

The BRI1-Associated Kinase 1, BAK1, Has a Brassinolide-Independent Role in Plant Cell-Death Control

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Summary

Programmed cell death (PCD) is a common host response to microbial infection [1–3]. In plants, PCD is associated with immunity to biotrophic pathogens, but it can also promote disease upon infection by necrotrophic pathogens [4]. Therefore, plant cell-suicide programs must be strictly controlled. Here we demonstrate that the *Arabidopsis thaliana* Brassinosteroid Insensitive 1 (BRI1)-associated receptor Kinase 1 (BAK1), which operates as a coreceptor of BRI1 in brassinolide (BL)-dependent plant development, also regulates the containment of microbial infection-

induced cell death. BAK1-deficient plants develop spreading necrosis upon infection. This is accompanied by production of reactive oxygen intermediates and results in enhanced susceptibility to necrotrophic fungal pathogens. The exogenous application of BL rescues growth defects of *bak1* mutants but fails to restore immunity to fungal infection. Moreover, BL-insensitive and -deficient mutants do not exhibit spreading necrosis or enhanced susceptibility to fungal infections. Together, these findings suggest that plant steroid-hormone signaling is dispensable for the containment of infection-induced PCD. We propose a novel, BL-independent function of BAK1 in plant cell-death control that is distinct from its BL-dependent role in plant development.

Results

Plant receptor-like kinases (RLKs) belong to the monophyletic interleukin-1 receptor-associated kinase (IRAK) or RLK/Pelle family [5]. Several leucine-rich repeat (LRR)-RLKs have roles in plant growth and development [6–8]. For example, *Arabidopsis* Brassinosteroid Insensitive 1 (BRI1), the receptor for the plant steroid hormone brassinolide (BL) [9], is an LRR-RLK that forms heterodimeric complexes with another LRR-RLK, BRI1-associated receptor kinase 1 (BAK1) [10, 11] in a hormone-dependent manner [12]. Plant LRR-RLKs also function as pattern-recognition receptors in basal and cultivar-specific host innate immunity [13–16]. Likewise, microbial pattern recognition by animal immune cells employs LRR transmembrane receptors, suggesting conceptual and mechanistic conservation in pattern recognition in different lineages [14, 17]. The size of the gene families of plant LRR-RLKs [5] and the involvement of some LRR-RLKs in plant immunity suggest important roles of these proteins in plant-pathogen interactions [13, 14].

To identify plant immunity-associated LRR-RLKs, we conducted experiments of gene-expression profiling with *Arabidopsis* Col-0 plants infected with various *Pseudomonas syringae* strains. Gene-expression analysis revealed increased transcript accumulation for 32 genes, including the BRI1 coreceptor *BAK1* [10, 11] (Figure S1 in the Supplemental Data available online). *BAK1* transcript levels in plants infected with avirulent (*P. syringae* pv. *tomato* DC3000AvrRpm1, *Pto*AvrRpm1) or nonpathogenic strains (*Pto*hrcC⁻, *P. syringae* pv. *phaseolicola*, *Pph*) increased by more than 2-fold compared to those in controls, whereas *BAK1* expression was repressed upon infection with virulent *Pto*DC3000 (Figures S1B and S1C). Two homozygous mutants carrying independent T-DNA insertions in *BAK1* (*bak1-3*, *bak1-4* in Col-0) that lacked the *BAK1* transcript were obtained (Figure 1A and B). As described for *Ws bak1-1* and *bak1-2* alleles [10, 11], both Col-0 *bak1* lines exhibited reduced leaf growth (~20%, Figure 1C), shorter hypocotyls in the dark, and partial BL insensitivity in root-growth assays (data not shown). Infection of Col-0 plants with

