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The *Pseudomonas syringae* effector HopF2 suppresses Arabidopsis immunity by targeting BAK1

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SUMMARY

Pseudomonas syringae **delivers a plethora of effector proteins into host cells to sabotage immune responses and modulate physiology to favor infection. The *P. syringae* pv. *tomato* DC3000 effector HopF2 suppresses Arabidopsis innate immunity triggered by multiple microbe-associated molecular patterns (MAMP) at the plasma membrane. We show here that HopF2 possesses distinct mechanisms for suppression of two branches of MAMP-activated MAP kinase (MAPK) cascades. In addition to blocking MKK5 (MAPK kinase 5) activation in the MEKK1 (MAPK kinase kinase 1)/MEKKs–MKK4/5–MPK3/6 cascade, HopF2 targets additional component(s) upstream of MEKK1 in the MEKK1–MKK1/2–MPK4 cascade and the plasma membrane-localized receptor-like cytoplasmic kinase BIK1 and its homologs. We further show that HopF2 directly targets BAK1, a plasma membrane-localized receptor-like kinase that is involved in multiple MAMP signaling. The interaction between BAK1 and HopF2 and between two other *P. syringae* effectors, AvrPto and AvrPtoB, was confirmed *in vivo* and *in vitro*. Consistent with BAK1 as a physiological target of AvrPto, AvrPtoB and HopF2, the strong growth defects or lethality associated with ectopic expression of these effectors in wild-type Arabidopsis transgenic plants were largely alleviated in *bak1* mutant plants. Thus, our results provide genetic evidence to show that BAK1 is a physiological target of AvrPto, AvrPtoB and HopF2. Identification of BAK1 as an additional target of HopF2 virulence not only explains HopF2 suppression of multiple MAMP signaling at the plasma membrane, but also supports the notion that pathogen virulence effectors act through multiple targets in host cells.**

Keywords: bacterial effector, pattern-triggered immunity, BAK1, BIK1, MAPK cascade, *Pseudomonas syringae*, *Arabidopsis thaliana*.

INTRODUCTION

Plants have evolved robust immune systems to protect them from pathogen invasions. Plant innate immunity is initiated with recognition of conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) through membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (Jones and Dangl, 2006; Boller and Felix, 2009). Pattern-triggered immunity (PTI) plays a pivotal role in defense against a broad spectrum of potential pathogens (Jones and Dangl, 2006; Boller and Felix, 2009). A 22 amino acid peptide from the N–terminus of bacterial flagellin, flg22, is perceived by Arabidopsis RLK flagellin-sensing 2 (FLS2), and induces FLS2 association with another plasma membrane-localized RLK, BAK1 (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). BAK1 was originally isolated as a RLK that interacts with the plant growth hormone brassinosteroid (BR) receptor BRI1 (Li *et al.*,

2002; Nam and Li, 2002). BAK1 has a relatively short extracellular leucine-rich repeat (LRR) domain and is not involved in flagellin or BR perception (Kinoshita *et al.*, 2005; Chinchilla *et al.*, 2007). Notably, BAK1 is required for signaling triggered by multiple MAMPs, including bacterial elongation factor Tu (EF–Tu), flagellin, harpin Z, lipopolysaccharide, peptidoglycan, necrosis-inducing *Phytophthora* protein 1 (NPP1), oomycete elicitor INF1 and bacterial cold-shock protein in Arabidopsis and *Nicotiana benthamiana* (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Shan *et al.*, 2008). In addition to FLS2, BAK1 has been shown to hetero-dimerize with EFR, an RLK for EF–Tu, and PEPR1/2, RLKs for the plant endogenous signal Pep1/2 (Postel *et al.*, 2010; Roux *et al.*, 2011). BAK1 is able to directly phosphorylate the plasma membrane-localized receptor-like cytoplasmic kinase (RLCK) BIK1 (Lu *et al.*, 2010a). In non-elicited cells, BIK1 interacts with BAK1, FLS2, EFR and PEPR1/2 (Lu *et al.*, 2010a; Zhang *et al.*, 2010; Liu *et al.*, 2013). flg22 induces rapid phosphorylation of BIK1, which further transphosphorylates FLS2–BAK1, and leads to the dissociation of BIK1 from the FLS2–BAK1 complex (Lu *et al.*, 2010a; Zhang *et al.*, 2010; Cao *et al.*, 2013). As a step toward attenuation of immune responses, flg22 induces FLS2 endocytosis in vesicles within approximately 30 min, and leads to FLS2 degradation (Robatzek *et al.*, 2006; Beck *et al.*, 2012). Protein ubiquitination often directs target proteins for degradation through the proteasome or vacuole, or mediates endosomal sorting of receptors. FLS2 is targeted by the plant U–box-containing E3 ubiquitin ligases PUB12 and PUB13 (Lu *et al.*, 2011). BAK1 phosphorylates PUB12/13 upon flg22 elicitation, and promotes FLS2–PUB12/13 association for ligand-induced FLS2 degradation. Despite specific recognition of MAMPs by their corresponding receptors, diverse MAMPs often elicit largely overlapping responses, including ion fluxes across the plasma membrane leading to membrane depolarization and medium alkalinization, production of reactive oxygen species (ROS), cytoplasmic calcium transients, callose deposition, stomatal closure, expression of defense-related genes and activation of mitogen-activated protein kinase (MAPK) cascades and Ca^{2+}-dependent protein kinases (CDPKs) (Boller and Felix, 2009; Tena *et al.*, 2011; Schwessinger and Ronald, 2012).

Successful pathogens have evolved the ability to interfere with plant physiology and immunity to favor infection. *Pseudomonas syringae* is a Gram-negative phytobacterial pathogen that causes a wide range of diseases, including blights, leaf spots and galls, in various plant species, and is also a model phytopathogen in molecular plant pathology (Preston, 2000). Extensive genetic and genomic studies of *P. syringae* have identified many key virulence determinants, including global virulence regulators, the type III secretion system, phytotoxins and exopolysaccharides (Block *et al.*, 2008). In particular, *P. syringae* delivers around 30 effectors into plant cells through the type III secretion system, and many of these effectors target important host components to sabotage plant immunity (Speth *et al.*, 2007; Block *et al.*, 2008; Gohre and Robatzek, 2008; Lewis *et al.*, 2009; Hann *et al.*, 2010). The *P. syringae* effector HopU1 is a mono-ADP-ribosyltransferase that targets several Arabidopsis RNA-binding proteins including GRP7 (Fu *et al.*, 2007). Interestingly, GRP7 interacts with both translational components and the MAMP receptors FLS2 and EFR, implying a role for GRP7 in plant immunity (Nicaise *et al.*, 2013). In addition, GRP7 directly binds to the transcripts of FLS2 and EFR, and HopU1 blocks this binding, thereby modulating receptor abundance at the translational level (Nicaise *et al.*, 2013). Two distinct effectors in lack of similarity with respect to sequences, AvrPto and AvrPtoB, are potent suppressors of multiple MAMP signaling by targeting RLKs, including BAK1 and FLS2 (He *et al.*, 2006; de Torres *et al.*, 2006; Gohre and Robatzek, 2008; Shan *et al.*, 2008; Xiang *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). AvrPtoB possesses E3 ubiquitin ligase activity, and targets certain RLKs, including FLS2 and CERK1, for degradation (Gohre *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). Other *Pseudomonas* effectors target components downstream of MAMP receptor complexes. For example, HopAI1 targets MPK3, MPK4 and MPK6 to disrupt MAPK activation upon MAMP perception (Zhang *et al.*, 2007, 2012). Interestingly, inactivation of MPK4 by HopAI1 activates defense responses mediated by the nucleotide binding leucine-rich repeat (NB-LRR) protein SUMM2 (Zhang *et al.*, 2012). Effector-mediated suppression of PTI signaling may also be defeated by NB-LRR-mediated effector-triggered immunity (ETI). HopM1 targets and degrades AtMIN7, a member of the ADP-ribosylation factor family of guanine nucleotide exchange factors involved in vesicle trafficking (Nomura *et al.*, 2006). Activation of ETI signaling by AvrRpt2, AvrPphB and HopA1 prevents HopM1-mediated degradation of AtMIN7 to suppress HopM1 virulence activity (Nomura *et al.*, 2011). Additionally, AvrRpt2 promotes auxin responses to facilitate pathogen virulence by stimulating turnover of auxin/indole acetic acid proteins, the key negative regulators in auxin signaling (Cui *et al.*, 2013).

