BACTERIAL EFFECTOR HOPF2 SUPPRESSES ARABIDOPSIS IMMUNITY BY TARGETING BAK1

A Thesis

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ABSTRACT

Pseudomonas syringae delivers a plethora of effector proteins into host cells to sabotage host immune responses and physiology to favor infection. We have previously shown that *P. syringae pv. tomato* DC3000 effector HopF2 suppresses *Arabidopsis* innate immunity triggered by multiple pathogen-associated molecular patterns (PAMP) at the plasma membrane. We show here that HopF2 possesses distinct mechanisms in the suppression of two branches of PAMP-activated MAP kinase cascades. In contrast to blocking MKK5 in MEKK1-MKK4/5-MPK3/6 cascade, HopF2 targets additional component(s) upstream of MEKK1 in MEKK1-MKK1/2-MPK4 cascade and 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 involved in multiple PAMP signaling. The interaction between BAK1 and HopF2 or two additional *P. syringae* effectors AvrPto and AvrPtoB, was confirmed in vivo and in vitro. Consistent with BAK1 as a physiological target of HopF2, the lethality of overexpression of HopF2 in wild-type *Arabidopsis* transgenic plants was largely alleviated in bak1 mutant plants. Identification of BAK1 as an additional HopF2 virulence target not only explains HopF2 suppression of multiple PAMP signaling at the plasma membrane, but also supports the notion that pathogen virulence effectors have multiple targets in host cells.

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NOMENCLATURE

BiFC	bimolecular fluorescence complementation
BR	brassinosteroids
CDPK	Ca^{2+}-dependent protein kinase
Co-IP	co-immunoprecipitation
DEX	dexmethithone
EF-Tu	elongation factor Tu
ETI	effector-triggered immunity
FLS2	flagellin-sensing 2
GFP	green fluorescence protein
GST	glutathione-S-transferase
HA	hemagglutinin
HrpZ	harpin Z
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MKK5	MAPK kinase 5
NB-LRR	nucleotide binding domain leucine-rich repeat
NPP1	necrosis-inducing *Phytophthora* protein 1
PAMP	pathogen-associated molecular pattern
PGN	peptidoglycan

PTI	PAMP-triggered immunity
RLK	receptor-like kinase
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TTSS	type III secretion system
WT	wild-type
½ MS	half-strength Murashige and Skoog medium

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

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) (Boller and Felix, 2009; Jones and Dangl, 2006). PAMP-triggered immunity (PTI) plays a pivotal role in defense against a broad spectrum of potential pathogens (Boller and Felix, 2009; Jones and Dangl, 2006). A 22-amino-acid peptide from N-terminus of bacterial flagellin, flg22, can be perceived by *Arabidopsis* RLK flagellin-sensing 2 (FLS2), and induces FLS2 association with another plasma membrane-localized RLK BAK1 (Chinchilla et al., 2007). BAK1 was originally isolated as a RLK interacting with plant growth hormone brassinosteroid (BR) receptor BRI1. BAK1 with a relatively short extracellular leucine-rich repeat (LRR) domain is not involved in flagellin nor BR perception (Chinchilla et al., 2007). Notably, BAK1 is required for signaling triggered by multiple PAMPs, including bacterial elongation factor Tu (EF-Tu), flagellin, harpin Z (HrpZ), lipopolysaccharide (LPS), peptidoglycan (PGN), 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). In addition to FLS2, BAK1 has been shown to hetero-dimerize with EFR, a RLK for EF-Tu and PEPR1/2, a RLK for plant endogenous signal Pep1/2. BAK1 is able to directly phosphorylate a plasma membrane-localized receptor-