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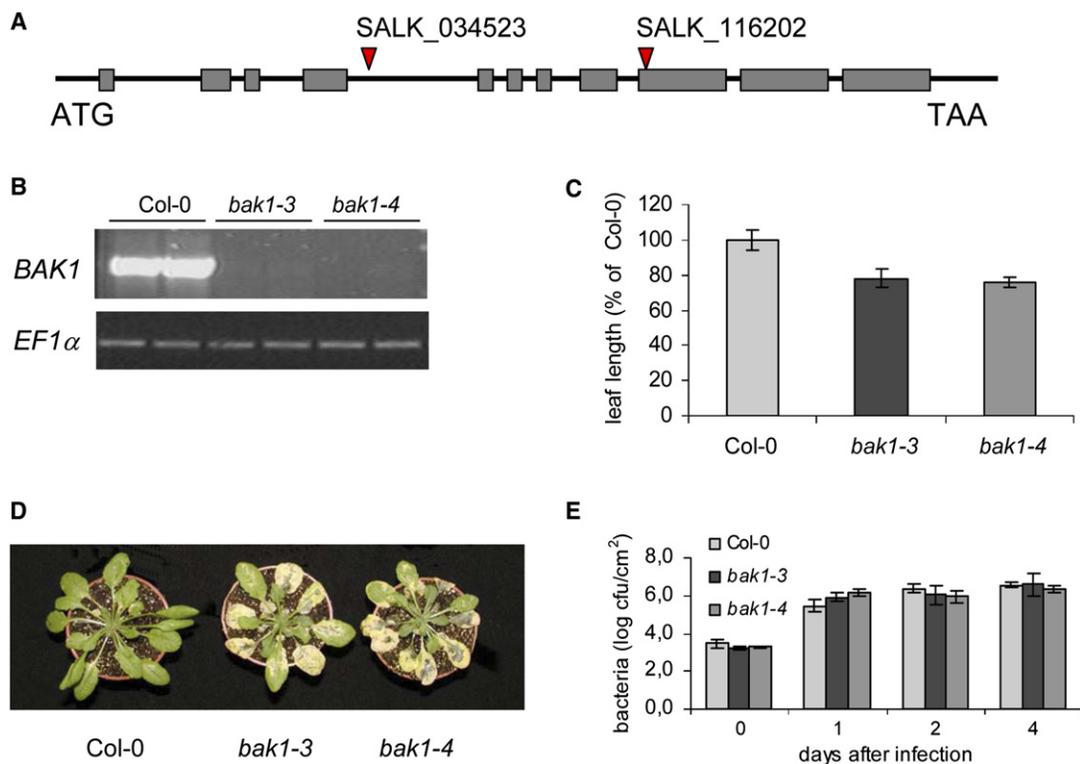


Figure 1. Inactivation of *BAK1* Causes Reduced Leaf Growth and Runaway Cell Death upon Bacterial Infection

(A) T-DNA insertion sites of *bak1-3* (SALK_034523) and *bak1-4* (SALK_116202).

(B) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *BAK1* and *EF1 α* (control) transcripts in wild-type Col-0 and *bak1* mutants.

(C) *bak1* mutants show reduced leaf growth. Leaf length is given relative to that of wild-type Col-0 plants. Results represent means \pm standard deviation (SD) ($n \geq 9$) of three independent experiments.

(D) Infection phenotypes of representative Col-0 wild-type and *bak1* mutant plants at 4 DA with *PtoDC3000*.

(E) Quantitative analysis of the growth of *PtoDC3000* in wild-type Col-0 and *bak1* mutant plants. Results represent means \pm SD ($n \geq 8$). Similar results were obtained in six independent experiments.

PtoDC3000 resulted in lesions that were restricted to infection sites, whereas infection of both mutant lines produced leaf chlorosis followed by spreading necrosis 4 days after infection (DAI) (Figure 1D). However, bacterial growth in Col-0 and *bak1* mutant lines did not differ (Figure 1E). Infection of *bak1* mutants with strains *PtoAvrRpm1* (Figure S2), *PtohrcC*⁻, or *Pph* (data not shown) resulted in symptoms and bacterial growth rates that were indistinguishable from those on Col-0.

