Functional divergence of $BAK1$ genes from $Brassica$ $rapa$ in regulating plant architecture

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ABSTRACT. BAK1 is a co-receptor of BRI1 in early signaling pathways mediated by brassinosteroids (BRs) and is thought to play a major role in plant growth and development. As the role of BAK1 has not yet been fully elucidated then further research is required to explore its potential for use in genetic modification to improve crops. In this study, three $BAK1$ genes from the amphidiploid species $Brassica$ $rapa$ were isolated and their kinase functions were predicted following DNA sequence analysis. A bioinformatic analysis revealed that two genes, $BrBAK1$-1 and $BrBAK1$-8, shared a conserved kinase domain and 5 tandem leucine-rich repeats (LRRs) that are characteristic of a BAK1 receptor for BR perception, whereas the third gene, $BrBAK1$-3, was deficient for a signal peptide, but had 4 leucine zippers and 3 leucine-rich repeats (LRRs) in an extracellular domain. All three $BrBAK1$ kinases localized on the cellular membrane. Ectopic expression of each $BrBAK1$ gene in BR-insensitive ($bri1$-5 mutant) Arabidopsis plants indicated that $BrBAK1$-1 and $BrBAK1$-8 were functional homologues of $AtBAK1$ based on the rescue of growth in the $bri1$-5 mutant. Overexpression of $BrBAK1$-3 caused a severe dwarf phenotype resembling the phenotype of null $BRI1$ alleles. The results here suggest there are significant differences among the three $BrBAK1$ kinases for their
effects on plant architecture. This conclusion has important implications for genetic modification of *B. rapa*.

**Key words**: *Brassica rapa*; Brassinosteroid; BAK1; LRR-RK

**INTRODUCTION**

Brassinosteroids (BRs) are important plant hormones that regulate multiple aspects of plant growth and development and play positive roles in plant defense mechanisms against biotic and abiotic stresses (Kauschmann et al., 1996). BRs can enhance cell elongation, promote differentiation of xylem vessels, increase ATPase activity, stimulate photosynthetic activity, adjust the generation and distribution of pores, change the balance of other endogenous phytohormones, increase the activity of antioxidant enzymes, and improve the ability to metabolize toxic compounds (Ryu and Hwang, 2013). The blocking of BR signaling pathways leads to serious deficiencies in plant growth and development. BR blockage causes abnormal phenotype in plants such as a dark green, dwarfed stature with epinastic round leaves and short petioles, male sterility, altered photomorphogenesis, delayed flowering, and senescence.

The specific receptor kinase for BRs was first cloned in the *bri1* mutant in *Arabidopsis* and named *BRASSINOSTEROID-INSENSITIVE 1* (BRI1) (Li and Chory, 1997). BAK1 (BRI1-associated kinase 1) was initially identified as a co-receptor of BRI1 in BR signaling (Li et al., 2002; Nam and Li, 2002). It is a leucine-rich repeat receptor kinase (LRR-RK) belonging to the LRR type II subfamily, which consists of 14 members in *A. thaliana* including SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (AtSERK1) and 4 homologous genes (Hecht et al., 2001). BAK1 is homologous to SERK1, and has been named SERK3. BAK1 has the typical structure of a plant receptor kinase, containing five LRR sequences of the extracellular domain, transmembrane region, and intracellular kinase domain.

Inactive BRI1 first interacts with BRs via the extracellular domain; this interaction triggers the rearrangement of the intracellular kinase domain, and may confer BRI1 basal kinase activity (Wang et al., 2005; Wang et al., 2008). Subsequently, the activated BRI1 releases the autoinhibitory C-terminal region and endogenous inhibitor protein, BRI1 kinase inhibitor (BKI1), and recruits BAK1 protein to form a co-receptor heterooligomer (Wang and Chory, 2006; Bucherl et al., 2013). In this complex, BRI1 and BAK1 transphosphorylate each other sequentially, which results in a fully activated pair of receptor kinases for amplifying and transducing BR signaling (Wang et al., 2008). BAK1 is therefore a major component in BR signal transduction.

