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Report

BAK1 and BKK1 Regulate Brassinosteroid-Dependent Growth and Brassinosteroid-Independent Cell-Death Pathways

Kai He,[1] Xiaoping Gou,[1] Tong Yuan,[1] Honghui Lin,[1,2] Tadao Asami,[3] Shigeo Yoshida,[3] Scott D. Russell,[1] and Jia Li[1,*]

[1] Department of Botany and Microbiology
University of Oklahoma
Norman, Oklahoma 73019

[2] College of Life Science
Sichuan University
Chengdu
P.R. China 610064

[3] RIKEN
2-1 Hirosawa
Wako, Saitama 351-0198
Japan

Summary

Brassinosteroids (BRs) are phytosteroid hormones controlling various physiological processes critical for normal growth and development. BRs are perceived by a protein complex containing two transmembrane receptor kinases, BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) [1–3]. *BRI1* null mutants exhibit a dwarfed stature with epinastic leaves, delayed senescence, reduced male fertility, and altered light responses. *BAK1* null mutants, however, only show a subtle phenotype, suggesting that functionally redundant proteins might be present in the *Arabidopsis* genome. Here we report that BAK1-LIKE 1 (BKK1) functions redundantly with BAK1 in regulating BR signaling. Surprisingly, rather than the expected *bri1*-like phenotype, *bak1 bkk1* double mutants exhibit a seedling-lethality phenotype due to constitutive defense-gene expression, callose deposition, reactive oxygen species (ROS) accumulation, and spontaneous cell death even under sterile growing conditions. Our detailed analyses demonstrate that BAK1 and BKK1 have dual physiological roles: positively regulating a BR-dependent plant growth pathway, and negatively regulating a BR-independent cell-death pathway. Both BR signaling and developmentally controlled cell death are critical to optimal plant growth and development, but the mechanisms regulating early events in these pathways are poorly understood. This study provides novel insights into the initiation and cross-talk of the two signaling cascades.

Results and Discussion

The model plant *Arabidopsis* contains a large family of proteins called leucine-rich-repeat receptor-like protein kinases (LRR-RLKs). A typical LRR-RLK contains a ligand-binding extracellular LRR domain and a cytoplasmic serine/threonine kinase domain. There are at least 223 LRR-RLKs in the *Arabidopsis* genome [4], only a handful of which have been functionally characterized. Those characterized have been shown to play critical roles in various processes directly modulating growth and development, as well as immunity responses [5–7]. The discoveries of two LRR-RLKs, BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), in controlling the early event of the brassinosteroid (BR)-signaling pathway, suggested that the heterodimerization of distinct LRR-RLKs after ligand binding could be a central paradigm in the activation of LRR-RLK-mediated signaling cascades [1–3].

The biological significance of BRI1 has been well documented. Genetic analyses indicated that *bri1* null alleles are extremely dwarfed and are insensitive to exogenous brassinolide (BL), the most active BR, but remain sensitive to other known phytohormones [8]. Biochemical analyses demonstrated that the island segment and the 22nd LRR of the BRI1 extracellular domain together confer BR-binding activity [9, 10], whereas the kinase domain of BRI1 activates downstream components by protein phosphorylation. BAK1 is a distinct LRR-RLK that contains only five LRRs and lacks the island region within its LRRs. BAK1 physically interacts with BRI1 in vivo [2, 3, 11]. In addition, BRI1 and BAK1 are able to phosphorylate each other. Both the interaction and the phosphorylation of BRI1 and BAK1 are BR dependent [12]. Unlike BRI1, however, null alleles of BAK1 display subtle *bri1*-like phenotypes, suggesting at least one additional protein in *Arabidopsis* is functionally redundant with BAK1 [2, 3].

