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Sequential Transphosphorylation of the BRI1/BAK1 Receptor Kinase Complex Impacts Early Events in Brassinosteroid Signaling

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SUMMARY

Brassinosteroids (BRs) regulate plant development through a signal transduction pathway involving the BRI1 and BAK1 transmembrane receptor kinases. The detailed molecular mechanisms of phosphorylation, kinase activation, and oligomerization of the BRI1/BAK1 complex in response to BRs are uncertain. We demonstrate that BR-dependent activation of BRI1 precedes association with BAK1 in planta, and that BRI1 positively regulates BAK1 phosphorylation levels in vivo. BRI1 transphosphorylates BAK1 in vitro on specific kinase-domain residues critical for BAK1 function. BAK1 also transphosphorylates BRI1, thereby quantitatively increasing BRI1 kinase activity toward a specific substrate. We propose a sequential transphosphorylation model in which BRI1 controls signaling specificity by direct BR binding followed by substrate phosphorylation. The coreceptor BAK1 is then activated by BRI1-dependent transphosphorylation and subsequently enhances signaling output through reciprocal BRI1 transphosphorylation. This model suggests both conservation and distinct differences between the molecular mechanisms regulating phosphorylation-dependent kinase activation in plant and animal receptor kinases.

INTRODUCTION

Brassinolide (BL) is the most biologically active member of a family of plant steroid hormones termed brassinosteroids (BRs), which are essential regulators of multiple aspects of plant growth and development (Clouse and Sasse, 1998). BL initiates a cascade of cellular events by binding to the extracellular domain of the BRASSINOSTEROID-INSENSTIVE 1 (BRI1) receptor kinase, which leads to phosphorylation and activation of the BRI1 cytoplasmic kinase domain (KD) and transduction of the signal via an intracellular kinase to the nucleus, where several novel BR-responsive transcription factors alter the expression of genes promoting cell elongation, division, and differentiation (Li and Nam, 2002; He et al., 2005; Kinoshita et al., 2005; Yin et al., 2005; Vert and Chory, 2006). Mutational analysis in *Arabidopsis thaliana* and several crop plants has shown that lesions in either the extracellular or cytoplasmic domains (CDs) of BRI1 can lead to severe developmental defects, including extreme dwarfism, altered leaf and vascular morphology, delayed senescence and flowering, and male infertility (Clouse et al., 1996; Li and Chory, 1997; Yamamuro et al., 2000; Montoya et al., 2002). These genetic data confirm the importance of BRI1-mediated BR signaling in normal plant development and have prompted intensive research on BRI1 function. However, the precise biochemical mechanism by which BRI1 activates this pathway in response to BL binding remains unclear (Gendron and Wang, 2007).

BRI1 belongs to the very large family of *l*eucine-*r*ich *r*epeat *r*eceptor-*l*ike *k*inases (LRR RLKs) found in plants that includes over 220 members in *Arabidopsis* and nearly 400 in rice (Shiu et al., 2004). LRR RLKs have an organization of functional domains similar to that of mammalian receptor tyrosine kinases (RTKs) and transforming growth factor-β (TGF-β) serine/threonine receptor kinases, including an extracellular domain potentially involved in ligand binding and receptor oligomerization, a single-pass transmembrane sequence, and a CD consisting of a catalytic KD and flanking regulatory sequences—the juxtamembrane (JM) region and the carboxy-terminal (CT) domain. The well-established paradigm for mammalian receptor kinase action begins with ligand-induced oligomerization, which promotes CD activation by autophosphorylation, followed by the generation of phosphorylation-dependent interaction motifs for binding specific downstream substrates (Massague, 1998; Schlessinger, 2002; Rahimi and Leof, 2007). Receptor kinase phosphorylation occurs on multiple sites in the KD, JM, and CT regions (Huse and Kuriyan, 2002). Phosphorylation of 1–3 residues in the conserved activation loop of kinase subdomains VII/VIII is a common mechanism of general kinase activation and, in some cases, also affects binding of kinase substrates (Adams, 2003). Phosphorylation of JM and CT regions, which show more sequence diversity among receptor kinases than the activation loop, generates docking sites for specific kinase

substrates (Pawson, 2004) and may also lead to general kinase activation by a variety of mechanisms (Pawson, 2002).

BRI1 appears to share some of the same general mechanistic features of kinase activation and function seen in mammalian receptor kinases. The extracellular domain of BRI1 consists of 25 tandem LRRs with a 70 amino acid island imbedded between LRR 21 and 22. The island domain in conjunction with LRR 22 directly binds BL, defining a new steroid binding motif (Kinoshita et al., 2005). We previously identified at least 11 sites of in vivo phosphorylation in the JM, KD, and CT domains of BRI1 by immunoprecipitation from BL-treated *Arabidopsis* seedlings followed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis and also found that BRI1 phosphorylation in planta on many of these residues was BL-dependent (Wang et al., 2005b). Moreover, BRI1 KD autophosphorylation sites identified in vitro (Oh et al., 2000) were highly predictive of in vivo phosphorylation. Functional characterization of each identified site by biochemical and genetic analyses showed that the highly conserved activation-loop residues, S1044 and T1049, were critical for kinase function in vitro and proper BR signaling in planta, while multiple JM and CT residues were required for optimal substrate phosphorylation by the BRI1 KD (Wang et al., 2005b). An autoinhibitory role of the CT region of BRI1 has also been reported (Wang et al., 2005a).

BRI1 can exist in plant membranes as a ligand-independent homodimer that is stabilized and activated by BL binding (Russinova et al., 2004; Wang et al., 2005a; Hink et al., 2008). However, full expression of BR signaling requires the association of BRI1 with other LRR RLKs. The SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) subfamily of LRR RLKs contains five closely related members (Hecht et al., 2001) that have small extracellular domains consisting of only five LRRs that lack the island domain of BRI1 and thus cannot bind BL directly. SERK3, also known as BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), interacts both in vitro and in vivo with BRI1 (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004), and we showed that this association was promoted by BR, as was in vivo phosphorylation of both LRR RLKs (Wang et al., 2005b). Moreover, overexpression of a K317E kinase-inactive mutant form of *BAK1* in *bri1-5* (a weak allele of *bri1*) led to a severe dwarf phenotype resembling *bri1* null mutant alleles, suggesting a dominant-negative effect arising from the disruption of a complex between BRI1 and BAK1 (Li et al., 2002). The *bak1-4* null allele shows only partial BR insensitivity and a weak *bri1*-like phenotype, indicating possible functional redundancy with other SERK-family members. SERK4, alternatively named BAK1-LIKE (BKK1), also interacts with BRI1 in vivo in a BL-dependent manner, and overexpression of kinase-inactive BKK1 in *bri1-5* leads to a dominant-negative effect similar to that observed with BAK1 (He et al., 2007). Thus, BAK1 and BKK1 functionally overlap in BR signaling. Surprisingly, the *bak1 bkk1* double mutant does not exhibit a strong *bri1*-like dwarf phenotype with altered leaf morphology, but, instead, it shows a novel seedling-lethal dwarfism. Biochemical and genetic analyses support a positive regulatory role for both BAK1 and BKK1 in BR signaling and a negative role for these two LRR RLKs in a currently undefined BR-independent cell-death pathway (He et al., 2007; Kemmerling et al., 2007). The seedling lethality of the *bak1 bkk1* double mutant is attributed to derepression of this cell-death pathway. Moreover, BAK1 heterodimerizes with another LRR RLK known to bind a peptide derivative of bacterial flagellin, FLAGELLIN-SENSITIVE2 (FLS2), and promotes its function in plant defense responses (Chinchilla et al., 2007; Heese et al., 2007). Thus, BAK1 functions in independent pathways by enhancing the signaling output of distinct LRR RLKs that bind different ligands. Finally, SERK1, known to be involved in embryogenesis, also physically interacts with BRI1 and enhances BR signaling (Karlova et al., 2006), suggesting that SERKs, in general, are coreceptors that regulate multiple independent pathways by heterodimerization or oligomerization with different LRR RLKs.

Although a great deal has been discovered regarding BR signaling during the past decade, a major gap in our understanding lies in the early molecular events that regulate BL-dependent BRI1/BAK1 asssociation and phosphorylation. In this study, we identify specific sites of in vivo phosphorylation of BAK1 and provide genetic and biochemical evidence for a sequential transphosphorylation mechanism for BRI1 and BAK1 activation that shares some similarities with, but also unique differences from, mammalian RTK and TGF-β models. Our data are consistent with a model in which BRI1 kinase function is initially activated to a basal level by BL binding to BRI1 in the absence of BAK1. The activated BRI1 then associates with BAK1 and transphosphorylates it on KD residues. BRI1-activated BAK1 subsequently transphosphorylates specific JM and CT residues in BRI1, thereby increasing BRI1 kinase activity and substrate phosphorylation, leading to enhanced BR signaling and increased plant growth.

RESULTS

The Role of Kinase Activity in the BRI1/BAK1 Association

To elucidate the detailed mechanisms of receptor kinase activation in response to BR, we examined the effect of kinase activity on the association and phosphorylation of epitope-tagged BRI1 and BAK1 in various genetic backgrounds. We previously used double transgenic *Arabidopsis* lines expressing both BRI1-Flag and BAK1-GFP in a wild-type background to show that BL strongly promoted coimmunoprecipitation of BRI1-Flag and BAK1-GFP and induced phosphorylation on Thr residues in both (Wang et al., 2005b). Here (Figure 1A), we demonstrate that inactivation of the BRI1-Flag KD by a K911E substitution (Oh et al., 2000) abolishes the BL-dependent increase in the association of BRI1-Flag with BAK1-GFP, suggesting that an active BRI1 kinase strongly enhances oligomerization with BAK1 in vivo. In contrast, when BAK1-GFP kinase activity was eliminated by a K317E substitution (Li et al., 2002), the mutated LRR RLK was still able to associate with wild-type BRI1-Flag in a BL-dependent manner (Figure 1A), although at slightly reduced levels. The extent of BRI1-Flag phosphorylation in planta was clearly affected by the level of BAK1 kinase activity. Overexpression of BAK1-GFP in double transgenic lines increased BRI1-Flag phosphorylation compared to single BRI1-Flag transgenic lines, in which only endogenous BAK1 activity was present. In contrast, overexpression of kinase-inactive BAK1(K317E)-GFP decreased BRI1-Flag phosphorylation below the level of the wild-type BAK1 background, most likely due

