Identification and Functional Analysis of Tomato BRI1 and BAK1 Receptor Kinase Phosphorylation Sites

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Brassinosteroids (BRs) are plant hormones that are perceived at the cell surface by a membrane-bound receptor kinase, BRASSINOSTEROID INSENSITIVE1 (BRI1). BRI1 interacts with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) to initiate a signal transduction pathway in which autophosphorylation and transphosphorylation of BRI1 and BAK1, as well as phosphorylation of multiple downstream substrates, play critical roles. Detailed mechanisms of BR signaling have been examined in Arabidopsis (Arabidopsis thaliana), but the role of BRI1 and BAK1 phosphorylation in crop plants is unknown. In Arabidopsis, we used liquid chromatography-tandem mass spectrometry to identify multiple in vitro phosphorylation sites of the tomato BRI1 and BAK1 cytoplasmic domains. Kinase assays showed that both tomato BRI1 and BAK1 are active in autophosphorylation as well as transphosphorylation of each other and specific peptide substrates with a defined sequence motif. Site-directed mutagenesis revealed that the highly conserved kinase domain activation loop residue threonine-1054 was essential for tomato BRI1 autophosphorylation and peptide substrate phosphorylation in vitro. Furthermore, analysis of transgenic lines expressing full-length tomato BRI1-Flag constructs in the weak tomato allele, 

Brassinosteroids (BRs) are plant hormones that are perceived at the cell surface by a membrane-bound receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) to initiate a signal transduction pathway in which autophosphorylation and transphosphorylation of BRI1 and BAK1, as well as phosphorylation of multiple downstream substrates, play critical roles. Detailed mechanisms of BR signaling have been examined in Arabidopsis (Arabidopsis thaliana), but the role of BRI1 and BAK1 phosphorylation in crop plants is unknown. In Arabidopsis, we used liquid chromatography-tandem mass spectrometry to identify multiple in vitro phosphorylation sites of the tomato BRI1 and BAK1 cytoplasmic domains. Kinase assays showed that both tomato BRI1 and BAK1 are active in autophosphorylation as well as transphosphorylation of each other and specific peptide substrates with a defined sequence motif. Site-directed mutagenesis revealed that the highly conserved kinase domain activation loop residue threonine-1054 was essential for tomato BRI1 autophosphorylation and peptide substrate phosphorylation in vitro. Furthermore, analysis of transgenic lines expressing full-length tomato BRI1-Flag constructs in the weak tomato allele, 

Brassinosteroids (BRs) are regulators of plant growth and development that signal through the membrane-bound receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1; Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002; Kim and Wang, 2010) to influence multiple physiological pathways involved in cell expansion and division, vascular differentiation, flowering, pollen development, and morphogenesis (Clouse, 2011b). Ligand-dependent phosphorylation of BRI1 and BAK1 modulates a cellular cascade of kinases and phosphatases (Kim et al., 2009; Tang et al., 2011) that ultimately results in the dephosphorylation and activation of two transcription factors, BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS SUPPRESSOR1 (BES1; He et al., 2005; Yin et al., 2005), which in the presence of different interacting partners regulate the expression of over 1,000 genes associated with specific BR responses (Sun et al., 2010; Clouse, 2011a; Yu et al., 2011).

To better understand the mechanism of receptor kinase action in BR signaling, a series of studies using Arabidopsis (Arabidopsis thaliana) identified specific sites of serine (Ser), threonine (Thr), and tyrosine (Tyr) phosphorylation in both BRI1 and BAK1 that are critical for kinase function and effective BR signal transduction in planta, and models of the BRI1-BAK1 interaction and transphosphorylation were presented (Oh et al., 2000, 2009b, 2010; Wang et al., 2005, 2008). More than a dozen sites of phosphorylation have been identified in the BRI1 cytoplasmic domain, including the juxta-membrane domain, the catalytic kinase domain, and the...
C-terminal region (Oh et al., 2000; Wang et al., 2005), and six or more sites have also been mapped in BAK1 (Wang et al., 2008; Karlova et al., 2009). Mutation of these essential regulatory sites to residues that are either nonphosphorylated or constitutively mimic phosphorylation can influence BRI1 and BAK1 kinase function and have dramatic effects on plant growth, development, and morphology. For example, mutation of the highly conserved BRI1 kinase domain activation loop residue Thr-1049 (and its corresponding residue Thr-455 in BAK1) to Ala results in complete loss of kinase activity in vitro and an extreme dwarf phenotype in vivo, suggesting that phosphorylation of this critical residue is required for BR signaling in planta (Wang et al., 2005, 2008). Phosphorylation of the BRI1 juxtamembrane residue Tyr-831 is not essential for kinase autophosphorylation in vitro but does confer regulatory properties in vivo, since the Y831F site-directed mutant has larger leaves and a higher photosynthetic rate than the wild type, suggesting that Tyr-831 is a negative regulatory site when phosphorylated (Oh et al., 2009a, 2011).

The identification of BR biosynthetic and insensitive mutants in tomato (Solanum lycopersicum; Bishop et al., 1999; Koka et al., 2000), rice (Oryza sativa; Yamamuro et al., 2000), barley (Hordeum vulgare; Chono et al., 2003), and pea (Pisum sativum; Nomura et al., 1997) clearly extended the importance of these compounds from the experimental plant Arabidopsis to crop plants. The Arabidopsis bri1 mutant was initially isolated by a root growth inhibition screen for BR insensitivity (Clouse et al., 1996), and several independent genetic screens revealed over two dozen alleles of bri1, most of which exhibited the extreme dwarfism and other phenotypic characteristics of severe BR-deficient mutants (Kauschmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999; Friedrichsen et al., 2000). We previously identified a BR-insensitive mutant in tomato, curl3 (cu3), that is phenotypically very similar to the Arabidopsis bri1-1 mutant (Koka et al., 2000), and cloning the CU3 gene from tomato showed that it encoded tomato BRI1 (SIBRI1), which also binds BR directly (Montoya et al., 2002; Holton et al., 2007). BRI1 homologs from several other crop species have also been cloned including rice, barley, and pea (Yamamuro et al., 2000; Chono et al., 2003; Nomura et al., 2003), and mutational analyses in both Arabidopsis and crop species have shown conclusively that the BRI1 receptor is required for normal BR perception and plant growth. However, a detailed analysis of BRI1 or BAK1 phosphorylation has not been reported in any plant other than Arabidopsis. The cytoplasmic kinase domains of tomato and Arabidopsis BRI1 are highly conserved (82% identical), and many phosphorylated Ser, Thr, and Tyr residues previously identified in Arabidopsis BRI1 (Wang et al., 2005) occur in the same position in tomato SIBRI1, based on sequence alignment (Montoya et al., 2002). However, there are numerous residues in SIBRI1 that are not conserved in Arabidopsis BRI1 as well as conserved residues that are not phosphorylated in Arabidopsis, providing potential for differential BRI1 phosphorylation between the two species. Moreover, SIBRI1 did not rescue the weak bri1-5 allele in Arabidopsis, indicating that BRI1 from the two species are not fully interchangeable (Holton et al., 2007). Differences between tomato and Arabidopsis in internal developmental cues, potential heterodimerization partners, and kinase domain substrates could yield differential patterns of BRI1 phosphorylation and gene expression between the two species. Thus, a detailed functional analysis of SIBRI1 phosphorylation sites and interacting proteins is required to determine the interspecies conservation and divergence of receptor kinase mechanisms involved in BR signaling. Here, we report the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify in vitro sites of phosphorylation in SIBRI1 and SIBAK1 along with a functional characterization of a subset of these sites in kinase function and tomato growth and development.