We previously reported that a *P. syringae* pv. *tomato* DC3000 effector, HopF2, suppresses Arabidopsis innate immunity at the plasma membrane (Wu *et al.*, 2011). Similar to AvrPto, HopF2 possesses a putative myristoylation modification motif that is required for its plasma membrane localization and virulence activity in Arabidopsis, tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) (Shan *et al.*, 2000; Robert-Seilaniantz *et al.*, 2006; Wu *et al.*, 2011). HopF2 suppresses immune responses triggered by multiple MAMPs, including flg22, elf18, lipopolysaccharide, peptidoglycan, harpin Z and chitin (Wu *et al.*, 2011). Structural analysis of the HopF2

homolog AvrPphF from *P. syringae* pv. *phaseolicola* identified several conserved surface-exposed residues, and mutational analysis indicated that the corresponding residues in HopF2 are required for its virulence and MAMP suppression activity (Shan *et al.*, 2004; Singer *et al.*, 2004; Wang *et al.*, 2010; Wu *et al.*, 2011). It has been shown that RIN4, a component involved in both PTI and ETI, is targeted and suppressed by HopF2 (Wilton *et al.*, 2010). HopF2 also targets MAPK kinase 5 (MKK5) and suppresses MKK5 phosphorylation of downstream MPK3/6 through its ADP-ribosyltransferase activity (Wang *et al.*, 2010). Interestingly, HopF2 suppresses flg22-induced BIK1 phosphorylation, an event that probably acts upstream or independently of MAPK cascades in flg22 signaling. HopF2 does not directly interact with BIK1 or affect BIK1 kinase activity (Wu *et al.*, 2011), suggesting that HopF2 targets additional host proteins upstream of BIK1 in flg22 signaling. We have extended that study, and found that HopF2 blocks flg22-induced phosphorylation of two BIK1 homologs, PBS1 and PBL1, and that HopF2 virulence is associated with its suppression of BIK1 phosphorylation. We have previously reported that HopF2 suppresses flg22-induced activation of MPK4 (Wu *et al.*, 2011). Consistent with its suppression upstream of BIK1, HopF2 does not affect MPK4 activation by MKK1/2 or MEKK1. Importantly, HopF2 directly interacts with BAK1 *in vivo* and *in vitro* in an FLS2-independent manner. We have previously shown that BAK1 is also a virulence target of AvrPto and AvrPtoB (Shan *et al.*, 2008). Recently the crystal structure of the AvrPtoB/BAK1 complex has been solved (Cheng *et al.*, 2011). The interaction between BAK1 and AvrPto or AvrPtoB was confirmed using *in vivo* co-immunoprecipitation (co–IP) and bimolecular fluorescence complementation (BiFC) assays, and an *in vitro* pull-down assay in this study. Expression of AvrPto, AvrPtoB or HopF2 under the control of the constitutive 35S promoter leads to lethality or causes severe growth defects in Arabidopsis wild-type (WT) plants, probably due to their strong virulence. Significantly, the growth defects/lethality caused by ectopic expression of AvrPto, AvrPtoB or HopF2 were dramatically reduced in *bak1* mutant plants, further indicating that BAK1 is their physiological target.

RESULTS

HopF2 virulence is associated with its suppression of BIK1 phosphorylation

flg22-induced BIK1 phosphorylation is indicated by a mobility shift on SDS–PAGE (Lu *et al.*, 2010a). The mobility shift of hemagglutinin (HA) epitope-tagged BIK1 is blocked by co-expression of GFP-tagged HopF2 in Arabidopsis protoplasts, suggesting that HopF2 suppresses flg22-induced BIK1 phosphorylation (Wu *et al.*, 2011). It has been reported that several BIK1 homologs, including PBL1 and PBS1, are also quickly phosphorylated upon flg22 treatment (Lu *et al.*, 2010b; Zhang *et al.*, 2010). Interestingly, HopF2 also blocked flg22-induced phosphorylation of PBL1 and PBS1 (Figure 1a), which probably have a redundant function with BIK1 in flg22-mediated signaling through association with FLS2 and BAK1 (Zhang *et al.*, 2010). These data suggest that HopF2 suppresses flg22-mediated signaling at an immediate early step upstream of BIK1/PBL1 phosphorylation in the FLS2/BAK1 receptor complex at the plasma membrane.

Structure analysis of HopF1 (AvrPphF) from *P. syringae* pv. *phaseolicola*, a homolog of HopF2, identified several conserved surface-exposed residues that are required for its virulence and avirulence functions in beans (*Phaseolus vulgaris*) (Singer *et al.*, 2004). The corresponding residues in HopF2 are essential for its suppression of flg22-induced expression of *pFRK1::LUC* (*FRK1* promoter fused to a luciferase reporter) (Wu *et al.*, 2011). In particular, HopF2 R71A and D175A mutants lost the ability to suppress flg22-induced *pFRK1::LUC* activation (Wu *et al.*, 2011). To determine whether these residues are also essential for HopF2 suppression of BIK1 phosphorylation, we examined the flg22-induced mobility shift of BIK1 in the presence of various HopF2 mutants. Significantly, HopF2 R71A and D175A mutants failed to suppress flg22-induced BIK1 phosphorylation (Figure 1b). Consistent with their suppression actions on flg22-induced *pFRK1::LUC* and MAPK activation (Wang *et al.*, 2010; Wu *et al.*, 2011), the S89A, H96A and

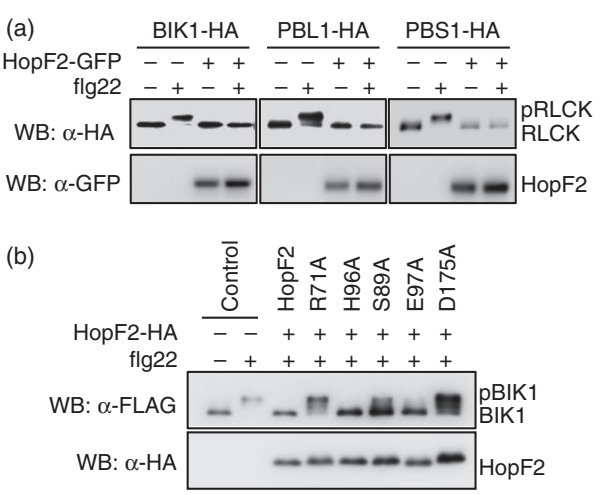

Figure 1. HopF2 suppresses flg22-induced phosphorylation of BIK1 and its homologs.

(a) HopF2 blocks the flg22-induced mobility shift of BIK1 and its homologs. Arabidopsis protoplasts were co-transfected with HA-tagged BIK1, PBL1 or PBS1 and GFP-tagged HopF2 for 10 h, and treated with 1 μM flg22 for 10 min. WB, Western blot; RLCK, receptor-like cytoplasmic kinase; pRLCK, phosphorylated RLCK.

(b) Conserved surface residues of HopF2 are required for suppression of flg22-induced BIK1 phosphorylation. Protoplasts were co-transfected with FLAG-tagged BIK1 and HA-tagged HopF2 or its mutants, and treated with flg22 as in (a). pBIK1, phosphorylated BIK1.

E97A mutants had little or no effect on the HopF2 suppression of flg22-induced BIK1 phosphorylation (Figure 1b). It has been shown that HopF2 R71 and D175 are required for its virulence function (Wang *et al.*, 2010; Wu *et al.*, 2011). Taken together, our data suggest that HopF2 virulence is associated with its suppression of BIK1 phosphorylation.

