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A Putative Leucine-Rich Repeat Receptor Kinase Involved in Brassinosteroid Signal Transduction

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Summary

Brassinosteroids are a class of growth-promoting regulators that play a key role throughout plant development. Despite their importance, nothing is known of the mechanism of action of these steroid hormones. We describe the identification of 18 Arabidopsis dwarf mutants that are unable to respond to exogenously added brassinosteroid, a phenotype that might be expected for brassinosteroid signaling mutants. All 18 mutations define alleles of a single previously described gene, *BRI1*. We cloned *BRI1* and examined its expression pattern. It encodes a ubiquitously expressed putative receptor kinase. The extracellular domain contains 25 tandem leucine-rich repeats that resemble repeats found in animal hormone receptors, plant disease resistance genes, and genes involved in unknown signaling pathways controlling plant development.

Introduction

Steroid hormones are crucial for embryonic development and adult homeostasis in animals (Evans, 1988). In the classic model of steroid hormone action, steroids bind to intracellular receptors, which act as ligand-dependent transcription factors that regulate gene expression. These receptors, members of the nuclear receptor superfamily, have a modular structure, consisting of a conserved DNA-binding domain, nuclear localization signals, a ligand-binding domain, and several transcriptional activation functions (Beato et al., 1995). In addition to these well-studied intracellular receptors, there is increasing evidence that steroids can exert rapid nongenomic effects in a variety of cells, including neurons, pituitary cells, heart, skeletal muscle, oocytes, sperm, and prostatic carcinoma cells by interactions with receptors on the cell surface. Little is known of the mechanism of action of these steroid membrane receptors (reviewed in McEwen, 1991).

In plants, many steroids have been identified, but only brassinosteroids (BRs) have wide distribution throughout the plant kingdom and cause biological effects on plant growth when applied exogenously (Mandava, 1988). Very recently, genetic evidence has implicated a role for the most active BR, brassinolide, in controlling plant growth and development. The Arabidopsis *deetiolated 2* (*DET2*) and *constitutive photomorphogenesis and dwarfism* (*CPD*) mutants have identified two steps in the synthesis of brassinolide and allowed an assignment for this steroid's role in plant development (Chory et al., 1991; Li et al., 1996; Szekeres et al., 1996). Loss-of-function mutations in *DET2* and *CPD* have pleiotropic effects. In the dark, these mutants develop as light-grown plants and inappropriately express light-regulated genes. In the light, the mutants are dwarfs and have reduced male fertility. *det2* mutant plants also have altered photoperiodic responses and display a significant delay in the senescence program. Such phenotypic differences establish that DET2 and CPD (and by extrapolation, brassinolide) are important throughout Arabidopsis development. Moreover, in the absence of hormone, Arabidopsis plants do not respond properly to fluctuations in their light environment.

The *DET2* locus encodes a protein that shares significant sequence identity with mammalian steroid 5α-reductases (Li et al., 1996, 1997). Mammalian steroid 5α-reductases catalyze a NADPH-dependent conversion of testosterone to dihydrotestosterone, a key step in steroid metabolism that is essential for the embryonic development of male external genitalia and the prostate (Russell and Wilson, 1994). Although the *DET2* steroid 5α-reductase functions in the formation of a plant-specific product, campestanol, steroid 5α-reductases are highly conserved in function between phylogenetic kingdoms. Recombinant DET2 can also catalyze the reduction of the androgens, progesterone, and testosterone, and expression of human steroid 5α-reductases from a plant promoter can rescue *det2* loss-of-function mutations (Li et al., 1997).

CPD encodes a protein that shares sequence identity with several mammalian cytochrome P450 proteins including steroid hydroxylases (Szekeres et al., 1996). Rescue studies with intermediates in the BR biosynthetic pathway suggest that CPD acts in the conversion of cathasterone to teasterone (Fujioka et al., 1995; Szekeres et al., 1996). Thus, CPD may encode a steroid 23-hydroxylase, an enzymatic activity that is also highly conserved with animal steroid biosynthetic enzymes. Other candidate BR biosynthetic genes include the Arabidopsis *DIM* gene, the tomato *Dwarf* locus and the pea *LKB* gene (Takahashi et al., 1995; Bishop et al., 1996; Nomura et al., 1997).

The pleiotropic effects of *det2* and *cpd* mutations on Arabidopsis development suggest the involvement of BRs in several processes throughout its life cycle. These include the expression of light- and stress-regulated genes, the promotion of cell elongation, normal leaf and chloroplast senescence, and flowering. To understand the mechanism by which BRs regulate plant development, it is necessary to identify components of the response pathway, including the receptor. Genetic approaches in Arabidopsis have proven to be fruitful in identifying components of plant growth regulator signaling. As examples, screens for mutants that are insensitive to high levels of exogenously applied auxins, ethylene, gibberellins, and abscisic acid have led to the identification of loci involved in signaling from these plant hormones (reviewed in Barbier-Brygoo et al., 1997). Among the ethylene-insensitive loci is *ETR1*, a gene that encodes the ethylene receptor, a member of

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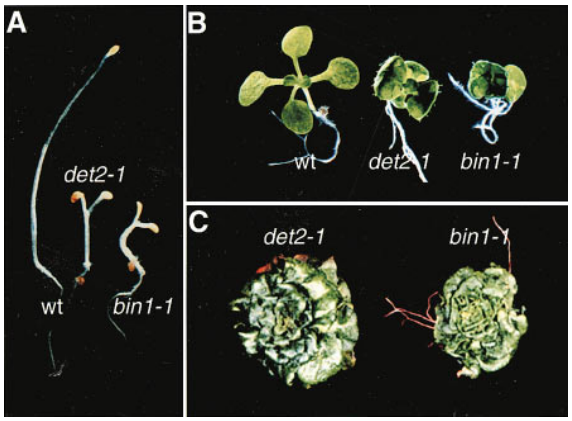

Figure 1. *bin* Mutants Display Similar Phenotypes to *det2* Mutants

(A) Dark-grown 10-day-old seedlings. (B) Light-grown 10-day-old seedlings. From left to right in (A) and (B), wild-type Col-0, *det2–1*, and *bin1–1/bri1–101*. (C) Two-month-old *det2–1* (left) and *bri1–101* (right) mutants grown in a 22°C growth room (9 hr light/15 hr dark).

a class of regulatory proteins known as sensor histidine protein kinases (Chang et al., 1993; Schaller and Bleecker, 1995).

