The Plant Cell, Vol. 23: 2831–2849, August 2011, www.plantcell.org

Biochemical and Genetic Requirements for Function of the Immune Response Regulator BOTRYTIS-INDUCED KINASE1 in Plant Growth, Ethylene Signaling, and PAMP-Triggered Immunity in *Arabidopsis* [C][W]

Kristin Laluk,[1] Hongli Luo,[2] Maofeng Chai,[3] Rahul Dhawan,[4] Zhibing Lai, and Tesfaye Mengiste[5]

Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

Arabidopsis thaliana **BOTRYTIS-INDUCED KINASE1 (BIK1) regulates immune responses to a distinct class of pathogens. Here, mechanisms underlying BIK1 function and its interactions with other immune response regulators were determined. We describe BIK1 function as a component of ethylene (ET) signaling and PAMP-triggered immunity (PTI) to fungal pathogens. BIK1 in vivo kinase activity increases in response to flagellin peptide (flg22) and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) but is blocked by inhibition of ET perception.** ***BIK1*** **induction by flg22, ACC, and pathogens is strictly dependent on EIN3, and the** ***bik1*** **mutation results in altered expression of ET-regulated genes. BIK1 site-directed mutants were used to determine residues essential for phosphorylation and biological functions in planta, including PTI, ET signaling, and plant growth. Genetic analysis revealed flg22-induced PTI to** ***Botrytis cinerea*** **requires BIK1, EIN2, and HUB1 but not genes involved in salicylate (SA) functions. BIK1-mediated PTI to** ***Pseudomonas syringae*** **is modulated by SA, ET, and jasmonate signaling. The** ***coi1*** **mutation suppressed several** ***bik1*** **phenotypes, suggesting that** ***COI1*** **may act as a repressor of** ***BIK1*** **function. Thus, common and distinct mechanisms underlying BIK1 function in mediating responses to distinct pathogens are uncovered. In sum, the critical role of BIK1 in plant immune responses hinges upon phosphorylation, its function in ET signaling, and complex interactions with other immune response regulators.**

INTRODUCTION

Mechanisms of plant defense against microbial infection have been studied extensively and the major immune response pathways identified along with many of their genetic components. Recognition of microbial effectors activates a highly specific and efficient form of plant defense known as effector-triggered immunity (ETI) (Jones and Dangl, 2006). Alternatively, pathogen-associated molecular patterns (PAMPs), evolutionarily conserved components of pathogens, can also elicit PAMP-triggered immunity (PTI), an attenuated but broad spectrum disease resistance. Bacterial flagellin and peptidoglycan, fungal chitin, and oligogalacturonides are some of the well-studied molecules that activate PTI in *Arabidopsis thaliana* (Boller and Felix, 2009). The idea that plant immune responses span a continuum between PTI and ETI rather than acting as distinct defense responses has recently been raised (Thomma et al., 2011). Although the recognition mechanisms and downstream responses in ETI and PTI are known, the intermediate genetic and biochemical events are less well understood. PTI and ETI converge on downstream immune responses, such as the oxidative burst, deposition of callose, and defense gene expression (Tsuda and Katagiri, 2010). Despite such convergence, the intensity, strength, and, thus, effectiveness of ETI and PTI responses are different albeit interaction dependent (Jones and Dangl, 2006).

Recognition of PAMPs or effectors is mediated by diverse plant proteins that confer specificity in the activation of immune responses. Effector recognition is mediated by R proteins, which have been widely studied and predominantly consist of nucleotide binding site–leucine-rich repeat proteins but also include receptor-like kinases (RLKs). Thus far, RLKs, such as the chitin receptor LysM/CERK1, the flagellin receptor FLS2, and the receptor for bacterial EF-TU EFR1, have been identified as critical early determinants of PTI (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Miya et al., 2007). BAK1, previously known for its role in brassinolide signaling, is an RLK central to PTI responses initiated by various PAMPs likely by acting as a coreceptor (Chinchilla et al., 2007). Receptor-like cytoplasmic kinases (RLCKs) are a subclass of RLKs that lack extracellular domains but due to their homology are classified within the RLK superclade (Shiu and Bleecker, 2001). The *Arabidopsis* genome

[1] Current address: Department of Biology, Colorado State University, Fort Collins, Colorado 80523.
[2] Current address: College of Agriculture, Hainan University, Haikou, Hainan 570228, China.
[3] Current address: Department of Plant Pathology, 201 Kottman Hall, 2021 Coffey Road, Ohio State University, Columbus, OH 43210.
[4] Current address: Monsanto Company, W212, 800 N. Lindberg Blvd., St. Louis, MO, 63167.
[5] Address correspondence to mengiste@purdue.edu.
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).
[C]Some figures in this article are displayed in color online but in black and white in the print edition.
[W]Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.111.087122

contains 610 RLKs and RLCKs that are predicted to function in plant response signaling to microbial infection, hormones, and other endogenous and environmental cues (Shiu and Bleecker, 2001; Becraft, 2002). RLCKs have also been implicated to function in ETI and PTI responses, acting in concert with surface-localized RLKs or indirectly as intracellular receptors of microbial effectors. The tomato (*Solanum lycopersicum*) Pto kinase serves as an intracellular receptor for the *Pseudomonas syringae* pv tomato (*Pst*) bacterial effector AvrPto (Tang et al., 1996). *Arabidopsis* RLCK PBS1 is a target of the *P. syringae* pv *phaseolicola* effector AvrPphB and a known component of ETI mediated by the nucleotide binding site–leucine-rich repeat R protein RPS5 (Shao et al., 2003). Tomato TPK1b and *Arabidopsis* BOTRYTIS-INDUCED KINASE1 (BIK1) are typical RLCKs localized to the plasma membrane that act early in the defense response pathways that contribute to defense against fungal necrotrophs (Veronese et al., 2006; Abuqamar et al., 2008). BIK1 and closely related kinases are also required for PTI to *Pst* downstream of FLS2 and BAK1 (Lu et al., 2010; Zhang et al., 2010). In addition, BIK1 regulates plant growth traits, demonstrating a dual function in plant immune and growth responses similar to BAK1 (Veronese et al., 2006). Both BAK1 and BIK1 appear to affect plant development and defense through their function in hormone biosynthesis and/or signaling.

Plant hormone synthesis and signaling modulates immune responses and development. Jasmonate (JA) and ethylene (ET) are generally regarded as the primary regulators of immune responses to necrotrophic pathogens (Glazebrook, 2005). By contrast, salicylate (SA) is central to resistance to biotrophic and hemibiotrophic pathogens (Dempsey et al., 1999), but its role in defense against necrotrophs is complex. In *Arabidopsis*, SA accumulation exceeding wild-type levels promotes susceptibility to necrotrophic fungi (Veronese et al., 2006), whereas deficiency in SA or SA signaling has no impact (Veronese et al., 2004) or only affects resistance at the site of inoculation (Ferrari et al., 2003). Biological or chemical activation of systemic acquired resistance, an SA-dependent immune response, has no effect on resistance to *B. cinerea* in *Arabidopsis* (Govrin and Levine, 2002). More recent data reveal that JA, SA, ET, gibberellin, and abscisic acid all contribute to plant immune responses to necrotrophic pathogens (Grant and Jones, 2009). The interaction between these pathways is a major factor in determining resistance, allowing plants to fine-tune immune responses depending on the invading pathogen. Mutual antagonism between SA and JA/ET-dependent defenses and its impact on immune responses to biotrophic and necrotrophic pathogens have been established (Spoel et al., 2007). Thus, hormone homeostasis is important for normal immune responses as well as typical growth and development.

Previously, we have shown that *Arabidopsis BIK1* is required for resistance to *Botrytis cinerea* and *Alternaria brassicicola* but suppresses defense against *Pst* (Veronese et al., 2006). More recently, data showing BIK1 function in the integration of PTI responses downstream of the PAMP receptors FLS2, EFR1, and CERK1 was published (Zhang et al., 2010). Consistent with this, BIK1 is a component of the flagellin-receptor complex that is a key determinant of flagellin-mediated immune responses (Lu et al., 2010). However, neither the role of BIK1 in PTI to fungal pathogens nor the role of BIK1 phosphorylation in any disease resistance was examined. In this study, the genetic, molecular, and biochemical function of BIK1 in PTI to bacterial and fungal pathogens was studied in detail. New functions for BIK1 in ET-mediated plant immune responses and growth traits were determined. In addition, epistasis analysis of the interactions between BIK1 and other *Arabidopsis* immune response regulators uncovered the genetic requirements for BIK1 function in PTI to fungal necrotrophs as well as virulent and nonpathogenic strains of *Pst*, ET, and flagellin responses.

RESULTS

Kinase Assays on Recombinant BIK1 Identifies Residues Required for in Vitro Phosphorylation Activity

The BIK1 amino acid sequence was analyzed to identify regulatory regions and putative phosphorylation sites. BIK1 has a large conserved catalytic kinase domain (KD) and nonconserved flanking regions predicted to contribute to its biological functions (Figure 1A). Within the KD is the activation domain (AD), a regulatory region important for general kinase activity and biological function (Hanks and Hunter, 1995). Analysis of the BIK1 AD identified seven phosphorylatable residues, of which, four (Ser-236, Thr-237, Thr-242, and Tyr-245) are invariant in BIK1-related kinases from *Arabidopsis* and other species (Figure 1A). Preceding the AD is an ATP binding site, the conserved Arg and Asp residues in domain VIb that classify BIK1 as an RD-type kinase (Hanks and Quinn, 1991; Hanks and Hunter, 1995) as well as additional putative phosphorylation sites.

To determine the biochemical basis for the biological functions of BIK1, highly conserved residues and predicted phosphorylation sites were substituted with Ala. Recombinant BIK1 proteins carrying site-specific substitutions in these 18 selected residues were made in and purified from *Escherichia coli* and assayed for auto- and transphosphorylation activities. Recombinant BIK1 phosphorylates itself as well as the commonly used artificial kinase substrate myelin basis protein (MBP), as previously shown (Veronese et al., 2006). The BIK1^{S33A}, BIK1^{T35A}, BIK1^{T42A}, BIK1^{T64A}, BIK1^{K105A}, BIK1^{Y234A}, and BIK1^{Y245A} substitutions eliminated all kinase activities (Figure 1B). Mutations at Thr-94, Ser-206, Ser-233, Ser-236, Ser-253, Ser-274, and Ser-333 did not alter BIK1 auto- or MBP phosphorylation. Autophosphorylation was lost in BIK1^{S71A} and BIK1^{D210A}, yet both proteins retained substrate kinase activity. By contrast, BIK1^{T237A} activity was restricted to self-phosphorylation.

BIK1 Phosphorylation Residues Are Required for ET-Induced BIK1 Kinase Activity in Vivo

To determine the biological functions of BIK1 residues, selected BIK1 substitutions, based on results from the experiments above and known functions in related proteins, were tagged with the hemagglutinin-epitope and expressed in the *bik1* mutant. The sites of the substituted residues used for in planta assays are indicated in Figure 1A. BIK1 phosphorylation is visible as a mobility shift after treatment with 1-aminocyclopropane-1-carboxylic acid (ACC) (Figures 1C to 1E). Phosphorylation of BIK1

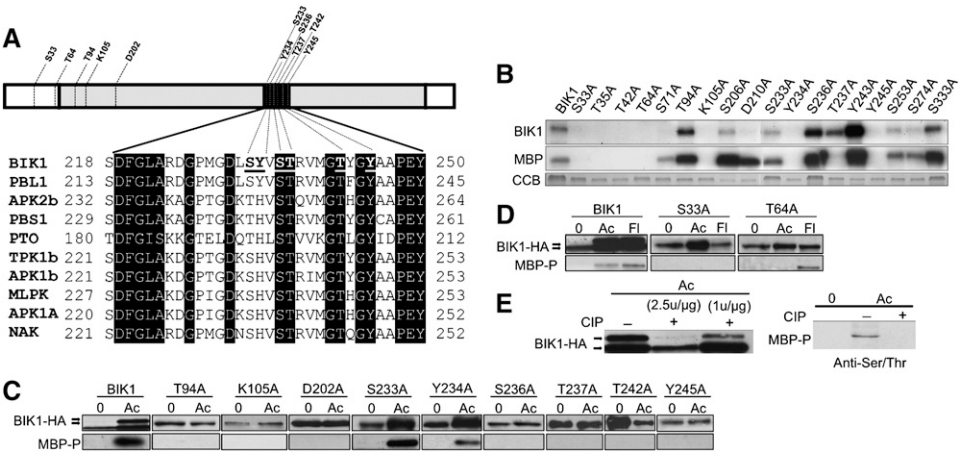

Figure 1. BIK1 Conserved Residues and in Vitro and in Vivo Kinase Assays.

(A) Structure of the BIK1 protein and comparison of residues in the ADs of BIK1 and related kinases. Gray region denotes the KD and black the AD. Residues noted in the BIK1 protein indicate those substituted for in planta assays.

(B) Kinase activity of recombinant BIK1 and Ala substitution mutants produced in *E. coli* detected by autoradiogram. CCB, Coomassie blue staining.

(C) and **(D)** BIK1 substitution mutants in vivo detected by a mobility shift on an HA-immunoblot or by phosphoserine/Thr-specific antibody.

(E) BIK1 and MBP phosphorylation is abrogated by phosphatase treatment. Protein dephosphorylation was performed according to the manufacturer's protocol (New England Biolabs) with ~1 to 2.5 units CIP/μg protein (left) or (~2.5 μ/μg) (right). −, Buffer; +, CIP.

In **(C)** and **(D)**, plants were treated with ACC (Ac) or flg22 (Fl) for 3 h and assayed for changes in BIK1 and MBP phosphorylation activity. In **(C)** to **(E)**, in vivo BIK1 phosphorylation was detected by ACC or flagellin-induced mobility shifts observable by HA-immunoblot. The top band corresponds to phosphorylated BIK1, which is migrating slower than the unphosphorylated form. MBP phosphorylation by BIK1 was detected by immunoblots with a phosphoserine/Thr-specific antibody.

and MBP increased after treatment with the ET precursor ACC (Figure 1C) or flagellin peptide flg22 (Figure 1D). Treatment with protein phosphatase restored mobility of BIK1-HA and eliminated MBP phosphorylation, indicating that these are phosphorylated forms (Figure 1E).