Distinct mechanisms of HopF2 suppression of two branches of flg22-induced MAPK cascades

MAPK activation is one of the early signaling events following MAMP recognition in both plants and animals (Barton and Medzhitov, 2003; Nurnberger *et al.*, 2004; Tena *et al.*, 2011). Accumulating evidence suggests that perception of flg22 activates two branches of MAPK cascades in Arabidopsis, MEKK1/MEKKs–MKK4/5–MPK3/6 and MEKK1–MKK1/2–MPK4 (Figure 2a) (Tena *et al.*, 2011). Although it has been reported that HopF2 directly targets and blocks MKK5 function (Wang *et al.*, 2010), HopF2 surprisingly did not directly affect MKK1 and MKK2 activity (Figure 2b). As shown in Figure 2(b), the constitutively active forms of Myc epitope-tagged MKK1 and MKK2 (MKK1ac–Myc and MKK2ac–Myc) activated HA-tagged MPK4 in Arabidopsis protoplasts in an immunocomplex kinase assay. Expression of HopF2 did not affect activation of MPK4 by MKK1ac or MKK2ac (Figure 2b). Furthermore, HopF2 did not interfere with MEKK1-mediated activation of MPK4 (Figure 2c). The data suggest that HopF2 suppresses flg22-induced MPK4 activation upstream of MEKK1–MKK1/2, consistent with HopF2 suppression of flg22-induced BIK1 phosphorylation. In agreement with the previous report (Wang *et al.*, 2010), HopF2 functions on MKK5 to suppress MPK3 activation. As shown in Figure 2(d), expression of HopF2 diminished active MKK5ac-mediated MPK3 activity. Thus, in addition to MKK5, HopF2 also targets additional component(s) upstream of MEKK1 and BIK1, probably immediately after flagellin perception by the FLS2–BAK1 receptor complex.

HopF2 interacts with BAK1

HopF2 suppresses *pFRK1::LUC* activation by multiple MAMPs, including elf18, peptidoglycan, lipopolysaccharide and harpin Z. As BAK1 is involved in signaling activated by multiple MAMPs, we tested whether HopF2 directly interacts with BAK1. Interestingly, similar to AvrPto and AvrPtoB, HopF2 co-immunoprecipitated with BAK1 in Arabidopsis wild-type protoplasts (Figures 3a and S1). We did not detect an interaction between AvrRpt2 and BAK1. However, HopF2 D175A interacted with BAK1 in Arabidopsis protoplasts (Figure S1). The association between BAK1 and HopF2, AvrPto or AvrPtoB was also detected in *fls2* mutant protoplasts, indicating that this association is independent of FLS2 (Figure 3a). To further confirm the *in vivo* association of HopF2 and BAK1 in intact plants, we transformed HA-tagged *HopF2* under the control of the dexamethasone (DEX)-inducible promoter (*DEX::HopF2-HA*) into *pBAK1::BAK1-GFP* transgenic plants. HopF2–HA co-immunoprecipitated with BAK1–GFP as detected using α–HA antibody upon immunoprecipitation using α-GFP antibody (Figure 3b). In addition, AvrPto–HA co-immunoprecipitated with BAK1–GFP in transgenic plants expressing *DEX::AvrPto-HA* and *pBAK1::BAK1-GFP* as detected using α–HA antibody upon immunoprecipitation using α-GFP antibody (Figure 3c). The GFP itself did not immunoprecipitate AvrPto or HopF2 (Figure S1). Consistently, the BiFC

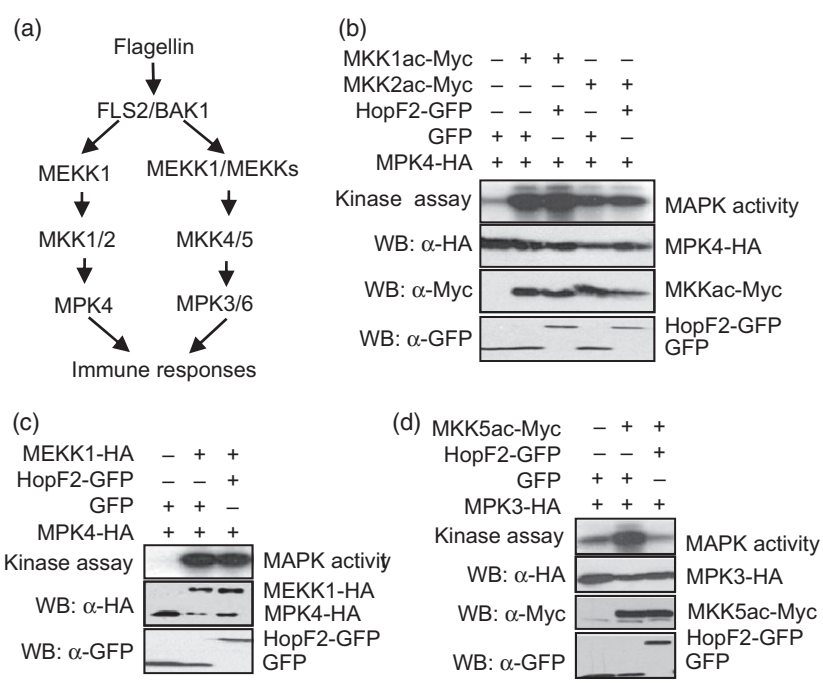

Figure 2. HopF2 suppresses two branches of the flg22-induced MAPK cascade.
(a) Scheme of the two branches of the MAPK cascade in Arabidopsis flagellin signaling.
(b) HopF2 does not suppress MKK1/2-mediated MPK4 activation. Arabidopsis protoplasts were co-transfected with the Myc-tagged constitutively active form of MKK1/2 (MKK1ac–Myc/MKK2ac–Myc), HA-tagged MPK4 (MPK4–HA) and GFP-tagged HopF2 (HopF2–GFP). MPK4–HA was immunoprecipitated using α–HA antibody, and subjected to an immunocomplex kinase assay using myelin basic protein as the substrate.
(c) HopF2 does not suppress MEKK1-mediated MPK4 activation. HA-tagged MEKK1 (MEKK1–HA) was co-expressed with MPK4–HA and HopF2–GFP, and MPK4–HA kinase activity was detected by an immunocomplex kinase assay as in (b).
(d) HopF2 suppresses MKK5-mediated MPK3 activation. MKK5ac-Myc was co-expressed with MPK3–HA and HopF2–GFP, and MPK3–HA kinase activity was detected by an immunocomplex kinase assay as in (b).