RESULTS

SIBRI1-CD and SIBAK1-CD Have Autophosphorylation and Transphosphorylation Activities in Vitro

To determine if SIBRI1 and SIBAK1 are active kinases in vitro, the cytoplasmic domain of each Leu-rich repeat receptor-like kinase (LRR RLK) was cloned into Escherichia coli expression vectors conferring either an N-terminal Flag epitope tag or an N-terminal maltose-binding protein (MBP) tag to yield Flag-SIBRI1-CD, MBP-SIBRI1-CD, and MBP-SIBAK1-CD. The cloned cytoplasmic domains consist of the complete juxtamembrane region, kinase domain, and C-terminal region. The BRI1 constructs contain amino acids 824 to 1,207 of the previously published tomato BRI1 sequence (Montoya et al., 2002), while the cytoplasmic domain of MBP-SIBAK-CD shares 100% sequence identity with amino acids 254 to 617 of SISERK3B, which has been shown by phylogenetic analysis to be the most likely tomato ortholog of Arabidopsis BAK1 (Mantelin et al., 2011). Putative kinase-inactive forms of tomato BRI1 (SlmBRI1-K916E) and BAK1 (SlmBAK1-K321E) were generated by mutating the conserved Lys residues in kinase subdomain II, which correspond to Arabidopsis BRI1 (K911E) and BAK1 (K317E) mutations, respectively, both of which lack kinase activity in vitro and in vivo (Wang et al., 2005, 2008).

Figure 1A shows that purified recombinant MBP-SIBRI1-CD, Flag-SIBRI1-CD, and MBP-SIBAK1-CD all exhibited substantial autophosphorylation activity when incubated under previously established assay conditions used for Arabidopsis BRI1 and BAK1 kinase analysis (Oh et al., 2000; Wang et al., 2005, 2008). Moreover, the mutated forms MBP-SlmBRI1-CD (K321E) and Flag-SlmBRI1-CD (K916E) showed no detectable kinase activity, as expected. When active Flag-SIBRI1-CD was incubated with kinase-inactive MBP-SlmBAK1, or when active MBP-SIBAK1-CD was incubated with...
kinase-inactive Flag-SlmBRI1-CD, both active kinases were able to transphosphorylate their kinase-inactive partners, similar to what has been previously shown for Arabidopsis BRI1 and BAK1 (Wang et al., 2008).

Recombinant Arabidopsis BRI1-CD phosphorylates synthetic peptides containing the previously determined consensus sequence for optimum BRI1 substrate phosphorylation, [RK]-[RK]-X(2)-[ST]-X(3)-[LMVIFY]-[RK], where one of the residues in brackets is the amino acid occurring at the indicated position and X represents any amino acid (Oh et al., 2000). To determine if SlBRI1-CD can recognize the same substrate sequences, peptide substrate phosphorylation assays were performed with Flag-SlBRI1-CD and a range of synthetic peptides previously characterized for Arabidopsis Flag-BRI1-CD (Oh et al., 2000). Flag-SlBRI1-CD phosphorylated synthetic peptides BR9, BR10, BR13, and BR15 at similar rates, while SP11 and BR14 supported reduced activity and BR11 and BR12 were both poor substrates (Fig. 1B). Overall, it appears that the sequence requirements for efficient substrate peptide phosphorylation by Flag-SlBRI1-CD are similar to those of Arabidopsis Flag-BRI1-CD, although some variations were evident. Flag-SlBRI1-CD phosphorylated BR9, BR10, BR13, and BR15 at higher levels than SP11 (Fig. 1B), while previous work in Arabidopsis showed that Flag-BRI1-CD relative velocity with respect to SP11 was greater than BR9, BR10, and BR15 and was exceeded only by BR13 (Oh et al., 2000). In fact, BR9, BR10, and BR15 had relative velocities at least 30% lower than BR13 in Arabidopsis, while all four were basically equally high in tomato. BR12 was a poor substrate for both tomato (Fig. 1B) and Arabidopsis Flag-BRI1-CD (Oh et al., 2000).

When the relative velocity of Flag-SlBRI1-CD against these SP11 variant peptides was compared (Fig. 1B), it was found that (1) changing the Lys at P+5, relative to the phosphorylated Ser (P = 0), in SP11 to an Arg (BR15) increases peptide phosphorylation by about 41%; (2) substituting Arg of SP11 at P_2 and P_4 with Lys (BR13) enhanced phosphorylation by about 35%, whereas replacement with His residues (BR14) decreased phosphorylation by approximately 34%; (3) substituting the

![Figure 1. SlBRI1-CD and SlBAK1-CD are active kinases that can autophosphorylate and transphosphorylate in vitro. A, Autophosphorylation assays contained 1.0 μg of affinity-purified Flag-SlBRI1-CD, MBP-SlBRI1-CD, or MBP-SlBAK1-CD and 20 μCi of [γ-32P]ATP in kinase buffer, followed by 10% (w/v) SDS-PAGE and autoradiography. B, Phosphorylation of synthetic peptides in vitro by 0.5 μg of Flag-SlBRI1-CD with 0.8 μCi of [γ-32P]ATP and 0.10 mg mL⁻¹ synthetic peptide in kinase buffer. J indicates nor-Leu, a nonoxidizing functional equivalent of Met. Error bars represent SE, and n = 3. C, Phosphorylation of the peptide substrate SP11 (GRKKIASVEJJKK, where J = nor-Leu) by Flag-SlBRI1-CD (1.0 μg) is increased over 2-fold by the addition of MBP-SlBAK1-CD (1.0 μg) compared with the expected additive activity and is dependent on an active SlBAK1 kinase. Error bars represent SE, and n = 3.](https://plantphysiol.org)
Arg residue at P−6 with Ala (BR10) increased peptide phosphorylation by 30%; (4) replacing the hydrophobic residue at P+4 of SP11 with Ala (BR9) also resulted in a 30% increase in phosphorylation; and (5) replacing Val, Glu, nor-Leu, Lys, and Gly at P+1, P+2, P+3, P+6, and P−7, respectively, of SP11 with Ala (BR12) reduced peptide phosphorylation by about 70%.