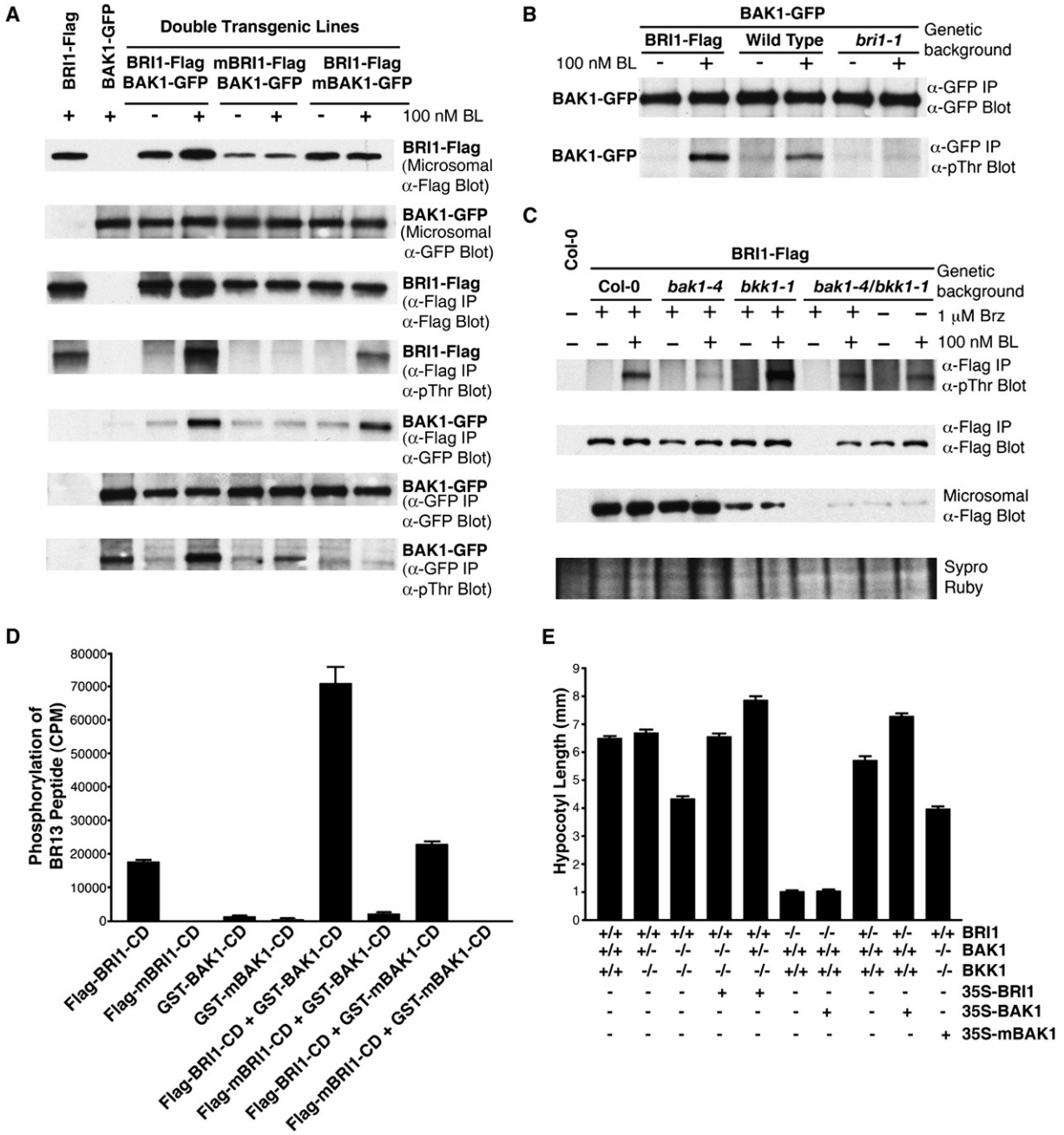

Figure 1. BL-Dependent Association of BRI1 and BAK1 Leads to Enhanced Kinase Activity and Increased BR Signaling

(A) T_3 homozygous transgenic *Arabidopsis* plants expressing both BRI1-Flag and BAK1-GFP were grown with the BR biosynthesis inhibitor Brz (2.0 μM added after 6 days of initial growth) and were then treated with BL or solvent for 90 min at day 11. Total membrane protein was immunoprecipitated and subjected to immunoblot analysis as indicated. Kinase-inactive constructs (mBRI1 = K911E and mBAK1 = K317E) were used to show that BRI1, but not BAK1, kinase activity is essential for BR-dependent in vivo association of the pair, as monitored by coimmunoprecipitation.

(B) BAK1-GFP in vivo phosphorylation is reduced in a *bri1-1* null mutant background and is enhanced in a BRI1-Flag-overexpressing line.

(C) BL-dependent in vivo phosphorylation of BRI1-Flag still occurs in single *bak1-4* and *bkk1-1* null mutants and in the double knockout. Sypro Ruby stain shows total membrane protein loading for the α-Flag microsomal blot. BRI1-Flag levels were reduced in the *bak1-4 bkk1-1* background and were eliminated in the presence of Brz without BL. BRI1-Flag protein levels were normalized after immunoprecipitation for the α-pThr blot, except for *bak1-4 bkk1-1* (+Brz/−BL), which contained no detectable BRI-Flag.

(D) Phosphorylation of the peptide substrate BR13 (GRJKKIASVEJJKK, J = norLeu) by Flag-BRI1-CD (1.0 μM) is increased over three-fold by the addition of GST-BAK1-CD (1.0 μM) and is dependent on an active BAK1 kinase. Error bars are standard error of the mean (SEM); n = 3.

(E) Hypocotyl length in 14-day-old light-grown *Arabidopsis* shows a dependence on BRI1 and BAK1 kinase activities and levels that correlate with (A–C) in vivo and (D) in vitro assays. Error bars are SEM; n = 50. All transgenic plants are T_3 homozygous lines; transgenic protein levels were verified by immunoblot analysis (data not shown). +/+, wild-type homozyogous; +/−, wild-type heterozygous; −/−, homozygous mutant; 35S, CaMV strong constitutive promoter. Transgenic constructs are the same as in (A).

to a dominant-negative effect resulting from the inactive BAK1(K317E)-GFP replacing wild-type endogenous BAK1 in a complex with BRI1-Flag. A similar dependence of phosphorylation levels of BAK1-GFP on the level of BRI1 kinase activity was also observed (Figure 1A).

The low baseline level of BAK1-GFP phosphorylation observed in mBRI1-Flag transgenics (Figure 1A) is likely due to the association of BAK1-GFP with endogenous BRI1. Therefore, to further investigate the role of BRI1 kinase activity on BAK1 phosphorylation levels in vivo, BAK1-GFP was expressed in a *bri1-1* null mutant that completely lacks BRI1 activity due to a point mutation in the KD. Figure 1B shows that BAK1-GFP in vivo phosphorylation is enhanced in a BRI1-Flag-overexpressing line and is reduced to an almost undetectable level in the *bri1-1* mutant background. Thus, BRI1 kinase activity is essential for BL-dependent BAK1 phosphorylation in vivo. The reciprocal experiment evaluating the role of BAK1 kinase activity on BRI1 phosphorylation levels in vivo is complicated by the functional redundancy of BAK1 and BKK1. As seen in Figure 1C, when monitoring equal amounts of immunoprecipitated protein, expression of BRI1-Flag in the null mutant allele *bak1-4* results in substantially reduced BL-dependent phosphorylation compared to expression in wild-type Col-0, which correlates well with the previously reported partial BR-insensitive phenotype of *bak1-4* (He et al., 2007). However, no such reduction in BRI1-Flag phosphorylation is seen after expression in the *bkk1-1* null mutant, which, again, correlates with the lack of observable developmental defects reported for the *bkk1-1* single mutant (He et al., 2007). Also, as previously reported, *bak1-4 bkk1-1* double mutants show an extreme phenotype, including seedling lethality, that is distinct from the *bri1-1* null allele (He et al., 2007). Therefore, it is significant that expression of BRI1-Flag in the *bak1-4 bkk1-1* double mutant results in BL-dependent phosphorylation of BRI1-Flag to at least the level of the *bak1-4* single mutant, indicating that BRI1 can recognize and respond to BL in the complete absence of both BAK1 and BKK1, although at reduced levels of phosphorylation when compared to wild-type. Interestingly, BRI1-Flag was unstable in the *bak1-4 bkk1-1* background when endogenous BL was reduced by treatment with the BR biosynthesis inhibitor brassinazole (Brz), which was not seen in either single mutant or in the double mutant in the absence of Brz.

Transphosphorylation of BRI1 by BAK1 Enhances BRI1 Kinase Activity in Substrate Phosphorylation Assays

To investigate in greater depth the biochemical mechanisms by which association of BRI1 and BAK1 leads to increased kinase activity of either receptor kinase, the complete CD of BAK1 fused to an N-terminal glutathione S-transferase (GST) sequence, was incubated with the entire CD of BRI1 fused to an N-terminal Flag epitope tag. Flag-BRI1-CD activity was monitored by phosphorylation levels of a synthetic peptide (BR13) containing the previously determined consensus sequence for optimum BRI1 substrate phosphorylation (Oh et al., 2000). GST-BAK1-CD alone did not phosphorylate BR13 significantly when compared to Flag-BRI1-CD alone. However, a mixture of equal protein amounts of GST-BAK1-CD and Flag-BRI1-CD resulted in over three times the level of BR13 phosphorylation with respect to Flag-BRI1-CD alone (Figure 1D). This stimulatory effect was dependent on BAK1 kinase activity since substitution of kinase-inactive GST-BAK1(K317E)-CD for GST-BAK1-CD in the mixture resulted in BR13 phosphorylation levels similar to those with Flag-BRI1-CD alone.

The positive regulatory interaction between BRI1 and BAK1 observed both in vivo and in vitro was also examined at the whole-plant level by using hypocotyl length as a read-out for BR signaling in a physiological response known to be strongly promoted by BR (Li et al., 2002; Wang et al., 2005a). The critical role of BRI1 in hypocotyl elongation is well documented (Clouse and Sasse, 1998) and is demonstrated in Figure 1E, in which a *bri1-1* null allele shows hypocotyl lengths less than one-seventh that of wild-type *Arabidopsis*. In contrast, *bak1-4 bkk1-1* double mutants have hypocotyls that are shorter than wild-type but substantially longer than *bri1-1* mutants, suggesting that BRI1 activity is more critical for hypocotyl elongation than BAK1 and BKK1. This is confirmed by the observation that overexpression of BRI1-Flag in the *bak1-4 bkk1-1* double mutant rescues hypocotyl length to wild-type length, whereas overexpression of BAK1-GFP in the *bri1-1* null mutant has no effect on hypocotyl elongation (Figure 1E). This is consistent with the observation in Figures 1B and 1C that BRI1 continues to function in a BL-dependent manner in the *bak1-4 bkk1-1* double mutant background, whereas BL-dependent BAK1 phosphorylation is lost in the *bri1-1* null mutant. However, BAK1 does stimulate BRI1-mediated hypocotyl elongation, since even a single active copy of BAK1 in the BRI1-Flag overexpression line leads to increased hypocotyl lengths, as does overexpression of BAK1-GFP in a genetic background containing at least one active copy of BRI1 (Figure 1E). This is consistent with the stimulation of BRI1 kinase activity by BAK1 seen in vitro (Figure 1D) and the enhanced phosphorylation of BRI1 in vivo promoted by overexpression of BAK1 (Figure 1A). Overexpression of kinase-inactive 35S-mBAK1-GFP in the *bak1-4 bkk1-1* double mutant, which has an active BRI1 allele, does not increase hypocotyl length when compared to the untransformed double mutant, confirming that BAK1 kinase activity is required for stimulation of BRI1 function (Figure 1E).