like cytoplasmic kinase BIK1. In non-elicited cells, BIK1 interacts with BAK1, FLS2, EFR, and PEPR1/2 (Lu et al., 2010; Zhang et al., 2010). Flg22 induces rapid phosphorylation of BIK1 which further transphosphorylates FLS2-BAK1, and dissociation of BIK1 from FLS2-BAK1 complex (Cao et al., 2013; Lu et al., 2010). As a step toward attenuation of the immune responses, flg22 induced FLS2 endocytosis in vesicles within ~30 minutes, leading to FLS2 degradation. Protein ubiquitination often directs target proteins for degradation through the proteasome or vacuole, or mediates receptor intracellular sorting in endosomes to other destinations. We found that FLS2 is a target of plant U-box containing E3 ubiquitin ligases PUB13 and PUB12. BAK1 phosphorylates PUB12/13 upon flg22 elicitation. Subsequently, PUB12/13 ubiquitinates FLS2, thus targeting it for degradation (Lu et al., 2011). Despite the diverse PAMPs and their corresponding receptor kinase complexes, PTI elicitation leads to often largely overlapping responses in different plant species, 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).

Successful pathogens 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 different plant species and is also a model system 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 (TTSS), phytotoxins and exopolysaccharides (Buell et al., 2003). In particular, *P. syringae* delivers around 30 type III effectors into plant cells through TTSS, and many of these effectors target important host components to sabotage plant immunity (Block et al., 2008; Gohre and Robatzek, 2008; Hann et al., 2010; Lewis et al., 2009; Speth et al., 2007). The *P. syringae* effector HopU1 is a mono-ADP-ribosyltransferase (ADP-RT) and targets several *Arabidopsis* RNA-binding proteins including GRP7 (Fu et al., 2007). Interestingly, GRP7 interacts with both translational components and PAMP receptors FLS2 and EFR, suggesting its role in plant immunity (Nicaise et al., 2013). In addition, GRP7 directly binds to FLS2 and EFR transcripts, and this binding was blocked by HopU1 to modulate FLS2 protein level (Nicaise et al., 2013). Two sequence-distinct effectors, AvrPto and AvrPtoB, are potent suppressors of multiple PAMP signaling by targeting RLKs, including BAK1 and FLS2 (de Torres et al., 2006; Gimenez-Ibanez et al., 2009; Gohre and Robatzek, 2008; He et al., 2006; Shan et al., 2008; Xiang et al., 2008). AvrPtoB possesses an E3 ubiquitin ligase activity and targets certain RLKs including FLS2 and CERK1 for degradation (Gimenez-Ibanez et al., 2009; Gohre et al., 2008). Other *Pseudomonas* effectors target components downstream of PAMP receptor complexes. For example, HopAI targets MPK3, MPK4 and MPK6 to disrupt MAP kinase activation upon PAMP perception (Zhang et al., 2007; Zhang et al., 2012). Interestingly, inactivation of MPK4 by HopAI1 activates nucleotide binding leucine-rich repeat (NB-LRR) protein SUMM2-mediated defense responses (Zhang et al., 2012).

HopQ1 is phosphorylated and associated with tomato 14-3-3 proteins, which regulates its subcellular dynamics (Giska et al., 2013). Effector-suppression of PTI signaling could also be overcome by NB-LRR mediated effector-triggered immunity (ETI). HopM1 targets and degrades a member of the ARF family of guanine nucleotide exchange factors, including AtMIN7, 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).

We previously reported a *P. syringae* pv *tomato* DC3000 effector, HopF2, suppresses *Arabidopsis* innate immunity at the plasma membrane (Wu et al., 2011). Similar with AvrPto, HopF2 also possesses a putative myristoylation modification motif which is required for its plasma membrane-localization and virulence activity in *Arabidopsis*, tobacco and tomato (Robert-Seilaniantz et al., 2006; Shan et al., 2000; Wu et al., 2011). HopF2 could suppress immune responses triggered by multiple PAMPs, including flg22, elf18, LPS, PGN, HrpZ and chitin (Guo et al., 2009). Structural analysis of HopF2 homolog AvrPphF from *P. syringae* pv. *phaseolicola* has identified several conserved surface-exposed residues, and mutational analysis indicates that the corresponding residues in HopF2 are required for its PAMP suppression activity (Jackson et al., 1999; Shan et al., 2004; Wang et al., 2010a; 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 to downstream MPK3/6 through its ADP-