Transphosphorylation of Flag-SIBRI1-CD by MBP-SIBAK1-CD Increases BRI1 Kinase Activity in Peptide Substrate Phosphorylation Assays

Previous results in Arabidopsis have shown that BAK1 transphosphorylation of BRI1 on specific juxtamembrane and C-terminal domain residues enhances the ability of BRI1 to phosphorylate synthetic peptide substrates by at least 3-fold (Wang et al., 2008). To determine if the transphosphorylation between tomato BRI1 and BAK1 leads to increased kinase activity of either protein, MBP-SIBAK1-CD was incubated with Flag-SIBRI1-CD and their kinase activities were monitored by assessing phosphorylation levels of the synthetic peptide SP11. Flag-SIBRI1-CD alone phosphorylated the SP11 peptide to an equivalent extent as MBP-SIBAK1-CD alone (Fig. 1C), which is in contrast to Arabidopsis BAK1, which had no apparent direct activity on BRI1 substrate peptides (Wang et al., 2008). However, as was previously observed in Arabidopsis, the mixture of equal amounts of MBP-SIBAK1-CD and Flag-SIBRI1-CD increased the level of peptide substrate phosphorylation significantly (Fig. 1C). Compared with their additive activity, the mixture of equal amounts of MB-SIBAK1-CD and Flag-SIBRI1-CD increased the level of peptide phosphorylation more than 2-fold. This enhancement in phosphorylation was dependent on MBP-SIBAK1-CD kinase activity, because substitution of the kinase-inactive form, MBP-SlmBAK1-CD, resulted in greatly reduced SP11 phosphorylation by BRI1. The kinase-inactive forms Flag-SlmBRI1-CD and MBP-SlmBAK1 were also unable to phosphorylate the SP11 peptide either alone or in combination.

Kinetics of Flag-SIBRI1-CD on Peptide Substrate Phosphorylation in Vitro

To determine if Flag-SIBRI1-CD phosphorylated substrate peptides SP11 and BR13 with realistic kinetics comparable to other known peptide substrates of plant kinases, we calculated $K_m$ and $V_{max}$ for both peptides with Lineweaver-Burk analyses using reaction velocities measured with seven substrate concentrations (Fig. 2). The $K_m$ values of BR13 (109 μM) and SP11 (193 μM) do indeed fall within the range of $K_m$ values for plant kinases with synthetic peptide substrates that reflect true physiological substrates, such as the cauliflower (Brassica oleracea) HMR kinase with a $K_m$ of 95 μM for the SAMS peptide (Weekes et al., 1993). Flag-SIBRI1-CD showed higher apparent affinity for BR13 than for SP11 ($K_m$ of 109 versus 193 μM, respectively) but lower $V_{max}$ for BR13 compared with SP11 (Fig. 2). The selectivity of Flag-SIBRI1-CD for the two substrates can be expressed as the $V_{max}/K_m$ ratio when the amount of enzyme is constant and was nearly identical for BR13 and SP11 (4.35 versus 4.86, respectively). It was previously reported that Arabidopsis Flag-BRI1-CD also had a higher affinity for BR13 than for SP11 ($K_m$ of 81.7 versus 427 μM, respectively), but the $V_{max}/K_m$ ratio for BR13 was 2.5 times higher than for SP11 (Oh et al., 2000). Overall, these data indicate that tomato and Arabidopsis BRI1 proteins are similar but not identical in terms of peptide substrate phosphorylation in vitro.

Tomato BRI1 Autophosphorylates on Multiple Ser, Thr, and Tyr Residues in Vitro

We previously identified at least 11 sites of in vivo phosphorylation in BRI1 by immunoprecipitation from BR-treated Arabidopsis seedlings followed by LC-MS/MS analysis and also found that Flag-BRI1-CD autophosphorylation sites identified in vitro were highly predictive of in vivo phosphorylation (Oh et al., 2000; Wang et al., 2005). To identify specific phospho (p)Ser, pThr, and pTyr phosphorylation sites in MBP-SIBRI1-CD, the autophosphorylated recombinant protein was subjected to SDS-PAGE, followed by gel extraction,
reduction, alkylation, and tryptic digestion. The digested peptides were extracted and subjected to quadrupole time-of-flight (Q-TOF) LC-MS/MS analysis, with or without immobilized metal affinity chromatography to enrich for phosphopeptides. The phosphorylated peptides identified are listed in Table I, and product ion spectra are shown in Supplemental Figure S1.

The sequence coverage of MBP-SlBRI1-CD was more than 85%, which resulted in five unambiguously assigned phosphorylation sites: Tyr-839, Thr-848, Ser-863 and Thr-877 in the juxtamembrane domain as well as Thr-1054 in the kinase domain activation loop (Table I). All of these juxtamembrane sites have been identified on the corresponding conserved Ser/Thr/Tyr residues in Arabidopsis BRI1 (Oh et al., 2000, 2009b; Wang et al., 2005). The highly conserved activation loop residue corresponding to tomato Thr-1054 (Thr-1049 in Arabidopsis BRI1) was shown by functional analysis to be critical for BRI1 kinase activity and signaling in Arabidopsis, and while likely phosphorylated, an unambiguous assignment of this site in Arabidopsis by LC-MS/MS analysis is not available (Oh et al., 2000; Wang et al., 2005). Our data provide tandem mass spectrometry spectral support that this residue is indeed phosphorylated in tomato. Moreover, based on manual inspection of product ion spectra, at least one and possibly up to three of the five sites (Thr-1040, Thr-1044, Ser-1047, Ser-1049, and Thr-1050) present in the activation loop of subdomain VII/VIII, spanning residues 1,032 to 1,061 of tomato BRI1, are likely to be phosphorylated (Table I). The length of the peptide, along with the presence of seven Thr and Ser residues in total, make unambiguous identification of the specific phosphorylation sites difficult. A similar situation occurred in the corresponding Arabidopsis activation loop peptide, where three of the six Ser/Thr residues were determined to be phosphorylated but their unambiguous location could not be determined (Oh et al., 2000; Wang et al., 2005).

