Interaction of Arabidopsis BRASSINOSTEROID-INSENSITIVE 1 receptor kinase with a homolog of mammalian TGF-β receptor interacting protein

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Summary

Brassinosteroids (BRs) regulate multiple aspects of plant growth and development and require an active BRASSINOSTEROID-INSENSITIVE 1 (BRI1) receptor serine/threonine kinase for hormone perception and signal transduction. In mammals, the transforming growth factor-beta (TGF-β) family of polypeptides modulate numerous aspects of development and are perceived at the cell surface by a complex of type I and type II TGF-β receptor serine/threonine kinases. TGF-β receptor interacting protein (TRIP-1) is a cytoplasmic substrate of the TGF-β type II receptor kinase and plays a role in TGF-β signaling. TRIP-1 is a WD domain protein that also functions as an essential subunit of the eIF3 eukaryotic translation initiation factor in animals, yeast and plants. We previously cloned putative TRIP-1 homologs from bean and Arabidopsis and found that transgenic Arabidopsis plants expressing antisense TRIP-1 RNA exhibited a broad range of developmental defects including some morphological characteristics that resemble the phenotype of BR-deficient and -insensitive mutants. We now show that the BRI1 kinase domain phosphorylates Arabidopsis TRIP-1 on three specific sites *in vitro* (Thr-14, Thr-89 and either Thr-197 or Ser-198). Co-immunoprecipitation experiments using antibodies against TRIP-1, BRI1 and various fusion proteins strongly suggest that TRIP-1 and BRI1 also interact directly *in vivo*. These findings support a role for TRIP-1 in the molecular mechanisms of BR-regulated plant growth and development, possibly as a cytoplasmic substrate of the BRI1 receptor kinase.

Keywords: brassinosteroids, BRI1, BAK1, TRIP-1, receptor kinase, TGF-β.

Introduction

Brassinosteroids (BRs) are required for the normal expression of a variety of developmental programs including cell elongation, vascular differentiation, seed germination, sensescence and male fertility (Altmann, 1999; Clouse and Sasse, 1998). The emerging picture of BR signal transduction reveals that plant steroids, in contrast to the primary mode of action of animal steroid hormones, are perceived at the cell surface by one or more members of the large family of leucine-rich repeat receptor-like kinases (LRR RLKs) found in plants (Bishop and Koncz, 2002; Clouse, 2002; Friedrichsen and Chory, 2001). The BRI receptor kinase has been shown by mutational analysis in Arabidopsis, rice, tomato and pea to be absolutely required for normal BR perception and plant growth (Clouse *et al*., 1996; Friedrichsen *et al*., 2000; Koka *et al*., 2000; Li and Chory, 1997; Montoya *et al*., 2002; Noguchi *et al*., 1999; Yamamuro *et al*., 2000). Furthermore, biochemical studies in Arabidopsis have shown that BRASSINOSTEROID-INSENSITIVE 1 (BRI1) is an essential component of the BR receptor complex (Wang *et al*., 2001), and that the extracellular domain of BRI1 can bind BRs directly (Kinoshita *et al*., 2005).

A common mechanistic property associated with many receptor kinases is ligand-dependent homo or heterodimerization, followed by kinase activation and subsequent

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phosphorylation of cytoplasmic substrates involved in the specific signaling pathway (Becraft, 2002; Heldin, 1995; Schlessinger, 2002). The recent discovery of BRI1-associated receptor kinase 1 (BAK1), a second LRR RLK that interacts with BRI1 *in vitro* and *in vivo*, suggests that receptor kinase heterodimerization may play an important role in BR signal transduction (Li *et al*., 2002; Nam and Li, 2002; Russinova *et al*., 2004). If dimerization of BRI1/BAK1 occurs in association with BR perception, it would suggest similarities to both animal receptor tyrosine kinase and TGF-β receptor activities. The TGF-β receptor complex consists of a heterotetramer of type I (RI) and type II (RII) receptor kinase pairs. Ligand binding induces formation of the heterotetramer and phosphorylation of RI by RII on specific Thr and Ser resides in a region immediately preceding the RI kinase domain. The activated RI kinase propagates the signal by phosphorylating signaling proteins, termed Smads, which translocate to the nucleus where they form complexes with transcription factors (Massague, 1998; Shi and Massague, 2003).

In addition to transphosphorylation of RI, RII also specifically phosphorylates several non-Smad cytoplasmic proteins, including TRIP-1, a WD-domain protein that has been shown by co-immunoprecipitation experiments to interact directly with the RII kinase domain *in vivo* (Chen *et al*., 1995; Choy and Derynck, 1998). We previously cloned putative TRIP-1 homologs from bean and Arabidopsis and found that antisense suppression of TRIP-1 in transgenic Arabidopsis plants resulted in extreme dwarfism, altered leaf morphology, delayed senescence and a bushy phenotype in mature plants (Jiang and Clouse, 2001). These morphological characteristics resemble BR-deficient and insensitive mutants, including severe alleles of *bri1*, which result from an inactive BRI1 receptor kinase.

Downstream components of BR signaling have now been identified, including BIN2 (BR Insensitive 2), a cytoplasmic Ser/Thr kinase whose catalytic domain shares 70% sequence similarity with the *Drosophila* shaggy kinase and mammalian glycogen synthase kinase-3 (GSK-3) (Li and Nam, 2002). Genetic and biochemical evidence suggests that BIN2 is a negative regulator of BR signaling which functions by phosphorylating and inactivating two critical proteins, BZR1 and BES1, required for BR-regulated gene expression in a process analogous to the Wingless/wnt signal transduction pathway in *Drosophila* and vertebrates (Choe *et al*., 2002; He *et al*., 2002; Li and Nam, 2002; Wang *et al*., 2002; Yin *et al*., 2002). BZR1 plays a dual role in regulating BR biosynthesis and downstream growth responses and acts as a transcriptional repressor that binds directly to the promoters of genes encoding BR biosynthetic enzymes (He *et al*., 2005). The structurally related BES1 is a transcription factor that binds to promoters of BR-activated genes, both independently and as a heterodimer with basic helix-loop-helix proteins (Yin *et al*., 2005).

A serious gap in our understanding of BR signal transduction remains between BR perception at the cell surface and subsequent inactivation of BIN2 in the cytoplasm, making identification of *in vivo* substrates of BRI1 and BAK1 a high priority for a complete understanding of BR signaling. Recently, a yeast two-hybrid screen was used to identify Arabidopsis transthyretin-like protein (TLL) as a potential BRI1 substrate (Nam and Li, 2004). The BRI1 kinase domain phosphorylates TLL *in vitro* and genetic evidence suggests that TLL is a negative regulator of BR-mediated plant growth. We report here that Arabidopsis TRIP-1, a protein with sequence similarity to a mammalian substrate of the TGF-β RII receptor kinase, is phosphorylated *in vitro* by BRI1 and associates directly with BRI1 *in vivo*, suggesting that TRIP-1 is also a potential BRI1 substrate.

