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Molecules and Cells

BAK7 Displays Unequal Genetic Redundancy with BAK1 in Brassinosteroid Signaling and Early Senescence in Arabidopsis

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BRI1-Associated kinase1 (BAK1), a five leucine-rich-repeat containing receptor-like serine/threonine kinase, has been shown to have dual functions: mediating brassinosteroid (BR) signaling and acting in the BR-independent plant defense response. Sequence analysis has revealed that BAK1 has two homologs, BAK7 and BAK8. Because BAK8 deviates from the canonical RD kinase motif, we focused on the functional analysis of BAK7. The expression pattern and tissues in which *BAK7* appeared partially overlapped with those observed for *BAK1*. Expression levels of *BAK7* increased in the *bak1* mutant. Overexpression of *BAK7* rescued the *bri1* mutant phenotype, indicating that BAK7 can compensate for BAK1 in BR-mediated processes, especially in the absence of BAK1. However, root and hypocotyl elongation patterns of transgenic plants overexpressing *BAK1* or *BAK7* appeared to be different from the patterns observed in a *BRI1* overexpressor. Furthermore, the sensitivity of transgenic plants overexpressing *BAK7* to brassinazole, a biosynthetic inhibitor of brassinolide (BL), did not change compared to that of wild-type plants. In addition, we generated transgenic plants expressing *BAK7 RNA* interference constructs and found severe growth retardation and early senescence in these lines. Taken together, these results suggest that BAK7 is a component of the BR signaling pathway, with varying degrees of genetic redundancy with BAK1, and that it affects plant growth via BL-independent pathways *in vivo.*

INTRODUCTION

The Arabidopsis genome contains more than two hundred leucine-rich repeat receptor-like serine/threonine kinases (LRR-RLKs), constituting one of the largest gene families (Shiu and Bleecker, 2001). Plant LRR-RLKs have structurally similar single transmembrane domains and intracellular cytoplasmic kinase domains, but their N-terminal extracellular domains differ greatly with regard to the number of leucine-rich-repeats (LRRs) (Shiu and Bleecker, 2003). Plasma membrane-localized LRR-RLKs play important roles in diverse plant developmental processes, such as above-ground organ growth (ERECTA) (Shpak et al., 2003), shoot meristem maintenance (CLAVATA1) (Dievart et al., 2003), resistance to pathogens in rice (Xa21) (He et al., 2000), abscission (HAESA) (Jinn et al., 2000) and brassinosteroid signaling (BRASSINOSTEROIDS-INSENSITIVE1 (BRI1), BRI1-ASSOCIATED KINASE1 (BAK1)) (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002). The genes encoding LRR-RLKs have thus far been identified via molecular genetic studies combined with mutant analyses. Among these genes, *BRI1* and its co-receptor *BAK1* provided the first example of heterodimerization between LRR-RLKs (Li et al., 2002; Nam and Li, 2002).

The phenotype of plants overexpressing *BAK1* is similar to that of *BRI1*- overexpressing plants and consists of more elongated and narrower leaves and petioles. *Bak1* mutant plants display a weak *bri1* phenotype and reduced BL-sensitivity (Nam and Li, 2002). *BAK1* has been found to be identical to *AtSERK3*, a homolog of *AtSERK1*. *AtSERK1* is an ortholog of *DsSERK,* which is involved in embryogenic competence in carrot tissue culture (Hecht et al., 2001).

Several recent reports have suggested that each LRR-RLKII subfamily member increases the potential for complex formation between different types of LRR-RLKs, resulting in functional diversity. For example, BAK1 can form a complex with FLS2, another type of LRR-RLK, to sense the flagellin, PAMP (pathogen-associated molecular patterns) (Chinchilla et al., 2007; Heese et al., 2007). The *bak1* mutant develops necrosis more easily upon infection with virulent strains of bacteria or the non-host fungus *A. brassicicola* through the increased production of reactive oxygen species (ROS) (Kemmerling et al., 2007). These results indicate that BAK1 participates in at least

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two different cellular functions: BR signaling and the BR-independent plant defense response. BRI1 can be co-immunoprecipitated by AtSERK1, and a mutant allele of AtSERK1 enhances the phenotype of the weak BRI1 allele *bri1-9* (Karlova et al., 2006). This finding suggests that AtSERK1 may be a component of the brassinosteroid signaling pathway.