**Table I. SIBRI1, SIBAK1 and STIRIP1 phosphorylation sites identified by Q-TOF LC-MS/MS or LC/MSE**

| Peptide          | Calculated M<sup>a</sup> | Measured M | Charge | Mascot Score<sup>c</sup> | PLGS Score<sup>d</sup> | Identified Site(s) | Correlating Arabidopsis Residue(s) |
|------------------|--------------------------|------------|--------|---------------------------|------------------------|-------------------|-----------------------------------|
| EALpSINLAAFEKPR  | 1,750.9018               | 1,750.8928 | 3      | 36 n/a                    |                        | Ser-863           | Ser-858                           |
| EAAALYMDGHSHApTANSAWK | 2,425.9998               | 2,425.9948 | 3      | 26 n/a                    |                        | Thr-848           | Thr-842                           |
| EEAApYMDGHSHApTANSAWK | 2,505.9661               | 2,505.9568 | 3      | 21 n/a                    |                        | Tyr-839, Thr-848  | Tyr-831, Thr-842                  |
| KpLTADILEATNFGHSVCSGGFpDVYK | 3,251.5176               | 3,251.4901 | 4      | 28 n/a                    |                        | Thr-877           | Thr-872                           |
| LMSMDTHLSVLpTAGTPGPVPEYQpSR | 3,397.5400               | 3,397.5490 | 3      | 20 n/a                    |                        | Thr-1054           | Thr-1049                          |
| LM*p*SAM*p*DHLSVT/LAGTPGPVPEYQpSR<sup>e</sup> | 3,429.5298               | 3,429.5374 | 3      | 17<sup>b</sup>n/a        |                        | Ambiguous Activation loop |
| SlBRI1-CD in vitro sites |                            |            |        |                           |                        |                   |                                   |
| GpTGIHAEPEypSTGGK | 1,622.7705               | 1,622.7724 | 2      | 40 10.29                  |                        | Thr-459           | Thr-455                           |
| GTGIHAEPEypSTGGK | 1,622.7705               | 1,622.7750 | 2      | 44 n/a<sup>b</sup>       |                        | Ser-469           | Thr-465<sup>e</sup>               |
| ELQApVApDEpSNFK | 1,444.6235               | 1,444.6279 | 2      | 22 7.44                   |                        | Thr-290           | Ser-286                           |
| ELQAvTDNpFPpSNKNLGR | 1,997.9571               | 1,997.9617 | 2      | 28 7.78                   |                        | Ser-294           | Ser-290                           |
| pTGqGLpEQQTEVEMISM*AVHR | 2,486.0971               | 2,486.1022 | 3      | n.d.<sup>d</sup>         | 6.84                   | Thr-328           | Thr-324<sup>2</sup>               |
| LLVYPMPAmGpSVASIR<sup>d</sup> | 1,719.8055               | 1,719.8178 | 2      | n.d.<sup>d</sup>         | 6.9                   | Ser-374/Ser-377   | Ser-370/Ser-373<sup>1</sup>       |
| LM*pDYKDHThpITTAVR | 1,744.7855               | 1,744.7680 | 3      | 50 7.12                   |                        | Thr-453           | Thr-449                           |
| LM*pDYKDHThpITTAVR<sup>e</sup> | 1,728.7906               | 1,728.7750 | 3      | 39 8.24                   |                        | Ambiguous         | Thr-446/Thr-449/Thr-450          |
| SIBRI1-CD × nBAK1-CD transphosphorylation |                            |            |        |                           |                        |                   |                                   |
| GpTGIHAEPEypSTGGK | 1,622.7705               | 1,622.7772 | 2      | 34 n.d.<sup>b</sup>      |                        | Thr-459           | Thr-455                           |
| SIBRI1-CD × STIRIP1 transphosphorylation |                            |            |        |                           |                        |                   |                                   |
| LAVpIpTDFpFM*GTLSAHpIK<sup>e</sup> | 2,123.0378               | 2,123.0361 | 3      | 45 n/a                    |                        | Thr-111/Th-112    | Thr-111/Thr-112                   |

<sup>a</sup>Met sulfoxide is denoted as M*; phosphoseryl, phosphotyrosyl, and phosphothreonyl residues are denoted as pS, pY, and pT, respectively. <sup>b</sup>M, Neutral monoisotopic mass. <sup>c</sup>Mascot scores are above the peptide identity threshold (P < 0.05) except as indicated. Spectra for all sites are provided in Supplemental Figures S1 (BRI1), S2 (BAK1), S3 (transphosphorylation), and S4 (TRIP1). <sup>d</sup>PLGS, ProteinLynx Global Server 2.4. <sup>e</sup>These data were analyzed by Mascot only. <sup>f</sup>At least one residue within the parentheses is phosphorylated, but the specific residue could not be assigned from spectral data. <sup>g</sup>This Mascot score was below the threshold level, but manual inspection of the spectrum confirmed phosphorylation of at least one of the residues within parentheses. See Supplemental Figure S1F for spectrum. <sup>h</sup>The PLGS search did not score this peptide. <sup>i</sup>Not previously reported as autophosphorylation sites in Arabidopsis BAK1. <sup>j</sup>The Mascot search did not score this peptide.

**Tomato BAK1 Autophosphorylates on Multiple Ser and Thr Residues in Vitro**

Tryptic digested peptides from in vitro autophosphorylated MBP-SlBAK1-CD were subjected to Q-TOF LC-MS/MS (data dependent tandem mass spectrometry) or Q-TOF LC/MS<sup>e</sup> (data independent mass spectrometry) analyses. The phosphorylated peptides identified are listed in Table I, and product ion spectra are shown in Supplemental Figure S2. Using these approaches, we identified tomato BAK1 phosphorylated residues Thr-290, Ser-294, Thr-453 and Thr-459, corresponding to Arabidopsis sites Ser-286, Ser-290, Thr-449, and Thr-455, which have been shown previously to be phosphorylated in vitro and in vivo (Wang et al., 2005, 2008). Phosphorylation at Arabidopsis

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Thr-455, an activation loop residue equivalent to BRI1 Thr-1049, is essential for BAK1 kinase function and BR signaling in vivo (Wang et al., 2008). In addition to Thr-459, the activation loop residue Thr-453 (equivalent to Arabidopsis Thr-449) is phosphorylated in tomato BAK1, and two additional activation loop residues, Thr-450 and Thr-454 (equivalent to Arabidopsis Thr-446 and Thr-450) might also be phosphorylated but could not be unambiguously assigned based on available tandem mass spectrometry or data independent mass spectrometry product ion spectra. Furthermore, we also identified tomato BAK1 residues Thr-328, Ser-374 (or Ser-377), and Ser-469 as phosphorylation sites. The corresponding Arabidopsis residues Thr-324, Ser-370 (or Ser-373), and Thr-465 have not previously been reported as BAK1 phosphorylation sites.

Besides being autophosphorylated, the critical activation loop residue Thr-455 is also transphosphorylated by BRI1 in Arabidopsis (Wang et al., 2008). To determine if a similar BRI1/BAK1 transphosphorylation occurs in tomato, we incubated kinase-inactive MBP-SlmBAK1-CD with kinase-active Flag-SlBRI1-CD and found that, as in Arabidopsis, the tomato BAK1 activation loop residue Thr-459 was transphosphorylated by tomato BRI1 (Table I; Supplemental Fig. S3).

Effect of Mutating Identified and Predicted SlBRI1 Phosphorylation Sites on in Vitro Kinase Function

To understand the biochemical functions of the identified SIBRI1 phosphorylation sites, we used site-directed mutagenesis to generate 14 in vitro mutant constructs with selected Flag-SlBRI1-CD Ser, Thr, and Tyr residues substituted with Ala (for Ser and Thr residues) or Phe (for Tyr residues). The functional importance of each substituted residue was assessed by determining the effect on Flag-SlBRI1-CD autophosphorylation and on phosphorylation of the BRI1 synthetic peptide. Mutating any of the S1040A, S1047A, and T1054A residues in the activation loop almost completely abolished kinase activity with respect to both autophosphorylation and peptide substrate phosphorylation (Fig. 3). The mutated activation loop residues T1044A and S1049A also showed dramatically reduced peptide substrate phosphorylation but did retain a residual amount of autophosphorylation activity. However, the T1050A activation loop mutation appeared to retain full activity in both assays.

Although the effects on kinase activity of mutating specific Ser/Thr residues in the activation loop of Flag-SlBRI1-CD were often similar to the effect of mutating...
the corresponding residues in Arabidopsis Flag-BRI1, there were differences as well (Wang et al., 2005). Mutating the highly conserved residue Thr-1054 (equivalent to Arabidopsis Thr-1049) resulted in almost complete loss of kinase activity in both species. Similarly, tomato T1044A and the corresponding Arabidopsis T1039A both showed reduced peptide substrate phosphorylation but significant autophosphorylation activities. However, tomato S1047A showed no autophosphorylation activity, while the corresponding Arabidopsis residue S1042A showed substantial autophosphorylation (Wang et al., 2005). This suggests that Ser-1047 is critical for tomato BRI1 kinase function but the corresponding Arabidopsis Ser-1042 is not essential for BRI1 activity in that species.

