Tyrosine phosphorylation of the BRI1 receptor kinase emerges as a component of brassinosteroid signaling in *Arabidopsis*

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Brassinosteroids (BRs) are essential growth-promoting hormones that regulate many aspects of plant growth and development. Two leucine-rich repeat receptor-like kinases (LRR-RLKs) are involved in BR perception and signal transduction: brassinosteroid insensitive 1 (BRI1), which is the BR receptor, and its coreceptor BRI1-associated kinase 1 (BAK1). Both proteins are classified as serine/threonine protein kinases, but here we report that recombinant cytoplasmic domains of BRI1 and BAK1 also autophosphorylate on tyrosine residues and thus are dual-specificity kinases. With BRI1, Tyr-831 and Tyr-956 are identified as autophosphorylation sites in vitro and in vivo. Interestingly, Tyr-956 in kinase subdomain V is essential for activity, because the Y956F mutant is catalytically inactive and thus this site cannot be simply manipulated by mutagenesis. In contrast, Tyr-831 in the juxtamembrane domain is not essential for kinase activity but plays an important role in BR signaling in vivo, because expression of BRI1(Y831F)-Flag in transgenic *bri1–5* plants results in plants with larger leaves (but altered leaf shape) and early flowering relative to plants expressing wild-type BRI1-Flag. Acidic substitutions of Tyr-831 restored normal leaf size (but not shape) and normal flowering time. This is an example where a specific tyrosine residue has been shown to play an important role in vivo in plant receptor kinase function. Interestingly, 6 additional LRR-RLKs (of the 23 tested) were also found to autophosphorylate on tyrosine in addition to serine and threonine, suggesting that tyrosine signaling should be considered with other plant receptor kinases as well.

It is well known that two leucine-rich repeat receptor-like kinases (LRR-RLKs) are involved in brassinosteroid (BR) perception and signal transduction: the receptor brassinosteroid insensitive 1 (BRI1) (1, 2) and its coreceptor, BRI1-associated kinase 1 (BAK1) (3, 4). Both of these receptor kinases have an extracellular domain, a single-pass transmembrane domain, and an intracellular cytoplasmic domain (CD) consisting of a juxtamembrane (JM) region, serine/threonine kinase domain (KD), and short carboxyl-terminal (CT) polypeptide (5–8). It is known that BRs bind to the extracellular domain of BRI1 (1) and thereby activate its KD and promote association with the coreceptor, BAK1 (3, 4, 6). This process results in release of BKI1, a recently described inhibitor protein (9), and phosphorylation of both BRI1 and BAK1 on multiple serine and threonine residues either as a result of autophosphorylation and/or transphosphorylation by their cytoplasmic KDs (6, 7, 10).

Autophosphorylation has been extensively studied in vitro by using recombinant CDs of the receptor kinases. Early studies demonstrated that when BRI1-CD was incubated with [γ-^{32}P]-ATP, phosphorylation of threonine and serine residues was readily detected (5–7, 11). More recently, staining with Pro-Q Diamond phosphoprotein stain and immunoblotting with antiphosphothreonine antibodies demonstrated that the CDs of BRI1 and BAK1 are highly autophosphorylated as isolated from *Escherichia coli*, and many of these sites have now been identified by mass spectrometry (6). During the course of our studies, we recently made the surprising observation that recombinant

BRI1-CD and BAK1-CD were also recognized by antiphosphotyrosine antibodies, suggesting that they may be dual-specificity kinases rather than serine/threonine kinases as classified (5–7, 11). The occurrence of tyrosine phosphorylation was confirmed for BRI1 both in vitro and in vivo, and we also identified two sites of tyrosine autophosphorylation. One of these sites (Tyr-831) is not essential for kinase catalytic activity but when manipulated by site-directed mutagenesis is demonstrated to play a key role in BR signaling in vivo.

Results

The key observation that served as the foundation of the present study was that recombinant BRI1-CD and BAK1-CD cross-reacted strongly with antiphosphotyrosine antibodies (Fig. 1*A*). This finding was surprising because both BRI1 and BAK1 are classified as serine/threonine protein kinases (12, 13) and have been shown to autophosphorylate only on serine and threonine residues (5–7, 11). Hence, it was critical to confirm the specificity of the antiphosphotyrosine antibodies, which we did in several different ways. First, addition of phosphotyrosine-conjugated BSA (pY-BSA) to the immunodetection buffer resulted in the disappearance of immunoblot bands indicating specificity of detection (Fig. 1*A*). Second, mBRI1, a kinase-inactive mutant of BRI1 resulting from a K911E substitution (6), was not autophosphorylated and did not cross-react with the antiphosphotyrosine antibodies, indicating that phosphorylated residues on BRI1 were specifically being detected (Fig. 1*B*). Third, pretreatment of BRI1-CD with a recombinant protein tyrosine phosphatase, PTP1B (14), eliminated the recognition of BRI1 by the antiphosphotyrosine antibodies without substantially affecting the staining with Pro-Q Diamond (Fig. 1*C*) or recognition by antiphosphothreonine antibodies (data not shown). Similar results to those shown in Fig. 1 for BRI1-CD were also obtained with recombinant BAK1-CD (data not shown). Collectively, these results indicated that the antiphosphotyrosine antibodies were specifically recognizing phosphotyrosine residues in the receptor kinases.

