

# The Membrane-Anchored *BOTRYTIS-INDUCED KINASE1* Plays Distinct Roles in *Arabidopsis* Resistance to Necrotrophic and Biotrophic Pathogens <sup>W</sup>

Paola Veronese,<sup>a</sup> Hirofumi Nakagami,<sup>b</sup> Burton Bluhm,<sup>a</sup> Synan AbuQamar,<sup>a</sup> Xi Chen,<sup>c</sup> John Salmeron,<sup>c</sup> Robert A. Dietrich,<sup>c</sup> Heribert Hirt,<sup>b</sup> and Tesfaye Mengiste<sup>a,1</sup>

<sup>a</sup>Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907-2054

<sup>b</sup>Gregor Mendel Institut, A-1010 Vienna, Austria

<sup>c</sup>Syngenta Biotechnology, Research Triangle Park, North Carolina 27709

**Plant resistance to disease is controlled by the combination of defense response pathways that are activated depending on the nature of the pathogen. We identified the *Arabidopsis thaliana* *BOTRYTIS-INDUCED KINASE1* (*BIK1*) gene that is transcriptionally regulated by *Botrytis cinerea* infection. Inactivation of *BIK1* causes severe susceptibility to necrotrophic fungal pathogens but enhances resistance to a virulent strain of the bacterial pathogen *Pseudomonas syringae* pv *tomato*. The response to an avirulent bacterial strain is unchanged, limiting the role of *BIK1* to basal defense rather than race-specific resistance. The jasmonate- and ethylene-regulated defense response, generally associated with resistance to necrotrophic fungi, is attenuated in the *bik1* mutant based on the expression of the plant defensin *PDF1.2* gene. *bik1* mutants show altered root growth, producing more and longer root hairs, demonstrating that *BIK1* is also required for normal plant growth and development. Whereas the pathogen responses of *bik1* are mostly dependent on salicylic acid (SA) levels, the nondefense responses are independent of SA. *BIK1* is membrane-localized, suggesting possible involvement in early stages of the recognition or transduction of pathogen response. Our data suggest that *BIK1* modulates the signaling of cellular factors required for defense responses to pathogen infection and normal root hair growth, linking defense response regulation with that of growth and development.**

## INTRODUCTION

Plant resistance to potential pathogens involves an elaborate system of signal perception and transduction to activate cellular defense. At least some host responses appear to be mediated by receptor molecules either associated with or embedded in the plant cell membrane, with the receptor domains on either the inner or outer surface of the membrane. These receptors may interact with various pathogen factors. These could include so-called pathogen-associated molecular patterns that are invariant between and are shared by many related pathogens (e.g., flg22, a conserved peptide domain in a bacterial flagella protein) and race-specific elicitors that are widespread among biotrophic plant pathogens (Nurnberger et al., 2004). After recognition, various host molecules, including protein kinases and transcription factors, relay the signal to activate downstream defense responses, including the synthesis of antimicrobial compounds. The speed and intensity of the activation of such responses are crucial for plant resistance to microbial infection. The signal recognition and early signal relay mechanisms appear to be

distinct depending on the nature of the pathogen, whereas downstream effector molecules show a large degree of overlap between different pathogens.

Plant resistance to pathogens is controlled by the combination of pathogen defense response pathways that are triggered depending on the nature of the pathogen and its modes of pathogenesis. Gene-for-gene resistance is based on the direct or indirect recognition of a race-specific pathogen elicitor molecule by the cognate plant resistance gene (Flor, 1971). These interactions frequently culminate in the activation of the hypersensitive response, which correlates with resistance to biotrophic fungal, nematode, viral, and bacterial pathogens. Basal defense pathways, which are activated by pathogen-associated molecular patterns (also called general elicitors), generally result in resistance without the hypersensitive response and appear to be effective against a broad range of pathogens (Asai et al., 2002; Nurnberger et al., 2004). General induced and systemic defense responses, such as systemic acquired resistance (SAR), jasmonate/ethylene-mediated resistance, and induced systemic resistance, have also been described (Nimchuk et al., 2003; Glazebrook, 2005). SAR is a form of resistance induced systemically in the plant after a localized pathogen challenge that provides protection against secondary infection by a broad spectrum of primarily biotrophic pathogens (Cao et al., 1997; Reuber et al., 1998; Dewdney et al., 2000). SAR can be activated by chemical agents or pathogens that cause cell death and correlates with the expression of a subset of pathogenesis-related proteins, some with antimicrobial activity. In dicotyledonous plants,

<sup>1</sup>To whom correspondence should be addressed. E-mail mengiste@purdue.edu; fax 765-494-0363.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Tesfaye Mengiste (mengiste@purdue.edu).

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salicylic acid (SA) is necessary and sufficient for SAR induction (Vernooij et al., 1994).

Necrotrophic pathogens are a destructive group of plant pathogens that have pathogenesis strategies distinct from biotrophic pathogens. Whereas necrotrophic pathogens induce cell death in their hosts by secreting toxic substances into host tissue before and during colonization, biotrophic pathogens require living cells to complete their life cycle. The necrotroph *Botrytis cinerea* produces cell wall-degrading enzymes (Prins et al., 2000), toxic levels of reactive oxygen intermediates (Edlich et al., 1989; Deighton et al., 1999; Muckenschnabel et al., 2002), and toxins (Tiedemann, 1997; Colmenares et al., 2002) that result in the death and maceration of tissue, leading to plant decay. Evidence to date strongly suggests a limited role for SAR and gene-for-gene (race-specific) resistance against necrotrophic pathogens in *Arabidopsis thaliana*. Mutants that are impaired in signaling required for the activation of SAR or disrupted in the biosynthesis of SA retain wild-type levels of resistance to *Botrytis* (Ferrari et al., 2003). Chemical or biological activation of SAR failed to contain *Botrytis* growth in *Arabidopsis* (Govrin and Levine, 2002). In addition, neither a race-specific fungal elicitor nor an *R* gene required for gene-for-gene resistance has been identified in any *Botrytis*-plant interactions. It also appears that resistance to *Botrytis* requires the concerted action of many genes, as opposed to the more usual single gene-mediated recognition-dependent resistance to biotrophic pathogens.

Induced general responses regulated by the plant hormones ethylene (ET) and jasmonate (JA) are required for defense responses to necrotrophic pathogens in some plant species (Thomma et al., 1999; Diaz et al., 2002; Ferrari et al., 2003). *Arabidopsis* and tomato (*Lycopersicon esculentum*) mutants altered in ET and JA signaling or biosynthesis show increased susceptibility to *Botrytis* and other necrotrophic pathogens (Thomma et al., 1999; Diaz et al., 2002). The expression of the plant defensin gene *PDF1.2* is activated in response to *Botrytis* and *Alternaria brassicicola* in an ET- and JA-dependent manner and correlates with resistance (Penninckx et al., 1998). However, susceptible responses to *Botrytis* and *A. brassicicola* have also been observed during normal activation of *PDF1.2*, suggesting that *PDF1.2* may not be sufficient to provide full plant protection (Ferrari et al., 2003; Mengiste et al., 2003). Here, we describe the identification and characterization of the *BOTRYTIS-INDUCED KINASE1 (BIK1)* gene of *Arabidopsis* as a critical component of the *Arabidopsis* pathogen response pathway required for full resistance to at least two necrotrophic pathogens, *Botrytis* and *A. brassicicola*. Although BIK1 is required for resistance to necrotrophic pathogens, our data show that it is a negative regulator of basal defense responses to virulent strains of the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) but has no role in race-specific and nonhost resistance.

## RESULTS

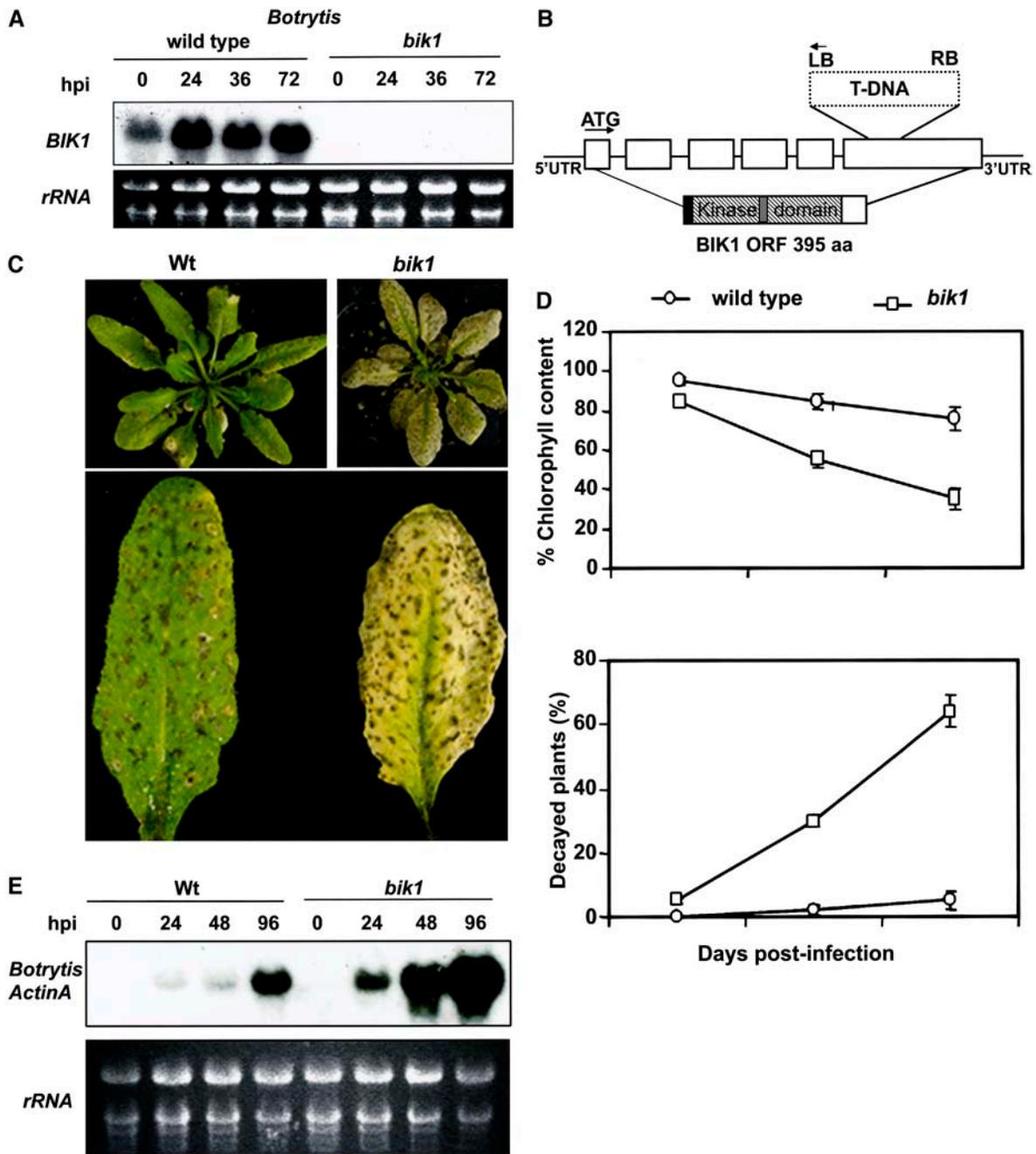
### Identification of *BIK1* and Characterization of Its Role in Disease Resistance