To test whether impaired cell-death control in *bak1* mutants also occurs upon infection by other pathogens, we conducted necrotrophic-fungal-infection assays. Infection with the virulent fungus *Botrytis cinerea* caused the complete decay of *bak1* plants (Figure 2A), and initial fungal growth was much higher on *bak1* mutants than on Col-0 (Figure 2B). Likewise, *Alternaria brassicicola* spore inoculation of *bak1* mutant plants resulted in increased lesion size and disease indices when compared to inoculated Col-0 (Figures 2C–2E). Enhanced susceptibility to *A. brassicicola* was also observed in seedlings grown under axenic conditions (Figures S3A and S3B). Conidial structures developed in *bak1* mutant lines but not in Col-0 plants (Figure S3C) and were found also in leaf areas beyond infection foci. These findings suggest that deregulated host cell death in *bak1* mutants affects both host immune responses to virulent pathogens and nonhost resistance to *A. brassicicola*.

Complementation assays confirmed that the phenotypes observed in the *bak1* mutants were due to disruptions of the *BAK1* gene. Transformation of a genomic DNA fragment containing the *BAK1* gene into the *bak1-4* background (*gBAK1*) restored wild-type growth and resistance to fungal infection (Figures 2G and 2H). Because the *bak1* mutation is recessive and the entire F₁ progeny from crosses of homozygous *bak1-3* and *bak1-4* mutants showed reduced leaf growth and enhanced susceptibility to *A. brassicicola* (data not shown), we concluded that mutated *BAK1* is responsible for both *bak1* phenotypes. Also, overproduction of *BAK1* green fluorescent protein (GFP) in Col-0 resulted in reduced lesion formation and disease development upon *A. brassicicola* infection (Figure S4) compared to that in wild-type Col-0. Accordingly, *gBAK1* lines exhibiting increased *BAK1* expression (Figure 2F) had reduced necrosis and susceptibility to fungal infection compared to that of Col-0 (Figures 2G and 2H), suggesting that *BAK1* levels control plant programmed cell death (PCD) and immunity to necrotrophic fungi.

Microscopic examination revealed that lesions (monitored by trypan blue) spread beyond fungal infection foci in *bak1* lines but remained restricted to inoculation sites in Col-0 (Figures 3A and 3B). This was not observed in another mutant, *bos1*, which is more susceptible to *B. cinerea* and *A. brassicicola* [18]. Cell death did not