One possible explanation for the lack of detection of phosphotyrosine residues in BRI1 and BAK1 after [^{32}P]ATP autophosphorylation in vitro is that the stoichiometry of tyrosine autophosphorylation is extremely low and may be detectable only with very sensitive immunoblotting protocols. However, the
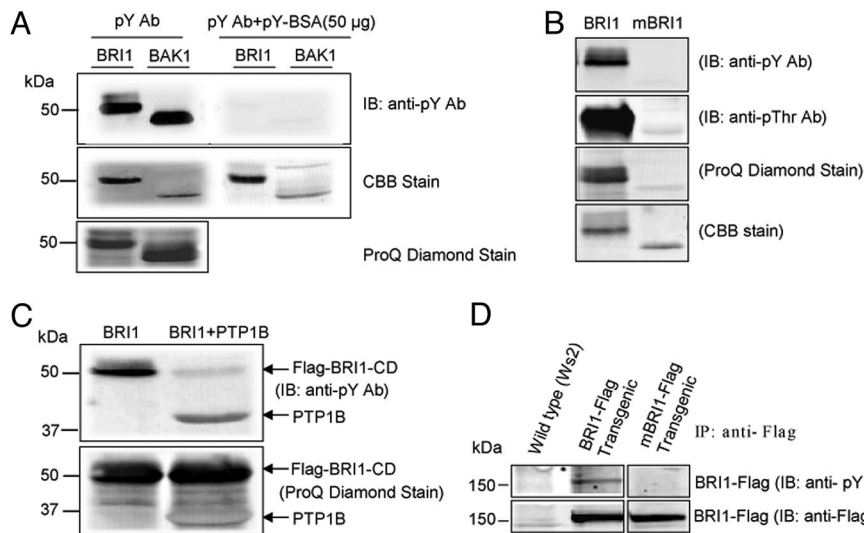
Fig. 1. BRI1 is phosphorylated on tyrosine residues in vitro and in vivo. (*A*) Detection of phosphotyrosine residues in recombinant BRI1 and BAK1 CDs by immunoblotting with antiphosphotyrosine (pY) antibodies. Preincubation of the antibodies with pY-BSA eliminated the immunoblot signal indicating specificity of the detection. Protein abundance was visualized by Coomassie Brilliant Blue (CBB) staining. (*B*) The kinase-inactive K911E mutant, mBRI1 (6), does not autophosphorylate as indicated by the lack of immunoblot signal with antiphosphotyrosine (pY) antibodies, antiphosphothreonine (pT) antibodies, or staining with Pro-Q Diamond phosphoprotein stain. (*C*) Treatment of the recombinant BRI1-CD with recombinant PTP1B dephosphorylates tyrosine residues without substantially reducing phosphorylation on serine/threonine residues, as indicated by staining with Pro-Q Diamond phosphoprotein stain. (*D*) Phosphorylation of BRI1-Flag on tyrosine residues in vivo. Nontransgenic wild-type plants served as a control to demonstrate specificity of the immunoprecipitation of BRI1-Flag from transgenic plants, and mBRI1-Flag served as a control to demonstrate specificity of the interaction with the antiphosphotyrosine antibodies.

stoichiometry of tyrosine phosphorylation on recombinant BRI1-CD was in fact quite high, because ≈50% of the BRI1-Flag protein could be immunoprecipitated with immobilized antiphosphotyrosine antibodies (Fig. S1). Thus, the failure to detect phosphotyrosine by [^{32}P]-phosphoamino acid analysis may be because the recombinant protein is already highly autophosphorylated on tyrosine residues as isolated from *E. coli*. Analysis of recombinant BRI1-CD tryptic peptides by liquid chromatography-tandem mass spectrometry (LC/MS/MS) was consistent with tyrosine phosphorylation of residue 831 (Fig. S2). We showed that S838 of BRI1-CD (which is on the same tryptic peptide as Y831) is heavily phosphorylated (6, 10) and may have suppressed the ionization efficiency for the phosphorylated Y831 peptide, thus complicating data-dependent LC/MS/MS identification of this residue.