Generally, BIK1 residues required for in vitro auto and MBP phosphorylation activities were also required for BIK1 phosphorylation in vivo. Similar to in vitro activities, Lys-105 and Tyr-245 were required for all kinase activities as were Thr-94, Asp-202, Ser-236, and Thr-237. Alternatively, BIK1^{S233A} and BIK1^{Y234A} show BIK1 and MBP phosphorylation comparable to BIK1. Intriguingly, Ala substitution at Thr-242 leads to BIK1 phosphorylation prior to elicitation and a loss of trans-kinase activity. Due to the differential responses of the substitution plant lines (see next section), the contributions of Ser-33 and Thr-64 to BIK1 kinase activity were further analyzed for their effects on flg22-induced phosphorylation in addition to ACC (Figure 1D). BIK1^{T64A} abolished BIK1 phosphorylation and ACC-induced MBP phosphorylation but did not affect MBP phosphorylation in response to flg22, indicating differential contributions to regulation of activity in response to different signals. BIK1^{S33A} blocked all MBP phosphorylation activity as well as flg22-triggered BIK1 phosphorylation.

BIK1 Mediates flg22 and Wound-Induced Immunity to *B. cinerea*

To determine the role of BIK1 regulatory residues and those required for phosphorylation in PTI responses, we assayed transgenic *bik1* plants expressing BIK1 Ala substitution mutants for basal (water-treated) and flagellin-induced resistance (flg22-PTI) to *B. cinerea*. Treatment with flg22 prior to inoculation significantly reduced disease lesion size in wild-type plants but failed to confer protection in the *bik1* background (Figures 2A and 2B). As wounding has also been found to confer strong resistance to *B. cinerea* in *Arabidopsis*, we examined this type of induced immunity in *bik1* and found the mutant is only partially protected relative to wounded wild-type plants (see Supplemental Figure 1 online).

Expression of BIK1^{S233A}, BIK1^{Y234A}, and BIK1^{Y245A} fully restored the basal and flg22-induced *B. cinerea* resistance of *bik1* to wild-type levels comparable to 35S:*BIK1-HA* (Figures 2A and 2B). BIK1^{S33A} partially restored basal resistance but completely abrogated flg22-induced resistance, whereas BIK1^{T64A} partially restored both. BIK1^{T94A}, the ATP binding site mutant, BIK1^{K105A}, BIK1^{D202A}, BIK1^{S236A}, BIK1^{T237A}, and BIK1^{T242A} inhibited resistance both with and without flg22 treatment, suggesting their critical importance in the PTI function of BIK1. Among these, BIK1^{D202A} disrupts the RD domain, and in RD kinases, the phosphorylated, negatively charged residues in the AD interact with the positively charged Arg of the RD domain, providing proper spatial arrangement for substrate access to the catalytic Asp (Johnson et al., 1996). Loss of BIK1 and MBP phosphorylation as well as the lack of complementation in *bik1*;BIK1^{D202A} are consistent with the role of the RD domain for the biochemical and biological functions of protein kinases (Johnson et al., 1996). Most residues that abrogated kinase activity also failed to complement the disease resistance and loss of PTI of *bik1*, suggesting that BIK1 phosphorylation is important for its function in immune responses.

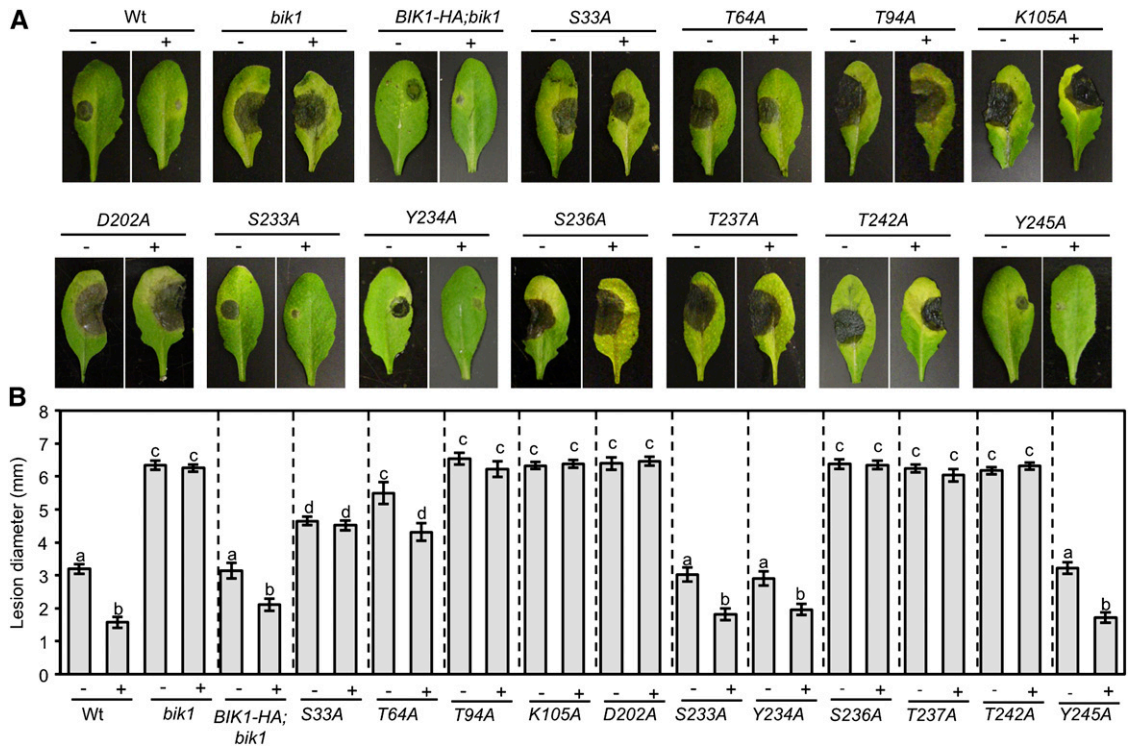

Figure 2. BIK1 Is Required for PTI to *B. cinerea.*

Disease symptoms **(A)** and mean lesion size **(B)** of water (−) and flg22-treated (+) *bik1* and *BIK1* substitution mutants after drop inoculation with *B. cinerea* (2.5×10^5 spores/mL). Data in **(B)** represent mean ± SE from a minimum of 30 disease lesions. The statistical significance of the mean lesion sizes was determined using analysis of variance and Tukey's test. The mean values followed by different letters are significantly different from each other (P = 0.01) Experiments were repeated at least three times with similar results. Images were taken 3 d after inoculation. The BIK1 site-directed mutants and the wild-type (wt) BIK1-HA are expressed in the *bik1* mutant background.

Resistance of the *bik1* substitution lines to *A. brassicicola* was similar to that observed for *B. cinerea* with the exception of $BIK1^{Y234A}$, which partially restored *bik1* resistance but fully rescued *bik1* susceptibility to *B. cinerea* (see Supplemental Figure 2 online). Induced resistance to *A. brassicicola* was not tested due to the high level of resistance in uninduced wild-type plants. In sum, BIK1 is a regulator of PTI required for flagellin-induced immunity to *B. cinerea* as well as full protection conferred by wounding.

BIK1 Is Required for Seedling Growth Responses to ET and Glc

In assaying for hormone-related functions that may explain the role of BIK1 in plant immune and growth responses, we observed that *bik1* is altered in the triple response (Figures 3A and 3B). The triple response is induced in the dark in response to ET or its precursor ACC, producing seedlings with exaggerated apical hooks and swollen hypocotyls that are inhibited in root/hypocotyl elongation (Guzmán and Ecker, 1990). *bik1* shows a clear insensitivity to ACC evidenced by a lack of growth inhibition in the hypocotyl compared with wild-type seedlings. The ET response mutant *ein2* shows typical impaired ET responses consistent with published data (Figures 3A and 3B). By contrast, *bik1* roots appear responsive to ACC, whereas *ein2* shows no significant changes in root length even at higher concentrations of ACC (40 and 100 μM). However, at these same concentrations, *bik1* hypocotyls show greater growth insensitivity than *ein2*. This variation is consistent with *ein2* being the only mutant that inhibits all aspects of seedling growth responses to ET and suggests BIK1 affects specific aspects of ET-mediated seedling growth responses (Guo and Ecker, 2004).

Genetic and molecular studies in *Arabidopsis* suggest that Glc and ET interact antagonistically (Yanagisawa et al., 2003). For instance, the ET-insensitive *etr1* and *ein2* mutants are sensitive to Glc, but the constitutive ET signaling mutant *ctr1* is insensitive (Zhou et al., 1998). In the presence of 2 or 6% Glc, *bik1* seedlings showed clear growth hypersensitivity, further confirming altered ET responses and lending support to BIK1 function in ET signaling (Figures 3C and 3D).

BIK1 Induction by ET and Flagellin Is EIN3 Dependent

To gain better insight into how BIK1 functions in ET responses, we studied its expression in *ein3* and other ET-signaling mutants. The transcription factor EIN3 is a member of a functionally redundant gene family involved in the regulation of ET and immune responses (Chao et al., 1997; Chen et al., 2009). In response to *B. cinerea*, *BIK1* shows a significant induction in wild-type plants that was completely abolished in *ein3* (Figure

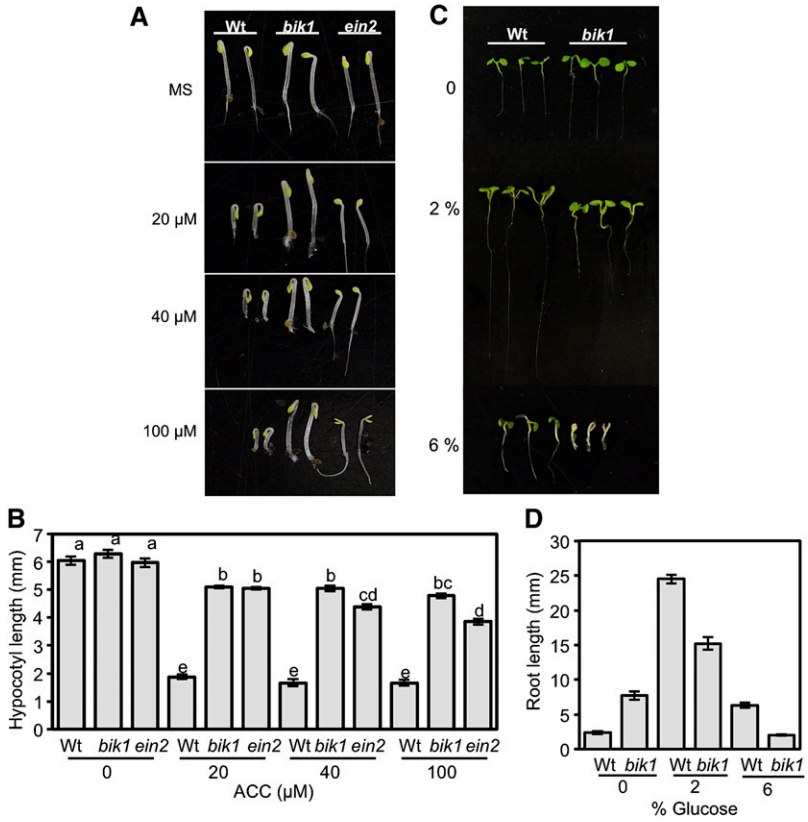

Figure 3. *BIK1* Is Required for Responses to ET and Glc.

(A) and **(B)** Triple response phenotype **(A)** and hypocotyl lengths **(B)** of *bik1* seedlings on different concentrations of ACC (μM) and unsupplemented MS media. wt, wild type.

(C) and **(D)** Sensitive growth response **(C)** and root length **(D)** of *bik1* on media containing increased levels of Glc.

Data in **(B)** and **(D)** represent mean ± SE from a minimum of 60 seedlings. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2.

4A). Similar to *B. cinerea*, ACC-induced *BIK1* expression also showed strict dependence on *EIN3* (Figure 4B). By contrast, *BIK1* was expressed at a higher level than the wild type in both *ein3-like* (*eil1*) and *ein2* plants in response to both treatments. Interestingly, *BIK1* induction in response to *Pst*DC3000 and flg22 requires *EIN3* and *EIL1* but shows only partial dependence on *EIN2* (Figures 4A and 4C). The strict dependence of *BIK1* expression on EIN3 is reinforced by the presence of EIN3 binding consensus sequences (Solano et al., 1998; Kosugi and Ohashi, 2000) in the *BIK1* promoter (see Supplemental Figure 3 online). Next, we performed chromatin immunoprecipitation–quantitative PCR (ChIP-qPCR) experiments using EIN3 antiserum. We show that the EIN3 protein is associated with the BIK1 promoter, suggesting that it directly controls BIK1 gene expression (Figure 4D). Recently, both EIN3 and EIL1 have been demonstrated to contribute to ET-mediated PTI responses through the direct transcriptional regulation of *FLS2* (Boutrot et al., 2010). Furthermore, *bik1* plants accumulate elevated levels of EIN3 that do not show any significant increase in response to ACC or flg22-treatment relative to wild-type seedlings, raising the possibility of feedback control of EIN3 by BIK1 adding another dimension to their regulatory relationship (Figure 4E). Interestingly, ACC- and flg22-induced BIK1 phosphorylation is attenuated by silver nitrate, which blocks ET perception, suggesting that ET signaling or perception is required for BIK1 phosphorylation (Figure 4F). β-Glucuronidase (GUS) activity in transgenic plants harboring a GUS reporter gene under the transcriptional control of the *BIK1* promoter showed a clear diffuse increase in response to flg22, ACC, and chitin in adult leaves (Figure 4G). In seedlings, ACC and flg22-induced GUS activity was also observed but largely localized to the vascular tissue (Figure 4H). Regulation of *BIK1* expression with ACC, flg22, and chitin supports its function in ET signaling and PTI. In addition, the coregulation of *FLS2* and *BIK1* by ET, flagellin, and EIN3 is consistent with their interaction as part of the flg22 receptor complex (Lu et al., 2010; Zhang et al., 2010).