Two BR-insensitive dwarf mutants (*bri1* and *cbb2*) of Arabidopsis have been described; each is an allele of a single locus that maps to the bottom of chromosome 4 (Clouse et al., 1996; Kauschmann et al., 1996). In this paper, we describe the identification of 18 new BR dwarf mutants with the inability to respond to exogenously applied brassinolide. The 18 new mutations are alleles of the previously described *BRI1/CBB2* gene. We used map-based cloning to identify the *BRI1* gene and show it encodes a putative leucine-rich repeat (LRR) receptor kinase. The extracellular LRR domain plays an important role in its function, suggesting that plant cells respond to BRs at the cell surface.

Results

Isolation of Brassinosteroid-Insensitive Mutants

Hormone-response mutants have been defined genetically as individuals that are phenotypically similar to hormone-deficient mutants yet cannot be rescued by addition of that hormone to their growth media. We screened for BR-response mutants by identifying individual mutagenized seedlings with a dwarf phenotype similar to that of *det2* or *cpd* mutants, followed by a secondary screen in which we determined the ability of brassinolide to restore the wild-type stature to such mutants. We screened approximately 80,000 ethyl methanesulfonate (EMS)-mutagenized M2 seedlings derived from six independent parental groups (1600 M1 plants/group) and isolated approximately 200 *det2*-like mutants. These mutants were then transferred to synthetic medium containing 1 μM brassinolide. Of the original 200 mutants, 18 showed no response to brassinolide, while all others were partially or fully rescued by the hormone treatment (data not shown).

The *bin* (*b*rassinosteroid-*in*sensitive) mutants had phenotypes similar to that of *det2* mutants. Figures 1A–1C present the dark-grown and light-grown phenotypes of wild-type, *det2–1*, and *bin1–1* mutant plants. In the dark, both *det2–1* and *bin1–1* mutants were short, had thick hypocotyls, accumulated anthocyanins, had open, expanded cotyledons, and developed primary leaf buds (Figure 1A). This is in contrast to wild-type etiolated seedlings, which had elongated hypocotyls and closed cotyledons (Figure 1A). In the light, both mutants were smaller and darker green than wild type, showed reduced apical dominance and male fertility, and exhibited a delay in flowering and leaf senescence (Figures 1B–1C). Of the 18 new *bin* alleles, only one, *bin1-1*, was able to produce homozygous seeds. The remaining 17 mutants were completely male-sterile under all growth conditions (data not shown).

All *bin* mutants were backcrossed to the isogenic wild-type strain, and analysis of the resulting F1 and F2 populations indicated that each mutant was caused by a monogenetic recessive mutation (data not shown). The segregating *bin* mutants in the F2 generation were retested for brassinolide-insensitivity, thereby verifying the hormone-insensitive phenotype. Complementation tests between homozygous *bin1–1* mutants and heterozygous wild-type-looking plants, derived from the F1 cross of *bin* mutants to wild type, indicated that all 18 *bin* mutants derived from our screen were alleles of a single gene. *bin1–1* was mapped to the bottom arm of chromosome 4 and showed tight linkage to the restriction fragment polymorphism marker, DHS1. A recently published brassinosteroid-insensitive mutation, *bri1*, also maps to this region of chromosome 4 (Clouse et al., 1996). *bin1–1* was crossed to *bri1*. The resulting F1 seedlings displayed a *bin/bri* phenotype, indicating that these are alleles of a single gene. Since our mutants represent new alleles of the previously characterized *bri1* locus, we have renamed them *bri1–101* to *bri1–118*. *bri1* is also allelic to *cbb2*. As such, all 20 brassinosteroid-insensitive mutations isolated to date correspond to a single gene.

Fine-Mapping of *BRI1*

bri1–101 was fine-mapped using cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) and simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). Based on the analysis of 1914 recombinant chromosomes derived from a mapping cross between polymorphic strains of Arabidopsis, *bri1–101* was mapped to a region flanked by the SSLP marker nga1107 and the CAPS marker DHS1 on the bottom of chromosome 4. The physical map of this region of chromosome 4 is published (Schmidt et al., 1996). Accurate positioning and orientation of the selected YACs within the nga1107-DHS1 region were established by hybridization of YAC DNAs with YAC-end probes obtained by thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier, 1995) (Figure 2). Two new CAPS markers, developed from cosmid CC15O17 (Schmidt et al., 1996) and the right end of EW4E8, respectively, were used to further delimit the *BRI1* gene to a 160 kb region. Hybridization of bacterial artificial chromosome (BAC) library filters (Choi et al., 1995) with several YAC-end sequences identified a number of BAC clones (Figure 2). Using a CAPS marker derived from the right end of YAC EW4E8 as a probe,

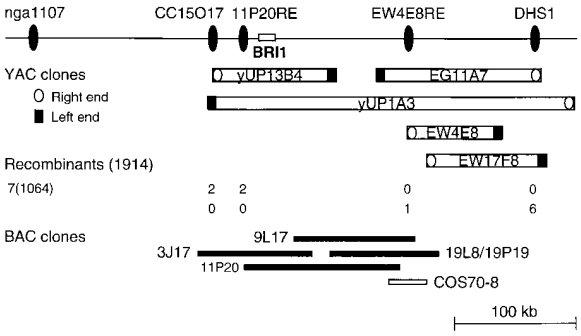

Figure 2. Physical Mapping of the *BRI1* Gene

The *BRI1* gene was mapped to an ~500 kb interval at the bottom of chromosome 4 by the identification of 7 recombinant chromosomes at marker nga1107 and 3 recombinant chromosomes at DHS1 (of 1064 total). The development of three new markers corresponding to the right end of YAC EW4E8, the right end of BAC11P20, and cosmid CC15O17 further defined this region to an ~150 kb region that was covered by DNA inserts of BAC11P20 and COS70–8 clones.

we identified a cosmid clone, COS70–8, that linked BAC11P20 to this marker. A CAPS marker converted from the right end of BAC11P20 allowed us to position the *BRI1* gene on either cosmid 70–8 or BAC11P20.