Importantly, BRI1 was also found to be phosphorylated on tyrosine residues in vivo. As shown in Fig. 1*D*, immunoprecipitated BRI1-Flag protein from transgenic *Arabidopsis* plants clearly cross-reacted with antiphosphotyrosine antibodies, establishing that tyrosine phosphorylation of BRI1 occurs in vivo. The failure to immunoprecipitate protein from wild-type plants with the anti-Flag antibodies and the failure to detect a phosphotyrosine signal in immunoprecipitated mBRI1-Flag served as critical controls to validate the detection of phosphotyrosine in BRI1-Flag in vivo (Fig. 1*D*).

Autophosphorylation of tyrosine residues in vitro was also observed in recombinant cytoplasmic domains of several other receptor kinases. Of the 23 LRR-RLKs that were tested, 11 were clearly autophophosphorylated (as evidenced by strong Pro-Q Diamond phosphoprotein staining) and of these 11, 9 contained phosphothreonine and 6 contained phosphotyrosine by immunoblotting (Fig. S3). Therefore, tyrosine phosphorylation may be of broad significance in plant receptor kinase signaling.

To investigate the role of specific tyrosine residues of BRI1, we produced site-directed mutants of BRI1-CD by individually replacing each of the 10 tyrosine residues with phenylalanine. The corresponding recombinant mutant proteins were compared with active wild-type BRI1-CD and the kinase-inactive

mutant, mBRI1-CD, for ability to catalyze autophosphorylation and transphosphorylation reactions. Substitution of tyrosine at positions 956, 1052, 1057, and 1072 with phenylalanine strongly inhibited autophosphorylation as indicated by almost complete elimination of Pro-Q Diamond staining and cross-reactivity with antiphosphothreonine and antiphosphotyrosine antibodies (Fig. 2 *A* and *B*). As expected, these substitutions also strongly inhibited kinase activity on a previously identified optimal peptide substrate for BRI1-CD (5) (Fig. 2*C*). Because these 4 tyrosine residues are essential for BRI1 kinase activity, the site-directed mutagenesis approach cannot be used to determine whether these residues are potential autophosphorylation sites. In contrast, substitution of the tyrosine residues at positions 898, 945, 961, 1058, and 1070 with phenylalanine did not reduce the phosphotyrosine immunoblot signal relative to wild-type BRI1 (Fig. 2*B*), and all retained considerable peptide kinase activity (Fig. 2*C*), suggesting that these tyrosine residues are not essential for activity and are also not major sites of tyrosine autophosphorylation. The only site-directed mutant that had reduced tyrosine autophosphorylation but retained significant autophosphorylation activity on serine and threonine residues and peptide kinase activity was the Y831F mutant (Fig. 2*A* and *B*). These results suggested that Tyr-831 is an autophosphorylation site. Interestingly, the inhibition of peptide kinase activity observed in the Flag-BRI1(Y831F) site-directed mutant was substantially ameliorated when Tyr-831 was substituted with an aspartate residue (Fig. S4), which is consistent with the notion that Tyr-831 is a major site of tyrosine autophosphorylation and that autophosphorylation stimulates kinase activity. However, because the BRI1-Y831F mutant still had ≈40% of the phosphotyrosine immunoblot signal of the wild-type protein, there is at least 1 additional tyrosine autophosphorylation site among the residues that when mutated resulted in the kinase-inactive phenotype.