BIK1 Regulates Expression of Genes Involved in PTI- and ET-Mediated Immune Responses

The *Arabidopsis* ETHYLENE RESPONSE FACTOR (ERF) domain transcription factors function in JA/ET signaling and defense

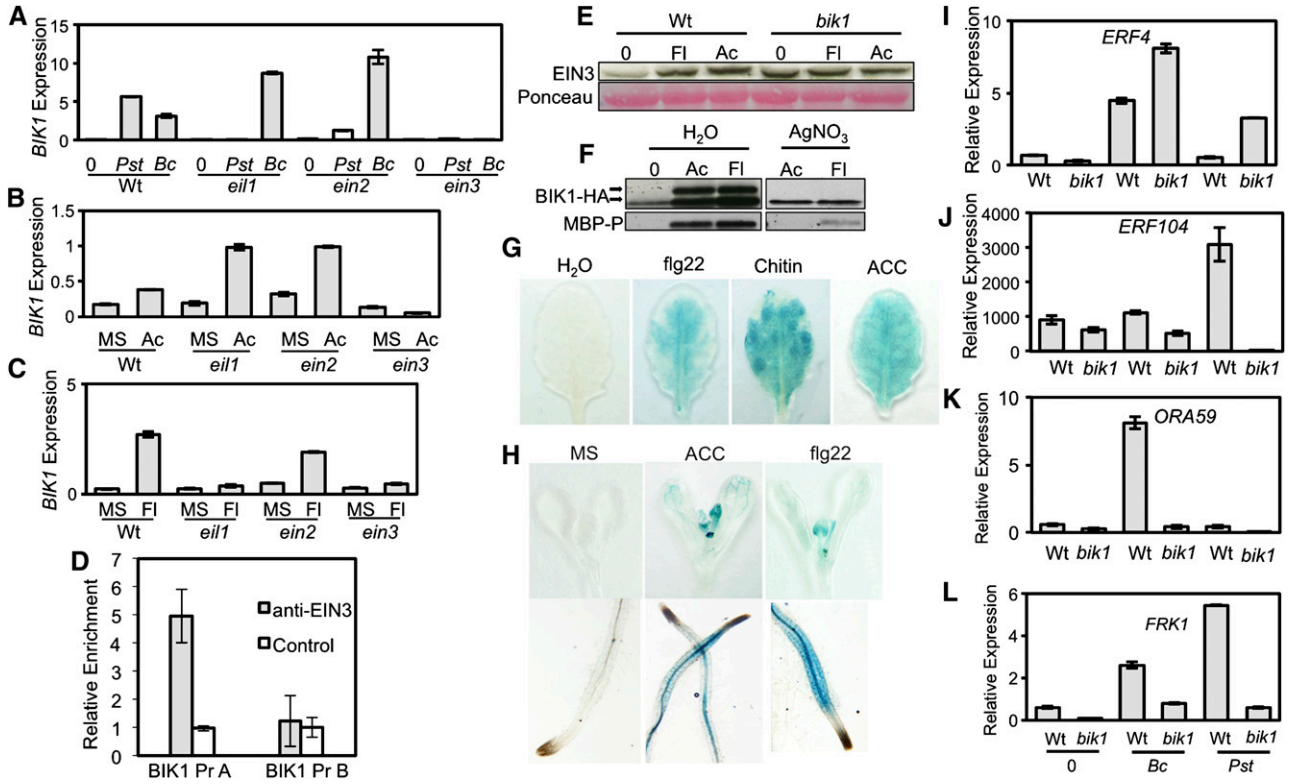

Figure 4. BIK1 Functions in the ET Response Pathway.

(A) to **(C)** Expression of *BIK1*, as determined by quantitative RT-PCR and normalized to actin following pathogen inoculation **(A)** and during the triple response **(B)** and flg22 (Fl) treatment **(C)** in ET response mutants. wt, wild type.

(D) ChIP-PCR showing the association of EIN3 protein with the BIK1 promoter. Chromatin from wild-type seedlings was immunoprecipitated with an anti-EIN3 polyclonal antibody. Enrichment of *BIK1* promoter region was verified by qPCR using *BIK1* promoter-specific primers. *BIK1* Pr region A refers to position −1221 to −774 and region B from −494 to −166 in the *BIK1* promoter region. The *BIK1* promoter sequence is shown in Supplemental Figure 3 online.

(E) EIN3 protein levels in *bik1* and wild-type seedlings in response to flg22 (Fl) and ACC (Ac) treatment.

(F) BIK1 phosphorylation is inhibited by blocking ET perception with silver nitrate ($AgNO_3$). Changes in BIK1 phosphorylation were visualized as a mobility shift observable by HA-immunoblot. The top band corresponds to the slowly migrating band caused by BIK1 phosphorylation. MBP phosphorylation was detected on an immunoblot with a phosphoserine/Thr-specific antibody.

(G) and **(H)** Histochemical assay showing GUS activity in transgenic *BIK1pr:GUS* plants **(G)** and seedlings **(H)** in response to flg22, chitin, and ACC.

(I) to **(L)** Quantitative RT-PCR determination of pathogen-induced expression of *ERF4* **(I)**, *ERF104* **(J)**, *ORA59* **(K)**, and *FRK1* **(L)** in wild-type and *bik1* plants. Phosphorylation detection was performed as described in the legend of Figure 1.

Experiments were performed as described in the Methods and repeated at least three times with similar results.

against fungal pathogens (Lorenzo et al., 2003). In *bik1* plants, *ERF4* expression was significantly upregulated in response to *B. cinerea* and *Pst* (Figure 4I). By contrast, *ERF104* induction in *bik1* shows a severe reduction in response to *B. cinerea* and is completely abolished in response to *Pst* (Figure 4J). *ERF104* contributes to plant immune responses, flg22 growth responses, and resistance to *B. cinerea* (Bethke et al., 2009). Similarly, *B. cinerea*–induced *ORA59* expression is significantly attenuated in *bik1* (Figure 4K). *ORA59* regulates JA- and ET-mediated expression of several defense genes and contributes to *B. cinerea* resistance through the integration of these two signaling pathways (Pré et al., 2008; Leon-Reyes et al., 2010; Zarei et al., 2011). Expression of *FRK1*, a marker for PTI, is also severely impaired in *bik1* in response to *B. cinerea* and *Pst* (Figure 4L). These data further strengthen the role of BIK1 in ET-dependent PTI responses.

BIK1 Residues Required for Phosphorylation Are Also Required for BIK1 Function in Seedling Growth Responses to ET and flg22

The *bik1*;35S:*BIK1-HA* line complemented all growth phenotypes of the *bik1* mutant as well as the seedling response to flg22 and ACC (Figures 5A and 5B). Among the substitution mutants, $BIK1^{S33A}$ partially restored growth sensitivity to ACC similar to its role in *B. cinerea* resistance, whereas $BIK1^{T64A}$, $BIK1^{T94A}$, $BIK1^{S233A}$, $BIK1^{Y234A}$, and $BIK1^{Y245A}$ led to full restoration, suggesting that these residues are dispensable for BIK1 function

in ET responses. By contrast, BIK1^{K105A}, BIK1^{D202A}, BIK1^{S236A}, BIK1^{T237A}, and BIK1^{T242A} failed to complement the *bik1* triple response phenotype, correlating with their importance in other BIK1 functions. Interestingly, whereas Thr-94 is required for basal and flg22-PTI to *B. cinerea*, it is completely dispensable for ET responses at the concentration tested.

BIK1 Residues Required for Phosphoryation Regulate PTI Responses to Virulent and Nonpathogenic Strains of *P. syringae*

Although *bik1* plants are resistant to *Pst*DC3000, they are susceptible to the nonpathogenic strain *Pst*DC3000 *hrcC*$^-$ *(Pst hrcC*$^-$*)*, which lacks Type III secretion but retains ability to activate PTI responses (Zhang et al., 2010) (Figures 6A and 6B). The *bik1*; BIK1-HA plants showed wild-type responses to both *Pst*DC3000 and *Pst*hrcC$^-$ strains. Interestingly, many of the substitution mutants retained full or partial resistance to *Pst*DC3000 relative to *bik1* (Figure 6A). BIK1^{S33A} partially restored wild-type responses to *Pst*DC3000 and flg22-PTI, whereas BIK1^{T64A} maintained *bik1* resistance yet partially complemented PTI in the mutant. Conversely, the BIK1 AD residue Ser-233 is partially required for basal resistance to *Pst*DC3000 but not flg22-induced protection. Only BIK1^{Y245A} and BIK1^{Y234A} fully restored wild-type responses to *Pst*DC3000 both before and after flg22-PTI, analogous to their rescue of *bik1* resistance to necrotrophic infection. Since *bik1* resistance to *Pst*DC3000 is largely due to high endogenous SA (Veronese et al., 2006), it is likely that substitutions other than BIK1^{Y245A} and BIK1^{Y234A} failed to restore normal SA levels. This may also suggest residues Tyr-234 and Tyr-245 are sufficient for suppression of SA accumulation.

When tested for basal resistance and flg22-PTI to *Pst*hrcC$^-$, BIK1^{T94A}, BIK1^{S233A}, BIK1^{Y234A}, and BIK1^{Y245A} fully restored both responses, the phenotypes of the latter three being consistent with their dispensability in BIK1-mediated ET growth responses and defense against *B. cinerea* (Figure 6B). BIK1^{S33A} and BIK1^{T64A} are required for PTI, yet BIK1^{S33A} restored wild-type level of resistance, whereas BIK1^{T64A} actually conferred increased basal resistance. Thus, the already heightened resistance of BIK1^{T64A} may account for its apparent unresponsiveness to flg22 as it may have already reached a threshold for resistance. This also suggests phosphorylation of Thr-64 may suppress plant responses to *PsthrcC*$^-$. BIK1 Lys-105, Asp-202, Ser-236, Thr-237, and Thr-242 are required for full resistance as

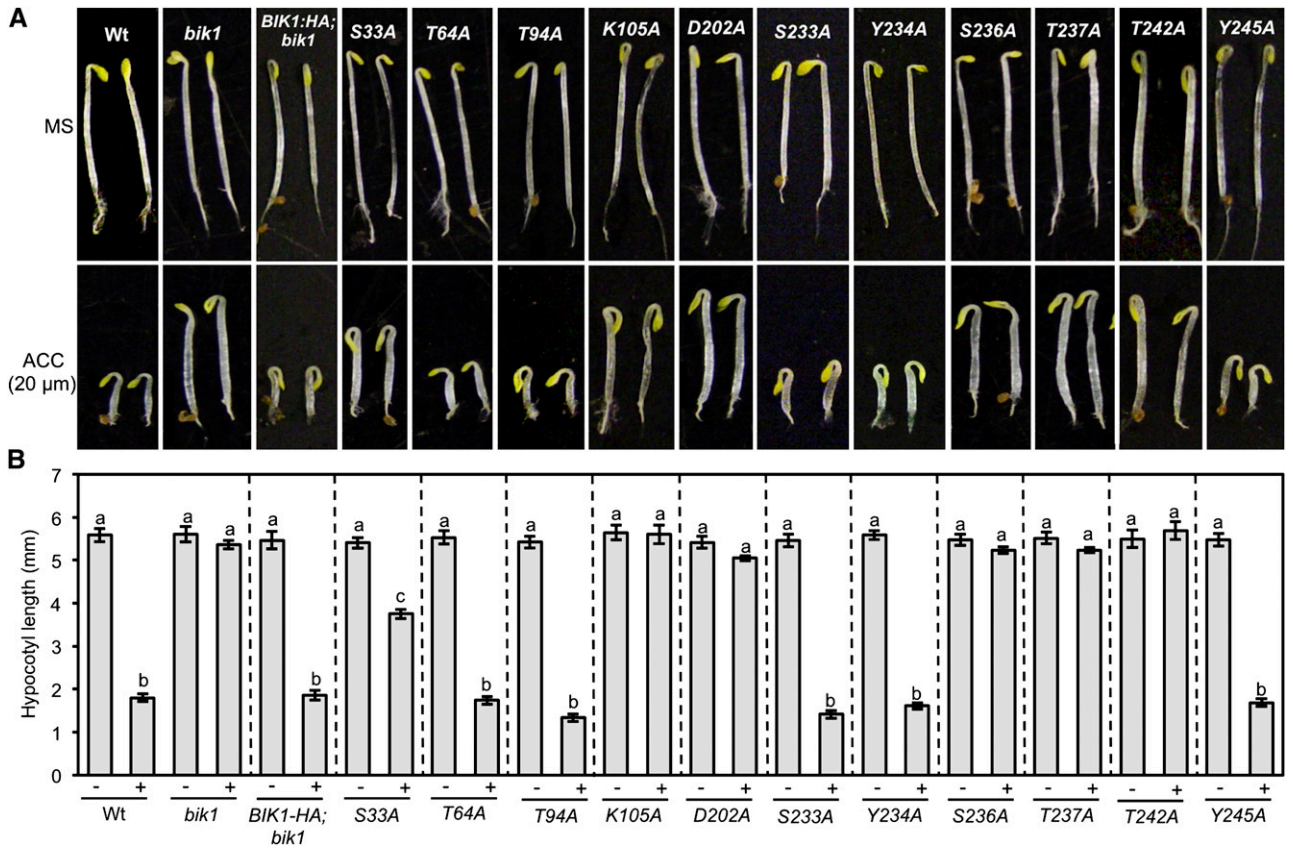

Figure 5. Ala Substitution Mutants Reveal BIK1 Residues Required for the *Arabidopsis* Triple Response.

Triple response phenotypes **(A)** and hypocotyl lengths **(B)** of *bik1* and *BIK1* substitution seedlings on 20 μM ACC (+) and unsupplemented MS media (−). Data in **(B)** represent mean ± SE from a minimum of 60 seedlings. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2. All BIK1 site-directed mutants are expressed in the *bik1* background. wt, wild type.

