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Molecules and Cells

Analysis of Phosphorylation of the BRI1/BAK1 Complex in Arabidopsis Reveals Amino Acid Residues Critical for Receptor Formation and Activation of BR Signaling

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The plasma membrane-localized BRASSINOSTEROID-INSENSITIVE1 (BRI1) and BRI1-ASSOCIATED KINASE1 (BAK1) are a well-known receptor pair involved in brassinosteroids (BR) signaling in Arabidposis. The formation of a receptor complex in response to BRs and the subsequent activation of cytoplasmic domain kinase activity share mechanistic characteristics with animal receptor kinases. Here, we demonstrate that BRI1 and BAK1 are BR-dependently phosphorylated, and that phosphorylated forms of the two proteins persist for different lengths of time. Mutations of either protein abolished phosphorylation of the counterpart protein, implying transphosphorylation of the receptor kinases. To investigate the specific amino acids critical for formation of the receptor complex and activation of BAK1 kinase activity, we expressed several versions of BAK1 in yeast and plants. L32E and L46E substitutions resulted in a loss of binding of BAK1 to BRI1, and threonine T455 was essential for the kinase activity of BAK1 in yeast. Transgenic *bri1* mutant plants overexpressing BAK1(L46E) displayed reduced apical dominance and seed development. In addition, transgenic wild type plants overexpressing BAK1(T455A) lost the phosphorylation activity normally exhibited in response to BL, leading to semi-dwarfism. These results suggest that BAK1 is a critical component regulating the duration of BR efficacy, even though it cannot directly bind BRs in plants.

INTRODUCTION

Brassinosteroids (BRs) are a group of plant-specific steroidal organic compounds (Mitchell et al., 1970); over 70 BR compounds have been isolated from various plants since the initial discovery of brassinolide (BL), the most bioactive BR, in 1973. BRs affect numerous developmental processes including cell expansion, vascular differentiation, and pollen elongation (Clause and Sasse, 1998). To date, the leucine rich-repeats receptor-like kinase (LRR-RLK), BRI1 (BRASSINOSTEROID-INSENSITIVE1) is the only protein that can directly bind to BL, triggering BR signaling at the plasma membrane in plants (Li and Chory, 1997; Wang et al., 2005b). Mutation of *BRI1* causes very severe dwarfism including compact rosette formation and male sterility in many plant species (Chono et al., 2003; Nomura et al., 1999; Yamamuro et al., 2000), indicating that BRs are essential compounds and that normal plant growth requires transduction of the BR signal to the interior of the cell. Yeast two hybrid screens for components interacting with BRI1, and genetic screens for *bri1* suppressors, yielded a single protein termed BRI1-ASSOCIATED KINASE1 (BAK1) that functions as a plasma membrane co-receptor with BRI1, and is required for BR transduction of the BR signal (Li et al., 2002; Nam and Li, 2002). Once the BR signal is transmitted into the cell from the BRI1/BAK1 complex, the key transcriptional factors BRI1 EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1) are activated (Wang et al., 2002; Yin et al., 2002). The transcriptional activities of BES1 and BZR1 depend on their phosphorylation status (He et al., 2002), which is controlled by BRASSINOSTEROID-INSENSITIVE2 (BIN2), a cytoplasmic serine/threonine kinase known to be a negative regulator of BR signaling (Li and Nam, 2002), and BRI1 SUPPRESSOR1 (BSU1), a plant-specific phosphatase (Mora-Garcia et al., 2004).

As BAK1 is also an LRR-RLK similar to BRI1, the BRI1/BAK1 heterodimer provided the first example of receptor heterodimerization among more than 400 RLKs in Arabidopsis (Shiu and Bleeker, 2001). This finding led to the hypothesis that, as in animals, ligand-induced (here BL-induced) heterodimerization of two different types of LRR-RLKs and subsequent activation of their kinase activities could occur in plants. Two classes of receptor kinases are found in animals (Pawson and

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Nash, 2000). The first, receptor tyrosine kinases (RTKs), act as ligand-activated homodimers or as heterodimers between related RTKs; subsequent phosphorylation of their kinase domains leads to activation of their kinase activities, and further phosphorylation of the tyrosine residues creates docking sites for downstream signaling components (Burgess et al., 2003; Ulrich and Schlessinger, 1990). The second class of receptor kinase comprises the TGFβ-receptor kinase, the only serine/threonine receptor kinase in animals. This kinase can generate ligand-independent heterotetramers consisting of (Tβ R1)$_2$/(Tβ R2)$_2$ on the cell surface. The constitutively active kinase Tβ R2 binds ligand and this initiates phosphorylation of TβR1, which can not bind ligand in the absence of TβR2. Phosphorylation of TβR1 is then recognized by components of a signaling cascade (Massgue, 1998; Tendijke and Hill, 2004).