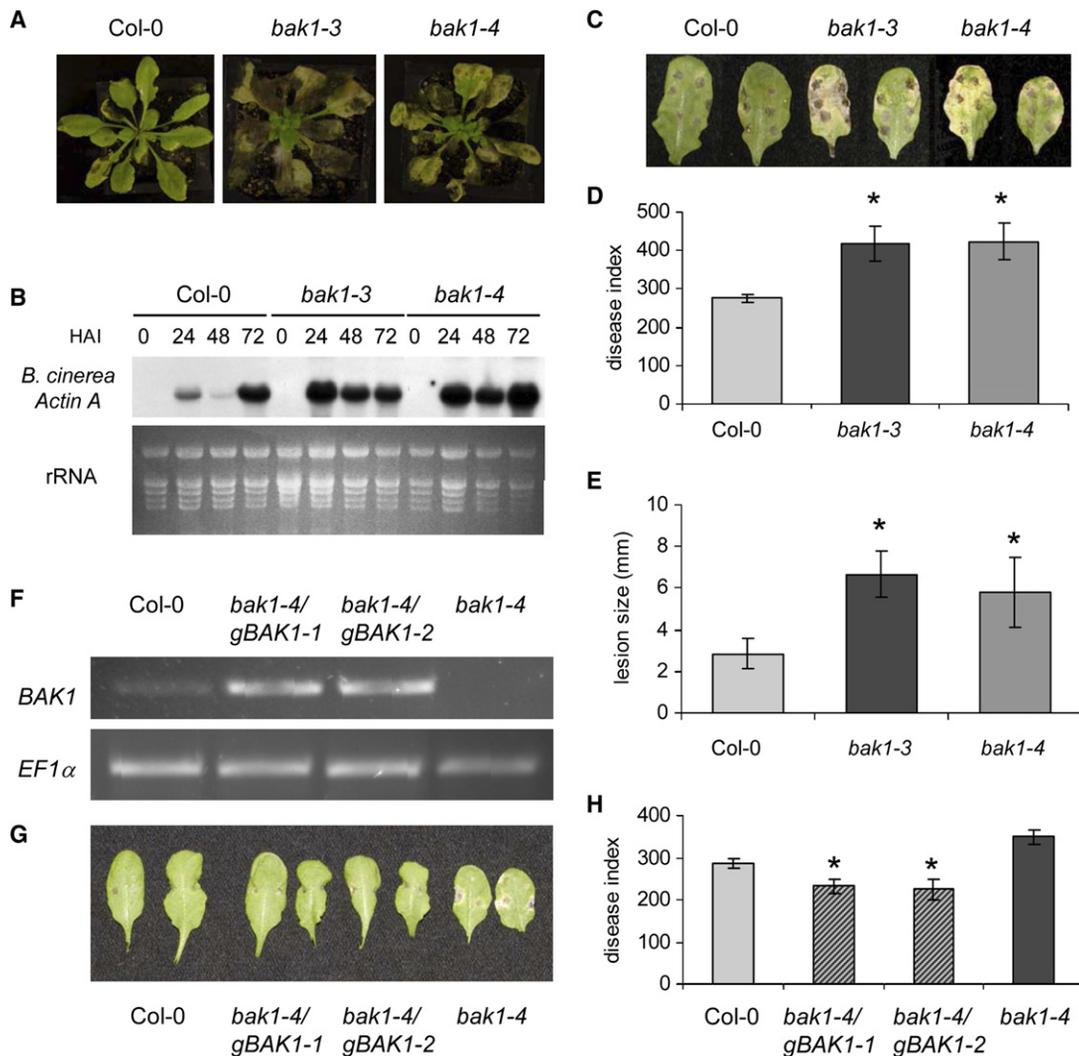


Figure 2. Loss of BAK1 Function Results in Enhanced Susceptibility to Infection by Necrotrophic Fungi

(A) Infection phenotypes of representative Col-0 and *bak1* mutant plants at 7 DAI by *B. cinerea*.
 (B) Quantification of fungal biomass in infected Col-0 and *bak1* plants by RNA-blot hybridization with a *B. cinerea*-Actin A-specific probe at the time points indicated (hours after infection [HAI]).
 (C) Infection phenotypes of leaves from representative Col-0 and *bak1* mutant plants at 7 DAI by *A. brassicicola*.
 (D) Calculation of disease indices on Col-0 and *bak1* plants at 7 DAI by *A. brassicicola*.
 (E) Lesion size determination at 7 DAI by *A. brassicicola*.
 (F) RT-PCR analysis of *BAK1* transcripts in Col-0, *bak1-4*, and *bak1-4* lines that were complemented with a genomic fragment encoding *BAK1* (*gBAK1-1*, *gBAK1-2*).
 (G) Infection phenotypes of leaves from representative lines as in (F) at 7 DAI by *A. brassicicola*.
 (H) Calculation of disease indices from experiments shown in (G). Similar results were obtained in three independent experiments ($n \geq 16$). Results represent means \pm SD; “*” indicates significant differences from Col-0 wild-type ($p < 0.05$).

occur in uninoculated *bak1* plants. Thus, *bak1* is not a spontaneous lesion-mimic mutant of the *lsd* class [2], and susceptibility to fungal infection is unlikely to be due to an enhanced saprophytic growth base for necrotrophic pathogens in *bak1* plants. Supporting this conclusion, salicylic acid caused necrosis in uninoculated *lsd1* mutants [19] but not in *bak1* lines (Figure S5).

The production of reactive oxygen intermediates (ROIs) is correlated with plant cell death [2] and can enhance susceptibility to necrotrophic pathogens [4]. We tested whether infection-triggered spreading necrosis in *bak1* mutants was accompanied by ROI formation. 3,3'-diaminobenzidine staining revealed H_2O_2 in

A. brassicicola-infected wild-type and *bak1* mutant plants (Figure 3C). However, ROI production was stronger in *bak1* lines and always spread into uninoculated areas. The same response was observed upon infection with *PtoDC3000* (Figure S6). Moreover, ROI microbursts appeared in infected leaves before the appearance of microscopic lesions (Figure S6). Notably, *bak1* mutant lines were not generally more sensitive to oxidative stress because their responses to paraquat or H_2O_2 were indistinguishable from those of Col-0 (data not shown).