Transphosphorylation within an oligomeric complex is one mechanism by which BRI1 and BAK1 could affect the activity of the corresponding receptor kinase. By pairing active BRI1 with kinase-inactive BAK1, and vice versa, Figure 2A clearly shows that transphosphorylation between BRI1 and BAK1 CDs occurs in vitro, in both directions. The importance of transphosphorylation of BRI1 by BAK1 in enhancing BRI1 activity in the BR13 peptide assay was further demonstrated by preincubating Flag-BRI1-CD bound to agarose beads with free GST-BAK1-CD in the presence or absence of ATP, followed by washing away soluble components before the BR13 peptide assay. Flag-BRI1-CD bound to beads gave the same three-fold increase in BR13 phosphorylation when GST-BAK1-CD was included in the peptide assay (Figure 2B), as was observed for soluble Flag-BRI1-CD (Figure 1D). However, preincubation of Flag-BRI1-CD beads with soluble GST-BAK1 and ATP resulted in a nearly six-fold increase in BRI1 activity, even when BAK1 was not added to the subsequent peptide assay. When ATP was removed from the preincubation solution, the resulting activation was dramatically reduced, confirming the importance of BAK1 phosphorylation of BRI1 in the activation mechanism.

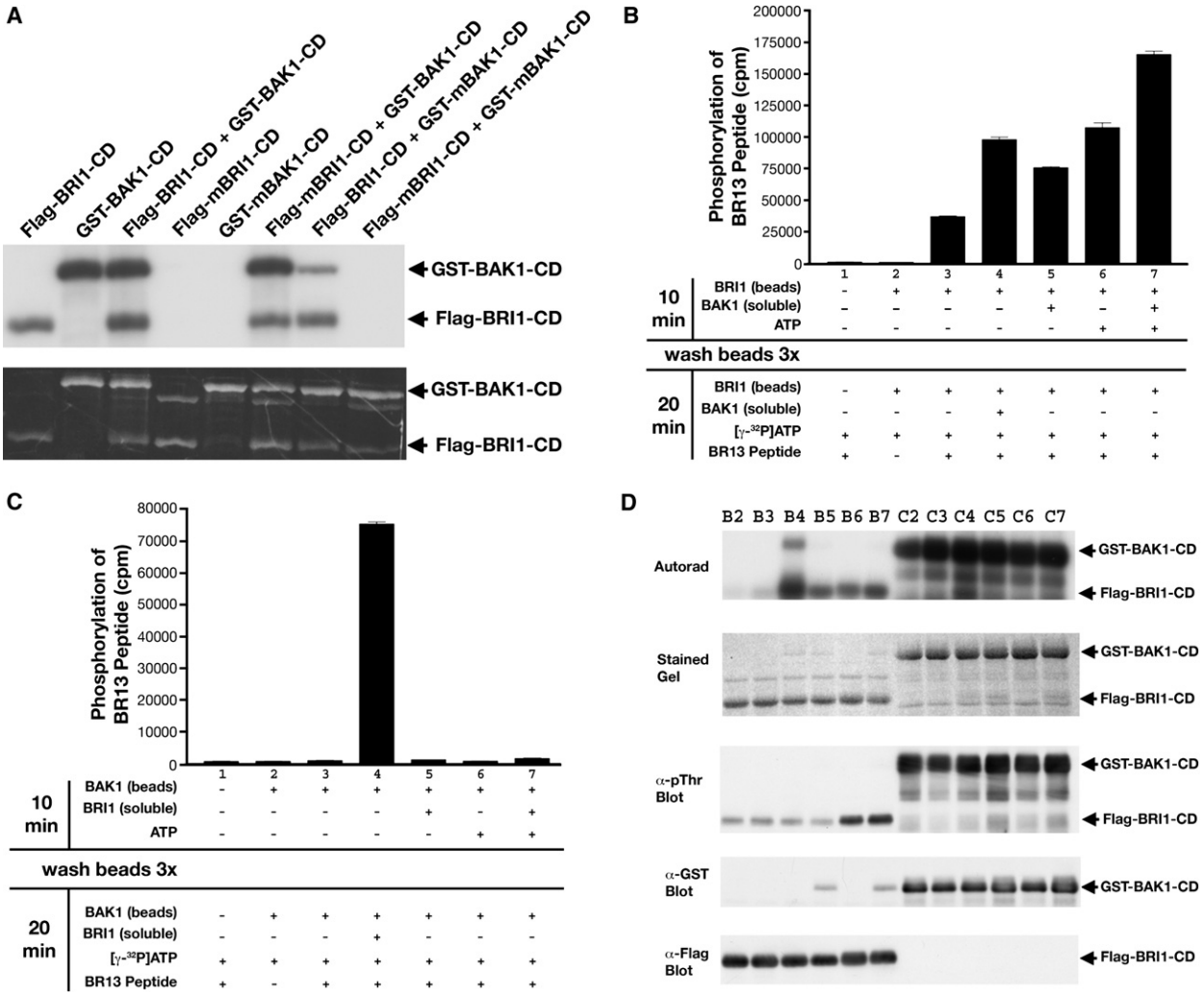

Figure 2. BAK1-CD Increases BRI1-CD Kinase Activity by Transphosphorylation

(A) Incubation of equal protein amounts (1.0 μM) of active and inactive (mBRI1 = K911E and mBAK1 = K317E) recombinant BRI1 and BAK1cytoplasmic domains with [γ-^{32}P]ATP shows that Flag-BRI1-CD and GST-BAK1-CD can both autophosphorylate and transphosphorylate each other. Sypro Ruby staining (bottom panel) shows protein loading for the autoradiograph (top panel).

(B) Preincubating Flag-BRI1 bound to agarose beads with soluble GST-BAK1 and ATP greatly enhances subsequent phosphorylation of the BR13 peptide by Flag-BRI1, even when GST-BAK1 is not included in the peptide assay, suggesting that BRI1 phosphorylation by BAK1 increases BRI1 activity toward specific substrates.

(C) GST-BAK1-CD by itself cannot phosphorylate BR13 directly under any condition tested, indicating that BAK1 enhances BR13 phosphorylation by activating BRI1.

(D) Protein levels and kinase activities for each of the reactions in (B) (B2–B7) and (C) (C2–C7) are shown.

For (B) and (C), error bars are SEM; n = 3.

Interestingly, preincubation of Flag-BRI1-CD with ATP alone also resulted in higher activities in the subsequent peptide assay than in controls without preincubation, suggesting that autophosphorylation also primed BRI1 for higher activity with respect to BR13 substrate phosphorylation, although not to the same extent as ATP + BAK1. Thus, the phosphorylation status of BRI1 at the time substrate is presented is important for optimal peptide phosphorylation, and BAK1 is clearly able to transphosphorylate BRI1, leading to increased activity of BRI1 toward specific substrates. The specificity of BRI1 for the BR13 peptide was also confirmed in this assay, since no preincubation treatment of GST-BAK1-CD resulted in significant BR13 phosphorylation unless Flag-BRI1-CD was included in the peptide assay (Figure 2C).

BAK1 Is Phosphorylated on Mulitple Kinase Domain Residues In Vivo and In Vitro

To refine the biochemical mechanisms by which BRI1 and BAK interact and activate each other, identification of specific sites of phosphorylation in each LRR RLK is necessary. Using a similar experimental approach to that used previously for BRI1 (Wang et al., 2005b), we identified five sites of in vivo phosphorylation in BAK1-GFP immunoprecipitated from BL-treated tissue (Table 1). In contrast to BRI1, which showed phosphorylation

Table 1. Identification of BAK1 Phosphorylation Sites by Ion Trap or Q-ToF LC/MS/MS