As an additional approach to localize sites of tyrosine autophosphorylation, we used the Flag- BRI1-CD construct to produce a set of deletion mutants lacking either the JM domain, the CT domain, or both. All constructs had the KD, which contains 9 of the 10 tyrosine residues, with a Flag-epitope tag at
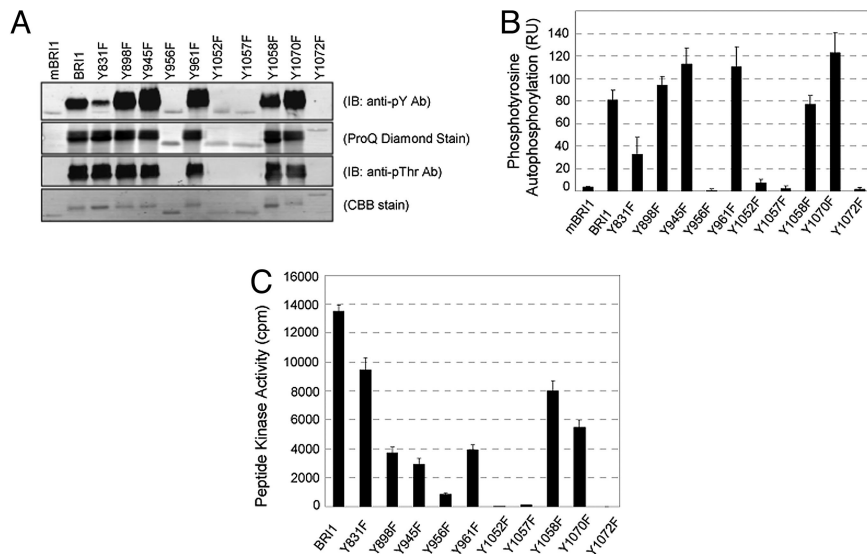
Fig. 2. Identification of tyrosine residues on BRI1 that affect autophosphorylation and transphosphorylation of a synthetic peptide substrate. (*A*) Effect of site-directed mutagenesis of tyrosine residues in the BRI1-CD on autophosphorylation of the recombinant protein. (*B*) Densitometry analysis of antiphosphotyrosine immunoblots similar to those shown in *A*. (*C*) Tyrosine mutagenesis inhibits transphosphorylation of the SP11 (5) synthetic peptide.

the N terminus (Fig. 3*A*). All of the recombinant proteins had significant autophosphorylation activity, as evidenced by Pro-Q Diamond staining (Fig. 3*B*). Interestingly, autophosphorylation of the constructs lacking the JM domain (BRI1-KC and BRI1-K) was primarily on serine residues as there was little or no evidence for phosphothreonine or phosphotyrosine by immunoblotting (Fig. 3*B*); only the constructs with the JM domain (BRI1-JKC and BRI1-JK) had robust autophosphorylation on both threonine and tyrosine residues. Furthermore, the constructs containing the JM domain also had the highest rates of peptide kinase activity (Fig. 3*C*), suggesting that the JM domain plays a greater role as an activator of kinase activity ($\approx$5-fold activation) than the CT domain plays as a negative regulator ($\approx$20% inhibition). These results are consistent with the in vivo result of Wang *et al.* (7) that the JM domain is essential for a functional BRI1 receptor, and with our previous in vitro results showing that specific JM residues played a critical role in peptide substrate phosphorylation by BRI1-KD (6).

Sequence- and modification-specific antibodies were developed to determine whether individual tyrosine residues in BRI1 were indeed autophosphorylation sites. As shown in Fig. 4*A*, recombinant BRI1-CD cross-reacted with antibodies directed against the sequences surrounding phosphotyrosine-831, phosphotyrosine-956, and phosphotyrosine-1072, suggesting that all three residues are sites of tyrosine autophosphorylation in vitro. The specificity of the modification-specific antibodies was indicated by: (*i*) the lack of cross-reaction with the kinase-inactive mutant, mBRI1, (*ii*) almost complete removal of the immunoblot signal by pretreatment of wild-type BRI1 with PTP1B (Fig. 4*A*), and (*iii*) specific inhibition of the immunoblot signals by only the appropriate phosphopeptide ([Fig. S5](#)). Furthermore, the BRI1 site-directed mutants also reacted with the antibodies in the expected manner. The Y956F and Y1072F mutants were kinase inactive as expected (Fig. 2) and accordingly did not cross-react with any of the phosphospecific antibodies (Fig. 4*A*). However, the Y831F mutant was kinase active as expected (Fig. 2) and cross-reacted with the anti-pY956 and anti-pY1072 antibodies but not the anti-pY831 antibodies. Importantly, full-length BRI1-Flag was phosphorylated on tyrosine residues in vivo (Figs. 1*D* and 4*B*) and the modification-specific antibodies identified Tyr-831 and Tyr-956 as in vivo sites of tyrosine phosphorylation (Fig. 4*B*). The failure to immunoprecipitate

protein from wild-type plants using anti-Flag antibodies and the failure to detect a specific phosphotyrosine signal in immunoprecipitated mBRI1-Flag, a kinase-inactive mutant, served as critical controls to validate the detection of site-specific tyrosine phosphorylation in BRI1-Flag in vivo. Likewise, the lack of cross-reaction of BRI1(Y831F)-Flag with the anti-pY831 antibodies confirmed the sequence specificity of these antibodies when applied to native full-length proteins. Interestingly, Tyr-1072 was not autophosphorylated in vivo as it was in vitro, and thus, autophosphorylation of this residue is apparently not required for BRI1 kinase activity. Rather it may be the hydroxyl function at residue 1072 that is needed for kinase function. However, the most important point to note is that Tyr-831 and Tyr-956 on BRI1 are clearly established as in vivo phosphorylation sites.

