University of Wisconsin, Madison (http://www.biotech.wisc.edu/NewServicesAndResearch/Arabidopsis/). Both mutants are in Ws-2 ecotype.

DNA Isolation and Analysis

Genomic DNAs of putative ucu1-3 suppressors were isolated as previously described (Li and Chory, 1998). The entire BIN2 gene was amplified using the Taq polymerase (Promega) with the BIN2F/R primer set (Supplemental Table S1). The amplified BIN2 genomic DNAs pooled from 4 independent PCR reactions of each putative suppressors was gel purified using the QIAquick gel extraction kit (Qiagen) and sequenced at the University of Michigan DNA sequencing core facility. The resulting BIN2 sequences were compared with those obtained from the parental ucu1-3 mutant to find single nucleotide changes.
Plasmid Construction and Plant Transformation

The gBIN2 genomic construct and its mutated version carrying the bin2-1 mutation were previously described (Li and Nam, 2002). To make gBIL1 and gBIL2 constructs, a 6.5-kb genomic fragment of At2g30980 and a 5.5-kb genomic fragment of At1g06390 gene were released from bacterial artificial chromosomes (BACs) F7F1 and T2D23 by digestion with Pst I and Afl II (followed by partial filling with dTTP) and cloned into the pPZP212 binary vector (Hajdukiewicz et al., 1994) digested with Pst I and EcoR I (followed by partial filling with dATP), respectively. The resulting pPZP212gBIL1 and pPZP212gBIL2 plasmids were subjected to site-directed mutagenesis using the QuickChange® II XL site-directed mutagenesis kit (Stratagene) to generate gBIL1(E295K) and gBIL2(E293K) plasmids. Both the wild type and mutated transgenes of BIN2, BIL1, and BIL2 were individually introduced into Agrobacterium tumefaciens strain GV3101 and transformed into Col-0 wild type Arabidopsis plants via a vacuum infiltration method (Bechtold and Pelletier, 1998).

Production of Fusion Protein and in vitro Phosphorylation Assay.

To express maltose-binding-protein (MBP)-fused BIN2, BIL1, or BIL2 in E. coli, each open-reading-frame of the three Arabidopsis GSK3-like kinases was individually cloned into pMAL-c1 (New England BioLabs). Induction and purification of MBP fusion proteins were carried out according to the manufacturer’s recommended protocol. Expression and purification of the GST-BES1 fusion protein and in vitro phosphorylation assay using the MBP-fused GSK3-like kinases were conducted as previously reported (Zhao et al., 2002).

Hypocotyl and Root Growth Measurement

In hypocotyl growth inhibition assay, seeds were sterilized and grown on half strength MS medium containing increasing concentration of Brz (from 0.1 to 2 μM) or DMSO [0.001% (v/v)] in the dark for 6 days. Etiolated seedlings were carefully removed from agar plates, placed on flat surface, and photographed at a resolution of 600 pixels/inch. Hypocotyl lengths of individual seedlings were measured by Image J (ver. 1.37 of Mac OS X; http://rsb.info.nih.gov/ij/) and the results were exported into Microsoft Excel for statistical analysis. The root growth inhibition assay to analyze the effects of loss-of-function mutations of BIN2, BIL1, and BIL2 on BR sensitivity was carried out as previously described (Li et al., 2001).
Treatment with Chemicals for Protein and RNA Analyses

18-day-old seedlings grown on half strength MS medium were gently removed from Petri dishes and submerged into liquid half strength MS medium containing different concentrations of BL, LiCl, KCl, or L690330 (Tocris, Ellisville, MO). Seedlings were removed at different time points and were either directly collected into 2X SDS buffer (200 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 0.2 M dithiothreitol) or immediately frozen in liquid nitrogen and stored in –80°C freezer for later analysis. To analyze the effect of Li+ on plant morphology, seeds of the bin2/+ heterozygous plants were germinated and grown on half strength MS medium with or without 10 mM LiCl at 22°C in a growth chamber under a 16h-light/8h-dark photoperiod condition. Individual seedlings were photographed for phenotype comparison and analyzed by PCR using the bin2-1 dCAPS primer set (Supplemental Table S1) to determine their genotypes.