Since the identification of BAK1 as a partner for BRI1 in the LRR-RLKII subfamily, sequence analysis and yeast two-hybrid screening have revealed additional LRR-RLKs in the same LRR-RLKII subfamily. We named BAK2 through BAK8, some of which have also been publicly annotated as AtSERKs (Albrecht et al, 2008), as follows: AtSERK2/BAK2, AtSERK4/BAK7, and AtSERK5/BAK8. Recently, BAK7 was named BKK1 (BAK1-LIKE 1) (He et al., 2007). In this report, we conducted a functional analysis of BAK7, which is the most homologous to BAK1. We detected a direct interaction between BAK7 and BRI1 using fluorescence resonance energy transfer (FRET) analysis and showed that overexpression of *BAK7* rescues the *bri1* mutant phenotype. These results indicate that BAK7 can compensate for BAK1 in BR-mediated processes, especially in the absence of BAK1. In addition, we generated transgenic plants expressing a *BAK7 RNA* interference construct to simultaneously down-regulate *BAK7* and *BAK1* to varying degrees, and we found severe growth retardation and early senescence in these lines. Taken together, these results suggest that members of the LRR-RLKII subfamily function diversely in plant development with different degrees of genetic redundancy.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana Columbia (Col-0) was used as the wild-type. Transgenic plants were made by floral dipping into suspensions of *Agrobacterium tumerfaciens* (GV3101) containing appropriate plasmid constructs. Seeds were sterilized with 75% ethanol containing 0.05% Tween-20, followed by washing twice with 95% ethanol, and were germinated in 1/2 MS (Duchefa) and 0.8% phytoagar supplemented with the appropriate antibiotics. To observe the rosette phenotypes of transgenic plants, seeds were planted directly onto Sunshine #5 top-layered with fine granules of vermiculite. All plants were grown at 22°C under long-light conditions (16 h L/8 h D).

Histochemical β-glucuronidase (GUS) reporter gene expression

Genomic fragments containing promoter sequences of 1.6 kb for *BAK1*, 1.8 kb for *BAK7,* and 1.6 kb for *BRI1* were PCR-amplified from the corresponding BAC DNA subclones and cloned into a modified *pPZP222-GUS* binary vector containing the *E. coli GUS*-encoding sequence derived from *pRTL2-GUS*. Homozygous T3 transgenic seedlings of the indicated developmental ages were tested for GUS expression using X-Gluc as a substrate. An incubation time of six hours at 37°C was used to assess GUS activity for all samples, as described by Stomp (1992). GUS signals were visualized by microscopy (Stemi 2000-C, Carl Zeiss).

Transient expression of CFP- and YFP-tagged constructs and FRET analysis

The open reading frames of *BRI1*, *BAK1*, and *BAK7* were PCR-amplified using gene-specific primer sets and cloned into the *pEZRK-LNC* vector for C-terminal fusion with CFP, and into the *pEZRK-LNY* vector for C-terminal fusion with YFP. *Agrobacterium tumerfaciens* (GV3101) cells transformed with each construct were cultured in LB medium containing selective antibiotics and 20 μM acetosyringone. After harvesting, bacterial cell pellets were re-suspended in infiltration buffer (500 μM MES, 500 μM $MgSO_4$, 100 μM acetosyringone) and mixed with the appropriate combinations in an equal volume. Prepared bacterial mixtures were injected into four-week old *Nicotiana benthamiana* leaves (Ryu et al., 2004). After 48 h of incubation under normal conditions, the lower epidermal leaf tissues were peeled off and bleached five times in the acceptor YFP channel with a 514-nm argon laser. Before and after acceptor photobleaching, CFP intensity images activated at 405-nm were monitored by confocal microscopy (LSM510, Carl Zeiss) (Karpova et al., 2003). FRET efficiency was calculated as E% = [1-(CFP emission before YFP photobleaching/ CFP emission after YFP photobleaching)] × 100 (Siegel et al., 2000).

Semi-quantitative RT-PCR analyses

For measurement of the expression of *BAK1* homologs in tissues other than roots, total RNA was isolated from the various tissues of 4-week-old soil-grown wild-type plants. Root RNA was obtained from 10-day seedlings grown vertically on 1/2 MS media. To determine the endogenous expression levels and expressional changes in response to brassinolide (BL), total RNA was isolated from 10-day-old seedlings of wild-type, *bak1*, and *bri1-9* mutant plants grown on 1/2 MS media with or without 1 μM BL. To monitor the expression of senescence-inducible genes, RNA was isolated from the rosette leaves of 4-week-old soil-grown transgenic plants. The RNA was treated with RNase-free RQ1 DNases (Promega) and first-strand cDNA was synthesized using Superscript^{III}-MMLV reverse transcriptase (Invitrogen) and oligo d(T_{15}) as a primer. The same aliquot of first-strand cDNA was used as a template in a second polymerase chain reaction, which was performed for 26 to 33 cycles with gene-specific primers to amplify the less homologous N-terminal region of each *BAK1* homolog. To monitor the senescence we performed RT-PCR for *SEN1*, *SAG12*, *SAG13*, and *CAB* as marker genes. The expression of *tubulin* was used to normalize the data. The sequences of the primers used are provided in Supplementary Table 1.

Measurement of root and hypocotyl growth

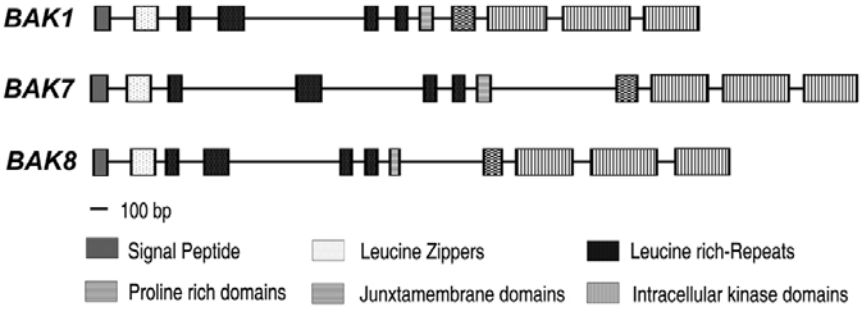
After sterilization, each seed was placed in a line on 1/2 MS (Duchefa) 0.8% phytoagar plates supplemented with or without 1 μM brassinazole. The seeds for all transgenic plants were seeded in the same plate to minimize ambient differences. Three sets of plates were positioned vertically at 22°C under long-light conditions (16 h L/8 h D) for root elongation or in the dark for hypocotyl growth. Root and hypocotyl length were measured for 20-30 seedlings in each line every 24 h after three days of growth. All experiments were repeated twice.

