BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*

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Accepted 16 August 2004

Development 131, 5341-5351
Published by The Company of Biologists 2004
doi:10.1242/dev.01403

Summary

Plant steroid hormones, brassinosteroids (BRs), are perceived by the plasma membrane-localized leucine-rich-repeat-receptor kinase BRI1. Based on sequence similarity, we have identified three members of the BRI1 family, named BRL1, BRL2 and BRL3. *BRL1* and *BRL3*, but not *BRL2*, encode functional BR receptors that bind brassinolide, the most active BR, with high affinity. In agreement, only BRL1 and BRL3 can rescue *bri1* mutants when expressed under the control of the *BRI1* promoter. While *BRI1* is ubiquitously expressed in growing cells, the expression of *BRL1* and *BRL3* is restricted to non-overlapping subsets of vascular cells. Loss-of-function of *brl1* causes abnormal phloem:xylem differentiation ratios and enhances the vascular defects of a weak *bri1* mutant. *bri1 brl1 brl3* triple mutants enhance *bri1* dwarfism and also exhibit abnormal vascular differentiation. Thus, *Arabidopsis* contains a small number of BR receptors that have specific functions in cell growth and vascular differentiation.

Key words: *Arabidopsis*, Brassinosteroids, Vascular differentiation

Introduction

Brassinosteroids (BRs) are plant steroid hormones crucial for cell growth and plant morphogenesis. They were first identified by their ability to elongate plant stems when applied exogenously (Grove et al., 1979; Mandava, 1988). Since then, *Arabidopsis* genetics has permitted the identification of a wealth of BR synthesis and signal transduction components. The phenotypic characterization of mutants affecting BR synthesis and signal transduction has revealed an essential role for BRs in cell elongation and differentiation. *bri1* is the only BR-insensitive loss-of-function mutant identified so far by genetic screens (Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory, 1997). Dark-grown *bri1* mutants resemble BR-deficient mutants, exhibiting severe dwarfism with dark green epinastic leaves, and reduced apical dominance and fertility (Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Azpiroz et al., 1998; Choe et al., 1999; Choe et al., 2001; Noguchi et al., 1999).

BRI1 encodes a membrane-localized leucine-rich repeat receptor-like kinase (LRR-RLK). The extracellular LRR domain is connected by a single pass transmembrane domain to a typical Ser/Thr kinase (Li and Chory, 1997; Friedrichsen et al., 2000). While more than 200 LRR-RLKs have been identified in the *Arabidopsis* genome (Yin et al., 2002b; Shiu and Bleecker, 2001), only a few have been assigned a function. Moreover, most of these receptors have no known ligand. Genetic and biochemical evidence has demonstrated that BRI1 is an essential component of a receptor complex that binds brassinolide (BL) with high affinity (Wang et al., 2001). Although understanding how BRs are perceived by the BRI1 receptor remains to be elucidated, other signaling components operating downstream of BRI1 have been identified. BRI1 associated receptor kinase 1 (BAK1), a much smaller RLK containing only five LRRs, was identified in an activation-tagging screen for *bri1* suppressors (Li et al., 2002) and in a yeast two-hybrid screen for BRI1 interacting proteins (Nam and Li, 2002). BAK1 thus acts in close proximity with BRI1, perhaps as its co-receptor. Brassinosteroid insensitive 2 (BIN2)/UCU1/DWF12, one of the 10 *Arabidopsis* GSK-3/Shaggy–like kinases, negatively regulates BR signaling (Li and Nam, 2002; Pérez-Pérez et al., 2002; Choe et al., 2002), while *bri1* Suppressor 1 (BSU1), a nuclear-localized Ser/Thr phosphatase, positively regulates BR signaling (Mora-García et al., 2004). When BR levels are low, proteins in the BES1/BZR1 family are hyperphosphorylated by BIN2 and targeted for degradation. Upon BR perception, dephosphorylated BES1/BZR1 proteins accumulate in the nucleus, where they presumably regulate the transcription of BR-regulated genes (Wang et al., 2002; Yin et al., 2002a). Many BR-induced genes are related to cell wall loosening enzymes, supporting the role of BRs in cell expansion

(Kauschmann et al., 1996; Nicol et al., 1998; Yin et al., 2002a; Goda et al., 2004).

Early physiological studies using *Zinnia* explants have shown that BRs play an important role in promoting xylem differentiation (reviewed by Fukuda, 1997). The BL synthesis inhibitors, uniconazole and brassinazole (BRZ) (Iwasaki and Shibaoka, 1991; Asami et al., 2000), prevent xylem differentiation in the *Zinnia* cell cultures, which can be restored by exogenous application of BL. Furthermore, the levels of BR intermediates have been shown to peak at the transition from undifferentiated cells to tracheary elements (Yamamoto et al., 2001). BRs also appear to promote xylem differentiation in cress plants (Nagata et al., 2001). In *Arabidopsis*, two mutants defective in BR-synthesis, *cpd* and *dwarf7*, exhibit vascular differentiation defects (Szekeres et al., 1996; Choe et al., 1999); however, vascular phenotypes in BR biosynthetic and signaling mutants have not been characterized in detail. How BRs regulate vascular development in *Arabidopsis* and whether xylem differentiation is controlled by specific BR-receptors are still unanswered questions.

We identified three proteins from the *Arabidopsis* genome, designated BRL1, BRL2 and BRL3, with high sequence identity to BRI1. The BRL2 gene corresponds to the previously identified Vascular Highway 1 (VH1), which appears to be required to maintain provascular differentiation in the leaves (Clay and Nelson, 2001). Here, we show that BRL1 and BRL3, but not BRL2, can complement *bri1* mutant phenotypes when expressed under the control of the *BRI1* promoter. We show that BRL1 and BRL3, but not BRL2, can bind BL with high affinity. Unlike BRI1, which is expressed ubiquitously, the *BRL* genes are predominantly expressed in the vascular tissues. Consistent with this observation, we found a *brl1* mutant had increased phloem and reduced xylem differentiation compared with the wild type. Double mutant combinations of *bri1 brl1* and triple *bri1 brl1 brl3* mutants displayed aberrant vascular differentiation. We propose that BRL1 and BRL3 are novel plasma-membrane-localized steroid receptors that function specifically in provascular differentiation to maintain proper xylem:phloem ratios.

Materials and methods

Plant material and growth conditions

Ecotypes Columbia (Col-0) and Wassilewskija (Ws-2) were used as the wild types. Seeds were germinated either on 1/2 MS medium (Caisson) plus 1% sucrose or directly in soil. Plants were grown under fluorescent light (12 hours light/12 hours dark cycles) except for the BL-binding assays, in which plants were grown under short-day conditions (9 hours light/16 hours dark). Seeds were kept at 4°C for at least 72 hours before light treatment.

Mutant isolation

Screening and isolation of a T-DNA insertion in the *brl1-1* line was done as described by the *Arabidopsis* knock-out Facility at the University of Wisconsin (http://www.biotech.wisc.edu/*Arabidopsis*/). The *brl1-2*, *brl2* and *blr3* mutants were isolated from the T-DNA Express Collection at the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress). The gene-specific primers used for PCR genotyping of the T-DNA insertions and the location of the T-DNA insertions for every allele are provided in Table S1 (in the supplementary material).