Results

When the Arabidopsis BRI cytoplasmic kinase domain is expressed in *Escherichia coli* (Flag-BRI1-KD), an active Ser/Thr kinase is obtained that undergoes autophosphorylation and phosphorylates synthetic peptide substrates with a conserved sequence motif (Oh *et al*., 2000). Arabidopsis TRIP-1 (Jiang and Clouse, 2001) was expressed in *E. coli* with a short His_7 N-terminal tag containing no Ser or Thr residues (H_7-TRIP-1) and was affinity purified on a Ni column. When affinity purified Flag-BRI-KD was incubated with H_7-TRIP-1 and [γ-^{32}P]ATP, significant phosphorylation of H_7-TRIP-1 was observed in addition to the expected autophosphorylation of Flag-BRI-KD (Figure 1a). No phosphorylation was detected when an inactive kinase mutant of Flag-BRI-KD was substituted, indicating that the phosphorylation of H_7-TRIP-1 resulted solely from an active BRI kinase domain. We also performed a standard phosphoamino acid analysis to show that Flag-BRI1-KD phosphorylated H_7-TRIP-1 primarily on Thr residues, very slightly on Ser and not detectably on Tyr (Figure 1b).

To determine if TRIP-1 protein purified from the plant was also a substrate for BRI1-KD, total protein was extracted from homozygous T_3 transgenic Arabidopsis lines expressing Flag-TRIP-1 and the extract was immunoprecipitated with anti-Flag antibody. The identity of the immunoprecipitated, PAGE-purified protein was verified by Western immunoblot analysis with a specific anti-TRIP-1 antibody and by identification of TRIP-1 typtic peptides in the gel band with quadrapole time of flight tandem mass spectrometry coupled with capillary liquid chromatography (Q-ToF LC/MS/MS). Figure 1(c) shows that Flag-TRIP-1 immunoprecipitated from plants was a substrate of Flag-BRI1-KD *in vitro*. The Flag-TRIP-1 phosphorylated band appeared more diffuse than the recombinant H_7-TRIP-1 protein phosphorylated under identical conditions, which might be explained by post-translational modifications of TRIP-1 *in vivo*, resulting

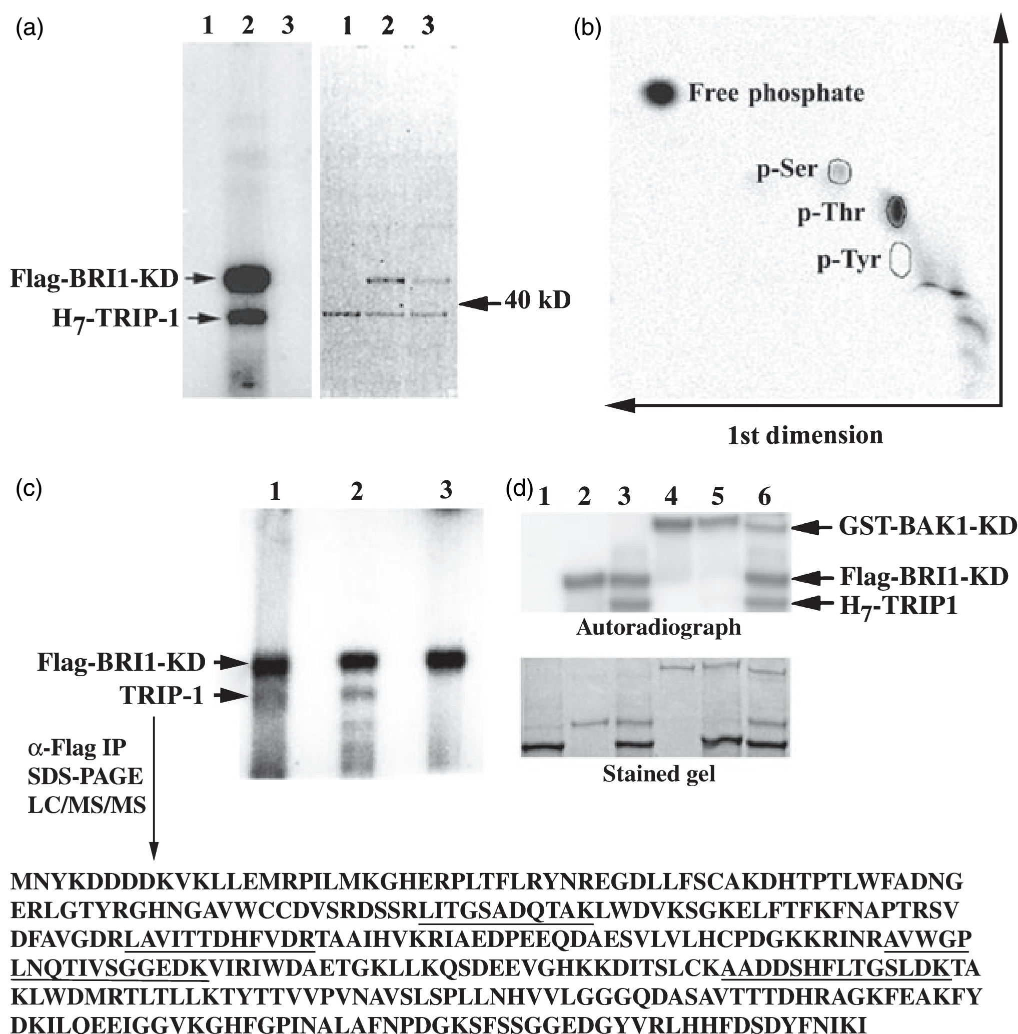

Figure 1. TRIP-1 is phosphorylated *in vitro* by recombinant BRI1-KD but not significantly by BAK1-KD.
(a) Autoradiograph (left panel), stained gel (right panel). Affinity purified recombinant proteins were incubated with [γ-^{32}P]ATP and kinase buffer. 1, H_7-TRIP-1 alone; 2, H_7-TRIP-1 plus Flag-BRI-KD; 3, H_7-TRIP-1 plus mutant Flag-BRI-KD (K911E mutant with no kinase activity).
(b) H_7-TRIP-1, was phosphorylated *in vitro* by Flag-BRI-KD and [γ-^{32}P]ATP. H_7-TRIP-1 was subsequently affinity purified away from Flag-BRI-KD, digested with HCl and subjected to phosphoaminoacid analysis by thin-layer two-dimensional electrophoresis. The position of p-Ser, p-Thr and p-Tyr was determined with phosphoamino acid standards.
(c) Transgenic Arabidopsis plants expressing Flag-TRIP-1 were subjected to total protein extraction and immunoprecipitation with anti-Flag antibody. Immunoprecipitated Flag-TRIP-1 was incubated with Flag-BRI-KD plus [γ-^{32}P]ATP and kinase buffer and subjected to SDS-PAGE followed by autoradiography (Lane 1). Lane 2, affinity purified H_7-TRIP-1 and Flag-BRI-KD; Lane 3, Flag-BRI-KD alone. Identity of the immunoprecipitated protein was verified as TRIP-1 by mass spectrometry. Underlined sequences indicate TRIP-1 tryptic peptides identified by Q-ToF LC/MS/MS.
(d) Equal amounts of H_7-TRIP-1 (6 μg) were incubated with 1 μg of Flag-BRI-KD or GST-BAK1-KD plus [γ-^{32}P]ATP and kinase buffer. 1, H_7-TRIP-1 alone; 2, Flag-BRI-KD alone; 3, H_7-TRIP-1 plus Flag-BRI1-KD; 4, GST-BAK1-KD alone; 5, GST-BAK1-KD plus H_7-TRIP-1; 6, Flag-BRI1-KD plus GST-BAK1-KD plus H_7-TRIP-1.

in a population of molecules with slightly different mobilities in SDS-PAGE.