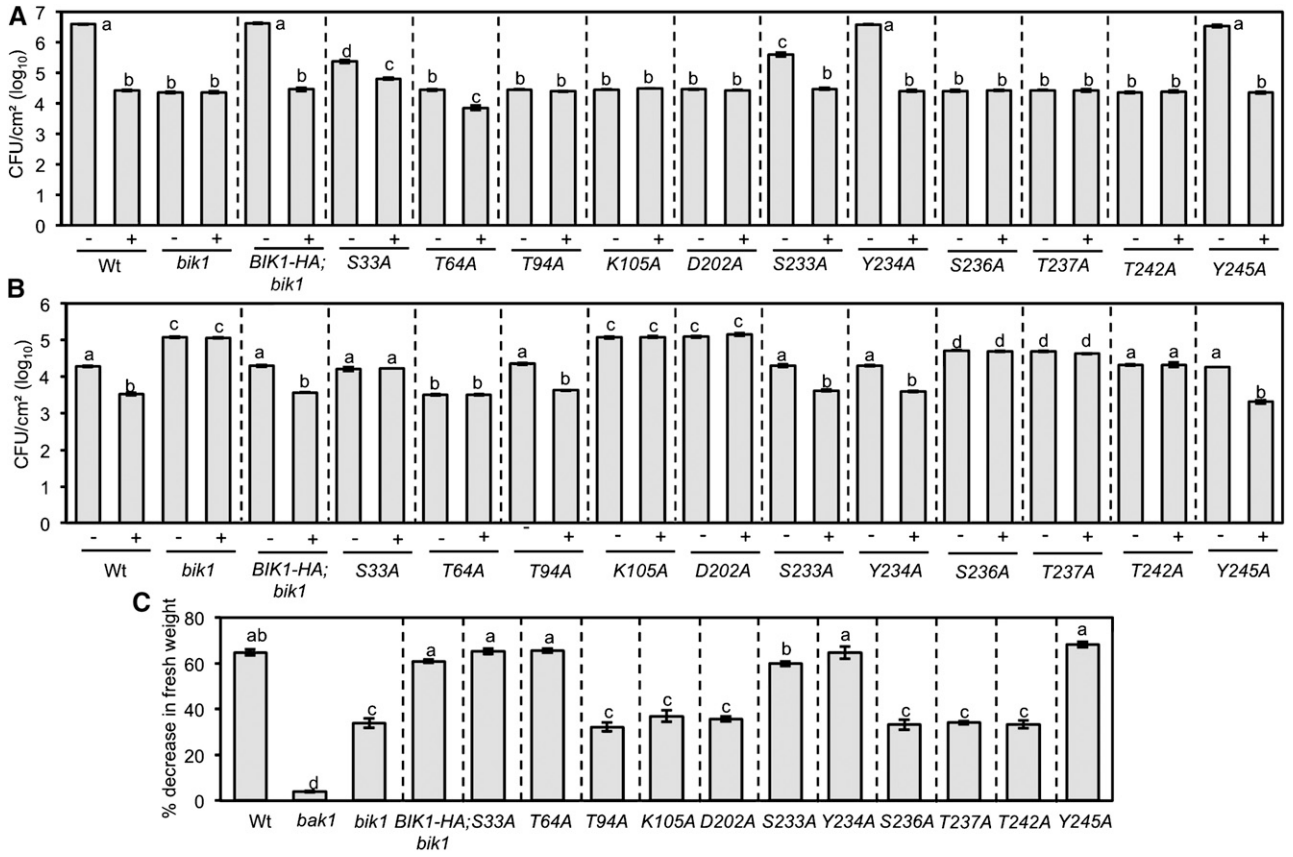

Figure 6. *BIK1* Mediates Plant Responses to *Pst* Strains and flg22.

(A) and **(B)** Bacterial growth ([cfu]/cm^2 leaf area) in water- (−) and flg22-treated (+) plants 3 d after inoculation with *Pst*DC3000 **(A)** or the nonpathogenic *Pst hrcC*$^-$ strain **(B)**, deficient in Type III secretion. CFU, colony-forming units; wt, wild type.

(C) Percentage decrease in fresh weight of seedlings after growth in 10 nM flg22. Data represent mean values ± SE from three experiments and a minimum of 120 seedlings for bacterial growth and fresh weights, respectively. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2. All BIK1 site-directed mutants are in *bik1*.

their Ala substitutions failed to rescue *bik1* susceptibility (Figure 6B). BIK1^{K105A} and BIK1^{D202A} were the only substitutions that completely failed to rescue both basal and flg22-PTI consistent with their conserved and critical role in kinase catalytic activity. Surprisingly, BIK1^{T242A} showed no difference from the wild type in basal defense against *Pst*hrcC$^-$ but was unresponsive in flg22-PTI, suggesting that this residue is required for flg22-induced resistance.

The Role of BIK1 in flg22 Growth Responses Correlated with Immune Response Function and Kinase Activity

To determine whether the role of BIK1 in flg22-induced immunity correlated with its function in flg22-induced growth responses, we assayed the BIK1 substitution lines for growth sensitivity to the flg22 peptide. The average decrease in fresh weight of *bik1* seedlings on flg22 was roughly half that observed for the wild type yet significantly higher than that of the insensitive *bak1* mutant (Figure 6C). BIK1^{K105A}, BIK1^{D202A}, BIK1^{S236A}, BIK1^{T237A}, and BIK1^{T242A} failed to restore wild-type sensitivity analogous to their failure to rescue the other *bik1* mutant phenotypes. This confirms a direct correlation between the role of BIK1 in ET and flagellin perception and regulation of immune responses. BIK1^{T64A}, BIK1^{T94A}, BIK1^{Y234A}, and BIK1^{Y245A} led to full restoration of flg22 sensitivity, whereas it was only partially restored in BIK1^{S233A}. The partial sensitivity of BIK1^{S233A} correlates only with its *Pst*DC3000 susceptibility as it fully rescued all other *bik1* phenotypes. As the majority of *bik1* resistance to *Pst*DC3000 is based on elevated SA, this may suggest S233 is required for BIK1 function in integrating flagellin and SA responses.

BIK1 Phosphomimic Mutation at Thr-242 Is Sufficient for Increased ET Sensitivity and Resistance to *B. cinerea*

The activation of protein kinases is regulated by phosphorylation events in the AD (Johnson et al., 1996). Among BIK1 AD phosphorylation sites, residues Thr-242 and Tyr-245 are invariant in BIK1-related kinases but also affect BIK1 kinase activity (Figures 1B and 1C). These two BIK1 residues were substituted with Asp

to create phosphomimic BIK1 mutations and transformed into *bik1* and wild-type plants. Acidic amino acids such as Asp can mimic the negative charge conferred by phosphorylation and lead to partial or constitutive activation of kinases (Johnson et al., 1996). In vivo kinase assays of BIK1^{T242D} and BIK1^{Y245D} suggest that they do not lead to constitutive activation but result in altered phosphorylation and plant phenotypes (Figure 7). Relative to *bik1;*BIK1-HA, the BIK1;BIK1-HA plants have higher basal BIK1 and MBP phosphorylation, which becomes further enhanced upon treatment with ACC or flg22 (Figure 7A). In both wild-type and *bik1* plants, BIK1^{Y245D} displayed ACC and flg22-induced kinase activity, with ACC eliciting a more robust increase in both BIK1 and MBP phosphorylation than flg22. BIK1;BIK1^{T242D} displays MBP phosphorylation comparable to that of BIK1;BIK1-HA yet has no observable BIK1 phosphorylation with or without induction. Similarly, BIK1 phosphorylation was not detected for *bik1;*BIK1^{T242D} despite clear phosphorylation of MBP. To further understand the role of T242 in BIK1 kinase activity and determine if the loss of BIK1^{T242D} phosphorylation is a result of substitution or artifact due to its activity being below the sensitivity threshold of our assay, we performed immunoblots on precipitated BIK1^{T242D} to compare basal and induced phosphorylation (Figure 7B). In wild-type and *bik1* plants, without ACC or flg22, BIK1^{T242D} shows phosphorylation that does not change after treatment, suggesting constitutive activation (Figure 7B). This may account for the lack of increase in BIK1 phosphorylation with [γ-^{32}P]ATP as BIK1^{T242D} phosphosites are already occupied with unlabeled phosphate; this also supports the ability of the protein to phosphorylate MBP as it is likely present in an activated state.

Subsequent phenotypic analyses revealed that BIK1; BIK1^{T242D} and *bik1;*BIK1^{T242D} plants have increased resistance to *B. cinerea* relative to their respective controls, with BIK1; BIK1^{T242D} further enhanced for flg22-PTI (Figure 7C). BIK1^{Y245D} had no effect on *B. cinerea* resistance in the presence of functional BIK1 but rescued susceptibility of the *bik1* mutant. Expression of BIK1^{Y245D} in both backgrounds also significantly enhanced the disease resistance conferred by flg22 treatment. Basal resistance to *Pst*DC3000 was restored to wild-type levels in BIK1;BIK1^{T242D}, BIK1;BIK1^{Y245D}, and *bik1;*BIK1^{Y245D} lines, whereas *bik1*;BIK1^{T242D} susceptibility was only partially restored (see Supplemental Figure 4A online). All lines showed flg22-PTI to *B. cinerea*, with resistance in *bik1*;BIK1^{Y245D} and BIK1; BIK1^{T242D} lower and exceeding that observed for wild-type plants, respectively. Both phosphomimic mutations also restored *bik1* PTI and susceptibility to *Pst hrcC*$^{-}$ with minimal gains of resistance observed for *bik1*;BIK1^{T242D} and BIK1; BIK1^{Y245D} plants (see Supplemental Figure 4B online). Growth responses to flg22 were restored to wild-type levels for all the Asp substitution lines other than *bik1;*BIK1^{Y245D}, which maintained slight insensitivity (see Supplemental Figure 4C online). In contrast with BIK1^{T242A}, which failed to complement *bik1*, the BIK1^{T242D} and all of the phosphomimic transgenic lines displayed wild-type triple responses on 20 μM ACC (see Supplemental Figure 5 online). However, at 2 μM ACC, BIK1^{T242D} conferred enhanced sensitivity observable as significantly shorter hypocotyls relative to both wild-type and *bik1* seedlings (Figures 7D and 7E).

BIK1 Thr-242 and Tyr-245 Are Required for Negative Regulation of Plant Growth Similar to Their Function in Suppression of *P. syringae* Resistance

Loss of *BIK1* function results in altered growth and development characterized by small statured early-flowering plants with exaggerated leaf serration and weak stems (Veronese et al., 2006). Overall, the growth patterns in *bik1* plants expressing the *BIK1* substitutions largely followed their contributions to other functions of BIK1, particularly ET sensitivity. Interestingly, both BIK1^{T242A} and BIK1^{Y245A} grew significantly bigger leaves and were larger in overall stature relative to wild-type plants (see Supplemental Figures 6A and 6B online). However, leaves of BIK1^{T242A} are more narrow and serrated than the *bik1* mutant, whereas those of BIK1^{Y245A} display wild-type morphology. Thus, it is possible that phosphorylation of these residues negatively regulate plant growth, with Thr-242 also contributing to leaf development. BIK1^{T64A} and BIK1^{T94A} were generally smaller than *bik1* plants.

flg22-Induced PTI to *B. cinerea* Requires BIK1, EIN2, and HUB1 but Is Antagonized by COI1

To determine the genetic interaction between BIK1 and other regulators of *Arabidopsis* immune responses, double mutants between *bik1* and *npr1* (Cao et al., 1994), *sid2* (Dewdney et al., 2000), *NahG* (Delaney et al., 1994), *pad4* (Glazebrook et al., 1997), *ein2* (Guzmán and Ecker, 1990), *coi1* (Xie et al., 1998), and *histone monoubiquitination1* (*hub1*) (Liu et al., 2007; Dhawan et al., 2009) were generated and assayed for their effects on BIK1 mediated basal and flg22-PTI to fungal and bacterial pathogens. The *npr1*, *pad4*, and *sid2* mutants and *NahG* transgenic plants showed wild-type responses to *B. cinerea* and were fully responsive to flg22-induced resistance, suggesting SA is dispensable for flg22-PTI to this pathogen (Figures 8A and 8B). The *bik1 sid2* plants have wild-type basal resistance but show no flg22-PTI, suggesting that the loss of induced resistance in *bik1* is independent of SA. These data were further confirmed by loss of flg22 PTI in *bik1 NahG* plants. Interestingly, loss of flg22-PTI in *bik1* requires the functions of PAD4. Basal and flg22 PTI of *bik1 npr1* plants was comparable to *bik1*, indicating that BIK1 function is independent or upstream of NPR1-dependent SA signaling during PTI.