A number of studies have elucidated the mechanism of activation for BRI1/BAK1. These two proteins can bind to each other independently of BL in yeast and plants (Nam and Li, 2002; Wang et al., 2005a), but the amount of complex formation is increased by BL treatment. BL treatment also induces threonine phosphorylation of both BRI1 and BAK1 (Wang et al., 2005a). The BL-induced increase in receptor oligomerization and BRI1/BAK1 phosphorylation activities is reminiscent of the activation of RTKs in animals. BRI is known to form a homodimer via an auto-inhibitory C-terminal domain. Binding of BL to the extracellular domain of BRI1 releases its C-terminal domain, leading to activation of its kinase activity and formation of multimeric complexes with BAK1 (Wang et al., 2005b). This process is similar to the activation of TGFβ-receptors in that only BRI1 can bind to BL, its cognate ligand, as in the case of Tβ R2; formation of hetero-multimeric complexes occurs on the cell surface.

The *in vivo* sites of BRI1 phosphorylation were identified by Wang *et, al.* using ion trap LC/MS/MS analysis (Wang et al., 2005b). Several serine and threonine residues in the juxtamembrane domain (S-838, S-858, T-872, T-880) and kinase domain (T-982, T-1049, either S-1044 or T-1045) of BRI1 were phosphorylated. Mutations of the threonines identified as phosphorylation sites in the activation loop abolished normal BRI1 signaling in plants. Wang et al. used the same method to show that phosphorylation of the three threonines (T-446, T-449, T-455) in the activation loop of the BAK1 kinase domain fused with GST.

Here we show that BRI1 and BAK1 are phosphorylated with different kinetics in response to BL. *bri1* and *bak1* mutants failed to phosphorylate the corresponding intact partner: BAK1 or BRI1, respectively. A mutational analysis of BAK1 also revealed that leucines (L32, L46) in the extracellular domain of BAK1 are important for complex formation with BRI1; in addition, T455 in the activation loop of BAK1 is critical for the kinase activity of BAK1 and for BR signaling.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis thaliana* lines used were from the Columbia ecotype (Col-0). Plants were grown in a growth room at 22°C under long light conditions (16L/8D) with white light. Plant transformation was performed by floral dipping into suspensions of *Agrobacterium tumefeciens* (GV3101) containing appropriate plasmid constructs. Seeds were sterilized with 75% ethanol containing 0.05% Tween-20 followed by washing twice with 95% ethanol, and germinated in 1/2 MS (Duchefa) and 0.8% phytoagar supplemented with appropriate antibiotics.

Construction of plasmids

We amplified entire BRI1 open reading frames with a C-terminal primer containing the coding sequence of an HA epitope tag, and cloned the resulting PCR product into the pYES2 vector (Clontech). We also amplified BAK1 and cloned it into the pESC vector (Stratagene), which has a Myc epitope (Nam and Li, 2002). To generate mutant BAK1, *in vitro* site-directed mutagenesis was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) with pESC-BAK1 as template. All the resulting plasmids were fully sequenced to confirm the presence of the intended changes as well as the absence of other alterations. For plant transformation with *BAK1(L46E), BAK1(T455A),* and *BAK1(3TA)*, we performed in vitro site-directed mutagenesis of the binary vector construct, *pPZP212-BAK1-BAK1:GFP* as template (Nam and Li, 2002) using the QuickChange Site-Directed Mutagenesis Kit. After confirmation, each plasmid was transformed into wild type and *bri1-301* plants by *Agrobacterium tumefaciens*-mediated floral dipping.

In vivo phosphorylation assay

We performed the phosphorylation assay *in vivo* following procedure by Wang et al. (2001) with some modifications. Seeds of the plants to be assayed were sterilized and grown in 1/2 MS liquid medium containing 1 μM brassinazole (BRZ), biosynthetic inhibitor of BRs (Asami et al., 2000), in a shaking incubator at 100 rpm with a light cycle of 9L/15D (in hours). After 10 days, seedlings were washed several times with distilled water, transferred to fresh 1/2 MS liquid medium containing 1 μM BL (Synthchem, Inc.), and incubated further in the same conditions for the indicated times. Total protein was extracted with extraction buffer [50 mM HEPES (pH 7.4), 10 mM EDTA, 0.1% Triton X-100, protease inhibitor cocktail (1 tablet/50 ml, Roche)]. Supernatants were recovered after centrifugation at 15,000 × *g* for 30 min at 4°C. Protein concentrations were measured with the Bio-Rad protein assay kit based on the Bradford method (Bradford, 1976). Equal amounts of total protein were run by 6% SDS-PAGE at 60 V overnight with the BIO-RAD PROTEAN II cooling system, 16 × 20 cm (W × L) gel size. Western blot analysis was carried out with primary antibodies [anti-GFP antibody (Molecular Probe), anti-BAK1 antibody, anti-BRI1 antibody] and peroxidase-conjugated secondary antibodies (Goat anti-rabbit IgG, Pierce). Protein bands were visualized with a SuperSignal West Pico Chemiiluminescence Substrate kit (Pierce).