Bak1 mutants develop deregulated cell death specifically in response to necrotizing pathogens (Figures 1–3). We tested whether responses to the biotrophic

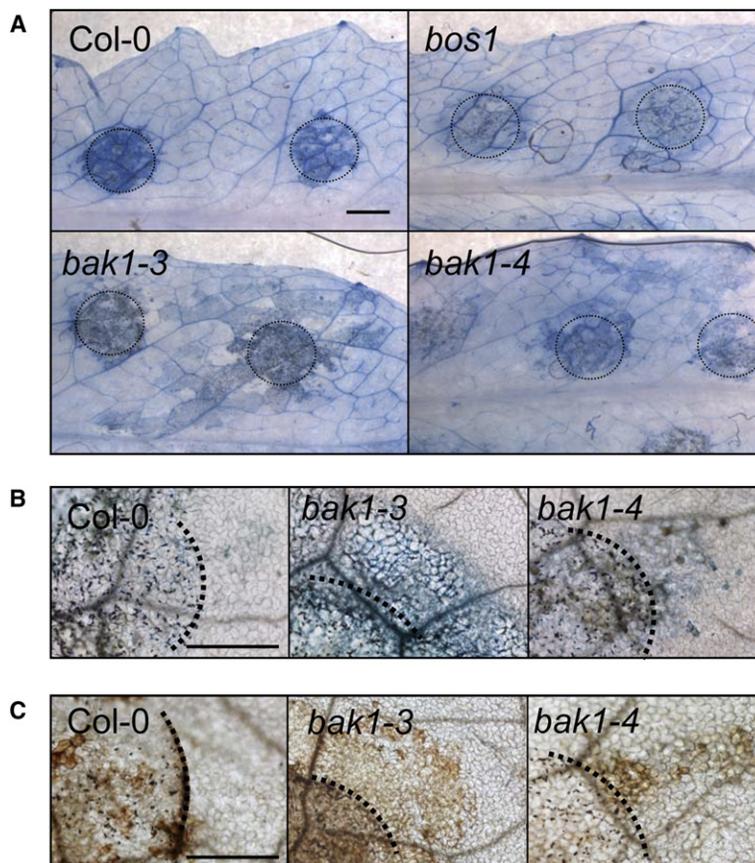


Figure 3. Micrograph Images of Wild-Type Col-0 and *bak1* Mutant Plant Reactions to *A. brassicicola* Infection

(A and B) Staining with trypan blue of representative leaves from wild-type Col-0, *bak1*, and *bos1* [18] mutant plants infected with *A. brassicicola* at 3 DAI.

(C) 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining of H₂O₂ production in *A. brassicicola*-infected wild-type Col-0 and *bak1* mutant plants. Dotted lines indicate borders of spore inoculation sites. Experiments were performed in triplicate with similar results. Scale bars represent 1 mm.

oomycete *Hyaloperonospora parasitica* were affected in *bak1* mutants. Infections of *bak1* plants with virulent or avirulent isolates did not induce spreading cell death or alter hyphal growth when compared to Col-0 (Figure S7). However, conidiophore formation indicative of pathogen reproduction was reduced on *bak1* mutants infected with the virulent isolate Noco2 (Figure S7). Growth of other virulent isolates was also impeded, whereas resistance gene-dependent immunity against avirulent isolate Cala2 was unaffected (unpublished data). Our findings suggest that BAK1 has opposing roles in resistance to necrotrophic and biotrophic pathogens.