To determine whether tyrosine phosphorylation is required for BRI1 function in vivo, we transformed the weak *bri1–5* mutant, which is an intermediate dwarf, with wild-type BRI1-Flag or site-directed mutants where Tyr-831 or Tyr-1052 was manipulated. The dwarf phenotype of the *bri1–5* mutant (15) was rescued by expression of wild-type BRI1-Flag (Fig. 5*A*), as demonstrated (6, 7), but only slightly by the BRI1(Y1052F)-Flag construct, which is consistent with the kinase-inactive phenotype of the corresponding recombinant protein (Fig. 2). However, whereas expression of BRI1(Y831F)-Flag clearly rescued the dwarf phenotype of the *bri1–5* mutant, the aberrant leaf shape (round leaves) of the *bri1–5* mutant (15) was not completely rescued. This result is visually apparent in the plants grown for 30 days under short days (Fig. 5*A*) and in plants grown for 32 days under long days (Fig. 5*B*). Quantitative analysis confirmed that whereas *bri1–5* leaves were small and nearly round, expression of the wild-type BRI1-Flag construct resulted in production of larger and elongated leaves as expected (Fig. 5*C*). In the case of the Y1052F site-directed mutant there was only a slight rescue of leaf size, whereas the Y831F site-directed mutant produced leaves that were even larger than wild-type BRI1 but the normal elongated leaf shape was only partially restored (Fig. 5*C*). Both of the acidic substitutions, BRI1(Y831D) and BRI1(Y831E), also rescued the dwarf phenotype but the elongated leaf shape was also not completely restored by the acidic substitutions. Interestingly, leaf size in the acidic substitutions was not in-
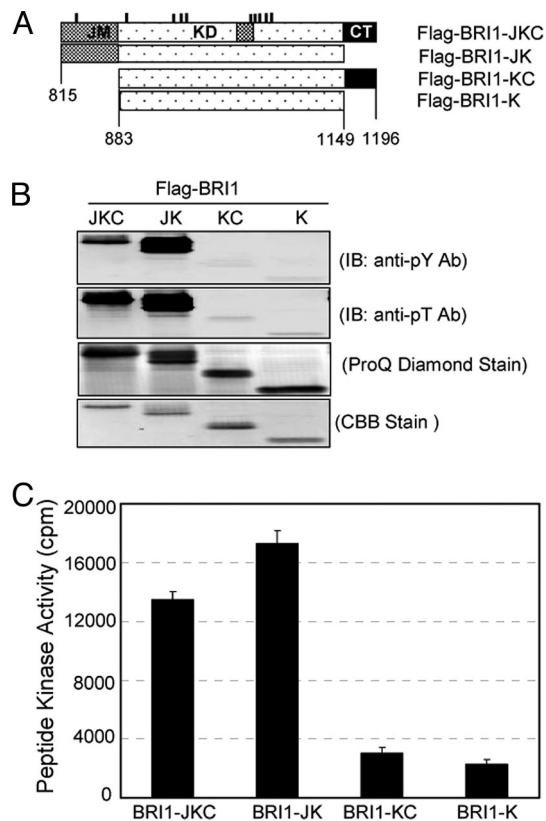
Fig. 3. The JM domain is a positive regulator of BRI1 kinase activity and is required for tyrosine phosphorylation. (*A*) Schematic representation of the Flag-BRI1-CD and the deletion mutants used in these experiments. J, K, and C refer to the JM, kinase, and C-terminal domains, respectively, and the hatch marks identify the location of the 10 tyrosine residues in BRI1-CD. The shaded portion of the KD depicts the activation loop. (*B*) Autophosphorylation of the recombinant proteins shown in *A* as measured with Pro-Q Diamond stain and immunoblotting with antiphosphotyrosine (pY) or antiphosphothreonine (pT) antibodies. (*C*) Transphosphorylation activity of the recombinant full-length and truncated proteins using the SP11 synthetic peptide as substrate.

creased above that observed with the wild-type BRI1-Flag construct (Fig. 5*C*).

Another important observation was that the *bri1-*5:BRI1(Y831F)-Flag plants flowered earlier than the other

genotypes (Fig. 5*B*). In Fig. 5*A*, plants were grown under short days and the BRI1(Y831F)-Flag transgenic plants were just beginning to bolt whereas the other plants were not, while under long days (Fig. 5*B*) BRI1(Y831F)-Flag plants clearly flowered earlier than wild-type BRI1. Thus, the early flowering phenotype was independent of photoperiod. As quantified in Fig. 5*C*, the *bri1–5*:BRI1(Y831F)-Flag plants flowered 7–10 days earlier than the *bri1–5* mutant control or *bri1–5* expressing wild-type BRI1-Flag or the Y1052F mutant. Importantly, the acidic substitutions at Tyr-831 restored the normal flowering time observed for wild-type BRI1-Flag plants. These results are consistent with the notion that there is a link between BR signaling and flowering (16) and suggests that phosphorylation of Tyr-831 may play some role in this linkage.