BAK1 belongs to the LRR type II subfamily, which contains 14 members [13], five of which were previously named SERK1 to SERK5 (Figure 1A; [14]) because of the similarity of their protein structures to that of the carrot DcSERK [15]. Phylogenetic analysis indicated that SERK4 and SERK5 are the two closest paralogs of BAK1/SERK3 (Figure 1A), and they might have biological functions similar to BAK1. Because overexpression of *BAK1* is able to suppress a weak *bri1* allele, *bri1-5* [2], it was predicted that a *BAK1* redundant gene would also suppress *bri1-5* phenotypes when overexpressed. *SERK4* and *SERK5* were subsequently transformed into *bri1-5* to test whether they can suppress *bri1-5* upon overexpression. Like *BAK1*, *SERK4*—but not *SERK5*—was able to partially rescue *bri1-5* when overexpressed (Figures 1B and 1C). *SERK4* was subsequently renamed *BAK1-LIKE 1* (*BKK1*). Further analyses indicated that Col-0 SERK5 bears an amino acid substitution of Leu for Arg at position 401 within the critical arginine/aspartate (RD) kinase motif, and this substitution might block the function of Col-0 SERK5 in the BR signaling pathway (Figure S1 in the Supplemental Data available online; [16]). Interestingly, the same mutation was not found in SERK5 from other *Arabidopsis* ecotypes, such as Wassilewskiji 2 (WS2) (data not shown).

*Correspondence: lij@ou.edu

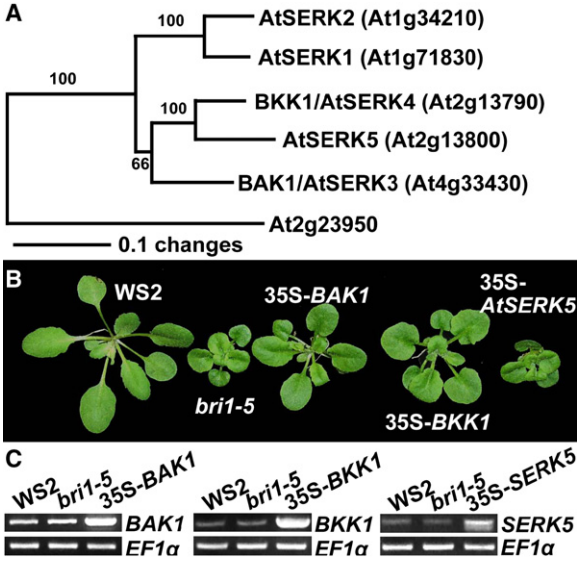

Figure 1. *BKK1* Plays a Redundant Role with *BAK1* in Suppressing *bri1-5* When Overexpressed

(A) Phylogenetic analysis suggesting BKK1/SERK4 is the closest paralog of BAK1 in *Arabidopsis*.
(B) Overexpression of *BAK1* or *BKK1*, but not *SERK5*, suppresses the phenotypes of *bri1-5*.
(C) RT-PCR analyses to confirm the elevated expression of the transgenes in the transgenic plants.

To examine the genetic significance of BAK1 and BKK1 in a real physiological setting, we isolated single T-DNA knockout lines from SALK T-DNA insertion pools. Several knockout lines were obtained for both genes. Only the lines directly relevant to this work are shown (Figure 2A). Whereas a *BAK1* null allele, *bak1-4*, showed a subtle *bri1*-like phenotype, a *BKK1* null allele, *bkk1-1*, did not exhibit any defective phenotypes (Figures 2B–2E). The double-null mutant, *bak1-4 bkk1-1*, however, illustrated extremely dwarfed phenotypes, distinct from typical *bri1* null mutants, such as *bri1-4*. During the first 4 days after germination, the double-null mutant showed no observable defective phenotypes compared to those of wild-type plants. A week after the germination, however, shoot apical meristem (SAM) growth of the double mutant almost completely ceased (Figure S2). Ten days after germination, the seedlings showed early senescence symptoms starting at the cotyledons. Careful analysis of embryos from the progeny of *bak1-4*$^{+/-}$ *bkk1-1*$^{-/-}$ or *bak1-4*$^{-/-}$ *bkk1-1*$^{+/-}$ plants failed to identify any abnormal embryos during embryogenesis. It was expected that 25% of the embryos would be aberrant if *bak1-4 bkk1-1* double mutations did affect zygotic embryogenesis. The onset of seedling lethality is, therefore, a postembryonic phenotype probably controlled by unknown developmental cues. The phenotypes observed in the double mutant were reproduced with different *bak1* and *bkk1* allele combinations from a Col-0 background (Figure S3) and were restored to wild-type-like seedlings by the expression of either *BAK1* or *BKK1* (Figures 2F and 2G). Various genetic segregation analyses further demonstrated that the observed phenotype was solely caused by the double *BAK1* and *BKK1* knockouts (Table S1).