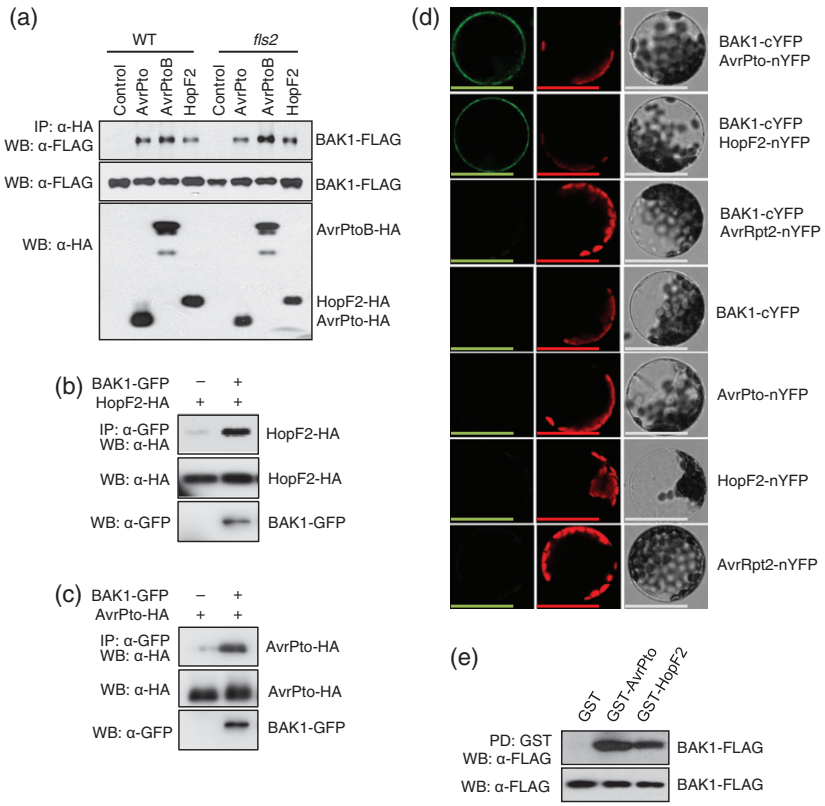

Figure 3. HopF2, AvrPto and AvrPtoB interact with BAK1.
(a) HopF2 and AvrPto/B interact with BAK1 in Arabidopsis protoplasts. The α–HA co–IP was performed using protoplasts co-expressing FLAG-tagged BAK1 and HA-tagged AvrPto, AvrPtoB or HopF2, and the immunoprecipitated proteins were analyzed by Western blotting (WB) using α–FLAG antibody. IP, immunoprecipitation.
(b, c) HopF2 and AvrPto interact with BAK1 in Arabidopsis plants. *pBAK1::BAK1-GFP* transgenic seedlings with DEX-inducible effector transgene were treated with 5 μM DEX for 12 h and subjected to an α–GFP co–IP assay, and the immunoprecipitated proteins were analyzed by Western blotting using α–HA antibody. The controls were transgenic Col–0 seedlings expressing the corresponding effector gene from the DEX-inducible promoter.
(d) HopF2 and AvrPto interact with BAK1 in BiFC assays. The various BiFC constructs were transfected into Arabidopsis protoplasts, and the fluorescence was visualized under a confocal microscope. Green color, yellow fluorescence signal; red color, chlorophyll autofluorescence signal. Scale bars = 50 μm.
(e) HopF2 and AvrPto interact with BAK1 in a pull-down assay. GST, GST–AvrPto and GST–HopF2 were expressed individually in *E. coli*, purified using glutathione agarose, and used to pull-down proteins from protoplasts expressing FLAG-tagged BAK1. The pull-downed proteins were analyzed by Western blotting using α–FLAG antibody.

assay also indicated *in vivo* association of HopF2 and BAK1 (Figure 3d). The fluorescence signal was detected when HopF2–nYFP (the N–terminal part of YFP fused to HopF2) was co-expressed with BAK1–cYFP (the C–terminal part of YFP fused to BAK1) (Figure 3d). Similarly, the *in vivo* AvrPto and BAK1 association was observed upon co-transfection of BAK1–cYFP and AvrPto–nYFP into protoplasts (Figure 3d). None of the individual constructs or BAK1–cYFP plus AvrRpt2–nYFP produced fluorescence signals in protoplasts (Figure 3d). Furthermore, HopF2 or AvrPto protein fused to glutathione *S*–transferase (GST) immobilized on agarose beads purified from *Escherichia coli* specifically pulled down BAK1–FLAG expressed from protoplasts (Figure 3e), suggesting a direct interaction between BAK1 and HopF2 or AvrPto. Thus, our results not only provide evidence that BAK1 is a target of HopF2, but also confirm our previous finding that AvrPto and AvrPtoB interact with BAK1 (Shan *et al.*, 2008).

HopF2 interacts with BAK1 via transmembrane and kinase domains

BAK1 consists of an extracellular LRR domain, a single transmembrane domain, a juxtamembrane domain and a kinase domain (Li *et al.*, 2002; Nam and Li, 2002). Using a yeast split-ubiquitin assay and co-immunoprecipitation assay, we previously reported that BAK1's transmembrane and kinase domains (BAK1TJK) are required for its interaction with AvrPto (Shan *et al.*, 2008). Similar to AvrPto, HopF2 immunoprecipitated with BAK1TJK in protoplasts co-transfected with HopF2–HA and BAK1TJK–FLAG (Figure 4a). In addition, GST–AvrPto or GST–HopF2 fusion proteins pulled down BAK1TJK–FLAG expressed from protoplasts (Figure 4b). These data suggest that HopF2 associates with BAK1 via the transmembrane domain and the kinase domain. The data are consistent with the observation that HopF2 functions inside plant cells, and

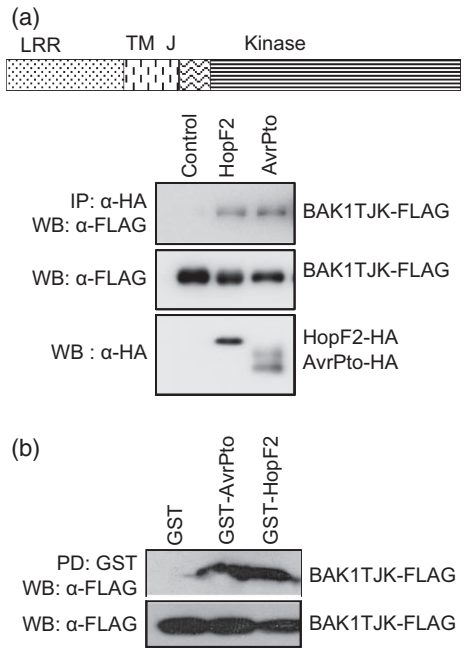

Figure 4. The transmembrane, juxtamembrane and kinase domains of BAK1 (BAK1TJK) are sufficient for BAK1–HopF2 or BAK1–AvrPto interaction.
(a) HopF2 and AvrPto interact with BAK1TJK in Arabidopsis protoplasts. The α–HA co–IP was performed using protoplasts co-expressing FLAG-tagged BAK1TJK with AvrPto–HA or HopF2–HA, and the immunoprecipitated proteins were analyzed by Western blotting using α–FLAG antibody. LRR, leucine-rich repeat; TM, transmembrane; J, juxtamembrane.
(b) HopF2 and AvrPto interact with BAK1TJK in a pull-down assay. The pull-down assay was performed using GST, GST–AvrPto and GST–HopF2 proteins to bind total proteins from protoplasts expressing BAK1TJK–FLAG.

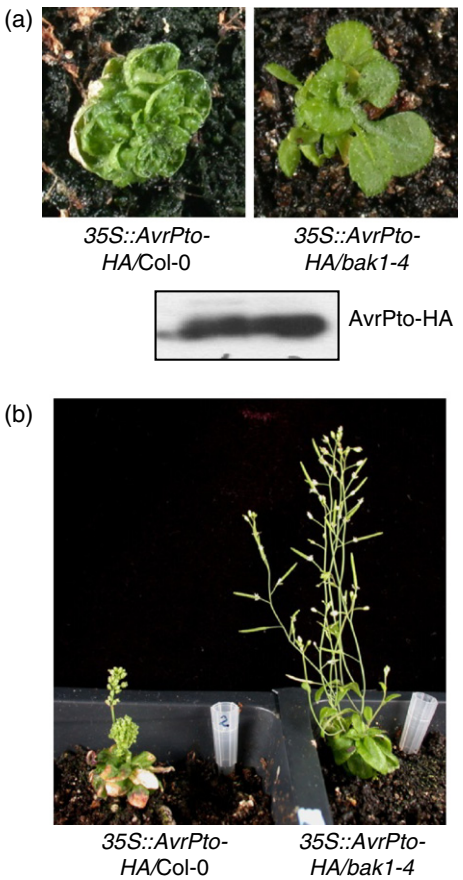

Figure 5. The toxicity of ectopic expression of *AvrPto*is alleviated in *bak1–4* mutant plants.
(a) Phenotype of 4-week-old *35S::AvrPto-HA*/Col–0 and *35S::AvrPto-HA*/*bak1–4* transgenic plants. The expression of AvrPto protein was demonstrated by α–HA Western blotting.
(b) Phenotype of 10-week-old *35S::AvrPto-HA*/Col-0 and *35S::AvrPto-HA*/*bak1-4* transgenic plants.

the plasma membrane localization is critical for its function in suppressing flg22-mediated signaling.