Generation of anti-BAK1 antibody

We amplified a part of the C-terminal kinase domain containing 173 amino acids of the *BAK1* open reading frame and cloned it into pMALc2 to create an MBP fusion. Recombinant proteins were purified according to the manufacturer's recommendation (New England BioLab). Purified proteins were used for antibody production (Lab Frontier, Inc).

Co-immunoprecipitation and *in vitro* kinase assay of BRI1 and BAK1 in yeast

Appropriate pairs of BRI1/BAK1 constructs were co-transformed into YPH499 yeast cells (Stratagene). The co-transformed yeast was grown in synthetic dropout medium containing 2% galactose to induce protein formation. Total proteins were extracted with extraction buffer [50 mM HEPES (pH 7.4), 10 mM EDTA, 0.1% Triton X-100, protease inhibitor cocktail (1 tablet/50 ml, Roche)] and crude extracts were immunoprecipitated with anti-BRI1 antibody overnight at 4°C. Protein A-Sepharose (nProtein A Sepharose 4 Fast flow, GE Healthcare) beads were added and incubation continued for 90 min more.

The beads were washed twice with IP Washing buffer A [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Triton X-100] and twice with IP Washing buffer B [50 mM Tris (pH 8.0), 0.1% Triton X-100]. We added 2× SDS sample buffer to the final bead-pellets; the precipitated proteins were separated by 7.5% SDS-PAGE. Western blot analysis was carried out with primary antibodies: anti-HA antibody (Roche), anti-MYC antibody (Calbiochem), and peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, rabbit anti-mouse IgG, Pierce). Immunoprecipitated and co-immunoprecipitated protein bands were visualized with the SuperSignal West Pico Chemiiluminescence Substrate kit. *In vitro* kinase assays were carried out with the final washed immunoprecipitates bound to Protein A Sepharose beads, as described in Nam and Li (2002).

RESULTS

BRI1 and BAK1 are phosphorylated with different kinetics in response to BL

BL-dependent phosphorylation of BRI1 at the plasma membrane is an initial step in the transduction of BR signals (Wang et al., 2001; 2005b). Binding of brassinolide to BRI1 triggers the association of BAK1 with BRI1; the resulting activation of BRI1 and BAK1 kinase activities leads to phosphorylation of threonine residues on both proteins (Wang et al., 2005a). We wished to establish the detailed mechanisms by which BRI1 and BAK1 transphosphorylate each other, and how phosphorylation status is changed in mutants of the corresponding genes.

To address these questions, we generated transgenic plants overexpressing *BRI1* or *BAK1* fused with *GFP* under the control of their own promoters. First, we checked whether the GFP-tagged BRI1 and BAK1 yielded the phenotypic changes known to result from overexpression of these genes. The transgenic plants indeed showed the typical phenotypic changes due to increased BR signaling, namely narrow and longer leaves and petioles, indicating that the C-terminal GFPs in BRI1 and BAK1 did not interfere with the function of BRI1 or BAK1 (Fig. 1A). To examine the kinetics of BRI1 and BAK1 phosphorylation, we collected seedlings of *pPZP212-BRI1-BRI1:GFP* transgenic plants treated with 1 μM BL for different length of time and analyzed total protein by SDS-PAGE and western blotting to assess the changes in BRI1 and BAK1 phosphorylation-mediated mobility (Wang et al., 2001). We found that the mobility of BRI1 decreased in response to BL treatment for 20 min; altered pattern was maintained for up to 2 h of BL treatment. BAK1 mobility changes were also detected in the sample exposed to BL for 20 min. However, the BAK1 mobility shift had disappeared by 80 min, and longer exposure to BL did not induce further phosphorylation of BAK1 (Fig. 1B). In the *BAK1-GFP* transgenic plants, the pattern of BRI1 phosphorylation was similar to the results obtained with *BRI1:GFP* plants, but phosphorylated BAK1 was still detected after 2 h of BL treatment. These results are probably due to transgenic BAK1-GFP protein still present in addition to the endogenous BAK1 (Fig. 1C). These results indicate that both BRI1 and BAK1 are phosphorylated in response to BL, and that BRI1 may respond to BL for longer than BAK1.