To identify genes that contribute to *Botrytis* resistance, we compared transcript profiles of *Botrytis*-infected and healthy

*Arabidopsis* leaves using an *Arabidopsis* microarray. We selected genes encoding potential components of signal transduction pathways that were significantly induced during *Botrytis* infection and focused on a putative Ser/Thr kinase (At2g39660) that we named *BIK1*. In the microarray experiments, the transcript of the *BIK1* gene increased an average of 6.5-fold in *Botrytis*-inoculated leaves compared with mock-inoculated controls (data not shown). This induction of *BIK1* expression after *Botrytis* inoculation was confirmed using RNA gel blots (Figure 1A). Healthy wild-type plants express low basal levels of *BIK1* RNA, but the transcript accumulates to high levels within 24 h of inoculation with *Botrytis* (Figure 1A).

To determine the function of *BIK1* in defense and nondefense response pathways, we searched for a loss-of-function mutant. A mutant allele of the *BIK1* gene containing a T-DNA insertion in the *BIK1* coding region was identified from the SALK T-DNA insertion collection (Alonso et al., 2003). The T-DNA inserted into the last exon of the *BIK1* gene and resulted in the loss of detectable *BIK1* transcript, indicating that *bik1* is a loss-of-function mutant (Figures 1A and 1B). To determine the role of BIK1 in resistance to *Botrytis*, homozygous *bik1* plants were inoculated by spraying a suspension containing  $2 \times 10^5$  *Botrytis* spores per milliliter, as described previously (Mengiste et al., 2003; Veronese et al., 2004). In wild-type (ecotype Columbia) plants, *Botrytis* infection caused restricted disease symptoms, with no significant damage to the plant (Figure 1C). Necrotic spots at the sites of primary infection were clearly visible, but the necrosis remained restricted and did not spread. On lower leaves, this infection occasionally led to the collapse of an entire leaf, although the infection rarely became systemic. In *bik1* plants, disease symptoms increased rapidly, showing chlorosis, necrosis, and complete plant decay compared with wild-type plants (Figures 1C and 1D). At 8 d after infection, *Botrytis* infection of wild-type plants caused a loss of chlorophyll ranging from 20 to 30% compared with the corresponding mock-inoculated control plants (Figure 1D, top). At the corresponding time point, *bik1* plants had lost 60 to 70% of their chlorophyll. In wild-type plants, *Botrytis* infection rarely moved to tissues that were not present at the time of infection, and <5% of the plants were killed. In the mutant, infection became systemic and resulted in the complete decay of 60 to 70% of the inoculated plants over a period of 8 to 12 d after infection, suggesting a severely impaired resistance mechanism (Figure 1D, bottom). These data suggest a clear role for *BIK1* in resistance to *Botrytis* infection.

The growth of the pathogen in inoculated plants was measured to determine whether the enhanced tissue death displayed by the *bik1* mutant was attributable to increased pathogen growth or to increased symptom development in response to normal pathogen growth. As a measure of fungal growth in planta, blots of RNA extracted from inoculated plants were hybridized with a probe made from the constitutively expressed *BOTRYTIS ACTIN A* gene as described (Benito et al., 1998). Fungal RNA accumulated in both wild-type and mutant plants as infection advanced and correlated well with the disease symptoms, including loss of chlorophyll (Figures 1C and 1E). In the *bik1* mutant, however, *Botrytis* RNA accumulated to significantly higher levels at 2 to 4 d after infection. At later stages, *Botrytis*



**Figure 1.** *Botrytis*-Induced Expression of *BIK1*, Structure of the *bik1* Mutant Allele, and Responses to *Botrytis*.

(A) RNA blot showing the induction of the *BIK1* gene in wild-type plants in response to *Botrytis* and the lack of the *BIK1* transcript in the *bik1* mutant. hpi, hours after inoculation.

(B) Genomic organization of the *bik1* mutant allele and the cDNA encoded by the *BIK1* open reading frame (ORF). The *BIK1* open reading frame is shown with the N-terminal myristoylation motif (black), the protein kinase domain (striped), and the Ser/Thr kinase active site signature (gray). aa, amino acids; LB, left border; RB, right border; UTR, untranslated region.

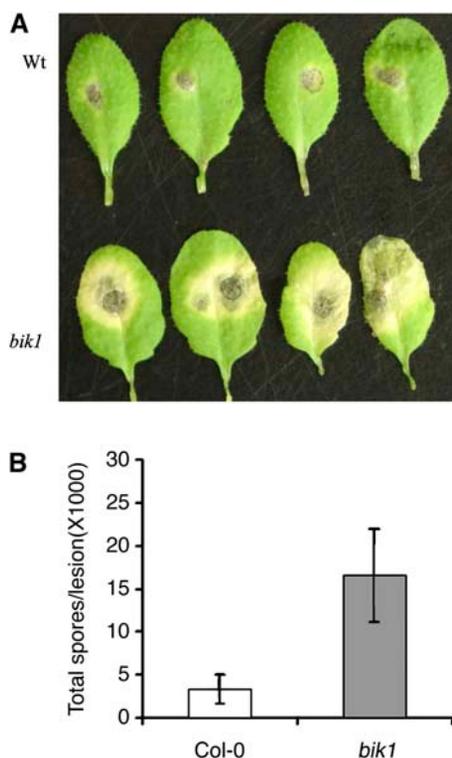
(C) Wild-type (left) and *bik1* (right) plants 5 d after inoculation with *Botrytis*. Four-week-old soil-grown plants were inoculated by spraying a fungal spore suspension ( $2 \times 10^5$  spores/mL).

(D) Susceptibility of *bik1* plants as measured by the loss of chlorophyll and plant decay. Top, chlorophyll content in inoculated plants is expressed as a percentage of the chlorophyll content of the corresponding mock-inoculated plants. Chlorophyll was extracted from entire rosette leaves of six mock-inoculated and six *Botrytis*-inoculated plants for each genotype. The data are means  $\pm$  SE from two independent experiments. Bottom, percentage of decayed wild-type and mutant plants caused by *Botrytis* infection as a function of time after inoculation. Plants were scored as dead when their centers were completely rotten. Data represent means  $\pm$  SE of three different experiments performed with 36 plants per genotype.

(E) Accumulation of the *BOTRYTIS ACTIN A* mRNA in inoculated plants. In (A) and (E), 20  $\mu$ g of total RNA was loaded per lane.

proliferated further, with the fungal hyphae becoming clearly visible on macerated leaf surfaces of *bik1*, whereas hyphae were not visible on the surfaces of wild-type leaves. Similar results were obtained by staining infected leaves with trypan blue to visualize the growth of the fungus in planta (data not shown). These data indicate that *bik1* is unable to restrict fungal growth, and in this respect it is phenotypically similar to the *Botrytis-susceptible1* (*bos1*) mutant (Mengiste et al., 2003).

The loss of *BIK1* function resulted in enhanced susceptibility to a second necrotrophic pathogen, *A. brassicicola*, the causal agent of early blight in cruciferous plants. As with *Botrytis*, the *bik1* mutation resulted in increased disease symptoms and pathogen growth in plants inoculated with *A. brassicicola* (Figures 2A and 2B). In wild-type plants, disease symptoms were limited to a lesion at the point of inoculation. In *bik1*, infection resulted in slightly larger lesions surrounded by extensive chlorosis. In addition, the fungus proliferated significantly more in mutant plants, with the production of spores on inoculated *bik1* leaves approximately fivefold greater than on wild-type leaves (Figure 2B). The loss of both *Botrytis* and *A. brassicicola* resistance in *bik1* plants suggests a broad role for *BIK1* in resistance to microbial necrotrophy.



**Figure 2.** *bik1* Plants Show Increased Susceptibility to the Necrotrophic Pathogen *A. brassicicola*.

(A) Leaves of drop-inoculated wild-type (top row) and *bik1* (bottom row) plants at 6 d after infection.

(B) Mean number of spores in *A. brassicicola*-inoculated plants at 6 d after infection. Data represent means  $\pm$  SE from three independent experiments. Each experiment contained the average spore counts from 20 inoculated leaves per genotype. Col-0, ecotype Columbia (wild type).

Plants were challenged with strains of the bacterial pathogen *Pst* to determine whether the resistance function of *BIK1* extends to bacterial pathogens. Interestingly, *bik1* plants showed increased resistance to the normally virulent strain *Pst*DC3000. No visible disease symptoms developed on inoculated *bik1* leaves, and bacterial growth was reduced significantly compared with that in wild-type leaves (Figure 3A). Resistance to an avirulent bacterial strain, *Pst*DC3000(*avrRpm1*), was unchanged relative to that in wild-type plants, suggesting a role for *BIK1* in basal defense response rather than in *R* gene-mediated resistance (Figure 3B). The *bik1* plants were not affected in their resistance to the nonhost pathogen *Pseudomonas syringae* pv *phaseolicola* (data not shown). These results suggest that *BIK1* acts as a negative regulator of basal resistance to virulent pathogens but has no role in resistance to the nonhost and avirulent pathogens tested.