BAK1 is a physiological target of AvrPto, AvrPtoB and HopF2

In addition to the above biochemical analyses, we also examined the dependence of AvrPto, AvrPtoB and HopF2 growth perturbation on BAK1 in transgenic plants. The strong growth perturbation by AvrPto prevented generation of viable transgenic plants expressing Avr-Pto under the control of the constitutive 35S promoter in the Col-0 (WT) background (Shan *et al.*, 2008). The occasionally surviving transgenic plants with detectable AvrPto protein expression showed dwarfed stature with small, round, thick leaves, short petioles and short inflorescences (Figure 5a,b) (Shan *et al.*, 2008). We never obtained viable seeds from *35S::AvrPto-HA* transgenic plants in the Col–0 background. A similar phenotype was observed when we transformed the *35S::AvrPto-HA* construct into *fls2* mutant plants. Interestingly, when the same *35S::AvrPto-HA* construct was transformed into the *bak1–4* null mutant, several transgenic lines with detectable AvrPto–HA protein expression were obtained and displayed considerably alleviated growth defects compared with transgenic plants in the WT Col–0 background. The *35S::AvrPto-HA* transgenic plants in the *bak1–4* mutant background had longer petioles, bigger leaves and longer inflorescences with viable seeds compared with *35S::AvrPto-HA* transgenic plants in the Col–0 background (Figure 5a,b). These physiological and genetic data suggest that BAK1 is avirulence target of AvrPto, and strongly support our previous observation that AvrPto targets BAK1 to suppress PTI signaling (Shan *et al.*, 2008).

Previously, we and others found that AvrPto associates with FLS2 (Shan *et al.*, 2008; Xiang *et al.*, 2008). We also transformed *35S::AvrPto-HA* into the Arabidopsis Ws–0 ecotype, which carries a natural mutation in the *FLS2* gene (Gomez-Gomez *et al.*, 1999). However, unlike *bak1–4* plants, we did not obtain any viable transgenic plants with detectable AvrPto expression in the Ws–0 background. In contrast, many transgenic plants with strong and constitutive AvrPto expression were obtained in the *bak1–1* mutant

background (*bak1-1* mutant is in the Ws–0 background). Significantly, multiple lines of *35S::AvrPto-HA* transgenic plants in the *bak1–1* mutant background were almost indistinguishable from *bak1–1* mutant plants at early developmental stages (Figure 6a). The *35S::AvrPto-HA* transgenic plants in the *bak1–1* mutant background displayed moderately reduced stem length and apical dominance at later developmental stages compared with the *bak1–1* mutant (Figure S2a). The observation that transgenic *bak1–1* and *bak1–4* mutant lines over-expressing AvrPto displayed ameliorated growth defects supports the view that BAK1 is a physiological target of AvrPto. Similarly, we never obtained any viable *35S::AvrPtoB-HA* or *35S::HopF2-HA* transgenic plants in the Col–0 or Ws–0 background with detectable protein expression, whereas many *35S::AvrPtoB-HA* or *35S::HopF2-HA* transgenic plants in the *bak1–1* background survived and set seed (Figures 6b,c and S2b, c), showing that BAK1 is also a physiological target of AvrPtoB and HopF2, consistent with our biochemical data on direct BAK1–AvrPtoB and BAK1–HopF2 interactions.

DISCUSSION

To achieve infections, successful pathogens have evolved deliberate virulence mechanisms to suppress host immunity and interfere with host physiological responses. The *P. syringae* type III effector HopF2 is injected into plant cells and blocks immune responses triggered by multiple MAMPs (Wu *et al.*, 2011). Here we show that HopF2 directly interacts with the plasma membrane-resident RLK BAK1 – a signaling partner of multiple MAMP receptors (Figure 7). This conclusion was supported by our comprehensive co–IP, BiFC and pull-down assays. The rapid heterodimerization of BAK1 with various MAMP receptors, including FLS2, EFR and PEPR1/2, constitutes an initial step in PTI signaling (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Postel *et al.*, 2010; Schulze *et al.*, 2010; Roux *et al.*, 2011). By targeting BAK1, HopF2 suppresses diverse early signaling events triggered by multiple MAMPs, including BIK1 phosphorylation, MAPK activation and immune gene expression. Our data are also consistent with the requirement for membrane localization for HopF2 virulence activity (Shan *et al.*, 2004; Robert-Seilaniantz *et al.*, 2006; Wu *et al.*, 2011). Interestingly, HopF2 possesses distinct mechanisms for suppression of two branches of MAMP-activated MAPK cascades. In addition to direct blocking of MKK5 in the MEKK1/MEKKs–MKK4/5–MPK3/6 cascade, HopF2 also functions at the plasma membrane and targets BAK1 upstream of the MEKK1–MKK1/2–MPK4, cascade as well as BIK1 and its homologs. In this study, we also confirmed our previous finding that BAK1 interacts with AvrPto and AvrPtoB using co–IP, BiFC and pull-down assays (Shan *et al.*, 2008). Importantly, the strong growth defects associated with the AvrPto transgene in WT Arabidopsis plants were largely alleviated in *bak1* mutant plants, providing genetic and physiological evidence that BAK1 is a virulence target of AvrPto. Thus, BAK1 is a virulence target of three sequence-distinct bacterial effectors: AvrPto, AvrPtoB and HopF2.

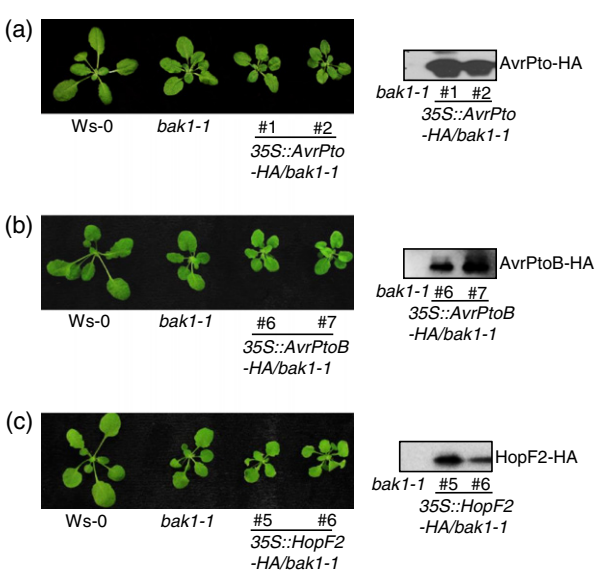

Figure 6. Toxicity of ectopic expression of *AvrPto*, *AvrPtoB* or *HopF2* is alleviated in *bak1–1* mutant plants.
Phenotype of 4-week-old Ws–0, *bak1–1*, *35S::AvrPto-HA/bak1–1* (a), *35S::AvrPtoB-HA/bak1–1* (b) and *35S::HopF2-HA/bak1–1* (c) plants. The expression of corresponding effector proteins was demonstrated by α–HA Western blotting.

BIK1 is rapidly phosphorylated upon flg22 perception, and is directly phosphorylated by BAK1 (Lu *et al.*, 2010a; Zhang *et al.*, 2010). Consistently, flg22-induced BIK1 phosphorylation depends on BAK1 (Lu *et al.*, 2010a). Although the detailed mechanisms remain elusive, the current model suggests that BIK1, together with its homologs, functions upstream or independently of MAPK cascades in flagellin signaling (Lu *et al.*, 2010a; Zhang *et al.*, 2010). Recently, it has been shown that a rice BIK1 homolog, OsRLCK185, acts upstream of MAPK cascades in chitin- and peptidoglycan-induced plant immunity (Yamaguchi *et al.*, 2013). Genetic analyses also indicate that the RLCK SSP (short suspensor) acts upstream of the YDA (MAPK kinase kinase/MEKK)–MPK3/6 cascade in the embryonic patterning process (Bayer *et al.*, 2009). Nevertheless, HopF2 suppression of flg22-induced phosphorylation of BIK1 and its homologs suggests that HopF2 targets an immediate early step in flagellin signaling. Importantly, the HopF2 virulence function is associated with its suppression of BIK1 phosphorylation. Notably, HopF2 did not interact with BIK1 or affect BIK1 *in vitro* kinase activity (Wu *et al.*, 2011). All these observations are consistent with HopF2 targeting BAK1, which functions upstream of BIK1.