Peptide[a]	Calc.[b] $[M+H]^+$	Measured $[M+H]^+$	Chg.	SEQUEST Xcorr[c]	ΔC_n[d]	Mascot Score[e]	Identified Site
In Vivo Phosphorylation Sites of Immunoprecipitated BAK1-GFP (Ion Trap)[f]							
280-R.ELQVASDNFpSNKNILGR.G-298	1986.1	1984.9	2	3.430	0.216	62	S290
307-R.LADGpTLVAVK.R-318	1067.2	1066.2	1	2.342	0.133	25[g]	T312
439-K.LM*DYKDpTHVTTAVR.G-454	1746.9	1747.3	3	3.567	0.189	48	T446
439-K.LM*DYKDTHVpTTAVR.G-454	1746.9	1745.7	2	3.063	0.103	48	T449
439-K.LMDYKDpTHVpTTAVR.G-454	1810.8	1810.1	2	3.440	0.132	n.d.[h]	T446, T449
453-R.GpTIGHIAPEYLSTGK.S-469	1624.7	1625.4	3	3.295	0.305	47	T455
In Vitro Phosphorylation Sites of GST-BAK1-CD (Ion Trap)							
280-R.ELQVApSDNFSNK.N-293	1432.4	1433.0	2	3.179	0.175	43	S286
280-R.ELQVASDNFpSNKNILGR.G-298	1986.1	1986.2	2	4.188	0.130	68	S290
307-R.LADGpTLVAVKR.L-319	1223.3	1222.8	2	3.462	0.610	71	T312
439-K.LM*DYKDpTHVTTAVR.G-454	1746.8	1746.2	2	4.134	0.139	47	T446
439-K.LMDYKDpTHVpTTAVR.G-454	1810.8	1810.2	2	4.927	0.118	42	T446, T449
439-K.LM*DYKDTHVTpTAVR.G-454	1746.8	1747.1	3	3.674	0.110	44	T450
453-R.GpTIGHIAPEYLSTGK.S-469	1624.7	1623.9	3	3.754	0.321	68	T455
In Vitro Phosphorylation Sites of GST-BAK1-CD (Q-ToF)							
280-R.ELQVApSDNFSNK.N-293	1431.616	1431.623	2	n/a[i]	n/a	26[g]	S286
280-R.ELQVASDNFpSNKNILGR.G-298	1984.949	1984.927	3	n/a	n/a	31[g]	S290
307-R.LADGpTLVAVKR.L-319	1222.656	1222.655	2	n/a	n/a	44	T312
439-K.LM*DYKDpTHVTTAVR.G-454	1745.793	1745.770	3	n/a	n/a	23[g]	T446
439-K.LM*DYKDTHVpTTAVR.G-454	1745.793	1745.798	2	n/a	n/a	28[g]	T449
444-K.DTHVTpTAVR.G-454	1079.489	1079.491	2	n/a	n/a	40	T450
453-R.GpTIGHIAPEYLSTGK.S-469	1623.778	1623.782	2	n/a	n/a	64	T455
In Vitro Transphosphorylation of GST-mbak1-CD × Flag-BRI1-CD (Ion Trap)							
280-R.ELQVASDNFpSNKNILGR.G-298	1986.1	1986.4	2	3.871	0.298	62	S290
307-R.LADGpTLVAVKR.L-319	1223.3	1224.1	2	2.405	0.262	63	T312
439-K.LM*DYKDpTHVTTAVR.G-454	1746.9	1745.8	2	3.048	0.134	51	T446
439-K.LM*DYKDTHVpTTAVR.G-454	1746.8	1746.4	2	4.755	0.043	58	T449
439-K.LMDYKDTHVTpTAVR.G-454	1730.8	1729.9	2	2.284	0.148	50	T450
453-R.GpTIGHIAPEYLSTGK.S-469	1624.7	1624.8	2	2.964	0.484	71	T455
In Vitro Transphosphorylation of GST-mbak1-CD × Flag-BRI1-CD (Q-ToF)							
439-K.LM*DYKDpTHVTTAVR.G-454	1745.793	1745.779	3	n/a	n/a	27[g]	T446
439-K.LMDYKDTHVpTTAVR.G-454	1729.798	1729.793	3	n/a	n/a	51	T449
In Vitro Transphosphorylation of Flag-mbri1-CD × GST-BAK1-CD (Ion Trap)							
824-K.EAELEMYAEGHGNpSGDR.T-842	1945.9	1945.9	2	1.793	0.254	46	S838
841-R.TANNpTNWKLTGVK.E-855	1527.6	1526.6	2	2.384	0.098	36[g]	T846
854-K.EALpSINLAAFEKPLR.K-870	1752.9	1752.2	2	1.778	0.277	37[g]	S858
1156-K.EIQAGSGIDpSQSTIR.S-1171	1642.6	1642.3	2	3.805	0.112	67	S1166
1171-R.SIEDGGFSpTIEMVDMSIK.E-1190	2040.2	2038.5	2	3.69	0.118	68	T1180
In Vitro Transphosphorylation of Flag-mbri1-CD × GST-BAK1-CD (Q-ToF)							
841-R.TANNpTNWK.L-850	1028.420	1028.428	2	n/a	n/a	8[g]	T846

[a] The amino acid residues appearing before and after the periods correspond to the residues preceding and following, respectively, the peptide in the protein sequence. Methionine sulfoxide is denoted as M*, and phosphoseryl and phosphothreonyl residues are denoted as pS and pT, respectively.
[b] The average mass for ion trap and the monoisotopic mass for Q-ToF.
[c] The SEQUEST crosscorrelation score (Xcorr) of the peptide is based on the match of the obtained product ion spectrum to the theoretical product ion spectrum for the corresponding peptide contained in the database.
[d] Difference of crosscorrelation scores between the top two Xcorrs for a given product ion spectrum.
[e] Mascot scores are above the peptide identity threshold ($p < 0.05$), except where indicated.
[f] Spectra for all in vivo sites are provided in the Supplemental Data.
[g] These Mascot scores were below the threshold level, but manual inspection of the spectrum confirmed phosphorylation at the residue indicated. See the Supplemental Data for spectra.
[h] The Mascot search did not score this peptide.
[i] Q-ToF data were analyzed by Mascot only.

throughout the CD, all of the identified BAK1 phosphorylation sites were within the KD, including S290 in subdomain I; T312 in subdomain II; and three residues, T446, T449, and T455, in the activation loop of subdomains VII/VIII. All of these in vivo sites were also found in recombinant GST-BAK1-CD autophosphorylated in vitro (Table 1 and Wang et al., 2005b). Two additional sites, S286 and T450, were detected in vitro but not in repeated in vivo analyses of BL-treated tissue (Table 1).

Specific targets of transphosphorylation between BRI1 and BAK1 were also identified in vitro by pairing kinase-active and -inactive recombinant proteins as described in Figure 2A. Flag-BRI1-CD transphosphorylated GST-BAK1(K317E)-CD on all of the same residues that were autophosphorylated by GST-BAK1-CD alone, with the exception of S286 (Table 1). In contrast, GST-BAK1-CD transphosphorylated Flag-BRI1(K911E)-CD on JM sites (S838, T846, and S858) and CT sites (S1166 and T1180), but no phosphorylated KD sites were detected. All of these residues were previously identified as potential in vitro autophosphorylation sites of BRI1-CD (Oh et al., 2000), and S838 and S858 were also verified as in vivo sites in BL-treated tissue (Wang et al., 2005b).

Mutating Specific BAK1 Phosphorylation Sites Alters Kinase Function

The functional significance of identified BAK1 phosphorylation sites (Table 1) was assessed by site-directed mutagenesis of specific Ser or Thr residues to Ala, to eliminate phosphorylation at that site, or to Asp, which can mimic constitutive phosphorylation under certain circumstances. Biochemical function of BAK1-CD was analyzed by autophosphorylation levels by using both incorporation of phosphate from [γ-^{32}P]ATP and immunobloting with α-pThr antibody. The activation-loop residue T455 is equivalent to T1049 in BRI1 and is highly conserved in over 100 LRR RLKs in *Arabidopsis* (Wang et al., 2005b). Like the T1049A substitution in BRI1, BAK1 T455A mutants lost nearly all kinase activity. Furthermore, T455D and T455E mutants, rather than mimicking constitutive phosphorylation, also led to loss of kinase function, suggesting that any change in this critical residue is likely to disrupt activity. A T1049D substitution in BRI1 also resulted in loss of kinase activity (Figure S5, available online), further supporting the view that D substitutions at this conserved residue do not have phosphomimetic properties.

The effect of mutating the remaining activation-loop residues, T446, T449, and T450, was more subtle. When examined by α-pThr blots, T-to-A substitutions in these residues reduced kinase activity compared to the wild-type kinase, whereas T-to-D substitutions increased activity above T-to-A mutant levels, but not to the level of the wild-type kinase. However, when monitored by kinase assays with [γ-^{32}P]ATP, all of these substitutions appeared to increase kinase activity above wild-type levels. This apparent discrepancy can be explained by our previous observations with recombinant BRI1-CD, which showed that the expressed KD can autophosphorylate to a very high degree within the bacteria during recombinant protein production, allowing for less uptake of phosphate from [γ-^{32}P]ATP during subsequent kinase assays in vitro (Wang et al., 2005b). The high level of wild-type BAK1-KD autophosphorylation in bacteria, as shown by the α-pThr blot (Figure 3A), resulted in reduced autophosphorylation levels with [γ-^{32}P]ATP, whereas the low level of bacterial autophosphorylation seen in T446, T449, and T450 mutants allowed for greater uptake of phosphate from [γ-^{32}P]ATP during subsequent assays. Those constructs that had high autophosphorylation within bacteria also exhibited reduced electrophoretic mobility and doublet-band formation. In contrast to activation-loop residues, substitutions at S290 or T312 had no observable effect on autophosphorylation when compared to wild-type BAK1-CD, nor did S286A. Surprisingly, the S286D substitution resulted in complete loss of kinase activity, similar to the K317E kinase-inactive mutant. If indeed S286D mimics constitutive phosphorylation, it suggests that S286 is a negative, phosphorylation-dependent regulatory site.

The effects of substitutions within Flag-BAK1-CD on phosphorylation of BR13 by Flag-BRI1-CD were also monitored (Figure 3B). As with autophosphorylation, kinase activity of substitutions at S290 or T312, as well as S286A, appeared similar to wild-type, and S286D was again equivalent to the K317E kinase-inactive mutation. T455A also resulted in complete loss of kinase activity, whereas T455D and T455E had slightly increased activity compared to T455A, but still much lower than wild-type. T446A and T450A substitutions did not reduce kinase activity compared to wild-type, whereas T449A was slightly lower. However, T-to-D substitutions at these activation-loop sites substantially increased Flag-BRI1-CD phosphorylation of BR13. In every case, Flag-BAK1-CD appeared to function by enhancing BRI1 function rather than by directly phosphorylating BR13, since wild-type BAK1 alone, or any of its mutants, did not significantly phosphorylate BR13.

Mutating Specific BAK1 Phosphorylation Sites Alters BR and Flagellin Signaling

The *bri1-5* mutant, a weak allele mutated in the extracellular domain (Noguchi et al., 1999), can be partially rescued by overexpression of wild-type BAK1, whereas overexpression of a BAK1(K317E) kinase-inactive mutant leads to a dominant-negative effect with severe *bri1-1* dwarf phenotypes (Li et al., 2002). To monitor in planta function, BAK1-GFP constructs mutated at six specific phosphorylation sites were overexpressed in *bri1-5*, and phenotypes were monitored visually in multiple independent T_1 transgenic lines (Figure 4A). Any change in T455 affected biological function and resulted in a dominant-negative effect almost as severe as the K317E mutant, which is consistent with in vitro autophosphorylation assays (Figure 3A). S286A mutants partially rescued *bri1-5*, whereas S286D caused a severe dominant-negative phenotype, which is also consistent with in vitro kinase results. This effect was evident at transgenic protein levels that were nearly undetectable by immunoblot analysis (Figure 4A), but it was not due to cosuppression of endogenous BAK1 gene expression (Figure S6). Examination of several independent transgenic lines at different growth stages indicated that the severity of the S286D phenotype was in fact correlated with levels of transgenic BAK1-GFP(S286D) (Figure S6).