### The *BIK1* cDNA Rescues the Disease Responses of *bik1* to Wild-Type Levels

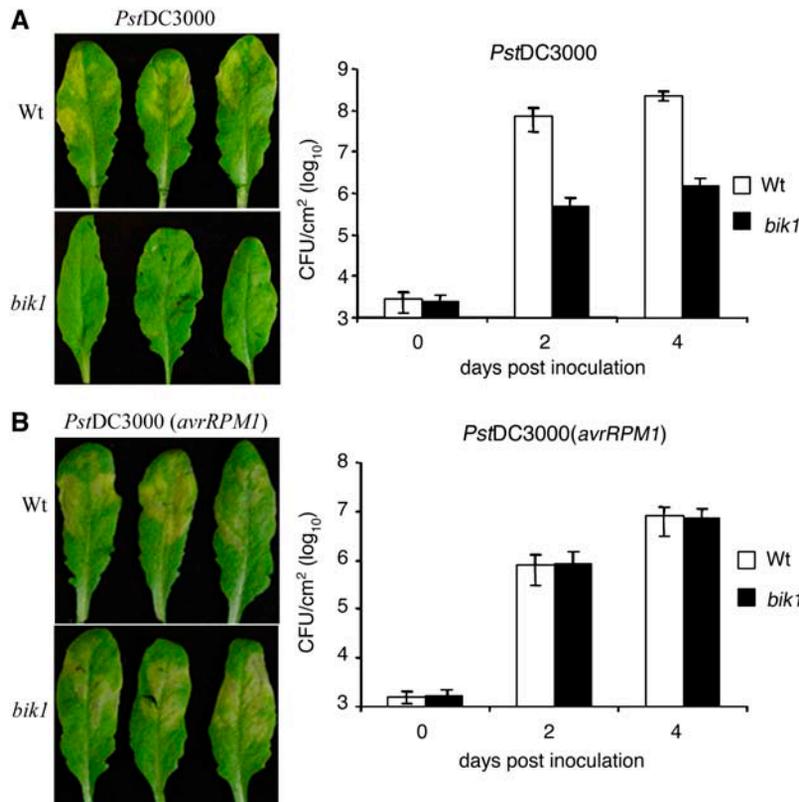
The observations that *BIK1* expression is upregulated during pathogen infection and that *bik1* plants exhibit altered responses to *Botrytis*, *A. brassicicola*, and *Pst* infection support the hypothesis that *BIK1* functions in a pathogen response pathway. To confirm that the observed phenotypes conferred by *bik1* are specifically attributable to the mutation in *BIK1*, we performed complementation experiments. Expression of the *BIK1* cDNA under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter in *bik1* resulted in transgenic plants with wild-type resistance levels to *Botrytis*, *A. brassicicola*, and *Pst* infection (Figures 4A and 4B). These data show that the mutation in *BIK1* is the sole cause of the *bik1* disease responses and confirm the identity of the *BIK1* gene.

### *bik1* Is Not a Lesion-Mimic Mutant

The presence of dead cells and necrotic tissues is thought to promote *Botrytis* infection by providing an initial saprophytic growth base from which the fungus further invades healthy tissue. Dead cells could provide nutrients for the growth of *Botrytis*, which is unable to obtain nutrients from living cells. However, no cell death lesions were observed before infection in *bik1* plants (Figure 5A), suggesting that the susceptibility of *bik1* to *Botrytis* is not attributable to the presence of spontaneous cell death lesions before inoculation. After *Botrytis* infection, patches of dead cells were visible in both wild-type and mutant plants. This is consistent with previously described *Arabidopsis* mutants that have increased *Botrytis* susceptibility in the absence of a lesion-mimic phenotype (Thomma et al., 1999; Mengiste et al., 2003; Veronese et al., 2004).

### Accumulation and Sensitivity to Reactive Oxygen Intermediates in *bik1*

Reactive oxygen intermediates (ROIs) have been implicated in both susceptible and resistance responses, depending on the nature of the pathogen (Levine et al., 1994; Lamb and Dixon, 1997; Govrin and Levine, 2000). ROIs are required for cell death



**Figure 3.** Responses of *bik1* to *Pst* Inoculation.

Representative leaves showing disease symptoms (left panels) and bacterial growth (right panels) after infiltration with *PstDC3000* (**A**) or *PstDC3000* (*avrRPM1*) (**B**). Bacterial growth (colony forming units [CFU]/cm<sup>2</sup> leaf area) at 0, 2, and 4 d after infection is plotted for wild-type and *bik1* plants. Plants were infiltrated with a bacterial suspension (OD<sub>600</sub> = 0.001). Photographs of diseased leaves are from 3 d after infection. Data represent average values ± SE from three experiments.

and resistance to biotrophic pathogens (Torres et al., 2002). In the case of necrotrophic pathogens, cell death exacerbates susceptibility (Govrin and Levine, 2000). We determined the levels of H<sub>2</sub>O<sub>2</sub> by 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997) 2 d after inoculation with *Botrytis*. Inoculated plants generated brown precipitate indicative of H<sub>2</sub>O<sub>2</sub> production that was absent in noninoculated plants (Figure 5B). There was a small but consistent increase in the intensity and size of the stained area in infected *bik1* plants. This may come from the increased *Botrytis* growth in the mutant, resulting in increased H<sub>2</sub>O<sub>2</sub> levels.

The previously described *bos1* mutant showed increased susceptibility to *Botrytis* and sensitivity to oxidative stress (Mengiste et al., 2003; Veronese et al., 2004). In the case of *bik1*, sensitivity to oxidative stress generated by paraquat (methyl viologen) was comparable to that in wild-type plants, suggesting that susceptibility to *Botrytis* is not linked to altered plant sensitivity to oxidative stress (data not shown).

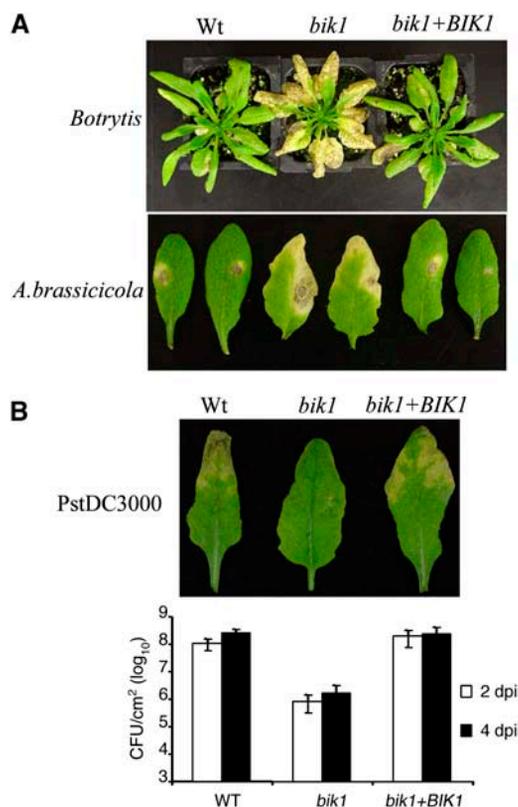
#### The *bik1* Mutation Affects SA Accumulation but Not Camalexin

To determine whether the altered disease responses of *bik1* are associated with altered levels of SA, we determined free SA

levels in *Botrytis*-inoculated and noninoculated plants (see Methods). In noninoculated *bik1* plants, basal SA levels were more than twofold greater than those in the corresponding wild-type plants. In both cases, SA levels increased after *Botrytis* infection, with the level of SA in the mutant being higher than that in the corresponding wild-type plants at 2 d after infection (Figure 6A). These data implicate BIK1 as a negative regulator of SA accumulation and hence also of the downstream responses to SA. To test whether BIK1 also regulates phytoalexin accumulation, we determined the levels of the phytoalexin camalexin after *Botrytis* infection. Despite the high degree of susceptibility to *Botrytis*, *bik1* mutant plants accumulated normal levels of camalexin during *Botrytis* infection (data not shown), indicating that BIK1 is not involved in regulating camalexin levels during *Botrytis* infection.

#### The *bik1* Mutation Affects the Expression of Defense-Related Genes

To relate the disease susceptibility of *bik1* to defects in defense response pathways, we studied the expression of molecular markers of JA/ET- and SAR-mediated disease response pathways. The expression of the plant defensin gene *PDF1.2* correlates with the activation of the ET/JA defense response pathway



**Figure 4.** The *BIK1* cDNA Rescues the *bik1* Disease Phenotypes.

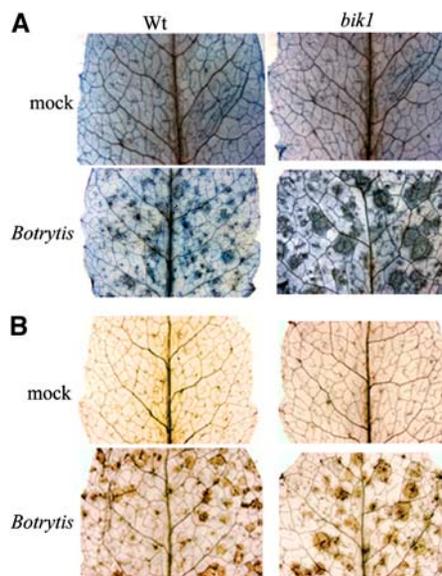
The *BIK1* cDNA expressed in *bik1* plants under the control of the CaMV35S promoter rescued susceptibility to *Botrytis* and *A. brassicicola* (A) and resistance to *PstDC3000* (B) to wild-type levels. The bottom panel in (B) shows bacterial growth. All assays were repeated three times on five independent lines. Data points for bacterial growth in (B) represent averages  $\pm$  SE from three experiments. Photographs were taken at 5 d after inoculation with *Botrytis* or *A. brassicicola* and at 3 d after inoculation with *PstDC3000*. *bik1+BK1*, *bik1* plants expressing the wild-type *BIK1* cDNA under the control of the CaMV35S promoter.

and is implicated in resistance to necrotrophic pathogens (Penninckx et al., 1996, 1998). The *bik1* mutation significantly reduced the expression of *PDF1.2* in response to *Botrytis* infection, paraquat, and methyl jasmonate (MeJA) relative to wild-type plants (Figure 6B). This finding implicates BIK1 as a positive regulator of a defense response pathway that controls *PDF1.2* expression. The transcript of the SAR marker gene *PR-1* increased through the first 48 h after *Botrytis* inoculation in wild-type plants. In *bik1*, this expression was significantly higher at 24 and 48 h after inoculation than in wild-type plants (Figure 6C). This may mean that *bik1* plants are more sensitized and respond faster in terms of *PR-1* expression, although the increased *PR-1* levels may be an indirect consequence of the increased rate of fungal growth in *bik1* plants. By contrast, the SA-induced expression of *PR-1* was comparable in wild-type and *bik1* plants, suggesting that BIK1 does not mediate SA signaling (Figure 6C). Limited *PR-1* expression under uninduced conditions was observed when  $>10$   $\mu$ g of RNA was loaded on an RNA gel (data not shown). In summary, the induction of some defense responses

and the inhibition of others in *bik1* suggest that *BIK1* is a regulatory factor that modulates defense responses.