Li *et al.* (2002) hypothesized that the BRI1/BAK1 interaction is mechanistically similar to the TGF-β signaling pathway. They suggested that BR binds to BRI1 which then activates BAK1 by transphosphorylation. The activated BAK1 would then phosphorylate downstream components. As mentioned above, mammalian type II, but not type I, TGF-β receptor phosphorylates TRIP-1 *in vivo*. If BRI1 is the mechanistic counterpart to the type II receptor, and BAK1 has a role similar to the type I receptor, one would expect BRI1 but not BAK1 to phosphorylate TRIP-1. Figure 1(d) shows that, at least *in vitro*, this is the case as H_7-TRIP-1 is a good substrate for Flag-BRI1-KD but GST-BAK1-KD shows an extremely weak phosphorylation of TRIP-1, visible only on long exposures of the autoradiograph, even though both kinases are active and capable of autophosphorylation.

To identify specific sites of TRIP-1 phosphorylation by Flag-BRI1-KD, we analyzed tryptic peptides derived from phosphorylated H_7-TRIP-1, with Q-ToF LC/MS/MS. This

analysis revealed that Thr-14 and Thr-89 of TRIP-1 are phosphorylated *in vitro* by the BRI1 kinase (Figure 2a,b). A second analysis using MALDI-ToF (matrix-assisted laser desorption ionization time of flight)-MS is consistent with the tryptic peptide DITSLCK having a single phosphorylation site (Figure 2c). Because the MALDI-ToF method of analysis did not yield a direct peptide sequence it was not possible to determine whether Thr-197 or Ser-198 was the phosphorylated residue.

We previously found that Flag-BRI1-KD phosphorylated synthetic peptides *in vitro* and defined a preliminary recognition motif of [RK]-[RK]-X(2)-[ST]-X(3)-[LMVIFY]-[RK], where residues in brackets indicate any of the listed amino acids can occur at that position (Oh *et al*., 2000). The most critical requirements for optimum phosphorylation are positively charged residues at P − 3, P − 4 and P + 5 relative to the phosphorylated residue at $P = 0$. To determine if short peptides corresponding to the phosphorylated sites in recombinant TRIP-1 also served as substrates for Flag-BRI1-KD, we synthesized the peptides TRIP2A (Thr-197/Ser-198 site), TRIP4.1 (Thr-14 site) and TRIP5.1 (Thr-89 site) and performed a standard peptide kinase assay (Figure 3a). All three peptides were phosphorylated by Flag-BRI1-KD, although TRIP2A appeared to be a rather poor substrate compared with the other two peptides. A positive residue at P – 3 or P – 4 is the most critical requirement for optimal phosphorylation of synthetic peptides by BRI1-KD and all three TRIP-1 phosphorylation site peptides satisfied this requirement. An additional requirement for optimal phosphorylation is a positive residue at P + 5.

None of the TRIP peptides have a positive residue precisely at P + 5, but all three have at least one positive residue between P + 4 and P + 8. The site at Thr-14 also has a Y at P + 4, which occurs in the consensus sequence. While the TRIP-1 peptides retain some of the key features of the *in vitro* consensus site, they are not a perfect match and it is not surprising that Trip4.1, the most active peptide substrate was phosphorylated at approximately 50% of the level of SP11 (Oh *et al*., 2000) a synthetic peptide with all of the conserved residues (data not shown). As the peptide recognition motif has not been verified by identification of BRI1 phosphorylation sites in kinase substrates *in vivo*, it is not certain if the peptide data will predict recognition sequences in the tertiary and quaternary environment of *in planta* protein complexes. However, it is interesting that all three of the phosphorylation sites identified thus far in the recombinant TRIP-1 protein contain an Arg or Lys at –3 and/or –4. Site directed mutagenesis of these sites will be required to determine if these residues are critical for phosphorylation by Flag-BRI-KD in the intact H_7-TRIP-1 protein.

Arabidopsis TRIP-1 shares 40–81% sequence identity over the complete 326 amino acids with other known eukaryotic TRIP-1 (eIF3i) homologs (Jiang and Clouse, 2001). To determine if the Thr residues phosphorylated by BRI-KD *in vitro* are conserved in TRIP-1 from other species, amino acid sequences around each phosphorylation site were aligned from Arabidopsis, bean, human, *Drosophila* and fission yeast (Figure 3c). A striking conservation of sequence was observed for the first phosphorylation site (Thr-14), with a corresponding Thr residue occurring in all species examined, as well as an invariant Arg at P − 3, an invariant Tyr at P + 4 and positive residues at P + 3 and P + 6. The sites represented by Thr-89 and Thr197/Ser198 were much less conserved, with the greatest similarity not surprisingly occurring between Arabidopsis and bean. The highly conserved sequence surrounding Thr-14 in all species examined suggests a possible important functional role for this segment of the protein and it would be of interest to determine the phosphorylation status of Thr-14 *in vivo* in the various species.

The *in vitro* phosphorylation data suggests TRIP-1 is a potential candidate for a cytoplasmic substrate of BRI1 *in planta*. To determine if TRIP-1 and BRI1 indeed interact directly *in vivo*, a variety of co-immunoprecipitation experiments were conducted. At least five different experiments with a range of antibodies against tagged and untagged protein were performed, and the results of each was consistent with an *in planta* TRIP-1/BRI1 interaction. The fact that untagged proteins in non-transgenic plants interacted in a similar way to tagged proteins expressed from strong promoters, indicates that the *in vivo* interaction of TRIP-1 and BRI1 in transgenic plants was not an artifact of overexpression of one or the other protein (data not shown). Moreover crude extracts centrifuged at 100 000 ***g*** to remove any insoluble membrane fraction gave similar results to extracts centrifuged at 10 000 ***g***, suggesting that that co-immunoprecipitation of TRIP-1 and BRI1-GFP was not the result of non-specific association of TRIP-1 with membrane vesicles (data not shown). Figure 4 illustrates two experiments with BRI1-GFP plants in which anti-Flag antibody co-immunoprecipitates Flag-TRIP-1 and BRI1-GFP, and anti-GFP antibody co-immunoprecipitates BRI-GFP and TRIP-1. These findings strongly suggest that TRIP-1 and BRI1 are part of the same immunoprecipitable complex in the plant cell.