As previously reported the *bik1*, *coi1*, *hub1*, and *ein2* mutants have increased susceptibility to *B. cinerea* (Thomma et al., 1998; Veronese et al., 2006; Dhawan et al., 2009) (Figure 8). The *coi1* and *hub1* mutants were competent in flg22-induced resistance to *B. cinerea*, whereas similar to *bik1*, flg22 PTI was blocked in *ein2* (Figures 8A to 8D). The *bik1 hub1* double mutant showed susceptibility similar to both parental lines but displayed a partial loss of flg22-induced resistance, suggesting that histone H2B monoubiquitination contributes to PTI. The disease susceptibility of *bik1 coi1* and *bik1 ein2* plants was comparable to *coi1* and *ein2*, respectively. The loss of flg22-PTI in *ein2 bik1* was comparable to that of its parental mutants. Despite the extreme susceptibility of the untreated *coi1* mutant, it was significantly protected by flg22, indicating that COI1 is not required for flg22-PTI. Intriguingly, the highly susceptible *bik1 coi1* double mutant was also significantly protected by flg22. Thus, it appears that loss of flg22-PTI in *bik1* plants is dependent on intact COI1-mediated JA responses,

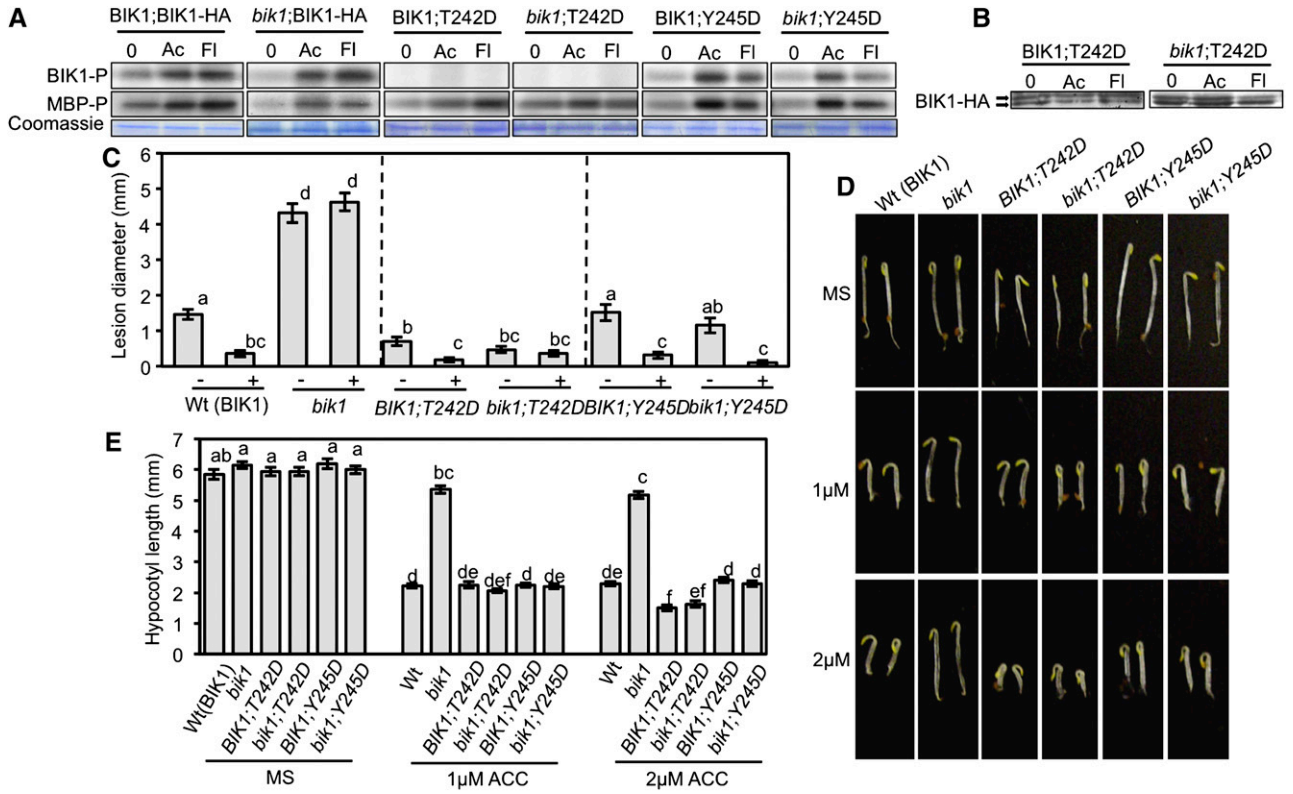

Figure 7. Phosphomimic Substitution at Thr-242 in the AD of BIK1 Confers Enhanced ET Responses and PTI to *B. cinerea*.

(A) In vivo kinase activity of the BIK1^{T242D} and BIK1^{Y245D} substitution mutants in response to ACC (Ac) and flg22 (Fl). Kinase activity of the phosphomimic mutants visualized by audioradiograms of radiolabeled proteins following incubation with [γ-^{32}P]ATP.

(B) Constitutive activation of BIK1 phosporylation by the BIK1^{T242D} phosphomimic mutation. BIK1 phosphorylation was visualized as a mobility shift detected by HA-immunoblot. Coomassie blue was used as a loading control.

(C) Lesion diameter in water (−) and flg22 (+) pretreated plants following drop inoculation with *B. cinerea* (2.5×10^5 spores/mL). Wt, wild type.

(D) and **(E)** Triple response **(D)** and average hypocotyl length **(E)** of seedlings on different concentrations of ACC (μM) (+) or unsupplemented MS media (−).

In **(A)**, protein staining with Coomassie blue was used as a loading control. Data in **(C)** and **(E)** represent mean ± SE from a minimum of 30 disease lesions or 60 seedlings, respectively. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2.

[See online article for color version of this figure.]

raising the intriguing possibility that COI1 may function as a repressor of BIK1. In sum, flg22-induced resistance to *B. cinerea* is independent of SA accumulation, but EIN2 and BIK1 are critical positive regulators of both basal and flg22-PTI and COI1 is either dispensable for PTI or suppresses the loss of PTI in *bik1*.

bik1 Susceptibility to *A. brassicicola* Is Dependent on SA Signaling and Synthesis

The role of the various defense genes on BIK1-mediated resistance to *A. brassicicola* was determined by comparing the level of in planta fungal DNA accumulation (as a measure of fungal growth) and the size of disease lesions (see Supplemental Figures 7A and 7B online). Similar to their responses to *B. cinerea*, *bik1 sid2* and *bik1 NahG* have wild-type resistance to *A. brassicicola.* Disease lesion size in *bik1 ein2* plants was comparable to that observed for *bik1* but fungal growth decreased relative to *bik1*. Resistance in *bik1 hub1* was comparable to both parental lines, indicating these two genes likely affect the same defense pathway during *A. brassicicola* infection. Based on lesion diameter, no significant difference was observed between *coi1* and *bik1 coi1* plants, yet the double mutant did support significantly lower fungal growth compared with *coi1*. The difference in fungal accumulation may be a result of nutrient depletion due to the extreme necrosis in *bik1 coi1*. Overall, the genetic requirements for *BIK1*-mediated resistance to *A. brassicicola* and *B. cinerea* were similar with the exceptions of *PAD4* and *NPR1*, which have different contributions.

Basal and Induced PTI Responses to Virulent and Nonpathogenic *Pst* Strains Are Modulated by SA, ET, and JA Responses

We assayed the *bik1* double mutants for basal and flg22-induced resistance to virulent and nonpathogenic *Pst* strains to compare

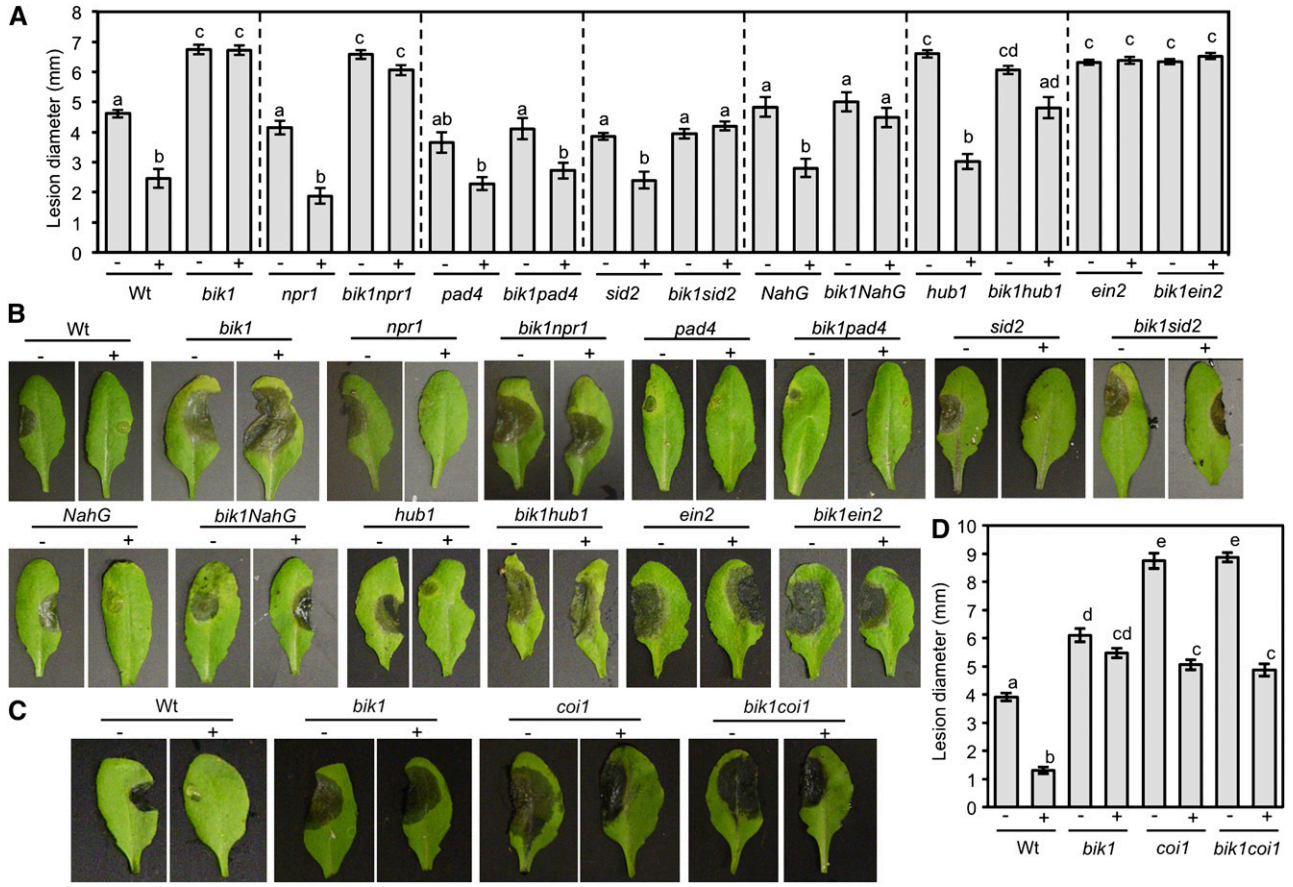

Figure 8. Epistasis Analysis of the Interaction between *BIK1* and Other Immune Response Genes in flg22-Induced PTI to *B. cinerea*.

Lesion diameter (**[A]** and **[D]**) and disease symptoms (**[B]** and **[C]**) in water (−) and flg22 (+) pretreated single and double mutants after drop inoculation with *B. cinerea* (2.5×10^5 spores/mL). Data in **(A)** and **(D)** represent mean ± SE from a minimum of 30 disease lesions. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2. Images were taken 3 d after inoculation. Wt, wild type.

the genetic requirements for responses to those during necrotrophic infection. In noninduced plants, *bik1* resistance is independent of NPR1 but requires PAD4, EIN2, HUB1, and SA accumulation (Figure 9A). Though the level of resistance varied, *hub1*, *npr1*, *pad4*, *sid2*, and *NahG* were responsive to flg22-induced PTI, supporting less bacterial growth than their respective water-treated controls. Flg22-PTI was fully lost in the *bik1 npr1*, *bik1 pad4*, *bik1 sid2*, and *bik1 hub1* double mutants. While *bik1 npr1* maintained resistance comparable to *bik1*, *pad4 bik1* and *sid2 bik1* were significantly more susceptible than *bik1* but less than *pad4* and *sid2*, respectively, suggesting that *bik1* resistance is partially dependent on SA signaling. Similarly, *bik1* responses to *Pst*DC3000 are partially dependent on *HUB1* as the double mutant shows a loss of resistance relative to *bik1*, with the disease phenotype of *bik1 hub1* plants being intermediate to both parental lines. *ein2* plants showed increased basal resistance relative to the wild type and remained unresponsive to flg22, mirroring the responses of *bik1. bik1 ein2* maintained resistance intermediate to both single mutants, while both the double mutant and *ein2* were unresponsive to flg22. Intriguingly, the *coi1* mutant showed enhanced susceptibility after flg22 treatment, suggesting that COI1 suppresses flg22-PTI to *Pst*DC3000 although the *coi1* mutant was responsive to flg22-PTI to *B. cinerea*, revealing a distinct role for *COI1* in PTI to bacterial and fungal pathogens. The *bik1 coi1* plants maintained *coi1* levels of susceptibility, showing a loss of resistance relative to *bik1* but lost flg22-PTI to *Pst*DC3000.

Inoculation with *Pst hrcC*$^-$ revealed a loss of basal resistance for all the mutants except *ein2*, *coi1*, and *bik1 coi1* (Figure 9B). This suggests that basal resistance to *Pst hrcC*$^-$ requires SA synthesis and signaling as well as HUB1 function. The *hub1* mutant, impaired in chromatin modifications, shows no altered responses to *Pst*DC3000 but has enhanced susceptibility to *Pst hrcC*$^-$ that is unresponsive to flg22-PTI. Interestingly, susceptibility of *bik1 hub1* is higher than that of its parental lines showing an additive action of HUB1 and BIK1 in resistance to *Pst hrcC*$^-$. Similar to *Pst*DC3000, flg22 treatment dramatically increased *coi1* susceptibility. However, whereas *Pst*DC3000-inoculated *bik1coi1* plants appeared unresponsive to flg22-induced PTI, flg22 increased susceptibility to *Pst hrcC*$^-$ in the double mutant to the same degree as observed for *coi1*.