### **BIK1 Encodes a Ser/Thr Protein Kinase**

The *BIK1* genomic region is composed of six exons and five introns (Figure 1B). The cDNA sequence of *BIK1* contains an open reading frame encoding a protein of 395 amino acid residues with an estimated molecular mass of 44.09 kD and a pI of 9.36 (see Supplemental Figure 1 online). The *BIK1* open reading frame contains a consensus plant *N*-myristoylation motif, Met-Gly-XXX-Ser/Thr (Arg, where X is any amino acid [MGXXXS/T(R)]) (Thompson and Okuyama, 2000), suggesting membrane localization. Protein *N*-myristoylation refers to the covalent attachment of myristic acid by an amide bond to the N-terminal Gly residue of a nascent polypeptide. Myristoylation is involved in directing and anchoring proteins to membranes. Membrane-anchored proteins can have various functions, including cellular regulation, signal transduction, and translocation (Podell and Gribkov, 2004). The central part of the BIK1 protein (residues 67 to 355) contains a kinase catalytic domain and harbors all 11 conserved subdomains of protein kinases (Hanks and Quinn, 1991; Hardie, 1999). Two consensus sequences, the Lys (K) residue in the DIKASN motif and the G-T/S-XX-Y/F-X-APE motif,

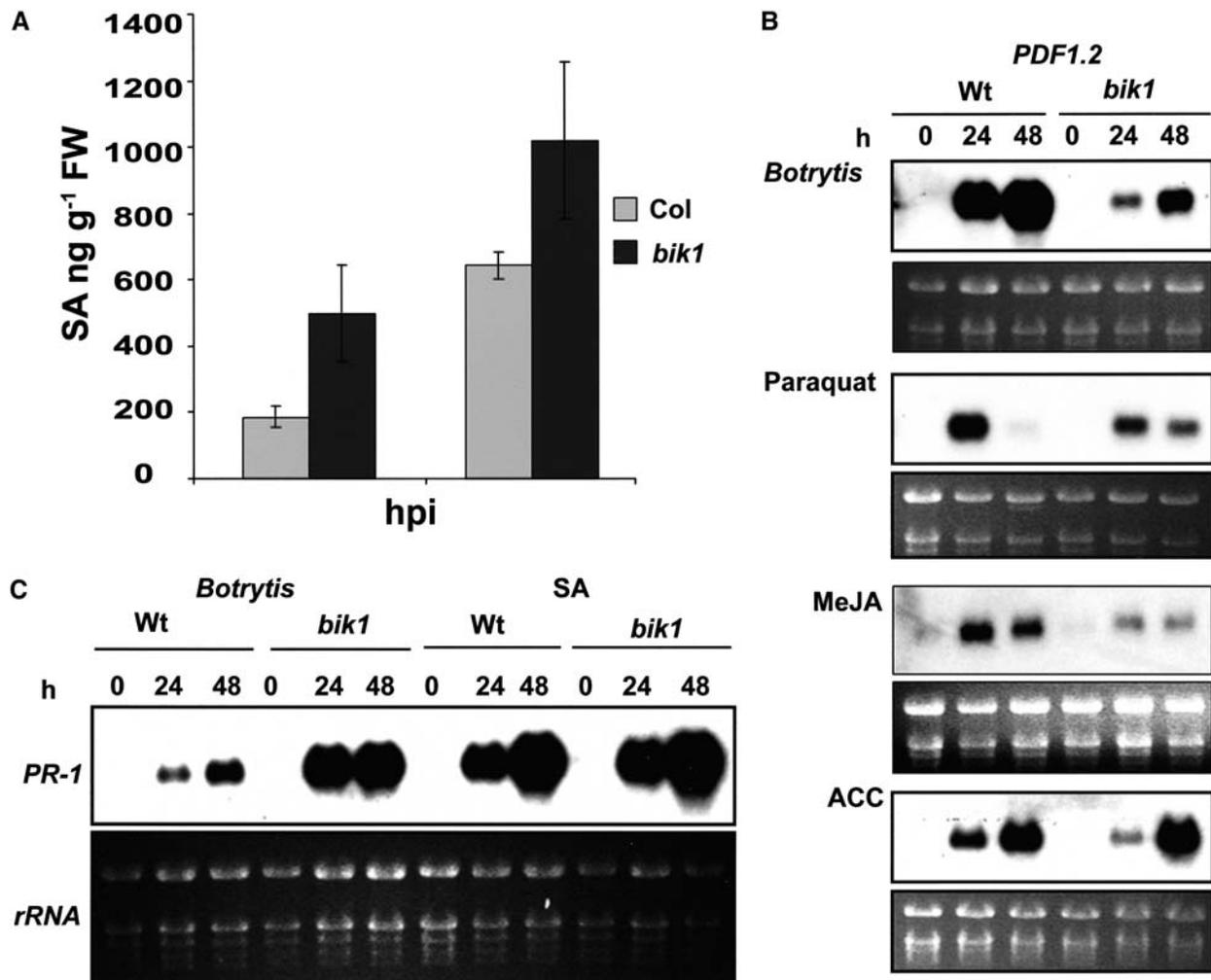


**Figure 5.** Cell Death and Production of H<sub>2</sub>O<sub>2</sub> in *Botrytis*-Inoculated Plants.

(A) Trypan blue staining of leaves from mock-inoculated (top row) or *Botrytis*-inoculated (bottom row) plants at 3 d after infection showing cell death triggered by pathogen infection.

(B) Production of H<sub>2</sub>O<sub>2</sub> in mock-inoculated (top row) or *Botrytis*-inoculated (bottom row) plants at 2 d after infection. Leaves were stained with DAB as described in Methods. The brown precipitate shows DAB polymerization at the site of H<sub>2</sub>O<sub>2</sub> production.

In both (A) and (B), representative leaves detached from inoculated plants are shown. The experiments were repeated three times with similar results.



**Figure 6.** *bik1* Plants Show Increased SA Accumulation and Altered Defense Gene Expression.

(A) SA levels before and after *Botrytis* infection in wild-type and *bik1* plants. FW, fresh weight.

(B) Expression of the *PDF1.2* gene in response to *Botrytis*, paraquat, MeJA, and ACC.

(C) Expression of *PR-1* in response to *Botrytis* infection and SA treatment.

SA levels shown in (A) represent means  $\pm$  SE from three independent experiments. In (B) and (C), the experiments were repeated three times with similar results. Each lane contained 10  $\mu$ g of total RNA.

identify BIK1 as a potential Ser/Thr kinase rather than a Tyr kinase (Stone and Walker, 1995). The protein kinase active site signature (YRDIKASNILL) and the conserved amino acid triplets D<sub>219</sub>FG (subdomain VII) and APE<sub>248</sub> (subdomain VIII) that define the borders of the activation domain segment of BIK1 are consistent with other protein kinases (Johnson et al., 1996) (see Supplemental Figure 1 online). Activation of protein kinases occurs by regulatory phosphorylation in this region (Morgan and De Bondt, 1994). There are six putative phosphorylatable residues within the activation segment of the *BIK1* kinase (residues 219 to 248): Ser (S), Thr (T), or Tyr (Y).

Sequence comparisons and database searches revealed that BIK1 shares sequence similarity with a large number of putative receptor-like cytoplasmic kinases in subfamily VII of the

receptor-like cytoplasmic kinase family of proteins (RLCK VII). These proteins have a common monophyletic origin with receptor-like kinases but have no apparent transmembrane domain (Shiu and Bleecker, 2001a, 2001b). The function of a large number of these protein kinases is unknown, including 46 members of the *Arabidopsis* RLCK VII subfamily. BIK1 is most similar to two putative proteins (At3g55450 and At5g02290) with 77 and 63% identity, respectively, over a stretch of >233 amino acids covering the kinase domain. In addition, two closely related proteins with Ser/Thr kinase activities, APK1a and APK1b (Hirayama and Oka, 1992), are similar to BIK1. The M locus protein kinase (MLPK) involved in *Brassica* self-incompatibility is closely related to BIK1, and APK1a and APK1b are the *Arabidopsis* homologs of MLPK (Murase et al., 2004). The *Arabidopsis*

disease resistance protein kinase PBS1 (Swiderski and Innes, 2001) is 51% identical to BIK1 in the kinase domain. These sequence similarities are further confirmed by the phylogenetic analysis of the relationship between BIK1 and other related plant protein kinases (see Supplemental Figures 2 and 3 online). BIK1 is closely related to *Arabidopsis* kinases, including APK proteins (Hirayama and Oka, 1992), *Brassica rapa* MLPK, and rice (*Oryza sativa*; XP\_4943889 and NP\_910058) and *Solanum demissum* (AAT40481) putative kinases (see Supplemental Figures 2 and 3 online). A comprehensive classification of *Arabidopsis* protein kinases, including BIK1 and related subfamilies, is available at <http://plantsp.genomics.purdue.edu/plantsp/family/class.html>.

### **BIK1 Is Induced by Paraquat but Not by SA, JA, or the ET Precursor 1-Aminocyclopropane-1-Carboxylate**

*BIK1* is expressed at low but detectable levels in leaf tissues. Expression of the gene was not induced in response to the plant defense-mediating hormones SA, JA, or 1-aminocyclopropane-1-carboxylate (ACC), the natural precursor of ET (Figure 7A). However, paraquat (methyl viologen), an herbicide that generates ROIs, strongly induced *BIK1* gene expression. In addition, inoculation with both virulent and avirulent strains of *P. syringae* induced *BIK1* transcript accumulation (Figure 7B). The *ein2*, *coi1*, *nahG*, and *pad2* genotypes of *Arabidopsis* are impaired in ET, JA signaling, SA, and camalexin accumulation, respectively, and show altered defense responses to *Botrytis* and/or other pathogens (Thomma et al., 1999). The *BIK1* transcript was induced at levels comparable to wild-type levels in these genotypes, suggesting that *BIK1* may be upstream of these genes or may function independently (see Supplemental Figure 4 online).

The sensitivity of root and/or hypocotyl elongation of *bik1* to the plant hormones JA, ACC, and auxins (2,4-D and indole-3-acetic acid [IAA]) was comparable to that of wild-type plants, suggesting that the phenotype conferred by *bik1* is not attributable to altered responses to any of these phytohormones (data not shown).