Constitutive over-expression of BAK1 in an *A. thaliana* plant carrying the weak *bri1-5* allele suppresses the dwarf phenotype and enhances plant height and petiole elongation (Li et al., 2002; Nam and Li, 2002). BAK1 therefore plays an important role in BR-mediated regulation of plant growth and development (He et al., 2013). However, *bak1* knock-out mutants present a phenotype that cannot easily be distinguished from the wild type. Thus, there must be functional redundancy of BAK1 and other SERKs, especially SERK1 and SERK4 (also known as BKK1, BAK1 like 1) (Gou et al., 2012). When BAK1 and BKK1 were knocked out at the same time, the double-mutant plants had a seedling lethal phenotype that differed from that of *bri1* null alleles. These findings indicate that BAK1 has important functions in other pathways, independently of BR signal transduction, for regulating plant growth and development (Schwessinger et al., 2011).

To date, *Arabidopsis* BAK1 has been shown to be extensively involved in several signal transduction pathways, such as BR signal transduction (Chaparro-Garcia et al., 2011), plant innate immunity (Chinchilla et al., 2007; Oh et al., 2010; Chaparro-Garcia et al., 2011; Yang et al., 2011;
Kim et al., 2013; Segonzac et al., 2014), and cell death control (He et al., 2007; Kemmerling et al., 2007; Gao et al., 2009; Wang et al., 2011). The demonstration that BAK1 plays multiple critical roles in A. thaliana suggests that it likely plays pivotal roles in many aspects of crop plant growth and development, such as resistance to biotic and abiotic stresses, growth period, final plant size and type, and crop yield. However, only further investigations will show the range of activities of BAK1 in crop species and determine its value for use in genetic modification of these species.

Here, we studied the popular vegetable crop B. rapa, which like A. thaliana belongs to the family Brassicaceae. The complete genome sequence of B. rapa was obtained in 2011 making gene cloning and functional studies a practical reality. Investigation of BrBAK1s will promote understanding of the molecular mechanisms of crop growth and development and provide crucial information for use in cultivar improvement.

MATERIAL AND METHODS

Plant material and plant growth conditions

B. rapa plants were grown under field conditions. A. thaliana (ecotype WS2 and bri1-5) plants were grown in a phytotron at 22°C under 16-h light/8-h dark, unless otherwise specified. A. thaliana seeds were sterilized in 75% ethanol for 1 min and 15% chlorine bleach (10% available chlorine) for 15 min. After sterilization, seeds were rinsed 3-5 times with sterile water and germinated on plates containing one-half MS medium (Nissui, China), 2% sucrose, and 0.7% agar. Chinese cabbage (B. rapa cv. Qingjiang) was grown in a greenhouse at approximately 25°C.

Genomic DNA and total RNA isolation

Genomic DNAs were extracted as described by Dellaporta et al. (1983). Total RNA from B. rapa was isolated from leaves. The RNA isolation procedure followed the manufacturer instructions for TaKaRa RNAiso Plus (Japan).

Gene cloning and Arabidopsis transformation

Three genes with high sequence homology to A. thaliana BAK1 (AtBAK1) (Nam and Li, 2002; Li et al., 2002) were identified in the Brassica Database using the online BLAST program. B. rapa cDNA was used as a template; the primers are listed in Table 1. PCR products were extracted using a gel extraction kit (TIANGEN, China), and the purified DNA fragments were cloned into the KpnI and XbaI sites of the binary expression vector CaMV35S-HYG-GFP. Constructs were transformed into A. thaliana bri1-5 plants by the floral dipping method (Clough and Bent, 1998).