ribosyltransferase activity (Wang et al., 2010a). Interestingly, HopF2 suppresses flg22-induced BIK1 phosphorylation, an event likely acts upstream or independently of MAPK cascades in flg22 signaling. HopF2 did not directly interact with BIK1 nor affected BIK1 kinase activity (Wu et al., 2011), suggesting that HopF2 targets additional host proteins upstream of BIK1 in flg22 signaling. We extended this study and report here that HopF2 blocks flg22-induced phosphorylation of two BIK1 homologs, PBS1 and PBL1, and HopF2 virulence is associated with its suppression of BIK1 phosphorylation. Consistent with its suppression upstream of BIK1, HopF2 did not affect MPK4 activation by MKK1/2 and MEKK1. Importantly, HopF2 directly interacts with BAK1 *in vivo* and *in vitro* in an FLS2-independent manner. Overexpression of HopF2 causes severe lethality in *Arabidopsis* wild-type (WT) plants likely due to its strong virulence. Significantly, HopF2-caused lethality is dramatically reduced in *bak1* mutant plants, further supporting that BAK1 is a physiological target of HopF2.

CHAPTER II

RESULTS

HopF2 virulence is associated with its suppression of BIK1 phosphorylation

Flg22-induced BIK1 phosphorylation is evidenced by a mobility shift on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Lu et al., 2010). The mobility shift of HA epitope tagged BIK1 is blocked by co-expression of green fluorescence protein (GFP) tagged HopF2 in *Arabidopsis* protoplasts, suggesting that HopF2 suppresses flg22-induced BIK1 phosphorylation (Fig. 1A, (Wu et al., 2011)). It has been reported that several BIK1 homologs, including PBL1 and PBS1, are also quickly phosphorylated upon flg22 treatment (Fig. 1A and (Zhang et al., 2010)). Interestingly, HopF2 also blocks flg22-induced phosphorylation of PBL1 and PBS1 (Fig. 1A). BIK1 and PBS1 are plasma membrane-associated RLCKs. Thus, our data are consistent with that HopF2 functions at the plant plasma membrane through a putative myristoylation modification and the myristoylation motif is required for its virulence functions (Robert-Seilaniantz et al., 2006). These data suggest that HopF2 suppresses flg22-mediated signaling at an immediate early step upstream of BIK1 phosphorylation in the FLS2/BAK1 receptor complex.

Structure analysis of HopF1 (AvrPphF) from *P. syringae* pv *phaseolicola,* a homolog of HopF2, identified several conserved surface-exposed residues which are required for its virulence and avirulence functions in beans (Singer et al., 2004). The corresponding residues in HopF2 are essential for its suppression of flg22-induced

expression of *pFRK1::LUC* (the FRK1 promoter fused with luciferase) (Wu et al., 2011). In particular, HopF2 R71A and D175A mutants completely lost the ability to suppress flg22-induced *pFRK1::LUC* activation (Fig. S1 and (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 presence of various HopF2 mutants. Significantly, HopF2 R71A and D175A mutants failed to suppress flg22-induced BIK1 phosphorylation (Fig.1B). Consistent with their suppression actions on flg22-induced p*FRK1::LUC* and MAPK activation (Fig. S1 and (Wang et al., 2010a; Wu et al., 2011)), the HopF2 S89A, H96A and E97A mutants had little or no effect on its suppression of flg22-induced BIK1 phosphorylation. 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 early signaling events following PAMP recognition in both plants and animals (Asai et al., 2002; Barton and Medzhitov, 2003; Nurnberger et al., 2004). Accumulating evidence suggests that perception of flg22 activates two branches of MAPK cascades in *Arabidopsis*, MEKK1-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4 (Fig. 2A, (Tena et al., 2011)). It has been reported that HopF2 directly targets and blocks MKK5 function, thereby suppressing downstream MPK3 and MPK6 activation (Wang et al., 2010a). In addition, HopF2 is able to suppress flg22-induced