The overexpression of *BKK1* suppresses *bri1-5* phenotypes, suggesting that BKK1 has a role in the BR signaling. To further substantiate the function of BKK1 in

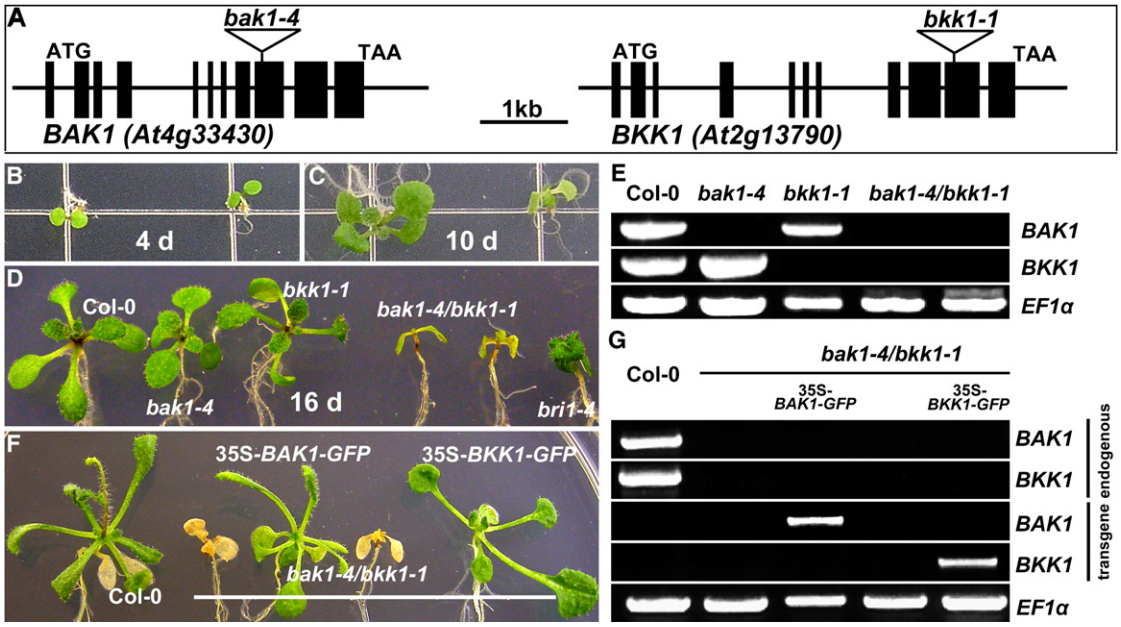

Figure 2. A *bak1-4 bkk1-1* Double-Null Mutant Shows a Seedling-Lethality Phenotype at an Early Developmental Stage

(A) T-DNA insertion sites of single knockout lines, *bak1-4* (SALK_116202) and *bkk1-1* (SALK_057955). Both are in a Col-0 background.
(B–C) Phenotypes of wild-type (in Col-0, left side) and double-null mutant (right side) seedlings at different developmental stages after germination.
(D) Phenotypes of wild-type, *bak1-4* and *bkk1-1* single-mutant, *bak1-4 bkk1-1* double-mutant, and *bri1-4* mutant seedlings.
(E) RT-PCR analyses to confirm the genotypes shown in (D). Genotypic analysis of *bri1-4* is not included.
(F) Overexpression of either *BAK1* or *BKK1* driven by the 35S promoter completely rescues the lethal *bak1-4 bkk1-1* double-null phenotypes.
(G) RT-PCR analysis verifies the genotypes of the plants shown in (F).

