Identification of Arabidopsis BAK1-Associating Receptor-Like Kinase 1 (BARK1) and Characterization of its Gene Expression and Brassinosteroid-Regulated Root Phenotypes

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Brassinosteroids (BRs) activate the BRI1 and BAK1/SERK3 membrane receptor complex, which leads to a wide range of changes in gene expression, plant growth and development. As an initial step to elucidate additional roles of BAK1, we cloned a BAK1-binding protein, BAK1-Associating Receptor-Like Kinase 1 (BARK1), and characterized its gene expression and root phenotypes. BARK1 is a putative membrane LRR-RLK (leucine-rich repeat receptor-like kinase) protein that specifically binds to BAK1 and its homologs. Careful examination of BARK1 expression using transgenic plants expressing a green fluorescent protein (GFP) reporter under the control of the native BARK1 promoter (BARK1p::GFP) revealed that this gene is ubiquitously expressed in most plant tissues, and shows especially strong expression in the xylem vasculature of primary and lateral roots as well as in mature pollen. Interestingly, the expression of the BARK1 gene was increased in the BR biosynthetic loss-of-function mutant, det2, and a loss-of-function mutant of BR signaling, bak1-3. In contrast, this gene was downregulated in the bizar1-1D plant, which is a BR signal gain-of-function mutant. BARK1-overexpressing transgenic plants clearly enhanced primary root growth in a dose-dependent manner, and their roots were hypersensitive to BR-induced root growth inhibition. In addition, both the number and density of lateral roots were dramatically increased in the BARK1 transgenic plants in a dose-dependent manner. Together with observations that ARF (AUXIN RESPONSE FACTOR) genes are upregulated in the BARK1 overexpressor, we suggest that the BARK1 overexpressor phenotype with more lateral roots is partly due to the increased expression of ARF genes in this genetic background. In conclusion, BAK1-interacting BARK1 protein may be involved in BR-mediated plant growth and development such as in lateral roots via auxin regulation.

Keywords: ARF • Auxin • BAK1 • Brassinosteroid • Lateral roots • Membrane receptor kinase.

Introduction

Brassinosteroids (BRs) are a class of polyhydroxysteroids that are widely known to play pivotal roles in plant growth and development, such as cell elongation and expansion, acceleration of senescence, resistance to biotic and abiotic stresses, vascular differentiation and pollen elongation (Clouse and Sasse 1998).

In the absence of BRs, BRI1 KINASE INHIBITOR 1 (BKI1) is associated with BRASSINOSTEROID INSENSITIVE 1 (BRI1), a membrane-localized leucine-rich repeat receptor-like kinase protein (LRR-RLK), such that binding of BRI1 to the BRI1-ASSOCIATED RECEPTOR KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (BAK1/SERK3) is inhibited (Wang and Chory 2006). In this physiological condition, GSK3/SHAGGY-like BRASSINOSTEROID-INSENSITIVE2 (BIN2),...
a well-characterized negative regulator of the BR signaling pathway, phosphorylates the positively regulating transcription factors, BRASSINAZOLE RESISTANT1 (BZR1) and BRASSINAZOLE RESISTANT2 (BZR2), and thus regulates the subsequent changes in plant growth and development (He et al. 2007, Roux et al. 2011). Upon binding of BRs to BRI1, BKI is dissociated from the intracellular kinase domain of BRI1 (Wang and Chory 2006), and BRI1 forms a heterodimeric complex with BAK1 to induce auto- and trans-phosphorylation on serine/threonine or tyrosine residues of BRI1 and BAK1 (Nam and Lee 2002, Wang et al. 2008, Oh et al. 2010, Jallias et al. 2011). The fully activated BRI1 induces activation of BRI1 SUPPRESSOR1 (BSU1) phosphatase, and BSU1 promotes the subsequent inactivation of the BIN2 kinase by dephosphorylating a conserved phospho-tyrosine residue (pTyr200) of BIN2 (Kim et al. 2009, Tang et al. 2010). The resulting dephosphorylated BZR1/BES2 and BZR2/BES1 then regulate a wide range of gene expression changes and the subsequent changes in plant growth and development (Sun et al. 2010, Yu et al. 2011). Recently, it was demonstrated that PP2A phosphatase activity and its interaction with BZR1 was also essential for BR-induced BZR1 dephosphorylation in vivo and normal plant growth (Tang et al. 2011). BAK1 was initially identified as a BRI1-associating protein that regulated BR-induced and BRI1-mediated growth responses. Recently, both BR-dependent growth and BR-independent negative roles for BAK1 and its homolog BAK1-LIKE 1 (BKL1/SERK4) in plant defense and programmed cell death control have been demonstrated (He et al. 2007, Kemmerling et al. 2007, Roux et al. 2011). Interestingly, a mutant BAK1 (bak1-5) plant with hypoactive kinase activity specifically blocked innate immune responses but was not impaired in BR signaling or cell death, which implies that BAK1-mediated and phosphorylation-dependent signaling pathways differentially regulate plant growth, cell death and innate immunity (Schwessinger et al. 2011). Plants sense potential microbial infection, thus directing FLS2 for degradation by the bacterial ubiquitin ligase AvrPtoB (Gohre et al. 2008, Shan et al. 2008). A BAK1-binding receptor-like cytoplasmic kinase, BOTRYTIS-INDUCED KINASE 1 (BIK1), is rapidly phosphorylated upon flagellin perception by BAK1, thus linking flagellin-induced FLS2-BAK1 complex activation to the intracellular signaling cascade leading to plant innate immunity (Lu et al. 2010). Other than these BAK1-binding proteins mentioned above, BAK1-interacting receptor-like kinase 1 (BIR1) and its suppressor protein, Suppressor of bir1-1-1 (SOBIR1), have been shown to be involved in multiple plant resistance signaling pathways including cell death and defense responses (Gao et al. 2009). BONZA11 (BON1) interacts with BIR1 and BAK1 to modulate temperature-dependent plant growth and cell death (Wang et al. 2011). Based on these and other reports, BAK1 has been proposed to be an adaptor molecule required for proper functionality of numerous RLKs (reviewed in Gendron and Wang 2007, Chinchilla et al. 2009, Postel et al. 2010). Roots of dicot plants typically consist of a primary root and its branched lateral roots (LRs) whose behavior and cell type arrangement are very similar to those of the parental roots. The apical regions of plant roots are composed of the mitotically inactive quiescent center (QC) cells and the surrounding meristematic stem cell initials (epidermal–lateral root cap initials, cortical–endodermal initials and stele initials). Each initial cell gives rise to the epidermis, cortex and endodermis, and pericycle and vascular tissues to make up a radial arrangement of root tissues, and many plant hormones are actively involved in the regulation of stem cell maintenance and differentiation (Bennett and Scheres 2010, Lee et al. 2013). The LRs are formed from pericycle cells adjacent to the protoxylem poles of the parent root (Kurup et al. 2005). Multiple auxin-regulated AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) and AUXIN RESPONSE FACTOR (ARF) modules control LR initiation and extension. For instance, both SOLITARY ROOT (SLR)/IAA14–ARF7–ARF19-dependent LR initiation and the BODENLOS (BDL)/IAA12–MONOPTROS (MP)/ARF5-dependent modules are required for proper LR organization (Smet et al. 2010). The SHORT HYPOCOTYL2–SUPPRESSOR OF HY2 (SHY2)/IAA3–ARF signalling module not only regulates the development and emergence of LR primordium after SLR/IAA14–ARF7–ARF19 module-dependent LR initiation but also cooperatively inhibits LR initiation by affecting auxin homeostasis (Goh et al. 2013). In addition, auxin regulated microRNA390 targets ARF3 and its homolog ARF4 in the autoregulatory control of LR growth (Marin et al. 2010, Yoon et al. 2010). The genome of Arabidopsis thaliana encodes >600 RLK genes, and some of those genes may be involved in the regulation of plant growth and development, probably by managing a wide range of signals originating from hormonal and environmental stimuli (Johnson and Ingram 2005, Chae et al. 2009, Smet et al. 2009). Here, we cloned a BAK1-binding protein, BAK1-Associating Receptor-Like Kinase 1 (BARK1), and characterized its gene expression and transgenic root phenotypes.
Results

