Brassinosteroid-Insensitive-1 Is a Ubiquitously Expressed Leucine-Rich Repeat Receptor Serine/Threonine Kinase[1]

Danielle M. Friedrichsen[2], Claudio A.P. Joazeiro[2], Jianming Li[2,3], Tony Hunter, and Joanne Chory*

Plant Biology Laboratory (D.M.F., J.L., J.C.), Molecular Biology and Virology Laboratory (C.A.P.J., T.H.), and Howard Hughes Medical Institute (J.C.), The Salk Institute for Biological Studies, La Jolla, California 92037; and Department of Biology, University of California at San Diego, La Jolla, California 92037 (D.M.F.)

Brassinosteroid (BR) mutants of Arabidopsis have pleiotropic phenotypes and provide evidence that BRs function throughout the life of the plant from seedling development to senescence. Screens for BR signaling mutants identified one locus, *BRI1*, which encodes a protein with homology to leucine-rich repeat receptor serine (Ser)/threonine (Thr) kinases. Twenty-seven alleles of this putative BR receptor have been isolated to date, and we present here the identification of the molecular lesions of 14 recessive alleles that represent five new mutations. BR-insensitive-1 (BRI1) is expressed at high levels in the meristem, root, shoot, and hypocotyl of seedlings and at lower levels later in development. Confocal microscopy analysis of full-length BRI1 fused to green fluorescent protein indicates that BRI1 is localized in the plasma membrane, and an in vitro kinase assay indicates that BRI1 is a functional Ser/Thr kinase. Among the *bri1* mutants identified are mutants in the kinase domain, and we demonstrate that one of these mutations severely impairs BRI1 kinase activity. Therefore, we conclude that BRI1 is a ubiquitously expressed leucine-rich repeat receptor that plays a role in BR signaling through Ser/Thr phosphorylation.

Receptor protein kinases (RPKs) activate a complex array of intracellular signaling pathways in response to the extracellular environment (van der Geer et al., 1994; Padgett, 1999). RPKs are single-pass transmembrane proteins that contain an amino-terminal signal sequence, extracellular domains unique to each receptor, and a cytoplasmic kinase domain. In general, ligand binding induces homo- or heterodimerization of RPKs, and the resultant close proximity of the cytoplasmic domains results in kinase activation by transphosphorylation. Although plants have many proteins similar to RPKs, no ligand has been identified for these receptor-like kinases (RLKs). The majority of plant RLKs belong to the family of Ser/Thr kinases, and most have extracellular Leu-rich repeats (LRRs; Becraft, 1998). The LRRs form a solvent-exposed parallel β-sheet, which creates a surface that mediates protein-protein interactions in other systems (Kobe and Deisenhofer, 1995). The known interactors for mammalian LRR receptors are peptide hormones, such as nerve growth factor and gonadotropin (Braun et al., 1991; Kobe and Deisenhofer, 1995).

Plant LRR-RLKs are involved in multiple processes including regulation of development (ERECTA, HAESA, and CLV1), disease resistance (Xa21), and steroid hormone signaling (brassinosteroid [BR]-insensitive-1 [BRI1]) (Song et al., 1995; Torii et al., 1996; Clark et al., 1997; Li and Chory, 1997; Jinn et al., 2000). ERECTA is important for proper shaping of organs originating in the shoot apical meristem (Torii et al., 1996). CLV1 is involved in the control of cell division and differentiation in the shoot apical meristem with CLV3 being the putative peptide ligand (Fletcher et al., 1999). Missense mutations in either the LRRs or the kinase domains of CLV1 and ERECTA lead to loss of function, confirming the importance of these domains for function (Torii et al., 1996; Clark et al., 1997). BRI1 encodes a putative BR receptor (Li and Chory, 1997), and *bri1* mutants display a BR-deficient phenotype but fail to be rescued by BR treatment (Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999). BRs are a unique class of plant steroids found throughout the plant kingdom that exhibit multiple effects when applied exogenously, including cell expansion of young aerial tissues, especially the hypocotyl and leaf petioles (Mandova, 1988). Classical animal steroid hormone receptors belong to a subfamily of nuclear receptors that are ligand-dependent transcription factors that regulate gene expression (Beato et al., 1995). There is also evidence for action of steroid hormones outside the nucleus involving membrane receptors and protein phosphorylation (Wehling, 1997). In *Xenopus* oocytes, progesterone

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[2] These authors contributed equally to the paper.

[3] Present address: Department of Biology, University of Michigan, Ann Arbor, MI 48109.