Histology and microscopy

Tissue samples were fixed overnight in 4% (v/v) glutaraldehyde in 25 mM NaPO4 buffer, pH 7.4, and then dehydrated with a graded series of ethanol and infiltrated with polymethacryl resin Technovit 7100 (Heraeus Kulzer, Germany), followed by embedding and polymerization in Technovit 7100. Sections (4 μm) were done using a Leica Jung Autocut microtome (Wetzlar, Germany). The tissue sections were stained with 0.1% Toluidine Blue in 0.1 M NaPO4 buffer, pH 7.0, and observed under bright-field illumination through a Zeiss Axioskop microscope. Images were processed using Photoshop 7.0. To calculate the number of phloem cells, pictures of each 4 μm transverse section were taken at 40× magnification under a Zeiss microscope. Then each vascular bundle was printed separately. The phloem cells were delimited with a pen and hand counted for each bundle of every plant. The average of phloem cells per plant was calculated using a total of 12 plants. Histochemical staining of plants expressing the GUS reporter fusions was basically done as reported in Baima et al. (Baima et al., 1995).

Visualization of green fluorescent protein (GFP)-tagged BRLs was done on 7-day-old light-grown hypocotyls using a BioRad Radiance 2100 microscope. The images were captured using the Kalman filter (n=5), and then analyzed with Confocal Assistant software.

Overexpression, localization and rescue constructs

bri1 rescue constructs

BRL1, *BRL2* and *BRL3* genes were amplified from bacterial artificial chromosome (BAC) clones F20N2, F14H20 and MRP15, respectively, and cloned into a modified pBluescript plasmid that contains a 1.7 kb *BRI1*-promoter sequence and the *RbcS*-E9 terminator. After sequencing verification, the *pBRI1-BRL-E9ter* fragments were cleaved from the pBluescript plasmids and cloned into the pPZP212 binary vector. Site-directed mutagenesis was carried out using Stratagene's QuickChange Site-Directed Mutagenesis kit to introduce G597E (corresponding to *bri1-113* mutation) or E1056K (corresponding to *bri1-1* mutation), mutation into the *pBRI1-BRL1-E9ter* construct. Plant transformation into the weak *bri1-301* mutants by floral dipping was done according to Clough and Bent (Clough and Bent, 1998). Due to unknown reasons, the *pBRI1-BRL2-E9ter* plasmid could not be transformed into *Agrobacterium* cells. Instead, we constructed a GFP-tagged version of the *BRL2* transgene and were able to transform the resulting *pBRI1-BRL2::GFP-E9ter* construct into the *bri1-301* mutants.

Construction of promoter GUS fusions

For *BRI1*, a 1.7 kb fragment including the 5′ utr and promoter region was amplified from BAC F23K16 and cloned between the *Hin*dIII/*Bam*HI sites in vector pBI101.3. Transgenic plants were selected on kanamycin. For *BRL* genes, a 1.72 kb *Spe*I/*Hin*dIII restriction fragment of the *BRL1* promoter and a 750 bp *Nsi*I/*Afl*II restriction fragment of the *BRL3* promoter were individually ligated in frame to the GUS gene of the binary vector pCB308. Transgenic plants were selected for resistance to the herbicide glufosinate. Homozygous plants were used for GUS histological staining.

Immunoprecipitation and ^{3}H-BL binding assays

These experiments were performed essentially as reported in Wang et al. (Wang et al., 2001), with slight modifications. For ^{3}[H]-BL binding assays with immunoprecipitated proteins, 4-week-old T3 plants expressing GFP-tagged BRLs were grown under short-day conditions. The membrane fractions were extracted from 15 g of rosette leaves using 2 ml/g tissue of BRI1 extraction buffer at 4°C (Wang et al., 2001). Membranes were immunoprecipitated using 5 μl of anti-GFP antibodies (Molecular Probes) and Protein A agarose beads (200 μl of 50% slurry immobilized protein A), (Pierce). After extensive washing, the immunoprecipitate was aliquoted in 50 μl/sample and was used for individual binding experiments. Specific BL binding and data analysis was done according to Wang et al. (Wang et al., 2001).

Immunoprecipitated GFP fusion proteins were analyzed by Western blotting with anti-GFP antibodies (1/3000 dilution).

Results

BRI1 has three close homologs in *Arabidopsis*

To identify novel receptors that may function in BR perception, a BLAST search of the *Arabidopsis* genome with the kinase domain of BRI1 was conducted, identifying three *BRI1*-like genes (Fig. 1A), designated *BRL1* (At1g55610), *BRL2* (At2g01950), and *BRL3* (At3g13380). These genes comprised large intronless open reading frames (ORF) of 3498, 3429 and 3483 base pairs encoding predicted proteins of 1166 (estimated molecular mass of 127 kD), 1143 (125 kD), and 1161 amino acids (127 kD), respectively. A full-length cDNA clone for each BRL had been identified from the *Arabidopsis* expressed sequence tag (EST) database (http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml).

The *BRI1-BRL* family encodes LRR-receptor kinases with several predicted domains: an N-terminal signal peptide followed by an extracellular domain consisting of a variable number of LRR-motifs in tandem, and a single transmembrane pass followed by an intracellular Ser/Thr kinase domain (Fig. 1B). The numbers of predicted LRR-motifs were 22 in BRL2 and 23 in BRL1 and BRL3, compared with 25 in BRI1. A