Mutation of BRI1 or BAK1 abolishes phosphorylation of its counterpart

The LRR21 (formerly LRR22) of BRI1, immediately following the 70 amino acid island (Vert et al., 2005), is known to be important for BL binding (Kinoshita et al., 2005). Since *bri1-9* has a mutation in LRR21, we expected that BL binding might be impaired. We therefore examined the phosphorylation status of BAK1 in the *bri1-9* mutant, and of BRI1 in the *bak1* mutant. BL

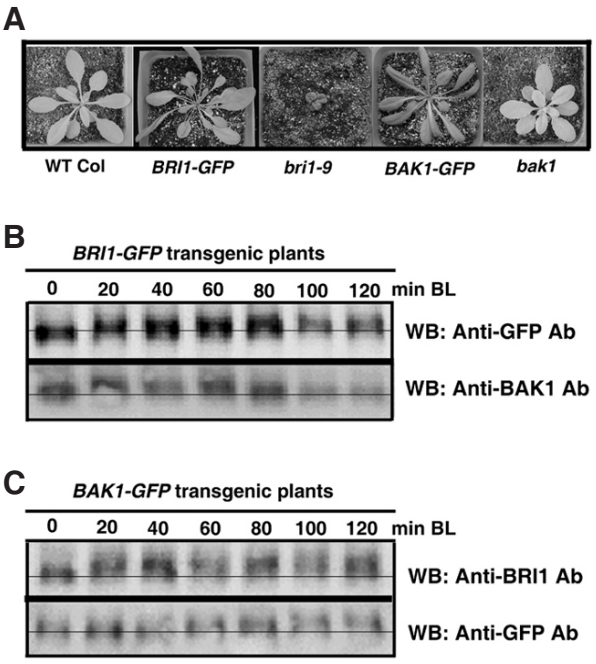

Fig. 1. *In vivo* phosphorylation of BRI1 and BAK1. (A) Comparison of phenotypes of *BRI1-* and *BAK1*-overexpressing plants containing *pPZP212-BRI1-BRI1:GFP* and *pPZP212-BAK1-BAK1:GFP*, respectively, with the wild type (Col-0), *bri1-9* and *bak1* mutants. The pictures are of 4-week-old plants. (B) *In vivo* phosphorylation status of BRI1 and BAK1 in *pPZP212-BRI1-BRI1:GFP* transgenic plants incubated with brassinolide (BL) for the indicated times. BRI1 and BAK1 were detected with anti-GFP and anti-BAK1 antibodies, respectively. (C) *In vivo* phosphorylation of BRI1 and BAK1 in BAK1-overexpressing plants (*pPZP212-BAK1-BAK1:GFP*) as in (B). BRI1 and BAK1 were detected with anti-BRI1 antibodies and anti-GFP antibodies, respectively.

treatment did not induce BAK1 mobility changes in *bri1-9*, or of BRI1 mobility changes in *bak1* (Figs. 2A and 2B), despite the fact that the partner of each mutant was wild type. These results show that both BRI1 and BAK1 are phosphorylated upon stimulation by BL, and that each protein is primarily transphosphorylated by its partner.

Specific residues of BRI1 and BAK1 critical for the binding and transphosphorylation activities of BL receptor complexes

We previously showed that expression of recombinant proteins in yeast was very useful for expressing intact proteins, especially plasma membrane proteins, which can be very difficult to express in prokaryotic cells (Nam and Li, 2002). Therefore, to examine which specific residues are important for the binding and transphosphorylation activities of BRI1 and BAK1, we expressed site-directed BRI1 and BAK1 mutants in yeast. Many of the known *bri1* alleles are mutated in either their extracellular domains or intracellular kinase domains. This implies that both domains are necessary for full activity of the BRI1 protein (Friedrichsen et al., 2000; Vert et al., 2005). We examined whether both domains of BAK1 also influence its binding to BRI1 and kinase activity. Based on previous publications and a comparison of the amino acid sequences of BAK1 and BRI1, we selected several bases for mutation as shown in Fig. 3A. The conserved cysteine residue in the first cysteine pair of BAK1

Table 1. Summary of the interaction between extracellular domain-mutagenized BAK1 and BRI1 in yeast

BRI1	BAK1	Binding to BRI1	Transphosphorylation
WT BRI1	L32E	Very weak	Yes
	L46E	Very weak	No (less than 10%)
	C64Y	Normal	Yes
	L94E	Normal	Yes
	L121E	Normal	Yes
	S172F	Normal	Yes

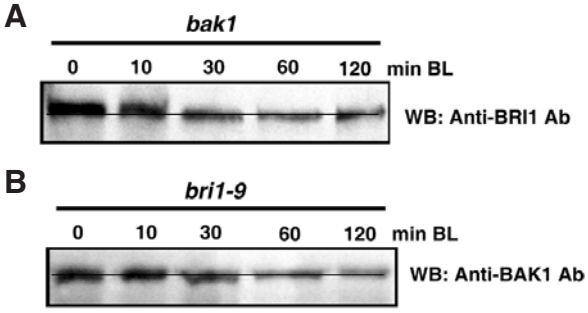

Fig. 2. Neither BRI1 nor BAK1 are phosphorylated by BL when their partner is mutated. *In vivo* phosphorylation of BRI1 in *bak1* (A) and of BAK1 in *bri1-9* mutants (B) was observed similarly as in Fig. 1. BRI1 and BAK1 were detected using anti-BRI1 and anti-BAK1 antibodies, respectively.