Discussion

We have shown that BRI1 is autophosphorylated on tyrosine residues, and we have demonstrated tyrosine phosphorylation of a plant receptor kinase in vivo. As noted in the Introduction, this finding is surprising because BRI1 has only been shown in previous studies to autophosphorylate on serine and threonine residues. None of the recognized consensus motifs for tyrosine kinases or dual-specificity kinases are contained in BRI1 (Table S1) or the other plant receptor kinases shown in the present study to have serine, threonine and tyrosine kinase activity (data not shown). Identifying the KD residues that control dual specificity of catalytic activity will be an important topic for future studies. It is relevant to note that two previous studies identified plant receptor kinases with tyrosine kinase activity in vitro: PRK1, a pollen-expressed receptor kinase that was shown to autophosphorylate on serine and tyrosine residues in vitro (17), and AtSERK1, which is transiently expressed during embryogenesis and was reported to phosphorylate myelin basic protein and casein on serine, threonine, and tyrosine residues in vitro (18). These results suggested that PRK1 and AtSERK1 are dual-specificity kinases; however, the occurrence of phosphotyrosine in vivo was not established nor was the significance of tyrosine phosphorylation elucidated, as was done in the present study with BRI1.

We also document the ability of several other receptor kinases to autophosphorylate on tyrosine residues. In addition to BRI1 and BAK1, many recognized receptor kinases, including BKK1 (19), HAESA (20), and the light-repressible receptor kinase (At3g21340) (21), all appear to be dual-specificity kinases (Fig. S3). Of particular interest is BAK1, which is a multifunctional
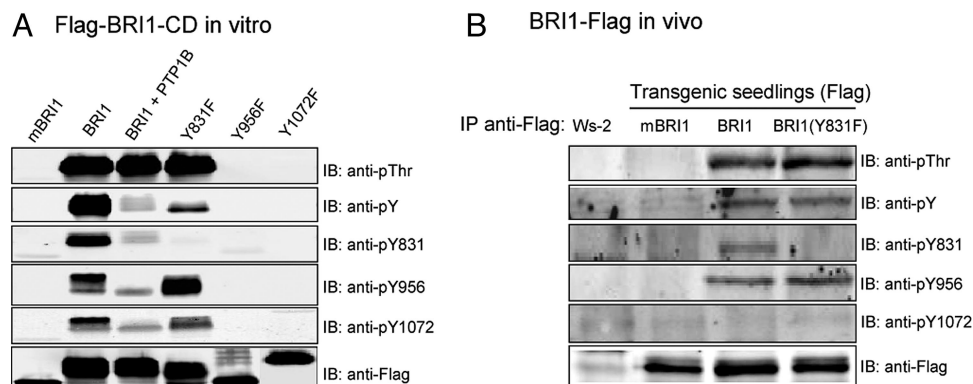