With respect to juxtamembrane domain mutations, Y839F and T848A both retained autophosphorylation activity and both had significantly enhanced peptide substrate phosphorylation activity of 2.9- and 5.8-fold, respectively (Fig. 3). However, the corresponding Arabidopsis residues, Y831F (Oh et al., 2009b) and T842A (Wang et al., 2005), while retaining autophosphorylation activity, had reduced rather than enhanced phosphorylation of the BR13 peptide, suggesting different functions of these residues in the two species with respect to downstream substrate phosphorylation. However, tomato T877A had increased autophosphorylation and dramatically enhanced peptide substrate phosphorylation by more than 20-fold, similar to the corresponding Arabidopsis T872A mutation, which was previously shown to have enhanced BR13 phosphorylation of at least 10-fold (Wang et al., 2005).

In Vivo Functional Analysis of the SIBRI1 Thr-1054 Phosphorylation Site

The Arabidopsis bri1-5 mutant is a weak allele that shows intermediate dwarfism and a smaller rosette size due to a C69Y amino acid substitution in the extracellular domain of BRI1 (Noguchi et al., 1999). Expression of wild-type Arabidopsis BRI1-Flag in the bri1-5 mutant led to full rescue, while expression of BRI1-Flag with a T1049A substitution of the essential activation loop residue not only failed to rescue bri1-5 but caused a dominant negative effect, with phenotypes similar to the extreme dwarfism of a bri1-1 null allele (Wang et al., 2005). To understand the importance of the corresponding tomato BRI1 residue Thr-1054 in vivo, we generated transgenic tomato plants expressing wild-type 35S::SIBRI1-Flag and 35S::SIBRI1-Flag carrying the T1054A mutation, both in the cu3-abs1 mutant background. The original cu3 allele of tomato BRI1 is a null allele with an extreme dwarf phenotype, aberrant leaf structure, and male sterility (Koka et al., 2000), similar to Arabidopsis bri1-1 (Clouse et al., 1996), and like bri1-1 in Arabidopsis, it is difficult to transform and propagate. However, cu3-abs1 is analogous to Arabidopsis bri1-5, which as a weaker allele is clearly distinguishable from the wild type but is easier to transform and propagate than the null allele (Montoya et al., 2002). Phenotypic analysis of transgenic plants expressing wild-type SIBRI1-Flag in the cu3-abs1 mutant demonstrated rescue of the dwarfism and curled, dark green, rugose leaf phenotype of the mutant plants (Fig. 4). However, SIBRI1-Flag(T1054A) failed to rescue the cu3-abs1 mutant and, in fact, enhanced the dwarfism and altered leaf phenotype in a dominant negative effect with phenotypes similar to the cu3 null allele (Fig. 4). Thus, as is the case for Arabidopsis Thr-1049, the BRI1 tomato activation loop residue Thr-1054 is essential for normal tomato growth and development.

SIBRI1-CD Phosphorylates Tomato TRIP1 in Vitro

We previously showed that Arabidopsis Flag-BRI1-CD could phosphorylate TGF-β Receptor Interacting Protein (TRIP1) in vitro on multiple Ser or Thr residues (Ehsan et al., 2005). To determine if Flag-SIBRI1-CD could also phosphorylate tomato TRIP1, a BLAST search of Arabidopsis TRIP1 (At2g46280) against the public tomato EST database was performed that resulted in the identification of an EST contig, TC195665, whose open reading frame shared 82% identity (91% similarity) to Arabidopsis TRIP1 across the entire 328-amino acid length of the TRIP1 protein. Conversely, a BLAST search of tomato unigene SGN-U575556 (the most recently updated and annotated version of TC195665, which corresponds to SolyCol1g0170071.1) showed 82% identity as expected with TRIP1 and only 30% identity with the next most closely related Arabidopsis sequence (transducin WD-40; At1g15470). Moreover, a BLAST search of SGN-U575556 against all nonredundant sequences in GenBank returned TRIP1 from many other
plant species, as well as yeast (Schizosaccharomyces pombe), Drosophila melanogaster, and human, as the most closely related proteins. Since many of these TRIP1 proteins have a proven functional role as translation initiation factor eIF3i, it is highly likely that SGN-U575556 encodes the tomato ortholog of TRIP1/eIF3i. The full-length complementary DNA (cDNA) represented by SGN-U575556 was expressed in E. coli with an N-terminal Flag epitope tag, and the purified recombinant protein was incubated with either MBP-SlBRI1-CD or MBP-SlBAK1-CD. As shown in Figure 5, both kinases phosphorylated tomato TRIP1, although BRI1 appeared to have greater activity toward this substrate than BAK1. LC-MS/MS analysis identified either Thr-111 or Thr-112 (either residue was equally likely based on the acquired product ion spectra) as a SlBRI1-CD phosphorylation site of Flag-SlTRIP1 (Supplemental Fig. S4A). Thr-112 is a highly conserved residue and appears in Arabidopsis, tomato, bean (Phaseolus vulgaris), Drosophila, human, and yeast TRIP1 (Supplemental Fig. S4B).

DISCUSSION

The BR signal transduction pathway is one of the best understood in plants (Kim and Wang, 2010; Wang et al., 2012). However, most of our mechanistic understanding is based on research done in the model plant Arabidopsis, and significantly less detail regarding BR signaling has been revealed in crop plants. Field experiments have shown that slight modulations in rice BRI1 expression can alter rice yields by up to 30% (Morinaka et al., 2006), suggesting that understanding the molecular mechanism of BR signal transduction in Arabidopsis. Brassinolide binding to the extracellular domain of BRI1 (Hothorn et al., 2011; She et al., 2011) results in activation of the BRI1 intracellular kinase domain and subsequent transphosphorylation and activation of BAK1, followed by reciprocal BAK1 transphosphorylation of BRI1, which enhances its kinase activity toward specific substrates (Wang et al., 2005, 2008). In order to compare the mechanisms of the early events in BR signaling between Arabidopsis and tomato, it is essential to identify and functionally characterize tomato BRI1 and BAK1 phosphorylation sites.

As expected, based on previous work with Arabidopsis BRI1 and BAK1, the recombinant Flag-SlBRI1-CD and SlBAK1-CD proteins had kinase activity in vitro, as evidenced by both autophosphorylation and also transphosphorylation of each other as well as of synthetic peptide substrates. The site-directed mutants Flag-SlBRI1-CD (K916E) and MBP-SlBAK1-CD (K321E), where the conserved Lys residue of subdomain II is substituted with Glu, produced inactive kinases, indicating that the activity observed with the native sequence proteins was the result of the tomato kinases and not a contaminating bacterial kinase. In order to identify the structural elements required for optimal substrate phosphorylation by Arabidopsis BRI1, peptide substrates having specific variation in their sequence were previously used for in vitro kinase assays (Oh et al., 2000). The optimal peptide sequences thus identified were further used to test the in vitro functional importance of BRI1 autophosphorylation sites (Wang et al., 2005) and also to understand the details of Arabidopsis BRI1 and BAK1 transphosphorylation and substrate phosphorylation (Wang et al., 2008). Similarly, we tested the ability of SIBRI1 to phosphorylate eight of these previously characterized peptide substrates.