It has been reported that HopF2 targets MKK5 and probably other MKKs to block flg22-triggered signaling (Wang *et al.*, 2010). The HopF2 homolog from *P. syringae* pv.

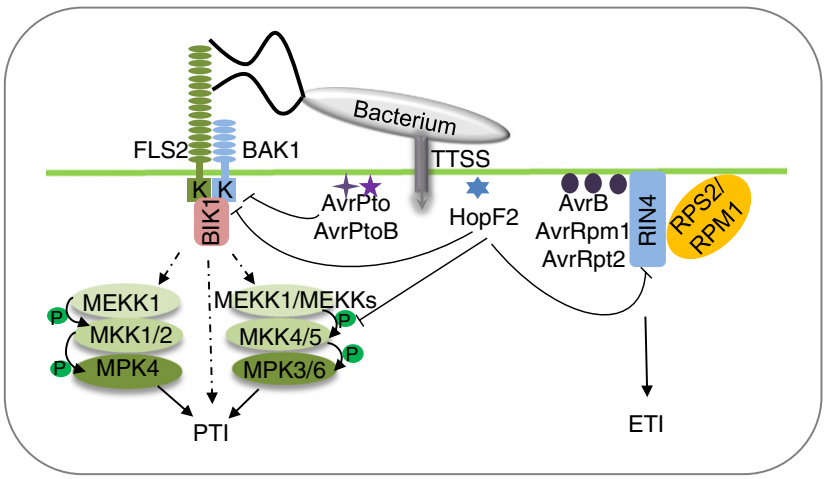

Figure 7. Model of the multiple host targets of HopF2.
Plant innate immunity includes pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Perception of bacterial flagellin by FLS2 activates phosphorylation of the FLS2/BAK1/BIK1 complex and two branches of the MAPK cascade (MEKK1–MKK1/2–MPK4 and MEKK1/MEKKs–MKK4/5–MPK3/6) in PTI signaling. The bacterial type III secretion system (TTSS) effectors AvrB, AvrRpm1 and AvrRpt2 modify host RIN4 protein, which is sensed by corresponding RPM1 and RPS2 proteins to activate ETI signaling. Bacterial effector proteins have the ability to suppress both PTI and ETI signaling. AvrPto and AvrPtoB target BAK1 to suppress PTI signaling. HopF2 suppresses PTI signaling by targeting BAK1 and MKK5, and suppresses ETI signaling by targeting RIN4.

phaseolicola, HopF1 (AvrPphF), was shown to possess marginal structural similarity to the catalytic domain of bacterial diphtheria toxin, an ADP-ribosyltransferase, although no ADP-ribosyltransferase activity was detected (Singer *et al.*, 2004). Wang *et al.* (2010) reported that HopF2 directly ADP-ribosylates MKK5 and blocks MKK5 kinase activity *in vitro*. The complex MAPK signaling plays pivotal roles in transmitting MAMP signaling (Tena *et al.*, 2011; Meng and Zhang, 2013). Two parallel MAPK cascades consisting of MEKK1/MEKKs–MKK4/5–MPK3/6 and MEKK1–MKK1/2–MPK4 have been proposed to function downstream of the MAMP receptor complex. Intriguingly, HopF2 did not directly interfere with MKK1 and MKK2 activity. Furthermore, HopF2 did not interfere with MEKK1-mediated MPK4 activation. Suppressing MKK5 but not MKK1/2 activity did not explain the fact that HopF2 suppresses flg22-induced MPK4 activation. Thus, HopF2 probably has an additional target upstream of the MEKK1–MKK1/2–MPK4 cascade. The identification of plasma membrane-resident BAK1 as a HopF2 target is consistent with these observations.

BAK1 is a multifunctional protein that plays important roles in plant growth and cell death control, in addition to plant immunity (Chinchilla *et al.*, 2009). BAK1 was originally isolated as an interacting protein of the plant growth hormone BR receptor BRI1, and plays positive roles in BR signaling (Li *et al.*, 2002; Nam and Li, 2002). Moreover, BAK1 was found to negatively regulate cell death, and double mutant plants of *bak1* and its closest homolog *bak1-like 1* (*bkk1*) exhibit a seedling lethality phenotype and constitutive defense responses (He *et al.*, 2007a). It is interesting that loss of BAK1 but not FLS2 alleviates the toxicity induced by ectopic over-expression of AvrPto, AvrPtoB or HopF2 in both the Col–0 and Ws–0 backgrounds. Plants with simultaneous loss of BAK1 and FLS2 (in the *bak1–1* mutant) largely tolerate over-expression of these three effectors, suggesting that this alleviation of toxicity may not be simply due to loss of BAK1's functions in immune signaling. It is possible that the toxicity caused by ectopic expression of these three effectors may be due to the modification of BAK1 that perturbs its functions in plant development and/or cell death control. Loss of BAK1 makes these three effectors less effective in suppression of plant growth and development.

In addition, HopF2 has also been found to directly interact with Arabidopsis RIN4, an important component in both PTI and ETI responses (Wilton *et al.*, 2010). Three additional *P. syringae* type III effectors (AvrRpt2, AvrRpm1 and AvrB) directly target and modify RIN4, which is sensed by the corresponding NB–LRR receptors RPS2 and RPM1, which initiate ETI signaling (Mackey *et al.*, 2002, 2003; Axtell and Staskawicz, 2003). HopF2 suppresses cleavage of RIN4 by AvrRpt2, thereby inhibiting AvrRpt2-mediated ETI responses (Wilton *et al.*, 2010). Thus, HopF2 suppresses both PTI and ETI responses by targeting BAK1/MKK5 and RIN4, respectively (Figure 7). This is consistent with the emerging theme that a single effector protein is able to target multiple host proteins to suppress innate immune signaling at multiple steps (Mukhtar *et al.*, 2011). We have previously shown that AvrPto and AvrPtoB suppress early defense signaling triggered by multiple MAMPs upstream of MAPK cascades, and proposed that AvrPto and AvrPtoB may target cell surface-resident RLKs that initiate MAMP signaling (He *et al.*, 2006). We further reported that AvrPto and AvrPtoB interacted with BAK1 and other RLKs, including FLS2, to block the initiation of MAMP signaling (Shan *et al.*, 2008). Biochemical and crystal structural analysis of the AvrPtoB–BAK1 complex indicates that $AvrPtoB_{250–359}$ is sufficient for BAK1 interaction (Cheng *et al.*, 2011). In this study, we demonstrated HopF2–BAK1, AvrPto–BAK1 and AvrPtoB–BAK1 interactions in transient assays and transgenic plants *in vivo* using co–IP and BiFC assays and *in vitro* using a GST pull-down assay. More importantly, the various growth defects associated with

constitutive expression of HopF2, AvrPto and AvrPtoB in Arabidopsis WT plants are significantly alleviated in the *bak1* mutant plants. These results collectively indicate that HopF2, AvrPto and AvrPtoB suppress MAMP-triggered signaling by targeting BAK1 as part of their virulence functions. Therefore, it appears that it is not uncommon that multiple structurally distinct effectors may target the same host protein to suppress host defense. Similar to the findings that AvrRpt2, AvrRpm1 and AvrB target the same host protein RIN4, our results from previous work and this study demonstrate that AvrPto, AvrPtoB and HopF2 all target BAK1 (Figure 7). We also predicted that additional targets exist, as AvrPto and AvrPtoB also suppress BAK1-independent immune signaling. Indeed, AvrPtoB is able to target Arabidopsis and the tomato chitin receptor CERK1 to promote bacterial virulence (Gimenez-Ibanez *et al.*, 2009; Zeng *et al.*, 2012). Thus AvrPto and AvrPtoB probably target multiple RLKs to impede plant immune signaling. It is possible that pathogenic bacteria have evolved a strategy to facilitate the infection by targeting the key components in plant immunity using multiple virulence factors. This is also consistent with the fact that only minute amounts of individual effectors are delivered into host cells, and multiple effectors may function synergistically or in a specific hierarchy to exhibit virulence activity.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *bak1-1* (Ws–0 background) and *bak1–4* (Col–0 background) mutants have been described previously (Li *et al.*, 2002; Lu *et al.*, 2010a). Arabidopsis plants were grown in soil (Metro Mix 360, Sun Gro Horticulture, http://www.sungro.com) in a growth chamber at 23°C, 65% relative humidity, 75 μE m^{-2} sec^{-1} light, and with a 12 h photoperiod for 4 weeks before protoplast isolation. To grow Arabidopsis seedlings, the seeds were surface-sterilized with 50% bleach for 15 min, and then placed on plates containing half-strength Murashige and Skoog medium with 0.5% sucrose, 0.8% agar and 2.5 mm MES [2-(N-Morpholino)ethanesulfonic acid, 4-Morpholineethanesulfonic acid monohydrate] at pH 5.7. The plates were first stored at 4°C for 3 days in the dark for seed stratification, and then moved to the growth chamber.