Fig. 4. Identification of Tyr-831, Tyr-956, and Tyr-1072 as phosphorylation sites on BRI1 using modification-specific antibodies. (*A*) Immunoblot analysis of recombinant Flag-BRI1-CD expressed in *E. coli*. The kinase-inactive mBRI1, wild-type BRI1 (treated or not with PTP1B), and the site-directed mutants Y831F, Y956F, and Y1072F were affinity purified and analyzed by immunoblotting (IB) with the indicated antibodies. (*B*) Immunoblot analysis of full-length BRI1-Flag constructs expressed in transgenic *Arabidopsis* plants. Protein was immunoprecipitated (IP) from nontransgenic wild-type plants (Ws-2) and transgenic plants using immobilized anti-Flag antibodies, and the purified protein was analyzed by immunoblot analysis as in *A*. Seedlings were grown in liquid culture and treated with 10^{-7} M brassinolide for 2 h before harvest.
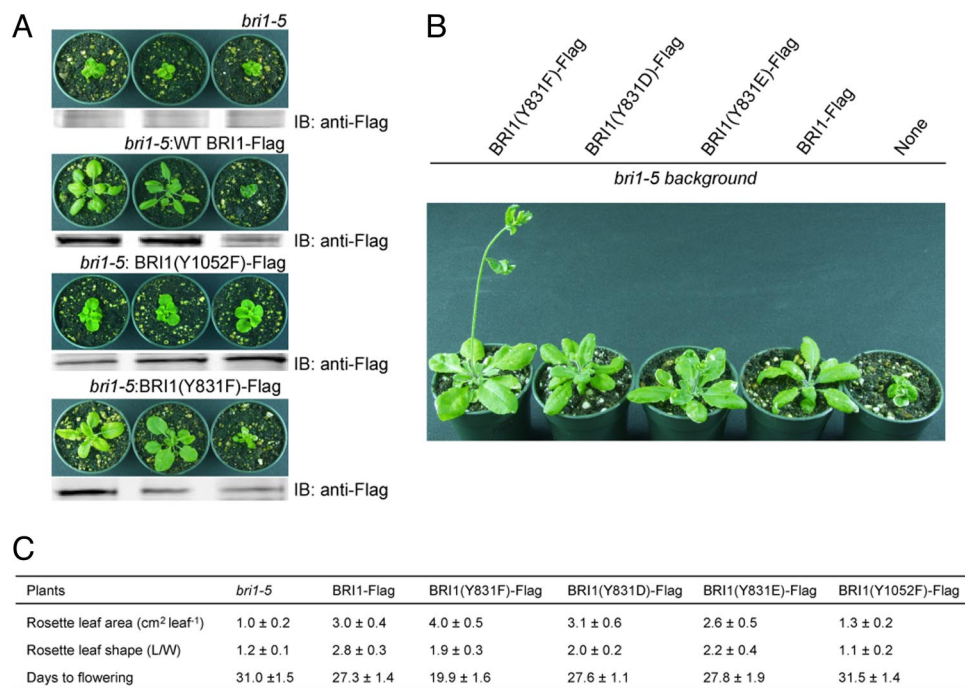
Fig. 5. Effect of mutating Tyr-831 or Tyr-1052 in the BRI1-CD on rescue of the weak *bri1–5* mutant. (*A*) Three independent transgenic lines (T2 generation) for each construct are shown after 30 days of growth under short days (8-h photoperiod). Immunoblotting with anti-Flag antibodies was used to demonstrate the level of transgenic protein in each line. (*B*) Tyr-831 plays an important role in BR signaling in vivo as revealed by rescue of some, but not all, growth abnormalities of the *bri1–5* mutant. Plants were grown for 32 days under long day conditions (16-h photoperiod). (*C*) Impact of tyrosine mutations of BRI1-Flag on leaf area (cm²·rosette leaf⁻¹), leaf shape (L/D, length/width), and timing of transition from vegetative to reproductive growth of plants grown on a long day (16 h) photoperiod for 32 days. The *bri1–5*, BRI1-Flag, BRI1(Y831D/E)-Flag, and BRI1(Y1052F)-Flag plants involved 10 independent transgenic lines (10 plants total) except for BRI1(Y831F)-Flag, which involved 12 transgenic lines (24 plants total). Transgenic lines, all in the *bri1–5* background, were used at the T2 stage and identical results were obtained with plants at the T1 stage (data not shown).

coreceptor involved in three distinct pathways: BR signaling, plant innate immunity (22, 23), and programmed cell death (19, 24). It is possible that the diverse functions of BAK1 are controlled by site-specific phosphorylation (10), and given our current findings, potential tyrosine autophosphorylation sites must be considered along with serine and threonine sites. The paradigm that plant receptor kinases are serine/threonine protein kinases, whereas in animal receptor kinases tyrosine phosphorylation often plays a prominent role (12, 25) must be modified.

Two specific tyrosine residues on BRI1 are shown to play an important role in BR signaling in vivo. Tyr-1052, which is located in the activation loop, is essential for kinase activity in vitro (Fig. 2) and in vivo, because the BRI1(Y1052)-Flag construct did not rescue the dwarf *bri1–5* mutant (Fig. 5). There is currently no evidence that Tyr-1052 is autophosphorylated, and if that is true, the hydroxyl function at residue 1052 may play a critical role in positioning of the activation loop so that the KD can assume an active conformation. In contrast, Tyr-831 is not essential for kinase activity but appears to be a major site of tyrosine autophosphorylation and plays an important role in BR signaling, as evidenced by the impact of the BRI1(Y831F) mutation on leaf shape and size and flowering time. The observation that acidic substitutions for Tyr-831 mimicked wild-type BRI1 suggests that phosphorylation of Tyr-831 is involved in controlling leaf size and flowering time (Fig. 5C). However, the acidic substitutions did not mimic wild-type BRI1 in terms of the impact on leaf shape, and thus it appears that the hydroxyl function of Tyr-831 may be the essential feature for this growth response. Moreover, it should be noted that acidic substitutions do not always mimic phosphoamino acids, especially when the function of phosphorylation is to promote protein/protein in-