Similar to the in vitro assays (Figure 3A), the biological effect of mutating T446, T449, and T450 was less pronounced, although T450A appeared consistently smaller than wild-type (Figure 4A). To determine if changes in these three residues might lead to subtle phenotypes, quantitative analyses of two BR-dependent growth responses, total plant height and leaf shape, were conducted on three independent T_3 homozygous transgenic lines for each mutation (Figure 4B). In each case, the extent of

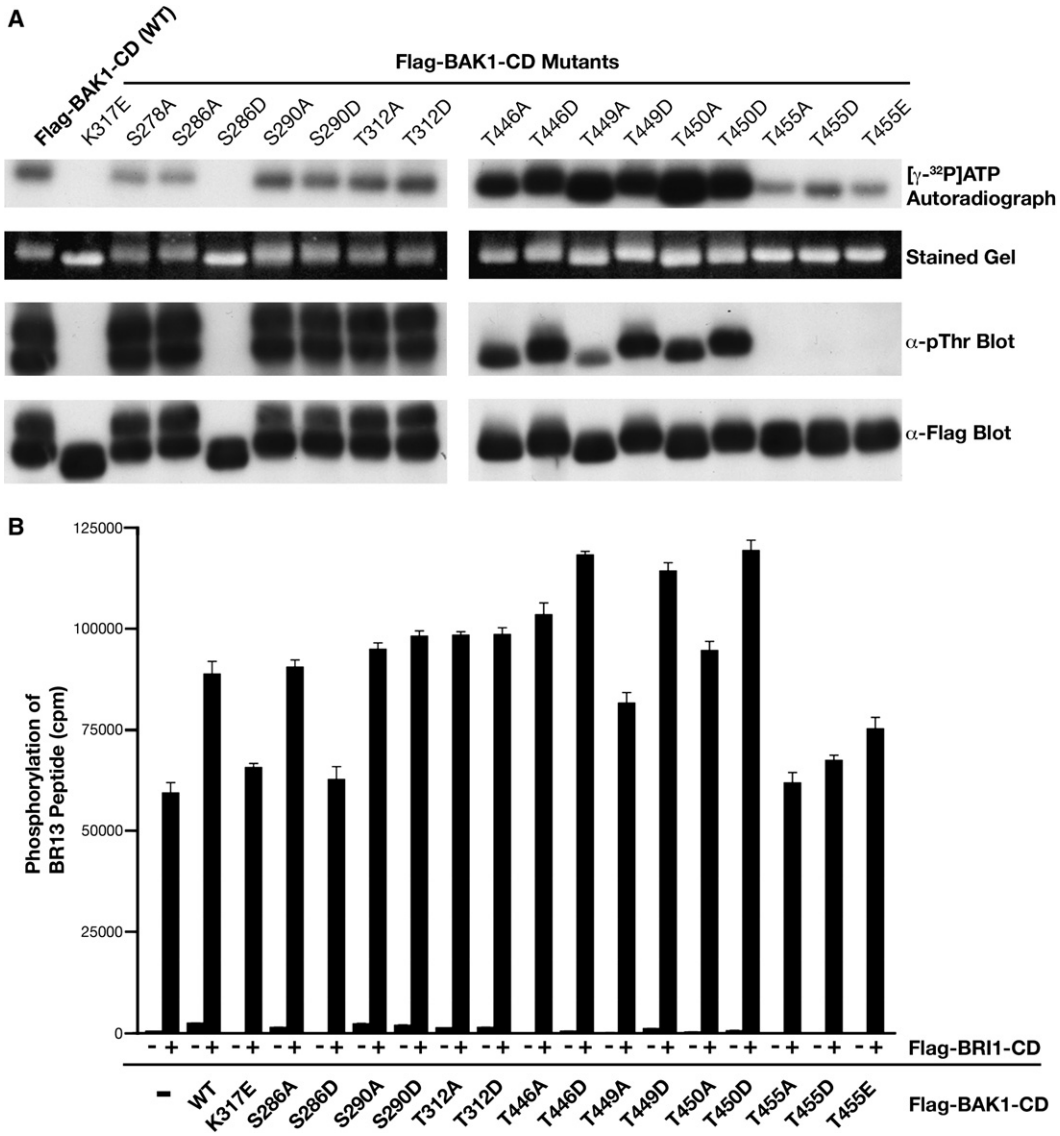

Figure 3. Effect of Mutating Specific BAK1 Phosphorylation Sites on Autophosphorylation and Stimulation of BRI1 Substrate Phosphorylation

(A) Site-directed mutagenesis of Flag-BAK1-CD was used to alter each identified site of in vivo and in vitro phosphorylation in BAK1. Equal amounts of recombinant protein (Stained Gel) were incubated with [γ-^{32}P]ATP (Autoradiograph) to determine autophosphorylation levels. Equal amounts of purified recombinant protein (α-Flag Blot) were also subjected to immunoblot analysis (α-pThr Blot).

(B) Equal protein amounts of wild-type or mutated Flag-BAK1-CD were incubated with [γ-^{32}P]ATP, BR13 peptide, and with or without equal amounts of Flag-BRI1-CD. Error bars are SEM; n = 3.

bri1-5 rescue for both characteristics was directly correlated with the level of transgenic protein, and T446A and T449A both rescued to the same extent as wild-type BAK1-GFP when highly expressed. T450A lines were expressed at intermediate levels and gave phenotypes intermediate between *bri1-5* and BAK1-GFP. However, comparison of T450A and T450D lines exhibiting equivalent expression showed that T450D enhanced biological function compared to T450A.

As another test of biological function, the ability to rescue the *bak1-4 bkk1-1* severe double mutant was examined in transgenic constructs driven by the native BAK1 promoter (Figure 5A). Double mutant seedlings die after 15–20 days of growth, and wild-type BAK1-Flag as well as S286A, S290A, T446A, T449A, and T450A were able to rescue this seedling-lethal phenotype. However, the kinase-inactive K317E mutation, as well as S286D and T455A, was ineffective, confirming the critical role of a fully active kinase in BAK1 signaling. The FLS2 peptide ligand flg22 inhibits *Arabidopsis* Col-0 seedling growth at low concentrations (10 nM), and *bak1* mutants show reduced sensitivity to flg22 (Chinchilla et al., 2007). Figure 5B shows that the *bak1-4 bkk1-1* double mutant (transformed with empty vector) is extremely dwarfed, and that wild-type BAK1::BAK1-Flag restores growth and flg22 sensitivity to levels equal to or greater than Col-0, as do the S286A, S290A, T446A, and T449A mutations.

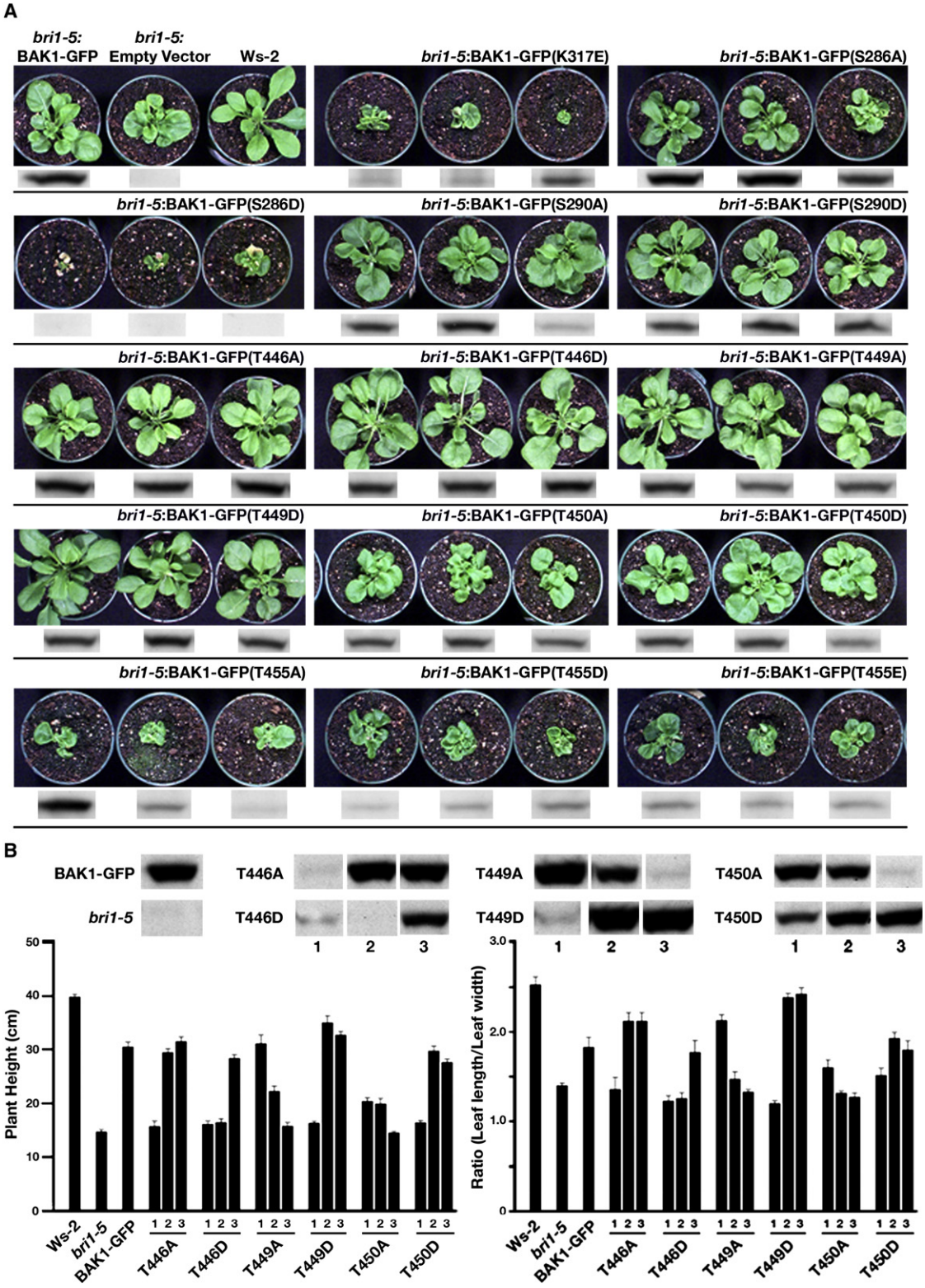

Figure 4. Functional Analysis of BAK1 Phosphorylation Sites In Planta

(A) Overexpression of CaMV35S::BAK1-GFP in the weak BR-insensitive mutant *bri1-5* leads to partial suppression of the *bri1-5* phenotype, whereas the kinase-inactive BAK1-GFP(K317E) produces a dominant-negative effect, confirming previous results (Li et al., 2002). Wild-type *Arabidopsis* (ecotype Ws-2) is shown for comparison. The effect of mutating specific phosphorylation sites in CaMV35S::BAK1-GFP on the suppression of the *bri1-5* phenotype is shown for three independent T_1 transgenic lines for each construct. The level of transgenic protein is shown by α-GFP immunoblot analysis of equal amounts of total protein extracted

This indicates that phosphorylation at these residues is not critical for BAK1 regulation of FLS2 function. However, a T450A substitution restores growth while retaining complete flg22 insensitivity, suggesting that phosphorylation at this residue may specifically enhance BAK1 regulation of FLS2 signaling. The kinase-inactive mutants K317E, S286D, and T455A were also tested in the flg22 assay, but since these constructs failed to rescue the seedling lethality of the *bak1-4 bkk1-1* double mutant, not enough viable seedlings remained at day 18 for a statistically significant analysis of growth response.