**BARK1 is a putative LRR-RLK protein specifically binding to BAK1 and its homologs**

To elucidate additional roles of BAK1 in plant growth and development, we screened an Arabidopsis cDNA library to identify potential BAK1-binding proteins, using the BAK1 kinase domain (BAK1KD) as bait in a yeast two-hybrid assay. Among the positive clones pulled out, there were nine overlapping cDNA clones encoding an LRR-RLK protein kinase (AT3g23750), and we named it BAK1-Associating Receptor-Like Kinase 1 (BARK1). After yeast two-hybrid analyses of the binding of BARK1 to other known BR signaling components such as BRI1, BSK1/2, BZR1, BIN2 and BSL1, we found that BARK1 interacts specifically with BAK1/SERK3 and its known homologs, BKK1/SERK4 (He et al. 2007) and SERK5 (Albrecht et al. 2008) (Fig. 1A). The specific interaction of BARK1 with BAK1 was also confirmed in tobacco leaves by showing that transient co-expression of the N-terminal green fluorescent protein (GFP)-tagged BARK1 (BARK1–GFPN) and the C-terminal GFP-tagged BAK1 (BAK1–GFPC) resulted in a GFP fluorescence signal in the epidermal cell membrane (Fig. 1B). In contrast, negative control pairs such as BARK1–GFPN/GFPc and BAK1–GFPN/BRI1–GFPc gave no GFP signal, whereas the positive control pair, BRI1–GFPN/BAK1–GFPc, showed a similar GFP signal.

BARK1 genomic DNA is composed of two exons and one intron (www.Arabidopsis.org), and it encodes a protein composed of 928 amino acids with a calculated molecular mass of 100 kDa and a pl of 5.46 (web.expasy.org/compute_pi/). The deduced protein sequence of BARK1 shows a typical structure of an LRR-RLK protein kinase; it has a long stretch of extracellular domain connected to a transmembrane domain and a cytoplasmic kinase domain in order (Fig. 1C). The extracellular domain contains a signal peptide at the N-terminus (amino acids 1–24), seven LRRs (indicated in Fig. 1C), four plant-specific LRR profiles (amino acids 69–139, 142–217, 238–308 and 369–441) and a glycine-rich region (amino acids 439–478). An active protein kinase ATP-binding region signature (amino acids 584–606) and a serine/threonine protein kinase active site signature (amino acids 703–715) at the cytoplasmic domain predict that BARK1 is an active ser/threonine kinase.