Protein and RNA Analysis

Freshly collected or frozen samples were ground thoroughly in 2X SDS sample buffer using plastic pestle and boiled at 100°C for 5 min. After 5 min centrifugation to get rid of insoluble tissue debris, soluble proteins were separated by a 12% SDS-PAGE gel, transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore), and analyzed by western blot with an anti-BES1 antibody (Mora-Garcia et al., 2004). The signals on the Immobilon-P membrane were detected using the horseradish peroxidase-conjugated goat-anti-rat-IgG secondary antibody (Abcam) and the Supersignal West Pico chemiluminescent substrate system (Pierce).

Total RNAs of freshly collected or frozen samples were extracted using the RNeasy Plant Mini kit (Qiagen). The resulting RNAs were separated electrophoresis, transferred to a nylon membrane, and hybridized with a 32P-labeled CPD probe as previously described (Li et al., 2001). For RT-PCR assays, 2-5 μg of total RNAs were converted using SuperScript® III First-Strand cDNA synthesis kit (Invitrogen) into single strand cDNA templates, which were used to amplify specific transcripts with gene-specific primer sets (see Supplemental Table S1). Northern blot autoradiographs and gel pictures of RT-PCR analysis were analyzed by Image J (ver. 1.37 of Mac OS X; http://rsbweb.nih.gov/ij/) to determine relative gene expression levels.
Sequence data from this article can be found in the GenBank database under the following accession numbers: BIN2, At4g18710, NP_193606; BIL1, At2g30980, NP_180655; BIL2, At1g06390, NP_973771.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. BIN2 and its two homologs display similar expression profiles.
Supplemental Figure S2. Conservation of amino acids critical for BR-mediated inhibition of GSK3-like kinase activity.
Supplemental Table S1. Primers used for genotyping, sequencing, and RT-PCR analysis.

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Figure Legends

Figure 1. Identification of bin2 Loss-of-Function Mutants.
A, ucu1-3 exhibits a weak bin2 phenotype. Shown here are 3-weeks-old wild type and the ucu1-3 mutant (both are in ecotype Ler) grown on half strength MS medium in the light.
B, ucu1-3 is a weak gain-of-function bin2 mutation. Shown here in the left is a 5-week-old wild type plant and in the right is a same age transgenic plant that expresses a mutated genomic gbin2 construct carrying the ucu1-3 mutation and displays the strong bin2-like phenotype (both are in Col-0 ecotype).
C, Phenotypic comparison between a 4-week-old wild type plant and a 4-week-old T-DNA insertional bin2 mutant (both in Ws ecotype).
D, Screening for ucu1-3 suppressors identified 8 bin2 loss-of-function mutations. Shown here from left to right in the top row are a Ler wild type plant, ucu1-3, and bin2-4 to bin2-6 mutants and from left to right in the bottom row are bin2-7 to bin2-11 mutants (all grown in soil for 4 weeks).
E, Molecular nature of 8 newly discovered bin2 alleles. Black bars represent protein-coding exon; grey bars indicate non-coding regions, while the thin line denotes introns. Arrows show the positions of the 8 single-nucleotide bin2 mutations, while ATG and TAA denote the start and stop codons, respectively.

Figure 2. Loss-of-BIN2-Function Stimulates BR Signaling.
A. The T-DNA insertional bin2-3 mutation partially suppresses the bri1-5 mutation. Shown from left to right are 4-week-old soil-grown plants of bri1-5, bin2-3bri1-5 and the Ws wild-type control.
B. The root lengths of two-week-old seedlings of wild type and bin2-3 grown on half-strength MS medium containing no or increasing concentrations of BL.
C. Six-day-old etiolated seedlings of the Ws wild type and bin2-3 grown on medium containing no or 2 μM Brz.
D. The hypocotyl elongation of the wild type and bin2-3 seedlings grown on medium containing different concentrations of Brz relative to seedlings of the same genotype grown on regular medium (see Materials and Methods for details). For both B and D, each data point represents average of 60 seedlings of two duplicate experiments. Error bars represent standard error.
E. Western blot analysis of BES1 phosphorylation status. 18-day-old seedlings were treated with 1 μM BL or mock solution for 1 h. Total proteins were extracted and analyzed by western blotting with anti-BES1 antibody. BES1-P is the phosphorylated BES1 and * indicates a non-specific band for loading control.