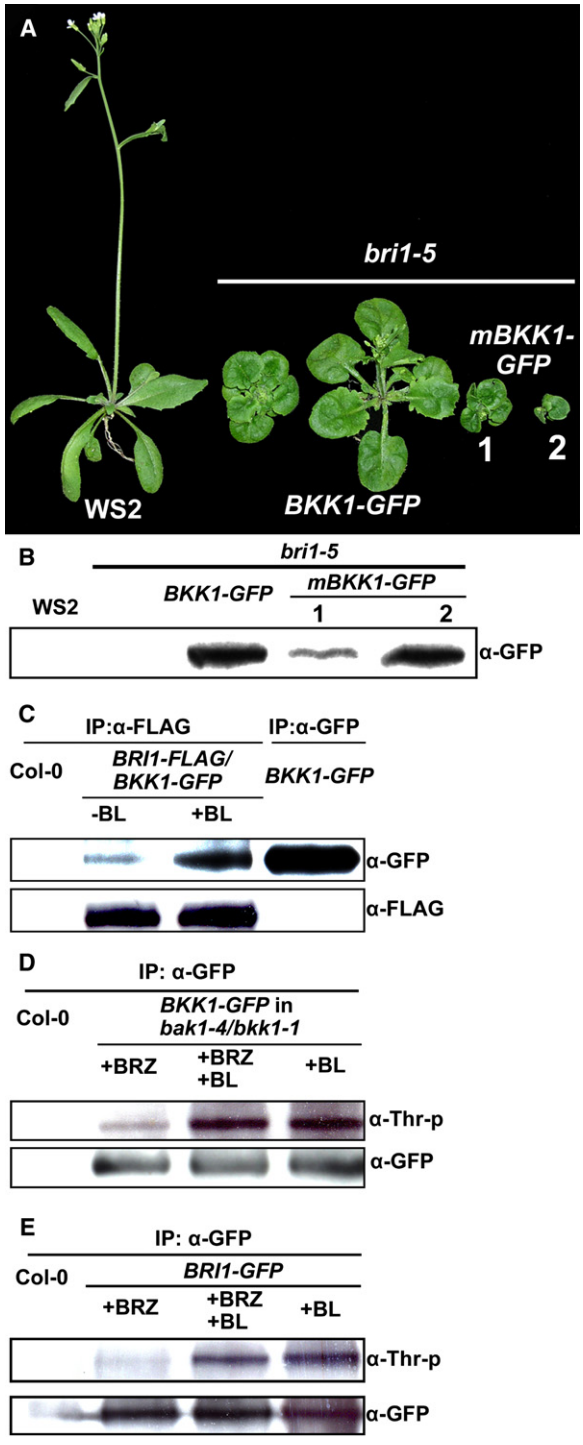

Figure 3. BKK1 Interacts with BRI1 and Mediates BR Signal Transduction

(A) The overexpression of *BKK1-GFP* suppresses *bri1-5* phenotypes, whereas the overexpression of *mBKK1-GFP* results in a dominant-negative effect, which greatly enhances *bri1-5* defective phenotypes.

(B) The severity of the transgenic plant phenotype is apparently correlated to the mBKK1-GFP protein levels. An equal amount of total protein was used for each sample for a western analysis to show differently expressed mBKK1-GFP levels.

(C) BKK1 interacts with BRI1 in vivo, and the interaction is BL enhanced. Wild-type and transgenic seeds harboring 35S-*BRI1-FLAG* and 35S-*BKK1-GFP* in Col-0 were grown in liquid culture

the BR pathway, we conducted a series of tests. To begin, we generated a *BKK1* kinase-dead mutant, *mBKK1* (K322E), and introduced it into *bri1-5*. Over 50% of the transgenic plants displayed dominant-negative phenotypes, with phenotypic severity positively correlated with the expression levels of the transgene (Figures 3A and 3B). The most severe plants resembled the phenotypes of null *bri1*, characterized by extreme dwarfism and complete male sterility (data not shown). This result suggested that BKK1 might associate with BRI1 in vivo; this was subsequently confirmed by a coimmunoprecipitation analysis with transgenic plants harboring *35S-BKK1-GFP* and *35S-BRI1-FLAG* (Figure 3C). The interaction was greatly enhanced by exogenously applied BL. Moreover, biochemical analysis indicated that the in vivo phosphorylation levels of BKK1 were also regulated by exogenously applied BL (Figure 3D), similar to that of BAK1 [12] and BRI1 (Figure 3E). The depletion of endogenous BRs by the treatment of seedlings (*BKK1-GFP* in *bak1-4 bkk1-1* and *BRI1-GFP* in Col-0) for a week with the specific BR-biosynthesis inhibitor brassinazole (BRZ) [17] showed basal levels of threonine phosphorylation on both BKK1 and BRI1. A 90 min treatment of these BRZ-pretreated seedlings with BL greatly increased phosphorylation levels on their threonine residues. These data demonstrated that BKK1 has biochemical properties similar to those of BAK1 in regulating the BR signal transduction. To further verify the physiological role of BKK1 in the BR signaling, we grew double and single mutants in the darkness. A typical BR mutant shows a de-etiolated phenotype when grown in darkness, including shortened hypocotyls and opened cotyledons. *bak1-4*, but not *bkk1-1*, showed weak de-etiolated phenotypes. Although the double mutants did not show additive effect on hypocotyl growth compared to that of the *bak1* single mutant, they showed enhanced cotyledon opening phenotype similar to that of the null *bri1* mutant, *bri1-4* (Figures S4A–S4F). These results confirmed that BKK1 has a clear role in the BR signal transduction, but there should be