Identification of the *BRI1* Gene

BAC 11P20 was used as a probe to screen for restriction fragment length polymorphisms from genomic DNA derived from several *bri1* alleles. As shown in Figure 3A, an 8 kb BspHI fragment was not detected in the *bri1–113* allele, while it was present in genomic DNA derived from other *bri1* mutants. Further examination of the hybridization signals suggested that *bri1–113* DNA had two new BspHI bands of 2 and 6 kb, respectively (indicated by two asterisks in Figure 3A). To confirm this observation, the 8 kb BspHI fragment derived from BAC11P20 was used to probe a duplicate Southern filter. As shown in Figure 3B, the probe hybridized only with an 8 kb BspHI fragment in all other alleles; however, it detected two BspHI fragments of 2 and 6 kb in the *bri1–113* allele.

A 3.5 kb EcoRI fragment that hybridized with the 8 kb BspHI DNA fragment and containing the BspHI polymorphism was used to probe an RNA gel blot with samples from various *bri1* alleles. As indicated in Figure 3C, there is no difference in the level of a 4.3 kb transcript detected by the probe between wild-type and *det2* plants. In contrast, the RNA level was reduced in most of the *bri1* alleles examined, especially in *bri1–105* seedlings where the RNA was below the detection limit. This result strongly suggests that the detected 4.3 kb transcript was derived from the *BRI1* gene.

To confirm that the candidate DNA corresponds to the *BRI1* gene, we sequenced a 5 kb genomic fragment that hybridized to the 4.3 kb transcript from wild type and mutants homozygous for five different *bri1* alleles. In each case, we identified a single base-pair change (Table 1). We concluded that the 5 kb genomic fragment that we sequenced encodes the *BRI1* locus.

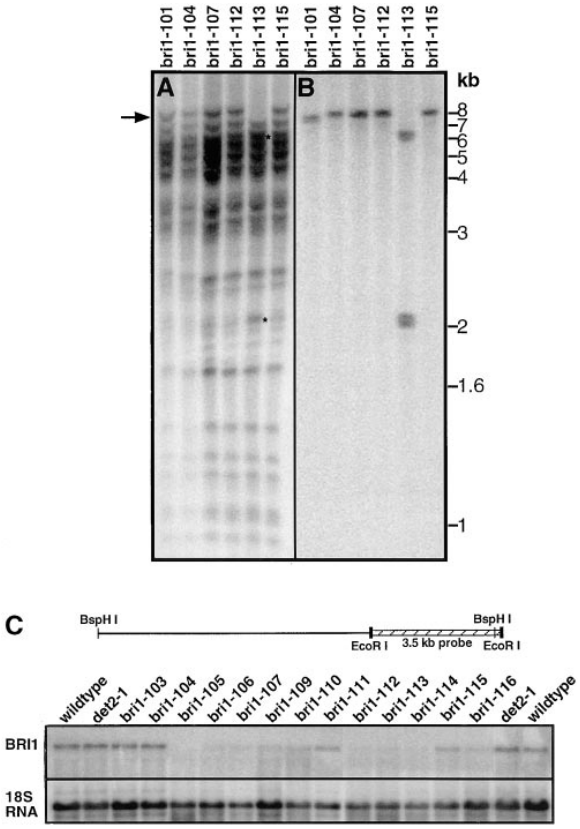

Figure 3. Identification of a Candidate DNA Fragment That Encodes the *BRI1* Gene

(A) DNAs of 6 indicated *bri1* alleles were digested with BspHI, separated on a 0.8% agarose gel, and blotted onto nylon membranes. The blotted filter was hybridized with a probe derived from HindIII-digested 11P20 BAC DNA. An 8 kb BspHI fragment is missing in *bri1–113* allele.

(B) The corresponding BspHI fragment was isolated from the same BAC DNA and used for probing a duplicate filter. The probe detected two new BspHI fragments (6 and 2 kb) in the *bri1–113* allele. Molecular length standards in kilobases are indicated to the right.

(C) The depicted 3.5 kb EcoRI fragment was used to probe a Northern filter containing RNA samples from wild-type, *det2–1*, and various mutants homozygous for the indicated *bri1* alleles. The expression level of a 4.3 kb transcript detected by the probe was reduced in most alleles examined when compared to those of wild type and *det2* mutants. Each lane contains 5 μg of total RNA isolated from two-month-old plants grown in a short-day growth room (9 hr light/15 hr dark). The same filter was reprobed with an 18S rDNA probe to compare the relative loading among samples.

BRI1 Encodes a Putative Leucine-Rich Repeat Receptor Kinase

The DNA sequence of *BRI1* revealed one large, intronless open reading frame (ORF) of 3588 bp encoding a predicted protein of 1196 amino acids with an estimated molecular mass of 130 kDa (Figure 4). The first ATG of this open reading frame was preceded by an in-frame stop codon (TGA) at the −6 to −4 position and an AGAA sequence that is a favorable context for translation initiation in eukaryotes (Lutcke et al., 1987). There is a typical TATA box sequence (TATATATA) at the −280 to −273 position. A cDNA clone (ATTS4702) identified from the Arabidopsis expressed sequence tag (EST) database

Table 1. Molecular Basis of *bri1* Mutations

Allele	Molecular Lesion	Polymorphism
bri1-101	G-A Glu-1078-Lys	Xho
bri1-104	G-A Ala-1031-Thr	
bri1-107	C-T Gln-1059-stop	
bri1-113	G-A Gly-611-Glu	BspHI
bri1-115	G-A Gly-1048-Asp	Rsa I

(http://www.tigr.org/tdb/at/at.html) contains a 420 bp 3′-untranslated region. The predicted mRNA size is, therefore, consistent with the transcript size (4.3 kb) detected by Northern analysis.

Database searches revealed that *BRI1* shared high identity to a family of proteins collectively called leucine-rich repeat (LRR) receptor kinases. The highest sequence identity was to two recently cloned Arabidopsis genes, *ERECTA* (BlastP score of 303, with a probability of 6.9e-142; Torii et al., 1996) and *CLV1* (BlastP score of 313, with a probability of 3.5e-126; Clark et al., 1997), which are believed to be involved in several developmental processes. Like many other family members, the predicted BRI1 protein has several distinct domains: a signal peptide, a putative leucine-zipper motif, an LRR domain, a transmembrane domain, and a cytoplasmic kinase domain.