Database searches revealed that three LRR-RLK proteins encoded by AT1g66159 (BLK1 representing BARK1-Like Kinase 1), AT2g01820 (BLK2) and AT1g24650 (BLK3) are most similar to the BARK1 in Arabidopsis, and they show 48.6, 47.7 and 44.4% identity to the full-length sequence of BARK1, respectively. The cytoplasmic kinase domain seems to be more conserved than the extracellular domain so that their identity at the C-terminal kinase domain exceeds 80% (Supplementary Fig. S1). It has been reported that BARK1 and the three BLK proteins belong to a small group of LRR protein kinases, RLK/Pelle-LRR IX (Shiu and Bleecker 2003), and they form a monophyletic gene family with BRI1 as an RLK (Shiu and Bleecker 2001). We compared the domain/motif structure of BAK1 and its known binding proteins and found that not only BAK1 but also most BAK1-binding proteins share domains and motifs similar to a typical RLK, except that BIK1 apparently lacks the transmembrane domain and BON1 does not carry both the transmembrane and kinase domains (Fig. 1D). Nonetheless, BIK1 and BON1 are membrane-associated proteins (Veronese et al. 2006, Wang et al. 2011).

We searched the BARK1 orthologs and paralogs using POGs (http://plantrbp.uoregon.edu/) and inferred cladistic relationships among 41 protein sequences obtained from 20 species (Fig. 2). We found that a group of dicots, monocots, Lycopsidophyta and Bryophyta formed a monophyletic group with 100% statistical support. The green alga, *Chlamydomonas reinhardtii*, has an outgroup relationship with this monophyletic group. This monophyletic group was divided into two clades: the first clade covering dicots and monocots and the second clade covering Lycopsidophyta and Bryophyta. We confirmed that the two clades have a sister group relationship with each other. Within the first clade, there are two subclades which are strongly supported by statistics. The first subgroup is a group of dicots that have >55% sequence similarity to BARK1. The second subgroup was a mixed group of dicots and monocots that have <50% sequence similarity. Based on this cladistic analysis, we concluded that the BARK1 gene family evolved before the separation of dicots and monocots.

**The BARK1 gene is ubiquitously expressed in most plant tissues, and shows specific expression in the pericycle and the xylem vasculature of primary and lateral roots as well as in mature pollen**

As an initial step to understanding BARK1-mediated plant development, we examined the general expression pattern of the BARK1 gene in diverse plant tissues by performing semi-quantitative reverse transcription–PCR (RT–PCR) analysis. BARK1 was ubiquitously expressed in all tissues examined, with relatively high expression in the stem and roots (Fig. 3A). To clarify the detailed tissue-specific expression of the BARK1 gene, we have generated transgenic plants expressing a GFP reporter under the control of the native BARK1 promoter (BARK1p:GFP) and examined its expression mainly in roots and anthers.

BARK1 was moderately expressed in floral buds and flowers (Fig. 3A). The BES1-mediated BR signaling pathway controls male sterility by regulating the expression of key genes involved in anther and pollen development. Thus, BR-related deficient or insensitive mutants are defective in the production of microspore mother cells, microspores and pollen, as well as in pollen release after anther dehiscence (Ye et al. 2010). We found that the BARK1 gene was also highly expressed in mature anther, especially in pollen grains, but weakly expressed in filament and was not expressed in...
Fig. 1 Interaction test of BARK1 with known brassinosteroid signaling proteins in yeast and plants, and schematic diagram of the domain structure of BARK1 and other BAK1-interacting proteins. (A) In vivo interaction test in yeasts. The kinase domain of BARK1 protein fused to the GAL4 DNA-binding domain (BARK1KD-pGBKT7) was used as a bait to test its interaction with other known proteins fused to the GAL4 activation domain (–pGADT7). The co-transformed yeast with these constructs were spotted on either a yeast drop-out medium lacking leucine and tryptophan or a medium lacking leucine, tryptophan and histidine in the presence of 1 mM 3-aminotriazole to test interactions. (B) In planta interaction test of proteins in tobacco leaves. Tobacco leaves were infiltrated with a mixture of Agrobacterium suspensions harboring constructs encoding the indicated fusion proteins. GFP epifluorescence and its merged bright field images of epidermal cells are shown in this image. (C) Amino acid sequence analysis of the BARK1 protein. The domain prediction was performed using programs provided by smart.embl-heidelberg.de/ and plantsp.genomics.purdue.edu/. (D) Schematic comparison of the domain structure of BARK1 and its homologs (BLK1, BLK2 and BLK3) and other BAK1-interacting proteins (BRI1, FLS2, BIR1, BIK1 and BON1) retrieved in Arabidopsis. LRR, leucine-rich repeat; TM, transmembrane domain; Pkinase, protein kinase domain; DCD, Development and Cell Death; C2, protein kinase C conserved region 2; VWA, von Willebrand factor type A domain.
Fig. 2  Phylogenetic analysis of the BARK1 protein and its orthologs and paralogs. A Neighbor–Joining tree constructed based on P-distance with pairwise deletion of gaps and missing data was generated from 41 BARK1 ortholog and paralog sequences obtained from 20 species of algae and plants using *Chlamydomonas reinhardtii* as an outgroup. The number on each branch is the Pb value from the bootstrap test (1,000 replications).
other maternal tissues such as tapetum and the outer wall layers (Fig. 3B). This expression pattern is partly consistent with our bioinformatic analysis performed using the PlantPAN database (http://plantpan.mbc.nctu.edu.tw; Chang et al. 2008), which predicts that transcription factors involved in floral determination and development bind to the promoter region of BARK1 (data not shown). These observations imply that BARK1 may be specifically involved in microspore and pollen development.