* Corresponding author; e-mail chory@salk.edu; fax 858–558–6379.

through an unidentified surface-associated receptor activates a Ser/Thr kinase, Eg2 (Andrésson and Ruderman, 1998). Additionally, progesterone stimulates Tyr phosphorylation in human sperm via a putative cell surface receptor (Tesarik et al., 1993; Mendoza et al., 1995), and in osteoblastic cells estrogen causes a rapid and transient MAP kinase activation (Endoh et al., 1997).

The BRI1 extracellular domain contains 21 tandem amino-terminal LRRs, a 70-amino acid island domain and four additional LRRs preceding the transmembrane domain. This organization is similar to that in tomato disease-resistance membrane-anchored LRR proteins, Cf-2, Cf-4, Cf-5, and Cf-9 (Jones and Jones, 1997; Dixon et al., 1998). These proteins contain "loop out" domains, similar to but smaller than the island domain, and these "loop out" domains interrupt tandem LRRs, creating four separate LRRs prior to the transmembrane domain. Three *bri1* mutations are in glycines of the island domain, and one is a missense mutation in the first LRR following the 70-amino acid island domain (Li and Chory, 1997; Noguchi et al., 1999), supporting the necessity of these regions for function. Four missense mutations occur in the cytoplasmic Ser/Thr kinase domain of BRI1, implicating another essential domain for BRI1 function (Li and Chory, 1997; Noguchi et al., 1999).

In this paper we present the identity of additional recessive alleles of *BRI1*, which highlights the importance of certain domains for BRI1 function. Additionally, we used a green fluorescent protein fusion to show the localization of BRI1 to the plasma membrane and to determine its pattern of expression within the plant. Finally, the function of BRI1 as a Ser/Thr kinase is directly demonstrated by an in vitro kinase assay. Therefore, we conclude that BRI1, the putative brassinolide receptor, is a ubiquitously expressed, plasma membrane-localized, LRR Ser/Thr kinase.

RESULTS

Sequencing of Additional *bri1* Alleles Implicates a New Domain in BRI1-Mediated Steroid Signaling

We previously conducted a screen for BR-insensitive mutants, and isolated 18 new *bri1* alleles (Li and Chory, 1997). To identify regions of functional significance, we sequenced *BRI1* from 14 of these mutants. The molecular lesions of *bri1* alleles are reported in Table I and are schematically represented in Figure 1, including already published *bri1* alleles (Li and Chory, 1997; Noguchi et al., 1999). We identified three new mutations in the kinase domain; *bri1-1* and *bri1-108* are missense alleles altering amino acids Ala-909 to Thr and Arg-983 to Gln, respectively. Ala-909 in subdomain II is present in all protein kinases, and Arg-983 in subdomain VIA is conserved among the putative plant LRR receptor kinases, including BRI1, CLV1, ERECTA, and Xa21 (Li and Chory, 1997). The *bri1-117* allele has a mutation at codon 1,139 of a non-conserved Asp to Asn in a region of the protein that generally contains negatively charged residues in protein kinases. These new mutants re-emphasize the necessity of the BRI1 kinase domain. In the BRI1 extracellular domain, we identified two new mutations: a nonsense mutation in *bri1-114* generates a stop codon early within the 70-amino acid island domain, and a missense mutation in *bri1-102* results in the substitution of Thr-750 with an Ile. The latter mutation occurs after the 25th LRR and before the second Cys pair, indicating a new region in BRI1 whose integrity is necessary for function.

BRI1 Is a Ubiquitously Expressed Plasma Membrane-Localized Protein

To determine the spatial pattern of expression and subcellular localization of BRI1, a green fluorescent

Table I. *bri1 alleles*

Allele	Lesion	Predicted Effect	Reference
bri1-1	G → A	Ala-909 → Thr	This work; Clouse et al. (1996)
bri1-3	4-bp deletion	Premature stop	Noguchi et al. (1999)
bri1-4	10-bp deletion	Premature stop	Noguchi et al. (1999)
bri1-5	G → A	Cys-69 → Tyr	Noguchi et al. (1999)
bri1-6, 119	G → A	Gly-644 → Asp	This work; Noguchi et al. (1999)
bri1-7	G → A	Gly-613 → Ser	Noguchi et al. (1999)
bri1-8	G → A	Arg-983 → Asn	Noguchi et al. (1999)
bri1-9	C → T	Ser-662 → Phe	Noguchi et al. (1999)
bri1-101	G → A	Glu-1078 → Lys	Li and Chory (1997)
bri1-102	C → T	Thr-750 → Ileu	This work
bri1-103, 104	G → A	Ala-1031 → Thr	This work; Li and Chory (1997)
bri1-105-107	C → T	Gln-1059 → Stop	This work; Li and Chory (1997)
bri1-108-112	G → A	Arg-983 → Gln	This work
bri1-113	G → A	Gly-611 → Glu	Li and Chory (1997)
bri1-114,116	C → T	Gln-583 → Stop	This work
bri1-115	G → A	Gly-1048 → Asp	Li and Chory (1997)
bri1-117,118	G → A	Asp-1139 → Asn	This work