The predicted BRI1 polypeptide has a 23–amino acid hydrophobic segment that presumably functions as a signal peptide to translocate the newly synthesized polypeptide into the ER membrane (von Heijne, 1990). The assigned cleavage site was based on the rules described by von Heijne (1986). The signal peptide is followed by a potential 4-heptad amphipathic leucine zipper motif (amino acids 32–52; Landschulz et al., 1988), which might be involved in forming homo- or heterodimers.

The major extracellular domain of the protein contained 25 tandem copies of a 24–amino acid leucine-rich repeat (LRR) (Figure 5A) with 13 potential N-glycosylation sites that are flanked by pairs of conservatively spaced cysteines (Figure 4). LRRs have been found in a variety of proteins with diverse function and cellular locations from human, flies, plants, and yeast and are believed to play a role in protein–protein interactions (Kobe and Deisenhofer, 1994). A unique feature of BRI1's LRR domain is the presence of a 70–amino acid island between the 21st and 22nd LRR. This island is essential for the function of BRI1. The mutation that gives rise to the BspHI polymorphism between *bri1–113* and other *bri1* alleles changes a glycine at codon 611 to a negatively charged glutamate.

The predicted BRI1 protein also contains two other domains of note. There is a predicted transmembrane domain (amino acids 793–814) flanked by two stop-transfer sequences that are rich in charged amino acids. The intracellular domain contains all 12 subdomains and all invariant amino acid residues found in almost all eukaryotic protein kinases (Figure 5B). The sequences of HRDMKSSN in subdomain VIb and GTPGYVPPEY in subdomain VIII are strong indicators that it functions as a serine/threonine kinase rather than a tyrosine kinase (Hanks and Quinn, 1991). This domain of BRI1 is most closely related to the kinase domains of several putative LRR receptor kinases from Arabidopsis and rice (Figure 5B). It shares 41%, 39%, 41%, 36%, and 37% sequence identity within the kinase domain to ERECTA (Torii et al., 1996), CLV1 (Clark et al., 1997), RLK5 (Walker, 1993),

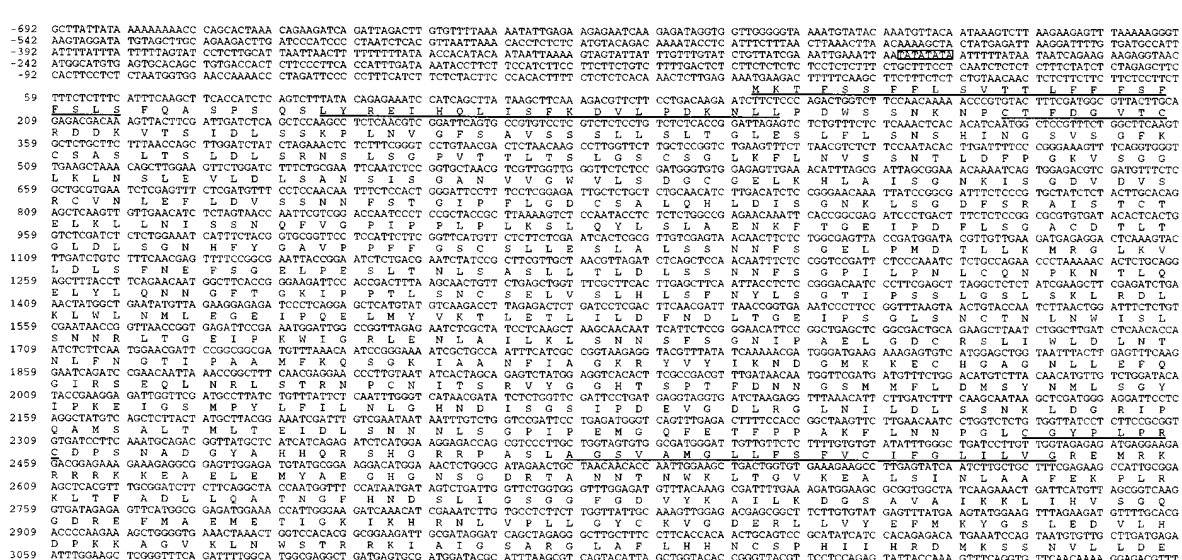

Figure 4. *BRI1* Encodes a Putative LRR Receptor Kinase

The DNA sequence of a 4.7 kb DNA fragment encoding the *BRI1* gene and the conceptual translational product of its longest open reading frame. A possible TATA box is boxed. The regions corresponding to a possible signal peptide, a putative leucine zipper motif, two conservatively spaced cysteine pairs, and a predicted transmembrane domain are underlined.