MPK4 activation which is mediated through MEKK1 and MKK1/2 cascade (Wu et al., 2011). Surprisingly, HopF2 did not directly affect MKK1 and MKK2 activity (Fig. 2B). As shown in Fig. 2B, the constitutively active form of Myc epitope tagged MKK1 and MKK2 (MKK1ac-Myc, MKK2ac-Myc) activated MPK4-HA in *Arabidopsis* protoplasts with an immuno-complex kinase assay. Expression of HopF2 did not affect activation of MKK1ac or MKK2ac on MPK4 (Fig. 2B). Furthermore, HopF2 did not interfere with the MEKK1-mediated activation of MPK4 (Fig. 2C). The data suggest that HopF2 suppresses flg22-induced MPK4 activation upstream of MEKK1-MKK1/2, which is consistent with HopF2 suppression of flg22-induced BIK1 phosphorylation. In agreement with the previous report, HopF2 functions on MKK5 to suppress MPK3 activation. As shown in Fig. 2D, expression of HopF2 diminished active MKK5ac-mediated MPK3 activity (Fig. 2D). Thus, in addition to MKK5, HopF2 also targets additional component(s) upstream of MEKK1 and BIK1, probably immediately after flagellin perception by FLS2/BAK1 receptor complex.

HopF2 interacts with BAK1

HopF2 suppresses *pFRK1::LUC* activation by multiple PAMPs, including elf18, PGN, LPS and HrpZ. Since BAK1 is involved in signaling activated by multiple PAMPs, we tested whether HopF2 might directly interact with BAK1. Interestingly, similar with AvrPto and AvrPtoB, HopF2 co-immunoprecipitated with BAK1 in *Arabidopsis* wild-type protoplasts (Fig. 3A). The association between BAK1 and HopF2, AvrPto or AvrPtoB was also detected in *fls2* mutant protoplasts, indicating that this

association is independent of FLS2 (Fig. 3A). To further confirm the *in vivo* association of HopF2 and BAK1 in intact plants, we transformed the HA-tagged *HopF2* under the control of dexmethithone (DEX) inducible promoter (*pDEX::HopF2-HA*) into the *pBAK1::BAK1-GFP* transgenic plants. HopF2-HA co-immunoprecipitated BAK1-GFP as detected with α-HA antibody upon α-GFP antibody immunoprecipitation (Fig. 3B). In addition, AvrPto-HA also co-immunoprecipitated with BAK1-GFP in transgenic plants carrying *pDEX::AvrPto-HA* and *pBAK1::BAK1-GFP* as detected with α-HA antibody upon α-GFP antibody immunoprecipitation. Consistently, bimolecular fluorescence complementation (BiFC) assay also indicated the *in vivo* association of HopF2 and BAK1 (Fig. 3D). The fluorescence signal was detected when HopF2-nYFP (the amino-terminal part of YFP fused with HopF2) was co-expressed with BAK1-cYFP (the carboxy-terminal part of YFP fused with BAK1) (Fig. 3D). Similarly, the *in vivo* AvrPto and BAK1 association was observed with co-transfection of BAK1-cYFP and AvrPto-nYFP in protoplasts and onion epidermal cells (Fig. 3D and Fig. 2S). None of the individual constructs emitted fluorescence signals in protoplasts or in onion epidermal cells. Furthermore, HopF2 or AvrPto protein fused to glutathione-S-transferase (GST) immobilized on agarose beads purified from *E.coli* specifically pulled down BAK1-FLAG expressed from protoplasts (Fig. 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 verify our previous finding that AvrPto and AvrPtoB interact with BAK1.

HopF2 interacts with BAK1 via transmembrane and kinase domain

BAK1 consists of an extracellular 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 with AvrPto, HopF2 immuno-precipitated with BAK1TJK in protoplasts co-transfected with HopF2-HA and BAK1TJK-FLAG (Fig. 4D). In addition, GST-AvrPto or GST-HopF2 fusion proteins also pulled down BAK1TJK-FLAG expressed from protoplasts (Fig. 4B). These data suggested that HopF2 associates with BAK1 via the transmembrane domain and kinase domain. The data are consistent with the observation that HopF2 functions inside plant cells, and the plasma membrane localization is critical for its function to suppress flg22 signaling.