Roots are a major source of BRs and, thus, these steroid hormones may play important roles in root growth and development (Shimada et al. 2003). We found that BARK1 was expressed in the periclinal dividing pericycle cells of LRs (Fig. 3C, * in the top left panel), which implies that BARK1 may contribute to LR initiation at an early stage. BARK1 was then redundantly expressed longitudinally from the meristematic zone to differentiating cells of the emerging and elongating LRs (Fig. 3C, top left panel). In terms of radial pattern, it was ubiquitously expressed across the tissue layers from the epidermis to the vascular cells. Interestingly, BARK1 expression disappeared in the meristematic zone of older roots and was restricted to the stele tissues of the differentiation zone including the protoxylem, xylem and pericycle cells, but was excluded from the phloem cells (Fig. 3C, top right panel). This is consistent with previous reports demonstrating that BRI1-LIKE 1 (BRL1) and BRL3 are specifically expressed in vascular tissues and drive vascular differentiation and growth in Arabidopsis (Caño-Delgado et al. 2004). The similar restricted expression pattern of BARK1 in the stele tissues was also found in the primary roots as early as 2 d after germination (Fig. 3B, bottom left panel). We are not certain at this point whether the BARK1 gene had been expressed in all root tissues before 2 d post-germination, as seen in the case of the early LRs.
The expression of the BARK1 gene is coordinately regulated by the strength of BR signaling pathways, and its regulation by BR and auxin varies, in contrast to the steady down-regulation of the gene by other plant hormones.

It has been reported that BR biosynthesis and its signaling pathways are jointly controlled by a BZR1-mediated negative feedback regulation on BR biosynthetic genes such as CPD and DWF4 to maintain BR homeostasis and growth responses (He et al. 2005). We then addressed the question of whether the component(s) of the BR signal transduction pathway influences the expression of BARK1 to evaluate BR-responsive regulation of gene function. Interestingly, the expression of the BARK1 gene was increased in the BR biosynthetic loss-of-function mutant, det2, and a loss-of-function mutant of BR signaling, bak1-3. In contrast, this gene was down-regulated in the bzr1-1D plant, which is a BR signal gain-of-function mutant (Fig. 4A). It is probable that, as noted by He et al. (2005), the expression of the BARK1 gene is coordinately regulated by the strength of BR signaling pathways. That is, low or moderate BR signaling induces up-regulation of the BARK1 gene and very strong signaling down-regulates the BARK1 gene by a negative feedback control. Indeed, a treatment with exogenous BR [1 μM epi-brassinolide (BL)] initially increased BARK1 gene expression but down-regulated it after a prolonged treatment with BL (Fig. 4B, C). Notably, BR-induced BARK1 and BAK1 gene expression showed a typical oscillating pattern in which gene expression fluctuated in a timely manner (Fig. 4B). The first peak was observed at 15 min (in the case of BAK1) or 30 min (in the case of BARK1) after epi-BL treatment, followed by a decrease in expression. However, there was a second peak of expression at 120 min after epi-BL treatment. In contrast, the expression of BIN2 started to increase 15 min after epi-BL treatment and remained at a relatively high level until 120 min after treatment. BZR1 expression reached a high peak at 30–60 min after the BR treatment and then rapidly decreased.

Other plant hormones also showed hormone-specific regulation of BARK1 gene expression. As described, epi-BL increased BARK1 gene expression >4-fold in a time-dependent oscillation.
manner. 2,4-D (an auxin) showed a similar induction effect on BARK1 gene expression, but its stimulation was <2-fold (Fig. 4C). In contrast, treatment with exogenous ABA (10 μM), gibberellic acid (GA₃, 10 μM) or cytokinin (6-benzylaminopurine, 10 μM) steadily decreased the expression of BARK1 immediately after the initial slight increase (Fig. 4C). These results imply that BARK1 gene expression and probably its functions are differentially regulated by hormones, and BRs and auxins work together in a positively coordinate manner, while ABA, gibberellin and cytokinins are antagonizing agents.

**Overexpression of BARK1 in Arabidopsis renders the plant hypersensitive to BR-induced root growth inhibition, and enhances LR development**

To evaluate the possible involvement of BARK1 in BR-responsive plant growth and development, we generated transgenic plants ectopically expressing BARK1 fused to the N-terminal region of GFP (35S::BARK1-GFPN, BARK1ox lines) and investigated their root phenotypes. Based on Western blot analysis performed using anti-GFP antibodies, we determined that BARK1ox-11, -14 and -15 lines overexpressed BARK1-fused transgenic proteins, ranging from moderate to high levels compared with the Columbia (Col-0) control (Fig. 5A).

There were no strikingly different aerial phenotypes of light-grown BARK1 overexpressor plants compared with the Col-0 plants, except that BARK1 transgenic plants were slightly larger. For dark-grown plants, their hypocotyls were 5–7% longer (Fig. 5B, C). Brassinazole (BRZ), a BR biosynthesis inhibitor, inhibited growth of hypocotyls in Arabidopsis, and this retarded growth was recovered by treatment with exogenous BRs (Asami et al. 2000). Transgenic plants overexpressing BRI1 and BAK1 are more sensitive to BR compared with the wild type and both show resistance against BRZ treatment (Wang et al. 2001, Nam and Li 2002). We found that transgenic plants overexpressing BARK1 showed no significant difference in the BRZ-induced inhibition of hypocotyl growth compared with the Col-0 wild type (Fig. 5D). This result implies that the longer hypocotyl phenotype may not be due to the overexpression of the BARK1 protein and its involvement in the BR signaling pathway downstream of BR perception.