### **BIK1 Is a Functional Protein Kinase That Is Localized to the Plasma Membrane**

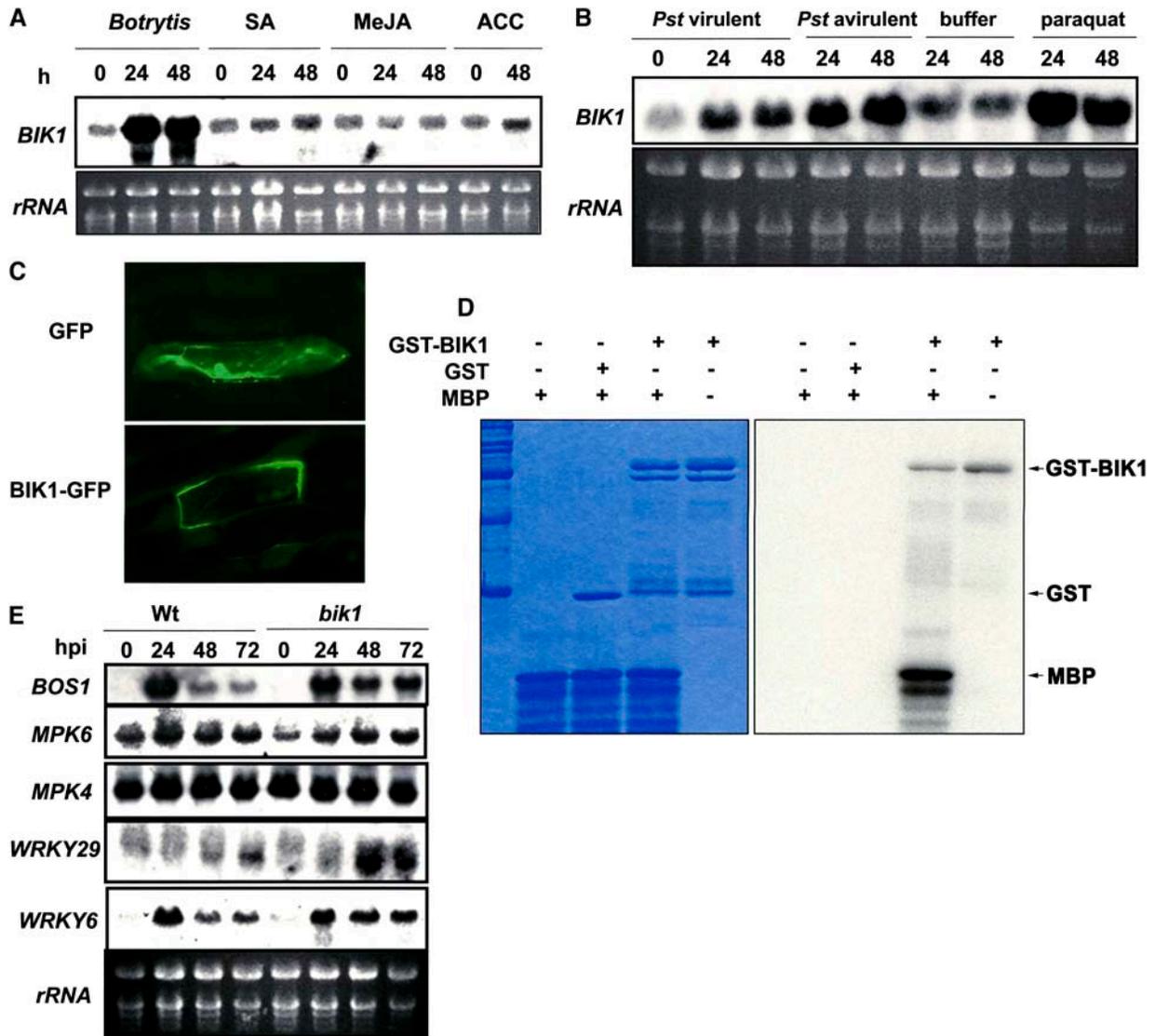
To better understand the mechanisms of *BIK1* function, we determined the subcellular localization of the BIK1 protein. Full-length BIK1 was translationally fused with green fluorescent protein (GFP), and the chimeric protein was expressed under the control of the CaMV35S promoter. Because *N*-myristoylated proteins are often translocated to the membrane, we expected the BIK1 protein to be localized to the membrane. Onion (*Allium cepa*) epidermal cells transiently expressing the BIK1-GFP fusion showed the GFP signal predominantly at the plasma membrane, whereas cells expressing GFP alone (control) exhibited signal around the nucleus and in the cytosol (Figure 7C). To confirm that BIK1 is a functional protein kinase, we performed a phosphorylation assay using BIK1 produced in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein. BIK1 exhibited autophosphorylation activity and also phosphorylated myelin basic protein as an artificial substrate (Figure 7D).

The structure of the BIK1 kinase suggests possible involvement in the early steps of recognition or signal transduction after contact with the pathogen. We hypothesized that BIK1 may function as an upstream component of the plant mitogen-activated protein kinase (MAPK) pathway. This hypothesis is consistent with the activation of the *Arabidopsis* MAPK pathway by bacteria, fungi, and ROIs, suggesting that signaling events initiated by diverse pathogens and external signals converge into a conserved MAPK cascade (Nühse et al., 2000; Asai et al., 2002). In addition, MPK4 and MPK6 were implicated as negative regulators of defense responses based on mutant phenotypes (Petersen et al., 2000; Menke et al., 2004). Recently, the OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) Ser/Thr protein kinase was described to function in the MAPK pathway upstream of MPK3 and MPK6 (Rentel et al., 2004). Loss of *OXI1* confers opposite disease resistance and root hair growth phenotypes (see below) to those of BIK1. To investigate the involvement of BIK1 in the early steps of pathogen recognition upstream of the plant MAPK pathways, we tested for MPK3, MPK6, MPK4, and OXI1 kinase activities using immunocomplex kinase assays. Although *Botrytis* infection induced the kinase activities of MPK3 and MPK6, no consistent differences between *bik1* and wild-type plants were observed (see Supplemental Figure 5 online). The *bik1* seedlings grown in tissue culture did not have significant constitutive activities of MPK3, MPK6, MPK4, and OXI1 relative to wild-type plants (data not shown). In addition, no direct physical interaction was observed between BIK1 and these MAPKs or OXI1 proteins in yeast two-hybrid assays (data not shown). There was also no difference detected in transcript amounts of *MPK4* and *MPK6* during *Botrytis* infection, regardless of the presence or absence of a functional *BIK1* (Figure 7E). These findings suggest that BIK1 functions independently of these components of the MAPK cascades and OXI1 kinase.

In addition, we examined the *Botrytis*-induced expression of potential target genes encoding transcription factors downstream of the MAPKs (Figure 7E). Recently, the transcription factors WRKY22 and WRKY29 were placed downstream of FLS2 and MPK3/MPK6 in a basal defense response pathway triggered by flagellin and also implicated in *Botrytis* resistance (Asai et al., 2002). WRKY6 regulates defense- and senescence-related genes in *Arabidopsis* (Robatzek and Somssich, 2002). The *WRKY6* and *WRKY29* genes showed increased transcripts in response to *Botrytis* infection in both wild-type and *bik1* plants (Figure 7E). *WRKY6* is strongly induced at 1 d after infection, whereas *WRKY29* transcript increased at later stages of infection in the wild type, with an early induction in the mutant. Similar results were obtained with the *BOS1* gene encoding an R2R3MYB transcription factor that is required to restrict *Botrytis* growth in inoculated plants (Mengiste et al., 2003). These data suggest that BIK1 may not be a direct upstream component of these transcription factors, either as a negative or a positive regulator.

### **BIK1 Is Required for Normal Plant Growth and Development**

*bik1* plants show defects in some aspects of plant growth and development. Mutant plants grown in tissue culture without disease or stress conditions produce shorter primary roots and longer and significantly more root hairs and lateral roots than



**Figure 7.** BIK1 Is a Functional Protein Kinase That Localizes to the Plasma Membrane.

(A) Accumulation of the *BIK1* transcript in response to *Botrytis*, SA, MeJA, and ACC.

(B) Accumulation of the *BIK1* transcript in response to *P. syringae* and paraquat.

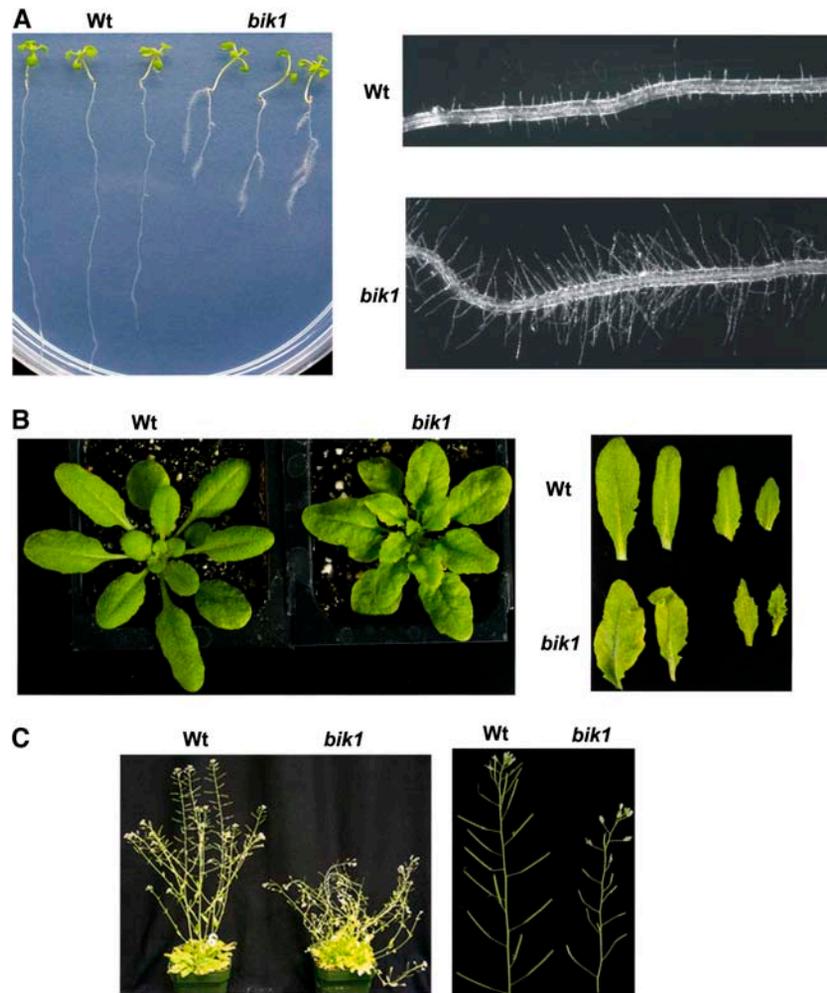
(C) Subcellular localization of the BIK1-GFP fusion protein in onion epidermal cells. Control GFP only (top) and BIK1-GFP (bottom) constructs were transiently expressed after particle bombardment. The micrographs were taken 24 h after bombardment of the cells. The experiment was repeated three times with similar results.