RESULTS

BAK1 has close homologs in the Arabidopsis genome

A search of the Arabidopsis genome database for genes homologous to *BAK1* revealed two genes that are close to *BAK1* but that were not obtained in the yeast two-hybrid screen in which *BAK1* was originally identified (which was a screen for genes encoding BRI1-interacting proteins) (Nam and Li, 2002). We named these two genes *BAK7* and *BAK8*. Recently *BAK7* was also named *BAK1-LIKE 1*, (*BKK1*) (He et al., 2007). *BAK1*, *BAK7* and *BAK8* each consist of 11 exons (Fig. 1A). Each exon forms a specific functional domain, and the numbers of amino acids encoded by the exons in these three genes are more or less similar. However, the number of nucleotides within each intron is variable (Figs. 1B and 1C). The second cysteine pair

A



B

	BAK1	BAK7	BAK8
Exon 1	28	32	27
Exon 2	45	48	48
Exon 3	24	24	24
Exon 4	48	48	48
Exon 5	24	23	23
Exon 6	23	23	23
Exon 7	25	26	19
Exon 8	45	42	35
Exon 9	114	114	114
Exon 10	132	132	132
Exon 11	107	107	107
Total Amino Acids	615	619	600

C

	BAK1	BAK7	BAK8
Intron 1	269	119	140
Intron 2	82	104	66
Intron 3	169	699	156
Intron 4	719	620	675
Intron 5	106	101	81
Intron 6	75	79	78
Intron 7	112	759	506
Intron 8	77	82	83
Intron 9	97	86	109
Intron 10	81	90	100
Total Nucleotides	1787	2739	1994

D

BAK1	411	KIIH**RD**VKAANILLDEEFEAVVGDFGLAKLMDYKD**T**HV**TT**AVRG**T**IGHIA	460
BAK7	416	KIIH**RD**VKAANILLDEEFEAVVGDFGLAKLMNYNDSHV**TT**AVRG**T**IGHIA	465
BAK8	397	KIIHL**D**VKAANILLDEEFEAVVGDFGLAKLMNYNDSHV**TT**AVRG**T**IGHIA	446

Fig. 1. The genomic structures of *BAK7* and *BAK8* in comparison with *BAK1*. (A) Comparison of the genomic organization of *BAK1* and *BAK1* homologs. Vertical squares indicate exons, and different patterns in the squares refer to different domains. Thick black lines indicate introns. (B, C) The numbers of amino acid encoded from each exon and the number of nucleotides in each intron of *BAK1*, *BAK7*, and *BAK8.* (D) Comparison of the amino acid sequences of subdomain VIb and subdomain VII of BAK1, BAK7 and BAK8. The conserved arginine and aspartic acid residues (RD) in subdomain VIb and the threonine residues in the activation loop of subdomain VII, are indicated in bold letters.

in exon 7, which usually defines the end of the LRR, is absent in *BAK1, BAK7/BKK1,* and *BAK8. BAK7* and *BAK8* are situated next to each other on chromosome 2, suggesting a recent gene duplication event. However, as pointed out in a recent report (He et al., 2007), BAK8 might be an inactive kinase, because it has a leucine residue instead of an arginine immediately downstream of the aspartic acid in the catalytic core of subdomain VIb, which is characteristic of the RD kinases (Fig. 1D).

Expression analysis of BAK1 and BAK7

Because the mutant phenotype of *bak1* is weak relative to that of *bri1*, it has been assumed that there may be proteins that are functionally redundant with BAK1. To determine whether BAK7 and BAK8 can perform the same cellular functions as BAK1, we first examined the expression patterns of these genes in different plant parts. Semi-quantitative RT-PCR analysis was performed using RNA isolated from the various tissues of Arabidopsis with gene-specific primers spanning the N-terminal part of each gene, which shows more divergence. *BAK1* was expressed most abundantly in all tissues, consistent with previous results (Nam and Li, 2002). Despite relatively low expression levels in all tissues except in roots, *BAK7* mRNA was as ubiquitous in the tissues we tested as was that of BAK1. The expression of BAK8 was barely detectable after long amplification cycles (Fig. 2A). Because the expression level of *BAK8* was very low and the characteristics of BAK8 protein deviate from those of canonical RD kinases, we focused our studies on the BAK7 and its functional interaction with BAK1.