teractions (26, 27). In such a case, substitution of tyrosine with phenylalanine or acidic residues would both result in loss of function. Important to the present study, however, is that two of the phenotypes associated with the BRI1(Y831F) mutant were restored to wild type by the acidic substitutions, which is consistent with a role for phosphotyrosine-831 in BR signaling in vivo. Although details remain unclear, our results are generally consistent with recent findings that BR signaling controls leaf size (28), leaf shape (29), and flowering (16). Molecular details of how Tyr-831 in its phosphorylated or dephosphorylated forms is involved in these processes remain to be elucidated, but our findings provide the foundation for further studies of tyrosine signaling involving BRI1 and other receptor kinases. It is worth noting that mutagenesis of serine/threonine autophosphorylation sites of BRI1 that have been tested to date have had only small effects on plant growth parameters (6), apart from residues in the activation loop such as Ser-1044 and Thr-1049 that are known to be essential for kinase activity. By comparison, the effect of mutagenesis of Tyr-831 on plant growth and development is striking. Establishing a role for tyrosine phosphorylation in BR signaling also adds to the similarities of plant receptor kinase function with the well-known models of animal receptor tyrosine kinase action (10).

Materials and Methods

Plant Growth. *Arabidospsis thaliana* ecotype Ws-2 was used as the wild type. Seeds were surface sterilized, kept at 4 °C for 2 days, and then sown on germination medium [1/4 strength Murashige and Skoog salt and vitamins medium (PhytoTechnology Laboratories), supplemented with 0.8% (wt/vol) agar, 30 mg·L⁻¹ hygromycin B (Sigma–Aldrich) and 2% sucrose, pH 5.7]. For phenotypic analysis between wild type, *bri1–5* allele, and tyrosine site-directed mutants, all plants were grown in the same temperature and light conditions to analyze phenotype. BRI-Flag and mBRI1-Flag transgenic lines in

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the *bri1–5* mutant background were as described (6). The same BRI1-Flag construct was used to generate the site-directed mutants, Y831F, Y831D, Y831E, and Y1052F, and each construct was transformed individually into the *bri1–5* mutant as described (6).

Preparation of Microsomal Membranes for Immunoprecipitation and Immunoblot Analysis. *A. thaliana* plants were grown in shaking liquid culture (30), and microsomal membranes were isolated as described (6). Membranes were solubilized with 1% (vol/vol) Triton X-100, clarified by centrifugation, and diluted so that protein concentration was adjusted to 0.5–1.0 mg/mL, and Triton X-100 was reduced to 0.1%.

BRI1-Flag was then immunoprecipitated with prewashed anti-Flag M$_2$ affinity gel (Sigma–Aldrich) and analyzed by immunoblotting after transfer to PVDF membranes using anti-Flag antibody (1:5,000 dilution), antiphosphotyrosine antibody (1:500), antiphosphothreonine antibody (1:500), or custom modification antibodies (anti-pY831, anti-pY956, or anti-pY1072 antibodies). The custom antibodies were produced against the following sequences: pY831, KKEAELEMpYAEGH; pY956, ERLLVpYEFMK; and pY1072, KGD-VYSpYGVV. The antibodies were produced by GenScript and sequentially affinity purified by using the nonphosphorylated and phosphotyrosine-containing antigen peptides. Immunoblots were scanned with an Odyssey Infrared Imaging System (LI-COR Bioscience) for visualization. As indicated, blots were also stained with ProQ Diamond phosphoprotein stain and scanned by using a Typhoon Molecular Dynamics phosphor/fluorescence imager.

Site-Directed Mutagenesis of Flag-BRI1-CD. The described Flag-BRI1-CD construct (5) was used as the template for site-directed mutagenesis with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). Ten individual constructs were generated with the following substitutions: Y831F, Y898F, Y945F, Y956F, Y961F, Y1052F, Y1057F, Y1058F, Y1070F, and Y1072F. All constructs were sequenced in both directions to verify specific mutations and lack of additional mutations.

Recombinant Protein Purification and In Vitro Peptide Kinase Assay. Flag-BRI1-CD and its mutations were expressed in BL21(DE3)pLysS cells and purified by using anti-Flag M2 affinity gel (Sigma–Aldrich). After elution from the beads, the protein solution was dialyzed against a 1,000× volume of buffer containing 20 mM Mops, pH 7.5, and 1 mM DTT. Peptide substrate phosphorylation assays were performed as described (5) using the SP11 peptide (sequence: GRJRRIASVEJJK, where J is norleucine; produced by Bethyl Laboratories).

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