[12]. One week after germination, one flask of seedlings was treated with mock (dimethyl sulfoxide [DMSO]), and a duplicated flask of seedlings was treated with 1 μM BL. The membrane fractions from these two treatments were extracted and immunoprecipitated with α-FLAG. The coimmunoprecipitated BKK1-GFP was detected by a western blot with α-GFP. A duplicated blot was hybridized with α-FLAG to confirm equal loading of the immunoprecipitated BRI1-FLAG (bottom panel). BKK1-GFP, immunoprecipitated with α-GFP from single-transgenic plants harboring 35S-BKK1-GFP, was used as a size reference.

(D) Exogenous application of BL increases the phosphorylation level of BKK1. Liquid-cultured seedlings harboring 35S-*BKK1-GFP* in a *bak1-4 bkk1-1* double-null background were either treated or untreated with 1 μM BL, after treatment with 1 μM BRZ to deplete endogenous BRs in liquid culture. Levels of threonine phosphorylation were detected with phosphoThr antibody (upper panel). The same amount of transgenic seedlings, as well as nontransgenic Col-0 as a negative control, were harvested and immunoprecipitated with anti-GFP antibody. Equal amounts of BKK1-GFP proteins were used, as demonstrated in a duplicated immunoblot with an anti-GFP antibody as shown in the lower panel.

(E) Exogenous BL application elevates the phosphorylation level of BRI1. Seedlings harboring *35S-BRI1-GFP* in Col-0 were used for the experiments. The treatments and detection were similar to those described in (D).

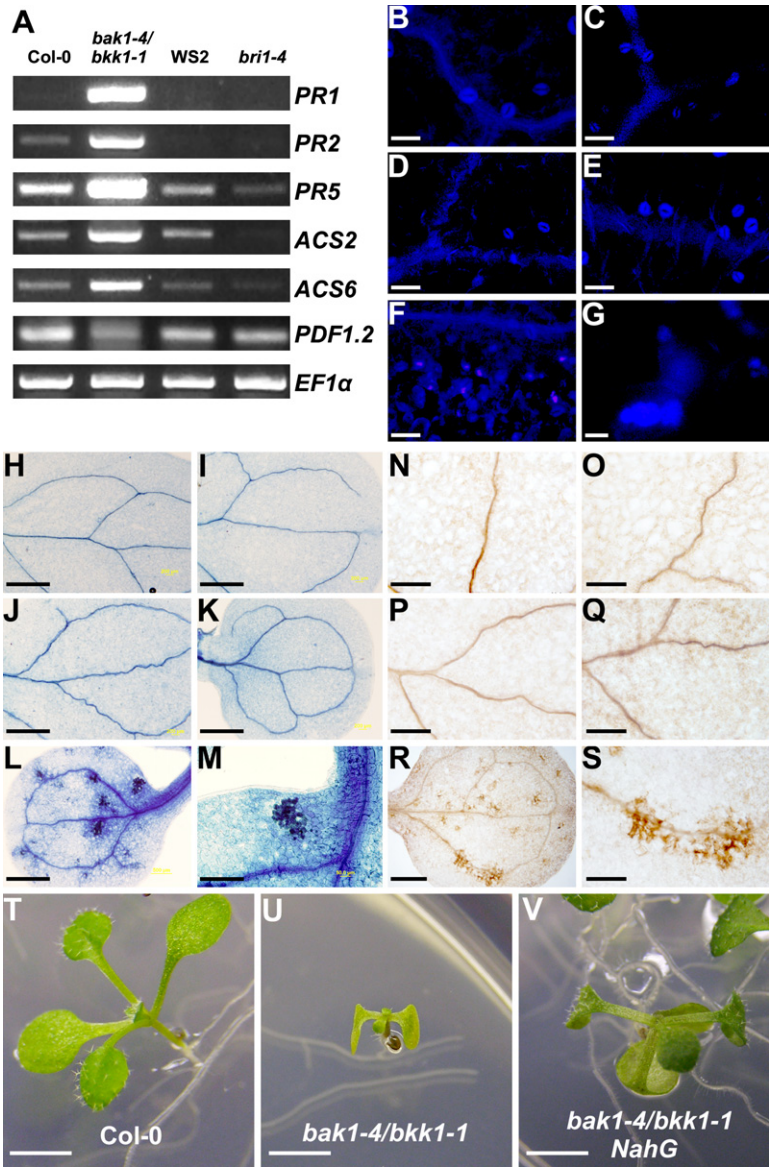

Figure 4. BAK1 and BKK1 Are Also Involved in a BR-Independent Cell-Death Signaling Pathway

(A) A number of defense-related genes are either upregulated or downregulated in *bak1-4 bkk1-1* relative to its background Col-0 plants, whereas these genes are not affected or are oppositely regulated in *bri1-4* compared to those of its background, WS2.