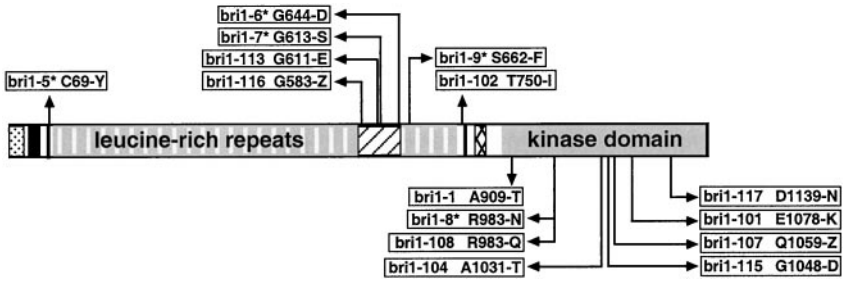

Figure 1. The majority of *bri1* mutations cluster in the island and kinase domains. A schematic representation of BRI1 including all the known *bri1* point mutations with their predicted effects. Symbols represent the following: ▒, Signal peptide; ▮, putative Leu-zipper motif; ▮, Cys pair; ▮▮, LRRs; ▨, 70-amino acid island; ▩, transmembrane domain; ■, kinase domain. Asterisk, These alleles were published by Noguchi et al. (1999).

protein (GFP) fusion with the C terminus of full-length BRI1 was made in the context of a BRI1 genomic clone containing, in addition to coding sequence, 1.7 kb of promoter DNA and upstream regulatory elements. This translational fusion (BRI1::GFP) was able to rescue *bri1-104* (data not shown), indicating that the fusion protein was functional. The expression of BRI1::GFP is ubiquitous in young tissue, especially in the meristem. Figure 2 presents a confocal microscopy analysis of BRI1 expression and shows that the BRI1::GFP fluorescence is localized to the cell surface in the hypocotyl, root, and cotyledons of young light-grown seedlings. The hypocotyl cells of wild-type (vector alone) seedlings (Fig. 2A) exhibit only the background chlorophyll autofluorescence, whereas in the hypocotyls of BRI1::GFP transgenic plants, the surface of each cell is outlined by the intense GFP fluorescence. The cotyledons show a similar pattern with the BRI1::GFP fluorescence illuminating the surface of epidermal cells (Fig. 2E). Roots have very low background fluorescence due to the lack of chloroplasts, and in Figure 2C, the fluorescence of the image was enhanced to show that a root-tip was in the field. In stark contrast to this wild-type root is the transgenic root (Fig. 2D), in which the surface of each cell is apparent. In these young cells in the root tip, the cytoplasm is not pushed up against the plasma membrane because the vacuoles are small. Therefore, we conclude that the BRI1-directed GFP fluorescence is at the cell wall/plasma membrane and not in the cytoplasm of these cells. The *BRI1* promoter drives expression of BRI1::GFP in all tissues in both light- and dark-grown seedlings (data not shown). Expression continues in younger tissue, but BRI1::GFP fluorescence decreases in non-growing older tissue (data not shown).

To determine if BRI1 is localized to the cell wall or the plasma membrane, a plasmolysis experiment was performed. Negative osmotic pressure results in the evacuation of fluid from inside the cell, and the plasma membrane is internalized with the cellular organelles, leaving the cell wall unaltered. When roots from a transgenic seedling were placed in 0.8 M mannitol, the BRI1::GFP fluorescence was internalized with the plasma membrane and the rest of the cell (Fig. 2F). Although many plant LRR-RLKs have putative signal sequences and transmembrane domains, this is the first evidence of visual localization of a LRR-RLK to the plasma membrane.