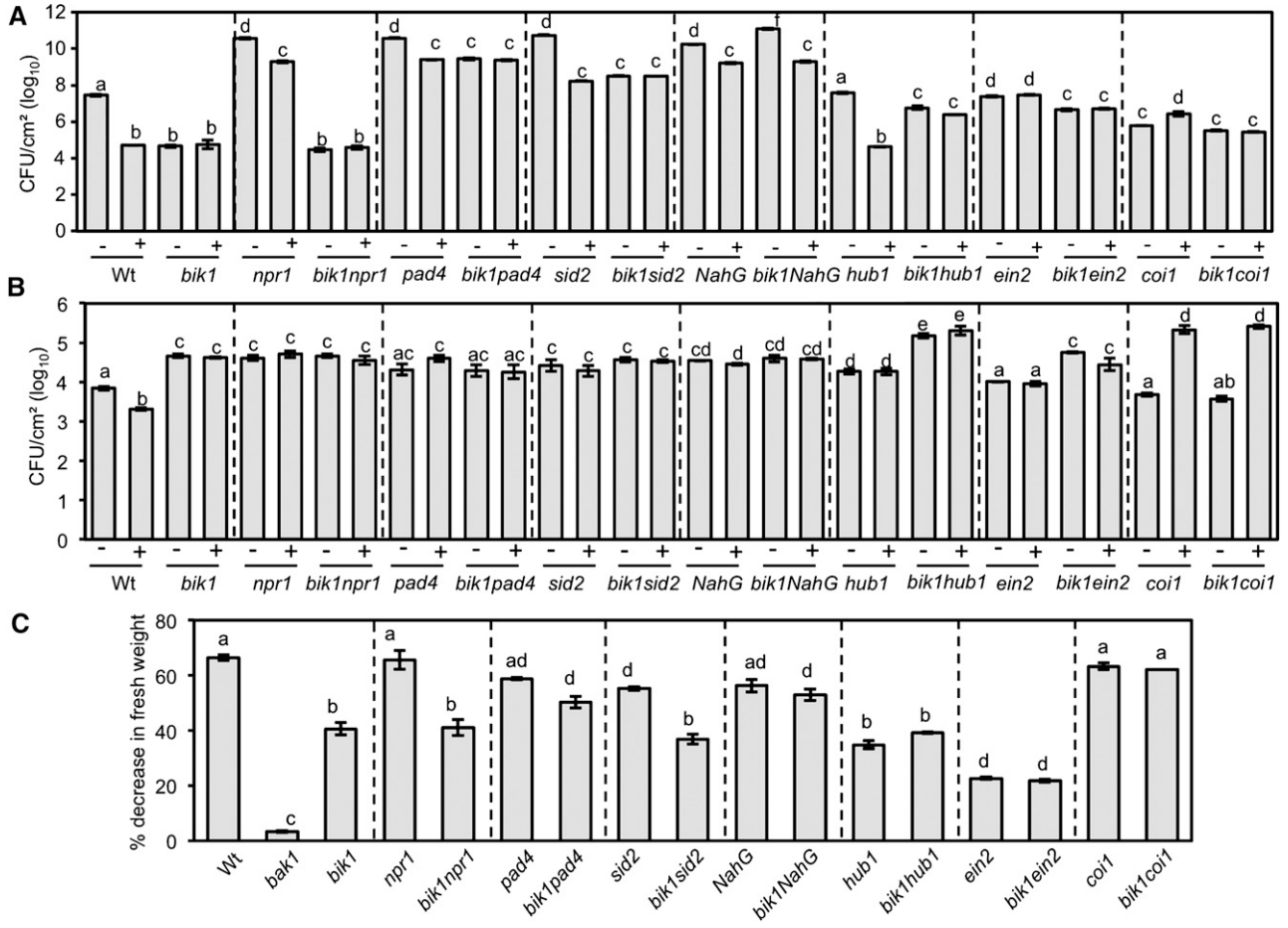

Figure 9. The Role of *Arabidopsis* Immune Response Genes on the Functions of BIK1 in flg22-Induced PTI to Virulent and Nonpathogenic Strains of *Pst*.

(A) and **(B)** Bacterial growth ([cfu]/cm^2 leaf area) in water (−) and flg22 (+) pretreated single and double mutants 3 d after inoculation with *Pst* DC3000 **(A)** or the nonpathogenic *Pst hrcC*$^-$ strain **(B)**, deficient in Type III secretion. CFU, colony-forming units; Wt, wild type.

(C) Percentage decrease in fresh weight of seedlings after growth in 10 nM flg22.

Data in **(A)** to **(C)** represent mean values ± SE from three experiments and a minimum of 120 seedlings, respectively. Experiments were repeated at least three times with similar results. Statistical analysis was performed as described in the legend of Figure 2.

The *coi1* Mutation Suppresses *bik1* Seedling Growth Insensitivity to flg22

The roles of SA, JA, and ET in BIK1-mediated seedling growth responses to flg22 were tested to determine the relationship between flg22-induced disease resistance and seedling sensitivity to the peptide (Figure 9C). *npr1*, *pad4*, *NahG*, and *coi1* seedlings showed no altered growth responses to flg22, whereas *sid2* was slightly but significantly insensitive to flg22. COI1 is dispensable for flg22 growth responses, but the *coi1* mutation restores *bik1* sensitivity to flg22 to wild-type level. This suggests insensitivity of *bik1* to flg22 is dependent on functional JA responses. The insensitivity of *bik1 npr1* and *bik1 sid2* plants mirrored *bik1*. By contrast and consistent with recent reports (Boutrot et al., 2010), *ein2* insensitivity exceeded that of *bik1*, with *bik1 ein2* seedlings showing *ein2* levels of flg22 growth inhibition. Interestingly, both *hub1* and *bik1 hub1* have insensitivity comparable to *bik1*. In sum, similar to PTI to *B. cinerea*, *bik1* growth responses to flg22 appear to be dependent on PAD4 functions and COI1-mediated JA responses. The growth responses to flg22 correlated with the susceptibility to *B. cinerea* and *Pst*hrcc$^-$ in *bik1*, *hub1*, and *ein2*. Furthermore, the insensitivity of *hub1* and *bik1 hub1* is consistent with HUB1 functioning in the same pathway as BIK1 in PTI to *Pst*hrcC$^-$ as well as defense against necrotrophic infection.

BIK1 Function in Seedling Growth Responses to ET Is Independent of SA and HUB1-Mediated Responses

All the double mutants with the exception of *bik1 ein2* are comparable to *bik1* in their insensitivity to ACC, suggesting that *bik1* growth responses to ET are independent of SA-related functions or ET is upstream of SA and HUB1 related functions

(see Supplemental Figures 8A and 8B online). At higher concentrations of ACC, *bik1 ein2* actually has increased insensitivity compared with both *bik1* and *ein2* based on hypocotyl length (see Supplemental Figures 8C and 8D online). The susceptibility of the double mutants to *B. cinerea* correlated with seedling growth responses to ET only in *npr1 bik1* (Figures 8A and 8B). SA accumulation has a significant impact on *bik1* responses to *B. cinerea*, whereas seedling ET responses are independent of SA.

BIK1 Regulates SA- and JA/ET-Regulated Defense Gene Expression

Previously, we showed that the increased *B. cinerea*–induced *PR-1* expression in *bik1* can be suppressed with removal of SA (Veronese et al., 2006). Interestingly, high *PR-1* expression is maintained in *bik1 npr1* plants but completely abolished in *bik1 sid2* and *bik1 pad4* (Figure 10A). This reaffirms the requirement of SA for BIK1-dependent *PR-1* expression and suggests that, in response to *B. cinerea*, BIK1 can bypass NPR1 regulation of *PR-1*. By contrast, combination with the *npr1* mutation abolished *bik1 PR-1* expression in response to *Pst*, as did *sid2*, *pad4*, *hub1*, and *ein2* (Figure 10A). The high level of *PR-1* expression in *B. cinerea*–infected *bik1 coi1* plants showed no significant difference from that of *bikl* and *coi1*; however, *Pst*-induced *PR-1* expression in the double mutant is higher than both parental lines. The *ein2* single and *bik1 ein2* double mutants have attenuated *PR-1* expression relative to that of *bik1* regardless of the pathogen, suggesting that EIN2 may positively function in the regulation of some SA responses during defense.

Arabidopsis defensin *PDF1.2* expression, a molecular marker for JA/ET responses, is induced in response to *B. cinerea*, ET, and JA in wild-type plants but reduced in *bik1*. In response to *B. cinerea* and ACC, *npr1* and *sid2* have significantly higher levels of *PDF1.2* expression than wild-type and *bik1* plants, indicating the suppressive role of SA on *PDF1.2* (Figure 10B). Although the *B. cinerea* susceptibility of the *bik1* mutant was rescued through removal of SA function by genetic crosses to *pad4*, *sid2*, or *NahG*, they did not restore *PDF1.2* expression. The decrease in *PDF1.2* expression correlates to *B. cinerea* susceptibility only for *bik1npr1* plants, suggesting that *B. cinerea* resistance can be uncoupled from *PDF1.2* expression. The expression of *PDF1.2* in response to ACC was comparable to that observed in response to *B. cinerea* in the single and double mutants.

BIK1 Is Unique among Closely Related Kinases for Its Role in Defense against Necrotrophic Infection and Responses to ET

Arabidopsis BIK1 shares high sequence similarity to many *Arabidopsis* RLCKs (Veronese et al., 2006). BIK-like protein (At3g55450; PBL1) shows the highest sequence identity followed by APK1b (At2g28930), APK1a (At1g07570), and APK2b (At2g02800) to BIK1 (Veronese et al., 2006). Most of these also harbor conserved residues targeted for cleavage by the bacterial effector protease AvrPphB, with PBS1 and BIK1 both suggested virulence targets (Zhang et al., 2010). However, mutation in the *BIK1*-related kinases resulted neither in increased susceptibility to *B. cinerea* or *A. brassicicola* nor ET insensitivity (see Supplemental Figure 9 online). Thus, BIK1 is distinct or has evolved an important regulatory role in plant growth and defense. Additionally, though seedlings of *pbs1* mutant alleles did not show enhanced insensitivity to ACC, some seedlings displayed altered growth featuring constitutive triple response in the dark on Murashige and Skoog (MS) media (see Supplemental Figure 9D online).

DISCUSSION

Here, we demonstrate the biochemical, molecular, and genetic bases of BIK1 function in PTI to the necrotrophic fungus *B. cinerea* and the hemibiotrophic bacterial pathogen *Pst*, extending our previous observations into the mechanisms underlying the role of BIK1 as an immune response regulator. In vitro and in vivo biochemical assays using BIK1 site-directed mutants identified BIK1 residues contributing to BIK1 and transphosphorylation as well as their biological roles in ET and PTI responses. The AD residues Ser-236, Thr-237, and Tyr-245 are critical for BIK1 and MBP phosphorylation and BIK1 biological function. $BIK1^{T242A}$ abrogated induced kinase activity and BIK1 function, whereas $BIK1^{T242D}$ was sufficient for enhanced PTI. Three KD residues had a differential role in seedling growth responses to ET and flg22, indicating the differential contributions of BIK1 phosphorylation sites. Mutation of the conserved Asp, which is part of the VIb domain in RD kinases, abrogated all functions of BIK1. Plants expressing $BIK1^{Y245A}$ grew significantly larger than the wild type, identifying Tyr-245 as important for negative regulation of plant growth. The effects of the site-directed mutants on phosphorylation and plant immune responses and growth phenotypes are summarized in Table 1. flg22-triggered immune response assays determined the genetic and biochemical requirements for BIK1-mediated PTI responses to *B. cinerea* and *Pst*. BIK1 is required for ET signaling that is important for its immune response as well as plant growth functions. Conversely, ET perception regulates BIK1 phosphorylation in response to ACC and flg22, further reinforcing the action of BIK1 in ET signal transduction. ET also regulates *BIK1* transcription through EIN3, which is found associated with the BIK1 promoter. Epistasis analysis defined interactions between BIK1 and other plant immune response regulators. Histone monoubiquitination was found to potentiate PTI to bacterial and fungal pathogens as well as seedling growth responses to flg22 synergistically with BIK1. COI1 antagonizes flg22-induced immunity, while EIN2 and BIK1 are central positive regulators of PTI and ET signaling. Elevated SA accumulation in the *bik1* mutant suppresses resistance to *B. cinerea*, but SA deficiency had no impact on flg22-PTI to this pathogen. Alternatively, SA is required for flg22-induced PTI to *Pst hrcC*$^{-}$. In sum, BIK1 is a regulatory component of immune responses to *P. syringae* and necrotrophic fungi, with its function in immune responses dependent on its kinase activities and ET signaling.

Multiple lines of evidence support BIK1 involvement in ET signaling and the ensuing function in plant immune responses: (1) *bik1* is impaired in typical seedling growth responses to ET, which is further confirmed by its hypersensitivity to Glc; (2) expression of several ET response genes, including *PDF1.2* and *ERF104*, is dependent on functional BIK1; (3) pathogen, ACC,

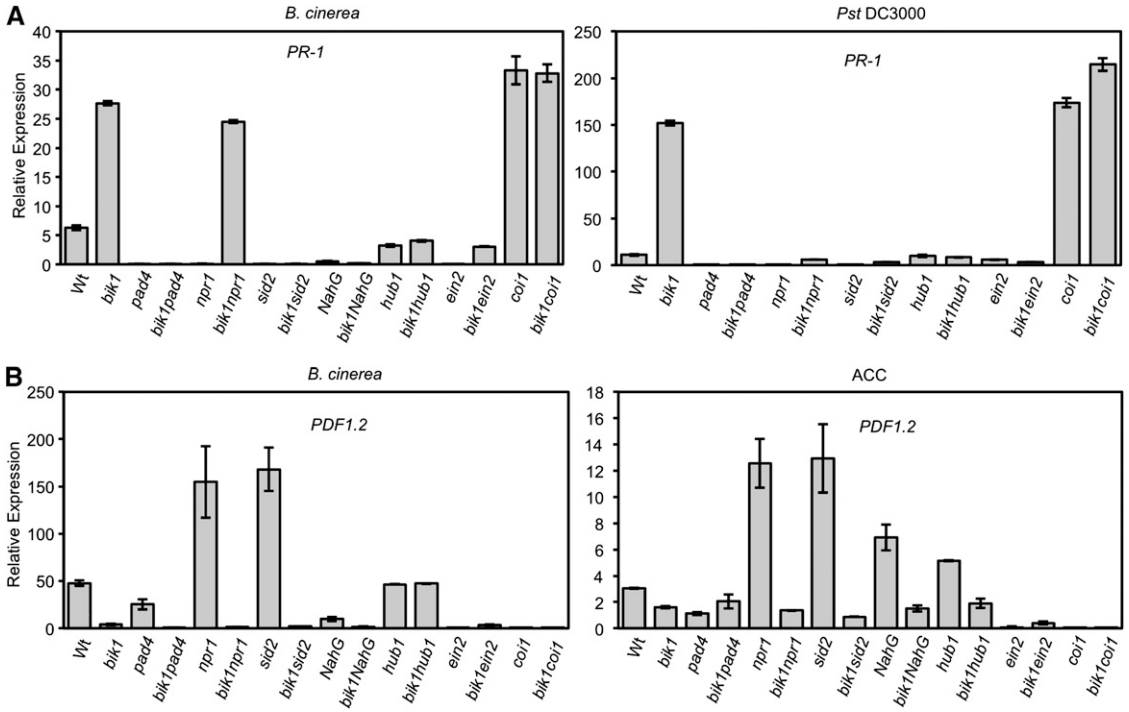

Figure 10. Expression of Defense Marker Genes in *bik1* and the Double Mutants in Response to Pathogen Infection and ACC.