(D) GST-BIK1 phosphorylates the myelin basic protein (MBP) in vitro. Left, Coomassie blue staining; right, autoradiogram of the gel shown at left.

(E) Expression of selected transcription factor and MAPK genes in response to *Botrytis* in wild-type and *bik1* plants. The RNA gel blots were repeated in three independent infection assays with similar results. Each lane contained 20  $\mu$ g of total RNA.

wild-type plants (Figure 8A). Mutant plants also have leaves with serrated margins and wrinkled surfaces that display occasional curling (Figure 8B). Mature *bik1* plants flower an average of 5 to 7 d earlier than wild-type plants and have a weaker stem strength, leading to reduced standing ability and lodging (Figure 8C, left). In addition, *bik1* plants have reduced fertility and smaller siliques (Figure 8C, right). These data suggest that wild-type BIK1 is required for normal plant growth and development.

To confirm that the altered growth traits of *bik1* are also attributable to the mutation in the *BIK1* gene, *bik1* plants expressing the *BIK1* cDNA were analyzed. The altered growth characteristics of *bik1*, including the length of the primary root and the number and length of the root hairs, were fully restored to wild-type levels, confirming that *BIK1* is required for normal plant growth and development and that the *bik1* mutation resulted in the altered growth traits (see Supplemental Figure 6 online).



**Figure 8.** *bik1* Plants Show Altered Growth Traits.

**(A)** Root growth phenotypes of wild-type and *bik1* plants. *bik1* plantlets growing on Murashige and Skoog (MS) medium show decreased primary root elongation and increased root hair growth compared with wild-type plants. At right are close-ups of the roots to show the root hairs.

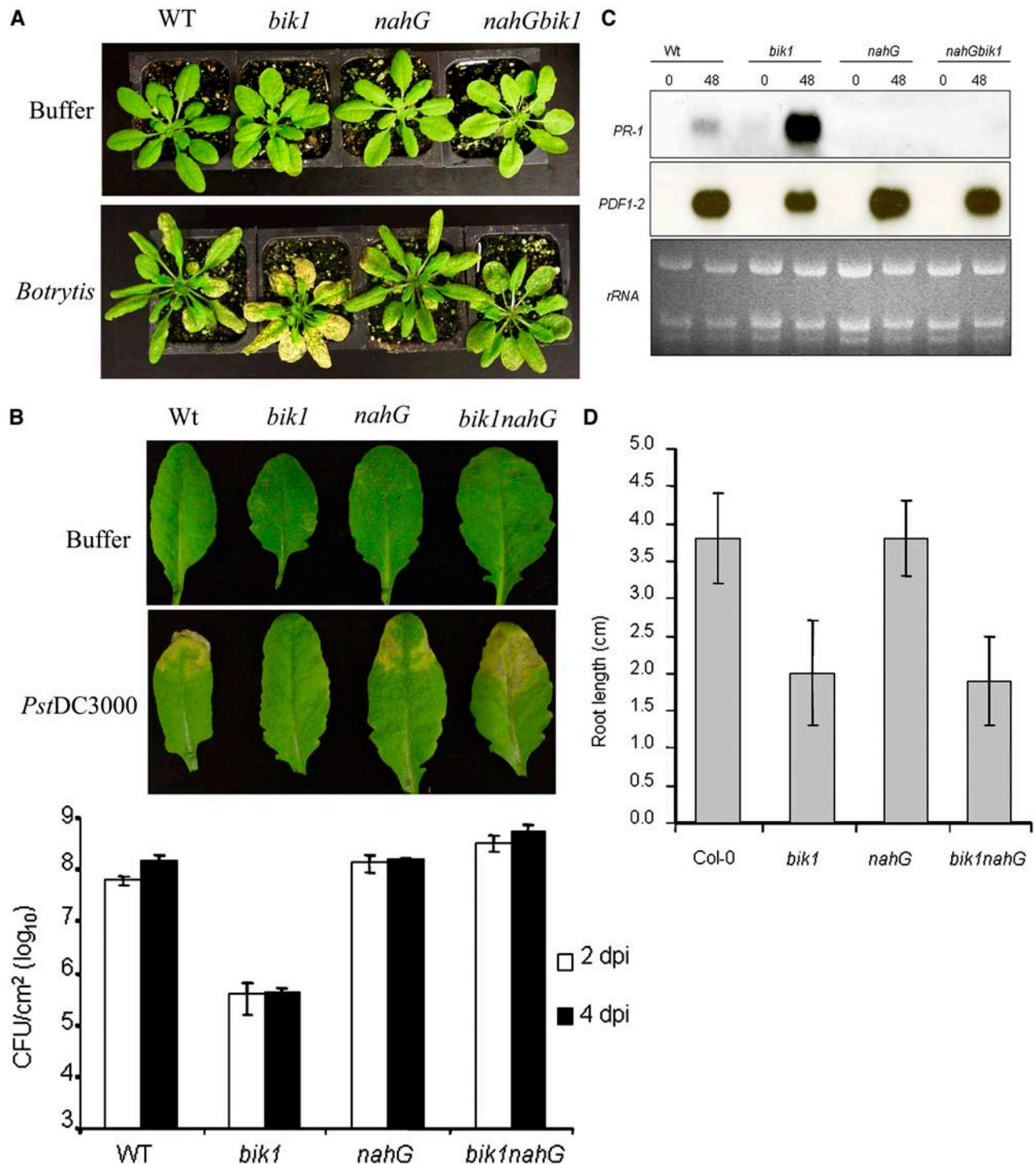
**(B)** Soil-grown plants revealing the wrinkled leaf surfaces in *bik1* (left), and close-ups of the leaves that emerged at different stages showing serrated leaf margins (right).

**(C)** Wild-type and *bik1* plants grown without stress conditions showing increased lodging in *bik1* plants (left), and close-ups of the inflorescences with smaller siliques (right).

### BIK1 Functions through SA-Dependent and SA-Independent Pathways

To investigate the role of SA in *bik1* disease responses and growth-related phenotypes, we crossed *bik1* to plants expressing the *nahG* gene encoding a bacterial salicylate hydroxylase that degrades SA to catechol (Delaney et al., 1994). F2 segregating plants were analyzed by PCR for the presence of the *bik1* mutation and the *nahG* gene. Homozygous *bik1* plants containing the *nahG* gene were identified, and their disease responses were studied (Figure 9). The *bik1 nahG* plants were comparable to wild-type and *nahG* plants in their resistance to *Botrytis* (Figure 9A). In the case of *PstDC3000*, *bik1 nahG* plants showed increased disease symptoms and supported significantly more bacterial growth than *nahG* or wild-type plants (Figure 9B)

(Duncan's test;  $P = 0.05$ ). These data suggest that the disease response of *bik1* is SA-dependent and that BIK1 may act through the SA pathway. The increased susceptibility of *bik1 nahG* plants to *PstDC3000* relative to *nahG* plants suggests that BIK1 may act through another pathway in addition to the SA pathway. The differences in bacterial growth between wild-type and *nahG* plants are consistent with previous reports (Delaney et al., 1994; Fouts et al., 2003; DebRoy et al., 2004). The patterns of SA levels in *bik1* are consistent with the pattern of expression of *PR-1* and the disease responses of *bik1 nahG* plants. As expected, expression of the *PR-1* gene was eliminated in *nahG* and *bik1 nahG* plants, whereas *PDF1.2* expression increased over that in *bik1* plants (Figure 9C). This finding is consistent with the suppression of JA/ET-regulated genes by increased SA levels and the antagonistic interactions between the JA/ET and SA response



**Figure 9.** The Role of SA in Disease and Growth Traits of *bik1*.

Comparisons of wild-type, *bik1*, *nahG*, and *bik1 nahG* are shown for the following features.

**(A)** Responses to mock (top row) or *Botrytis* (bottom row) inoculation.

**(B)** Responses to buffer infiltration (top row) and *PstDC3000* (bottom row). The bar graph shows bacterial growth at 2 and 4 d after infiltration with *PstDC3000* ( $OD_{600} = 0.001$ ). Duncan's multiple range test for counts was performed to separate mean values to determine the significance of the differences. The mean values of bacterial counts in *nahG* and *bik1 nahG* were significantly different from each other at  $P = 0.05$ . Data on bacterial counts represent averages  $\pm$  SE from four experiments.

**(C)** Expression of *PR-1* and *PDF1.2* genes after *Botrytis* inoculation. Each lane contained 6  $\mu$ g of total RNA.

**(D)** Primary root length. Root length was measured at 10 d of growth on MS plant growth medium. Each data point represents the average from measurements of 12 plants  $\pm$  SE. The experiments were repeated at least three times.

pathways (Kunkel and Brooks, 2002; Spoel et al., 2003). By contrast, the growth phenotypes of *bik1* were not affected by *nahG*. The primary root length and root hair phenotypes in *bik1 nahG* plants were comparable to those of wild-type *bik1* plants, suggesting that the function of BIK1 in root growth is independent of SA (Figure 9D). Among 190 F2 seedlings of the cross between *bik1* and *nahG* evaluated for root length and root hair patterns, 54 were similar to *bik1* and 136 showed wild-type root growth patterns. This segregation is consistent with no effect from the *nahG* gene and a 3:1 segregation ratio for a single recessive mutation rather than 15:1 if *nahG* had an effect ( $\chi^2 = 1.011$ ;  $P \leq 0.01$ ). The mean primary root length of *bik1* plants was approximately twofold shorter than that of wild-type plants after 10 d on MS plates. *nahG* plants did not differ from wild-type plants in root growth characteristics (Figure 9D). The early flowering and reduced fertility of *bik1* were also not restored to wild-type levels by *nahG*.