To investigate whether *BAK1* and *BAK7* are expressed in specific tissues or regions, we constructed *β-glucuronidase* (*GUS*) reporter genes driven by the each promoters of *BAK1*, *BAK7*, and *BRI1* (Fig. 2B). To observe *GUS* reporter gene expression, we used four different T3 homozygous transgenic plants lines for *BRI1-GUS*, two lines for *BAK1-GUS*, and four lines for *BAK7-GUS*. *GUS* expression driven by the *BRI1* promoter was widespread in young seedlings, with stronger expression observed at the junction of the root and hypocotyls. The elevated *GUS* signal in the roots was maintained as plants developed. In contrast, *GUS* expression driven by the promoters of *BAK1* and *BAK7* was relatively low and the expression pattern was more limited compared to expression driven by the *BRI1* promoter. However, *GUS* expression patterns driven by the promoters of *BAK1* or *BAK7* overlapped in certain regions. Weak expression of *BAK1-GUS* and *BAK7-GUS* was detected in the root tip and leaf primordia on day three and in the vascular strand of the hypocotyls and leaves on days five and seven.

Changes in the expression of *BAK7* in *bak1* mutants

The overlapping regions of temporal/spatial expression of *BAK7* and *BAK1* shown above led to us to hypothesize that *BAK7* may be able to substitute for *BAK1* in the *bak1* mutant. To assess whether the expression of *BAK7* is influenced by mutations in *BAK1,* RT-PCR was performed using RNA isolated from the *bak1* mutant. We also tested whether treatment with brassinolide (BL) affects the expression of these genes (Fig. 2C). In wild type plants, *BAK1* was expressed more strongly than *BAK7,* and *BAK1* was induced about 6-fold by BL treatment, consistent with the previous result that *BAK1* is likely to be the major component among *BAKs* (Fig. 2A). However, in a *bak1* mutant, in that the expression of *BAK1* is completely nullified, the transcript level of *BAK7* was elevated compared to wild type plants. Also, BL treatment increased the expression of

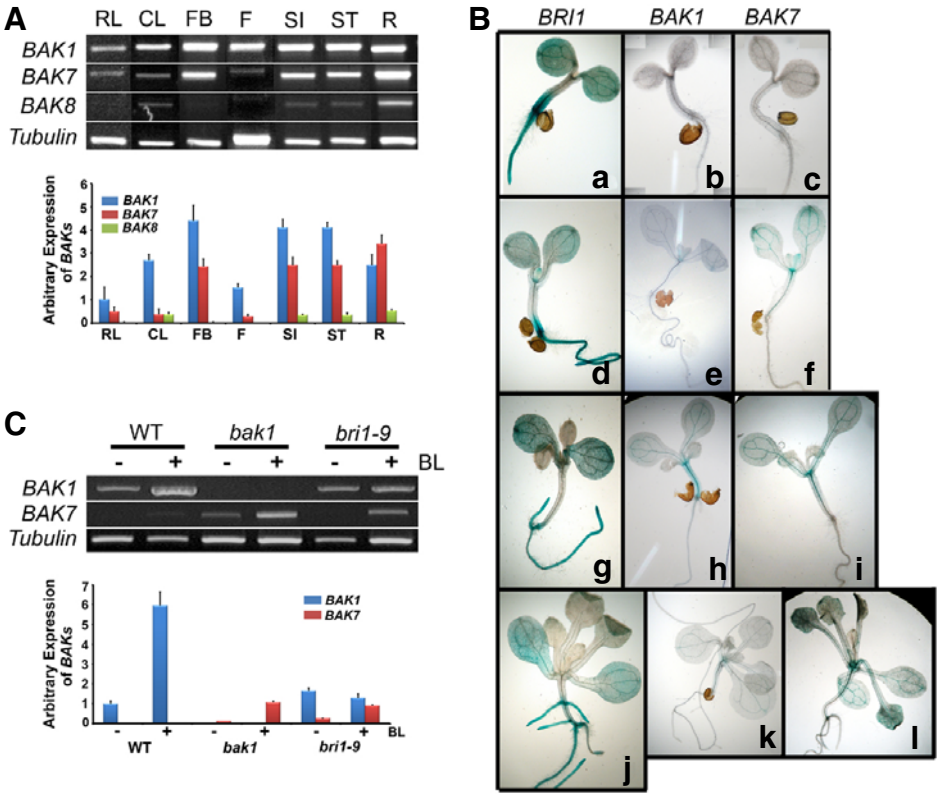


Fig. 2. Expression of *BAK1* and *BAK1* homologs. (A) Different expression levels of *BAK1, BAK7* and *BAK8* are shown in all plant tissue parts. Semi-quantitative RT-PCR was carried out with RNA isolated from the various tissues of wild-type Arabidopsis grown for four weeks in the soil (upper panel). Results of semi-quantita-tive RT-PCR were normalized to tubulin using the DNR-Bio Imaging System (Gelquant Version 2.7.0) and quantified in arbitrary units with the expression of YL in wild-type set to 1 (lower panel). YL, young rosette leaves; CL, cauline leaves; FB, floral buds; F, mature flowers; SI, siliques; ST, stems; and R, roots. (B) *GUS* reporter gene expression revealed distinct and partially overlapping regions of tissue expression and the relative expression levels of *BAK1* and *BAK7* compared with *BRI1*. *GUS* reporter gene expression driven by the promoters of *BAK1*, *BAK7*, and *BRI1* was observed in 3-day (a, b, c), 5-day (d, e, f), 7-day (g, h, i), and 10-day (j, k, l) old transgenic plants. (C) RT-PCR analysis of *BAK1* and *BAK7* expressed in *bak1* and in *bri1-9* grown on 1/2 MS plates for ten days with or without 1 μM brassinolide (BL) (upper panel). Semi-quantitative RT-PCR results were normalized as described above, and the expression of BAK1 in wild-type plants un-treated with BL was set to 1 (lower panel).