Table 1. Primers used in this study. Numbers in parentheses indicate amplicon sizes for the BrBAK1 genes.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-3′)</th>
<th>Amplicon size (bp)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrBAK1-1-Kpn-F</td>
<td>CCGGTACCATGGACGAGGATGAGATAG</td>
<td>1867</td>
<td>Cloning of ORF</td>
</tr>
<tr>
<td>BrBAK1-1-XbaI-R</td>
<td>CTTGGACCCGAGGGGTAA</td>
<td>1851</td>
<td></td>
</tr>
<tr>
<td>BrBAK1-3-Kpn-F</td>
<td>CCGGTACCATGGAGGATGAGATAG</td>
<td>1456</td>
<td></td>
</tr>
<tr>
<td>BrBAK1-3-XbaI-R</td>
<td>CTTGGACCCGAGGGGTAA</td>
<td>1440</td>
<td></td>
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<tr>
<td>BrBAK1-5-Kpn-F</td>
<td>CCGGTACCATGGAGGATGAGATAG</td>
<td>1861</td>
<td></td>
</tr>
<tr>
<td>BrBAK1-5-XbaI-R</td>
<td>CTTGGACCCGAGGGGTAA</td>
<td>1845</td>
<td></td>
</tr>
</tbody>
</table>
Western blot analysis

Proteins were extracted from leaves of 3- to 4-week-old seedlings with 1× SDS sample loading buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, and 5% β-mercaptoethanol). After boiling for 5 min, the lysate was centrifuged at 13,000 rpm for 5 min to remove debris. The proteins were quantified using Bio-Rad Protein Assay reagent (TIANGEN). Six micrograms of protein per lane was used for PAGE. The anti-green fluorescent protein antibody and secondary antibody anti-mouse were obtained from Transgen Biotech (China). All antibodies and detection reagents were used as suggested by the manufacturers.

Transient expression and subcellular localization in *Nicotiana benthamiana*

*Agrobacterium tumefaciens* GV3101 containing the respective constructs were grown for 2 days at 28°C in Luria broth medium supplemented with the appropriate antibiotics. Cultures were pelleted and resuspended in infiltration medium (0.5% d-glucose, 50 mM MES, 2 mM Na$_3$PO$_4$·12H$_2$O, 100 μM acetosyringone) to OD$_{600}$ = 0.5. Agrobacteria carrying different constructs were inoculated into the abaxial sides of the leaves of 3-week-old *N. benthamiana* using a 1 ml syringe without a needle (Sparkes et al., 2006). Samples were collected 3 to 4 days after inoculation.

Morphometric analysis

Approximately 20 *A. thaliana* seeds were planted in round pots (5.5 cm in diameter) with medium (Pindstrup Plus:Vermiculite, 1:1) presoaked in water; the pots were covered with plastic wrap. A growth chamber maintained at a constant 22°C with 16-h light/8-h dark was used. The plastic wrap was removed after the seedlings were established (5-7 days). The pots were sub-irrigated with water or nutrient solution as necessary. Plant height was measured to the nearest millimeter, and the lengths of siliques were measured to the nearest half-millimeter.

RESULTS

Sequence analysis of three *BAK1* genes from *B. rapa*

It is estimated that *Arabidopsis* and *Brassica* diverged from a common ancestral plant 17-18 mya (Yang et al., 2006). After separation, the *B. rapa* genome triplicated at approximately 13-17 mya (Yang et al., 2006). Therefore, three homologues of *AtBAK1* are predicted to exist in the *B. rapa* genome. This prediction was confirmed by the identification of three genes in a screen for high sequence homology to *A. thaliana* BAK1 in the *Brassica* database (http://brassicadb.org/brad/): gene IDs Bra037006, Bra011439, and Bra034562.

The three *BAK1* genes cloned from *B. rapa* cDNA were named *BrBAK1-1*, *BrBAK1-3*, and *BrBAK1-8* and were found to locate to chromosomes A01, A03, and A08, respectively, of the *B. rapa* genome. Their relative positions on the chromosomes are shown in Figure 1A. As mentioned above, *AtBAK1/SERK3* has 4 homologues and *SERK1*, *SERK2*, and *SERK4* show partial functional redundancy in mediating BR signaling. Alignments of the three *BrBAK1* genes with these 4 *Arabidopsis* genes using Vector NTI showed that *AtBAK1* was the closest ortholog (Figure 1B).
Analysis of function and structure showed that both \textit{BrBAK1-1} and \textit{BrBAK1-8} polypeptides include the functional domains present in \textit{AtBAK1}. The entire DNA sequences of \textit{BrBAK1-1} and \textit{BrBAK1-8} had 90.5 and 90.4\% identity, respectively, with \textit{AtBAK1}. The conserved amino acid residues were present at the expected positions in the deduced \textit{BrBAK1-1} and \textit{BrBAK1-8} protein sequences (Figure 1C) suggesting the two genes possess similar functions as \textit{AtBAK1}. By contrast, the entire DNA sequence of \textit{BrBAK1-3} had only 63.5\% identity with \textit{AtBAK1} and the kinase domain DNA sequence identity was just 75.1\%. The polypeptide sequence of \textit{BrBAK1-3} lacked the signal peptide and had only 4 leucine zippers and 3 LRRs in the extracellular domain (Figure 1D). In light of the differences between \textit{BrBAK1-3} and \textit{AtBAK1}, these proteins may show functional differentiation; \textit{BrBAK1} may have lost some of the biological function of \textit{AtBAK1}.