Virulence of HopF2 and AvrPto/AvrPtoB are additive

It has been reported that HopF2, AvrPto and AvrPtoB suppress plant immunity, and AvrPto and AvrPtoB show additive virulence activity (He et al., 2006; Wu et al., 2011). It is unclear whether HopF2 has additive virulence with AvrPto/AvrPtoB. We carried out a bacterial growth assay to test the virulence of DC3000 deletion mutants. Three days after inoculation on Col-0 plants, population of *ΔAvrPtoΔAvrPtoB* double mutant was 10-fold lower than that of DC3000, whereas the bacterial number of *ΔAvrPtoΔAvrPtoBΔHopF2* triple mutant was reduced by 100-fold (Fig. 5A). Compared

with plants infected with DC3000 double mutant, plants infected withDC3000 *ΔAvrPtoΔAvrPtoBΔHopF2* triple mutant showed reduced disease symptoms with less chlorosis and necrosis (fig. 5B). The data indicate that the virulence of HopF2/AvrPto/AvrPtoB are additive.

BAK1 is a physiological target of HopF2

In addition to the above biochemical analyses, we also tested whether HopF2 virulence depends on BAK1 in transgenic plants. We transformed *35S::HopF2-HA* into *Arabidopsis* WS ecotype, and we did not obtain any viable transgenic plants with detectable HopF2 expression. The strong virulence of HopF2 prevented the generation of viable transgenic plants carrying HopF2 under the control of constitutive 35S promoter in WS plants. Interestingly, when the same *35S::HopF2-HA* construct was transformed into *bak1-1* mutant (a BAK1 null mutant), several transgenic lines with detectable HopF2-HA protein expression have been obtained (Fig. 6). These physiological and genetic data suggest that BAK1 is the virulence target for HopF2 and strongly support our observation that HopF2 targets to BAK1 in planta.

CHAPTER III

MATERIALS AND METHODS

Plant materials and growth conditions

The *bak1-1* (the BAK1 null mutant in the WS background lacking a functional FLS2) and wild-type WS *Arabidopsis* were reported previously (Li et al., 2002; Lu et al., 2010). *Arabidopsis* plants were grown in soil (Metro Mix 360) in a growth chamber at 23 °C, 60% relative humidity, 75 μE m^{-2} s^{-1} light and with a 12-hr 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 the plates with half-strength Murashige and Skoog medium (½ MS) containing 0.5% sucrose, 0.8% agar and 2.5 mm MES at pH 5.7. The plates were first stored at 4 ℃ 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 of *HopF2, AvrPto, AvrPtoB, MAPKs, MEKK1, BIK1* and *BAK1* in plant expression vector or protein expression vector were previously reported (He et al., 2006; Lu et al., 2010; Shan et al., 2008; Wu et al., 2011). The constructs of *PBL1* and *PBS1* were made by cloning PCR fragments from *Arabidopsis* cDNA library into a plant expression vector with a HA tag at the C terminus. The constitutively active forms of *MKKs* were generated by site-specific mutagenesis replacing the threonine or serine residues in the activation loop domains and were previously reported (Asai et al., 2002).

BAK1, AvrPto and *HopF2* were sub-cloned into the modified BiFC vectors (a kind gift from Dr. F. Rolland) with BamHI and StuI digestion. The *HopF2* transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation of WS or *bak1-1* with the construct of *HopF2* under the control of a constitutive cauliflower mosaic virus *35S* promoter with an HA tag. Dexamethasone (DEX)-inducible *AvrPto*-HA and *HopF2-HA* transgenic plants were previously reported (He et al., 2006; Wu et al., 2011). Transgenic plants carrying both DEX-inducible *HopF2-HA* and *BAK1::BAK1-GFP* were generated by transforming *HopF2* construct into the *BAK1::BAK1-GFP* transgenic plants. Transgenic plants carrying both DEX-inducible *AvrPto-HA* and *BAK1::BAK1-GFP* were previously reported (Shan et al., 2008). The transgenic plants were confirmed by western blot with an α-HA or α-GFP antibody.

Pathogen assay

Pseudomonas syringae different strains used in this study include *P.s.* pv tomato DC3000, DC3000 Δ*AvrPto*Δ*AvrPtoB*, and DC3000 Δ*AvrPto*Δ*AvrPtoB*Δ*HopF2* deletion mutant, that were provided by Dr. Hai-Lei Wei at Cornell University.