was changed to tyrosine, BAK1(C64Y), which mimics the mutation in *bri1-5*; several leucines in the leucine zipper and LRR domains of BAK1 were changed to glutamic acids (BAK1 (L32E), BAK1(L46E), BAK1(L94E), BAK1(L121E)). Serine 172 of BAK1 was mutagenized to phenylalanine, BAK1(S172F), which is reminiscent of the *bri1-9* mutation (Noguchi et al., 1999). We co-transformed each of these extracellular domain-mutagenized *BAK1* constructs with wild type *BRI1* into yeast, and then detected the immunoprecipitated BRI1 with anti-HA antibody and the co-immunoprecipitated mutated BAKs with anti-Myc antibody. *In vitro* kinase assays of the immunoprecipitates were also carried out. The results are summarized in Table 1. BAK1(C64Y), BAK1(L94E), BAK1(L121E) and BAK1 (S172F) bound to BRI1 and retained kinase activity. However, the L32E and L46E substitutions in the leucine zipper of BAK1 led to a reduction of binding between BRI1 and BAK1, and barely any phosphorylated BRI1 and BAK1 were detected when BAK1(L46E) was co-expressed with BRI1 (Table 1 and Fig. 3B).

We also examined the effects of mutating the cytoplasmic kinase domain of BAK1 on its binding to BRI1 and the subsequent activation of kinase activity (Table 2). We previously showed that substitution of the lysine in the catalytic core (K317E), and of the threonine in the activation loop of the kinase domain (T455A) abolished its transphosphorylation activity, even though the mutated BAK1 still bound wild type BRI1 (Nam and Li, 2002). We tested the effect of changing each of the other three threonines in the activation loop of BAK1 kinase subdomain VII. Single changes of the threonines at positions 446, 449, and 450 did not affect binding affinity or kinase activity. However, when we changed all three threonines to alanines (3TA), BAK1 lost its kinase activity, although it still bound BRI1 (Fig. 3C). This indicates that the threonine residues at 446, 449 and 450 contribute to BAK1 activity.

Table 2. Summary of the interaction between intracellular serine/threonine kinase domain-mutagenized BAK1 with BRI1 in yeast

BRI1	BAK1	Binding to BRI1	Transphosphorylation
WT BRI1	K317E	Normal	No
	T455A	Normal	No
WT BRI1	T446A	Normal	Yes
	T449D	Normal	Yes
	T450D	Normal	Yes
	T455D	Normal	No
	3TA	Normal	No (10%)

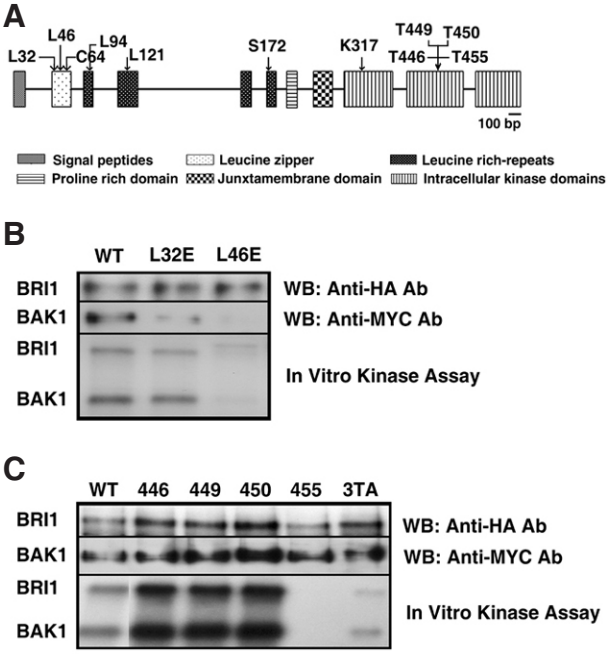

Fig. 3. Identification of specific amino acids critical for the formation and activation of BRI1/BAK1 receptor pairs. (A) Schematic feature of BAK1 genomic structure. Squares represent exons and lines between them introns. Specific mutagenized amino acid residues are marked. (B) Mutations in the leucine zipper of BAK1 affect the binding affinity of BRI1 and BAK1 in yeast. Anti-HA antibody immunoprecipitates HA-tagged BRI1 and co-immunoprecipitated BAK1 proteins from yeast extracts were detected by western blot analysis. Phosphorylation activities of the immunoprecipitates were determined using γ-[^{32}P]-ATP. (C) Mutations of threonine residues in the activation loop of the kinase domain of BAK1 were analyzed in yeast as in (B).

Transgenic plants reveal T455A in BAK1 critically impacts activity

To investigate the roles of important residues of BAK1 in BR signaling, we generated transgenic plants containing the L46E, 3TA and T455A mutations.

Neither wild type nor *bri1-301* plants transformed with *BAK1(L46E)* displayed any noticeable phenotypic change in development, although a very small number of the transgenic *bri1-301* plants had severe *bri1* phenotypes (Figs. 4A-4E). Later in development, however, more than 60% of the trans-

genic *bri1-301* plants displayed reduced apical dominance, and defects in seeds development (Figs. 4F, 4G, and 4H).