Plasmid construction and generation of transgenic plants

The constructs for *HopF2*, *AvrPto*, *AvrPtoB*, *MAPKs*, *MKK*, *MEKK1*, *BIK1* and *BAK1* in plant expression vectors or protein expression vectors have been described previously (He *et al.*, 2006; Shan *et al.*, 2008; Lu *et al.*, 2010a; Wu *et al.*, 2011). *BAK1*, *AvrPto* and *HopF2* were sub-cloned into modified BiFC vectors by *Bam*HI and *Stu*I digestion. The *AvrPto*, *AvrPtoB* or *HopF2* transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation in Col–0, Ws–0, *bak1–1* or *bak1–4* plants with the corresponding construct under the control of a constitutive CaMV 35S promoter with an HA epitope tag. The DEX-inducible *AvrPto-HA* and *HopF2-HA* transgenic plants have been described previously (He *et al.*, 2006; Wu *et al.*, 2011).Transgenic plants carrying both DEX-inducible *HopF2-HA* and *pBAK1::BAK1-GFP* were generated by transforming the *HopF2* construct into *pBAK1::BAK1-GFP* transgenic plants. Transgenic plants carrying both DEX-inducible *AvrPto-HA* and *pBAK1::BAK1-GFP* have been described previously (Shan *et al.*, 2008). The transgenic plants were confirmed by Western blotting using α–HA or α–GFP antibody.

Co-immunoprecipitation (co–IP) assay

Protoplast isolation and transfection were performed as described previously (He *et al.*, 2007b). For the protoplast co–IP assay, total proteins from 2×10^5 transfected protoplasts were isolated using 0.5 ml extraction buffer [10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X–100, 1× protease inhibitor cocktail (Roche, http://www.roche.com/)]. The samples were vortexed vigorously for 30 sec, and then centrifuged at 12 000 ***g*** for 10 min at 4°C. The supernatant was incubated with α–HA antibody for 2 h, and was further incubated with agarose beads for another 2 h at 4°C with gentle shaking. The beads were collected and washed three times in 1.5 ml tubes at 4°C with washing buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X–100, 1× protease inhibitor cocktail) and once more with 50 mM Tris/HCl pH 7.5, 1 min per wash. Bound protein was released from beads by boiling in SDS–PAGE sample loading buffer, and analyzed by Western blotting using an α–FLAG antibody.

For co–IP assay in plants, 7-day-old seedlings grown on plates containing half-strength Murashige and Skoog medium were treated with 5 μM DEX overnight to induce HopF2 or AvrPto expression, and were then ground with liquid nitrogen. The total proteins from 50 seedlings were isolated using 1 ml extraction buffer. The samples were centrifuged twice at 12 000 ***g*** for 10 min at 4°C to remove cell debris. The supernatant was subjected into an α–GFP co–IP assay, and the immunoprecipitated proteins were analyzed by Western blotting using α–HA antibody.

GST pull-down assay

GST, GST–AvrPto and GST–HopF2 were individually expressed in the *E. coli* BL21 strain and purified using standard glutathione agarose. Protoplasts (2×10^5) were transfected with the full-length or truncated version of the BAK1 construct tagged with an FLAG epitope at its C–terminus. Total proteins were isolated using 0.5 ml extraction buffer. The samples were vortexed vigorously for 30 sec, and then centrifuged at 12 000 ***g*** for 10 min at 4°C. The supernatant was inoculated with pre-washed GST or GST-tagged protein for 2 h at 4°C with gentle shaking. The beads were collected by centrifugation at 1000 ***g*** for 1 min at 4°C and washed three times with washing buffer and once with 50 mM Tris/HCl pH 7.5. Bound protein was released from beads by boiling in SDS–PAGE sample loading buffer, and analyzed by Western blotting using α–FLAG antibody.

BiFC assay

Protoplast isolation and transfection were performed as described previously (He *et al.*, 2007b). Arabidopsis protoplasts were co-transfected with various BiFC constructs as shown in the figures. The fluorescence signal was visualized under a confocal microscope (Leica Microsystems, http://www.leica-microsystems.com/) 18 h after transfection. The following filter sets were used for excitation and emission: GFP, 488 nm (excitation)/Band Pass (BP)505–530 nm (emission); chlorophyll, 543 nm (excitation)/Long Pass (LP)650 nm (emission); bright field, 633 nm. Images were captured in multichannel mode, and were analyzed and processed using Leica LAS AF Life and Adobe Photoshop (Adobe Systems, http://www.adobe.com/).

Immunocomplex kinase assays

Protoplasts (2×10^5) transfected with various DNA constructs were lysed using 0.5 ml IP buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 2 mM NaF, 2 mM Na_3VO_3, 1% Triton X-100, and 1× protease inhibitor cocktail). The samples were vortexed vigorously for 30 sec, and then centrifuged at 13 000 rpm for 10 min at 4°C. The supernatant was incubated with α–HA antibody for 2 h, and then with protein G–agarose beads for another 2 h at 4°C with gentle shaking. The beads were harvested and washed once with IP buffer and once with kinase buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 100 mM NaCl, 1 mM dithiothreitol). The kinase reactions were performed in 20 μl kinase buffer using 2 μg myelin basic protein as a substrate, 0.1 mM cold ATP, and 5 μCi [^{32}P]-γ–ATP at room temperature for 1 h with gentle shaking. The phosphorylation of proteins was analyzed by 12% SDS–PAGE.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. HopF2 and AvrPto interact with BAK1 in Arabidopsis protoplasts.

Figure S2. The toxicity of ectopic expression of *AvrPto*, *AvrPtoB* and *HopF2* is alleviated in *bak1–1* mutant plants.

REFERENCES

Axtell, M.J. and Staskawicz, B.J. (2003) Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. *Cell*, **112**, 369–377.

Barton, G.M. and Medzhitov, R. (2003) Toll-like receptor signaling pathways. *Science*, **300**, 1524–1525.

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T. and Lukowitz, W. (2009) Paternal control of embryonic patterning in *Arabidopsis thaliana*. *Science*, **323**, 1485–1488.

Beck, M., Zhou, J., Faulkner, C., MacLean, D. and Robatzek, S. (2012) Spatio-temporal cellular dynamics of the Arabidopsis flagellin receptor reveal activation status-dependent endosomal sorting. *Plant Cell*, **24**, 4205–4219.

Block, A., Li, G., Fu, Z.Q. and Alfano, J.R. (2008) Phytopathogen type III effector weaponry and their plant targets. *Curr. Opin. Plant Biol.* **11**, 396–403.

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.

Cao, Y., Aceti, D.J., Sabat, G., Song, J., Makino, S., Fox, B.G. and Bent, A.F. (2013) Mutations in FLS2 Ser938 dissect signaling activation in FLS2-mediated Arabidopsis immunity. *PLoS Pathog.* **9**, e1003313.

Cheng, W., Munkvold, K.R., Gao, H.S., Mathieu, J., Schwizer, S., Wang, S., Yan, Y.B., Wang, J.J., Martin, G.B. and Chai, J.J. (2011) Structural analysis of *Pseudomonas syringae* AvrPtoB bound to host BAK1 reveals two similar kinase-interacting domains in a type III effector. *Cell Host Microbe*, **10**, 616–626.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, 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.