(B–G) Aniline-blue-stained cotyledons of 8-day-old seedlings grown on ½ Murashige and Skoog medium (MS) plates under sterile conditions. Only vascular tissues and guard cells were stained in wild-type (Col-0, [B]), *bak1-4* (C), *bkk1-1* (D), and *bri1-4* (E) seedlings. Additional clustered signals on *bak1-4 bkk1-1* (F and G) double mutants were stained, indicating callose accumulation, a typical spontaneous defense response.

(H–M) Trypan-blue-stained cotyledons of 8-day-old seedlings grown on ½ MS medium under sterile conditions. No cell death was found in cotyledons of wild-type (Col-0, [H]), *bak1-4* (I), *bkk1-1* (J), and *bri1-4* (K) seedlings. Significant microscopic mesophyll cell death (shown as blue-stained cells) was detected in the cotyledons of the *bak1-1 bkk1-1* double mutants (L and M).

(N–S) DAB-stained cotyledons of 8-day-old seedlings grown on ½ MS medium under sterile conditions. H_2O_2 was only detected in the vascular tissues of wild-type (Col-0, [N]), *bak1-4* (O), *bkk1-1* (P), and *bri1-4* (Q) seedlings; relatively little was detected in their mesophyll cells. H_2O_2 accumulation was observed in the clustered mesophyll cells of the *bak1-1 bkk1-1* double mutants near the vascular tissues (R and S).

(T–V) Expression of bacterial *NahG* in the double mutant partially rescues its seedling-lethality phenotype. Phenotypes of 9-day-old Col-0 (T), *bak1-4 bkk1-1* (U), and *bak1-4 bkk1-1 NahG* (V) seedlings.

Scale bars represent 50 μm (B–F), 10 μm (G), 40 μm (H–K), 150 μm (L), 40 μm (M), 1 mm (N–R), 200 μm (S), and 2 mm (T–V).

additional proteins, aside from BAK1 and BKK1, involved in BR signal transduction. It was recently reported that SERK1 was part of the BRI1 and BAK1 complex and that it might also participate in BR signal transduction [18]. Reverse-transcription polymerase chain reaction (RT-PCR) analysis indicated that *BKK1* is expressed considerably less than are *BAK1* and *SERK1* in darkness, and this explains why BKK1 plays less of a role in controlling hypocotyl growth under the dark condition (Figure S4C). Because the *bak1-4 bkk1-1* double mutant showed a seedling-lethality phenotype, a triple mutant was not generated in this study.

To investigate the molecular mechanisms leading to the seedling-lethality phenotype of *bak1-4 bkk1-1*, we employed Affymetrix arrays to compare the global gene expression patterns of the 8-day-old double mutant with those of the Col-0 wild-type. Among the most significantly upregulated genes were those involved in defense responses. Representative upregulated defense- and senescence-related genes, *PR1*, *PR2*, *PR5*, *ACS2*, *ACS6*, and the downregulated gene, *PDF1.2*, were further analyzed by RT-PCR to examine whether the expression patterns were similar to those shown in *bri1-4*. If BAK1 and BKK1 are involved exclusively in the BR signaling, one would expect these genes to show similarly dramatic expression changes in both *bri1-4* and *bak1-4 bkk1-1*. Interestingly, these defense- and senescence-related genes showed either no or opposite expression changes in *bri1-4* (Figure 4A). Because extreme dwarfism, constitutive defense-gene expression, early senescence, and seedling lethality are common phenotypes of cell-death mutants [19, 20], various tissue-staining approaches were conducted to determine whether cell death is involved in the double-mutant seedlings. Interestingly, callose deposition (aniline-blue staining [21]), cell death (trypan-blue staining [22]), and the accumulation of reactive oxygen species (ROSs) (3, 3′-diaminobenzidine [DAB] staining [23]) were all seen in the double-mutant seedlings but not in other seedlings from the same developmental