Overexpression of *BAK1(T455A)* led to severe phenotypic changes. Many of the transgenic wild type plants (37.2%, 16 out of 43 plants) exhibited *bak1* mutant phenotypes (Figs. 5A and 5B), indicating that a dominant-negative mutation of the BAK1 protein bound to endogenous BRI1. When we measured various growth parameters quantitatively, we found that the transgenic plants containing *BAK1(T455A)* were a little bit shorter (10%) than the wild type. Leaf petiole length was more strongly affected (≥ 40% reduction), making the plants look like *bak1* mutants (Fig. 5B). Furthermore, some of the transgenic plants were more like *bri1-301* mutant, one of the weak *bri1* alleles (Figs. 5A-5C). We sorted them based on severity of phenotypic changes and designated them arbitrarily as exhibiting a phenotype 1) similar to that of *bak1*, 2) similar to that of *bri1-301*, or 3) with leaves that were severely twisted (Figs. 5A-b, c, and d). We then asked whether phenotypic severity was correlated with the level of expression of mutated BAK1(T455A) protein using C-terminally GFP-tagged BAK1(T455A). We performed western blot analyses with anti-GFP antibody of total proteins extracted from transgenic plants of the representative phenotypes. We found, as anticipated, that there were higher levels of BAK1(T455A) in transgenic plants exhibiting a *bak1*-similar phenotype (Fig. 5C); moreover, BRI1 and BAK1 were not phosphorylated in these plants (Fig. 5D). We observed similar effects in the *bri1-301* background, as well; indeed most of the transgenic *bri1-301* (50 out of 52 plants) looked like strong *bri1* mutants with severe dwarfism (Figs. 5A-f, g, and h). Taken together, these results and previous data indicate that T455 is critical for the activity of BAK1 in Arabidopsis.

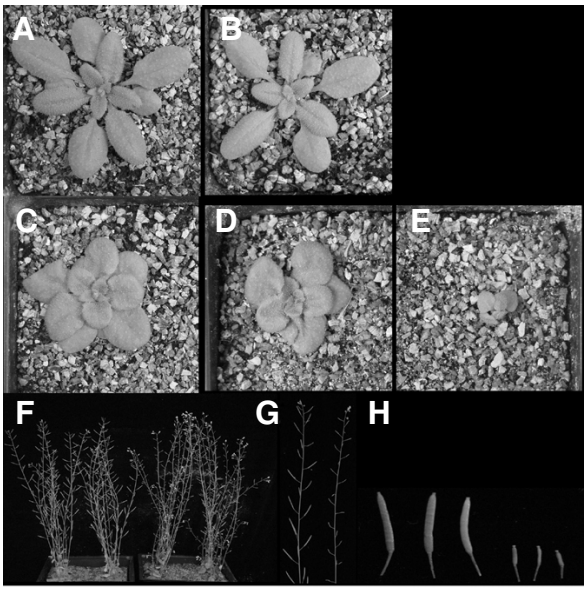

Fig. 4. Phenotypic analysis of transgenic plants expressing BAK1(L46E). (A, B) A wild type plant (A) and a transgenic plant containing *BAK1(L46E)* (B). (C, D, and E) A *bri1-301* mutant plant (C) and two independent transgenic *bri1-301* plants containing *BAK1(L46E)* (D and E). Pictures (A to E) are of plants grown side by side for 4 weeks in long-day conditions. (F) A 9-week old *bri1-301* (in the left pot) and transgenic *bri1-301* containing *BAK1(L46E)* (in the right pot). (G, H) Influorescence and silique phenotypes of the plant in panel F displaying poor seed development in *bri1-301*-containing *BAK1(L46E)*.

DISCUSSION

Since BRI1 and BAK1 were identified as essential components of plasma membrane BR receptor complexes (Li et al., 2002; Nam and Li, 2002), they have become one of the best known protein pairs in plant hormone signaling. Because they encode plasma membrane-localized leucine rich-repeats serine/threonine receptor-like kinases, it has been assumed that BRI1 and BAK1 signal via protein phosphorylation/dephosphorylation.