Interestingly, SIBRI1 transphosphorylated synthetic peptides and was generally similar to Arabidopsis BRI1 in specificity. However, SIBRI1 was less discriminating with respect to the nature of the basic residues at the P-3 and P-4 positions. With Arabidopsis BRI1, there was a stronger preference for Lys over Arg and His (Oh et al., 2000) compared with SIBRI1 (this study). Accordingly, Arabidopsis BRI1 prefers the BR13 peptide (with Lys at P-3 and P-4) over the SP11 peptide (with Arg at both positions) and strongly favors the BR14 peptide with His at these two positions.

Figure 5. Both tomato BRI1-CD and BAK1-CD can phosphorylate Flag-SlTRIP1 in vitro. Assays containing 1 μg each of affinity-purified recombinant BRI1 or BAK1 cytoplasmic domains (the wild type or kinase-inactive mutants) and 5 μg of affinity-purified Flag-SlTRIP1 were incubated with 20 μCi of [γ-32P]ATP in kinase buffer for 1 h, followed by 10% (w/v) SDS-PAGE and autoradiography.
In contrast, SIBRI1 does not display as dramatic a preference for BR13 over SP11, either in assays at fixed peptide concentrations (Fig. 1B) or when selectivity is assessed as the ratio of $V_{\text{max}}$ to $K_m$ (Fig. 2). Also, SIBRI1 had higher relative activity with the BR14 peptide, with His at P–3 and P–4, compared with Arabidopsis BRI1 (0.4 versus 0.2, respectively). Although not directly tested with SIBRI1, previous studies with Arabidopsis BRI1 indicated that basic residues at P–3 and P–4 were almost essential for transphosphorylation activity (Oh et al., 2000). It is likely that tomato BRI1 is similar but simply less discriminating in terms of the nature of the basic residue at these positions. Moreover, it is also worth noting that SIBAK1 had activity with the SP11 peptide that was significant and generally equivalent to SIBRI1 (Fig. 1C). This is in contrast to Arabidopsis BAK1, which has little or no activity with these synthetic peptides in vitro (Wang et al., 2008).

Although tomato BAK1 was not further characterized in terms of peptide preferences, the differences between tomato and Arabidopsis BRI1 and BAK1 highlight the importance of studies from different species.

Another important similarity with Arabidopsis is that the combination of SIBRI1 with SIBAK1 resulted in reciprocal transphosphorylation of each of the partner kinases, assessed using kinase-inactive site-directed mutants of either SIBRI1 or SIBAK1 (Fig. 1A). We previously expressed different combinations of kinase-inactive and wild-type tagged versions of Arabidopsis BRI1 and BAK1 in the same transgenic plant and found that an active BRI1 kinase, but not BAK1 kinase, was required for BR-dependent association of the pair. Moreover, when BAK1-GFP was expressed in the bri1-1 null mutant background, phosphorylation levels were dramatically reduced in BAK1-GFP (Wang et al., 2005, 2008). A range of in vitro kinase assays also showed that BAK1 stimulates BRI1 activity and that both BRI1 and BAK1 can transphosphorylate each other on specific residues. Based on these findings, we proposed a sequential transphosphorylation mechanism of BR signaling in which ligand-activated BRI1 heterodimerizes with BAK1, leading to BAK1 kinase activity on BRI1-mediated transphosphorylation of BAK1 activation loop residues, including the critical Thr-455 residue. The activated BAK1 in turn transphosphorylates BRI1 on juxtamembrane and C-terminal residues, increasing BR signaling by enhancing the phosphorylation of specific BRI1 substrates (Wang et al., 2008). In tomato, we have now shown that SIBRI1 transphosphorylates SIBAK1 on Thr-459, the residue equivalent to Thr-455 in Arabidopsis BAK1 (Table I). Furthermore, the end result of the transphosphorylation between active forms of both tomato kinases is enhanced SP11 peptide phosphorylation activity (Fig. 1C). However, unlike the situation with Arabidopsis BRI1 and BAK1, it cannot be concluded that the transphosphorylation activity of SIBRI1 was specifically increased by SIBAK1 transphosphorylation, as enhanced SIBAK1 activity directed against the SP11 peptide itself could also be involved. Further studies will be required to resolve this important point and to determine the extent to which the sequential transphosphorylation model applies to tomato BR signaling.

We previously examined the functional significance of each identified and predicted phosphorylation site in Arabidopsis BRI1 by site-directed mutagenesis of specific Ser, Thr, or Tyr residues followed by biochemical analysis in vitro and testing for the ability of the altered construct to rescue the weak bri1-5 BR-insensitive mutant in planta. For biochemical function, we assessed the effect of mutagenesis on autophosphorylation of the BRI1 cytoplasmic domain in vitro and on phosphorylation of the BRI1 synthetic peptide (Wang et al., 2005; Oh et al., 2009b). A similar analysis was conducted with tomato BRI1 to determine the functional significance of identified phosphorylation sites and their role in the biochemical function of SIBRI1. The juxtamembrane phosphorylation site Thr-872 appears to serve a negative regulatory role in Arabidopsis BRI1, as the T872A site-directed mutant has 10-fold higher activity against the BR13 peptide substrate compared with wild-type BRI1. The corresponding residue in tomato BRI1, Thr-877, is also phosphorylated, and the T877A site-directed mutant showed 20-fold higher activity with the BR13 peptide compared with the wild-type kinase. In contrast, Arabidopsis BRI1 juxtamembrane mutants Y831F, T842A, and S858A all showed substantially reduced peptide substrate phosphorylation (Wang et al., 2005; Oh et al., 2009b), while the corresponding tomato BRI1 residues showed increased (Y839F and T848A) or similar (S863A) peptide phosphorylation (Fig. 3B). Thus, there appears to be both conservation and divergence in the functional role of juxtamembrane phosphorylation between tomato and Arabidopsis BRI1, at least in vitro.

The activation of many protein kinases occurs by autophosphorylation of one to three residues within the activation loop of subdomain VII/VIII, which is thought to allow substrate access to the catalytic site in subdomain VIIb (Johnson et al., 1996). We previously confirmed that both Arabidopsis BRI1 and BAK1 autophosphorylate on at least three residues within the activation loop in vivo and thus may share this activation mechanism with many animal kinases (Wang et al., 2005, 2008). Moreover, sequence alignment of plant RLKs reveals that the activation loop is highly conserved, including several Ser and Thr residues that are routinely present. In fact, the activation loop residue corresponding to Thr-1049 in Arabidopsis BRI1 and Thr-455 in BAK1 is nearly invariant in 100 of the most closely related Arabidopsis RLKs. This conserved residue is also present in tomato BRI1 (Thr-1054) and BAK1 (Thr-459), and we showed here that this residue is phosphorylated in vitro in both tomato BRI1 and BAK1. Furthermore, functional analysis shows that tomato BRI1 Thr-1054 is essential for both autophosphorylation and peptide substrate phosphorylation in vitro (Fig. 3) and for normal BRI1 signaling and plant development in vivo (Fig. 4), as was previously demonstrated for...
Arabidopsis residue Thr-1049 (Wang et al., 2005). However, the function of other BRI1 activation loop residues appears to differ between Arabidopsis and tomato. Tomato BRI1 site-directed mutant T1047A shows complete loss of kinase activity for both auto-phosphorylation and peptide substrate phosphorylation (Fig. 3), while the corresponding Arabidopsis BRI1 mutant, T1042A, exhibits substantial autophosphorylation activity and limited peptide substrate phosphorylation (Wang et al., 2005). Thus, as with juxta-membrane residues, there appears to be both conservation and divergence in the functional role of activation loop residue phosphorylation when comparing tomato and Arabidopsis BRI1.