BAK7 in the *bak1* mutant. These results suggest that *bak1* plants increased the expression of *BAK7* to compensate for the lack of *BAK1*.

BAK7 can interact with BRI1 and overexpression of *BAK7* rescues the *bri1* mutant phenotype

FRET analysis was performed using CFP/YFP pairs to compare the *in vivo* interaction of BRI1/BAK7 to that of BRI1/BAK1. The presence of FRET is a good indicator of close proximity, implying biologically meaningful protein-protein interactions. *Agrobacteria* transformed with pairs of constructs containing BRI1 tagged with YFP as a C-terminal fusion and BAK1 or BAK7 tagged with CFP as a C-terminal fusion, were introduced into leaves of *Nicotiana benthamiana* (Fig. 3A). To quantify the efficiency of FRET between the donor (CFP-tagged proteins) and acceptor (YFP-tagged proteins), we measured CFP emission before and after selective photobleaching of YFP at the epidermal surface of leaf guard cells by confocal microscopy (Siegel et al., 2000). The increase, or de-quenching, of CFP emission is a direct measure of FRET efficiency. First, we evaluated the FRET efficiency of CFP and YFP alone. After photobleaching of YFP, emission fluorescence of CFP for this pair demonstrated significant quenching. When BRI1-YFP was photobleached, fluorescence intensities obtained for CFP-tagged BAKs emission were increased by up to 32% (Fig. 3B). In this condition, the BRI1/BAK7 pair displayed the highest FRET efficiency. BRI1-YFP did not interact with CFP alone. These results suggest that BAK7 can interact specifically with BRI1 and can possibly form receptor complexes for BR.

To confirm this hypothesis genetically, we also generated *bri1* plants transformed with a *BAK7* overexpression construct and observed that overexpression of *BAK7* clearly rescued the phenotype of *bri1-301*, as did overexpression of *BAK1* (Fig. 3C).

Phenotypic analysis of transgenic plants overexpressing BAK1 homologs

We further examined the physiological roles of BAK7 by analyzing transgenic plants overexpressing *BAK7* compared to those overexpressing *BAK1*. We previously reported that transgenic plants overexpressing a *BAK1* genomic fragment containing the native promoter had narrower and longer leaves and more elongated petioles than wild-type plants (Nam and Li, 2002). Here we found that transgenic plants overexpressing *BAK7* also had an elongated leaf phenotype in rosettes, but to a lesser degree than plants overexpressing *BAK1* (Supplementary Fig. 1, Fig. 4A). We also examined root and hypocotyl growth in the seedling stages. Overexpression of *BAK1* and *BAK7* resulted in the reductions in both root and hypocotyl growth over the period examined relative to wild-type plants, in contrast to the rosette phenotype described above. However, *BRI1*-overexpressing transgenic plants still showed increased growth of roots and hypocotyls compared to wild-type plants (Figs. 4B and 4C). These results suggest that the rosette growth of *Arabidopsis* is not coupled to the development of roots and hypocotyls at the seedling stage, during establishment of the body plan.

To examine whether overexpression of *BAK7* is associated with BR signaling, we further analyzed root and hypocotyl elongation using the brassinosteroid biosynthesis inhibitor, brassinazole (BRZ). Transgenic plant overexpressing BAK7 did not