Discussion

The importance of BRI1 and BAK1 in the initial stages of BR signaling has been clearly established, and there has been significant recent progress in understanding downstream signaling events (reviewed in Clouse, 2002; Peng and Li, 2003). However, identification of cytoplasmic binding partners and kinase domain substrates of BRI1 and BAK1, remains a high priority for completing the overall model of BR signal transduction. Molecular genetic and biochemical approaches have been used to identify *in vivo* substrates of several other plant receptor kinases (Bower *et al*., 1996; Gu

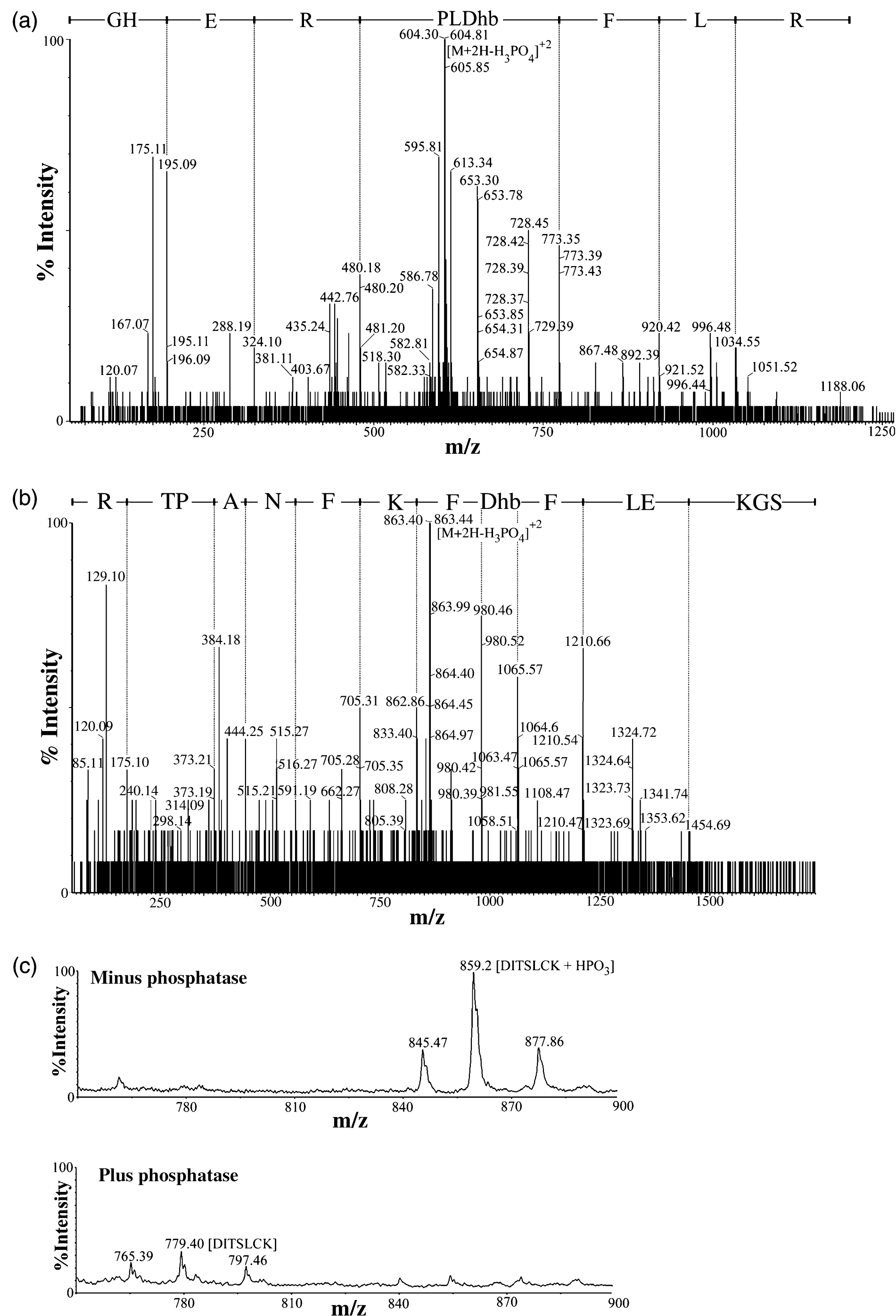

Figure 2. Identification of TRIP-1 *in vitro* phosphorylation sites by mass spectrometry.

(a) H_7-TRIP-1 was phosphorylated by Flag-BRI1-KD, digested with trypsin and subjected to Q-ToF LC/MS/MS analysis after phosphopeptide enrichment on a gallium spin column. Shown is the ion product spectrum (prominent b ions labeled) corresponding to fragmentation of the doubly charged peptide GHERPLpTFLR ($m/z = 653.29$). The peak labeled $[M + 2H\text{-}H_3PO_4]^{+2}$ represents the neutral loss of 98 Da from phospho-Thr to yield 2-aminodehyrobutyric acid (Dhb).

(b) H_7-TRIP-1 was phosphorylated by Flag-BRI1-KD, digested with trypsin and subjected to Q-ToF LC/MS/MS analysis after phosphopeptide enrichment on an iron affinity column. Shown is the ion product spectrum (prominent y ions labeled, sequence shown C to N terminus) corresponding to fragmentation of the doubly charged peptide SGKELFpTKFKNAPTR ($m/z = 911.87$). The peak labeled $[M + 2H\text{-}H_3PO_4]^{+2}$ represents the neutral loss of 98 Da from phospho-Thr to yield 2-aminodehyrobutyric acid (Dhb).

(c) H_7-TRIP-1 was phosphorylated by FLAG-BRI1-KD, digested with trypsin and subjected to MALDI-ToF MS analysis after phosphopeptide enrichment on an iron affinity column. The peak at 859.2 Da is consistent with the tryptic peptide DITSLCK containing a single phosphorylation site. A shift of 80 Da (loss of HPO_3) after phosphatase treatment confirms phosphorylation of the peptide.