BAK1 Quantitatively Enhances BRI1 Phosphorylation Levels and Kinase Function

Taken together, the results in Figures 1–3 and Table 1 are consistent with a model in which BAK1 transphosphorylates BRI1 on specific JM and CT residues, which then increases the ability of BRI1 to phosphorylate its substrates. If this model is correct, phosphomimetic mutants in which specific JM and CT S/T residues are replaced by D should reduce or eliminate the need for BAK1 to increase BRI1 phosphorylation of the BR13 peptide substrate. To test this model, JM and CT residues in BRI1 known to be transphosphorylated by BAK1 (Table 1), plus additional residues previously identified as BRI1 phosphorylation sites (Wang et al., 2005b), were mutated. Figure 6A shows that a mutant construct (CT5D) of Flag-BRI1-CD containing S1166D, S1168D, S1172D, S1179D, and T1180D displays a substantial BAK1-independent increase in activity and phosphorylates BR13 in the absence of GST-BAK1-CD to the same level as wild-type Flag-BRI1-CD in the presence of GST-BAK1-CD. However, addition of BAK1 to CT5D increases BR13 phosphorylation even further, suggesting that additional CT5D sites (perhaps in the JM region) are being phosphorylated by BAK1 to enhance CT5D activity above that of wild-type BRI1 plus BAK1. Similarly, JM4D, containing S838D, T842D, T846D, and S858D in the JM region of Flag-BRI1-CD enhances kinase function significantly in the absence of BAK1 (Figure 6A). Addition of BAK1 increases JM4D activity even further, again most likely by additional phosphorylation of unmutated sites (perhaps in the CT region). The combined construct (JMCT9D) again shows enhanced function without BAK1 and a response to added BAK1 that is less than either JM4D or CT5D. The slight response to BAK1 addition suggests that other residues in JMCT9D are phosphorylated in response to BAK1 either in the JM, CT, or even the KD region. These may be real transphosphorylation sites that went undetected in our LC/MS/MS analysis, or they may be sites that are only phosphorylated significantly when the primary sites of BAK1 transphosphorylation are mutated. The BAK1 plus:BAK1 minus ratios for wild-type (2.4x) and JMCT9D (1.2x) clearly show that phosphomimetic mutations confer significant BAK1 independence on BRI1 with respect to enhanced phosphorylation of BR13.

To further confirm that BAK1 quantitatively enhances BRI1 phosphorylation levels, we used an advanced label-free quantification procedure for Q-ToF LC/MS/MS analysis termed LC/MS^E, which allows for accurate determination of phosphorylation levels at individual residues (Silva et al., 2006; Vissers et al., 2007). Focusing on the BRI1 S1166 residue, which is a site of BAK1 transphosphorylation (Table 1), we found that the average percent phosphorylation of S1166 minus BAK1 was 40.1%, which increased significantly to 64.5% upon the addition of BAK1 (Figure 6B). Thus, BAK1 quantitatively enhances BRI1 phosphorylation at a specific residue, although the contribution of direct BAK1 transphosphorylation versus BAK1-stimulated BRI1 autophosphorylation could not be determined in this experiment.

Specific JM Mutations Quantitatively Affect BR Signaling In Vivo

We previously demonstrated that eliminating phosphorylation at specific BRI1 JM sites with the mutants S838A, T846A, and S858A (the three JM sites in BRI1 shown here to be transphosphorylated by BAK1) resulted in BR13 peptide phosphorylation at levels of only 12%–16% of that seen with wild-type BRI1-KD (Wang et al., 2005b). Measurement of hypocotyl length in increasing concentrations of the BR biosynthesis inhibitor Brz has been successfully used to quantitatively assess the effect of BRI1 mutations on BR signaling (Wang et al., 2005a). Therefore, we used this system to determine if the S838A, T846A, and S858A substitutions, would quantitatively affect BR signaling in planta. As shown in Figure 6C, overexpressing wild-type 35S-BRI1-Flag in the *bri1-5* mutant resulted in hypocotyl lengths greater than untransformed wild-type *Arabidopsis*. However, in independent transgenic lines showing approximately equal transgenic protein levels, S838A, T846A, and S858A all exhibited a quantitative reduction in BR signaling output, as shown by hypocotyl lengths intermediate between wild-type BRI1-Flag and the untransformed *bri1-5* mutant (Figure 6C). The only exception, line 1 of T846A, had much higher levels of transgenic protein, which confirms the correlation of phenotype with levels of transgenic protein. Thus, altering specific BRI1 residues that are the target of BAK1 transphosphorylation quantitatively affects BR signaling in planta and BRI1 kinase function in vitro.

DISCUSSION

The ligand-dependent association of BRI1 with BAK1 and other members of the SERK family of LRR RLKs supports the hypothesis that BR signaling shares some mechanistic similarities to mammalian RTK and TGF-β receptor kinase signaling. In order to investigate the detailed mechanism of BRI1/BAK1 interaction and determine the extent of conservation with mammalian receptor kinase modes of action, we used transgenic plants expressing epitope-tagged BRI1 and BAK1 (either native proteins or kinase-inactive mutants) in different genetic backgrounds to study BR dependence of the physical association of the pair and the ability to auto- and transphosphorylate in the complex.

from plants of the same age. All plants were grown under the same conditions for 39 days before photographing. Even very low levels of S286D expression led to dominant-negative effects, which were not the result of cosupression of endogenous BAK1, BKK1, or BRI1 (Figure S5).

(B) Total plant height and leaf shape, two BR-dependent morphological characteristics, are partially rescued by overexpression of CaMV35S::BAK1-GFP in *bri1-5*. The effects of mutating the activation-loop residues T446, T449, and T450 on this rescue were examined in three independent T_3 homozygous transgenic lines expressing varying levels of transgenic protein (determined by α-GFP immunoblot analysis). Measurements were taken at 64 days. Error bars are SEM; n = 7.

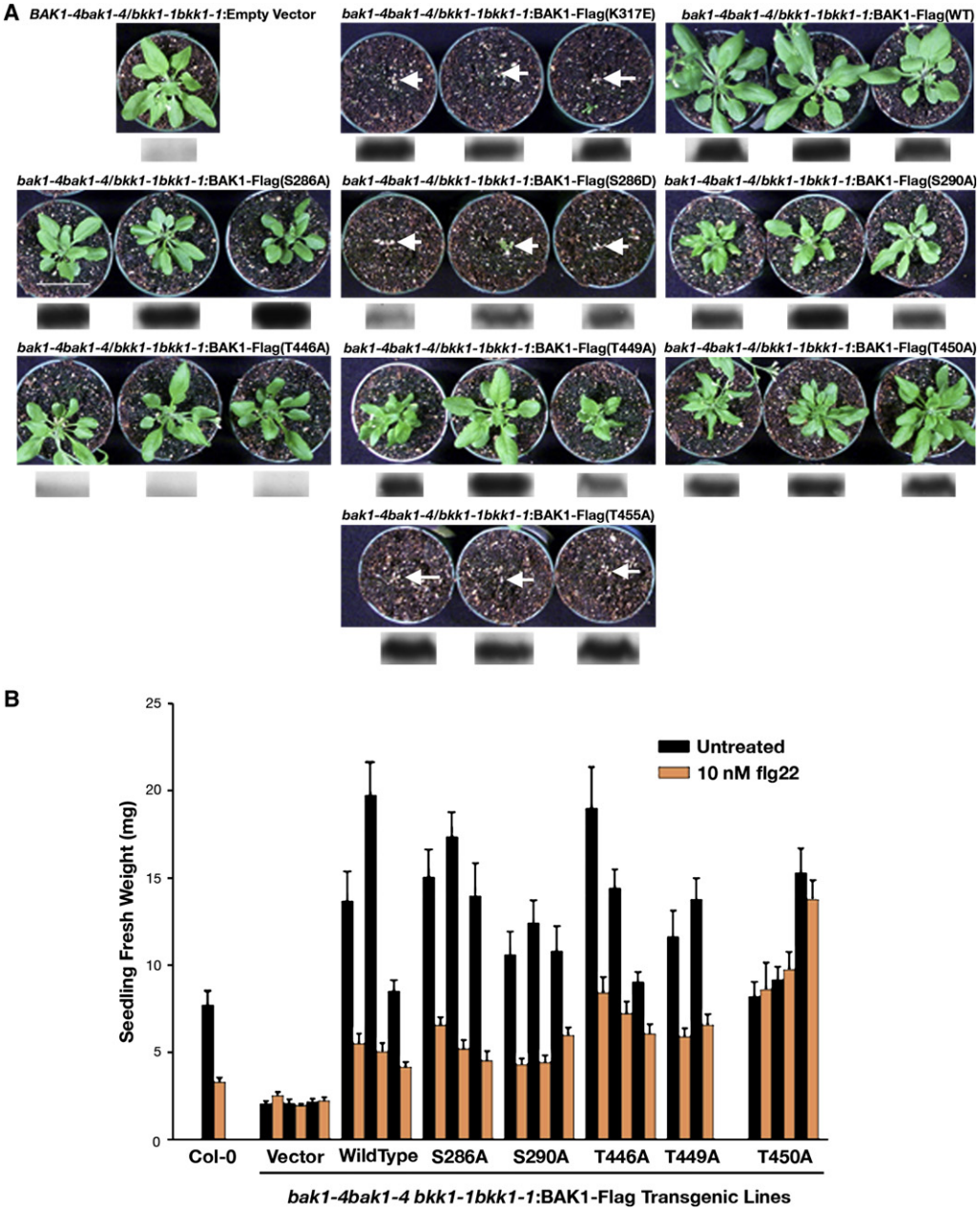

Figure 5. Effect of Mutating Specific Phosphorylation Sites of BAK1 on the Rescue of the Severe *bak1-4 bkk1-1* Double Mutant

(A) Expression of BAK1::BAK1-Flag in *bak1-1 bkk1-1* rescued the seedling-lethal phenotype of the double mutant. Kinase-inactive forms of BAK1, including K317E, S286D, and T455A, failed to rescue *bak1-1 bkk1-1*, and plants died after 20–25 days of growth (white arrows). Three independent T_2 transgenic lines for each construct are shown, and the level of transgenic protein was determined by α-Flag immunoblot analysis of equal amounts of total protein extracted from plants of the same age. All plants were grown under the same conditions for 36 days before photographing.