BRI1 Is a Ser/Thr Kinase

The BRI1 cytoplasmic domain is predicted to contain Ser/Thr kinase activity, and we performed an in vitro kinase assay to determine if BRI1 is an active kinase. Wild-type and mutant *BRI1* cDNA constructs containing a C-terminal HA tag were expressed in human embryonic kidney 293T cells by transient transfection. The mutants tested were *bri1-101*, a kinase domain mutant altering Gly-1078 to Glu in subdomain IX, and *bri1-113*, which has a mutation in the extracellular island domain (Fig. 1). Transfected BRI1 is readily detectable as an approximately 150-kD protein by immunoblot of whole cell lysates using the 12CA5 monoclonal antibody and enhanced chemiluminescence (data not shown). The kinase activity of BRI1 proteins was determined by an in vitro kinase assay. In this assay, BRI1 is immunoprecipitated from transiently transfected 293T cells and is incubated with [γ-^{32}P]ATP in a buffer containing Mg^{2+} and Mn^{2+}. ^{32}P-Labeled proteins are separated by SDS-PAGE and detected by autoradiography. Figure 3A shows that although the wild-type and bri1-113 proteins are competent for autophosphorylation, the kinase activity of bri1-101 is significantly reduced. Quantitation revealed that the kinase activity of bri1-101 was 45 times lower than that of the wild type in this assay, whereas bri1-101 expression was only one-half of the wild-type levels (Fig. 3A). The kinase-associated protein phosphatase (KAPP) binds in a phosphorylation-dependent manner to the kinase domains of the LRR-RLKs, HAESA (RLK5) and CLV1 (Stone et al., 1994, 1998; Williams et al., 1997). BRI1 was shown to phosphorylate KAPP as a substrate in vitro (J. Li, R. Williams, E. Meyerowitz, and J. Chory, unpublished data). The kinase domain sequence predicts that BRI1 belongs to the Ser/Thr kinase family (Li and Chory, 1997). Phosphoamino acid analysis performed with the in vitro autophosphorylated receptor showed that phospho-Ser and phospho-Thr were present, thus confirming this prediction (Fig. 3B).

DISCUSSION

BRI1 Is a Functional Ser/Thr Kinase

Five plant LRR receptor Ser/Thr kinases have been shown to be active protein kinases. The kinase do-