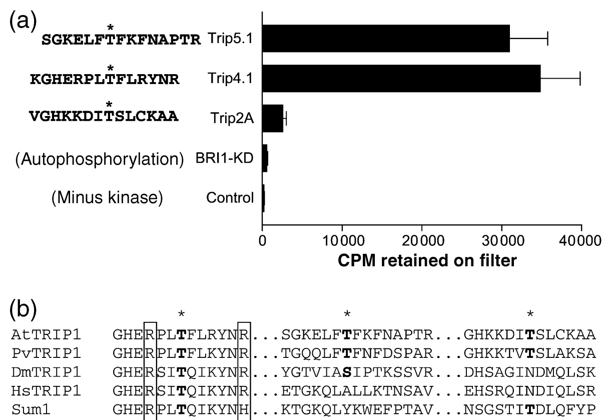

Figure 3. Flag-BRI1-KD phosphorylates synthetic peptides corresponding to the *in vitro* TRIP-1 sites.
(a) Flag-BRI1-KD was incubated with the indicated peptides in the presence of [γ-^{32}P]ATP and kinase buffer. Aliquots of the reactions were applied to P81 phosphocellulose paper and washed extensively with dilute phosphoric acid. Incorporation of ^{32}P into synthetic peptides was quantified using liquid scintillation spectrometry.
(b) Alignment of the sequence surrounding each identified phosphorylation site (*) of Arabidopsis TRIP-1 (AtTRIP1, AAC62878.1) with TRIP-1 from bean (PvTRIP1, AF335551), *Drosophila* (DmTRIP1, AAF52183), human (HsTRIP1, S60335) and fission yeast (SUM1, P79083) is shown.

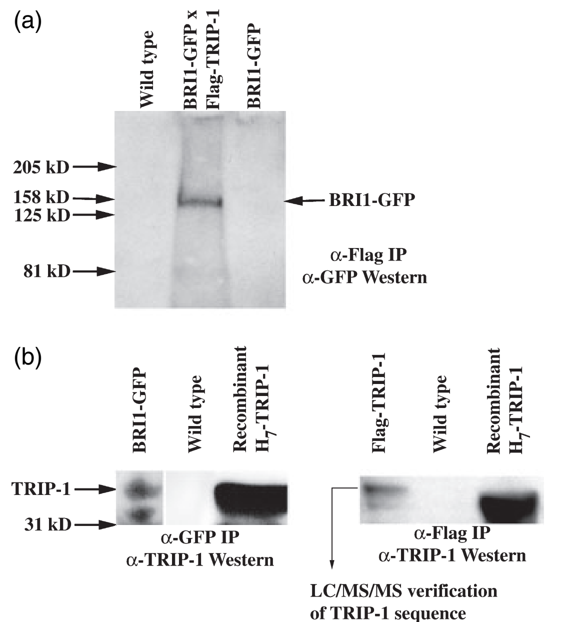

Figure 4. TRIP-1 and BRI1 interact *in vivo*.
(a) Protein extracts from wild-type Columbia plants and transgenic Arabidopsis plants expressing BRI1-GFP alone or both BRI1-GFP and Flag-TRIP-1, were immunoprecipitated with anti-Flag antibody. After SDS-PAGE and transfer to a PVDF membrane, Western blot analysis was performed with anti-GFP antibody. The arrow marks a band of the correct size for BRI1-GFP that does not occur in wild-type or single BRI1-GFP transgenics, suggesting that BRI1-GFP co-immunoprecipitated with Flag-TRIP-1 in the double transgenic line.
(b) BRI1-GFP and wild-type protein extracts were immunoprecipitated with anti-GFP antibody. After SDS-PAGE and transfer to a PVDF membrane, Western blot analysis was performed with anti-TRIP-1 antibody. Recombinant H_7-TRIP-1 is included as a marker. The arrow marks a band of the correct size for TRIP-1 that does not occur in wild-type, suggesting that TRIP-1 co-immunoprecipitated with BRI1-GFP. Numbers represent migration of molecular mass markers (kDa). The panel on the right shows the specificity of the anti-TRIP-1 antibody. The TRIP-1 antibody interacted only with a protein that was immunoprecipitated specifically from Flag-TRIP-1 transgenic plants with anti-Flag antibody and which was confirmed by Q-ToF LC/MS/MS to be Flag-TRIP-1. The antibody interacted with native TRIP-1 from wild-type plants in a similar manner. A doublet was routinely observed for both native and Flag-tagged TRIP-1, which is likely due to post-translational modification, or a specific degradation product. The approximately 160 kDa protein immunoprecipitated by anti-GFP antibody was previously shown by LC/MS/MS to be BRI1-GFP (Kinoshita *et al.*, 2005).

et al., 1998; Stone *et al.*, 1994; Trotochaud *et al.*, 1999). Arabidopsis TTL, a protein with substantial sequence identity to the vertebrate thyroid-binding protein tranthyretin, was recently reported as a potential cytoplasmic substrate of BRI1 (Nam and Li, 2004). BRI1 and TTL interact in yeast cells in a kinase-dependent manner and TTL is phosphorylated by recombinant BRI1 kinase domain *in vitro*. Overexpression of the TTL gene results in a semi-dwarf phenotype similar to weak *bri1* and null *bak1* mutants, while null mutants of TTL enhance BR sensitivity and promote plant growth. Thus, genetic evidence suggests that TTL is a negative regulator of BR signaling, while *in vitro* evidence supports a role for TTL as a putative BRI1 substrate (Nam and Li, 2004). However, a direct interaction of the two proteins *in planta* has not been demonstrated, nor have the specific sites of phosphorylation in TTL been identified.

The data reported here showing TRIP-1 is an *in vitro* substrate of the BRI1 kinase domain coupled with the finding that BRI1 and TRIP-1 are associated *in vivo*, make TRIP-1 another promising candidate for a substrate of the BRI1 kinase domain *in planta*. Moreover, the fact that the mammalian homolog of Arabidopsis TRIP-1 is a substrate of the TGF-β RII receptor kinase is intriguing and lends further experimental support to the previously proposed similarities between early events in BR and TGF-β signaling. Several known facts about BRI1 and BAK1 structure and function are consistent with a TGF-β type mechanism (Figure 5). First, BRI1 has a much larger extracellular domain than BAK1, including a 70 amino acid island domain between Leu-rich repeats 21 and 22 that has been shown by mutational and biochemical analysis to be important for BRI1 function and BR binding (Friedrichsen *et al.*, 2000; Kinoshita *et al.*, 2005; Li and Chory, 1997; Noguchi *et al.*, 1999). BAK1 lacks this domain. If BR binds BRI1 first, it might initiate heterodimerization with BAK1, much like RII binding of TGF-β followed by oligomerization with RI. Second, while transphorylation in both directions between BRI1 and BAK1 was shown in yeast cells, previous *in vitro* experiments with recombinant kinase domains showed that BRI1 stimulated BAK1 phosphorylation (Li *et al.*, 2002; Nam and Li, 2002) and that *in vitro* autophosphorylation of BRI1-KD most likely