LRR-RLKs have been implicated in signaling processes and thereby probably control stimulus-specific transcriptional reprogramming. We addressed whether BAK1 controls the expression of genes associated with plant PCD and immunity by performing transcriptome analyses of noninfected (Table S1) or *A. brassicicola*-infected *bak1-3* and Col-0 plants (Table S2). Expression of 38/8 genes was differentially induced/repressed in noninfected, nonnecrotic *bak1-3* lines compared to that in Col-0 (Table S1). Importantly, the conditional expression of 41 of these genes, including pathogenesis-related (PR) genes *PR-2* and *PR-5*, is associated with microbial infection (Table S1, Figure S8). Thus, BAK1 likely contributes to the control of infection-induced plant transcriptional responses. Comparison of gene expression patterns in *A. brassicicola*-infected *bak1-3* and Col-0 lines revealed that expression of 39/54 genes was induced/repressed by more than 2-fold in *bak1-3*

lines relative to those in Col-0; 47% of these expression patterns are associated with microbial infection (Table S2). Relative to those in Col-0, deregulated gene expression patterns in noninfected and infected *bak1-3* mutants did not significantly overlap (the expression of 3/46 genes that were deregulated in noninfected *bak1-3* versus Col-0 was also deregulated in infected *bak1-3* versus Col-0; Table S1 and Table S2), suggesting that BAK1 controls the expression of distinct sets of genes in noninfected and infected plants.

The *bak1* mutant phenotypes suggested a link between steroid-hormone activity and cell-death control. We therefore tested whether defects in brassinosteroid signaling cause runaway cell death upon infection with necrotizing pathogens. We found that (1) treatment of wild-type plants with 1.5 μ M brassinolide did not increase resistance to bacterial or fungal infection (Figure S9, Figure 4C). The same result was obtained after foliar application of lower (300 nM) or higher (15 μ M) BL concentrations (data not shown). Our findings contrast with moderately increased levels of pathogen resistance reported in tobacco or rice upon treatment with 20–200 μ M BL [20], suggesting plant-species-specific differences in BL activities. (2) Spraying *bak1* mutant plants with 1.5 μ M BL restored wild-type growth (Figures 4A and B) but failed to complement cell death (data not shown) and resistance-associated mutant phenotypes in infection assays (Figure 4C). (3) Analysis of other BL-signaling mutants for enhanced disease susceptibility to *A. brassicicola* infection revealed that mutants impaired in BL perception (*bri1-5* [21], *bri3*