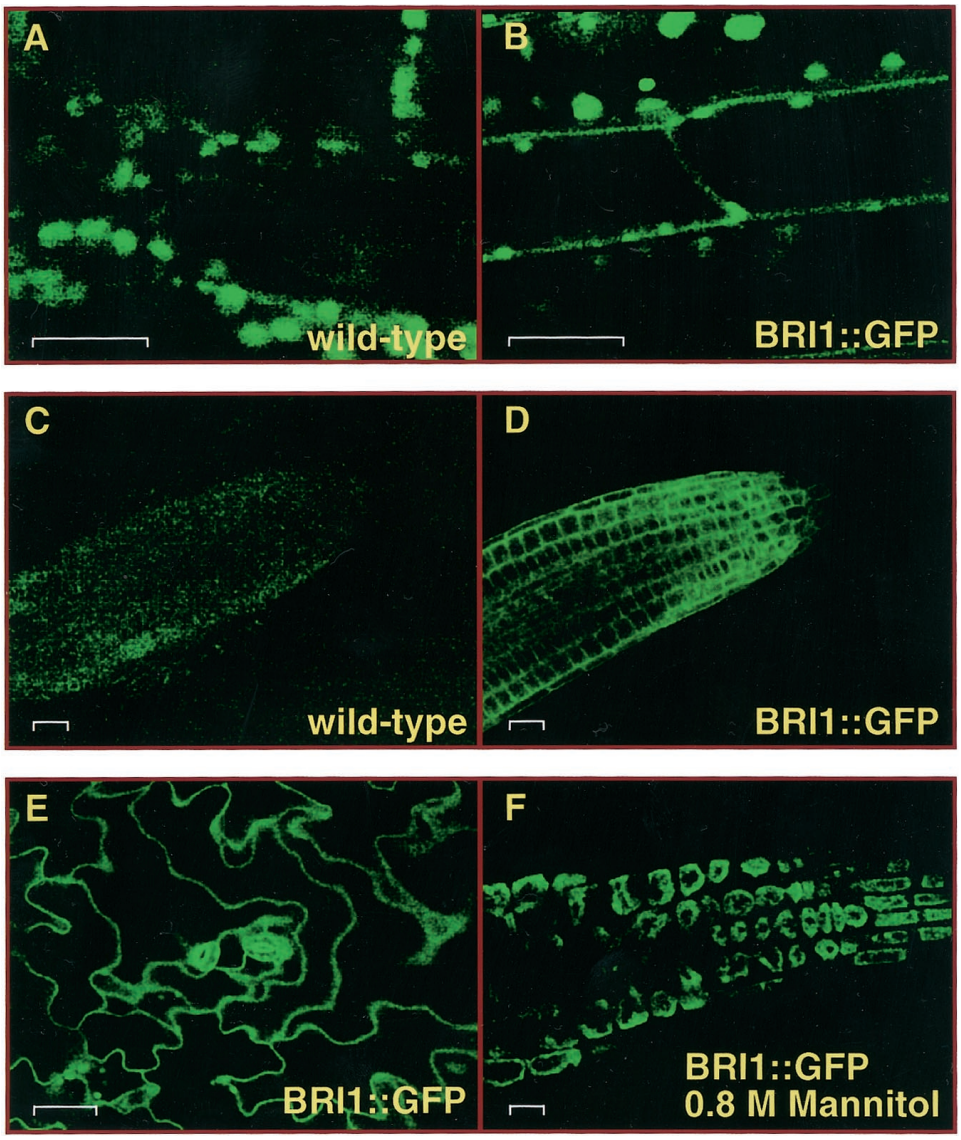

Figure 2. BRI1-GFP is expressed ubiquitously during early seedling development and is localized to the plasma membrane. A BRI1::GFP fusion protein was expressed from the *BRI1* promoter in stably transformed wild-type Arabidopsis. A, Background chlorophyll fluorescence in a wild-type hypocotyl; B, BRI1::GFP fluorescence along the cell surface of hypocotyl cells; C, background fluorescence of a wild-type Arabidopsis root; D, BRI1::GFP at the cell surface in young root; E, BRI1::GFP at the cell surface in cotyledon epidermal cells (wild type has only stomata and guard cell chloroplast autofluorescence, which is not shown); F, BRI1::GFP localizes with the cytoplasm if cells are collapsed in 0.8 M mannitol, indicating that BRI1::GFP is in the plasma membrane. Bar = 20 μm.

main of HAESA (RLK5), for example, was expressed in *Escherichia coli* as a fusion protein and then was purified and used in in vitro kinase assays (Horn and Walker, 1994). The results from these assays showed that the fusion protein possessed kinetic parameters typical of protein kinases. We chose to express BRI1 in an animal cell system because similarities exist between animal and plant cells with regard to the expression and the peptide signal-mediated membrane targeting of large receptor proteins. We reasoned that proper membrane expression of the full-length protein in a cellular context would allow the study of hormonal regulation of receptor activation. BRI1 expressed in 293T cells by transient transfection was used for in vivo labeling experiments (data not shown) and was also immunoprecipitated and used for an in vitro kinase assay.

The in vitro kinase assay revealed that both the wild-type protein and the product of an allele that contains a mutation in the extracellular domain were active protein kinases, at least in vitro, under conditions of antibody-mediated dimerization. On the other hand, the product of the *bri1-101* allele, which contains a mutation in the kinase domain, exhibited greatly reduced kinase activity. This assay confirms the predicted activity of the kinase domain of BRI1

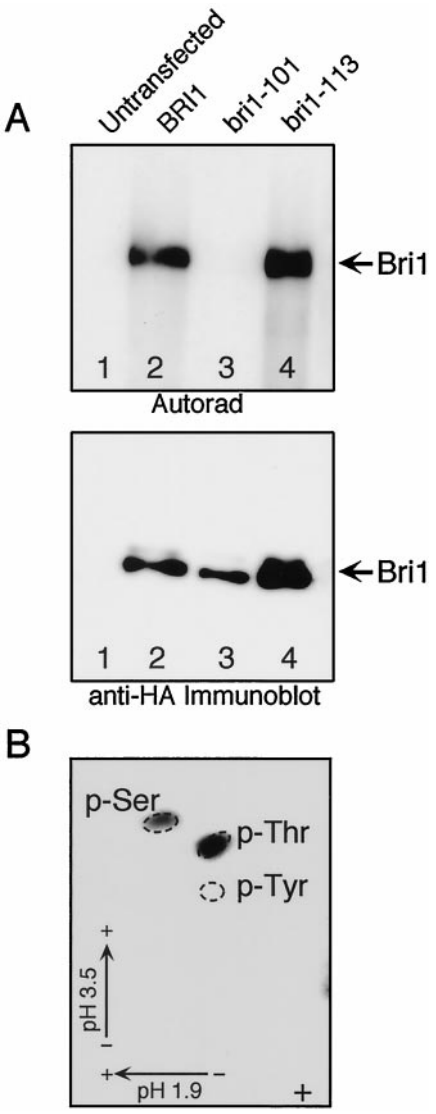

Figure 3. BRI1 is a Ser/Thr kinase. Immunoprecipitated HA-BRI1, wild type, bri1-101, and bri1-113 were used for an in vitro kinase assay. A, The top presents an autoradiogram of the kinase assay. The bottom presents an immunoblot using anti-HA antibody of the same gel. Lane 1, Untransfected 293T cells; lane 2, wild-type BRI1; lane 3, bri1-101 kinase mutant; lane 4, bri1-113 island domain mutant. B, Autophosphorylation of BRI1 occurs on Ser/Thr residues. Anti-HA-BRI1 autophosphorylation was subjected to phosphoamino acid analysis. The positions of the internal phospho-Ser, phospho-Thr, and phospho-Tyr standards (visualized by ninhydrin staining) are indicated. The origin (+) and the directions of electrophoresis with the pH are as indicated.

and can now be used as a biochemical tool to identify direct substrates of the activated receptor.