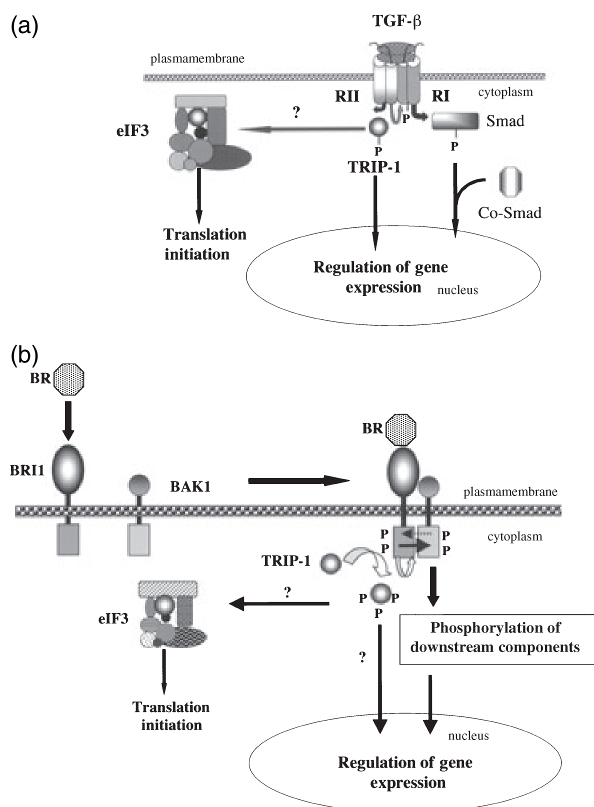

Figure 5. Model for early events in BR signaling.
(a) TGF-β signaling pathway. Binding of the TGF-β polypeptide ligand by the receptor Ser/Thr kinase RII leads to assembly of the heterotetramer and unidirectional phosphorylation of RI by RII. RI phosphorylates signaling proteins termed Smads which move to the nucleus where they associate with transcription factors and other regulatory proteins to modulate gene expression. RII phosphorylates TRIP-1 directly which is also involved in regulation of gene expression. TRIP-1 has a dual role as an essential component of the eIF3 translation initiation factor. It is currently not known whether phosphorylation of TRIP-1 by RII plays a role in the assembly or activity of eIF3.
(b) TGF-β model for interaction of BRI1 and BAK1. In this model, BRI1 binds BR first, which leads to association with and phosphorylation of BAK1. BAK1 phosphorylates unknown downstream components which eventually leads to regulation of gene expression. In a manner analogous to TGF-β RII, BRI1 phosphorylates TRIP-1. While *in vitro* phosphorylation of TRIP-1 by BRI1 has been shown, as well as *in vivo* association of the two proteins, it is not yet clear what functional role TRIP-1 plays in BR signaling. For simplicity, recently discovered downstream components of BR signaling are not shown.

occurred by an intramolecular mechanism (Oh *et al.*, 2000). Moreover, we have shown here that while TRIP-1 is a good *in vitro* substrate for BRI1, BAK1 does not phosphorylate TRIP-1 to any significant extent. The critical *in planta* experiments have not been undertaken, but a useful working hypothesis is one in which BR binds BRI1 first, leading to autophosphorylation, followed by dimerization with BAK1 and predominant phosphorylation of BAK1 by BRI1 in a manner similar to RII phosphorylation of RI (Figure 5).

Our findings that Arabidopsis TRIP-1 is an *in vitro* substrate of BRI1, but not BAK1, and associates with BRI1 *in vivo*, are consistent with the mechanistic similarity of BRI1 and RII. While these general mechanistic similarities are interesting, only TRIP-1 has significant sequence similarity between BR and TGF-β signaling components. There is not extensive sequence identity between BRI and RII, or BAK1 and RI, beyond what one would expect of conserved elements of the Ser/Thr kinase domains found in all eukaryotes. In fact our current knowledge of BR signaling suggests a pathway that has marked similarity to the overall signal transduction logic of several eukaryotic pathways, with only limited conservation of individual signaling components at the sequence homology level (reviewed in Peng and Li, 2003).

In mammals, TRIP-1 has been shown to have both positive and negative regulatory effects on TGF-β responsive gene expression. Moreover some of the TRIP-1 regulatory effects are Smad independent, suggesting multiple functional roles (Choy and Derynck, 1998; Sheu *et al.*, 2003). Our data supports a direct *in vivo* interaction between TRIP-1 and BRI1, but the functional outcome of this association is not yet clear. Preliminary experiments with GFP-TRIP-1 fusions transiently expressed in onion epidermal cells show nuclear localization of the chimeric protein (H. Ehsan and S.D. Clouse, unpublished data). WD domains are known to be associated with protein–protein interaction (Neer *et al.*, 1994), and if nuclear localization can be confirmed in Arabidopsis, it is possible that TRIP-1 might associate with nuclear transcription factors to affect BR-regulated gene expression, much like BES1 and BZR1 (He *et al.*, 2005; Yin *et al.*, 2005). Examination of BR-regulated gene expression in antisense TRIP-1 lines would be informative in this respect. The multiple morphological similarities between TRIP-1 antisense lines and BR-insensitive mutants is striking and is consistent with a positive role for TRIP-1 in BR signaling (Jiang and Clouse, 2001). However, these morphological characteristics are not conclusive, as loss of TRIP-1 activity could also affect BR-independent pathways, possibly resulting in a similar phenotype.

Our finding that BRI1, but not BAK1, phosphorylates TRIP-1 *in vitro* on three specific Thr or Ser residues, strongly suggests that TRIP-1 is a substrate of the BRI1 kinase domain. We previously showed using synthetic peptides that an Arg or Lys at −3 and/or −4 relative to the phosphorylation site was critical for BRI1 substrate phosphorylation (Oh *et al.*, 1998). Examination of sequences around the *in vitro* phosphorylation sites in recombinant TRIP-1 extend our previous findings with synthetic peptides to an intact, folded protein as all three TRIP-1 sites show this feature. Interestingly, TTL also has several Ser and Thr residues in a sequence context consistent with several key elements of the consensus sequence, but it is not yet known if these are sites of phosphorylation by BRI1. We are currently examining the *in vivo* phosphorylation sites of TRIP-1 in wild-type and BR-deficient backgrounds to confirm the *in vitro* sites *in planta* and their possible BR dependence. Preliminary results using immunoprecipitation and phosphatase

treatment show that TRIP-1 is a phosphoprotein *in vivo* (data not shown), but specific sites have yet to be identified by mass spectrometry.

Besides its role in TGF-β signaling, TRIP-1 functions as a subunit (eIF3i) of the eIF3 translation initiation factor in mammals, yeast and plants (Burks *et al.*, 2001). Several Arabidopsis and mammalian eIF3 subunits have functional roles outside of the translation initiation complex (Asano *et al.*, 1997; Karniol and Chamovitz, 2000; Yahalom *et al.*, 2001) and SUM1, the TRIP-1 homolog in fission yeast, also interacts with the 26S proteasome during heat shock (Sheu *et al.*, 2003). Such dual roles of eIF3 components may present possible avenues of interaction with multiple signaling pathways allowing direct regulation of the global cellular growth process of translation initiation. It is possible that *in vivo* phosphorylation of TRIP-1 by BRI1 might enhance its competence to initiate assembly or activation of the eIF3 complex, thus representing a non-genomic effect of BR on translation initiation. Interestingly, it was recently shown that BR treatment limits the loss of several components of the translational apparatus in *Brassica napus* during a prolonged heat stress, which correlates with a more rapid resumption of cellular protein synthesis following the stress (Dhaubhadel *et al.*, 2002).