Chinchilla, D., Shan, L., He, P., de Vries, S. and Kemmerling, B. (2009) One for all: the receptor-associated kinase BAK1. *Trends Plant Sci.* **14**, 535–541.

Cui, F., Wu, S., Sun, W., Coaker, G., Kunkel, B., He, P. and Shan, L. (2013) The *Pseudomonas syringae* type III effector AvrRpt2 promotes pathogen virulence via stimulating Arabidopsis auxin/indole acetic acid protein turnover. *Plant Physiol.* **162**, 1018–1029.

Fu, Z.Q., Guo, M., Jeong, B.R., Tian, F., Elthon, T.E., Cerny, R.L., Staiger, D. and Alfano, J.R. (2007) A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. *Nature*, **447**, 284–288.

Gimenez-Ibanez, S., Hann, D.R., Ntoukakis, V., Petutschnig, E., Lipka, V. and Rathjen, J.P. (2009) AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. *Curr. Biol.* **19**, 423–429.

Gohre, V. and Robatzek, S. (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu. Rev. Phytopathol.* **46**, 189–215.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W. and Robatzek, S. (2008) Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* **18**, 1824–1832.

Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18**, 277–284.

Hann, D.R., Gimenez-Ibanez, S. and Rathjen, J.P. (2010) Bacterial virulence effectors and their activities. *Curr. Opin. Plant Biol.* **13**, 388–393.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T. and Sheen, J. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. *Cell*, **125**, 563–575.

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

He, P., Shan, L. and Sheen, J. (2007b) The use of protoplasts to study innate immune responses. *Methods Mol. Biol.* **354**, 1–9.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C. and Rathjen, J.P. (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl Acad. Sci. USA*, **104**, 12217–12222.

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

Kinoshita, T., Cano-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S. and Chory, J. (2005) Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature*, **433**, 167–171.

Lewis, J.D., Guttman, D.S. and Desveaux, D. (2009) The targeting of plant cellular systems by injected type III effector proteins. *Semin. Cell Dev. Biol.* **20**, 1055–1063.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E. and Walker, J.C. (2002) BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell*, **110**, 213–222.

Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, Q., Tian, X. and Zhou, J.M. (2013) BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proc. Natl Acad. Sci. USA*, **110**, 6205–6210.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010a) 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.

Lu, D., Wu, S., He, P. and Shan, L. (2010b) Phosphorylation of receptor-like cytoplasmic kinases by bacterial flagellin. *Plant Signal. Behav.* **5**, 598–600.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T.P., He, P. and Shan, L. (2011) Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science*, **332**, 1439–1442.

Mackey, D., Holt, B.F. 3rd, Wiig, A. and Dangl, J.L. (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell*, **108**, 743–754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L. (2003) Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*, **112**, 379–389.

Meng, X. and Zhang, S. (2013) MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* **51**, 245–266.

Mukhtar, M.S., Carvunis, A.R., Dreze, M. *et al.* *(2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. Science*, **333**, 596–601.

Nam, K.H. and Li, J. (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell*, **110**, 203–212.

Nicaise, V., Joe, A., Jeong, B.R., Korneli, C., Boutrot, F., Westedt, I., Staiger, D., Alfano, J.R. and Zipfel, C. (2013) *Pseudomonas* HopU1 modulates plant immune receptor levels by blocking the interaction of their mRNAs with GRP7. *EMBO J.* **32**, 701–712.

Nomura, K., Debroy, S., Lee, Y.H., Pumplin, N., Jones, J. and He, S.Y. (2006) A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science*, **313**, 220–223.

Nomura, K., Mecey, C., Lee, Y.N., Imboden, L.A., Chang, J.H. and He, S.Y. (2011) Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **108**, 10774–10779.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* **198**, 249–266.

Postel, S., Kufner, I., Beuter, C., Mazzotta, S., Schwedt, A., Borlotti, A., Halter, T., Kemmerling, B. and Nurnberger, T. (2010) The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. *Eur. J. Cell Biol.* **89**, 169–174.

Preston, G.M. (2000) *Pseudomonas syringae* pv. *tomato*: the right pathogen, of the right plant, at the right time. *Mol. Plant Pathol.* **1**, 263–275.

Robatzek, S., Chinchilla, D. and Boller, T. (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes Dev.* **20**, 537–542.

Robert-Seilaniantz, A., Shan, L., Zhou, J.M. and Tang, X. (2006) The *Pseudomonas syringae* pv. *tomato* DC3000 type III effector HopF2 has a putative myristoylation site required for its avirulence and virulence functions. *Mol. Plant–Microbe Interact.* **19**, 130–138.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F.G., Tor, M., de Vries, S. and Zipfel, C. (2011) The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell*, **23**, 2440–2455.

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.

Schwessinger, B. and Ronald, P.C. (2012) Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* **63**, 451–482.

Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M. and Tang, X. (2000) The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell*, **12**, 2323–2338.

Shan, L., Oh, H.S., Chen, J., Guo, M., Zhou, J., Alfano, J.R., Collmer, A., Jia, X. and Tang, X. (2004) The *HopPtoF* locus of *Pseudomonas syringae* pv. *tomato* DC3000 encodes a type III chaperone and a cognate effector. *Mol. Plant Microbe Interact.* **17**, 447–455.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nurnberger, T., Martin, G.B. and Sheen, J. (2008) Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe*, **4**, 17–27.

Singer, A.U., Desveaux, D., Betts, L., Chang, J.H., Nimchuk, Z., Grant, S.R., Dangl, J.L. and Sondek, J. (2004) Crystal structures of the type III effector protein AvrPphF and its chaperone reveal residues required for plant pathogenesis. *Structure*, **12**, 1669–1681.

Speth, E.B., Lee, Y.N. and He, S.Y. (2007) Pathogen virulence factors as molecular probes of basic plant cellular functions. *Curr. Opin. Plant Biol.* **10**, 580–586.

Tena, G., Boudsocq, M. and Sheen, J. (2011) Protein kinase signaling networks in plant innate immunity. *Curr. Opin. Plant Biol.* **14**, 519–529.

de Torres, M., Mansfield, J.W., Grabov, N., Brown, I.R., Ammouneh, H., Tsiamis, G., Forsyth, A., Robatzek, S., Grant, M. and Boch, J. (2006) *Pseudomonas syringae* effector AvrPtoB suppresses basal defence in Arabidopsis. *Plant J.* **47**, 368–382.

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X. and Zhou, J.M. (2010) A *Pseudomonas syringae* ADP-ribosyltransferase inhibits Arabidopsis mitogen-activated protein kinase kinases. *Plant Cell*, **22**, 2033–2044.

Wilton, M., Subramaniam, R., Elmore, J., Felsensteiner, C., Coaker, G. and Desveaux, D. (2010) The type III effector HopF2Pto targets Arabidopsis RIN4 protein to promote *Pseudomonas syringae* virulence. *Proc. Natl Acad. Sci. USA*, **107**, 2349–2354.

Wu, S., Lu, D., Kabbage, M., Wei, H.L., Swingle, B., Records, A.R., Dickman, M., He, P. and Shan, L. (2011) Bacterial effector HopF2 suppresses Arabidopsis innate immunity at the plasma membrane. *Mol. Plant Microbe Interact.* **24**, 585–593.

Xiang, T., Zong, N., Zou, Y. *et al.* *(2008) Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr. Biol.* **18**, 74–80.

Yamaguchi, K., Yamada, K., Ishikawa, K. *et al.* *(2013) A receptor-like cytoplasmic kinase targeted by a plant pathogen effector is directly phosphorylated by the chitin receptor and mediates rice immunity. Cell Host Microbe*, **13**, 347–357.

Zeng, L., Velasquez, A.C., Munkvold, K.R., Zhang, J. and Martin, G.B. (2012) A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB. *Plant J.* **69**, 92–103.

Zhang, J., Shao, F., Li, Y. *et al.* *(2007) A Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe*, **1**, 175–185.

Zhang, J., Li, W., Xiang, T. *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.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J. and Zhang, Y. (2012) Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB–LRR protein SUMM2. *Cell Host Microbe*, **11**, 253–263.