The demonstration that BRI1 encodes an active protein kinase provides more substantial ground to explain the effects of the intracellular domain mutations in BRI1. Although mutations in the intracellular domain of a transmembrane receptor might affect several processes, such as receptor homo- and heterodimerization, interaction with other proteins, receptor stability, and enzymatic activity, most mutations found in the intracellular domain of BRI1 are consistent with the latter possibility: *bri1-1* substitutes an Ala residue in the protein kinase subdomain II (Hanks et al., 1988) that is conserved in all protein kinases; *bri1-8* and *bri1-108* substitute an Arg in subdomain VIa that is conserved in LRR kinases; *bri1-104* substitutes an Ala in subdomain VII that is frequently found at that position in other protein kinases; *bri1-115* changes a Gly residue in subdomain VIII that is thought to be involved in substrate recognition and Ser/Thr specificity; *bri1-107* creates a stop codon that truncates the kinase domain in subdomain VIII; *bri1-117* replaces an Asp with Asn in a segment of subdomain XI in which negatively charged residues are frequently observed among protein kinases; and *bri1-101*, which revealed greatly reduced kinase activity in vitro, substitutes a Glu that is frequently found at that position in subdomain IX of protein kinases.

Phosphoamino acid analyses of in vitro phosphorylated BRI1 revealed the presence of phosphorylated Ser and Thr only, as originally predicted from the inspection of primary amino acid sequence of this kinase. Based on data from other protein kinases, we anticipate that some of the phosphorylated residues will lie in the activation loop of the kinase domain, which is in fact Ser/Thr–rich in BRI1. In addition to activation loop phosphorylation, which generally serves to increase kinase activity, sites elsewhere may be phosphorylated to recruit phosphopeptide-binding proteins into an activated receptor complex, as happens for receptor protein-Tyr kinases. Three modular protein domains are now known that selectively bind to peptide sequences containing phosphorylated Ser or Thr: FHA, WW, and 14-3-3 (Yaffe and Cantley, 1999). All of these domains are found in plants and, in fact, an FHA domain-containing protein phosphatase, KAPP, has been shown to selectively bind to activated LRR receptors (Stone et al., 1994; Williams et al., 1997; Braun et al., 1997; Li et al., 1999).

We have not detected either basal or steroid-induced phosphorylation of BRI1 in 293T cells labeled with [^{32}P]orthophosphate, despite the fact that many variables have been explored and that phosphorylation of several other protein kinases and kinase substrates was readily detected under similar conditions (data not shown). Based on what has been learned from RPKs in other systems and on the observation that at least part of the autophosphorylation of HAESA (RLK5) is intermolecular (Horn and Walker, 1994), we speculate that BRI1 is found in the inactive monomeric state when expressed in 293T cells and that the formation of kinase-active BRI1 dimers is induced during the immunoprecipitation reaction that precedes the in vitro kinase assay. This model explains why, under the conditions of the latter assay, a ligand is not required to promote stimulation of receptor kinase activity. Brassinolide and

other steroids may have failed to promote BRI1 dimerization and activation in intact cells for several reasons, such as a missing accessory steroid carrier protein or presenting molecule in the heterologous 293T cell system.

Role of the Extracellular Domain

There are several putative domains in the extracellular region of BRI1. Analysis of mutant alleles indicates domains of functional importance, including the amino-terminal Cys pair, the 70-amino acid island, the LRR domain, and the region between the LRR and the second Cys pair (Fig. 1).

The island domain contains three missense mutations, *bri1-6*, *bri1-7*, *and bri1-113*, which affect three separate Gly residues. Although the *bri1-113* mutant fails to respond to the steroid, it was shown in this paper to be an active kinase in vitro. This mutant could be an active kinase in vitro due to antibody-mediated dimerization, but in vivo a mutation of Gly in the island domain Gly might inhibit positive regulation of the kinase domain. The requirement for this Gly could be due to the small size of this residue, which allows for a conformational change of the protein when the ligand is bound to activate the kinase domain. Alternatively, mutation of these glycines could interfere with protein/ligand binding to BRI1 or with extracellular dimerization. Although the importance of the Gly residues is unclear, the Cf-2, Cf-4, Cf-5, and Cf-9 defense genes contain "loop out" domains that are similar to the island domain of BRI1 but that are much smaller, 32, 27, and 39 amino acids, respectively. All of these proteins contain a Gly for the −6 amino acid relative to the most carboxy-terminal amino acid in each domain. This Gly is mutated in the *bri1-6* allele, thereby supporting the hypothesis that the Glys represent a structural requirement for LRR transmembrane protein function.