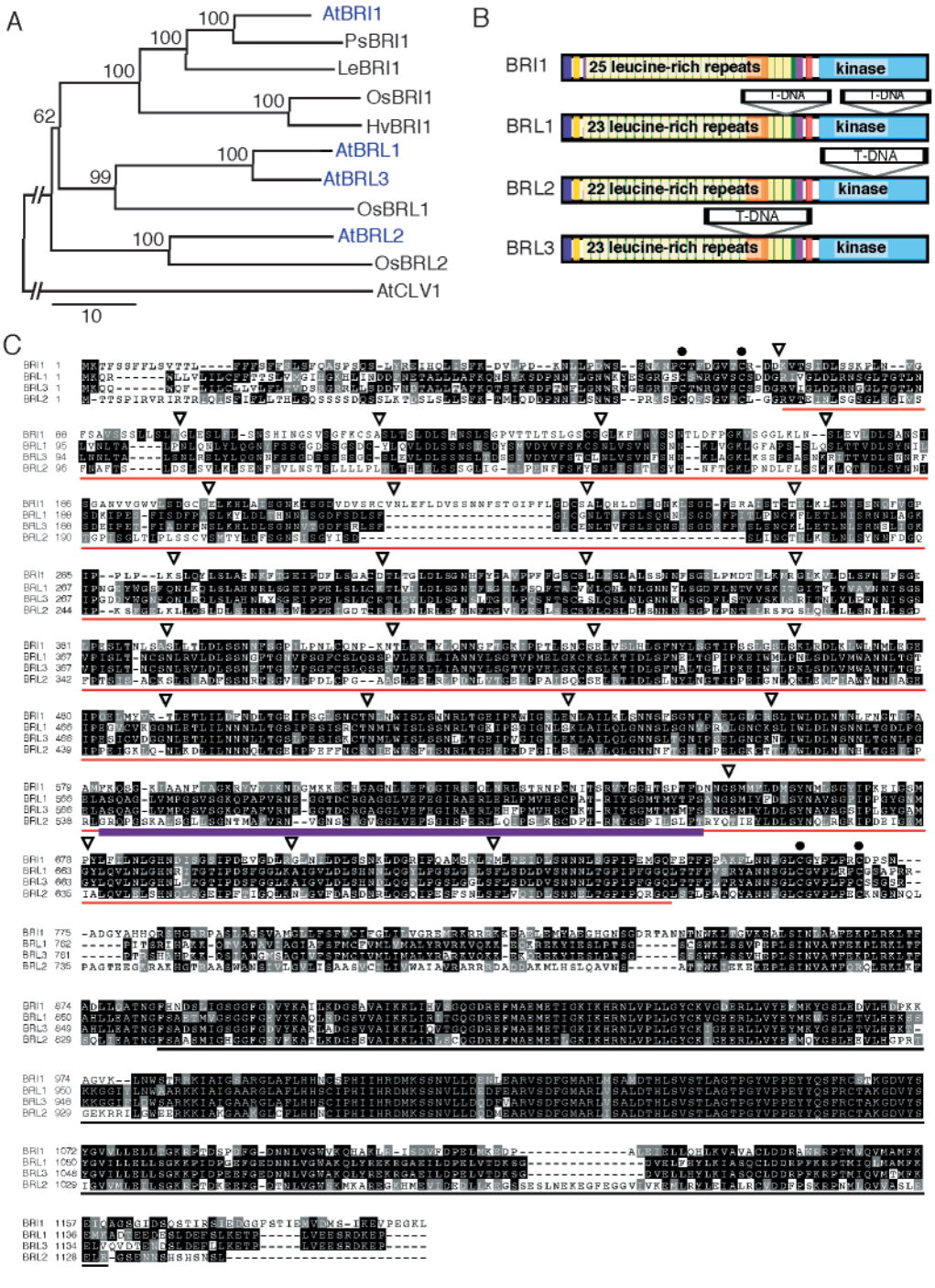

Fig. 1. BRL genes encode homologs of BRI1. (A) Phylogenetic tree of members of the BRI1-family. Full-length protein sequences were aligned with ClustalX and manually adjusted. The N-J tree was calculated with MEGA2.1 (Kumar et al., 2001) for 1000 bootstrap repetitions. Branch lengths are proportional to the number of substitutions per 100 residues (indicated by the bar below the tree) and the branchpoint values indicate the percentage bootstrap support. The comparison included the *Arabidopsis* family members BRI1 [AtBRI1 (Li and Chory, 1997), AAC49810], AtBRL1 (NP_175957), AtBRL2 [(Clay and Nelson 2001), NP_178304], AtBRL3 (NP_187946), the corresponding sequences from rice, OsBRI1 [(Yamamuro et al., 2000), BAB68053], OsBRL1 (BAD01717) and OsBRL2 (AAK52544), and those reported for tomato [LeBRI1, *cu3* (Montoya et al., 2002), Q8GUQ5], pea [PsBRI1, *lka* (Nomura et al., 2003), Q8GUQ5] and barley [HvBRI1, *uzu* (Chono et al., 2003), BAD06331]. *Arabidopsis* CLV1 (Q9SYQ8) was included as an outgroup RLK. The *Arabidopsis* proteins are highlighted in blue. (B) Diagram of the BRI1 and BRL proteins showing the location of the T-DNA insertions. Like *BRI1*, no introns are present in any of the *BRL* genes. (C) Alignment of the *Arabidopsis* proteins. In the extracellular region, the LRR domain is underlined in red, the arrowheads indicate the beginning of each LRR, the 70-amino acid island domain region is underlined in purple and the paired cysteines flanking the LRR-domain are indicated by black dots. The Ser/Thr kinase domain is underlined in black. Identical and similar amino acids are highlighted by black and gray boxes, respectively.

distinctive feature of all BRI1-like proteins is a 70-amino acid island domain essential for BRI1 to bind BRs, which was located four LRRs away from the transmembrane domain in all four proteins (Fig. 1B).

The amino acid sequence alignment is shown in Fig. 1C. BRL1 and BRL3 were the closest homologs, both sharing 43% sequence identity with BRI1, followed by BRL2 with 41%. Among the four LRR-RLKs, BRL1 and BRL3 shared the highest homology, being 80% identical. It is noteworthy that BRL1 and BRL3 contained island domains that were 93% identical, while BRL2 is the most divergent member (Fig. 1C). The finding that BRI1, BRL1, BRL2 and BRL3 constituted a subfamily of the LRR-RLK proteins led us to hypothesize that the BRLs may be BR receptors with distinct functions.

BRL1 and *BRL3*, but not *BRL2*, encode functional BR receptors

To test whether the *BRL* genes encode functional BR receptors, three fusion constructs containing the *BRI1* promoter, the complete ORF for each *BRL*, and the 3′ terminator of the pea *RbcS-E9* gene were generated. Each *pBRI1::BRL::E9* transgene was then transformed into a weak *bri1* (*bri1-301*) mutant background (Fig. 2). Unlike many *bri1* mutants that are male sterile, *bri1-301* displays a weak mutant phenotype and is fertile, and therefore is directly transformable (Fig. 2C). We found that transgenic plants expressing *pBRI1-BRL1* (Fig. 2D) and *pBRI1-BRL3* (Fig. 2F) were able to suppress the dwarf *bri1* phenotype, whereas the *pBRI1-BRL2* transgenic plants were indistinguishable from the *bri1-301* mutants (Fig. 2E). In addition, transgenic plants expressing *pBRI1-BRL1* and *pBRI1-BRL3* in the Col-0 background conferred a long and curled petiole phenotype, similar to the one described for the overexpression of BRI1 under its own promoter (Wang et al., 2001), while wild-type plants transformed with *pBRI1-BRL2* looked similar to wild-type controls (data not shown). Thus, BRL1 and BRL3 can substitute for BRI1 function when expressed in the expression domain of *BRI1*. By contrast, BRL2 does not appear to function in BR signaling, despite its sequence homology with BRI1.

Sequence analyses of various *bri1* alleles have previously identified several regions that are essential for BRI1 function, including the 70-amino acid island domain and the cytoplasmic kinase domain (He et al., 2000; Wang et al., 2001). To test whether the island domain and a functional kinase domain were required for BRL1 to carry out BRI1's function, two mutated *pBRI1-BRL1* constructs, *BRI1-BRL1-GE* and *BRI1-BRL1-EK*, containing mutations in the 70-amino acid island and the kinase domain, respectively, were generated by PCR-based site-directed mutagenesis. The mutations G597E and E1056K correspond to *bri1-113* and *bri1-1* alleles (Li and Chory, 1997). These constructs were transformed individually into *bri1-301*. As indicated in Fig. 2G,H, neither mutated construct was able to fully rescue the mutant phenotype, confirming that both the integrity of the island domain and a functional kinase are essential for BRL1 to substitute for BRI1 function in BR-regulated plant growth.

BRL1 and *BRL3* encode membrane localized receptors that bind BL with high affinity

To test whether BL is the ligand for the BRL receptors, full-length *BRL1*, *BRL2*, *BRL3* were expressed as fusion proteins with GFP under the control of the strong CaMV 35S promoter. These constructs were transformed into *Arabidopsis* wild-type Col-0. Transgenic plants expressing *35S-BRL1::GFP*, *35S-BRL2::GFP* and *35S-BRL3::GFP* did not show any apparent phenotypic alteration. Light-grown seedlings of these transgenic plants were examined for BRL subcellular localization using a confocal scanning laser microscope (CSLM), revealing that all three proteins were localized at the plasma membrane (Fig. 3D and data not shown). Accordingly, these membrane fractions were used to test if the BRLs were able to bind specifically to BL, using a previously defined assay for BRI1 (Wang et al., 2001). As shown in Fig. 3E, only BRL1 and BRL3 were able to bind BL, whereas no binding could be detected in *BRL2*-overexpressing lines. BRL1::GFP and BRL3::GFP proteins were immunoprecipitated by anti-GFP