(A) *B. cinerea* and *Pst*DC3000-induced expression of *PR-1*. Wt, wild type.
(B) *B. cinerea* and ACC-induced expression of *PDF1.2*.
Expression was determined using quantitative RT-PCR 24 h after inoculation/treatment as described in Methods. Experiments were repeated at least two times with similar results.

and flg22-induced *BIK1* expression is strictly dependent on EIN3, a transcription factor central to ET signaling, suggesting that *BIK1* is an EIN3 target; and (4) BIK1 kinase activity increases in response to ACC and flg22, with inhibition of ET perception blocking induction. In addition, BIK1 site-directed mutants that abrogate the triple response also abrogate immune responses and seedling flg22 growth, so (5) BIK1 kinase activity correlated with ET responses such that mutations that attenuate ET responses also eliminate plant immune responses. Consistent with this, *ein2* was recently isolated as a flagellin-insensitive mutant and shown to have defects in PTI to *Pst* (Boutrot et al., 2010). In addition, expression of *FLS2*, encoding the flagellin receptor, is directly regulated by EIN3. Thus, ET regulates the different components of the flg22-receptor complex.

Table 1. Summary of Phenotypes and Kinase Activities of BIK1 Ala Substitution Mutants

Phenotype/Activity	BIK1-HA	S33A	T64A	T94A	K105A	D202A	S233A	Y234A	S236A	T237A	T242A	Y245A
B. cinerea (basal/flg22 PTI)	+/+	IN/-	-/IN	−/−	−/−	−/−	+/+	+/+	−/−	−/−	−/−	+/+
A. brassicicola	+	IN	IN	-	-	-	+	IN	-	-	-	+
*Pst*DC3000 (basal/flg22 PTI)	+/+	IN/IN	-/D	−/−	−/−	−/−	IN/+	+/+	−/−	−/−	−/−	+/+
Pst hrcC$^-$ (basal/flg22 PTI)	+/+	+/−	D/+	+/+	−/−	−/−	+/+	+/+	IN/IN	IN/IN	+/−	+/+
Growth sensitivity to flg22	+	+	+	-	-	-	IN	+	-	-	-	+
Triple response	+	D	+	+	-	-	+	+	-	-	-	+
Plant growth	+	D	D	-	-	-	+	+	-	-	D	D
In vivo kinase activity (BIK1/MBP)	+/+	IN/-	-/IN	−/−	−/−	−/−	+/+	+/+	−/−	−/−	D/-	−/−

+, The wild type, complementation; −, *bik1*, no complementation; IN, intermediate; D, differential responses relative to the wild type and *bik1*. For kinase activity, (+) retains activity, while (−) loss of activity

Plant RLKs function in diverse physiological processes but show remarkable sequence conservation. Comparisons of RLK sequences revealed 80% or greater identity in phosphorylatable residues in the AD- of RD-type RLKs (Wang et al., 2005). Variation in biological functions is thus attributed to differences in posttranslational regulation, including phosphorylation and interactions with up- or downstream partners. Both general and specific regulatory mechanisms have been associated with the control of physiological processes by RLKs. For instance, autophosphorylation of less conserved N- and C-terminal KD regions is suggested to create docking sites for kinase substrates to achieve specificity (Johnson et al., 1996). Accordingly, mutations in the KD phosphorylation sites Ser-33, Thr-64, and Thr-94 have differential roles in BIK1-mediated PTI, flg22, and ET growth responses. The differential impact of S33A and T64A on BIK1 phosphorylation in vitro as well as BIK1 phosphorylation in response to flg22 or ACC in vivo is also consistent with the role of this region in conferring specificity to BIK1 function. The phenotypes of the BIK1^{S33A} and BIK1^{T64A} lines also suggest that BIK1 phosphorylation sites have differential functions. BIK1^{T64A} eliminated disease resistance but was dispensable for phosphorylation activities. BIK1 Thr-94 is required for basal and induced resistance to *B. cinerea* and growth responses to flg22 but not the triple response. It is not clear why BIK1 T64A impairs PTI as it maintains kinase activity. The equivalent residue in Pto (Pto Thr-38) is the main site of autophosphorylation and required for hypersensitive response (Sessa et al., 2000).

The AD of protein kinases occupies the catalytic cleft harboring the regulatory elements, including the T-loop, where activating phosphorylation events occur, and the C-terminal P+1 loop, which plays a role in recognition and binding of protein substrates (Johnson et al., 1996). *bik1* substitution mutants reveal the importance of the conserved AD residues Ser-236, Thr-237, and Thr-242 for kinase activity and BIK1 function in planta. In other protein kinases, phosphorylation of two to three AD residues is required for kinase activation. BIK1 Thr-242 falls in the T-loop, suggesting its phosphorylation may activate BIK1. T242A abolished induced kinase activity in vivo, PTI responses to all pathogens tested, and seedling sensitivity to flg22 and ACC, suggesting its critical contributions to the function of BIK1. The increased phosphorylation of BIK1^{T242A} without elicitation suggests it may negatively regulate phosphorylation. Pto Thr-204 corresponds to BIK1 Thr-242 and is similarly important to Pto function, suggesting that the biological relevance of these residues is conserved. Phosphomimic mutation at BIK1 Thr-242 was sufficient for increased basal and flg22-induced resistance to *B. cinerea*. BIK1^{Y245A} and BIK1^{Y234A} were the only substitutions that completely rescued resistance to *Pst*DC3000. This suggests these residues are sufficient for BIK1 function in the negative regulation of resistance to *Pst*DC3000 and may possibly act by affecting SA synthesis. The phosphomimic Pto mutants Y207D (BIK1 Y245) and T204D (BIK1 T242) activate hypersensitive response in the absence of AvrPto (Sessa et al., 2000), whereas the Pto Tyr-207 and Thr-204 Ala substitution mutants failed to interact with AvrPto (Frederick et al., 1998; Rathjen et al., 1999). Thus, BIK1 Tyr-245 may influence substrate binding, whereas Thr-242 is likely required for substrate recognition and specificity. These observations suggest the C-terminal region of the AD is an important regulatory region in RLCKs. Overall, the loss of biochemical activity in BIK1 correlated with loss of basal and flg22-PTI as well as ET responses in the site-directed mutants. Finally, the phosphorylation results from BIK1-HA plants may have been influenced by proteins that coprecipitate with BIK1. Since BIK1 interacts or is likely to interact with many RLKs, including FLS2, BAK1, CERK1, and ERF1 (Lu et al., 2010; Zhang et al., 2010), it will be difficult to completely exclude this possibility. However, most of our results from the in vitro and in vivo experiments correlated, and the genetic data from the different genotypes support our conclusions.

Our genetic studies uncovered interactions between BIK1 and other components of the immune response pathways. The blunted PTI in the *bik1* mutant is primarily due to inhibition of ET signaling. In support of this, the immune response functions of BIK1 mirror that of EIN2, a protein that regulates ET signaling and plays a central role in plant immune responses (Thomma et al., 1999; Boutrot et al., 2010). The responses of the *ein2 bik1* double mutant to *B. cinerea*, *Pst*DC3000, *Pst*hrcC$^-$, flg22, and ACC are consistent with BIK1 and EIN2 functioning in the same pathway. Furthermore, the defects in ET signaling function of BIK1 may be the underlying cause of upregulated SA synthesis in the mutant. EIN3 and EIL proteins were recently shown to suppress SA synthesis through regulation of *SID2* expression (Chen et al., 2009). Interestingly, EIN3 also directly regulates *FLS2* transcription, thereby modulating PTI (Boutrot et al., 2010). Thus, components of ET signaling suppress SA and promote FLS2-mediated responses similar to BIK1. *BIK1* expression is also EIN3 dependent through the direct transcriptional control by the later. The interaction between SA and ET in mediating PTI is complex, similar to the role of SA in BIK1-mediated immune responses. On one hand, BIK1 is a negative regulator of SA, with high SA accumulation in the *bik1* mutant linked, at least partially, to its resistance to *Pst*DC3000 and susceptibility to *B. cinerea*. On the other hand, SA is dispensable for flg22-PTI to *B. cinerea* but required for induced resistance to *Pst* hrcC$^-$. The *bik1* mutant, the single mutants in SA synthesis and signaling, and the double mutants with *bik1* are susceptible to *Pst hrcC*$^-$, and the flg22-induced resistance is also completely abrogated. Overall, SA is important for flg22-PTI to *Pst hrcC*$^-$, but the basal susceptibility of *bik1* to this strain is not linked to SA levels. NPR1-mediated SA signaling was completely dispensable for BIK1-mediated PTI responses. Alternatively, SA responses mediated by PAD4 contribute to the loss of basal and induced resistance to *B. cinerea* in the *bik1* mutant. These observations are consistent with the role of SID2 and PAD4 in resistance to *Pst hrcC*$^-$ (Tsuda et al., 2008).

The growth response of *bik1* seedlings to ACC was not affected by SA, suggesting that ET acts upstream of SA in the function of BIK1. Thus, the increased SA accumulation in *bik1* may have resulted from defects in ET signaling. The expression of *PDF1.2* was also not restored in *bik1* by removal of SA, further supporting this conclusion. By contrast, *bik1* seedling growth insensitivity to flg22 was modulated by SA. The susceptibility of *bik1*, *hub1*, *ein2*, *ein2 bik1*, and *bik1 hub1* to *Pst hrcC*$^-$ correlated with their insensitivity to flg22. Overall, defects in ET signaling and histone H2B monoubiquitination are accompanied by flg22 insensitivity and impaired PTI responses.

The most unexpected result from the genetic analysis was the interaction between COI1 and BIK1. COI1 was dispensable for flg22-PTI to *B. cinerea* but essential for resistance in uninduced plants. Intriguingly, *bik1 coi1* plants were responsive to flg22-mediated protection, implying that *coi1* mutation suppresses the loss of flg22-PTI in *bik1*. Both *coi1* and *bik1coi1* showed resistance comparable to the wild type for *Pst hrcC*$^-$, again suggesting that *coi1* mutation suppresses *bik1* susceptibility. In addition, flg22 actually enhanced the susceptibility of *coi1* and *bik1 coi1* plants to *Pst hrcC*$^-$. Furthermore, the flg22-insensitivity of *bik1* seedlings was rescued by the *coi1* mutation, consistent with flg22 enhancing susceptibility in *coi1*. Taken together, these data reveal that COI1 is either dispensable or suppresses PTI but also negatively regulates BIK1-mediated PTI. The higher basal resistance of nonelicited *bik1* plants to *B. cinerea* relative to *coi1* may indicate the increased contributions of COI1 to basal resistance in the absence of BIK1 and points to the role of BIK1 in limiting the contributions of COI1. The molecular mechanisms through which BIK1 suppresses COI1 function need to be investigated in the future. In the case of *Pst*DC3000, *coi1* failed to rescue *bik1* responses to flg22 protection likely due to effector-mediated suppression of PTI. The E3 ligase HUB1, required for Histone H2B monoubiquitination, contributes to resistance to necrotrophic fungi but is dispensable for flg22-PTI to *B. cinerea.* By contrast, HUB1 contributes immensely to flg22-PTI to *Pst hcC*$^-$, with *bik1 hub1* significantly more susceptible than any other double mutant, revealing that the role of HUB1 in flg22-induced PTI is additive to BIK1. It is likely that HUB1 is required for activation of PTI responses at the transcriptional level.

In conclusion, the central role of BIK1 in immune responses and plant growth is tightly linked to its signaling function in ET responses. Flagellin, a bacterial PAMP, induces PTI responses to distinct class of pathogens, suggesting a convergence of downstream immune responses regardless of the trigger consistent with recent reports that show BIK1 integrates immune responses from different upstream regulators (Zhang et al., 2010). Remarkably, our results reveal the complexity of PTI as well as the common and distinct genetic requirements underlying plant immune responses to pathogens with distinct pathogenesis strategies. Based on the documented biochemical data, activation of BIK1 may be linked to promotion or suppression of disease as well as PTI.

METHODS

Plant Growth Conditions, Diseases Assays, and flg22 Growth Responses

Plant growth conditions, fungal growth media, and fungal disease assays were done as previously described (Dhawan et al., 2009). Plants were grown in soil in growth chamber (Percival AR75L) with light intensity (140 to 150 $\mu E\, m^{-2}\, s^{-1}$), temperature (24°C), relative humidity (70%), and a 12-h-light/12-h-dark cycle. flg22- and wound-induced resistance to *Botrytis cinerea* was performed as described (Ferrari et al., 2007; Chassot et al., 2008). Bacterial cultures and disease assays were performed as described by infiltrating 4-week-old plants with 10^6 colony-forming units (cfu)/mL of *Pseudomonas syringae* DC3000 or the *hrcC*$^-$ mutant derived from DC3000 1 d after infiltration with 1 μM flg22 or double deionized water (Zheng et al., 2006; Zhang et al., 2010). Assays for seedling growth sensitivity to flg22 were performed as previously described using the *bak1* mutant as a positive control for insensitivity (Chinchilla et al., 2007). Silver nitrate treatment to inhibit ET signaling was performed on 4-week-old plants as described (Colville and Smirnoff, 2008). Seeds for mutants were obtained from the ABRC. *ein3* and *ein2* seeds were obtained from Joe Ecker's lab at the SALK institute.