In contrast to the hypocotyl phenotype, different levels of BARK1 overexpression clearly enhanced primary root growth in a dose-dependent manner. A >35% increase in root growth was observed in the BARK1ox-15 line, while about 3–5% statistically insignificant root growth was observed in the BARK1ox-11 line, which expresses the transgenic BARK1 protein at a relatively low level (Fig. 5C). BRs regulate root growth in a concentration-dependent manner; they promote root growth at low concentrations and inhibit it at high concentrations (Müssig et al. 2003, Kim et al. 2007). Our root growth inhibition assay showed that growing Columbia plants in a medium containing >10⁻¹⁵ M epi-BL increased the length of primary roots by 5–10%. As expected, there was a gradual inhibition of root growth for plants grown in a medium containing >10⁻¹³ M epi-BL in a concentration-dependent manner (Fig. 5E). Notably for BARK1 transgenic plants, this root growth inhibition can be seen already in plants grown in medium with >10⁻¹⁵ M epi-BL. Our results indicate that overexpression of BARK1 renders the roots of plants more sensitive to BR inhibition.

In this report, we revealed that the promoter activity of the BARK1p::GFP transgenic plants was high during LR formation, especially in xylem and pericycle cells. Consistent with this expression pattern, both the number and density of LRs were dramatically increased in BARK1 transgenic plants in a dose-dependent manner. For example, the BARK1ox-15 line had >6-fold more LRs compared with the Col-0 plants (Fig. 5C), and their density along the primary root was also more than four times higher (Fig. 5F). These results indicate that BARK1 may be involved in the growth and development of LRs.

The establishment of the auxin response maximum by GNOM/FEWER ROOTS, a regulator of the PIN (PIN-FORMED) auxin efflux carriers, is a major trigger of LR initiation and development (Okumura et al. 2013). It is well known that downstream components of the auxin signaling pathway, such as AUX/IAA and ARFs, are actively involved in the control of this root development. Therefore, we examined whether the BR- and BARK1-regulated LR phenotype is accompanied by a transcriptional modulation of any AUX/IAA and ARF genes. As expected from Fig. 4B and C, BARK1 was highly up-regulated by the BR treatment (Fig. 5G). At the same time, the expression of BDL/IAA12 was greatly decreased while that of MP1/ARF5, a module partner in the regulation of LR initiation, was dramatically increased by >20-fold by treatment with 5 × 10⁻¹⁵ M exogenous BL. A similar but far less extensive regulation was observed in gene expression of the LR-controlling modular proteins of SLR/IAA14 and ARF7-ARF19. Among other ARF proteins involved in the regulation of LR development, expression of ARF2 and ARF3 genes was moderately increased 2- to 5-fold compared with the mock control, while that of the ARF4 gene was not significantly changed or slightly decreased by the BL treatment. Interestingly, the BARK1 overexpressor showed a similar up-regulated expression pattern of ARF genes, ranging from 3.2 to 5.5 times (Fig. 5H). In this transgenic line, ARF4 expression was also enhanced. It was previously shown that ectopic expression of ARF4 increased the number of LRs. In contrast, the loss-of-function mutant, arf4-2, showed a decreased number of LRs (Yoon et al. 2010). Similarly, the arf7 arf19 null mutant exhibited a dramatically decreased number of LRs, and its defective LR phenotype was rescued by the introduction of their direct transcriptional downstream targets such as LATERAL ORGAN BOUNDARIES DOMAIN 16 (LBD16), LBD18 and LBD29 (Okushima et al. 2007, Lee et al. 2009). Based on these reports, we suggest that the BARK1 overexpressor phenotype with enhanced LR number is partly due to the increased expression of ARF genes in this genetic background. Unfortunately, we were not able to confirm opposite root phenotypes in BARK1 knock-out plants as we have failed to isolate homozygous progeny from several lines of.
Fig. 5 Phenotypic analysis of BARK1-overexpressing 35S::BARK1-GFPN transgenic plants. (A) Western blot analysis of BARK1–GFPN expression in different 35S::BARK1-GFPN transgenic lines. Total protein extracts were isolated from 3-week-old plants, size-fractionated by SDS–PAGE and probed against anti-GFP antibodies. (B) Pictures of light- and dark-grown Columbia plants and a BARK1-overexpressing transgenic line, BARK1ox-15. (C) Roots and hypocotyl phenotypes of BARK1ox lines. The lengths of primary roots (12 d after germination) and hypocotyls (6 d after germination) of BARK1-overexpressing transgenic plants and their number of lateral roots were normalized as a percentage of that of the Col-0 plants, which was set to 100%. (D) Brassinazole (BRZ)-driven inhibition of hypocotyl growth. Seedlings were grown on half-strength MS medium in the presence or absence of BRZ, and their hypocotyl length was normalized as a percentage of the untreated mock control for each corresponding plant line. (E) Brassinosteroid-induced root growth inhibition assay. Seedlings were grown on a half-strength MS medium in the presence or absence of the indicated concentration of epi-BL for 12 d after germination. The length of primary roots was normalized as a percentage of the untreated mock control for each corresponding plant line. (F) Measurement of lateral root density of BARK1ox lines. Lateral root density of BARK1-overexpressing transgenic plants was normalized as a percentage of that of the Col-0 plants, which was set to 100%. More than 50 seedlings were used for each treatment for measurement in C–F. (G) Effects of BR on the expression of genes involved in...
T-DNA-tagged heterozygote plants. It will be of value in the future to clarify the molecular mechanisms that block the production of the homozygous knock-out plants.