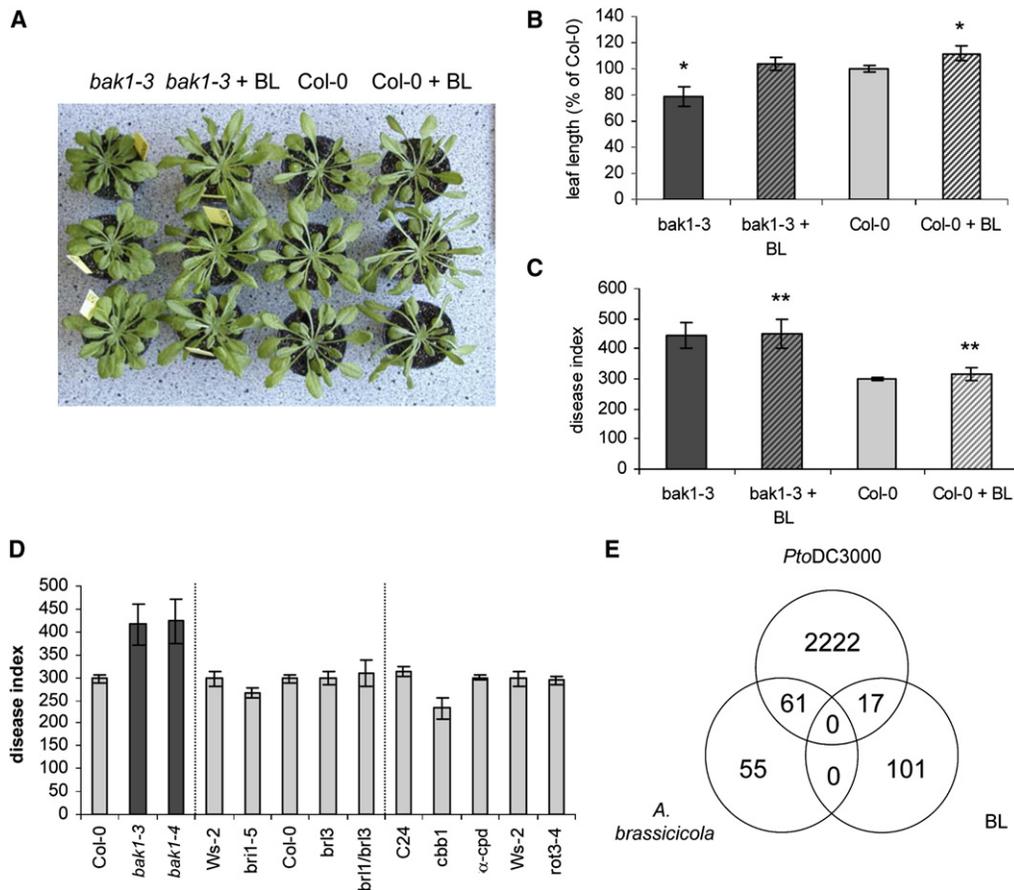


Figure 4. Disease Resistance Phenotypes in *bak1* Mutants Are Brassinolide-Independent

(A) Growth phenotypes of representative wild-type Col-0 and *bak1* individuals 7 days after treatment with 1.5 μ M brassinolide (“+BL”) or water as control.

(B) Leaf length is given relative to that of wild-type Col-0 plants. Results represent means \pm SD ($n \geq 9$) of three independent experiments. “***” indicates significant differences from Col-0 controls ($p < 0.01$).

(C) Calculation of disease indices of wild-type Col-0 and *bak1* plants at 7 DAI with *A. brassicicola* in the presence (“+BL”) or absence (“-BL”; water used as control) of 1.5 μ M brassinolide. “****” indicates no significant difference from -BL controls ($p < 0.01$).

(D) Calculation of disease indices of wild-type Col-0, *Ws-2*, and C24 plants and *bak1*, BL-insensitive, and BL-deficient mutant plants at 7 DAI with *A. brassicicola*. Experiments were performed in triplicate with similar results.

(E) Gene-expression-profiling analysis of wild-type Col-0 plants infected with *PtoDC3000* (2, 6, 24 hr) or *A. brassicicola* (24 hr) or treated with BL (0.5, 1, 3 hr). Expression analysis of genes that are coordinately upregulated by several treatments (overlap) or that are upregulated by individual treatments at any time point (Venn diagram). For each treatment versus the control condition, genes that changed were assigned based on a one-way analysis of variance (ANOVA) test. The numbers given as insets refer to those genes of which expression was statistically significantly induced by more than 2-fold. Gene expression data derived from *A. brassicicola*-infected Col-0 plants were taken from [26].

[22], *brl1brl3* [22]) or in BL biosynthesis (*cbb1* [23], α -cpd [23], *rot3-4* [24]) were not more susceptible to fungal infection (Figure 4D). We purposefully performed these assays on weak BL mutants to avoid severe growth defects that might arise from hormone insufficiency affecting infectivity of the fungus. (4) Because hormone activities are often linked to changes in gene expression patterns [25], we investigated whether early changes in transcript profiles of Col-0 plants treated with BL (0.5, 1, 3 hr) or infected with *PtoDC3000* (2, 6, 24 hr) overlapped. Of 2300 genes that were induced upon bacterial infection, only 17 were coincided by BL treatment (Figure 4E). Similarly, BL-induced gene expression patterns did not overlap with those of *A. brassicicola*-infected plants (24 hr) [26], suggesting that BL-induced gene expression is unlikely to govern infection-induced plant responses. We further assessed whether any of the

136 genes whose expression in noninfected or infected *bak1-3* mutants was deregulated relative to Col-0 was also regulated by BL. Only three genes (2.2%) showed BL-induced/repressed expression patterns (Tables S1 and S2). Together, our data suggest that BAK1 activity in plant cell-death control is BL-independent.