Rapid progress has recently been made in understanding BR perception and downstream signaling, but a thorough understanding of BR signal transduction requires characterizing substrates of BRI1 and BAK1 and their functional significance. The identification of TRIP-1 as a putative cytoplasmic substrate of the BRI1 receptor kinase presents numerous experimental approaches to understanding early events in BR signaling and examining their similarity to other eukaryotic signal transduction pathways.

Experimental procedures

Construction of plasmids for TRIP-1 expression in E. coli *and Arabidopsis*

EST clone 134K13T7 harboring a full length Arabidopsis *TRIP-1* cDNA within the pSPORT1 vector (Arabidopsis Biological Resource Center, Ohio State University) was amplified with primers 5′-GAA-ACTGCATATGAGGCCGATCCTGATGAAG-3′ and 5′-AGTAGTCGG-ATCCTAAATCTTGATGTTGAAGTAGTCGG-3′, using previously described PCR conditions (Oh *et al.*, 2000). The PCR product was digested with *Nde*I and *Bam*HI and ligated into the bacterial expression vector pET-15b (Novagen, Madison, WI, USA) digested with the same enzymes. The presence of cloned *TRIP-1* was confirmed by restriction digestion and sequencing. The His_7 tag of pET-15b contains a number of Ser residues that could potentially complicate phosphorylation site analysis. This tag was removed by digestion with *Nco*I and *Nde*I and replaced with the tag MVPHHHHHHH generated by annealing the two oligonucleotides, 5′-CATGGTACCACATCACCATCACCATCACCA-3′ and 5′-TATGGTG-ATGGTGATGGTGATGTGGTAC-3′. The reading frame and junction of the new tag with *TRIP-1* was verified by sequencing.

Flag-TRIP-1 was generated by an initial PCR amplification of 134K13T7 as described above, except that the PCR primers contained *Xho*I or *Kpn*I restriction sites. The PCR product was then digested with *Xho*I and *Kpn*I and ligated into *Xho*I/*Kpn*I digested pFlag-MAC (Sigma-Aldrich, St Louis, MO, USA). A small portion of this ligation reaction was PCR amplified with the primers 5′-CAGGAGGGATCCTATGGACTACAAGGACGACGAT-3′ and 5′-CAG-GCTGAAAATCTTCTCTCACTCGACGAGCTC-3′. This PCR product was gel-purified, digested with *Bam*HI and *Sac*I and introduced into the binary vector pBI121 (Clontech, Palo Alto, CA, USA) that had also been digested with *Bam*HI/*Sac*I and gel purified to remove the *uidA* gene. The resulting plasmid was transformed into Agrobacterium strain GV3101 and then transferred into Arabidopsis as previously described (Jiang and Clouse, 2001). All constructs were verified by sequencing.

Purification of recombinant proteins

Flag-BRI-KD was purified as previously described (Oh *et al.*, 2000). To purify H_7-TRIP-1, the bacterial pellet from a 500-ml culture overexpressing the protein was thawed on ice and resuspended in 10 ml of 25 mM Tris–HCl (pH 8), 8 M urea, 80 mM sodium chloride, 10 mM imidazole, 10 mM β-mercaptoethanol, 1 mM PMSF and Complete, EDTA-free, Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). The solution also contained 15 mg lysozyme from a freshly prepared stock solution and 0.5 mg DNase I. The resuspended pellet was sonicated 5×30 sec on ice with 1 min of cooling between each sonication. Insoluble material was removed by centrifugation at 4°C for 1 h (23 736 ***g***). The supernatant was applied to 1 ml Ni-NTA agarose charged with $NiCl_2$ as described by the manufacturer (Qiagen, Valencia, CA, USA) and equilibrated in the above resuspension buffer minus protease inhibitors, enzymes and reductant. The column was washed with 20 ml of equilibration buffer plus reductant. TRIP-1 was eluted using 5 ml of resuspension buffer with 500 mM imidazole (minus enzymes). Purified H_7-TRIP-1 was stored in aliquots at −20°C.

Radiolabeled TRIP-1 for *in vitro* phosphorylation analysis, was prepared by adding 4 volumes of methanol to 1 volume of affinity-purified H_7-TRIP-1, followed by centrifugation. The pellet was dried and resuspended in approximately 1 ml of buffer [100 mM Tris–HCl (pH 8), 8 M urea, and 10 mM β-mercaptoethanol]. The protein was bound to 0.3 ml Ni-NTA resin (Qiagen) charged with $NiCl_2$ as described above. The resin was washed with resuspension buffer then equilibrated in kinase reaction buffer [50 mM HEPES-KOH (pH 7.9), 10 mM $MnCl_2$]. The resin was transferred to a new tube with 100 μl 5X kinase reaction buffer, 20–25 μg purified Flag-BRI1-KD (approximately 200 μl) and 100 μl 2 mM ATP (containing 250 μCi γ-^{32}P-ATP, 3000 Ci mmol^{-1}). Dithiothreitol was added to a final concentration of 1 mM and the reaction was incubated with shaking at 37°C for 1–2 h. After incubation, the resin was washed sequentially in a disposable column with 15 ml kinase reaction buffer to remove unincorporated ATP and 15 ml wash buffer [25 mM Tris–HCl (pH 8), 8 M urea, 80 mM sodium chloride, 10 mM imidazole, 10 mM β-mercaptoethanol] to remove Flag-BRI1-KD. Radiolabeled, phosphorylated H_7-TRIP-1 was eluted from the resin and methanol precipitated as described above. The purified protein was resuspended at approximately 10 mg ml^{-1} in 100 mM Tris–HCl (pH 8) containing 8 M urea and 10 mM dithiothreitol.

Kinase assays

In-vitro phosphorylation assays of recombinant H_7-TRIP-1 were performed in 40 μl reactions containing 1–2 μg of Flag-BRI1-KD,

approximately 5 μg of H_7-TRIP-1, 20 μCi of [γ^{32}P]ATP (3000 Ci mmol^{-1}; Perkin-Elmer Life Sciences, Boston, MA, USA) with 40 nM unlabeled ATP, and 1X kinase buffer (50 mM HEPES, pH 6.8, 0.15 M NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$). The reactions were incubated at 37°C for 2 h and terminated by adding 6× Laemmli sample buffer followed by boiling for 5 min. Reactions were centrifuged, concentrated to 20–30 μl with a Microcon filter device (Millipore, Lincoln Park, NJ, USA) and separated on 10% SDS-PAGE gels. After fixing in 50% MeOH/12% HAc gels were exposed to a phosphorimager screen or autoradiographed. Kinase reactions with TRIP-1 immunoprecipitated from plants were performed as described above except Flag-TRIP-1 was still attached to M_2 agarose beads (Sigma-Aldrich) in a total reaction volume of 100 μl. TRIP-1 peptides were synthesized by Invitrogen (Carlsbad, CA, USA) and purified by reverse phase HPLC on an Ultremex 5 C18 column (Phenomenex, Torrance, CA, USA). Solvent A contained 0.1% (v/v) TFA in ultrapure water (Sigma-Aldrich) and solvent B contained 0.1% TFA in 100% acetonitrile (Fisher, Atlanta, GA, USA). A 45–60 min gradient from 0 to 100% B was employed and purified peptide fractions were lyophilized and resuspended at 0.1 mg ml^{-1} in Mops buffer (pH 7.5). Peptide kinase assays with Flag-BRI1-KD and phosphoamino acid analysis of recombinant TRIP-1 were performed as previously described (Oh *et al.*, 2000).