(B) BAK1 T450 is a critical residue for flg22 sensitivity. Seedlings were grown on agar plates ± 10.0 nM flg22 peptide (QRLSTGSRINSAKDDAAGLQIA) for 18 days. Independent T_2 transgenic lines are the same as in (A) and are presented in the same order (left to right). The aerial portion of each seedling was weighed individually. Error bars are SEM; n = 10.

Our LC/MS/MS analysis identified individual phosphorylation sites in BRI1 and BAK1, and functional analysis of these sites both in planta and in vitro helped to define the specific role of BRI1 and BAK1 kinase function in the interaction of these LRR RLKs. The data presented here are consistent with a sequential transphosphorylation mechanism of BR signaling in which BRI1 can act independently from BAK1 (and its homolog BKK1) with respect to ligand binding, initial kinase activation, and participation in a basal level of BR signaling in hypocotyl elongation, a typical BR-promoted growth response. However, the full potential of BR signaling is realized when ligand-activated BRI1 interacts directly with BAK1, leading to BAK1 kinase activation by

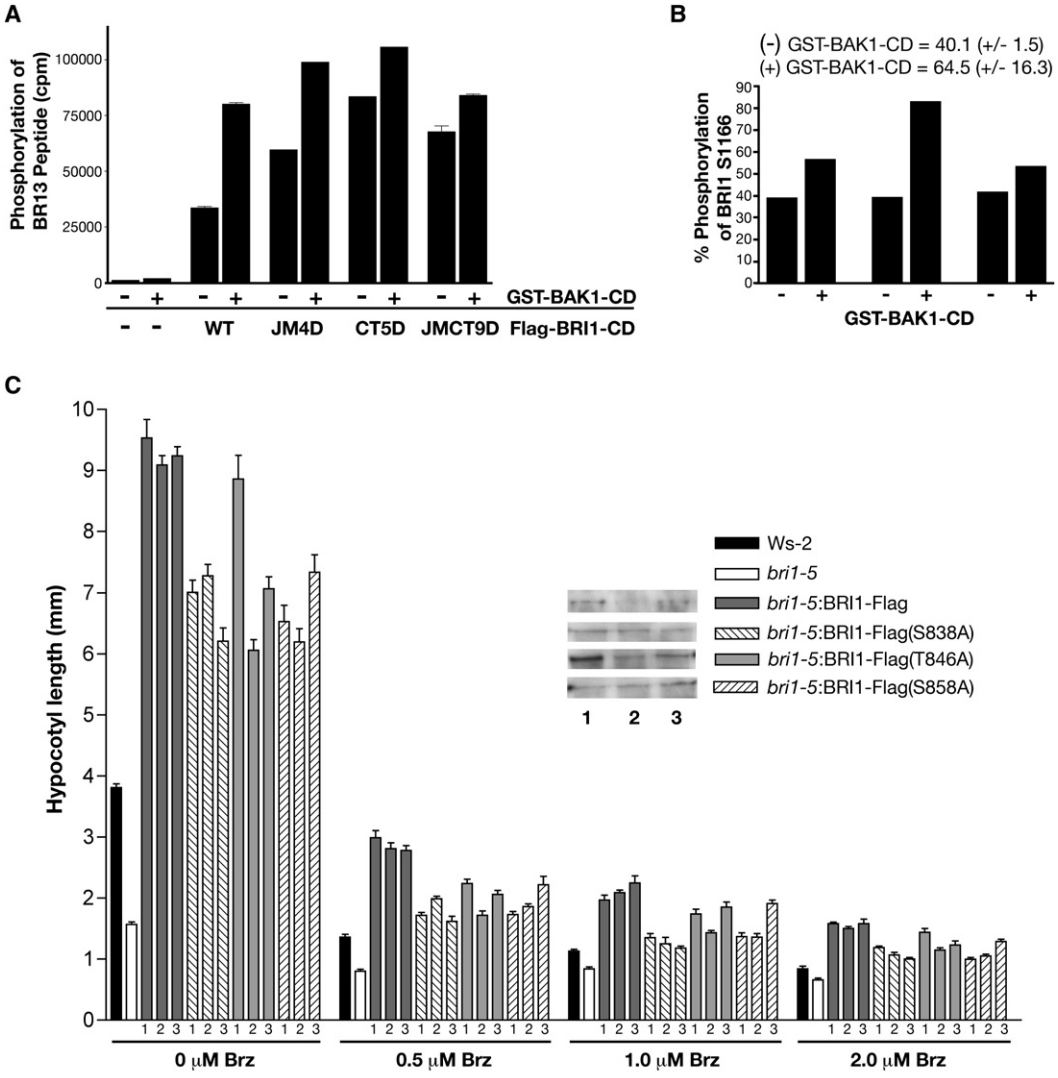

Figure 6. BRI1 Residues that Are Targets of BAK1 Transphosphorylation Quantitatively Affect BRI1 Kinase Activity and BR Signaling Output

(A) Incubation of equal protein amounts (1.0 μM) of GST-BAK1-CD with [γ-^{32}P]ATP and several Flag-BRI1-CD constructs shows that phoshomimetic mutants can substitute for BAK1 in enhancing BRI1 phosphorylation of the BR13 peptide substrate. JM4D = S838D, T842D, T846D, S858D; CT5D = S1166D, S1168D, S1172D, S1179D, T1180D; JMCT9D = JM4D + CT5D. Error bars are SEM; n = 3.

(B) Three independent LC/MS^E experiments show that incubation of BRI1 with BAK1 quantitatively increases the level of phosphorylation at the BRI1 CT residue S1166. A Student's t test showed that the difference in mean percent phosphorylation between BAK1 plus and BAK1 minus was significant (p value of 0.06).

(C) Hypocotyl length was measured in 7-day-old seedlings (3 days total darkness followed by 4 days in 16 hr light:8 hr dark) for three independent T_2 transgenic lines plus controls. Transgenic protein levels were determined by α-Flag immunoblot. Error bars are SEM; n = 10.

BRI1-mediated transphosphorylation of BAK1 activation-loop residues. The activated BAK1, in turn, transphosphorylates BRI1 on JM and CT residues, quantitatively increasing BR signaling by enhancing the phosphorylation of specific BRI1 substrates (Figure 7).

This model has several similarities with, but also distinct differences from, mammalian receptor kinase function. Members of the TGF-β family of peptides modulate numerous aspects of mammalian development and are perceived at the cell surface by a complex of Type II (RII) and Type I (RI) receptor serine/threonine kinases. TGF-β-RII receptors homodimerize in the absence of ligand, and TGF-β binding by RII homodimers induces the formation of a heterotetramer with TGF-β-RI, resulting in phosphorylation of RI by RII on specific Thr and Ser residues in the JM domain (Massague, 1998; Rahimi and Leof, 2007). Although it has a ligand-binding domain, TGF-β-RI cannot bind ligand in the absence of TGF-β-RII, but it does so cooperatively in the presence of the TGF-β-RII ligand complex to generate the active heterotetramer. In contrast, RI receptors in the related bone morphogenetic protein family do bind ligand independently of RII and exhibit a different mechanism of heterotetramer assembly (Massague, 2008). BRI1 appears to be analogous to TGF-β-RII, in that it homodimerizes in the absence of ligand (Russinova et al., 2004; Wang et al., 2005a; Hink et al., 2008) and binds BL in the absence of its coreceptor BAK1 (Kinoshita et al., 2005), whereas BAK1 is similar to TGF-β-RI, in that it

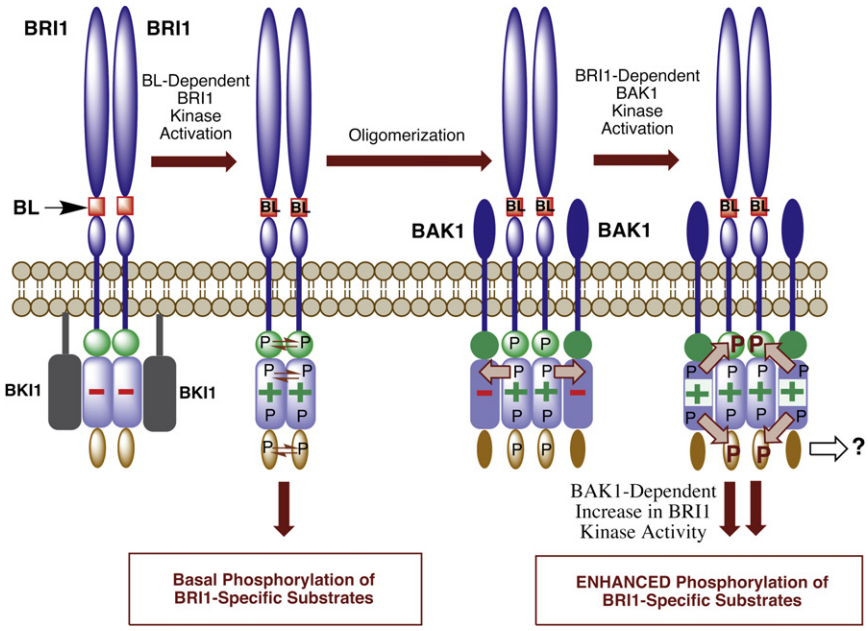

Figure 7. The Sequential Transphosphorylation Model of BRI1/BAK1 Interaction and Activation

BL binds inactive BRI1 homodimers, resulting in kinase activation and release of the BKI1 (Wang and Chory, 2006) negative regulator. BRI1, activated by BL binding and transphosphorylation in the homodimer (double arrows), can signal independently of BAK1 to promote plant growth or oligomerize with inactive BAK1 and transphosphorylate it on KD residues. This BRI1-activated BAK1 then transphosphorylates BRI1 on JM and CT residues, thus quantitatively enhancing BRI1 phosphorylation of downstream substrates and increasing BL-dependent signaling. Our model does not rule out the possibility that BAK1 also transphorylates BRI1 on KD residues that were below the level of detection in our LC/MS/MS analyses. Only BL signaling is shown; the known interaction of BAK1 with BL-independent signaling components such as the FLS2 receptor kinase is omitted from this model for simplicity. The question mark represents the possibility that BAK1 may have as yet undiscovered cytoplasmic substrates in addition to the BRI1 CD. BKK1 and SERK1 (not shown) may substitute for BAK1 in these complexes.

cannot independently bind ligand but requires ligand-dependent activation of its partner before formation of a putative heterotetramer can proceed. However, BAK1 differs from TGF-β-RI in that BAK1 does not possess a BR-binding site, and thus cooperative binding with the BRI1-BR complex may not be critical for BRI1/BAK1 complex formation (Kinoshita et al., 2005). Our data indicate that a key feature in BRI1/BAK1 complex formation is an active BRI1 kinase, which could be directly involved in binding or might function through release of the BKI1 repressor (Wang and Chory, 2006). Although BRI1 and BAK1 may form a simple heterodimer, recent data (Wang et al., 2005a; Karlova et al., 2006) tend to support a heterotetrameric model as shown in Figure 7.