## DISCUSSION

In this study, we identified BIK1 as a crucial component of host response signaling required to activate the resistance responses to *Botrytis* and *A. brassicicola* infection in *Arabidopsis*. *BIK1* encodes a regulatory protein, specifically a protein kinase, predicted to be specific to Ser/Thr residues, that is similar to receptor-like cytoplasmic protein kinases and may act early in the disease response pathway. Additionally, the *bik1* mutant phenotypes suggest that the BIK1 kinase function cannot be fully complemented by other endogenous kinases. The *BIK1* gene transcript is strongly upregulated in response to challenge with *Botrytis* and a ROI-generating compound. The *bik1* mutant is susceptible to *Botrytis* and *A. brassicicola* and shows attenuated expression of the plant defensin *PDF1.2* gene, but it is competent for the activation of SAR. Race-specific resistance to *Pst* is not altered, based on plant resistance to a bacterial strain with the *Avr* protein. However, *bik1* plants exhibit increased resistance to a virulent bacterial pathogen that is based on SA-dependent and SA-independent pathways and independent of cell death lesions. Interestingly, *bik1* plants accumulate increased levels of SA before and after *Botrytis* infection, suggesting that BIK1 acts upstream of SA accumulation. These data implicate BIK1 as a negative regulator of SA accumulation and basal defense against virulent bacterial pathogens, because the *bik1*-null mutant supported less bacterial growth and a total absence of disease symptoms.

The function of BIK1 as a disease resistance factor may be to regulate normal levels of SA required for resistance to necrotrophs. Upon infection, BIK1 may be activated to trigger the *Botrytis* and *A. brassicicola* resistance response, including the regulation of optimal levels of SA synthesis. In the absence of BIK1, this regulation is relieved and SA level increases, which may lead to suppression of the mechanisms required for necrotrophic resistance, consistent with previous reports (Penninckx et al., 1996; Clarke et al., 1998; Gupta et al., 2000; Kunkel and Brooks, 2002; Spoel et al., 2003). Accordingly, *bik1* contains increased SA levels, reduced *PDF1.2* expression, and distinct disease resistance responses to biotrophic and necrotrophic pathogens. These distinct responses are further sub-

stantiated by data from *bik1 nahG* plants that show wild-type levels of resistance to *Botrytis* infection and expression of *PDF1.2*. The ET/JA functions independent of defense responses are normal, as *bik1* plants exhibited wild-type sensitivity to plant hormones and lacked hormone-related phenotypes. The loss of resistance to necrotrophic pathogens but enhanced resistance to virulent pathogens suggests that BIK1 modulates the cross-talk between different defense signaling pathways.

The role of SA in regulating responses to *Botrytis* and other necrotrophs appears to be complex and may be multidimensional, with both positive and negative regulatory roles. Increased SA associated with lesion-mimic mutants correlated with susceptibility to *Botrytis*, and this may be an indirect effect rather than a defect in a bona fide resistance mechanism. In addition, SA synthesized via Phe ammonia lyase, and not via isochorismate synthase, was required for local resistance in *Arabidopsis*, whereas defects in SA signaling had no effect (Ferrari et al., 2003). Increased SA also suppresses the expression of JA-responsive genes (Spoel et al., 2003) normally required for full resistance to necrotrophic pathogens (Thomma et al., 1998, 1999). The function of BIK1 as a positive regulator of resistance to *Botrytis* and a negative regulator of resistance to *Pst* is dependent on normal levels of SA. When SA increases above a certain threshold level, it may trigger the suppression of mechanisms required for resistance to *Botrytis* and *A. brassicicola* while still promoting mechanisms for resistance to *Pst*. Alternatively, BIK1 may suppress SA accumulation and basal resistance to *Pst*, and when removed, it may lead to the activation of defense responses that some necrotrophs (*Botrytis* and *A. brassicicola*) exploit for their benefit. Consistent with this notion, the hypersensitive cell death, a particularly strong defense response, promotes susceptibility to *Botrytis* (Govrin and Levine, 2000). Our data and emerging data suggest no effect or limited effects linked to SA deficiency, whereas increased SA (and *PR-1* expression) is positively correlated with susceptibility to *Botrytis* in *Arabidopsis*.

Localization of BIK1 to the plasma membrane suggests that BIK1 may act as an early component of the plant defense response, either directly in pathogen recognition or early in the signaling cascade. A protein with a similar structure to BIK1, the OXI1 protein kinase, is activated in response to active oxygen species and functions through the positive regulation of MPK3 and MPK6 (Rentel et al., 2004). The loss-of-function *oxi1* mutant has reduced root hair growth and increased susceptibility to virulent strains of the oomycete pathogen *Peronospora parasitica*, which was attributed to altered ROI signaling (Rentel et al., 2004). The root hair phenotypes and disease responses of *bik1* and *oxi1* mutants suggest that the two proteins act antagonistically or that BIK1 functions upstream of OXI1, MPK3, and MPK6 as a negative regulator. In response to *Botrytis* infection, the kinase activity of MPK3 and MPK6 increased, but no consistent differences were observed between *bik1* and wild-type plants. Similarly, tissue culture-grown 14-d-old seedlings of *bik1* showed no significant difference from wild-type plants for MPK3, MPK4, MPK6, and OXI1 kinase activities. This, coupled with the lack of evidence for direct physical interaction between BIK1, MAPKs, or OXI1, implicates BIK1 as a negative regulator of root hair growth and basal resistance to pathogens independent of the

MAPK pathway. We cannot exclude the possibility that BIK1 functions through other components of the MAPK pathway and that MAPK activity may be transient and tissue-specific. However, further and detailed examination of the relationship between BIK1 and this group of proteins and other MAPKs is warranted.

BIK1 may be activated by pathogen-derived signals or signals resulting from pathogen-plant interactions regardless of the strain. The increased transcript accumulation of *BIK1* in response to *Botrytis*, *Pst* strains, and paraquat is consistent with this hypothesis. The kinase activity of BIK1 may be required to transduce pathogen-derived signals to downstream molecules that are targeted for negative or positive regulation depending on the pathogen. The reduced activation of the ROI-responsive *PDF1.2* in response to paraquat suggests an altered ROI signaling in *bik1* plants. Previously, the attenuated expression of *PDF1.2* in response to paraquat was linked to altered ROI signaling and susceptibility to necrotrophic pathogens in *Arabidopsis* (Tierens et al., 2002). Paraquat treatment leads to the production of  $H_2O_2$ ,  $O_2^-$ , and  $OH^-$  in plant cells (Suntres, 2002). ROI production and signaling are required for plant defense responses and normal root hair growth in *Arabidopsis* (Levine et al., 1994; Lamb and Dixon, 1997; Foreman et al., 2003; Rentel et al., 2004). Reduced ROI levels resulted in reduced cell death and resistance to biotrophic pathogens (Torres et al., 2002). In the case of *Botrytis*, host susceptibility positively correlated with the levels of  $O_2^-$  or  $H_2O_2$  produced (Govrin and Levine, 2000). Consistent with the disease responses of *bik1*,  $H_2O_2$  levels in *Botrytis*-infected leaf tissue was slightly higher than that in wild-type plants, based on histochemical assays. The sensitivity to ROI-generating compounds was not different between *bik1* and wild-type plants. This finding excludes the possibility that susceptibility to *Botrytis* is the result of plant sensitivity to ROIs.

Among defense responses associated with resistance to necrotrophs in *Arabidopsis*, in *bik1*, camalexin accumulation was not affected, whereas *PR-1* was expressed more strongly and the induced expression of *PDF1.2* was reduced. *PR-1* expression positively correlates with resistance to biotrophic pathogens but negatively correlates with resistance to *Botrytis* in some mutants, including *bos3* and *ssi2*, and provides a clear distinction between host responses to the two classes of pathogens (Kachroo et al., 2001; Veronese et al., 2004). In the *bos* mutants described previously, expression of *PR-1* and camalexin accumulation varied, with no consistent correlations to enhanced *Botrytis* susceptibility (Mengiste et al., 2003; Veronese et al., 2004). This lack of consistent correlation in different mutants may indicate that the roles of the pathways represented by the defense markers are not clear and may vary in the different mutants, because there may be cross-regulation of the different pathways. Similarly, the *bos* mutants varied in terms of their disease responses to biotrophic pathogens, with no consistent antagonistic or coordinated genetic control of resistance (Mengiste et al., 2003; Veronese et al., 2004).

The *Arabidopsis* genome encodes many putative Ser/Thr kinases whose functions are not yet determined. The *R* genes *PBS1* and *PTO* from *Arabidopsis* and tomato, respectively, encode Ser/Thr protein kinases that have similar structures to that of BIK1 and show significant sequence similarity. In contrast

with BIK1, however, *PBS1* and *PTO* are involved in race-specific resistance to *Pst* (Martin et al., 1993; Swiderski and Innes, 2001). It is possible that the BIK1 protein may be a cellular target for an effector protein(s) and may interact with and be guarded by an unknown R protein, consistent with the guard hypothesis (Dangl and Jones, 2001). In that case, inactivation of BIK1 could result in upregulated basal resistance similar to the responses of the *rin4* mutant (Mackey et al., 2003; Belkhadir et al., 2004). In sum, the *bik1* mutation represents a defect in a novel gene affecting disease responses and plant growth. Our data suggest that *bik1* is affected in signaling required for the activation of cellular mechanisms involved in plant development and the pathogen response, and they provide a novel avenue for further dissection of the components of disease response pathways.

## METHODS

### Plant Cultivation

Procedures for growing plants in tissue culture and in soil have been described (Mengiste et al., 2003). *Arabidopsis thaliana* plants were grown in soil under fluorescent light ( $150 \mu E \cdot m^{-2} \cdot s^{-1}$ ) at  $22 \pm 4^\circ C$  with 60% RH and a 12-h-light/12-h-dark cycle. For axenic growth, seeds were sterilized and sown on a medium solidified with 1% agar that contained MS salts (PhytoTechnology Laboratories) and 2% (w/v) sucrose. Conditions for axenic growth were 12 h of light of  $60 \mu E \cdot m^{-2} \cdot s^{-1}$ .

### Fungal Culture and Disease Assays

The *Botrytis cinerea* strain BO5-10 used in all of our experiments was cultured on  $2 \times V8$  agar (36% V8 juice, 0.2%  $CaCO_3$ , and 2% Bacto-agar) and incubated at 20 to  $25^\circ C$ . Collection of conidia and plant inoculation were as described (Mengiste et al., 2003). Conidia were resuspended in 1% Sabouraud maltose broth buffer (Difco) before inoculation. *Alternaria brassicicola* strain MUCL20297 was grown on potato dextrose agar, and spores were suspended in distilled water for plant inoculation. The procedures for inoculation and determination of susceptibility have been described (Mengiste et al., 2003; Veronese et al., 2004). All of the *Botrytis* disease assays described here were performed on whole plants by spraying a spore suspension ( $2 \times 10^5$  spores/mL) in Sabouraud maltose broth buffer. The disease assay for *A. brassicicola* was performed on detached leaves. A single  $5\text{-}\mu L$  spore suspension ( $5 \times 10^5$  spores/mL) in water was deposited on each detached leaf. Inoculated plants were kept under a transparent cover to maintain high humidity. *A. brassicicola* spores in inoculated plants were counted as described with minor modifications (van Wees et al., 2003).