Characterizing BR-dependent phosphorylation sites in cytoplasmic targets of BRI1 is essential for a complete understanding of BR action. In mammals, the TGF-β family of polypeptides are perceived at the cell surface by a complex of type I (RII) and type II (RIII) TGF-β receptor Ser/Thr kinases. Mammalian TRIP1 binds to the cytoplasmic domain of the TGF-βRII receptor, is phosphorylated on Ser and Thr residues by the receptor kinase, and functions as a modulator of TGF-β receptor signaling in vivo (Choy and Derynck, 1998). Subsequently, TRIP1 was also shown to function as eIF3i in mammals (Asano et al., 1997), wheat (Triticum aestivum), and Arabidopsis (Burks et al., 2001). We previously found that the majority of Arabidopsis TRIP1 antisense and overexpression lines exhibited strong developmental phenotypes (Jiang and Clouse, 2001), that TRIP1 and BRI1 interact directly in planta, and that the BRI1 kinase domain phosphorylates Arabidopsis TRIP1 in vitro on at least three specific residues (Ehsan et al., 2005). These findings support a possible role for TRIP1 in the molecular mechanisms of BR-regulated growth and development in Arabidopsis. Here, we show that tomato BRI1 can also phosphorylate tomato TRIP1 on specific residues in vitro. Demonstration that BRI1 and TRIP1 also interact in planta will be necessary to show a functional role for the BRI1-TRIP1 interaction in tomato. Interestingly, the Tomato Expression Database (http://ted.bti.cornell.edu) shows that TRIP1 (represented by probes 1-1-6.20.15 and 1-1-7.4.13.21 on the TOM1 microarray) is expressed in immature fruit. Furthermore, genes encoding BR biosynthetic enzymes are expressed, endogenous BRs accumulate to significant levels in developing tomato fruit during the cell expansion phase (Montoya et al., 2005), and application of exogenous BRs promotes tomato fruit ripening (Vidya Vardhini and Rao, 2002). Therefore, SIBRI1-mediated BR signaling is likely to be active during the same period when TRIP1 is showing increased expression in developing tomato fruit, suggesting that studies of the interaction and phosphorylation of SIBRI1 and SITRIP1 at various stages of tomato fruit development might be a productive avenue of future experiments that would not be possible in Arabidopsis.

In summary, we have identified several specific sites of phosphorylation in both tomato BRI1 and BAK1 receptor kinases and have initiated a functional characterization of selected BRI1 sites and their role in kinase function and BR signaling. This work provides a foundation for future characterization of additional sites in vivo and their role in regulating BR signaling pathways involved in vegetative and reproductive development in tomato, an important horticultural crop.

MATERIALS AND METHODS

Cloning SIBRI1 and SIBAK1 Cytoplasmic Domains and SITRIP1 for Bacterial Expression

The tomato (Solanum lycopersicum) BRI1 cytoplasmic domain was amplified from tomato genomic DNA with sense (5'-CGATTCGAGGACGCAA- GAGGGGAGGAGG-3') and antisense (5'-GATGATCAGCTCAAGAGTTGT- TTGCTCAGCCT-3') primers. The purified PCR product was digested with Xhol/KpnI (New England Biolabs), gel purified, and ligated with Xhol/KpnI-digested, gel-purified pFlag-MAC (Sigma) expression vector to yield Flag-SIBRI1-CD, containing a N-terminal Flag epitope tag (DYKDDDDKVKL) followed by amino acids 824 through 1,207 of SIBRI1. The construct was completely sequenced to verify 100% identity with the SIBRI1 genome sequence. Transformation of Flag-SIBRI1-CD into pG-T2/BL21 Escherichia coli competent cells using CaCl2-mediated transformation (Bio-Rad) yielded single colonies (Promega). A second E. coli expression construct, MBP-SIBRI1, was generated in pMAL-cX4 expression vector (New England Biolabs) using the procedures described above. To generate a kinase-inactive mutant, Flag-SIBRI1(K916E), the correct mutation.

The SIBAK1 cytoplasmic domain was amplified by PCR from cDNA of tomato cv Heinz 5-d-old seedlings using primers MALBAK1-CD_F (5'-AGGAATTCCGTCGAAAACCGGAAGACCAC-3') + MALBAK1-CD_R (5'-ATAAGCTTTCATCTTGGCCCTGATAACTCATC-3'). The Flag-SIBAK1 kinase-inactive mutation (K321E) was generated by PCR-induced mutagenesis using the following primer pairs, MALBAK1-CD_F + mBAK1-CD_R (5'-CTTTCATTTCCACTCCAGAACCAGAACCAGAC-3') and mBAK1-CD_F (5'-TAGCTGCTTGATGAAATGGAAGGACC-3') + MALBAK1-CD_R, to produce two PCR products. The two PCR products were pooled, and primers MALBAK1-CD_F + MALBAK1-CD_R were used to generate mutant BAK1-CD. Both wild-type and mutant BAK1-CD were cloned into pGEM-T-easy (Promega) and sequenced. Wild-type and mutant BAK1-CD were digested from pGEM-T-easy with HindIII and EcoRI and were cloned into pMALc2E (New England Biolabs) to form N-terminal fusions with the MBP tag. Clones were sequenced to confirm MBP fusions.

SITRIP1 was amplified by PCR from cDNA of tomato cv Cobra seedlings using forward primer 5'-CGATATCGATGCTGAGCCATATGAGATTGCGGCCC-3' and reverse primer 5'-GACTCTCGACATATGATGTTGAGGAAATGAG-3'. The gel-purified PCR product was digested with HindIII/Xhol (Promega), gel purified, and ligated with HindIII/Xhol-digested, gel-purified pFlag-MAC (Sigma) to obtain Flag-SITRIP1. The construct was confirmed by DNA sequencing. The Flag-SIBRI1-CD construct was transformed into E. coli BL21 (DE3) cells (Stratagene).

Purification and Biochemical Analysis of Recombinant Proteins

The purification of Flag-tagged fusion constructs with M2 agarose beads was conducted essentially as per the manufacturer’s instructions (Sigma-Aldrich), using Flag peptide (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 0.25 mg/ml 12 Flag peptide) to elute Flag-SIBRI1-CD from the agarose beads. Purification of the MBP-tagged proteins using amylose resin was carried out per the manufacturer’s instructions (New England Biolabs) except that the concentration of maltose in the elution buffer was 20 mM rather than 10 mM.