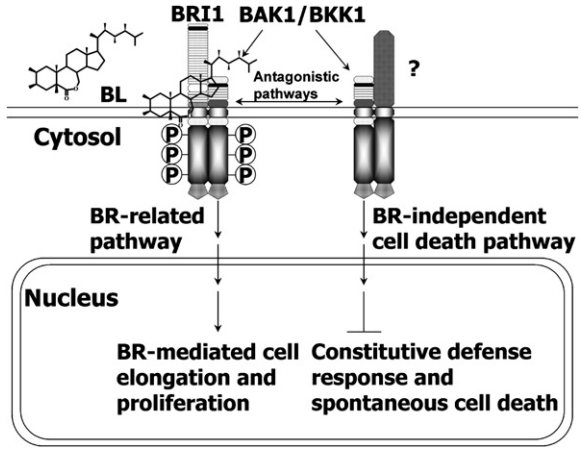

Figure 5. A Proposed Model Indicating that BAK1 and BKK1 Positively Regulate a BR Signaling Pathway and Negatively Regulate a Spontaneous Cell-Death Pathway

Cell death was observed on *bak1-4*$^{-/-}$ *bkk1-1*$^{-/-}$ seedlings grown under sterilized conditions, suggesting that the death signal is produced by the plant itself. The production of this cell-death signal is likely controlled by unknown developmental cues.

stages (Figures 4B–4S). Many cell-death mutants, such as *lsd1*, showed a cell-death phenotype in a salicylic acid (SA)-dependent manner [24]. To test whether the cell-death phenotype observed in *bak1-4 bkk1-1* was also SA-dependent, we introduced the bacterial *NahG* gene into the double mutant by performing a genetic crossing with a transgenic Col-0 *Arabidopsis* plant expressing *NahG* [25]. *NahG* encodes a salicylate hydroxylase that converts SA to catechol. The presence of the *NahG* transgene partially rescues the *bak1-4 bkk1-1* double-mutant phenotype, suggesting that the cell-death phenotype in the double mutant was likely SA-dependent (Figures 4T–4V).

Our extensive analyses demonstrated that BAK1 and BKK1 are not only involved in a BRI1-mediated pathway, but also involved in a BR-independent signaling pathway. The two pathways mediated by BAK1 and BKK1 showed some antagonistic features. For example, null *bri1* mutants usually displayed prolonged life spans and a dark-green phenotype, whereas *bak1 bkk1* double-mutant plants exhibited shortened a life span, early cotyledon senescence, and a cell-death phenotype. Expressions of senescence-related genes such as *ACS2* and *ACS6* are reduced in null *bri1* mutants but enhanced in *bak1 bkk1* double-null seedlings, relative to their wild-type backgrounds. The seedling-lethality phenotype of the double mutant is likely a consequence of the blocking of a second unknown BR-independent pathway.

We hypothesize that BAK1 and BKK1 regulate BR-dependent and BR-independent pathways via an alternating interaction with BRI1 and/or another defense-related LRR-RLK (Figure 5). In wild-type plants, BAK1 and BKK1 positively regulate the BR-mediated cell-growth pathway and negatively regulate a defense-related cell-death pathway. In *bak1-4 bkk1-1*, the cell-death pathway is constitutively activated, leading to spontaneous cell death. This hypothesis is partially supported by an independent study from Nürnberger and colleagues [26], who recently found that BAK1 knockout alleles, *bak1-3* and *bak1-4*, were more susceptible than the wild-type to several different pathogens. They similarly concluded that BAK1 was probably involved in a BR-independent immunity pathway [26]. This model can also be used to explain several early observations. For example, it was reported that overexpression of *CPD*, a key gene regulating multiple steps in BR biosynthesis, could induce the expression of a number of defense-related genes [27]. Early experiments also indicated that the application of BL induced senescence. It is likely that when the BR signaling pathway was enhanced, its antagonistic pathway was automatically reduced, leading to the upregulation of defense-related genes and cell death (Figure 5). Developmentally controlled programmed cell death is critical for normal plant growth and development, as well as a defense against numerous biotic and abiotic stresses. It would be interesting to further define the second signaling pathway in which BAK1 and BKK1 are involved. If the hypothesis is correct, the alternative BAK/BKK1 binding partner should have roles in plant defense and/or cell-death control. To date, at least two LRR-RLKs are known to be involved in plant defense against pathogens in *Arabidopsis*. For instance, ERECTA has roles in both plant development and immunity [28, 29], and FLS2 is involved in defense responses [30]. In the future, it will be intriguing to investigate whether BAK1 and BKK1 dimerize with ERECTA or FLS2 to mediate their corresponding signaling pathways.