**Discussion**

Many BR-deficient or -insensitive mutants are defective in root growth and show a short-rooted phenotype (Müssig et al. 2003, Mouchel et al. 2004). Actually, it seems that roots are one of the major sources of BRs, and thus play an important role in root growth and development. BRs were successfully isolated from the roots of several plants, including Arabidopsis (Shimada et al. 2003). The asymmetric division of the endodermis/cortex initial separates daughter cells into two ground tissue layers, the endodermis and cortex layers, due to the action of SCARECROW (SCR) and SHORT-ROOT (SHR) (Helariutta et al. 2000). Whole-genome microarray analysis revealed that BR biosynthetic enzyme (BR6ox2/Cyp85A2 Cyt P450) is a direct endogenous target of SHR, and its expression domain in roots overlapped with that of SHR, which implies that BRs together with SHR are important regulators of vascular development in roots (Levesque et al. 2006). This finding is consistent with a previous report demonstrating that BR1-like gene 1 (BRL1) and BRL3 function in vascular differentiation in Arabidopsis, especially in promoting xylem differentiation (Caño-Delgado et al. 2004). In our study, we also demonstrated that BAK1-interacting BARK1 is specifically expressed in the vasculature cells of maturing roots. In addition, transgenic plants ectopically expressing the BARK1 protein have enhanced growth and development of both primary roots and LRs. In contrast, ectopic expression of Arabidopsis BAK1 (AtBAK1) in rice resulted in semi-dwarfism and shortened primary roots (Wang et al. 2007), and this root phenotype is opposite to that of the BARK1 transgenic plants. Nonetheless, the authors concluded that the observed phenotypes in the AtBAK1-overexpressing plants were presumably caused by hypersensitivity to endogenous levels of BRs, different from BR-insensitive and -deficient rice mutants. In line with this scenario, the BARK1 transgenic plants were also hypersensitive to BR-induced root inhibition (Fig. 5E).

BRs interact with multiple hormone signaling pathways in the regulation of root growth and development. They act synergistically with auxin to promote LR development by increasing acropetal auxin transport in Arabidopsis; thus, BR induction of LR formation is suppressed by an auxin transport inhibitor (Bao et al. 2004). Conversely, in br1-119 mutants defective in BR perception or after treatment with a BR biosynthetic inhibitor, BRZ, auxin levels are reduced in the tip of both primary roots and LRs (Asami et al. 2000). In contrast to reports on the interactive action of BRs and auxin in LR development, enhancement of root growth by a low concentration of BRs is independent of auxin transport (Müssig et al. 2003). The BREVIS RADIX (BRX) protein is a plasma membrane-associated protein, and its loss-of-function mutant, brx, shows a root-specific deficiency of BRs resulting in retarded root growth (Mouchel et al. 2006). Cytokinin inhibits initiation of LRs, but the brx-2 mutant is insensitive to this cytokinin-induced inhibition. Interestingly, the br1-6 plant shows normal cytokinin-mediated inhibition of LR growth, suggesting that cytokinin-mediated inhibition may not be directly dependent on the BR signaling pathway initiated by BR perception to BRI1 (Li et al. 2009). ABA inhibits root growth at a micromolar concentration, but stimulates it at a lower concentration (Zeevaart and Creelman 1988). A BR biosynthetic mutant, hypersensitive to abscisic acid and to auxin (sax1), has a short-rooted phenotype and, at the same time, is hypersensitive to auxin- and ABA-induced root inhibition (Ephtihikine et al. 1999). Additionally, both BR gain-of-function positive signaling mutants (bes1-D, brzr1-1D and bsu1-D) and BR biosynthetic mutants (brx2, det2 and cpd) are hypersensitive to ABA-mediated root growth inhibition. These results imply that modulation of the plant response to BR strongly affects ABA sensitivity of roots (Rodrigues et al. 2009). In our study, BARK1 gene expression was differentially regulated by hormones; BR and auxin increased gene expression while ABA, gibberellin and cytokinin decreased gene expression (Fig. 4). Nonetheless, it is still unclear how a modulation in BARK1 levels induced by different hormones and/or strength of BR signaling pathway mediated by BAK1/BARK1 cooperation is related to an alteration in Arabidopsis root growth and development.

BARK1 differentially regulates the development of roots and hypocotyls in young seedlings. Its regulation was root specific as the growth of their transgenic roots was controlled by BRs in a concentration-dependent manner while hypocotyls were unaffected or affected very little (Fig. 5). Actually, BRs cause diverse changes in plant growth and development in a tissue-dependent manner (reviewed in Yang et al. 2011). For example, both auxin and BRs promote gene expression of a subset of the cell wall-modifying XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE (XTH), by which they could regulate hypocotyl elongation induced by low-intensity blue light (Sun et al. 2010, Keuskamp et al. 2011). Recently, it was reported that a BR response dependent on the receptor-like kinase FERONIA antagonizes the effect of ethylene on hypocotyl growth of etiolated seedlings.
In developing primary roots, plants treated with exogenous BR or mutants with enhanced BR signaling, such as bes1-D, prematurely exit the cell cycle, and this exit results in early differentiation of meristematic cells and thus negatively influences meristem size and overall root growth (Gonzalez-Garcia et al. 2011). In line with these observations, an Arabidopsis BR biosynthetic mutant, dwarf7-1, has a smaller number of dividing cells undergoing DNA synthesis and mitosis in the roots (Cheon et al. 2010). Considering that BAK1 is proposed to be an adaptor molecule required for differential functionality of numerous RLKs (reviewed in Gendron and Postel et al. 2010), it seems likely that BARK1 is a partner of BAK1 in the regulation of BR-induced root growth and development, but not for control of hypocotyl elongation.

The Arabidopsis SERKs family of plasma membrane receptors consists of five closely related members, annotated as SERK1–SERK5 (Hecht et al. 2001). SERK1 and SERK2 have been shown to be involved mainly in embryogenesis (Hecht et al. 2001, Salaj et al. 2008), development of reproductive organs (Albrecht et al. 2005) presumably in a BR-independent fashion (Albrecht et al. 2008), separation of floral organs (Lewis et al. 2010) and Mi-1-mediated resistance to potato aphids (Mantelin et al. 2011). SERK3/BAK1 and SERK4/BKK1 are the well-studied SERKs whose activities regulate diverse BR-induced plant growth and BR-dependent/independent multiple plant resistance signaling pathways as noted in the Introduction. So far, not much is known about the functional roles of SERK5 in plants. Recently, it was demonstrated that coordinative functions of SERKs are indispensable in BR-induced early signaling leading to plant growth phenotypes (Gou et al. 2012). Also, a minimal homo- or heteromeric configuration of the SERK proteins determines independent functional specificity of SERK pairs such as in the regulation of plant growth, immune responses against pathogens and formation of reproductive organs (Albrecht et al. 2008). The Arabidopsis SERKs control root development via both BR-dependent and BR-independent pathways. SERKs are possibly involved in the BR-independent pathway via their interactions with unknown ligand-binding RLKs, and the subsequent modulation of genes regulating auxin transport and root development (Du et al. 2012). Similarly, our report shows that BARK1 interacts with diverse SERK proteins in yeasts, and its ectopic expression in Arabidopsis modulates root development and gene expression of AUX/IAA and ARFs. It would be interesting in the future to investigate how BARK1 is involved in plant growth and development related to the diverse SERK proteins, especially in root and flower development.

Materials and Methods

Plant materials and growth conditions

Wild-type Arabidopsis thaliana (Col-0), its BR-related mutants (det2, bak1-3 and bZR1-D), a promoter-driven reporter plant BARK1p::GFP and the BARK1-overexpressing 3SS: BARK1-GFP transgenic plants were used for gene expression and phenotype analysis in this study.

Seeds were surface-sterilized by washing them with 70% EtOH/0.1% Triton X-100 for 20 min, 70% EtOH for 10 min and 95% EtOH for 10 min. Finally, seeds were dried on a clean bench and cold-treated in the dark at 4°C for 72 h. These sterilized seeds were then sown on half-strength Murashige and Skoog (MS) agar medium containing 0.8% phytoagar (pH 5.7) in the presence or absence of the indicated chemicals in each experiment. For experiments examining tissue expression of the BARK1 gene by performing RT–PCR (Fig. 3A) and using a confocal microscope (Fig. 3B), seeds were sterilized/cold-treated and sown in pots containing Sunshine No. 5 soil (Pollysciences). For other experiments, plants were grown in a growth chamber or growth room operating under a cycle of 16 h light and 8 h dark at 23–25°C unless they were grown under total darkness to analyze the hypocotyl phenotype (Fig. 5B–D). All seedlings were grown vertically.

In vivo interaction tests in yeast

All procedures for yeast transformation and the protein interaction test were performed according to Clontech’s manual “Yeast Protocols Handbook” (PT3024-1) using pGBK7T as the GAL4 DNA-binding domain vector and pGAD7 as the GAL4 DNA activation vector (Clontech).

To test interactions between BARK1 and other known BR signaling components, a cDNA encoding the BARK1 kinase domain (BARK1KD; Val498 to Arg928) was amplified by PCR using EcoRI and SalI (New England Biolabs) tagged primers and subsequently subcloned into pGBK7 to generate the BARK1KD-pGBK7T construct. Other cDNAs encoding the kinase domain (KD) of BRI1, BAK1 (AT4g33430), BKK1 (BAK1-like kinase 1; AT2g13790) or SERK5 (AT2g13800) and the full coding region of BSU1 (AT4g35230), BSK2 (AT5g46570), BZR1 (AT1g75080), BIN2 (AT4g18710) or BSU1 (AT1g03445) were subcloned into pGBK7 to generate test constructs. Each test construct was co-transformed with BARK1KD-pGBK7T into AH109 yeast cells pre-conditioned with TE/LiAc solution (10 mM Tris–HCl/1 mM EDTA/0.1 M lithium acetate, pH 7.5). Co-transformed yeast with equal density, determined by measuring the optical density at 600 nm, were then spotted on either a yeast drop-out medium lacking leucine and trytophan or a medium lacking leucine, tryptophan and histidine in the presence of 1 mM 3-aminotriazole to test interactions. Primers used for yeast constructions are listed in Supplementary Table S1.