BL-dependent transphosphorylation of BRI1 and BAK1

There has been controversy about how phosphorylation of BRI1 and BAK1 occurs, and whether each protein acts as a substrate for its partner. We demonstrated here that BRI1 and BAK1 are phosphorylated by their respective partners after 20 min of incubation with BL. If this time lag represents the delay in response to BL, it would support the finding that BR-responsive genes are induced after 20 to 30 min of BR treatment (Goda et al., 2002; 2004). The lag time for BR is known to be longer than that for other plant hormones, such as auxin or GA. Usually auxin-inducible genes begin to be expressed after less than 10 min in soybean and Arabidopsis (Liu et al., 1994; Zurek et al., 1994). We also showed that phosphorylation of BRI1 persists longer than does phosphorylation of BAK1: phosphorylation of BRI1 persisted for 2 h regardless of BRI1 overexpression, whereas BAK1 in BRI1 overexpressing plants was dephosphorylated within 80 min, suggesting that BL treatment for longer than 80 min leads to desensitization of the plants with respect to BAK1 phosphorylation. It is also possible that the amount of BAK1 available for transducing the BR signal was insufficient, because dephosphorylated BAK1 was not detected in BAK1-overexpressing plants containing sufficient BAK1. We suggest that these observations indicate that BAK1 is critical for regulating the duration of effectiveness of BR in plants.

It appears that BRI1 and BAK1 phosphorylate each other via preferential transphosphorylation. Although the autophosphorylation activity of BAK1 was reported using recombinant proteins *in vitro*, the degree of its own activity was weak. However, the amount of phosphorylated BAK1 was dramatically increased in the presence of recombinant BRI1 (Li et al., 2002). Wang et al. (2005a) confirmed that only when BRI1 and BAK1 were simultaneously present, did phosphorylation on the two proteins increase in response to BL. We demonstrated above that BAK1 was not phosphorylated in *bri1* plants containing wild type BAK1, nor was BRI1 phosphorylated in *bak1* plants (Fig. 2), implying that upstream kinases capable of phosphorylating BRI1 and BAK1 do not exist. Therefore, BAK1 appears to be a substrate of BRI1, and BRI1 a substrate of BAK1. Database search revealed that there are four close BAK1 homologs in Arabidopsis genome. Among them, AtSERK1 was shown to be involved in BR signaling as a putative interactor with BRI1, too (Karlova et al., 2006). However, none of the single mutant of BAK1 homologs reveals noticeable phenotypic changes as did *bak1* mutant. This suggests that BAK1 is major co-receptor of BRI1, even though other BAK1 homologs play minor roles in BR signaling in physiological conditions.

BRI1 can also phosphorylate other downstream proteins. It has been reported to phosphorylate a homolog of mammalian TGF-beta receptor interacting protein (TRIP1) (Ehsan et al, 2005), trans-thyretine like protein (TTL) (Nam and Li, 2004) and BRI1 kinase inhibitor 1 (BKI1) (Wang and Chory, 2006). BKI1 and TTL are thought to be negative regulators that are phosphorylated by BRI1 upon BL treatment; TRIP1 may be a growth regulator. There has been no report of any substrate of BAK1