Our in vivo data clearly show that BL-dependent phosphorylation of BAK1 on Thr residues requires an active BRI1 kinase, and thus the initial direction of phosphorylation is from BRI1 to BAK1, similar to the RII-to-RI transphosphorylation in TGF-β signaling. However, in contrast to the RII/RI heterotetramer, our in vitro data suggest that BAK1 is phosphorylated by BRI1 in the KD, including three residues in the activation loop, rather than the JM region, and these sites were verified as in planta phosphorylation sites for BAK1. Moreover, BAK1 autophosphorylates the same sites in vitro in the absence of BRI1. However, the extremely low level of BAK1-GFP phosphorylation in vivo in the *bri1-1* null mutant background suggests that initial activation by BRI1 phosphorylation is required for BAK1 to function in BL signaling, which then may be further amplified by BAK1 autophosphorylation on the same residues. Our data cannot rule out the possibility that JM or CT residues are phosphorylated in BAK1 at levels below detection by our LC/MS/MS analysis, but the predominant phosphorylation on BAK1 residues in the KD represents a clear difference between BR and TGF-β mechanisms. Interestingly, phosphorylation of the activation-loop residue T455 appears to be essential for BAK1 function, and we previously found that the corresponding BRI1 residue T1049 was also required for kinase function and BR signaling in planta (Wang et al., 2005b). A Ser or Thr residue is conserved in this position in over 100 *Arabidopsis* LRR RLKs and thus may represent a fundamental site for kinase activation in these proteins.

Once activated by phosphorylation, RI propagates the TGF-β signal by phosphorylating proteins, termed Smads, which translocate to the nucleus and form complexes with transcription factors to regulate the expression of TGF-β-responsive genes (Massague, 1998; Rahimi and Leof, 2007). Although we cannot rule out that BAK1 may have as yet undiscovered substrates that propagate the BR signal downstream, our in vitro and in vivo data clearly show that BAK1 can transphosphorylate BRI1, and that elimination of BAK1 kinase function reduces BRI1 phosphorylation levels in planta, which is distinctly different from the primarily sequential direction of phosphorylation from RII to RI in TGF-β signaling. Our data are consistent with previous demonstrations of BRI1/BAK1 transphosphorylation (Li et al., 2002; Nam and Li, 2002) but extend these findings by clarifying the directional flow of phosophorylation between BRI1 and BAK1 in planta and by identifying individual sites of transphosphorylation in each LRR RLK. Although there may be KD sites of low stoichiometry not detected in our LC/MS/MS analysis, the predominant sites of BRI1 phosphorylation by BAK1 are clearly JM and CT residues that we previously showed were essential for BRI1 phosphorylation of an optimal peptide substrate (Wang et al., 2005b).

Based on our in vitro kinase assays, transphosphorylation of these BRI1 JM and CT residues by BAK1 stimulates BRI1 kinase function and increases its activity toward downstream substrates. Moreover, phosphomimetic mutants in which these specific residues are changed to D show significantly enhanced BRI1 activity and peptide substrate phosphorylation in the absence of BAK1 in vitro. A focused LC/MS/MS analysis of a selected BRI1 site (S1166) confirms that BAK1 association increases the level of BRI1 phosphorylation at that residue.

Furthermore, elimination of phosphorylation at these same residues by substitution with A quantitatively reduces BR signaling output in vivo. Thus, all of our data are consistent with a model in which BAK1 quantitatively enhances BRI1 function by transphosphorylation of specific BRI1 residues that are essential for kinase function. The in planta confirmation of this aspect of our model is hampered by the lack of demonstrated BRI1 in vivo substrates. Besides BAK1, three other proteins have been shown to interact with BRI1 in vivo and be phosphorylated by BRI1-KD in vitro (Nam and Li, 2004; Ehsan et al., 2005; Wang and Chory, 2006), but in vivo phosphorylation of these proteins by BRI1 is not confirmed and thus the role of BAK1 in enhancing phosphorylation of these proteins via BRI1 cannot be examined.

The transphosphorylation of BRI1 and BAK1 within the associated complex in some aspects more closely resembles mammalian RTK mechanisms than the TGF-β model. Ligand binding to the extracellular domain of RTKs induces dimerization, which activates the KD of each member of the pair either by phosphorylation or allosteric conformational change of the activation loop. The activated kinases within the dimer transphoshorylate on Tyr residues in the CT region to generate docking sites for downstream signaling proteins (Schlessinger, 2002). The apparent phosphorylation of BRI1, but not BAK1, on CT residues may represent a modification of the transphosphorylation mechanisms for BR signaling versus mammalian RTKs. The best understood RTKs mechanistically are members of the epidermal growth factor (EGF) receptor family, which includes four members, ErbB1 through ErbB4. ErbB2 cannot itself bind EGF ligand, but it heterodimerizes with the other three members of the family in a ligand-dependent manner, significantly enhancing the signaling output of each (Hubbard and Miller, 2007). In this respect, ErbB2 resembles BAK1, in that it does not determine signaling specificity by directly binding a ligand, but instead enhances activity of its ligand-binding partner. BAK1 has now been shown to associate with other LRR RLK partners besides BRI1, including FLS2 (Chinchilla et al., 2007; Heese et al., 2007), and differential BAK1 phosphorylation may be involved in its participation in distinct signaling pathways, including BR-promoted growth, regulation of cell death, and defense responses to pathogens. For example, our data show that the T450A mutation in BAK1 does not suppress the *bri1-5* mutant to the extent that S290A, T446A, and T449A mutations do, but it does fully rescue the BR-independent growth defects of the *bak1 bkk1* double mutant, suggesting that phosphorylation at T450 may be important in BR signaling, but not the enhanced cell death phenotype observed in the *bak1 bkk1* mutant (He et al., 2007). In our LC/MS/MS analysis of in vivo BAK1 phosphorylation sites, the b and y ions generated from the detected phosphopeptides suggest that pT449 was the modified residue, although pT450 could not be unequivocally excluded. Thus, BL-induced phosphorylation of T450 in vivo cannot be ruled out and in fact is supported by the transphosphorylation of BAK1 T450 by BRI1-CD in vitro. The highly specific loss of flagellin sensitivity in the T450A mutant observed here, is also quite striking and suggests that phosphorylation of BAK1 T450 may be crucial for interaction with and activation of FLS2. A quantitative comparative analysis of BAK1 in vivo phosphorylation sites in BL versus flagellin-treated tissue would be informative in this respect.

Finally, the extreme kinase-inactive phenotype of BAK1 S286D mutants suggests that phosphorylation at this residue might be a negative regulator of BAK1 function in a specific signaling pathway. Phosphorylation at S286 was not detected in BL-treated tissue in repeated experiments, and it was not a site of in vitro transphosphorylation of BAK1-CD by BRI1-CD. Therefore, if S286 is indeed phosphorylated in vivo, it may be in response to a non-BL ligand in partnership with an undiscovered LRR RLK.

The very large number of LRR RLKs found in *Arabidopsis* present immense possibilities for heterodimerization or oligomerization and thus the diversification and amplification of signaling pathways regulating plant growth and development. Our analysis of the BRI1/BAK1 interaction helps clarify early events of BR signaling and serves as a foundation for analysis of other LRR RLKs to determine if a sequential transphosphorylation mechanism exists in other heteromeric complexes. To this end, we have cloned the entire LRR RLK family in a variety of plant and bacterial expression vectors to aid in studies of kinase function, phosphorylation-site analysis, and protein-protein interactions (Clouse et al., 2008). Such global studies should help to reveal whether sequential transphosphorylation, as observed here in BRI1/BAK1 interactions, is a general mechanism of plant receptor kinase action.

EXPERIMENTAL PROCEDURES

Plant Growth, Transgenic Lines, and Protein Analysis

Plants were grown in shaking liquid culture or growth chambers and were treated with 100 nM BL or 2.0 μM Brz 220 as previously described (Wang et al., 2005b). Transgenic lines BRI1-Flag, BAK1-GFP, mBRI1-Flag (K911E), and mBAK1-GFP (K317E) are described elsewhere (Li et al., 2002). Details of cloning, in vitro mutagenesis, and plant transformation for other lines used are provided in the Supplemental Data. Total membrane protein isolation, immunoprecipitation, and immunoblot analysis were performed as previously described (Wang et al., 2005b).

Recombinant Protein Production and In Vitro Kinase Assays

Generation of Flag-BRI1-CD and Flag-mBRI1-CD is described elsewhere (Oh et al., 2000). Construction of GST-BAK1-CD, Flag-BAK1-CD, and associated site-directed mutants is described in detail in the Supplemental Data. Purification of recombinant protein and general conditions for kinase autophosphorylation and peptide substrate assays are as previously described (Oh et al., 2000; Wang et al., 2005b); details for specific experiments are provided in the Supplemental Data.

Determination of In Vivo and In Vitro Phosphorylation Sites by LC/MS/MS

For in vivo and in vitro phosphorylation-site analysis, BAK1-GFP was immunoprecipitated from solubilized total membrane protein extracts isolated from *Arabidopsis* plants treated with 100 nM BL, and recombinant GST-BAK1-CD and Flag-BRI1-CD were expressed and purified as previously reported (Wang et al., 2005b), with minor modifications that are described in the Supplemental Data. Microcapillary reversed-phase ion trap LC/MS/MS analysis of peptide samples was performed in a data-dependent MS/MS mode with an Agilent 1100 capillary LC system (Agilent Technologies, Inc., Palo Alto, CA) coupled to a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA), by using previously described conditions (Wang et al., 2005b). For Quadrupole Time-of-Flight (Q-ToF) LC/MS/MS, aliquots of in-gel digests were subjected to data-dependent analysis by using a NanoAcquity UPLC coupled to a Premier Q-ToF mass spectrometer equipped with a nanolockspray source (Waters Corporation, Milford, MA). Label-free quantitative analysis of the phosphorylation levels of BRI1 S1166 was performed on the Premier Q-ToF by using the LC/MS^E procedure and algorithms (Silva et al., 2006; Vissers et al., 2007). Further details of sample preparation, LC/MS/MS analysis, and search algorithm parameters are provided in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include detailed Supplemental Experimental Procedures, six figures, and Supplemental References and can be found at http://www.developmentalcell.com/cgi/content/full/15/2/220/DC1/.

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