For autophosphorylation assays, a 40-μL reaction containing 1 μg of affinity-purified Flag-SIBRI1-CD, MBP-SIBRI1-CD, or MBP-SIBAK1-CD was incubated with 20 μCi of [γ-32P]ATP in kinase buffer (50 mM HEPES-KOH, pH 8.0, 10 mM MgCl2, 5 mM DTT, and 10% glycerol). After incubation, samples were resolved by SDS-PAGE and analyzed by phosphorimaging.
Cloning of SIRBI1-Flag into Plant Transformation Vectors

SIRBI1 was PCR amplified from tomato genomic DNA with sense (5'-CGATCCCGGGAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCATCA-3') and antisense (5'-GACTGACTCAGCTTTTCCCTTCCTCTCTCTCTAG3') primers. The sense primer contained a 37-bp untranslated region sequence from Alfalfa mosaic virus upstream of the SIRBI1 start sequence that has been shown previously to enhance the expression of transgenes in transformed tomato plants (Krasnayanski et al., 2001). The PCR product was gel purified, digested with Smal and SacI, and introduced into the binary vector pBI121 (Clontech) that had also been digested with Smal/SacI and gel purified to remove the uidA gene, to yield the plasmid (35S:SIRBI1-Flag/pBI121). To obtain full-length SIRBI1 with the T1054A mutation, the first 2,484 bp of SIRBI1 was PCR amplified from 35S:SIRBI1-Flag/pBI121 using the sense primer 5'-CGATCCCGGGAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCATCA-3' and antisense primer 5'-GACTGACTCAGCTTTTCCCTTCCTCTCTCTCTAG3'. The resulting PCR product was gel purified and cloned into the pGemT-Easy vector (Promega). The SIRBI1-Flag gene carrying the T1054A mutation was amplified from the mutant Flag-SIRBI1-CD construct using the PCR primer pair 5'-AGGCCAAAGAGCCGGTTCGC-3' (forward) and 5'-GACTGAGCTCCTTACCATGCTCTGTCGTCGGGTT TC-3' (reverse). To obtain the full-length mutant SIRBI1-Flag construct in the pGemT-Easy vector, the amplified PCR product was gel purified, digested with Stul/SacI (Promega). gel purified, and ligated with a Stul/SacI-digested, gel purified pGemT-Easy vector containing the first 2,484 bp of SIRBI1. All constructs were sequenced completely.

The EcoRI/HindIII nosp-uidA segment of pGPTV-KAN (Becker et al., 1992) was replaced with 35S:GUS containing the EcoRI/HindIII fragment of the plasmid pBI121 to obtain pGPTV-35S:KAN. Full-length SIRBI1-Flag-T1054A was released from pGemT-Easy using Smal/SacI restriction enzymes, gel purified, and ligated into SacI-digested, gel purified pGPTV-35S:KAN. The wild-type SIRBI1-Flag construct was transferred from 35S:SIRBI1-Flag/pBI121 into pGPTV-35S:KAN by Smal/SacI digestion and ligation.

Transformation of the Tomato cuc3-abs Mutant with SIRBI1-Flag Constructs

Agrobacterium tumefaciens-mediated transformation was performed essentially as described by Krasnayanski et al. (2001) with modifications. Seeds of the wild-type lines of SIRBI1, cuc3-abs, and cuc3-dk (Gage and Bajwa, 2002) were autoclaved for 15 min with shaking at 50 rpm. Five to six weeks later, 1-cm-long shoots were excised and placed (15–20 explants per plate) on tomato shoot regeneration (TSR) medium (1× MS salts + MS vitamins, 30 g L−1 Suc, 0.1 mg L−1 indole-3-acetic acid, 2.0 mg L−1 zeatin, 300 g L−1 timentin, and 100 g L−1 kanamycin, 40 mg L−1 acetyosyringone, and 8.0 g L−1 agar, pH 5.6) for 2 weeks. The cultures were transferred to differentiation medium (1× MS salts + MS vitamins, 30 g L−1 Suc, 2.0 g L−1 Glc, 0.5 mg L−1 naphthylacetic acid, 1.0 mg L−1 benyalaldehyde, 700 g L−1 MES, 40 mg L−1 acetyosyringone, and 12 g L−1 agar, pH 5.6) and wounded with multipronged needles. Wounded cotyledons were subcultured in differentiated liquid A. tumefaciens suspension for 15 min with shaking at 50 rpm. Following inoculation, cotyledons were blotted dry on sterile paper towels and placed back on tomatococultivation medium for 48 h in the dark at 25°C.

After cocultivation, explants were washed twice to three times with LR medium containing 300 mg L−1 timentin, blotted dry on sterile paper towels, and placed (15–20 explants per plate) on tomato shoot regeneration (TSR) medium (1× MS salts + MS vitamins, 30 g L−1 Suc, 0.1 mg L−1 indole-3-acetic acid, 2.0 mg L−1 zeatin, 300 g L−1 timentin, 100 mg L−1 kanamycin, 40 mg L−1 acetyosyringone, and 8.0 g L−1 agar, pH 5.6). After 2 weeks on TSR medium, the explants were subcultured onto fresh TSR medium and further cultured under a 16-h photoperiod (provided by 20-W cool-white fluorescent tubes yielding a light intensity of 10 mmol m−2 s−1) for up to 6 months. Explants were excised and placed on a tomato rooting medium (1× MS salts + MS vitamins, 30 g L−1 Suc, 300 mg L−1 timentin, 100 mg L−1 kanamycin, and 8.0 g L−1 agar, pH 5.6) for secondary selection. Tomato plants with well-developed roots were transferred into small plastic pots with sterile soil and covered with clear.
PCR to Monitor SIBRI1-Flag Transgene Expression

Total RNA was isolated from 300 mg of leaves harvested from transgenic tomato plants of identical age followed by grinding in liquid nitrogen, and isolation of RNA using the RNeasy kit (Qiagen). Two micrograms of total RNA was used for the synthesis of first-strand cDNA with the RETROscript reverse transcription system using oligo(dT) primer (Ambion). PCR amplification of cDNA was used the following primers: 5′-ATGACTCAAATCATGGTACGAC-3′ and 5′-GATCTTCTGGCCTTGGACG-3′ for actin; 5′-GAACCCAGATAGTGATGATGACC-3′ and 5′-GATCTTCTGGCCTTGGACG-3′ for endogenous SIBRI1; and 5′-GAACCCAGATAGTGATGATGACC-3′ and 5′-GATCTTCTGGCCTTGGACG-3′ for transgenic SIBRI1-Flag. PCR was performed for 35 cycles of 94°C for 1 s, 55°C for 1.5 min, and 72°C for 1.5 min, followed by a single extension of 10 min at 72°C.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. BRII phosphorylation product ion spectra.
Supplemental Figure S2. BAKI phosphorylation product ion spectra.
Supplemental Figure S3. BAKI phosphorylation product ion spectrum.
Supplemental Figure S4. TRIP1 transphosphorylation product ion spectrum.

Received May 16, 2013; accepted July 1, 2013; published July 10, 2013.

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Plant Physiol. Vol. 163, 2013

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