### Bacterial Disease Assay

Leaves of 4-week-old plants were infiltrated with bacterial suspensions ( $OD_{600} = 0.001$  in  $10 \text{ mM } MgCl_2$ ) as described (Mengiste et al., 2003). The bacterial strain *Pseudomonas syringae* pv *tomato* DC3000, strain DC3000 harboring a plasmid carrying the *avrRpm1* gene, and the bean-infecting strain *Pseudomonas syringae* pv *phaseolicola* strain 1448A race 6 used in our assays were from J. Dangl (University of North Carolina, Chapel Hill). To determine bacterial growth, leaf discs from infected leaves were collected at 0, 2, and 4 d after inoculation. Each experiment for bacterial growth assay was performed in three replicates. At each time point, 10 leaves were collected from each wild-type plant and other genotypes tested for each replicate. Leaf discs of the same size were made using a hole puncher, and bacterial titer per leaf area was determined. Analysis of variance and Duncan's multiple range test for

counts were performed on the bacterial count data in Figure 9 using the SAS program.

### SA Analysis

Free SA levels in leaves were determined by grinding tissue (1 g) to a fine powder under liquid nitrogen and extracting with 100% methanol (2.5 mL) for 20 min with shaking. Extracts were then passed through a 0.45- $\mu$ m filter (Nylon Acrodisc 13; Pall Corporation) and analyzed with an HPLC apparatus equipped with an analytical C-18 column (Econosphere, 150 mm  $\times$  4.6 mm; Alltech) and a fluorescence detector (Shimadzu Scientific Instruments). The HPLC program consisted of a linear gradient from 25% acetonitrile and 75% 0.1 M sodium phosphate buffer, pH 3.3 (v/v), to 75% acetonitrile and 25% 0.1 M sodium phosphate buffer, pH 3.3 (v/v), over the course of 14 min, with the detector set at 305-nm excitation and 407-nm emission. SA levels were quantified by comparing peak areas of samples with a standard curve generated from a SA standard (Sigma-Aldrich).

### Chlorophyll Extraction and Assays

Chlorophyll was extracted essentially as described (Veronese et al., 2003). Briefly, shoot tissues were ground into powder in liquid nitrogen and weighed. Chlorophyll was extracted in 80% acetone, and the concentration per gram fresh weight was calculated as described by Lichtenthaler (1987). Chlorophyll was extracted from pools of six uninoculated and six inoculated plants for each genotype.

### Induction Treatments

Induction treatments were performed by spraying 4-week-old soil-grown plants with  $2 \times 10^5$  *Botrytis* spores/mL, 5 mM SA (Aldrich), 100  $\mu$ M MeJA (Aldrich), 0.5 mM ACC (Sigma-Aldrich), or 100  $\mu$ M paraquat (methyl viologen; Sigma-Aldrich). The chemical compounds were dissolved in 0.1% ethanol. The controls were sprayed with 0.1% ethanol.

### Sensitivity to Plant Hormones

Surface-sterilized seeds were sown on 1.0% (w/v) agar medium containing MS salts, 2% (w/v) sucrose, and different concentrations of MeJA, ACC, 2,4-D, or IAA, pH 5.7. The plant hormones were added to the autoclaved medium from filter-sterilized stock solutions. Plates were incubated in a vertical position. The effects of MeJA and ACC on primary root growth and/or hypocotyl elongation were determined essentially as described (Ton et al., 2002). The primary root length was measured after 8 d of growth in culture. In each case, 25 randomly selected seedlings were measured. The sensitivity of root elongation to auxin (2,4-D or IAA) was done in a similar manner. IAA was tested at 0.01, 0.1, 1, 10, and 100  $\mu$ M; 2,4-D was tested at 100, 200, 300, 400, and 500 nM; ACC was tested at 1, 3, and 6  $\mu$ M; and MeJA was tested at 0.2, 0.4, 0.6, 0.8, and 1  $\mu$ M. The experiments were repeated at least twice on different seed lots.

### RNA Blots

RNA was extracted from harvested leaf tissue frozen in liquid nitrogen using phenol/chloroform followed by lithium chloride precipitation (Lagrimini et al., 1987). RNA was separated on formaldehyde agarose gels. The gels were then blotted onto Hybond N<sup>+</sup> nylon membranes (Amersham Pharmacia Biotech). Probes were labeled with <sup>32</sup>P by random priming using a commercial kit (Sigma-Aldrich). Hybridization of probe and subsequent washings were performed as described (Church and Gilbert, 1984).

### Identification of *bik1 nahG* Plants

The *bik1 nahG* plants were identified using PCR. Plants carrying the *nahG* gene were identified using primers specific to *nahG* (5'-ATGAAAAACAA-TAAACTTGGCTTG-3' and 5'-CTGACCTCCAGCACATGACTAC-3'). The primers from the *BIK1* gene (5'-CAGGTCACTGAATGCAAGAAGCG-3' and 5'-GGGTATGGGACATGTAACCGGAAA-3') and primers from the T-DNA insertion in *bik1* (5'-TGGTTCACGTAGTGGGCCATCG-3') (Alonso et al., 2003) were used to identify plants carrying the *bik1* mutation. The procedure for the extraction of plant genomic DNA for PCR was described previously (Veronese et al., 2004).

### Histochemical Assays

The presence of H<sub>2</sub>O<sub>2</sub> in leaf samples was determined using the DAB staining method (Thordal-Christensen et al., 1997). Leaves were detached and placed in 1 mg/mL DAB-HCl, pH 3.8 (D-8001; Sigma-Aldrich). In the presence of endogenous peroxidase activity, DAB generates a reddish-brown DAB polymer that can be detected at the site of H<sub>2</sub>O<sub>2</sub> formation. After staining, leaves were cleared in 96% boiling ethanol and observed with a microscope. Trypan blue staining of leaves was performed as described (Koch and Slusarenko, 1990).

### Subcellular Localization

For the analysis of the subcellular localization of BIK1 using the GFP reporter gene, the *BIK1* coding sequence was cloned into a C-terminal GFP fusion vector kindly provided by Ron Coolbaugh (Purdue University). The vector was constructed by assembling the CaMV35S promoter from pBI121 (Clontech), the multicloning site from the vector pSP72 (Promega), and the GFP from the vector pUHGFP3-N (Carlsberg Laboratory). *BIK1* coding sequence was amplified using the BD Advantage-HF 2 PCR kit (BD Biosciences, Clontech) using the primers 5'-CGGGATCCCGA-TGGGTTCTTGACAG-3' and 5'-GCCTAGGACACAAGGTGCCTGCCA-3'. The correct amplification was verified by sequencing.

### In Vitro Kinases and Immunocomplex Kinase Assays

The open reading frame of *BIK1* was cloned into GST fusion protein expression vector pGEX4T-1 (Pharmacia). Expression of the GST fusion protein and affinity purification were performed as described previously (Cardinale et al., 2002). The protein concentrations of the recombinant proteins were determined with the Bio-Rad detection system using BSA as a standard. Kinase reactions were performed in 15  $\mu$ L of kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 5 mM EGTA, and 1 mM DTT) containing 5  $\mu$ g of GST fusion protein, 5  $\mu$ g of myelin basic protein, 0.1 mM ATP, and 2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. The protein kinase reactions were performed at room temperature for 30 min, and the reactions were stopped by adding 4 $\times$  SDS loading buffer. The phosphorylation of myelin basic protein was analyzed by autoradiography after separation by 12.5% SDS-PAGE. Immunocomplex kinase assays were performed as described (Cardinale et al., 2002).

### Complementation Experiments

The full-length *BIK1* cDNA was obtained from the ABRC (clone F17A14). The cDNA was reamplified using the BD Advantage-HF 2 PCR kit (BD Biosciences, Clontech), sequenced, and inserted after the CaMV35S promoter into a modified version of the binary vector pCambia 1200 kindly provided by Huazhong Shi (Texas Tech University). Hygromycin-resistant homozygous T2 plants were identified from progeny of primary transformants and selfed to produce the T3 generation of *bik1* plants expressing the *BIK1* cDNA. Five independent transformants were examined for the various phenotypes of *bik1*, as described in Results.

### Phylogenetic Analysis

For construction of the BIK1 phylogenetic tree (see Supplemental Figure 2 online), the full-length amino acid sequences were aligned using ClustalW (Thompson et al., 1994) (see Supplemental Figure 3 online) with default gap penalties, and the alignment was manually adjusted where necessary. Mean character distances were used to construct an unrooted neighbor-joining phylogeny (Saitou and Nei, 1987) from the PAUP version 4.0 package (Swofford, 2000). Statistical support of the branches was tested with 1000 bootstrap resamples.

### Accession Numbers

Sequence data for the genes described in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: BIK1 (At2g39660), NAK (At5g02290), PBS1 (AT5g13160), APK1B (At2g28930), APK1A (At1g07570), APK2B (At2g02800), MPLKe (BAD12263), PTO (A49332), putative protein kinase (At3g55450), *Solanum demissum* putative kinase (AAT40481), *Oryza sativa* putative protein kinases (NP\_910058, XP\_493889), and *Solanum demissum* putative protein kinase (AAT40481).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Deduced Amino Acid Sequence of the BIK1 Protein.

**Supplemental Figure 2.** Phylogenetic Tree Showing the Relationship between BIK1 and Related Plant Protein Kinases.

**Supplemental Figure 3.** Multiple Sequence Alignment Used for Phylogenetic Analysis.

**Supplemental Figure 4.** Expression of *BIK1* in *Arabidopsis* Wild-Type, *ein2*, *coi1*, *nahG*, and *pad2* Plants during *Botrytis* Infection.

**Supplemental Figure 5.** Immunocomplex Assays Showing the Activation of MPK3 and MPK6 in Wild-Type and *bik1* Plants in Response to *Botrytis* Infection.

**Supplemental Figure 6.** The *BIK1* cDNA Rescues the *bik1* Altered Growth Traits to Wild-Type Levels.

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