Preparation of peptides for mass spectrometry

A 100 μl aliquot (typically about 1 mg) of radiolabeled, phosphorylated TRIP-1 in 100 mM Tris–HCl (pH 8) containing 8 M urea was reduced and denatured by adding dithiothreitol to a final concentration of 50 mM followed by incubation at 60°C for 1 h. The sample was then brought to a volume of 1 ml to give final concentrations of 100 mM freshly prepared ammonium bicarbonate, 10% acetonitrile, 0.8 M urea, 5 mM dithiothreitol, 10 mM Tris–HCl (pH 7.8) and 10 μg sequencing-grade modified trypsin (Promega, Madison, WI, USA). After overnight incubation at 37°C, the digest was centrifuged to remove insoluble material. Peptides were precipitated from the supernatant with four volumes of acetone and recovered by a 30-min, 14 000 ***g*** room-temperature centrifugation following a 1-h incubation at −80°C. Peptides were dissolved in 5% acetonitrile, 0.1% trifluoroacetic acid and separated by reverse-phase HPLC as previously described (Oh *et al.*, 2000). Radioactive fractions were identified by scintillation counting or by spotting 1–2 μl on a TLC plate followed by phosphorimager analysis.

Enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) was performed using either one of two methods. The gallium-based phosphopeptide isolation spin-column kit (Pierce Biotechnology, Rockford, IL, USA) was used as described by the manufacturer. Eluted fractions were acidified (pH < 3) using acetic acid and dried using a vacuum centrifuge. Samples were dissolved in 5% acetonitrile/1% trifluoroacetic acid, cleaned using C18 zip tips (Millipore, Bedford, MA, USA) and concentrated again in a vacuum centrifuge to reduce acetonitrile concentration prior to MS analysis. Alternatively, Ni-NTA agarose (Qiagen) was used for phosphopeptide enrichment following a previously published protocol (Zhou *et al.*, 2000). Briefly, 0.3 ml Ni-NTA agarose was loaded into a disposable chromatography column and washed with 0.3 ml 100 mM EDTA to remove the bound nickel. After equilibration in 0.1 M acetic acid, the column material was charged with iron using 0.3 ml 100 mM $FeCl_3$ in 0.1 M acetic acid then reequilibrated in 0.1 M acetic acid. After being diluted to 0.3 ml with 0.1 M acetic acid, a sample was applied to the column and washed with 3 × 0.5 ml water followed by 3 × 0.5 ml 0.1 M acetic acid. Phosphopeptides were eluted using 3 × 0.3 ml 20 mM sodium phosphate (dibasic). Eluted fractions were processed as described above. For some samples, peptide acidic residues were converted to methyl esters by methanolic hydrochloride treatment using a previously described procedure, prior to enrichment of phosphopeptides (Ficarro *et al.*, 2002).

Phosphorylation site identification by MS

Peptide samples were analyzed by MALDI-ToF-MS essentially as previously described (Oh *et al.*, 2000) except the accelerating voltage used was 25 kV and the 2,5-dihydroxybenzoic acid matrix was supplemented with 25 mM spermidine. To verify phosphopeptides by loss of a phosphate group, 4 μl 50 mM ammonium bicarbonate was added to 2 μl peptide sample aliquots which were then incubated for 4 h at room temperature with or without 2 units of alkaline phosphatase. Both samples were cleaned using C18 zip-tips (Millipore) and analyzed by MALDI-ToF-MS as described above.

Nanoscale capillary LC-MS/MS analysis of peptide samples was performed using a Waters CapLC (Waters, Milford, MA, USA) coupled to a Waters Q-ToF Ultima mass spectrometer. Samples were loaded on a peptide CapTrap (Michrom Bioresources, Auburn, CA, USA) and washed with approximately 60 μl 2% acetonitrile/0.1% trifluoroacetic acid. The trap was then switched in line and the trapped peptides were separated using a 75 μm × 20-cm picrofit column (New Objective, Woburn, MA, USA) packed with Magic AQ-C18 reverse-phase material (Michrom Bioresources). Solvents used for fractionation were 0.1% formic acid (solvent A) and 95% acetonitrile/0.1% formic acid (solvent B), with a 16-min linear gradient from 5 to 40% solvent B at approximately 250 nl min^{-1}. The Q-ToF was operated in a data-dependent mode with a 1-sec precursor scan of *m*/*z* values from 400 to 1200 followed by data-dependent acquisition of tandem MS/MS data of the three most intense multiply charged (+2, +3 and +4) ions. MS/MS spectra were processed and subsequently analyzed with Mascot (Matrix Science, London, UK) using an in-house server.

Immunoprecipitation and Western blot analysis

Sterilized and vernalized Arabidopsis seeds (100 mg) from wild type or transgenic lines were placed in 1 l flasks containing 75 ml of B5 media (Sigma-Aldrich) with 2% sucrose and grown under continuous light at room temperature with constant shaking (60 rpm). Plants were harvested after 11 days by rapidly suctioning the media and freezing the tissue in liquid nitrogen. Frozen tissue was ground to a fine powder in liquid nitrogen and extracted by vortexing with 10 mg glass beads (5 × 3 min, 4°C) in Lysis Buffer 250 [50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% nonidet P-40, 1 mM dithiothreitol, 100 μg ml^{-1} PMSF plus complete protease inhibitor cocktail (Roche Applied Science)]. The resulting crude extracts were centrifuged 1 h at 10 000 ***g*** and supernatants were centrifuged again for 15 min at 10 000 ***g*** to remove remaining debris. Total protein extract (typically 16.5 mg ml^{-1} of protein from approximately 20 g of tissue) was immunoprecipitated with anti-Flag M_2 agarose beads (Sigma-Aldrich) according to the manufacturer's instructions.

Western blot analysis was performed by standard techniques after transfer of proteins from 10% SDS-PAGE gels to PVDF membranes (Bio-Rad, Richmond, CA, USA), with 3% BSA (in 50 mM Tris, pH 7.5, 150 mM NaCl) as blocking agent. Anti-GFP antibody (Molecular Probes, Eugene, OR, USA) was used at 1:1000 dilution and detected with 1:5000 dilution of donkey anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Amersham Bioscience, Piscatway, NJ, USA). Secondary antibody was

visualized by chemiluminescence (ECL Plus Kit; Amersham Bioscience) followed by autoradiography.

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