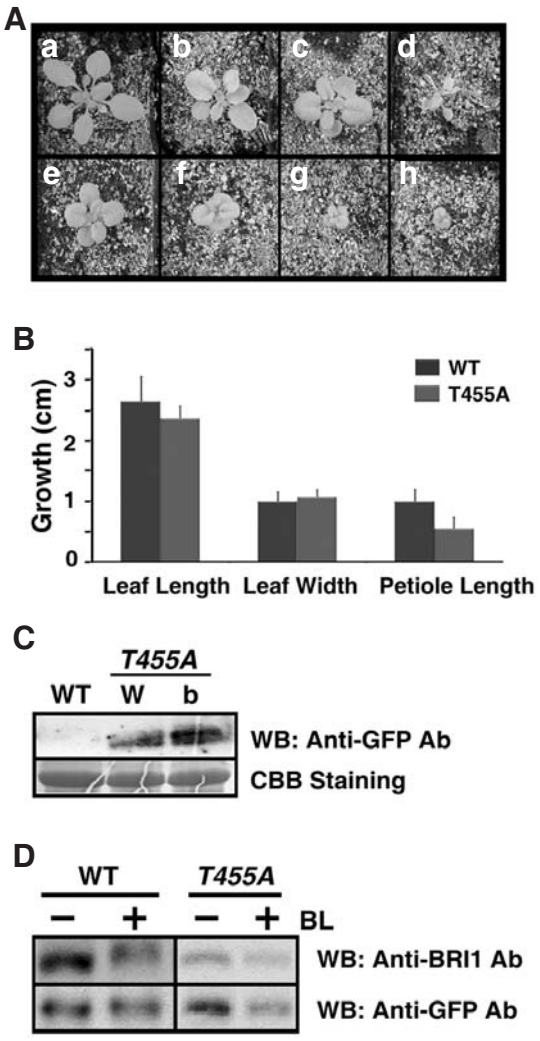

Fig. 5. T455 of BAK1 is critical for activity. (A) Phenotypes of three independent transgenic wild type plants containing *BAK1(T455A)* (b, c, and d) and the wild type (Col-0) (a) in the upper row. In the lower row, are three independent transgenic *bri1-301* plants containing *BAK1(T455A)* (f, g, and h), and the parental *bri1-301* (e). Pictures are of 3-week-old plants. (B) Growth of the wild type and transgenic plants containing *BAK1(T455A).* The three longest leaves in each of 15 plants grown for 4 weeks were used to measure leaf length and width, as well as petiole length (n = 45). Bars denote standard errors. (C) Western blot analysis of the expression of the mutated BAK1 proteins. Total proteins were extracted from the leaves of transgenic plants phenotypically similar to wild type (T455A-W) or to *bak1* plants, (T455A-b); proteins were detected with anti-GFP antibodies. (D) *In vivo* phosphorylation assays of the transgenic plant containing *BAK1(T455A).* Ten-day seedlings grown in brassinozole-containing 1/2 MS were treated with BL for 1 h as described in "Materials and Methods". BRI1 and BAK1 were detected with anti-BRI1 antibodies and anti-GFP antibodies, respectively.

other than BRI1. Screening for BAK1 interactors using various approaches may reveal additional components of BR signaling. We cannot rule out the possibility that some BAK1 substrates and/or interactors have different functions unrelated to BR signaling, because BAK1 has been reported to form a complex with FLS2, a pattern-recognition receptor for flagellin (Chinchilla et al., 2007; Hesse et al., 2007). We have also detected binding between BAK1 and BAK1 homologs in plant cells (data not shown here). However, it seems unlikely that similar kinds of transphosphorylation events to those occurring in the BRI1/BAK1 complex also take place in any other complexes involving BAK1.

Specific residues of BAK1 are critical for the binding and transphosphorylation activities of the BL receptor complex

Using a combination of site-directed mutagenesis and recombinant protein induction in yeast, we investigated residues that might be critical for binding and kinase activation. Leucines 32 and 46 are localized in the leucine-zipper region preceding the LRR domain in BAK1. An L32E mutant displayed partial loss of binding to BRI1 in yeast, while L46E lost all binding activity. We expected that overexpression of BAK1(L46E) would not affect binding of the endogenous BAK1 to BRI1, because BAK1 (L46E) does not bind to BRI1. This assumption seemed to be correct in a wild type background. However, it was somewhat surprising that *BAK1(L46E),* transformants in a *bri1-301* background exhibited severe *bri1* phenotypes. We presume that the BAK1(L46E) protein binds to the wild type BAK1, and prevents it from binding to BRI1. In wild type plants, BRI1 is fully functional, so that even though some of the endogenous BAK1 may be prevented from binding to BRI1, sufficient numbers of BRI1/BAK1 complexes are formed to maintain cellular functions.

In our work on the cytoplasmic kinase domain of BAK1, we focused on three additional threonine residues in the activation loop to extend our previous work on the K317E and T455A mutations (Nam and Li, 2002). MALDI-TOF analysis using the GST-tagged recombinant kinase domain of BAK1 has shown that threonines 446 and 449 are phosphorylated (Wang et al., 2005a). However, defects at any of these sites including T450 are not likely to affect the activation of BRI1 and BAK1 kinase activities, since BAK1 proteins mutated at these sites have normal BRI1-mediated transphosphorylation activity in yeast (Fig. 3C). T446, T449, and T450 in BAK1 corresponds to T459, T462,and T463 in Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (AtSERK1) (Shah et al., 2001). AtSERK1 is highly homologous to BAK1 and is a marker gene for embryogenic competence. Shah et al. (2001) demonstrated the autophosphorylation and substrate phosphorylation activities of the recombinant AtSERK1 kinase domain. They found that mutation of T462 (corresponding to T449 of BAK1) partially impaired its autophosphorylation activity, and abolished its action on casein and myelin basic protein (MBP) *in vitro*. We did not observe this kind of partial inhibitory effect following mutation of T449 in BAK1. The double mutations T446A/T449A, T446A/T450A, and T449A/T450A also failed to affect phosphorylation of BRI1 and BAK1 (data not shown). Only when all three threonines were mutated did BAK1 lose most of its kinase activity, implying that these threonines may play minor roles in regulating the activation of BAK1 kinase activity. This assumption is supported by the fact that transgenic plants containing BAK1(3TA) had no phenotypic defects (data not shown). In contrast, alteration of T455 in BAK1 was closely related to the loss of BAK1 kinase activity and the resultant phenotypic abnormalities, since the severity of the phenotypic changes was correlated with the expression level of mutated BAK1. T455 in BAK1 corresponds to T1049 in the activation loop of BRI1, and overexpression of BRI1(T1049A) in weak *bri1-5* mutants results in smaller plants due to a dominant negative effect (Wang et al., 2005a).

Based on the results presented here, we suggest that BRI1 and BAK1 transphosphorylate each other, with different kinetics,

once they form a complex in response to BL. Mutations in either partner protein abolish phosphorylation of both proteins. Leucines in the leucine zipper region of BAK1 are required for interaction between the two proteins, indicating that the extracellular domains of these LRR-RLKs are important for their interaction. T455, in the activation loop of BAK1, in addition to the catalytic K317, is critical for activation of its kinase activity.

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