Transient expression and bimolecular fluorescence complementation (BiFC) assay in tobacco

Interaction of proteins in planta was examined as previously described with minor modifications (Walter et al. 2004). In brief, full-length genomic DNAs encoding BRI1, BAK1 and BARK1 were amplified from Col-0 plants by PCR using MluI- and XhoI- (New England Biolabs) tagged primers. The amplified
DNA fragments were then cut by these enzymes and subcloned into two binary vectors, pmGFPN173C and/or pmGFPC155c in fusion with the N-terminal half (GFPN) or the C-terminal half (GFPC) of GFP, respectively. For the in planta interaction assay, *Agrobacterium tumefaciens* GV301 carrying the BiFC binary expression constructs was infiltrated into the abaxial air space of 2- to 4-week-old tobacco leaves. Epidermal cell layers were then assayed for fluorescence 36–48 h after the infiltration using a Leica TCS SP2 AOBS laser-scanning confocal microscope. Primers used for these constructions are listed in Supplementary Table S1.

**Generation of transgenic plants**

To build a promoter-driven reporter construct of BARK1 (BARK1p::GFP), a 2 kb fragment of genomic DNA residing immediately before the first methionine of BARK1 was amplified by PCR from Col-0 plants, cloned into pENTR/SD/D-TOPO and then into the upstream region of the C-terminal sgfp-expressing binary vector pGWB4 (Nakagawa et al. 2007), according to the manufacturer’s instructions for Gateway cloning (Invitrogen). For generation of a BARK1 overexpressor, the GFP-tagged BARK1-expressing pmGFPN173C construct (35S::BARK1-GFPN) described above was used to express BARK1–GFP fusion protein ectopically under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Primers used for these constructions are listed in Supplementary Table S1.

All binary vector constructs were transformed into *A. tumefaciens* GV3101 and then transformed into *A. thaliana* using the floral dipping method (Clough and Bent 1998). T1 seeds from primary transformants were screened on half-strength MS solid medium containing 50 μg ml−1 kanamycin to select for transgenic progeny. Transgenic T2 seeds from individual T1 plants were planted on the same medium to identify homozygous T2 seeds. The transformation was verified by genotype analysis, and T1 homozygous seeds were used for all experiments.

**Microscopic analysis**

A Zeiss LSM 510 Meta NLO-UV confocal laser-scanning microscope equipped with an argon 2 ion laser (488 nm) and a He/Ne 1 ion laser (543 nm) was used. An HFT488 chromatic beam splitter and a BP500–550 IR bandpass filter were used to generate the GFP signal, and an HFT 543 beam splitter and a BP565–615 bandpass filter aided in the observations of the propidium iodide (PI) staining. Images were compiled with the Leica confocal software. For PI staining, tissues were incubated for 30 min in 5 μg ml−1 PI (Sigma-Aldrich).

**Total RNA isolation and RT–PCR analysis**

Total RNAs were isolated from plant tissues using the Plant RNA Extraction Kit (Intron biotechnology) and then treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer’s instructions. To examine semi-quantitative RNA expression, the first-strand cDNA of a gene was synthesized using the Impron II reverse transcription kit (Promega) and then amplified by PCR using G-Taq polymerase (Labopass) according to the manufacturer’s instructions. For quantitative real-time RT–PCR (qRT–PCR), total RNA was extracted from >10 individuals of 2-week-old plant root tissues. The qRT–PCR was performed by the SYBR green method using the Applied Biosystems Step One Plus System with appropriate primers. The expression of each transcript was normalized against the amount of UBC1 control in each sample. The results were reported as the relative expression relative to the mock control (Fig. 5G) or Col-0 control (Fig. 5H). Three biological replicates were included in each experiment, and the data were statistically analyzed by the Student’s t-test. Primers used for RT–PCRs are summarized in Supplementary Table S1.

**Protein isolation and Western blot analysis**

To examine expression of BARK1–GFPN in transgenic plants, the rosette leaves of 3-week-old seedlings were harvested and frozen in liquid nitrogen. Frozen leaves were then pulverized using a mortar and pestle and total proteins were extracted using a homogenization buffer (50 mM Tris-acetate, 100 mM potassium acetate, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, pH 7.9). The homogenate was centrifuged at 12,000 r.p.m. for 10 min at 4 °C and the supernatant was used for size fractionation of total proteins by 10% SDS–PAGE. The size-fractionated total proteins were then transferred onto a nitrocellulose membrane (Whatman) and probed against anti-GFP rabbit polyclonal antibodies in 5% milk/TBST (50 mM Tris-acetate, 150 mM NaCl, 0.05% Tween-20, pH 7.6) for 2 h at room temperature. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) was used to quantify BARK1–GFP protein. Peroxidase activity was detected using the ECL system (Thermo Fisher Scientific Inc.) according to the manufacturer’s instructions.

**Phylogenetic analysis**

Amino acid sequences were aligned using the MEGA 5.05 ClustalW program and its parameters. A penalty of 10 for the gap opening and 0.2 for the gap extension were given for this alignment, and the phylogenetic clade was constructed using the Neighbor–Joining method. The P-distance model was selected for the analysis.

**Measurement and statistical analysis**

Plants grown on a half-strength MS agar medium in the presence of the indicated chemicals were photographed, and the images were saved in a TIFF or JPEG format. The saved images were transferred to the Image J program (NIH), and the length of hypocotyls and primary roots together with the number and density of LRs were measured. The density of LRs represents the number of roots divided by the length of the primary root from which the LRs were derived. All experiments were done at least in triplicate, and the data were statistically analyzed by the Student’s t-test.
Supplementary data
Supplementary data are available at PCP online.

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Disclosures
The authors have no conflicts of interest to declare.

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