The LRR domain is another region possibly involved in protein/ligand interactions. The predicted site for this interaction is in the solvent exposed parallel β-sheet (Kobe and Deisenhofer, 1995). The LxxLxLxx (x is any amino acid) domain within the LRR corresponds with the solvent face of the protein with the Leu resides facing away from the solvent face (Jones and Jones, 1997). The x or variable amino acids could lead to specificity of the protein binding to the LRR. The strong *clv1* alleles, *clv1-4* and *clv1-8*, are both missense mutations in this solvent face domain. In contrast to this, *bri1-9* is a weak allele (Noguchi et al., 1999) altering a Ser in the conserved BRI1 solvent face, LxxLxLSx (Li and Chory, 1997). This mutation could alter protein/ligand interactions or dimerization. It is interesting that this mutation is in the first of the four LRR after the island domain. This could indicate that many of the important molecular interactions occur close to the transmembrane domain. This idea is supported by *bri1-102*, a mutation also in this region. Conversely, the clustering of mutations closer to the transmembrane domain could indicate that the protein interactions with the first 21 LRRs are strong enough that altering one amino acid does not significantly destabilize binding.

BRI1 Expression Is Not Spatially Regulated

BRI1 is expressed in all tissues in the seedling (root, hypocotyl, cotyledons, and leaves; Fig. 2 and data not shown) and in adult organs, including cauline leaves and inflorescent stems (data not shown). The expression pattern of BRI1-GFP fusion protein is consistent with previous mRNA expression data (Li and Chory, 1997), which revealed the presence of *BRI1* transcripts in all tissues. Although there is no tissue-specific expression of BRI1, there is temporal regulation. Fully expanded leaves and elongated root or inflorescence cells express BRI1::GFP at low levels (data not shown). This expression of the transgene correlates with physiological data that showed that exogenously applied BRs promote growth only in younger tissues (Mandova, 1988). The lack of response of older tissues to BRs may be because BRI1, the putative BR receptor, is present at significantly diminished levels in fully expanded tissues.

Model of BRI1 Signaling

We propose two models for the function of BRI1 in brassinolide signaling. First, similar to animal RPKs, the binding of ligand to the LRR or the island domain may result in the dimerization of BRI1 with itself or another receptor kinase. This dimerization would result in transphosphorylation and activation of the kinase domain. The activated kinase would then send a phosphorylation signal to alter gene expression and induce cell expansion, among other effects. Conversely, BRI1 may not be the receptor itself but may be a protein in the brassinolide receptor complex whose extracellular domain is involved in interactions with other receptor complex proteins. The formation of an active complex results in activation of the kinase phosphorylation signal.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis Columbia was the wild-type ecotype. Seeds were surface sterilized by washing for 20 min in 70% (v/v) ethanol containing 0.05% (v/v) Triton X-100, followed by a wash with 95% (v/v) ethanol. Seeds were dried on filter papers under sterile conditions and sown on 0.5× Murashige-Skoog medium (Gibco-BRL, Cleveland) supplemented with 1% (v/v) Suc and 0.8% (v/v) phytoagar. The plates were wrapped in aluminum foil and left at 4°C overnight to induce germination. Seedlings were grown in growth chambers at 21°C under long-day conditions (16 h of light).

Sequence Analysis of *bri1* Alleles

The alleles described were isolated from ethyl methanesulfonate-mutagenized Arabidopsis Columbia seeds carrying the homozygous mutation *glabrous1* (Lehle Seeds, Round Rock, TX), except for *bri1-119*, which was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus; stock no. CS399, ecotype Enkheim-2). Genomic DNA was isolated from various *bri1* mutants using a plant DNA miniprep method (Li and Chory, 1997). Based on the wild-type *BRI1* sequence, three pairs of gene-specific primers (forward 1, 5′-AGTTACCATTGCAGACGA-3′; reverse 1, 5′-AACCCAACCAACGACGTT-3′; forward 2, 5′-GAATTCAATCTCCGGTGCTA-3′; reverse 2, 5′-GAAGAGGATAACCACAGA-3′; forward 3, 5′-TGGTTCGATTCCTGATGA-3′; and reverse 3, 5′-GAATTAATAGGTCACGTGC-3′) were designed to amplify three overlapping PCR fragments covering approximately 2.0 kb of 5′-untranscribed/untranslated sequence, the complete protein-coding sequence (3, 588 bp), and 420 bp of 3′-untranslated region. PCR amplifications were conducted in 100-μL reaction volumes containing 50 mM Tris [Tris-(hydroxymethyl)-aminomethane]-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% (w/v) gelatin, approximately 100 ng of Arabidopsis DNA, 200 μM dNTPs, 250 ng each of a specific pair of *BRI1* primers, and 5 units of *Taq* DNA polymerase (Fisher Scientific, Pittsburgh).