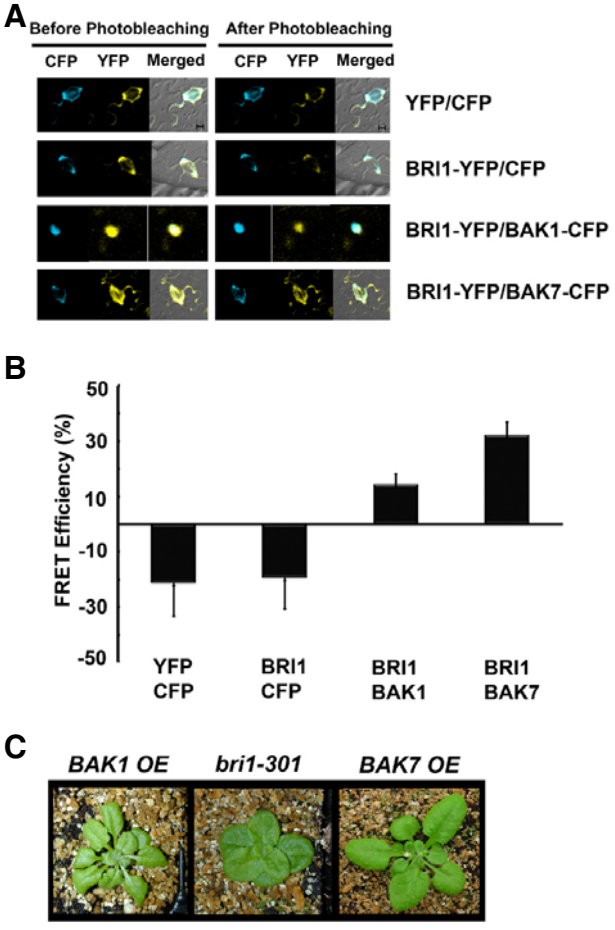


Fig. 3. BAK7 interacts with BRI1 and rescues the *bri1* mutant. (A) FRET analysis between BRI1 and BAK1 or BAK7 before and after YFP photobleaching. Scale bars indicate 10 μm. (B) Quantitative mean FRET efficiency of BRI1/BAK1 and BRI1/BAK7 pairs compared with that of non-relevant controls. Each value was calculated for three to five repeated experiments and the error bars denote standard errors. (C) Overexpression of *BAK7* rescued the *bri1-301* phenotype. Pictures were taken from the plants grown for four weeks.

show reduced sensitivity to BRZ in root and hypocotyl growth compared to wild-type plants, while transgenic plant overexpressing *BRI1-GFP* and *BAK1* were less sensitive to BRZ, especially with regard to root growth (Fig. 4D). About 45% of the root growth was inhibited in wild-type and BAK7 transgenic plants in the presence of BRZ, but only 25% and 35% inhibition of root growth occurred in *BRI1-GFP* and *BAK1* overexpressing plants treated with BRZ.

Down- regulation of BAK7 expression leads to developmental defects

The phenotypic changes induced by overexpression of *BAK7* were weak compared to those induced by *BAK1*. Furthermore, a single T-DNA insertion mutant of *BAK7* was reported to have no effect on growth (He et al., 2007). Therefore, we used the RNA interference approach to simultaneously knock-down the expression level of *BAK7* and potentially homologous genes, including *BAK1*, in order to further determine whether *BAK7* acts redundantly with *BAK1*. Transgenic *BAK7-RNAi* plants displayed a few phenotypic changes. Many of the seeds germinated well, but they hardly grew beyond two weeks. This is consistent with a recent report about *bak1/bak7* (*bkk1*) double knockout lines (He et al., 2007). We chose two lines among the few plants that still grew after two weeks for further examination. One transgenic line (*7-i1*) had even smaller rosettes and more compact shape than *bak1*, while the other line (*7-i2*) looked similar to the wild-type (Fig. 5A). At later stages of development, both lines displayed reduced growth and early senescence. To assess whether the phenotypic changes were attributable to the reduction in *BAK7* and any homologous transcripts, we checked the levels of *BAK7* and *BAK1* and found that both were reduced in *7-i1* plants and that *BAK1* was reduced in the *7-i2* line (Fig. 5B). In addition, we noticed that the rosette leaves of the *7-i1* plant exhibited early senescence even during early developmental stages. We checked the expression of the *SEN1*, *SAG12*, and *SAG13* in both lines and found that these senescence-associated genes were up-regulated in the *BAK7* RNAi transgenic plants compared to un-transformed wild-type plants of the same developmental stages. Especially, expression of the *SAG13* was detected only in the *BAK7* RNAi transgenic plant displaying more severe senescent phenotype. Moreover, the expression of *CAB*, which is usually abundant in photosynthetically active cells, showed an opposite expression pattern to the senescence-related genes (Fig. 5C). Early senescence features were more distinct in the *bak1* background. Several independent *bak1* plants transformed with the *BAK7-RNAi* construct displayed higher proportions of senescent and dying leaves during early developmental stages of growth compared to wild-type plants (Fig. 5D), indicating that decreased levels of both genes enhanced the early senescence phenotype.

DISCUSSION

Formation of complexes between different LRR-RLKs may be a general phenomenon in plants

Since BAK1 was reported to be an LRR-RLK interacting with BRI1, which is also an LRR-RLK, the formation of complexes between different combinations of LRR-RLKs has been examined using different methods in plants. Although BAK7 and BAK8 were not identified as BRI1 interactors in our yeast two-hybrid screen, the possibility that they have roles in BR signaling that are redundant with BAK1 was suggested by their high sequence homology with BAK1. BAK7 is also named AtSERK4 and BKK1, while BAK8 is also named AtSERK5 (Albrecht et al., 2008).

In this study, we confirmed the lower endogenous expression levels of *BAK7* and *BAK8* compared to *BAK1* and *BRI1* in all tissues (Fig. 2A), and this might have prevented detection of these two gene products in our yeast two-hybrid screen. We subsequently showed that *BAK7* and *BAK1* share specific regions of expression (Fig. 2B), implying a physical interaction between BRI1 and BAK7 functionally comparable to the interaction between BRI1 and BAK1. Our FRET analyses further support direct interactions between BRI1/BAK1 and BRI1/BAK7 (Figs. 3A and 3B). BRI1 was also reported to be a component of the AtSERK1 signaling complex *in vivo* (Karlova et al., 2006). These results suggest that BRI1 can form complexes with various LRR-RLKs that belong to the LRR-RLKII subfamily.