**Figure 1.** Characterization of \textit{BrBAK1} genes. \textbf{A.} The relative positions of the three \textit{BrBAK1} genes on \textit{Brassica rapa} chromosomes. \textbf{B.} Phylogenetic analysis of the \textit{AtSERK} gene family and \textit{BrBAK1} genes. A guide tree was generated using Align X (Vector NTI). The values in brackets represent the length of the tree. \textbf{C.} Multiple alignment of the deduced polypeptide sequences of \textit{AtBAK1}, \textit{BrBAK1-1}, \textit{BrBAK1-3} and \textit{BrBAK1-8}. Predicted functional domains are marked and labeled. Black and gray backgrounds represent identical and similar amino acid residues, respectively. \textbf{D.} Schematic diagram of the amino acid motifs of \textit{AtBAK1} and \textit{BrBAK1} proteins. The different shading and hatching of the boxes indicate the different motifs.
Subcellular localization of BrBAK1 proteins in vivo

Previous reports suggest that BAK1 is a transmembrane protein located in the plant cell membrane surface (Li et al., 2002; Nam and Li, 2002). Analysis of the three BrBAK1 protein sequences and their hydrophobicity profiles predicted a single transmembrane (TM) helix between the receptor-like part and the kinase domain, suggesting that all of the BrBAK1 proteins are transmembrane proteins that are likely anchored to the plasma membrane, in an analogous manner to the AtBAK1 protein. The subcellular localizations of BrBAK1 proteins were determined in vivo using constructs carrying translational fusions to green fluorescent protein (GFP), namely, p35S-BrBAK1-1-GFP, p35S-BrBAK1-3-GFP, and p35S-BrBAK1-8-GFP. Microscopic examination of N. benthamiana leaves transiently expressing these constructs revealed that all of the BrBAK1 proteins were localized on the plasma membrane (Figure 2).

Figure 2. Subcellular localization of BrBAK1 fused to GFP at its C-terminus. The construct was inoculated into leaves of N. benthamiana with Agrobacterium. Left panels, middle panels, and right panels show structure of cells from the same set using fluorescent light to visualize GFP, bright field, and the merged image, respectively. All of the BrBAK1 proteins localized to the plasma membrane. Bar indicates 10 μm.

Analysis of BrBAK1 functions in Arabidopsis

The mutation bri1-5 generated a “weak” allele of BRI1 and has some significant phenotypic differences compared to the original wild-type WS2 ecotype, such as a severe dwarf stature, round rosette leaves, and short petioles. Overexpression of functional AtBAK1 protein in bri1-5
partially suppresses the bri1-5 dwarf phenotype by promoting leaf petiole elongation and shaping a narrow leaf blade in the seedling stage. To confirm that the proteins encoded by BrBAK1 genes are functional, each of the three BrBAK1 coding sequences, under the control of a CaMV 35S promoter, was transformed into bri1-5 plants. Transgenic plants expressing BrBAK1 showed the expected specific target bands in the western blot analysis, while the negative control had no bands.