The PCR reactions were performed in a thermocycler (Trio-Thermoblock, Biometra, Germany) by denaturing the template DNA for 5 min at 95°C followed by 40 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, and a 10-min extension at 72°C. The PCR products were size-fractionated by electrophoresis in 0.8% (w/v) agarose gel, purified using the QIAEX II gel extraction kit (Qiagen, Chatsworth, CA), and directly sequenced on an ABI PRISM 310 genetic analyzer using the dRhodamine terminator cycle sequencing kit (PE-Applied Biosystems, Foster City, CA). Putative mutations were identified by comparing the DNA sequences of mutant *bri1* alleles with the wild-type *BRI1* sequence using the Lasergene Sequence Analysis System (DNASTAR, Inc., Madison, WI), and they were confirmed by sequencing at least two independently amplified PCR fragments or by conducting cleaved-amplified polymorphic sequence/derived cleaved-amplified polymorphic sequence analysis (Konieczny and Ausubel, 1993; Neff et al., 1998). The wild-type *BRI1* gene of the ecotype Enkheim-2 was sequenced and used to identify the *bri1-119* mutation.

Construction of BRI1::GFP

The entire *BRI1* coding region, including 1,690 bp of promoter relative to the translation start, was fused to GFP5.1 by replacing the BRI1 stop codon with a three-amino acid linker: Trp, Asp, Pro. *GFP5.1* was created by inserting the *Nde*I-*Bst*BI fragment from *mGFP5* (GenBank accession no. U87974; Siemering et al., 1996) into a S65T mutated version of GFP. This fusion construct was cloned into pCHF4, a pPZP212-derived vector with a pea ribulose 1,5-bisphosphate carboxylase terminator *Sac*I-*Eco*RI fragment. An ASE *Agrobacterium tumefaciens* strain containing the *BRI1::GFP* translational fusion construct was used to transform wild-type Arabidopsis plants by vacuum infiltration. Transformed seedlings were selected on 0.5× Murashige-Skoog medium, 1% (w/v) Suc, 0.8% (w/v) phytagar, and 50 μg/mL kanamycin and were propagated on soil.

Fluorescence Microscopy

Confocal and conventional fluorescence microscopy were performed on an IX70 inverted microscope (Olympus, Tokyo). Fluorescence was filtered with fluorescein isothiocyanate filter sets (Olympus).

In Vitro Kinase Assay

Hemagglutinin (HA) tag was added to the 3′ end of *BRI1*, *bri1-101*, and *bri1-113* by cloning the PCR product (BRI1-N, 5′-CCCCGGGTACCTTGAGAAATGAAGACT-3′; BRI1-HAC, 5′-GGGCTAGCGTAATCTGGAACATCGTATGGGTATAATTTTCCTTCAGGAACTTC-3′) from wild-type *BRI1* DNA and *bri1-101* and *bri1-113* mutant DNA into the pCMX-PL2 vector. The products were sequenced to confirm that no mutations were introduced by the PCR. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. Five micrograms of HA-tagged *BRI1* plasmid DNA and 5 μg of carrier DNA were transfected into 2×10^6 cells with the calcium phosphate method (Sambrook et al., 1989). Forty-eight hours after transfection, cells were lysed in buffer containing 25 mM Tris-Cl (pH 8.0), 1% (v/v) Nonidet-P40, 10 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 units/mL aprotinin, 20 μg/mL leupeptin, 20 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 10 mM sodium fluoride for 20 min on ice. Cell lysates were cleared by centrifugation after a 20-min incubation with fixed *Staphylococcus aureus* at 4°C. HA-tagged BRI1 proteins were immunoprecipitated with the 12CA5 anti-HA tag monoclonal antibody and protein A agarose beads (RepliGen, Needham, MA). The immune complexes were washed twice with lysis buffer, once with phosphate-buffered saline, and twice with kinase reaction buffer (20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol). After a 20-min incubation at room temperature in 20 μL of kinase reaction buffer containing 5 μM ATP and 5 μCi [γ-^{32}P]ATP (1 Ci = 37 GBq), the kinase reaction was terminated by the addition of 1 mL of phosphate-buffered saline containing 20 mM EDTA. Immune complexes were collected by centrifugation, resuspended in sample buffer, separated on denaturing SDS/polyacrylamide gel, transferred to Immobilon-P membrane (Millipore, Bedford, MA), reacted with primary and secondary antibodies (12CA5 and horseradish peroxidase-conjugated sheep anti-mouse, respectively), and visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Kinase reaction products were detected in the same membrane by autoradiography.

Phosphoamino Acid Analysis

The experiment was performed as described by Boyle et al. (1991).

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