Experimental Procedures

Materials and Plant Growth Conditions

bri1-5 is in ecotype WS2. *bak1-4* (SALK_116202), *bkk1-1* (SALK_057955), and *NahG* transgenic plants were all in Col-0. Plants were grown at 22°C under 16 hr light/8 hr dark, unless otherwise specified.

Gene Cloning and *Arabidopsis* Transformation

The *BAK1* expression vector used was the same as previously reported [2]. *BKK1* and *AtSERK5* cDNAs were amplified by RT-PCR from Col-0. The following primers were used: BKK1-fw 5′-TCTAGATCTATGGAACAAAGATCACTCCTTTGCT-3′, BKK1-rv 5′-TCTAGATCTTTATCTTGGACCCGAGGGGTAATCGT-3′, *AtSERK5*-fw 5′-TCTAGATCTATGGAACATGGATCATCCCGTGGCT-3′, and *AtSERK5*-rv 5′-TCTAGATCTTTATCTTGGCCCCGAGGGGTAATCGT-3′. The PCR products were cloned into the KpnI site of the binary vector *pBIB-BASTA-35S*. Constructs were transformed into *bri1-5* by the floral dipping method [31].

BAK1 and *BKK1* cDNAs were also cloned into the KpnI and BamHI sites of the binary vector *pBIB-BASTA-35S-GFP* with the primers *BAK1*-fw 5′-TCTAGATCTATGGAACGAAGATTAATGATCCCT-3′, *BAK1*-rv 5′-TCTGGATCCTCTTGGACCCGAGGGGTATTCGTT-3′, *BKK1*-fw and *BKK1*-rv2 5′-TCTGGATCCTCTTGGACCCGAGGGGTAATCGT-3′. The constructs were all confirmed by sequencing analysis.

RT-PCR Analysis

Two micrograms of total RNA was reverse transcribed in a 20 μl volume with Superscript III reverse transcriptase (Invitrogen). Two microliters of first-strand cDNA was used for RT-PCR with Ex Taq polymerase (Takara). A preliminary experiment was performed to determine the exponential range of each individual gene. The PCR cycles used were *BAK1* 22, *BKK1* 22, *AtSERK5* 28, *PR1* 30, *PR2* 30, *PR5* 22, *ACS2* 26, *ACS6* 22, *PDF1.2* 30, and *EF1α* 19, respectively. The primers for *BAK1* and paralogs were the same as those used for cloning. The primers for defense-related genes and senescence-related genes were the same as previously reported [32–34].

Protein Extraction and Immunoprecipitation

Five grams of liquid-cultured seedlings was ground in liquid N_2 as previously described [12]. Various treatments with 1 μM BRZ or 1 μM BL and membrane-protein isolation were the same as previously reported [2, 12]. BKK1-GFP was immunoprecipitated from solubilized total membrane protein with anti-GFP mouse antibody (Invitrogen) followed by a pull-down process with protein G beads (Roche).

Western-Blot Analysis

GFP-immunoprecipitated membrane proteins were separated with 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Various antibodies of α-GFP, α-FLAG, and α-phosphothreonine and western-analysis procedures were all the same as previously described [2, 12].

Site-Directed Mutagenesis

BKK1 was cloned into the Gateway donor vector pDONR/zeo (Invitrogen) to generate pENTR-*BKK1*. PCR was conducted with pENTR-*BKK1* as a template and primers 5′-AATCTAGTGGCTGTCGAAAGG CTAAAAGAAGAA-3′ and 5′-TTCTTCTTTTAGCCTTTCGACAGCCAC TAGATT-3′. The mutation was confirmed by sequencing analysis. The obtained pENTR-*mBKK1* was further cloned into pBIB-BASTA-35S-*GFP* with a Gateway strategy for transformation.

Tissue Stainings

Tissue stainings with aniline blue (0.01%, Sigma), trypan blue (1.25mg/ml, Sigma), and DAB (1mg/ml, Sigma) were the same as previously reported [19, 22, 23].

Supplemental Data

Experimental Procedures, four figures, and one table are available at http://www.current-biology.com/cgi/content/full/17/13/1109/DC1/.

Acknowledgments

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