BRI1 is not the only promiscuous LRR-RLK in Arabidopsis. The fact that BAK1 can interact with FLS2 to increase innate immunity (Chinchilla et al., 2007, Heese et al., 2007) could be another example of heterodimerization between different families of LRR-RLKs. Moreover, homodimerization of AtSERK1 and AtSERK2 was reported to play a role in the development of

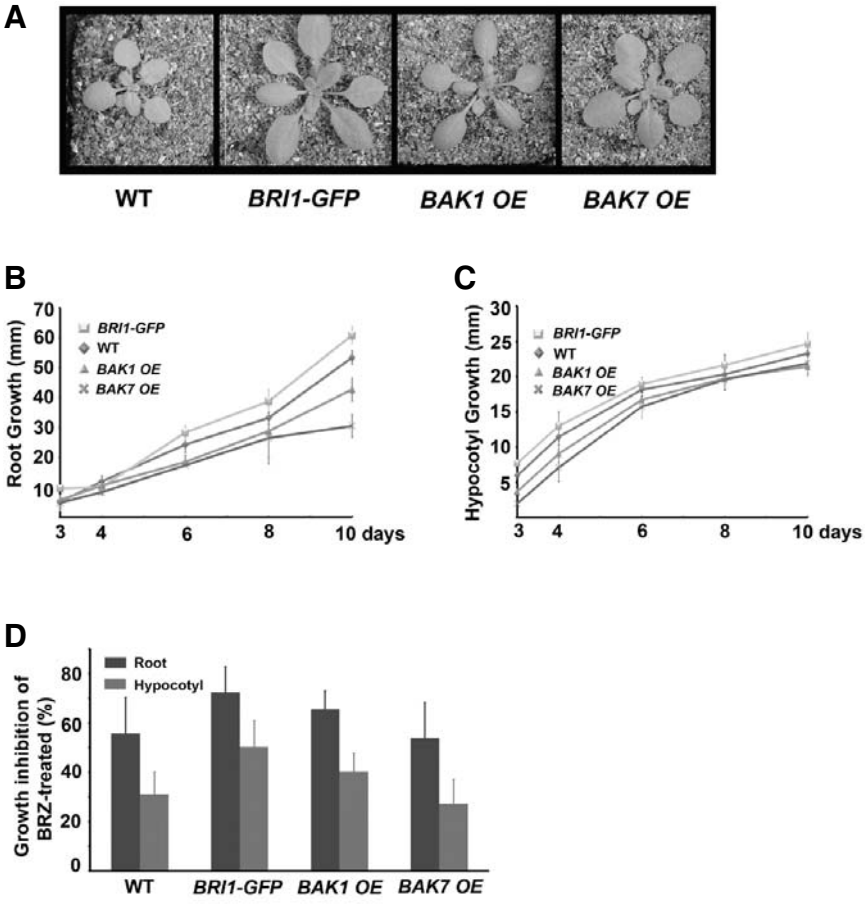


Fig. 4. Transgenic analysis of plants overexpressing *BAK7.* (A) Phenotypes of transgenic plants transformed with overexpression constructs for *BAK1* and *BAK7* driven by their own promoters. Pictures were taken of plants grown for three weeks. (B) and (C) Root and hypocotyl elongation of seedlings grown on 1/2 MS medium over the growth period (n = 30). Error bars denote standard errors. (D) Sensitivity to brassinazole (BRZ) of root and hypocotyl growth of seedlings grown on medium containing 1 μM BRZ. Growth is represented as a relative value compared to that of seedlings grown on media without BRZ. Error bars denote standard errors.

the male gametophyte (Albrecht et al., 2005, Colcombet et al., 2005). We also detected heterodimer formation between BAK1 and BAK7 (data not shown). Together these results suggest that the formation of complexes between different LRR-RLKs may be a general phenomenon, and this could explain the huge diversity of protein complexes composed of relatively small numbers of subunits in plants.

BAK7 acts redundantly and non-redundantly in BR signaling with BAK1

The binding capacity of BAK7 with BRI1 (Figs. 3A and 3B) and the compensatory higher expression of *BAK7* in the *bak1* mutant (Fig. 2C) strongly suggest that BAK7 functions redundantly with BAK1 in BR signaling. Overexpression of *BAK7* resulted in phenotypic changes similar to *BAK1* overexpression, and moreover, it rescued the *bri1* mutant phenotype (Fig. 3C). However, we hypothesized that BAK7's physiological roles in BR signaling in plants may be exerted under specific conditions *in vivo*. First, we did not observe any phenotypic changes in a single T-DNA insertion mutant of *BAK7,* indicating that BAK7 is not likely to be an essential genes for normal plant development. Second, overexpression of *BAK7* did not affect the sensitivity of root and hypocotyl growth to BRZ inhibition, which is a way of determining whether certain genes may be involved in BR signaling or not. By comparison, *BAK1* overexpression led to a reduction in sensitivity to BRZ under the same conditions (Fig. 4D). Therefore, in wild-type plants, the functional contribution of the BRI1/BAK7 pair to BR signaling is much less than that of the BRI1/BAK1 pair. Only in the absence of *BAK1*, BAK7 function as a BRI1 co-receptor more actively.