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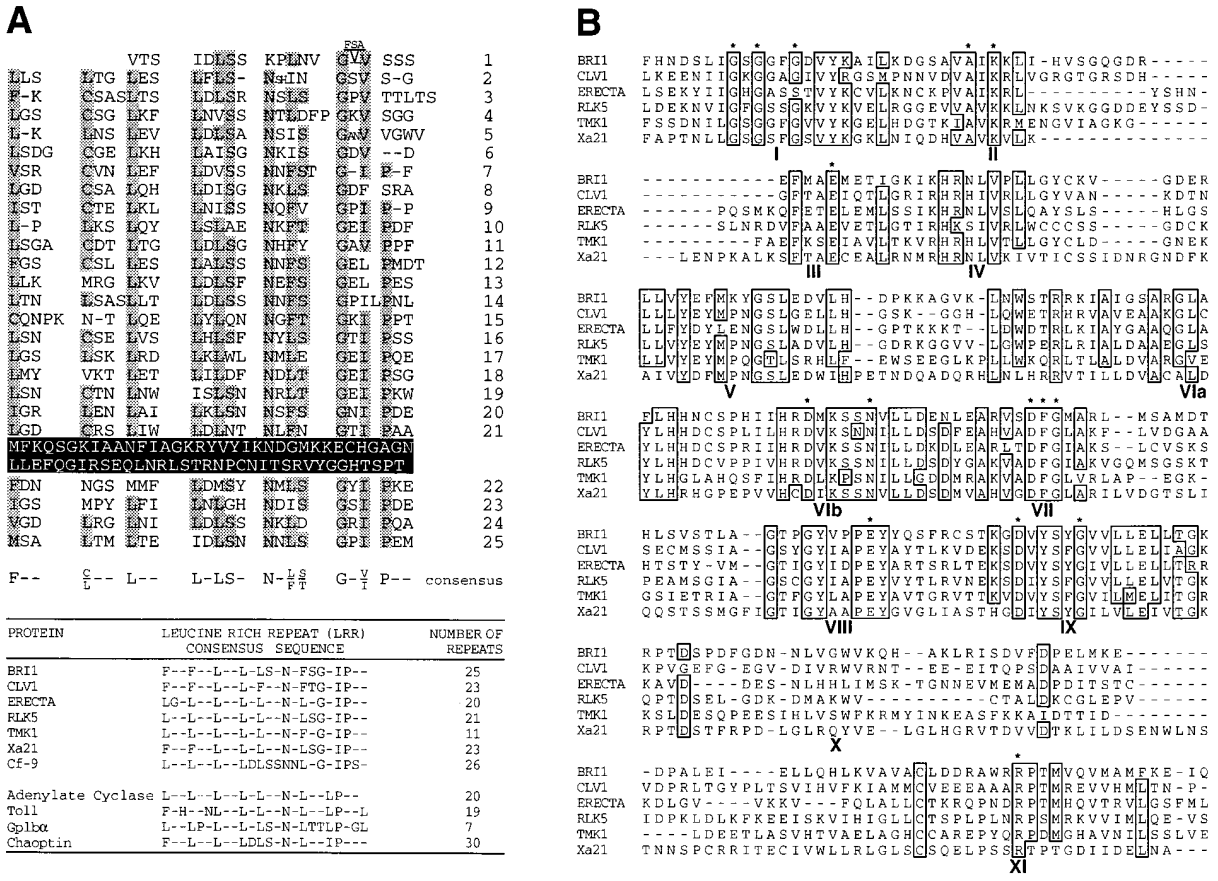

PROTEIN	LEUCINE RICH REPEAT (LRR) CONSENSUS SEQUENCE	NUMBER OF REPEATS
BRI1	F--F--L--L-LS-N-FSG-IP--	25
CLV1	F--F--L--L-F--N-FTG-IP--	23
ERECTA	LG-L--L--L-L--N-L-G-IP--	20
RLK5	L--L--L--L-L--N-LSG-IP--	21
TMK1	L--L--L--L-L--N-F-G-IP--	11
Xa21	F--F--L--L-L--N-LSG-IP--	23
Cf-9	L--L--L--LDLSSNNL-G-IPS-	26
Adenylate Cyclase	L--L--L--L-L--N-L--LP--	20
Toll	F-H--NL--L-L--N-L--LP--L	19
Gplbα	L--LP-L--L-LS-N-LTTLP-GL	7
Chaoptin	F--L--L--LDLS-N-L--IP---	30

Figure 5. Sequence Analysis and Comparisons of the Predicted BRI1 Protein

(A) The main extracellular domain comprises 25 leucine-rich repeats with a unit length of 24 amino acids. Numbers to the right of LRR domain indicate the specific LRR number. The bottom line indicates the consensus sequence for the *BRI1* LRR. Dashes stand for any amino acid and F indicates an aliphatic amino acid residue. The amino acid residues that match the deduced consensus sequence are shaded. A 70–amino acid island that does not fit the consensus sequence was buried between the 21st and 22nd LRR. These amino acids are shown by black boxes. The LRR consensus sequence of BRI1 was compared to those of other LRR-containing proteins, including Arabidopsis CLV1, ERECTA, RLK5, and TMK1, rice Xa21, tomato Cf-9 (Jones et al., 1994), yeast adenylate cyclase (Kataoka et al., 1985), Drosophila Toll (Hashimoto et al., 1988), human chaoptin (Reinke et al., 1988), and GalPα (Lopez et al., 1987).

(B) Alignment of the kinase domains among several putative LRR receptor kinases in plants, including BRI1, CLV1, ERECTA, TMK1, RLK5, and Xa21. The 12 conserved protein kinase domains are indicated I to XI (Hanks and Quinn, 1991). Residues that are conserved among at least five of the compared sequences are boxed. The 15 invariant amino acids present in all protein kinases are indicated by asterisks.

TMK1 (Chang et al., 1992), and Xa21 (Song et al., 1995), respectively.

The kinase domain appears to be essential for BRI1 function. Of the 5 alleles we sequenced, 4 have mutations in this domain, and they are clustered in a 50–amino acid segment (amino acids 1031–1080). *bri1–107* has a nonsense mutation at codon 1059. Two other alleles have substitutions of conserved residues. *bri1–115* has a mutation in subdomain VIII in which a conserved glycine is changed to aspartate. *bri1–101* contains a mutation at codon 1078, changing glycine to glutamate. Alanine 1031 is changed to threonine in the *bri1–104* allele.

BRI1 Expression Patterns

The phenotypes of brassinosteroid biosynthesis and response mutants suggest that brassinosteroids play a key role in Arabidopsis development throughout the life cycle of the plant. In addition, brassinosteroid mutants have a severe phenotype in the dark, indicating a role for this hormone during seedling etiolation. We examined the expression of *BRI1* throughout development and in seedlings grown in the light and dark. As expected, *BRI1* RNA accumulated ubiquitously and constitutively in light-grown plants (Figure 6A). Moreover, we detected no major alterations in RNA accumulation in either dark- or light-grown Arabidopsis seedlings (Figure 6B).