F. RT-PCR and northern blot analyses of SAUR-AC1, DWF1 and CPD gene expression in 18-day-old seedlings treated with or without 1 μM BL. See “Materials and Methods” for experimental details. β-Tubulin was used as a control for RT-PCR analysis of both SAUR-AC1 and DWF4 while ethidium bromide staining of rRNAs serves as a loading control for northern blot analysis of CPD.

**Figure 3.** Both BIL1 and BIL2 Are Capable of Blocking BR Signaling.
A. Sequence alignment of BIN2 and BIL1 and BIL2. The sequence alignment was done by the Mcoffee program at http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi and identical/similar amino acids are shaded with black/grey background using the BoxShade Server at http://www.ch.embnet.org/software/BOX_form.html. Eight gain-of-function bin2/ucu1/dwf12 mutations are indicated by black triangles and the conserved TREE motif is boxed with red line.

B. Both BIL1 and BIL2 are able to phosphorylate BES1 in vitro. The top panel is the Coomassie Blue staining of purified MBP-AtSKs and GST-BES1 fusion proteins while the low panel is an autoradiograph of 32P-labelled AtSKs and GST-BES1.

C. Both BIL1 and BIL2, when carrying a mutation equivalent to bin2-1, can inhibit BR signaling. Shown here clockwise are transgenic plants expressing a wild type gBIN2 genomic construct, a mutated gbin2-1 transgene carrying the bin2-1 mutation, a genomic gBIL2 transgene with an E293K mutation, and a genomic gBIL1 construct with a E295K mutation, respectively. The percentage number at the left bottom corner in each panel indicates the percentage of T1 transgenic plants (out of >150 lines) exhibiting the displayed morphology.

**Figure 4.** Genetic Analysis of T-DNA Insertional Mutations of BIN2 and Its Two Closest Homologs.
A. Schematic representation of T-DNA insertions in the BIL1 and BIL2 genes. Black bar represents protein-encoding exon, grey bars denotes untranslated regions, while the thick line indicates introns. Arrows indicate orientations of T-DNA insertions from its left to right boarder and the texts above or below the arrows show the junction between
flanking genomic DNAs and the inserted T-DNAs. The color arrows represent the primers used for RT-PCR analysis of BIL1 or BIL2 gene expression.

B, RT-PCR analysis of BIL1 and BIL2 gene expression in wild type, bil1, and bil2 mutants. β-Tubulin was used as a control.

C, Shown here from left to right are soil-grown 4-week-old Ws wild type plant, bin2-3, bil1, bil2, bil1bil2, and two individual bin2-3bil1bil2 mutants.

D, Genetic analysis of bri1-5 with loss-of-function mutations of BIN2, BIL1, and BIL2. Shown here from left to right are 4 week-old soil-grown bri1-5, bin2-3bri1-5, bil1bri1-5, bil2bri1-5, bil1bil2bri1-5, bin2-3bil1bri1-5, bin2-3bil2bri1-5, bin2-3bil1bil2bri1-5, and the Ws wild type control.

**Figure 5.** Physiological, Biochemical, and Molecular Analyses of the T-DNA Insertional Mutations of BIN2, BIL1, and BIL2.

A, The average root lengths of 2-week-old seedlings of the Ws wild type, bin2-3, bil1bil2, and bin2-3bil1bil2 mutants grown on half-strength MS medium containing no or increasing concentrations of BL.

B, The hypocotyl elongation of wild-type, bin2-3, bil1bil2, and bin2-3bil1bil2 mutants grown on medium containing no or increasing concentrations of Brz. Inhibition of hypocotyl elongation by Brz is expressed relative to the average hypocotyl length of the same genotype grown on the regular half strength MS medium. For both A and B, each data point represents average of 60 seedlings of two duplicate experiments. Error bars represent standard error.

C, Western blot analysis of BES1 phosphorylation status in BL or mock treated seedlings. Total proteins were extracted with 2 X SDS buffer, separated by 10% SDS-PAGE, and analyzed by western blotting with anti-BES1 antibody. BES1-P is the phosphorylated BES1, and * indicates a non-specific band used for loading control.