Discussion

Higher eukaryotes have evolved PCD mechanisms that play important roles in development and immunity [1, 2]. PCD is a common host response in plant-pathogen interactions and mediates both disease resistance and susceptibility [4]. Hypersensitive cell death is frequently associated with plant cultivar-specific host immunity [3, 14], whereas PCD in susceptible plants is caused by necrotizing pathogens that utilize virulence factors

to trigger host cell-death programs [2, 4]. The timely induction of PCD presents a formidable barrier to biotrophic pathogens, but defense strategies that culminate in PCD render the plant more susceptible to necrotrophic pathogens and must therefore be tightly controlled.

The nature and activities of core regulators of plant PCD are poorly understood [2]. How many PCD programs operate in plant development and immunity is also unclear. In animal or yeast cells, multiple cell-death pathways that control growth, differentiation, and immunity have been defined [1, 27]. However, plant proteins with a negative regulatory role in PCD, such as LSD1, ACD1, ACD2, and ACD5, are not related to metazoan PCD regulators [28], and members of the known classes of animal cell-apoptosis regulators (BCL-2/CED-9, APAF1/CED-4, and caspase/CED-3) are not found in plant genomes [2]. These findings suggest that alternative, structurally distinct regulators have evolved to control PCD in plants [2]. We show here that BAK1 constitutes a novel negative control element of microbial-infection-induced cell death in plants. BAK1 differs from other known negative regulators of plant PCD in several respects: (1) *bak1* is a conditional, propagative cell-death mutant that does not produce spontaneous lesions during normal development. (2) *bak1* mutants are not more sensitive to oxidative stress than are wild-type plants. (3) *bak1* does not develop salicylic-acid-inducible PCD. And (4) PCD in *bak1* plants is triggered after infection with virulent, necrotizing pathogens, whereas cell death associated with host cultivar-specific immunity or caused by necrotizing elicitors is unaffected (unpublished data). The molecular mechanisms by which negative regulators of plant PCD operate might be diverse. These proteins might regulate PCD directly or control processes whose perturbation leads to altered cellular homeostasis and cell death [28]. How BAK1 keeps in check this potentially destructive defense mechanism will be investigated with the information provided by our microarray experiments.

BAK1 represents a second example of a plant LRR-RLK with dual functions in plant development and immunity. *ERECTA* encodes an LRR-RLK with a defined role in flower development but was recently also implicated in plant pathogen resistance [15, 16]. Involvement of LRR-type proteins in developmental and innate immune programs was also reported in animals, suggesting that multitasking of proteins in development and immunity is evolutionarily conserved [29]. A prime example is the *Drosophila* plasma-membrane LRR protein TOLL, which controls embryonic patterning and immunity against fungal infections in adult insects [30].

We show that BAK1 function in PCD control is independent of the steroid hormone, BL. Therefore, we propose that BAK1 serves a BL-independent function in plant immunity, in addition to its established BL-dependent role in development [7, 8] (Figure S10). This conclusion is supported by the concomitant inactivation of two closely related members of the small somatic embryogenesis receptor kinase gene family in *Arabidopsis* [31], BAK1/SERK3 and BAK1-like Kinase 1/SERK4 (BKK1) [32] causing spontaneous cell death and post-embryonic lethality [32]. Importantly, no known plant mutant with defects in BL-dependent development exhibited cell death, favoring an additional role of BAK1 in controlling host PCD. Inactivation of BKK1 or other

SERK gene family members did not result in spontaneous PCD [32] or infection-induced spreading necrosis (data not shown). Thus, BAK1 activity is crucial for the containment of plant PCD in response to necrotizing pathogens, whereas BKK1 might have an accessory role in controlling cell death.

Proteins with dual functions must be strictly controlled. BAK1 activity in plant development is regulated by BKI1, which prevents BAK1/BRI1 heterodimerization in the absence of BL [33]. Identification of additional proteins that interact with BAK1 and control its BL-independent activity should provide insight into how specificity in plant developmental and immunity programs is maintained.

Supplemental Data

Experimental Procedures, ten figures, and two tables are available at <http://www.current-biology.com/cgi/content/full/17/13/1116/DC1/>.

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