Discussion

A screen for brassinosteroid-insensitive mutants with a stature similar to BR biosynthetic mutants resulted in the isolation of 18 new alleles of a single genetic locus, *BRI1*. We cloned *BRI1* and found that it encodes a putative LRR-containing receptor kinase. A mutation in the extracellular domain and four other mutations in the cytoplasmic kinase domain abolish the in vivo activity

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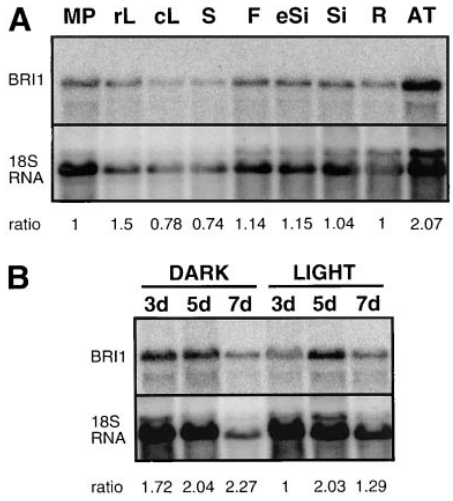

Figure 6. Expression Pattern of *BRI1*

(A) *BRI1* is ubiquitously expressed in different organs. RNAs were isolated from different tissues. MP, 4-week-old mature plants; rL, rosette leaves; cL, cauline leaves; S, inflorescent stems; F, flowers; eSi, emerging siliques; Si, mature siliques; R, roots; and AT, aerial tissues. The roots and aerial tissues were collected from 14-day-old seedlings grown in liquid medium.

(B) Expression of *BRI1* is slightly affected by the light conditions and developmental age of seedlings. RNAs were isolated from both dark- and light-grown seedlings at 3, 5, and 7 days postgermination. In (A) and (B), 5 μg of total RNA was loaded per lane. Both filters were hybridized with probes derived from the 3.5 EcoRI fragment as shown in Figure 3C and reprobed with 18S rDNA as a loading control. After normalizing with rRNA, a ratio was calculated by comparing the *BRI* expression level of different tissues with that of roots in (A) or by comparing the *BRI1* expression level of different seedlings with that of 3-day light-grown seedlings in (B).

of *BRI1*. The striking phenotypic changes caused by mutations in *BRI1* and the significant homologies of its encoded protein with several known receptor kinases strongly suggest the involvement of this receptor kinase in the brassinosteroid signal transduction cascade.

It is somewhat surprising that all 18 new alleles and two previously described BR-insensitive mutations isolated by different screening strategies (Clouse et al., 1996; Kauschmann et al., 1996) are all alleles of a single gene. This suggests that the LRR receptor kinase is the only unique component in the BR-signaling pathway or that downstream components are redundant. Several LRR receptor kinases have been described previously in Arabidopsis (Chang et al., 1992; Walker, 1993; Torii et al., 1996; Clark et al., 1997; Hong et al., 1997). In each case, the ligand is not known yet. It is possible that these receptors phosphorylate common signal transduction intermediates to control plant growth and differentiation. If this were true, then mutants in these common signaling intermediates might die as embryos and would not be identified in specific genetic screens.

A transmembrane receptor kinase is a central theme in many different signal transduction pathways in animals (Ullrich and Schlessinger, 1990). The homology of the predicted structure of the *BRI1* gene with those of animal receptor kinases immediately leads to a possible mechanism of BR action in plants. Like its animal counterparts, the BRI1 protein, upon binding to BRs, either directly or indirectly, may lead to its homo or heterodimerization and activate its intrinsic cytoplasmic kinase activity, which in turn phosphorylates both itself and several other intracellular targets, thereby propagating and amplifying the BR signals.

The presence of the LRR sequences in the extracellular domain of BRI1 is very intriguing. Found in a functionally and evolutionarily diverse set of proteins, LRRs are used in many molecular recognition processes as diverse as signal transduction, cell adhesion, cell movement, DNA repair, and RNA processing (Kobe and Deisenhofer, 1994). At least half of the known LRR-containing proteins participate in signal transduction (Kobe and Deisenhofer, 1994). For example, the human G protein–coupled receptor for glycoprotein hormones, including chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone, and the Trk tyrosine kinase receptors for nerve growth factors are involved in signal transduction of peptide hormones (Kobe and Deisenhofer, 1994). The LRR-containing proteins encoded by many plant disease resistance genes such as *Cf-2* (Dixon et al., 1996), *Cf-9* (Jones et al., 1994) of tomato, and *Xa21* (Song et al., 1995) of rice take part in transducing pathogen signals, leading to plant resistance to diseases, while the Arabidopsis CLV1 (Clark et al., 1997) and ERECTA (Torii et al., 1996) proteins participate in developmental signaling pathways. In many cases, LRRs are believed to be specific ligand binding sites for either peptidic hormones in animals (Kobe and Deisenhofer, 1994) or pathogenesis elicitors (Baker et al., 1997) and developmental signals in plants (Torii et al., 1996; Clark et al., 1997). The specificity of ligand binding in those LRR-containing transmembrane receptors are most likely provided by the nonconsensus residues within LRRs (Kobe and Deisenhofer, 1994). Although all known ligands for these LRR-containing receptors are small peptides or glycoproteins, it is formally possible that LRRs may also bind small molecules, such as brassinolide. This is known to be true for proteins that contain PAS repeats. PAS domains were originally defined as protein–protein interaction domains, but PAS repeats can also bind small molecules, such as dioxin (Hoffman et al., 1991).

A unique feature of *BRI1*'s LRR domain is the presence of a 70– amino acid island buried between the 21st and 22nd LRR. This island bears no resemblance to any known LRR or other sequence in the database. The island is, however, essential for the function of BRI1 in brassinosteroid signal transduction. In *bri1–113*, a severe allele, Gly-611 is changed to a negatively charged glutamate. It is possible that this 70–amino acid motif is important for direct ligand binding or for maintaining the structure of the ligand binding domain.

Another possibility to explain the LRRs found in BRI1 is that they mediate interactions with a steroid-binding protein that presents BRs to the cell surface. In animals, it has been shown that sex steroids can bind to the cell surface through a protein called sex hormone binding globulin (SHBG), which stimulates a cyclic AMP-dependent signaling pathway that spurs growth (Nakhla et al., 1997). SHBG is a 90 kDa protein that occurs in blood plasma and binds to both androgens and estrogens (Lewin, 1996). Some cells (e.g. prostate, endometrium,

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liver, and testis) bind SHBG specifically, presumably via a cell surface receptor whose identity is not known. Sequences with homology-to-steroid binding proteins have been found in the Arabidopsis genome. It is possible that these proteins are involved in extracellular interactions with BRI1 to stimulate growth by brassinosteroids.