Compared with bri1-5 mutant plants, both bri1-5:BrBAK1-1 and bri1-5:BrBAK1-8 transgenic plants had significantly longer petioles and a partial suppression of the mutant dwarf phenotype (Figure 3A). The petiole length: leaf length ratio resembled that of bri1-5:AtBAK1, i.e., plants with ectopic expression of BrBAK1-1 were significantly different from the bri1-5 mutant. Although the BrBAK1-8 transgenic plants had obviously extended petioles, they did not show any significant difference in petiole length: leaf length ratios in contrast to the bri1-5 host plants. This suggests that the two BrBAK1 genes from B. rapa had a parallel function to AtBAK1 in promoting the elongation of petioles. However, overexpression of BrBAK1-3 could not restore the petiole length in the bri1-5 mutant (Figure 3B), and the plant phenotype resembled that of the bri1-5 mutant; we conclude that BrBAK1-3 does not function in promoting petiole elongation.

BrBAK1-1 and BrBAK1-8 genes also displayed similar function to AtBAK1 in leaf shape control. Constitutive over-expression of either B. rapa gene could be responsible for suppressing the rounded leaves phenotype of the bri1-5 mutant. Although BrBAK1-1 and AtBAK1 transgenic plants had narrow leaf shapes, their leaf length: width ratios had no significant differences compared to the bri1-5 mutant; a Student t-test indicated that the ratio between the BrBAK1-8 transgenic plants and bri1-5 mutant was significantly different (P < 0.05) (Figure 3C). This indicated that BrBAK1-8 had a stronger function in regulating leaf shape than AtBAK1 and the other two BrBAK1 genes. BrBAK1-3 transgenic plants had rounded leaves that resembled those of the bri1-5 mutant; leaf length: width ratios were even lower than those of the bri1-5 mutant. Thus, the BrBAK1-3 gene did not have a role in promoting blade elongation in Arabidopsis.

Interestingly, bri1-5 mutant plants show severe dwarfing at the bolting stage. This dwarf phenotype can be significantly restored by over-expression of functional AtBAK1. Overexpression of BrBAK1-1 or BrBAK1-8 promoted elongation of the main inflorescences and led to a large increase in plant height in transgenic lines (Figure 3D). The function of BrBAK1-1 in increasing plant height was significantly weaker than AtBAK1. Compared to AtBAK1 and BrBAK1-1 and BrBAK1-3 proteins, BrBAK1-8 was more effective in promoting inflorescence elongation, suppressing the bri1-5 dwarf phenotype, and enabling a plant height closer to the wild type. After ectopic expression of BrBAK1-3, the height of the transgenic plants was significantly smaller than bri1-5 mutant plants (P < 0.01) (Figure 3E). This finding confirmed that the BrBAK1-3 gene was incapable of promoting elongation of the main inflorescence in bri1-5 plants to increase their final height.

In addition to dwarfishm, mature bri1-5 plants also had abnormal siliques. Even constitutive overexpression of ectopic AtBAK1 in bri1-5 had little restorative effect on phenotype. However, both BrBAK1-1 and BrBAK1-8 caused an increase in the longitudinal growth of the siliques. Thus, BrBAK1-8 transgenic plants had a significant increase in the length of the siliques compared with bri1-5 (P < 0.01). Moreover, both BrBAK1-1 and BrBAK1-8 had a stronger effect on promoting silique elongation than AtBAK1. BrBAK1-3 transgenic plants produced only an average of 7 siliques per plant. The siliques were flat and rarely contained seeds; the lengths of these siliques were significantly shorter than bri1-5 plants (P < 0.01) (Figure 3F). This result suggested that BrBAK1-3 gene overexpression did not promote silique growth, but did disturb the normal development of the siliques leading to plant sterility.
In conclusion, based on the observation of the phenotypes of transgenic plants, BrBAK1-8 had the strongest regulatory function in narrowing leaves, promoting main inflorescence elongation, and accelerating the silique longitudinal growth; consequently, BrBAK1-8 rescued Arabidopsis bri1-5 mutant plants better than AtBAK1. Only with regard to promoting petiole elongation did BrBAK1-1 show a stronger effect than BrBAK1-8. However, BrBAK1-3 did not show a positive function in any aspect of these sections, and its ectopic expression gave rise to sterility of the transgenic plants. The experimental results demonstrated that there was considerable functional differentiation among the three BrBAK1 receptor kinases.