D, RT-PCR analysis of SAUR-AC1 expression. The numbers on the right represent numbers of PCR cycles. RT-PCR gel pictures were analyzed by Image J (ver 1.3.7) to determine signal intensity of individual bands. The expression level of SAUR-AC1 in the bar graph was expressed as percentage of the ratio of the SAUR-AC1 transcript abundance vs. the β-Tubulin transcript level relative to that of the mock-treated Ws wild type control. Each data represents the average of three independent RT-PCR experiments, and error bars denote standard errors.
E, Northern blot analysis of *CPD* gene expression. Ethidium bromide stained rRNAs were used as loading control. The autoradiograph was photographed and analyzed by Image J (ver. 1.3.7; http://rsb.info.nih.gov/ij/). The signal intensity of each *CPD* band in the bar graph below is expressed as percentage relative to that of mock-treated Ws wild type seedlings.

**Figure 6.** Simultaneous Elimination of BIN2, BIL1, and BIL2 Results in Constitutive Activation of BR Signaling.
A, Phenotypic comparison between the *bin2-3bil1bil2* triple mutant and with a transgenic line expressing a stabilized BZR1 protein grown in soil for 6 weeks.
B, The etiolated 6-day-old *bin2-3bil1bil2* triple seedlings are morphologically similar to etiolated wild type Ws seedlings grown on half-strength MS medium containing 1 μM BL.
C, The light-grown 12-day old seedlings of Ws and *bin2-3bil1bil2* mutant grown on half strength MS medium supplemented with no or 1 μM BL.

**Figure 7.** The BES1-Phosphorylation Activity in the *bin2-3bil1bil2* Mutant Is Likely Catalyzed by Other GSK3s.
A, Li⁺ treatment rescued the short hypocotyl phenotype of etiolated *bin2-1* seedlings. Shown here are 5 *bin2-1* seedlings grown in the dark on half-strength MS medium supplemented with or without 10 mM LiCl.
B, Li⁺ treatment rescued the petiole length defect of the *bin2-1* mutation in the light. Phenotypic comparison between 2-week-old *bin2-1* mutants grown in the light on half-strength MS medium supplemented with or without 10 mM LiCl.
C, Complete elimination of phosphorylated BES1 by treatment with 100 mM LiCl.
D, Treatment of 100 mM KCl had no effect on the BES1 phosphorylation status.
E, Li⁺ treatment led to rapid inhibition of *CPD* gene expression. Northern blot analysis of *CPD* expression in Ws wild-type seedlings treated with 100 mM LiCl for 0, 10, 30, or 60 min. The upper panel is an autoradiograph of ³²P-labelled *CPD* hybridization while the lower panel is ethidium bromide stained RNA gel picture showing the amounts of rRNAs of each sample for control of equal loading of total RNAs.
F, Treatment with an inositol monophosphatase inhibitor L690330 had no effect on the *in vivo* BES1 phosphorylation activity.
G, The BES1-phosphorylation activity in the *bin2bil1bil2* mutant can be completely inhibited by treatment with BL or Li⁺.
For C, D, F, and G, total protein crude extracts from treated seedlings were separated by 10% SDS-PAGE and analyzed by immunoblotting with an anti-BES1 antibody. The arrows indicates positions of phosphorylated BES1 (BES1-P) and non-phosphorylated BES1 (BES1) on western blots, while * denotes non-specific bands used for loading control.

Figure 8. LiCl treatment failed to suppress a strong BR receptor mutation.
A. Shown here are 3-week-old bri1-101 seedlings grown in the light on half-strength MS medium supplemented with or without 10 mM LiCl.
B. Shown here are 7-day-old bri1-101 seedlings grown in the dark on half-strength MS medium with or without 10 mM LiCl.
A

...ttaccggt/TDNA

BIL1

ATG

BIL2

ATG

...ttggcaca/TDNA

TAG

TAG

B

Ws  bil1  bil2

BIL1

BIL2

Tubulin

C

Ws  bin2-3  bil1  bil2  bil1bil2  bin2-3bil1bil2.a  bin2-3bil1bil2.b

D

bri1-5  bin2-3bri1-5  bil1bri1-5  bil2bri1-5  bil1bil2bri1-5  bin2-3bili1bri1-5  bin2-3bili2bri1-5  bin2-3bili1bili2bri1-5  Ws
A  LIGHT  

10 mM Li+  1/2 MS  

bri1-101

B  DARK  

10 mM Li+  1/2 MS  

bri1-101