Like many well-studied receptor kinases, the cytoplasmic kinase activity is indispensable for transducing extracellular signals to intracellular targets. Although there is no direct biochemical evidence that the cytoplasmic domain of the *BRI1* gene encodes a functional kinase, the homologies of this region with those of *CLV1*, *TMK1*, and *RLK5* (see Figure 5B), whose activities have already been demonstrated in vitro (Chang et al., 1992; Horn and Walker, 1994; Clark et al., 1997), strongly argue for such a possibility. Molecular analyses of several *bri1* alleles further strengthen this argument. Out of the 5 alleles we sequenced, 4 have mutations in the presumed kinase domain and all display similar mutant phenotypes. *bri1–101* has a mutation at codon 1078, resulting in a substitution of a conserved negatively charged glutamate to a positively charged lysine in subdomain IX. Although this glutamate is not one of 15 invariant or nearly invariant residues among all protein kinases, it is absolutely conserved through the LRR receptor kinase family in plants. *bri1–115* contains a G1048-D mutation in subdomain VIII, which is important to determine substrate specificity. The sequence G(T/S) xx(Y/F)xAPE (the first G is mutated in *bri1–115* allele) in this subdomain is one of two signature sequences that differentiate the protein serine/threonine kinases from the protein tyrosine kinases. It is interesting to note that the exact same mutation has been identified in two *clv1* alleles (*clv1–1* and *clv1–5*). The *bri1–107* mutation causes premature termination at codon Q1059, thereby deleting 138 amino acids at the C terminus predicting a polypeptide missing the last three subdomains of the kinase domain. *bri1–104* changes an alanine to threonine at codon 1031, the second residue immediately after the conserved DFG triplet in subdomain VII, which is implicated in ATP binding. Although valine, serine, and threonine have been found in its place, there is precedence for the importance of this alanine. The mutation in the *clv1–9* allele causes a substitution of this alanine by valine, resulting in a weak *clv1* phenotype.

The overall similarity between *BRI1* and *Xa21*, a disease resistance gene isolated from rice (Song et al., 1995), and the similarities between the extracellular LRR domain of *BRI1* and those of many plant disease resistance genes (Baker et al., 1997) suggests possible interactions between the steroid-signaling pathway and disease resistance pathways. It has been known that BRs can enhance disease resistance in several crop plants (Mandava, 1988). A recent study showed that overexpression of *CPD*, an Arabidopsis gene encoding a key enzyme in BR biosynthetic pathway, resulted in induction of several pathogenesis-related proteins (PRs; Szekeres et al., 1996). Several possible mechanisms can explain the interaction between these two pathways. First, BRI1 and the disease resistance receptor kinase might share substrates for kinase activity or share similar second messengers. Second, the same pathway might be involved in two different processes by interacting with specific downstream components. In Drosophila, the Toll/Dorsal-mediated patterning pathway plays an important role in the activation of the antifungal pathway (Lemaitre et al., 1996). Third, heterodimerization of BRI1 and an LRR-containing transmembrane receptor that is specific for disease resistance could lead to the activation of both signaling pathways.

The cloning of *BRI1* may also shed some light on the signaling pathways of other plant hormones. Many studies have suggested that plant hormones are perceived on the cell surface and that protein kinases are components of their signaling pathways. The Arabidopsis genome encodes a large family of transmembrane receptor kinases, and some of them might be involved in plant hormone signaling pathways. Two LRR-containing receptor kinases have recently been identified to be induced by either ABA (Hong et al., 1997) or GA (van der Knaap et al., 1996). These receptor kinases transduce extracellular signals by activation of their intrinsic kinase activities and phosphorylation of their downstream targets. A recent study identified a type 2C phosphatase (KAPP) as a substrate for the cytoplasmic kinase activity of the Arabidopsis RLK5 (Stone et al., 1994). Recently, two abscisic acid–insensitive loci, *ABI1* and *ABI2*, have been cloned, and each of them encodes a type 2C phosphatase (Leung et al., 1994, 1997; Meyer et al., 1994). Although both ABIs lack a kinase interaction (KI) domain that is identified in the Arabidopsis KAPP, it is still possible that both proteins might be involved in an ABA signaling pathway initiated on the cell surface by a transmembrane receptor kinase. Both the Arabidopsis CLV1 and ERECTA are implicated in regulating several developmental processes (Torii et al., 1996; Clark et al., 1997); however, their corresponding ligands remain unknown. It is possible that their ligands are one of the well- known plant hormones and each receptor kinase represents a tissue-specific or developmental stage–specific form of these hormone receptors. Thus, it is tempting to speculate that some of the previously characterized, as well as uncharacterized or unidentified, transmembrane receptor kinases might be the long-sought-after cell surface receptors for the well-characterized plant hormones.

In animal systems, steroid hormones are generally thought to produce their major long-term effects on differentiation and homeostasis via intracellular receptors that regulate gene expression. There is evidence, however, that steroids can also affect the surface of cells where they alter ion permeability and release of neurohormones and neurotransmitters (reviewed in McEwen, 1991). There are also physiologically relevant actions of progestins on the maturation of spermatozoa and of oocytes (Kwon and Schuetz, 1986; Blackmore et al., 1991; Wistrom and Meizel, 1993). Several studies have implicated hormone binding on the surface of these cells, although the relevant binding activities have not been purified. Steroid binding at the cell surface mediates calcium uptake and tyrosine phosphorylation, suggesting that protein tyrosine phosphorylation is involved in signal transduction through these cell surface receptors (Tesarik et al., 1993; Mendoza et al., 1995). Plants are not known to contain receptor tyrosine kinases, but

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it is intriguing that BRI1 has many of the properties expected for the animal steroid membrane receptors that are thought to mediate nongenomic effects of steroids. It will be worthwhile to look for the involvement of the BRI class of receptor kinases in animal responses to steroids.

BRI1 appears to be constitutively and ubiquitously expressed throughout Arabidopsis development and in response to different light conditions. This expression pattern of *BRI1* parallels that of *DET2*, a steroid reductase that functions in the synthesis of brassinolide (D. Friedrichsen and J. C., unpublished data). Coexpression of these two genes at the organ level raises the question of why plant cells respond to BRs by proteins that are on the cell surface. Although the precise cell type–specific expression patterns of *BRI1* and *DET2* are not known, the ubiquitous expression of both these genes reinforces the classical physiological observations that plant growth regulators are distinct from animal hormones for which there are discrete sites of synthesis and action. How a plant cell affects a specific response to a growth regulator remains a future challenge in the study of signal transduction in plants.