Generation of Recombinant Proteins and Transgenic Lines

For purification of recombinant BIK1 and BIK1 substitution mutants, the open reading frame of *BIK1* was cloned into glutathione *S*-transferase fusion protein expression vector pGEX4T-1 (Pharmacia). To generate transgenic lines expressing *BIK1* and *BIK1* substitution mutants, the full-length *BIK1* cDNA (GeneBank: AEC09703) was cloned after the cauliflower mosaic virus 35S promoter into a modified version of binary vector pCAMBIA 99-1. Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis thaliana* wild-type or *bik1* plants (Clough and Bent, 1998). Transgenic plants were selected on medium containing hygromycin and lines selected based on protein expression following immunoblot analysis using an anti-HA antibody (Covance). Primers used are listed in Supplemental Table 1 online.

Construction of Double Mutants and Mutant Alleles

Generation of the *bik1 NahG* double mutant was previously described (Veronese et al., 2006). Generation and selection of all other *bik1* double mutants were performed as previously described (Dhawan et al., 2009). In addition, DNA from ACC-selected *bik1ein2* plants was sequenced to ensure plants were homozygous for the *ein2* point mutation. The *ein3-1* and *eil1-3* (*SALK_049679C*) mutant alleles were obtained from the ABRC.

Immunoprecipitation, Kinase Assays, and Immunoblot Analysis

Tissue from 4-week-old plants 3 h after treatment with double deionized water, 5 μM flg22, or 20 μM ACC by homogenization was frozen and homogenized in cold immunoprecipitation buffer with added protease inhibitor cocktail (Sigma-Aldrich) (50 mM HEPES, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% Brij 58, 50 mM β-glycerol phosphate disodium salt pentahydrate, 2 mM DTT, and 10% glycerol at pH 7.5). Subsequent immunoprecipitation steps were performed as previously described using an anti-HA affinity matrix (Roche) with the exception of overnight incubation of the supernatant (Schulze et al., 2010). In vivo kinase assays were performed on immunoprecipitated proteins as previously described with or without the addition of 2 μCi of [γ-^{32}P]ATP (Lu et al., 2010). Phosphorylation of MBP was analyzed by immunoblot using an antiphosphoserine/Thr antibody (ECM Biosciences) or autoradiography after separation on 12% SDS-PAGE. Autophosphorylation of BIK1 and mutated BIK1 proteins was analyzed by immunoblot using an anti-HA antibody (Covance) or autoradiography after separation on 12% SDS-PAGE. Expression, purification, and in vitro kinase assays for the recombinant BIK1 proteins was performed as previously described (Abuqamar et al., 2008). Total protein extracted from 4-week soil-grown *Arabidopsis* or 10-d-old seedlings treated for 24 or 3 h, respectively, with 5 μM flg22 or 20 μM ACC as described (Zhang et al., 2010) was analyzed via immunoblots using anti-EIN3, WRKY33 (Qiu et al., 2008), or MPK4 antibodies. Total protein staining with Ponceau S (Sigma-Aldrich) or Coomassie Brilliant Blue R 250 (Thermo Scientific) was used as a loading control. Protein dephosphorylation was performed using calf intestinal alkaline phosphatase (CIP) according to the manufacturer's protocol (New England Biolabs) with ~1 to 2.5 units of CIP (as indicated)/μg protein.

RNA Extraction and Expression Analysis

Total RNA was isolated with Trizol reagent according to the manufacturer's instructions (Invitrogen). DNase treatment, cDNA synthesis, and

quantitative RT-PCR were performed according to the manufacturer's instructions using gene-specific primers with *Arabidopsis Actin2* as an endogenous reference for normalization (Promega, Biotium). A minimum of three technical replicates of the quantitative RT-PCR assay was used for each sample with a minimum of two biological replicates. Expression levels were calculated by the comparative cycle threshold method (Applied Biosystems). Primers used are listed in Supplemental Table 1 online.

ChIP-PCR

ChIP was performed essentially as described (Saleh et al., 2008) using EIN3 antibody. Chromatin was immunoprecipitated from 3-week-old wild-type seedlings treated with 20 μM ACC for 4 h. ChIP-qPCR was performed using specific primers to the predicted EIN3 binding regions on the BIK1 promoter. A control ChIP lacking the EIN3 antibody was run in parallel. The EIN3 antibody used for the immunoprecipitation of EIN3-associated DNA was from Joe Ecker (SALK Institute). Actin was used as internal control for normalization. The primers used for the ChIP-qPCR are listed in Supplemental Table 1 online.

GUS Staining

Histochemical staining for GUS was done as previously described (Liu et al., 2007) on 4-week soil-grown *Arabidopsis* or 10-d-old seedlings treated for 24 or 3 h, respectively, with double deionized water, 5 μM flg22, 20 μM ACC, or 100 mg/L chitin.

Accession Numbers

Sequence data for the genes described in this article or used in the phylogenetic analysis can be found in the GenBank/EMBL data libraries under the following accession numbers: *TPK1b* (GenBank accession number *EU555286*), *CAO21648*, *BIK1* (*At2g39660*), *NAK* (At5g02290), *PBS1* (At5g13160), *APK1b* (At2g28930), *APK1a* (At1g07570), *APK2b* (At2g02800), *MPLKe* (*BAD12263*), *PTO* (*A49332*), and *At3g55450.*

Supplemental Data

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

Supplemental Figure 1. Wound-Induced Immunity to *B. cinerea* Requires BIK1.

Supplemental Figure 2. Response of the *BIK1* Substitution Mutants to *A. brassicicola* Infection.

Supplemental Figure 3. The BIK1 Promoter Sequence and Location of Putative EIN3 Binding Sequences in the *BIK1* Promoter.

Supplemental Figure 4. Bacterial and Flagellin Responses of the BIK1 Phosphomimic Mutants.

Supplemental Figure 5. The Triple Response of BIK1 Thr-242 and Tyr-245 Phosphomimic Mutations at 20 μM ACC.

Supplemental Figure 6. Growth Morphology of the *BIK1* Substitution Mutants.

Supplemental Figure 7. Disease Responses of the *bik1* Double Mutants to *A. brassicicola.*

Supplemental Figure 8. The BIK1-Regulated ET Growth Response Is Independent of SA.

Supplemental Figure 9. BIK1 Function in Necrotrophic Defense and ET Responses Is Unique among Related Protein Kinases.

Supplemental Table 1. Primers Used in the *BIK1* Study.

ACKNOWLEDGMENTS

This research was funded by a grant (IOS-0749865) from the National Science Foundation to T.M. We thank Joe Ecker and Katherine Chang (The SALK institute) for the EIN3 antibodies and *ein3-1* mutant seeds.

AUTHOR CONTRIBUTIONS

K.L. performed research and wrote the article. H.L., M.C., R.D., and Z.L. performed research. T.M. designed the research, analyzed data, and wrote the article.

Received May 10, 2011; revised July 31, 2011; accepted August 9, 2011; published August 23, 2011.

REFERENCES

Abuqamar, S., Chai, M.F., Luo, H., Song, F., and Mengiste, T. (2008). Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. Plant Cell **20:** 1964–1983.

Becraft, P.W. (2002). Receptor kinase signaling in plant development. Annu. Rev. Cell Dev. Biol. **18:** 163–192.

Bethke, G., Unthan, T., Uhrig, J.F., Pöschl, Y., Gust, A.A., Scheel, D., and Lee, J. (2009). Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. Proc. Natl. Acad. Sci. USA **106:** 8067–8072.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. **60:** 379–406.

Boutrot, F., Segonzac, C., Chang, K.N., Qiao, H., Ecker, J.R., Zipfel, C., and Rathjen, J.P. (2010). Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc. Natl. Acad. Sci. USA **107:** 14502–14507.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:** 1583–1592.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89:** 1133–1144.

Chassot, C., Buchala, A., Schoonbeek, H.J., Métraux, J.P., and Lamotte, O. (2008). Wounding of Arabidopsis leaves causes a powerful but transient protection against *Botrytis infection*. Plant J. **55:** 555–567.

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., Cai, R., Zuo, J., Tang, X., Li, X., Guo, H., and Zhou, J.M. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in *Arabidopsis*. Plant Cell **21:** 2527–2540.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448:** 497–500.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Colville, L., and Smirnoff, N. (2008). Antioxidant status, peroxidase activity, and PR protein transcript levels in ascorbate-deficient *Arabidopsis thaliana* vtc mutants. J. Exp. Bot. **59:** 3857–3868.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266:** 1247–1250.

Dempsey, D.A., Shah, J., and Klessig, D.F. (1999). Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. **18:** 547–575.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M., Drummond, E.P., and Ausubel, F.M. (2000). Three unique mutants of Arabidopsis identify eds loci required for limiting growth of a biotrophic fungal pathogen. Plant J. **24:** 205–218.

Dhawan, R., Luo, H., Foerster, A.M., Abuqamar, S., Du, H.N., Briggs, S.D., Mittelsten Scheid, O., and Mengiste, T. (2009). HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in *Arabidopsis*. Plant Cell **21:** 1000–1019.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M., and Dewdney, J. (2007). Resistance to Botrytis cinerea induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiol. **144:** 367–379.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003). Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J. **35:** 193–205.

Frederick, R.D., Thilmony, R.L., Sessa, G., and Martin, G.B. (1998). Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase. Mol. Cell **2:** 241–245.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43:** 205–227.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R., and Ausubel, F.M. (1997). Phytoalexin-deficient mutants of Arabidopsis reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics **146:** 381–392.

Gómez-Gómez, L., and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol. Cell **5:** 1003–1011.

Govrin, E.M., and Levine, A. (2002). Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). Plant Mol. Biol. **48:** 267–276.

Grant, M.R., and Jones, J.D. (2009). Hormone (dis)harmony moulds plant health and disease. Science **324:** 750–752.

Guo, H., and Ecker, J.R. (2004). The ethylene signaling pathway: New insights. Curr. Opin. Plant Biol. **7:** 40–49.

Guzmán, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2:** 513–523.

Hanks, S.K., and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. FASEB J. **9:** 576–596.

Hanks, S.K., and Quinn, A.M. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. Methods Enzymol. **200:** 38–62.

He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S.D., and Li, J. (2007). BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr. Biol. **17:** 1109–1115.

Johnson, L.N., Noble, M.E., and Owen, D.J. (1996). Active and inactive protein kinases: Structural basis for regulation. Cell **85:** 149–158.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444:** 323–329.

Kosugi, S., and Ohashi, Y. (2000). Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. Nucleic Acids Res. **28:** 960–967.

Leon-Reyes, A., Du, Y., Koornneef, A., Proietti, S., Körbes, A.P., Memelink, J., Pieterse, C.M., and Ritsema, T. (2010). Ethylene signaling renders the jasmonate response of Arabidopsis insensitive to future suppression by salicylic acid. Mol. Plant Microbe Interact. **23:** 187–197.

Liu, Y., Koornneef, M., and Soppe, W.J. (2007). The absence of histone H2B monoubiquitination in the *Arabidopsis* hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy. Plant Cell **19:** 433–444.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165–178.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. Proc. Natl. Acad. Sci. USA **107:** 496–501.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc. Natl. Acad. Sci. USA **104:** 19613–19618.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M., and Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. **147:** 1347–1357.

Qiu, J.L., et al. (2008). Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO J. **27:** 2214–2221.

Rathjen, J.P., Chang, J.H., Staskawicz, B.J., and Michelmore, R.W. (1999). Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of avrPto. EMBO J. **18:** 3232–3240.

Saleh, A., Alvarez-Venegas, R., and Avramova, Z. (2008). An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants. Nat. Protoc. **3:** 1018–1025.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J. Biol. Chem. **285:** 9444–9451.

Sessa, G., D'Ascenzo, M., and Martin, G.B. (2000). Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response. EMBO J. **19:** 2257–2269.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., and Innes, R.W. (2003). Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science **301:** 1230–1233.

Shiu, S.H., and Bleecker, A.B. (2001). Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc. Natl. Acad. Sci. USA **98:** 10763–10768.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12:** 3703–3714.

Spoel, S.H., Johnson, J.S., and Dong, X. (2007). Regulation of trade-offs between plant defenses against pathogens with different lifestyles. Proc. Natl. Acad. Sci. USA **104:** 18842–18847.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science **274:** 2060–2063.

Thomma, B.P., Eggermont, K., Penninckx, I.A., Mauch-Mani, B.,

Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95:** 15107–15111.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol. **121:** 1093–1102.

Thomma, B.P., Nürnberger, T., and Joosten, M.H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell **23:** 4–15.

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Curr. Opin. Plant Biol. **13:** 459–465.

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J.D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. Plant J. **53:** 763–775.

Veronese, P., Chen, X., Bluhm, B., Salmeron, J., Dietrich, R., and Mengiste, T. (2004). The BOS loci of Arabidopsis are required for resistance to *Botrytis cinerea* infection. Plant J. **40:** 558–574.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H., and Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell **18:** 257–273.

Wang, X., Goshe, M.B., Soderblom, E.J., Phinney, B.S., Kuchar, J.A., Li, J., Asami, T., Yoshida, S., Huber, S.C., and Clouse, S.D. (2005). Identification and functional analysis of in vivo phosphorylation sites of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 receptor kinase. Plant Cell **17:** 1685–1703.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094.

Yanagisawa, S., Yoo, S.D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature **425:** 521–525.

Zarei, A., Körbes, A.P., Younessi, P., Montiel, G., Champion, A., and Memelink, J. (2011). Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the PDF1.2 promoter in Arabidopsis. Plant Mol. Biol. **75:** 321–331.

Zhang, J., et al. (2010). Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. Cell Host Microbe **7:** 290–301.

Zheng, Z.Y., Qamar, S.A., Chen, Z.X., and Mengiste, T. (2006). Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. **48:** 592–605.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc. Natl. Acad. Sci. USA **95:** 10294–10299.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125:** 749–760.