Here we identified additional functions of *BAK7* and *BAK1* that may indicate a major role of these genes in plant development. Seedlings of transgenic plants overexpressing *BAK1* or *BAK7* exhibited different pattern of hypocotyls and root growth compared with those of *BRI1* overexpressors (Figs. 4B and 4C). However, the rosette part of the soil-grown *BAK1* and *BAK7* overexpressors showed a similar enhanced growth pattern (longer petioles and narrower leaves) to that of BRI1-overexpressing transgenic plants. These results are consistent with the finding that the overexpression of *AtSERK4* in *bri1-301*, one of the mutant alleles of *bri1*, only rescued the compact rosette leaves to longer ones without affecting *bri1-301*'s defects in hypocotyl growth (Albrecht et al., 2008). Taken together, these results imply that rosette growth in *Arabidopsis* is a separate process from root and hypocotyl growth. Moreover, BAK1 and BAK7 may be negative regulators controlling the development of roots and hypocotyls at the seedling stage. Through knock-down of the expression of both *BAK1* and *BAK7* using *RNA* interference directed against *BAK7,* we found that the lack of both BAK1 and BAK7 led the plant to develop early senescence. The *BAK7 RNAi* approach produced many transgenic lines and thus overcame the previous reported seedling-lethal phenotype of the *bak1/bkk1* double mutant (He et al., 2007) because the degree of reduction of *BAK7* and *BAK1* expression was variable in each transgenic plant. This feature made it possible to analyze the function of these genes in later stages of development. We found that the transgenic plants in which the levels of both *BAK7* and *BAK1* were reduced had small and compact rosette leaves and senesced early (Figs. 5A, 5B, and 5C). Leaf senescence was often caused by the environmental

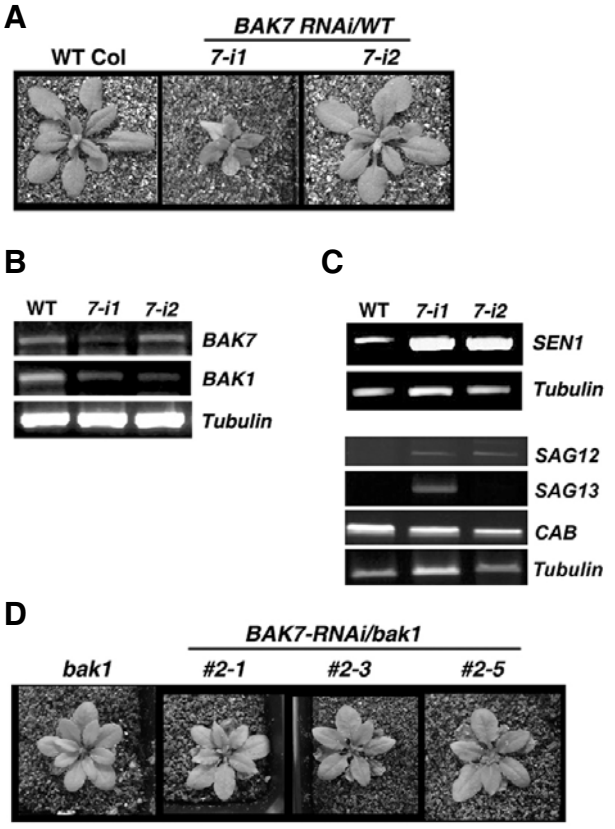


Fig. 5. Transgenic analysis of plants with down-regulated levels of *BAK7*. (A) Some transgenic plants harboring the *BAK7RNAi* construct displayed more severe phenotypes than *bak1*. (B) RT-PCR analysis of *BAK7RNAi* plants demonstrating reduced levels of *BAK7* and *BAK1*. (C) Increased senescence-associated gene expression in *BAK7RNAi* plants. (D) *Bak1* mutants transformed with the *BAK7RNAi* construct displayed early senescence in rosette leaves. Pictures in (A, D) were taken from plants grown for four weeks.

stress (Yoon et al., 2008). However, the early senescence phenotype was observed even more frequently in transgenic *bak1* mutant plants expressing the *BAK7 RNAi* construct (Fig. 5D) in normal growth condition. The small and compact rosette leaves are characteristics of mutants with decreased BR signaling capacity. However, senescent leaves in young rosettes have not been observed even in *bri1* or *bin2*, the two strongest BR signaling mutants so far. Because cell death eventually accompanies senescence, these results indicate that BAK7 may have roles in cell death that are redundant with those of BAK1 as previously reported (He et al., 2007).

In conclusion, LRR-RLKs, the biggest family of RLKs in plants, participate in a huge diversity of signaling processes through the formation of complexes among individual members of different subfamilies. It seems clear that the LRR-RLKII subfamily to which BAK1 and BAK7 belong is prominently involved in complex formation. Elucidation of the developmental cues or environmental signals that drive the specific pairs of LRR-RLKs to form complexes at the correct time will be among the next challenges to overcome in our understanding of plant signaling processes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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