Figure 3. Overexpression of BrBAK1 in bri1-5 mutant Arabidopsis plants. A. Partial suppression of the effect of the bri1-5 mutation on leaf and petiole lengths at the seedling stage. Top panel shows the phenotypes of transgenic plants harboring 35S::BAK1-GFP or 35S::BrBAK1-GFP. From the top left, the first three plants are wild-type (WS2 ecotype), bri1-5:AtBAK1-GFP, and bri1-5 which was used as the recipient for transformation. The next three rows show transgenic plants harboring 35S::BrBAK1-1-GFP, 35S::BrBAK1-3-GFP and 35S::BrBAK1-8-GFP, respectively. AtBAK1, BrBAK1-1, and BrBAK1-8 overexpression lead to a BRI1-overexpression phenotype, while overexpression of BrBAK1-3 on the bri1-5 background resulted in a dominant-negative phenotype. All plants were grown for 5 weeks and then photographed. Bottom panel shows the corresponding transgene products. Anti-GFP polyclonal antibodies were used as primary antibodies and anti-mouse rabbit serum as the secondary antibodies. B, C. Seedlings were grown for 5 weeks and then measured. Quantitative analyses of leaf length, width and petiole length were made on at least 10 representative BrBAK1 transgenic lines. Data represent means and standard errors. The asterisks indicate significant differences compared to the bri1-5 mutant (*P < 0.05 and **P < 0.01 by the t-test). D. Rescue of normal growth by ectopic expression of BrBAK1-1 and BrBAK1-8 in dwarf brassinosteroid insensitive bri1-5 Arabidopsis plants; overexpression of BrBAK1-3 led to a serious dwarf phenotype. Seedlings were grown for 9 weeks and then photographed. E, F. Measurements of plant height and silique length were made on at least 10 plants, and the silique length was measured using 10 siliques from each plant except the BrBAK1-3 transgenic plants that had only 7 siliques on average per plant. Data represent mean and standard errors. BrBAK1-1 and BrBAK1-8 rescued the bri1-5 in a similar fashion to AtBAK1. The asterisks indicate significant differences compared to the bri1-5 mutant (*P < 0.05 and **P < 0.01 by the t-test).
DISCUSSION

In this study, three BrBAK1 genes were cloned, and their functions were studied in the model plant Arabidopsis. The experiments showed that BrBAK1-1 and BrBAK1-8 proteins were functional in BR signal transduction, and that both had the ability to regulate leaf and petiole development processes in plants. Previous studies of AtBAK1 crystal structure showed that the extracellular domain of the BAK1 protein was indispensable for BL, BRI1, and SERK1 to combine in the BR signaling pathway (Santiago et al., 2013; Sun et al., 2013). Considering the absence of an extracellular domain in the BrBAK1-3 protein and the similarity of the phenotype of plants with ectopic BrBAK1-3 expression to that of the dominant-negative phenotype of AtBAK1, then this BAK1 protein likely has no function in BR signal transduction pathways. As mentioned above, previous studies indicated that BAK1 is a ubiquitous adaptor protein involved in various signal transduction pathways including BR signaling, PAMP-triggered immunity, cell-death control, and anther development regulation. Although recent research on the structural basis of flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex revealed that the 5 LRRs of the BAK1 extracellular domain directly interact with FLS2 but are also an indispensable component in the recognition of the C terminus of FLS2-bound flg22 (Sun et al., 2013). We speculate that among the various complex interactions there may be some receptor proteins that are independent of the BAK1 extracellular domain and may instead be involved in pathways mediated by BrBAK1-3.

Our analyses show that there are significant differences among the three BrBAK1 kinases in their roles in regulating plant size and type, and plant architecture. This information will be of value for future molecular genetic approaches to breeding of B. rapa. However, given that bak1 mutants have yet to be found in B. rapa and that generating transgenes is still problematical, it will be difficult in the immediate future to examine in more detail the roles of these three genes in B. rapa growth and development. Future studies that differentiate the functions of the three BAK1 proteins will help unravel the mechanisms of BAK1-mediated metabolic pathways.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES