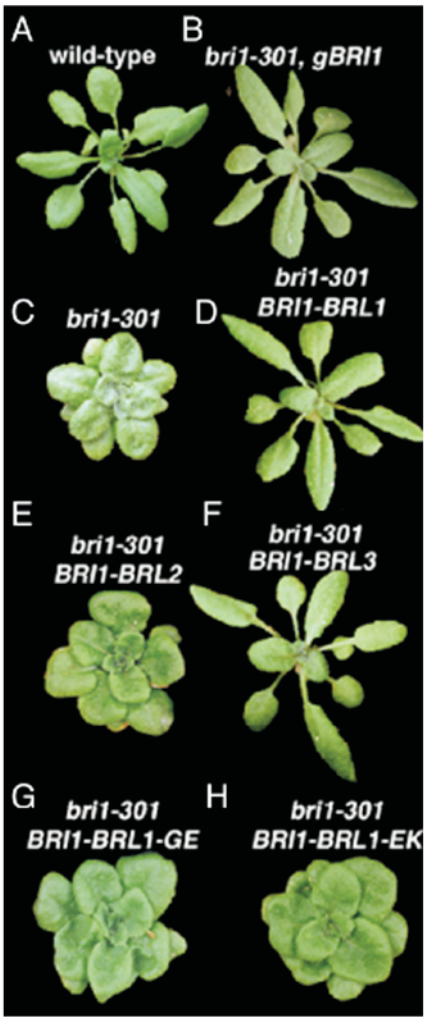

Fig. 2. BRL1 and BRL3, but not BRL2, are able to rescue the phenotype of a weak *bri1* mutant. (A) *Arabidopsis* wild-type rosette phenotype. (B) Complementation of *bri1-301* mutant by *BRI1* genomic DNA. (C) *bri1-301* shows a weak dwarf phenotype. (D) *pBRI-BRL1* rescues the dwarf *bri1-301* phenotype. (E) *pBRI-BRL2* fails to rescue the *bri1-301* phenotype. (F) *pBRI-BRL3* rescues the dwarf *bri1-301* phenotype. (G,H) Point mutations in the island domain (G597E, G) and the kinase domain (E1056K, H) of BRL1 destroy the *bri1*-rescuing activity of *pBRI1-BRL1*.

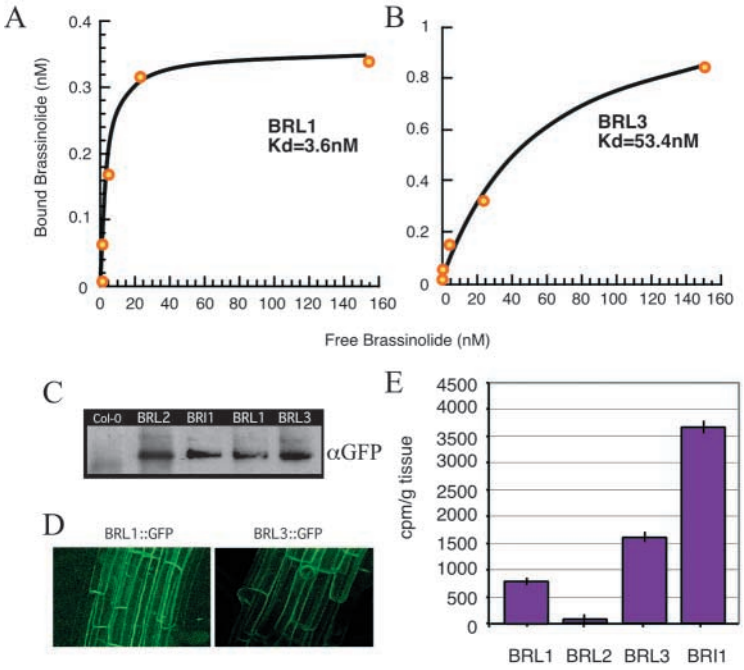

Fig. 3. BRL1 and BRL3 are membrane-localized receptors that bind BL. (A) Specific [^{3}H] –BL binding for immunoprecipitated membrane fractions of BRL1 and (B) BRL3. Shown are the Kd values from one representative experiment of three replicates. BRI1-GFP was used as a positive control in our experiments (Kd=55.5 nM±0.08). (C) Western blot developed with anti-GFP antibodies showing the amount of BRI1::GFP, BRL1::GFP, BRL2::GFP and BRL3::GFP fusion proteins in the immunoprecipitated membrane fractions used for the [^{3}H] –BL binding assays. (D) Subcellular localization of the BRL receptors. Confocal images of hypocotyl epidermal cells of light-grown 7-day-old seedlings carrying *35S::BRL1-GFP* and *35S::BRL3-GFP* fusion constructs show that both proteins are localized to the plasma membrane. Scale bar: 25 μm. (E) The specific [^{3}H] –BL binding activity for each BRL protein and BRI1. BRL2 does not bind BL specifically. In these graphs, BRL1 binding appears lower than BRL3 or BRI1 due to the level of protein expressed in the transgenic lines used for this experiment.

antibodies (Fig. 3C) and the specific binding activity of ^{3}[H]-BL was calculated (Fig. 3A,B). The dissociation constants of the immunoprecipitated binding activity were calculated to be 3.6±0.07 nM for BRL1 and 53.4±0.04 nM for BRL3, as shown in Fig. 3A,B. As a positive control, we obtained a Kd=55±0.08 nM for the *BRI1::GFP* transgenic plants, which are known to have a high BL-binding activity (Wang et al., 2001).

BRL1 and BRL3 receptors are specifically expressed in the vascular tissues in a complementary fashion

Having established that *BRL1* and *BRL3* encode functional BR receptors, we further investigated the organ- and tissue-specific expression patterns of these genes. Transgenic wild-type *Arabidopsis* containing intergenic 5′ regions of *BRI1*, *BRL1* and *BRL3* were used in transcriptional fusions to the histochemical *GUS* reporter gene (Fig. 4). The expression of *pBRI1::GUS* was detected postembryonically, in dividing and elongating cells of roots and hypocotyls of young seedlings, and in the emerging leaves and petioles of mature plants (Fig. 4C,F,I), consistent with the previously described role for BRI1 in BR-promoted cell elongation (Friedrichsen et al., 2000). However, *pBRI1::GUS* expression was not particularly enriched in vascular cells (Fig. 4C). Conversely, both *BRL1* and *BRL3* appeared to be predominantly expressed in the vascular tissues. Seven-day-old transgenic seedlings showed activity of the *BRL1* promoter in the columella cells of the root tip, in the vascular initials in the meristematic region of the roots, and in the vascular tissues (Fig. 4A). This expression pattern appeared during late embryogenesis when *pBRL1::GUS* was expressed in the root tip (Fig. 4J), specifically in the columella cells and in the vascular initial cells. After germination, GUS staining was detected in the stele cells, and in the early differentiation zone of the root (Fig. 4K), where the expression continues from the root to the hypocotyls and cotyledons following the mid-vein (Fig. 4D). In mature plants, *pBRL1*::GUS expression traced the vasculature of the leaf, predominantly in the mid-vein (Fig. 4G), and was found in the vascular bundles of inflorescence stems. Histological examination of inflorescence stems revealed that the *BRL1* expression is associated with the procambial cells of the vascular bundles (Fig. 4M), located between the differentiating xylem and the phloem.

By contrast to *BRL1*, the *pBRL3* activity became apparent only during postembryonic development. It exhibited a very discrete pattern of expression, preferentially in the two protophloem cell files at the elongation zone of the root (Fig. 4B). The expression in these two cell files attenuated as the phloem cells differentiated in the upper root. In cotyledons and leaves, *pBRL3*::GUS was expressed in phloem cells (shown in Fig. 4E,H and detailed in 4N,O). The GUS expression started at the cotyledons and shoot apex, moving toward the basal part of the leaves, where the expression became attenuated (Fig. 4E,H). Complementary to *pBRL1*::GUS, which is expressed in the main vein, the expression domain of *pBRL3*::GUS was more intense in the secondary and tertiary veins and in the upper part of the cotyledons and leaves (Fig. 4N). No GUS staining could be detected in the inflorescence stems (data not shown).

To summarize, the vascular tissue-preferential expression of *BRL1* and *BRL3* was common in all organs. In addition, the spatial complementation of this expression pattern was related to the level of maturation of vascular development, with *BRL1* preferentially expressed in more mature cells than *BRL3*. In agreement, *BRL1* and *BRL3* appeared to be preferentially expressed in the provascular tissues, in a tissue-specific microarray analysis (Birnbaum et al., 2003) available at the AREX database (www.arex.org). This unique expression pattern suggests that BR signaling through BRL1 and BRL3 may play a role in provascular organogenesis in plants.

BRL1 and BRL3 play distinct and overlapping roles with BRI1 in vascular differentiation and growth

To investigate the role of *BRL1* and *BRL3* genes in vascular

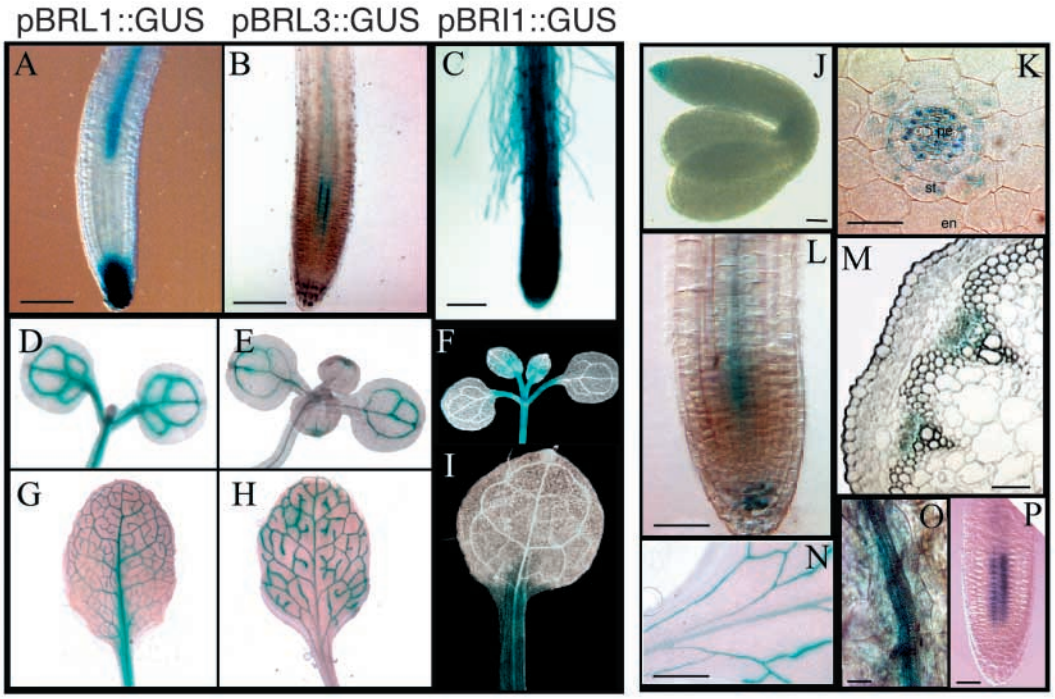

Fig. 4. *BRL1* and *BRL3* are expressed in the vascular tissues. *BRL* expression was assessed by GUS staining in 7-day-old seedlings, in embryos and in expanded leaves. (A) *pBRL1::GUS* is expressed in the columella root cap cells and in mature vascular tissues. (B) *pBRL3::GUS* is expressed in the roots in the two protophloem cell files in the elongation zone. (C) *pBRI1:GUS* is ubiquitously expressed in roots, especially in the elongation zone. (D) *pBRL1::GUS* is expressed in the mid-vein and lower part of the cotyledons. (E) *pBRL3::GUS* is preferentially expressed in the secondary veins at the tip of cotyledons. (F) *pBRI1::GUS* expression in the petioles and the lower part of cotyledons and leaves. (G) *pBRL1::GUS* expression in leaves is predominantly in the mid-vein. (H) *pBRL3::GUS* expression in leaves is predominantly in the secondary and tertiary veins and reduced in the mid-vein. (I) Strong expression of *pBRI1::GUS* in the petioles. (J) GUS staining in the embryos of *pBRL1::GUS* transgenic seeds. (K) Transverse section of the root tip of seedlings shows that *pBRL1::GUS* expression is confined to the vascular cylinder. en, endodermis; pe, pericycle; st, stele. (L) The root tip of a five-day-old *pBRL1::GUS* transgenic seedling showing that, after germination, *BRL1* expression appears associated with the stele. (M) Transverse section at the base of the inflorescence stem in mature plants shows *pBRL1::GUS* expression in the procambial cells in the vascular bundles. (N) Detail of a leaf expressing *pBRL3::GUS* shows higher expression in the secondary and tertiary veins compared to the mid-vein. (O) Close-up showing the leaf vasculature stained with *pBRL3::GUS* reveals specific expression in the phloem sieve elements. (P) The root tip of a 5-day-old *pBRL3::GUS* transgenic seedling showing that, after germination, *BRL3* expression appears associated with the protophloem. (Scale bars: 100 μm in A,B,C; 10 μm in J; 25 μm in K,L,M,N,P.

development, we identified T-DNA tagged lines for each gene. A total of three mutants, two alleles of *brl1*, *brl1-1* in the Ws-2 background and *brl1-2* in the Col-0 background, and a single *brl3* allele in the Col-0 background, were identified. The location of the T-DNA insertions is shown in Fig. 1B, and all alleles are null, based on Northern blotting analysis (data not shown).

All the *brl* single mutants identified exhibited wild-type appearance at the seedling and mature plant stages (Fig. 5 A-C and data not shown). However, due to the specific expression of these promoters in vascular cells, we looked for vascular defects by histological analysis. Vasculature in the *Arabidopsis* inflorescence stem follows a radial pattern in which vascular bundles alternate with interfascicular parenchyma. The vascular pattern in the stem arises from the meristematic activity of the procambial cells, which give rise to the phloem (in the outer side) and the xylem (in the inner side of the bundle; stained in dark blue by Toluidine Blue in Fig. 5I), thus creating a collateral pattern (Esau, 1965; Turner and Somerville, 1997).

brl1-1 displayed a clear vascular development phenotype (Fig. 5E,F). The number of phloem cells in *brl-1* was increased (93±5, *n*=12) compared with wild-type Ws-2 (62±5, *n*=12). These alterations were associated with a concomitant reduction of xylem cells in the *brl1-1* mutant (Fig. 5E,F). These results indicate that BRL1 promotes xylem differentiation and represses phloem differentiation in the inflorescence stem in the Ws-2 background. In addition to the phenotypic defects associated with reduced elongation previously described for the *bri1* mutants, we found that *bri1* mutants were also altered in vascular development, affecting the xylem/phloem patterning in the shoot. *bri1*-5 plants, like *brl1-1* mutants, had increased phloem differentiation and reduced xylem differentiation compared with wild-type vascular bundle (Fig. 5E-G). Consistent with this, a BR-deficient mutant, *det2-1* (Li et al., 1996), also exhibited reduced xylem and increased phloem differentiation, as well as enlarged phloem cap cells (Fig. 5L). Conversely, we found that plants overexpressing BRI1 exhibited a striking increase in the procambial activity that leads to increased xylem differentiation in the vascular bundles (Fig. 5M).

In addition, we found that *brl1-1* enhanced the dwarf

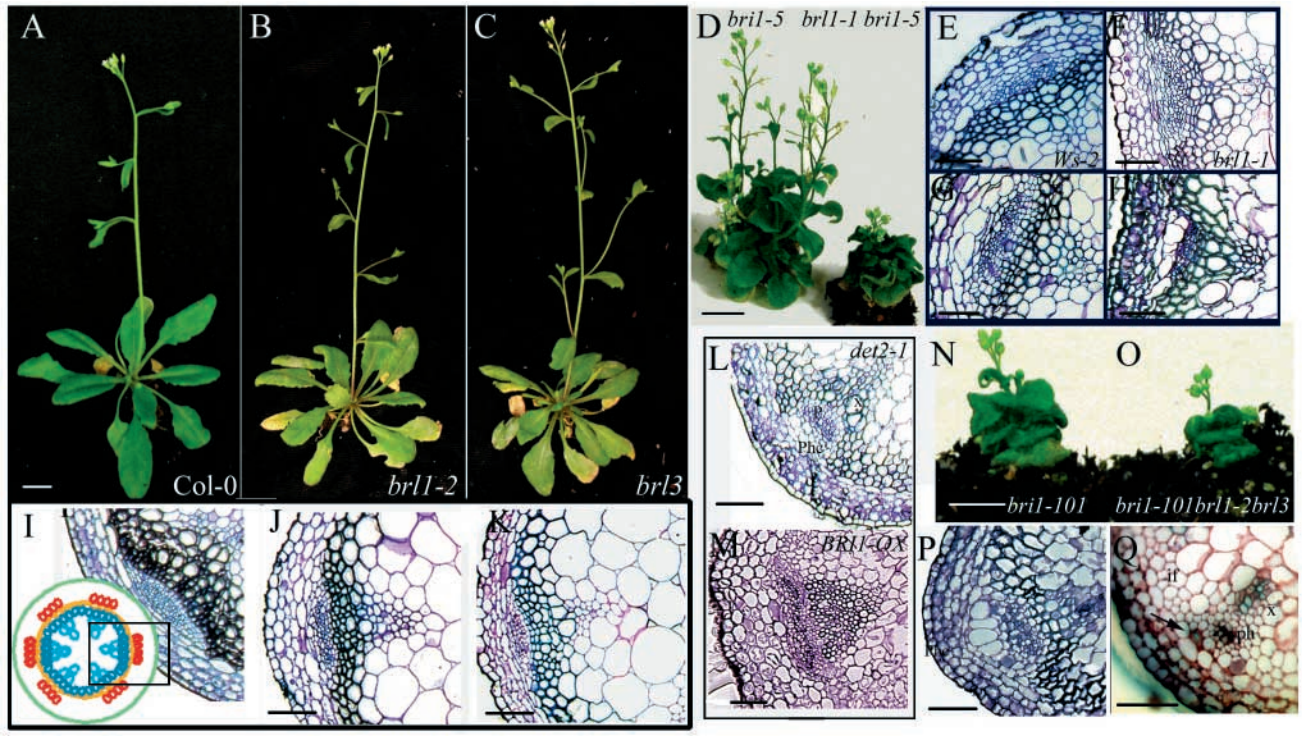

Fig. 5. Phenotypic analysis of *brl* mutants and genetic combinations of double and triple mutants with *bri1*. Mature plants of (A) Col-0 wild type, (B) *brl1-2* (C), and *brl3* mutants were taken for histological analysis of the vascular tissues. Since no apparent phenotype was observed for any of the *brl1* alleles, only *brl1-2* is shown. (D) *bri1-5* mature plants (left) compared with the double mutant *bri1-5 brl1-1* (right), where *bri1-5* dwarfism is enhanced. (E) Cross section of an inflorescence stem showing the organization of a Toluidine Blue-stained vascular bundle of Ws-2 ecotype. (F) *brl1-1* shows an increased amount of phloem compared to the xylem in the vascular bundles. (G) *bri1-5* shows an increased differentiation of the phloem at the expense of the xylem and enlarged phloem cap cells compared with wild-type plants. (H) The aberrant vasculature in the *bri1-5 brl1-1* double mutant consists of small vascular bundles where the small phloem cells are placed between the xylem and less differentiated than the wild type, and has dramatically enlarged phloem cap cells. (I) Schematic representation of the *Arabidopsis* vascular system at the basal part of the inflorescence stem of a mature plant. In wild-type plants, there are six to eight vascular bundles arranged in a radial pattern. The activity of the procambial cells (yellow) gives rise to phloem (red) tissue in the outer part and the xylem (blue) in the inner part of the vascular bundle. Pictures shown in this figure show the anatomy of a single vascular bundle (black box). Cross section of an inflorescence stem showing the organization of a Toluidine Blue-stained vascular bundle of Col-0 ecotype. (J,K) *brl1-2* and *brl3* in Col-0 background show normal vascular bundles. (L) A biosynthetic mutant, *det2-1*, shows similar defects to those of the *bri1* mutants. (M) Plants overexpressing the BRI1 receptor (Wang et al., 2001) show increased xylem differentiation in the vascular bundles. (N,O) *bri1-101* mutant phenotype and triple mutants *bri1-101 brl1-2 brl3*. (P) Vascular bundle in *bri1-101* mutants. (Q) Vascular bundle *bri1-101 brl1-2 brl3* mutants showing reduced vascular differentiation and unusual formation of phloem fibers. Phc, phloem cap cell; ph, phloem; x, xylem; if, interfascicular xylem). Scale bar: 10 mm in A-D,N,O; 20 μm in E-M,P,Q.

phenotype of *bri1-5*, a weak allele of *bri1* (Fig. 5D). *bri1-5brl1-1* double mutants exhibited an overall abnormal vascular organization, affecting the differentiation of both the phloem and xylem (Fig. 5H). The small phloem cells appeared collapsed between the enlarged phloem cap cells and xylem. The vascular defects of the *bri1-5brl1-1* double mutants in the Ws-2 ecotype resembled those found in *bri1-101*, a strong *bri1* allele in the Col-0 ecotype (Fig. 5P), indicating that BRL1 and BRI1 function redundantly in provascular cell differentiation in the Ws-2 background.

It is noteworthy that the *brl1-1* vascular phenotype described above appears to be background-dependent. *brl1-2* and *brl3*, as well as double mutants *brl1-2 brl3*, did not display any significant vascular defects in the root and the shoot in the Col background. Furthermore, the number of phloem cells found in *brl1-2* (83±5, *n*=12) and *brl3* (82±5, *n*=12), as well as double mutants *brl1-2 brl3* (79±5, *n*=10), was similar to the Col wild type (81±5, *n*=12). Similarly, the *bri1-101 brl1-2 and bri1-101 brl3* vasculature resembled that of the single mutant *bri1-101* (Fig. 5I-K and data not shown), altogether suggesting that BRI1, BRL1 and BRL3 function redundantly in vascular development, especially in the Col-0 background. To test this possibility, we generated *bri1-101 brl1-2 brl3* triple mutants in the Col-0 background. The triple mutant was smaller than the single *bri1-101* mutant (Fig. 5N,O). The mutant plants were not fertile and most died after making a single inflorescence stem with a few small flowers (Fig. 5O). We observed enhanced vasculature defects in the triple mutant compared with the *bri1-101* single mutant (Fig. 5P,Q). Xylem development in the basal part of the stem appeared to be delayed in the triple mutant, resulting in decreased xylem differentiation and an absence of differentiated interfascicular fibers (Fig. 5Q). By contrast, the small phloem cells differentiated to give rise to an unusual formation of phloem

fibers (arrowhead), and bundles were not fully differentiated. The presence of phloem fibers suggests abnormal coordination in vascular bundle development, in which the phloem may differentiate at the expense of xylem due to lack of BR perception in these cells. These results indicate that BR signal transduction through the BRI1, BRL1 and BRL3 receptors functions redundantly to regulate provascular cell growth and differentiation.

Discussion

In this study, we showed that BRs are perceived by a small family of receptors in *Arabidopsis*. Previous studies have shown that BRI1 is responsible for the major BR-binding activity in *Arabidopsis* (Wang et al., 2001). Here we demonstrate that two closely related LRR-receptor kinases, BRL1 and BRL3, were also capable of binding BL with high affinity. Moreover, we found that these genes were expressed exclusively in vascular cells, and that a synergistic interaction of BRL1 and BRL3 with BRI1 was required to achieve normal vascular differentiation in the plant shoot.

BRL1 and BRL3 are members of the BRI1 family of plant steroid receptors

Biochemical and genetic evidence demonstrates that BRL1 and BRL3, but not BRL2, are bona fide BR receptors. First, BRL1 and BRL3 can rescue the *bri1* mutant phenotype when expressed under the control of the *BRI1* promoter (Fig. 2) and they conferred long petiole phenotypes when expressed in wild-type plants, both suggesting that functional specification of these two receptors may reside, at least in part, in their *cis*-regulatory elements. Second, immunoprecipitates of BRL1 and BRL3, but not of BRL2, can bind BL with high affinity (Fig. 3A,B), indicating that both BRL1 and BRL3, like BRI1, are involved in BR perception. However, it is plausible that additional factors may specify the action of these receptors. In fact, signaling components downstream of BRI1 belong to multigene families, whose members probably have overlapping, as well as specific, functions (Li et al., 2002; Nam and Li, 2002; Yin et al., 2002a; Wang et al., 2002; Mora-García et al., 2004). Future studies should investigate the signal transduction pathway(s) associated with the BRL receptors in comparison to what is known for BRI1. Moreover, differences in the amino acid sequences of these receptors may be functionally relevant. In the intracellular part, while the core sequences of the kinase domain are highly homologous among all members, the sequences are more divergent in the regions flanking the catalytic domain of the kinase, i.e. in the juxtamembrane region and the C-terminal extension. In the extracellular domain, the functional relevance of the number of LRRs is unclear, since BRI1 orthologs from other species with seemingly equivalent functions show some degree of variability. The intervening 70-amino acid islands probably play a more significant role, given that mutations in the extracellular domain of BRI1 cluster to this region and abolish binding of BL (Wang et al., 2001). The sequences of the island domains share a number of conserved residues, those of BRL1 and BRL3 being highly similar and distinct from those of BRI1 and BRL2. However, BRL1 binds BL with a 10-fold higher affinity than that of either BRI1 or BRL3, which suggests that regions in addition to the island domain may modulate the affinity for the ligand.

The BRL2 protein, previously described as Vascular Highway 1 (VH1), has been proposed to regulate provascular cell specification (Clay and Nelson, 2001). VH1 is expressed in the vascular tissues and loss-of-function of *BRL2*/*VH1* in the L*er* ecotype results in abnormal phloem transport due to a discontinuous phloem network that results in premature leaf senescence. Despite the sequence similarity to the BR receptors, we have shown that BRL2/VH1 does not function as a BR receptor. Sterols have been implicated in the establishment of the vascular pattern, in correct auxin transport and distribution, and more recently in leaf senescence (Carland et al., 2002; Willemsen et al., 2003; Suzuki et al., 2004; Motose et al., 2004). Since sterol-mediated vascular development appears to be required to maintain the continuity of the vascular network, it is therefore conceivable that BRL2 may bind a sterol. Our studies underscore the importance of performing biochemical assays prior to assigning a function to proteins identified through database searches.

Evolutionary implications of the BRI1-receptor family in mono- and dicotyledonous species

The completed sequence of the rice genome offers the possibility of establishing a comparison between the *Arabidopsis* and rice BRI1 families. Fig. 1A depicts the phylogenetic relationship between all described or annotated members of the BRI1 family. Several inferences can be extracted from this tree, inasmuch as it accurately represents the evolution of the BRI1 family in angiosperms. First, all major branches include sequences of both rice and *Arabidopsis*, implying that the diversification inside the family predates the split between monocots and dicots. In addition, the whole family seems to have undergone an early functional division between receptors that bind BL (BRI1 and BRL1/3) and those that do not (BRL2). Finally, a relatively recent duplication event in the *Arabidopsis* lineage accounts for the presence of the highly homologous *BRL1* and *BRL3* genes, encoded in duplicated chromosomal regions. Their complementary expression pattern probably reflects a process of subfunctionalization from an ancestral function throughout the vascular tract (Lynch and Force, 2000). Analysis of the expression pattern and function of the unique rice *BRL1* homolog will help to address this question.

The analysis of recessive, dwarf mutants insensitive to BL has led to the identification of *BRI1* orthologs in several species other than *Arabidopsis*, such as *OsBRI1* in rice (Yamamuro et al., 2000), *UZU* in barley (Chono et al., 2003), *CU3* in tomato (Montoya et al., 2002) and *LKA* in pea (Nomura et al., 2003). It is noteworthy that all of them cluster in a highly supported group (Fig. 1A), suggesting that these proteins are the major BL receptors among angiosperms. Consistently, loss-of-function of *Arabidopsis BRL1* and *BRL3*, described here, and *BRL2* (Clay and Nelson, 2001) caused relatively subtle effects. It is remarkable that the expression of members of the *BRI1* family, except *BRI1* itself, is restricted to vascular tissues. It remains to be established whether this vascular specificity is an ancestral feature of these receptors, and by extension, of BL signaling, or rather a derived trait related to the functional specialization among paralogous genes.

It should be pointed out that other members of the *Arabidopsis* RLKs have been reported to behave similarly. In the case of the *ERECTA* family, both *ERL1* and *ERL2* act

synergistically with *ERECTA* in defining aerial organ size (Shpak et al., 2003; Shpak et al., 2004). Like *BRL1* and *BRL3*, *ERL1* and *ERL2* are also located in duplicated chromosomal regions (Shpak et al., 2004), suggesting that distinct RLK families may have evolved similarly in plants.

A role for BRs in vascular development in the shoot

The molecular mechanism by which procambial cells differentiate into phloem or xylem remains largely unknown. The inner location of the vascular tissues has hampered the identification of vascular development mutants and often led to the identification of cell wall synthesis genes (Turner and Somerville, 1997; Caño-Delgado et al., 2000; Caño-Delgado et al., 2003; Taylor et al., 1999). To date, only a few mutants have been identified in *Arabidopsis* that specifically affect vascular patterning, (Clay and Nelson, 2001; Koizumi et al., 2000; Clay and Nelson, 2002; Bonke et al., 2003; Parker et al., 2003; Motose et al., 2004) (reviewed by Fukuda, 2004).

The vascular-specific expression of *BRL1* and *BRL3* genes (Fig. 4) implies that these BR receptors play important roles during vascular development. Furthermore, studies using *Zinnia* mesophyll cell cultures have shown that exogenous application of BRs induces tracheary element differentiation in the terminal stage of xylogenesis (Yamamoto et al., 1997). Our studies in intact *Arabidopsis* establish that BR signaling in the provascular cells through BRL1, BRL3 and BRI1 receptors contributes to the collateral organization of the vascular bundles in the plant shoot (Fig. 5). The analysis of *bri1* and *brl1* mutant combinations revealed an increase in phloem and a decrease in xylem differentiated cells (Fig. 5F-H,Q), indicating that BR signaling in the procambial cells induces xylem cell differentiation and, at the same time, represses phloem differentiation. It is noteworthy that the *bri1* null mutant (Fig. 5P) and the *bri1-101 brl1 brl3* triple mutant (Fig. 5Q), exhibited phloem cells that are either collapsed with dramatically enlarged phloem cap cells or prematurely differentiated to phloem fibers, suggesting that different BR-signaling strength has different effects on phloem cell differentiation in the stem. Altogether, our data support the hypothesis that BRs contribute to the establishment of the collateral xylem/phloem pattern rather than just inducing xylem differentiation per se. In agreement, APL, a MYB transcription factor that determines phloem cell identity in the root, functions in promoting phloem differentiation and repressing xylem differentiation (Bonke et al., 2003), also suggesting that the differentiation of these two cell types is tightly linked.

We want to point out that the phenotypes of *brl* mutants appear to be affected by natural variation between different *Arabidopsis* accessions. In the Ws-2 background, *brl1-1* null mutants have an increased number of phloem cells compared with the wild type, while no significant differences were observed between the *brl1-2* allele and the Col-0 wild type. Since no *brl3* alleles were available in the Ws-2 ecotype, we attribute the differences in phenotype to the observed ecotype variation, rather than to an allele-specific effect. Furthermore, natural variation in genes that affect vascular development has been described previously for *REV/IFL1*, a member of the homeodomain-leucine zipper transcription factor family that controls interfascicular fiber differentiation (Talbert et al., 1995; Zhong and Ye, 1999; Emery et al., 2003). Interestingly, *Zinnia* members of this family of transcription factors were found to be positively regulated by BRs (Demura et al., 2002; Ohashi-Ito and Fukuda, 2003). In addition, while the *vh1* mutants in the L*er* accession produced clear leaf senescence defects (Clay and Nelson, 2001), we did not observe these phenotypes in our mutant allele *brl2* in the Col-0 ecotype under the same experimental conditions (data not shown). Several differences in allele variation between Col-0 and Ws-2 related to vascular development in the inflorescence stem of *Arabidopsis* have also been reported (Altamura et al., 2001). For example, phloem cap cells appeared to be larger in Ws-2 than in Col-0. We found increased size in the phloem cap cells in *bri1*, *brl1-1*, *bri1-5brl1-1* and *bri1-101brl1-2brl3* mutants, suggesting that natural variation in these genes may account for the differences in vascular structure between the Ws-2 and Col-0 accessions.

A model for BR function in vascular patterning

Several plant hormones have been implicated in vascular pattern formation. Auxin, the only hormone that has been shown to exhibit polar transport, acts as a major signal in vascular differentiation (Aloni, 1987; Sachs, 1991). In the ‘canalization hypothesis’, the diffusion of the hormone from an auxin source induces the formation of a polar auxin transport system along a procambial cell file that differentiates into a xylem strand. However, additional molecules have been proposed to be required for vascular differentiation. Our data suggest that BR may be one of these additional molecules required for the induction of vascular cell differentiation in plants. The expression pattern of *BRL1* in the provascular cells correlates with an inductive role of BRs in xylem differentiation; by contrast, the discrete expression of *BRL3* in the phloem cells suggests that BRL3 may repress phloem differentiation, thereby controlling the formation of the collateral pattern in the vascular bundles. This hypothesis would be possible if BRs are transported from one cell to its neighbors. However, while polar auxin transport has been widely demonstrated, the long distance transport of BRs awaits to be demonstrated. Knowledge of where BRs are synthesized and whether they are perceived as part of a paracrine or autocrine system in the plant is crucial to further understand the role of BRs in vascular ontogeny.

We propose a model for the function of BRs in vascular cell differentiation (Fig. 6). BRs function through vascular-specific receptors BRL1 and BRL3 and the ubiquitously expressed receptor BRI1 to promote xylem differentiation and to repress phloem cell differentiation (Fig. 6A). In support of this model, phenotypes of BR-biosynthesis and -perception mutants exhibit a reduced number of xylem cells at the expense of phloem cells (Fig. 6B), indicating that the differentiation of these two cell types is reciprocally linked. Thus, BR perception by the procambial cells may act as an inductive signal through BRI1/BRL receptors in the acquisition of vascular cell fate and thus in the mutual regulation of xylem/phloem differentiation in the vascular bundle. However, it is also possible that BRs promote the division of the procambial cells in the shoot apex that will later differentiate into xylem cells. This hypothesis is supported by the phenotypes observed in the stems of BRI1 overexpressing plants (Fig. 5M), where procambial activity appeared to be enhanced compared with the wild type (Fig. 5I). By contrast, we observed that the *bri1* null mutant (Fig. 5H)

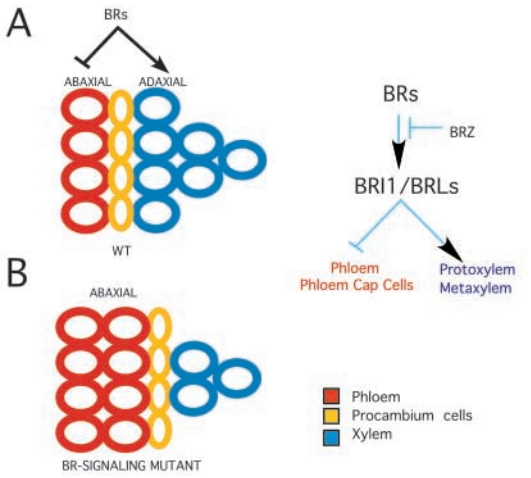

Fig. 6. A model for BR action in vascular development. Schematic representation of the vascular bundles in which BRs act as a vascular patterning signal that promotes xylem while repressing phloem differentiation. (B) In the case of defective BR-signaling (as shown in *bri1* and *brl1-1* mutants), increased phloem differentiation occurs at the expense of xylem. This model supports the idea that the specification of phloem and xylem in the vascular bundles is tightly linked. Whether BRs are perceived by receptors of the BRI1-family on the plasma membrane of cells that secrete BRs (autocrine) or cells that do not secrete BRs (paracrine) remains unknown.

and *bri1 br1 brl3* triple mutants (Fig. 5Q) had small bundles, probably due to a reduced division of the procambial cells.

Understanding the molecular mechanism by which plant steroid signaling induces vascular cell specification and how this signal is integrated with other signaling molecules such as sterols and auxin in the meristem is an outstanding question for the following years. Future molecular and computational analyses on BR action will be crucial to elucidate the regulatory mechanism of vascular cell specification leading to xylem/phloem differentiation.

We thank Jeff Long, Niko Geldner and Jennifer Nemhauser for critical reading of the manuscript. We thank Juan Carlos Izpisua-Belmonte for use of his confocal microscope, Gabriel Sternik for confocal assistance, and the T-DNA KO facilities at the Salk Institute and University of Wisconsin for making the mutants available. This work was funded by grants from the USDA and HFSP to J.C., a grant from NIH (GM60519) to J.L., and by HFSP fellowships to A.C.D. and S.M.G. J.C. is an Investigator of the Howard Hughes Medical Institute.

Note added in proof

Recently, Zhou et al. (Zhou et al., 2004) reported that the overexpression of BRL1 partially complements the phenotype of a weak *bri1* mutant, thus confirming our results that BRL1 acts as a functional BR receptor.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/21/5341/DC1

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