Experimental Procedures

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was the wild type used for backcrossing and comparison with mutant plants. Ecotype Landsberg carrying the erecta mutation (Ler) was used for mapping purposes. *det2–1* mutants (Chory et al., 1991) were used for morphological comparison with the newly identified BR response mutants. Seed sterilization, seedling growth media, and plant growth conditions were described before (Li et al., 1997).

Isolation of *bin* Mutants

EMS mutagenized M2 seeds of Arabidopsis thaliana (Col-0) carrying the homozygous recessive mutation *glabrous1 (gl1)* were purchased from Lehle Seeds (Round Rock, TX). Approximately 80,000 M2 seeds derived from six independent parental groups (1600 M1/group) were screened on 0.5 × MS medium (GIBCO–BRL, Grand Island, NY) at a density of ~1,000 seeds per petri plate (150 × 15 mm). After growing in the light for 10 days, seedlings displaying light-grown *det2* (Chory et al., 1991) or cpd phenotypes (Szekeres et al., 1996) were transferred to fresh MS medium containing 1 μM brassinolide (a gift from Dr. Trevor McMorris, Department of Chemistry, University of California, San Diego), and mutants that showed no response to the hormone were picked after growing for 2 additional weeks on the brassinolide-containing MS medium, transferred to soil and grown to maturity. Since most BR-insensitive mutants are male-sterile, they were backcrossed to the wild-type Col-0 in order to maintain these lines as heterozygotes. Brassinolide-insensitivity was retested with the segregating F2 mutants. A total of 28 putative *bin* mutants were initially identified, 7 of them died without producing any seeds and 3 others failed the BR-insensitivity test in the F2 generation.

Genetic Analysis

bin mutants were crossed to wild-type Col-0 to generate F1 plants, which were then allowed to self-pollinate to produce F2 seeds. The number of wild-type and *bin* mutant plants in the resulting F2 populations was counted. Statistical analysis indicated that all *bin* mutations segregated with a 3:1 ratio expected for a recessive mutation.

To test for allelism, pollen of heterozygous plants of other *bin* mutants were used to pollinate homozygous *bin1–1* mutants. The phenotype of the resulting F1 progeny was scored 10 days after germination. Complementation between *bin1–1* and *bri1–1* (Clouse et al., 1996) was done by pollinating homozygous *bri1–1* plants with pollen from homozygous *bin1–1* mutants. Since the *bri1–101* mutation causes a XhoI polymorphism within the *BRI1* gene (see Results), the heterozygosity of the *bri1–101* allele in the resulting F1 plants was verified by CAPS analysis.

Mapping of *BRI1*

A homozygous *bri1–101* mutant was pollinated with L*er* pollen, and the resulting F1 plants were self-pollinated to generate F2 plants segregating the *bri1* mutation. DNAs isolated from 957 individual F2 *bri1* mutants were used for SSLP (Bell and Ecker, 1994) and CAPS analysis (Konieczny and Ausubel, 1993). After scoring 1064 chromosomes, *bri1* was mapped to a region flanked by SSLP marker nga1107 (http://cbil.humgen.upenn.edu/~atgc/SSLP_info/comingsoon.html) and CAPS marker DHS1. The cosmid CC15O17 (Schmidt et al., 1996; kindly provided by Dr. Caroline Dean, John Innes Center, Norwich, UK) was converted into a CAPS marker to identify two recombinant events out of 1914 meioses, thus placing *BRI1* on yUP1A3. The TAIL-PCR (Liu and Whittier, 1995) method was used to isolate both the right and left ends of several selected YAC clones (see Figure 2) overlapping with yUP1A3, and these YAC ends were then used for hybridization of YAC DNAs to determine their correct orientations and relative positions. The right end of EW4E8 was converted into a CAPS marker and used to further delimit the *BRI1* gene. The YAC ends were also used for hybridization to bacterial artificial chromosome (BAC) filters (Choi et al., 1995; kindly provided by Dr. Joe Ecker at the University of Pennsylvania). The right end of the largest positive BAC clone, 11P20, isolated by TAIL-PCR, was converted into a CAPS marker that allowed us to place the gene onto a contig consisting of this BAC and the cosmid clone isolated from a library (Schulz et al., 1995; distributed by the Arabidopsis Biology Resources Center, Ohio State University) using the right end of EW4E8 as a probe.

DNA and RNA Analysis

Plant DNAs were isolated from frozen tissues as described (Li et al., 1997) for both PCR reactions and Southern blot analysis. Yeast and BAC DNAs and plant RNA were isolated according to standard protocols (Ausubel et al., 1994).

BAC Filter Hybridization and Screening of Cosmid Library

Hybridization of BAC DNA filters using various YAC ends generated by TAIL-PCR was performed as described (http://http.tamu.edu:8000/~creel/BACVEC.html). A cosmid chapter library (Schulz et al., 1995) was screened by a two-step procedure to identify clones that link BAC11P20 to the right end of EW4E8. First, PCR reactions were done with frozen E. coli cells using primers corresponding to the right end of EW4E8 to identify individual chapters that can amplify this DNA. Standard procedures for screening cosmid libraries were then used to isolate a single cosmid clone COS70–8.

DNA Sequence Analysis

A 3.5 kb EcoRI fragment and a 2.5 kb SalI/EcoRI fragment were isolated from cosmid clones that were derived from 11P20 BAC DNA and were subcloned and sequenced by standard procedures. This information was used to design primers to sequence the complete *BRI1* gene using an ABI automated sequencer. The *BRI1* gene from various *bri1* alleles was amplified as three overlapping fragments of 1.7–2.5 kb in length using the Pwo polymerase (Boehringer Mannheim, Indianapolis, IN). Products of five independent PCR reactions were pooled together to run preparative agarose gel electrophoresis, and DNA fragments of the right sizes were isolated using the QIAEXII gel extraction kit (Qiagen Inc., Chatsworth, CA) and then sequenced directly. The primary sequencing data were analyzed using the LaserGene programs (DNASTAR Inc., Madison, WI), and database searches were performed at the U. S. National Center for Biotechnology Information with the BLAST program (Altschul et al., 1990).

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