Different *P. syringae* strains were grown at 28 ℃ for overnight in King's B (KB) medium with antibiotic rifamycin (50 mg/L). Bacteria were collected by centrifugation, washed, and diluted to the density of 5 x 10^5 cell/ml with 10 mM $MgCl_2$. *Arabidopsis* leaves were inoculated with bacteria with a needleless syringe for bacterial counting. To measure bacterial numbers, two leaf disks were harvested with a cork borer and ground in 100 μL H_2O, and a series of bacterial dilutions were plated on KB medium with the

appropriate antibiotic. Bacterial colony forming units recovered on plates were enumerated after 2 days.

Co-immunoprecipitation (co-IP) assay

Protoplasts isolation and transfection were performed as described (He et al., 2007). For the co-IP assay in protoplasts, the total proteins from 2 x 10^5 transfected protoplasts were isolated with 0.5 ml of extraction buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 1 x protease inhibitor cocktail from Roche). The samples were vortexed vigorously for 30 s, and then centrifuged at 13,000 rpm for 10 min at 4 ℃. The supernatant was inoculated with an α-HA antibody for 2 hr and was further inoculated with agarose beads for another 2hr, at 4 ℃ with gentle shaking. The beads were collected and washed three times with washing buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, and 1 X protease inhibitor cocktail) and once more 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 blot with an α-FLAG antibody.

For the co-IP assay in plants, 7-day-old seedlings grown on ½ MS medium plates were treated with 5 μM DEX for overnight to induce HopF2 and AvrPto expression and were then ground with liquid nitrogen. The total proteins from 50 seedlings were isolated with 1 mL of extraction buffer. The samples were centrifuged twice at 13,000 rpm for 10 min at 4 ℃ to completely remove cell debris. The supernatant was subjected into an α-GFP

co-IP assay and the immunoprecipitated proteins were analyzed by western blot with an α-HA antibody.

GST pull-down assay

GST, GST-AvrPto and GST-HopF2 were individually expressed in *Escherichia coli* BL21 strain and purified with glutathione agarose with standard protocol. 2 x 10^5 protoplasts were transfected with full length or truncated version of BAK1 construct tagged with FLAG epitope at its C-terminus. The total proteins were isolated with 0.5 ml of extraction buffer. The samples were vortexed vigorously for 30 s, and then centrifuged at 13,000 rpm for 10 min at 4 ℃. The supernatant was inoculated with prewashed GST or GST-tagged protein for 2 hr at 4 ℃ with gentle shaking. The beads were harvested and washed 3 times with washing buffer and once with 50 mM Tris-HCl pH7.5. Bound protein was released from beads by boiling in SDS-PAGE sample loading buffer and analyzed by western blot with an α-FLAG antibody.

BiFC assay

Arabidopsis protoplasts were co-transfected with various BiFC constructs. Complementation of fluorescence signal was visualized under a confocal microscope (Leica Microsystems CMS GmbH) 18 hr after the transfection. Following are the filter sets used for excitation (Ex) and emission (Em): GFP, 488 nm (Ex)/BP505 nm to 530 nm (Em); chlorophyll, 543 nm (Ex)/LP650 nm (Em); bright field, 633 nm. Images were

captured in a multichannel mode, and were analyzed and processed with Leica LAS AF Life and Adobe Photoshop (Adobe Systems).

Immunocomplex kinase assays

2×10^5 protoplasts transfected with various DNA constructs were lysed with 0.5 ml of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_3, 1% Triton, and protease inhibitor cocktail). The samples were vortexed vigorously for 30 s, and then centrifuged at 13,000 rpm for 10 min at 4 ℃. The supernatant was incubated with an α-HA antibody for 2 hr and then with protein-G–agarose beads for another 2 hr at 4 ℃ 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 MgCl2, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT). The kinase reactions were performed in 20 μl of kinase buffer with 2 μg of myelin basic protein as a substrate, 0.1 mM cold ATP, and 5 μCi of [^{32}P]-γ-ATP at room temperature for 1 hr with gentle shaking. The phosphorylation of proteins was analyzed by 12% SDS-PAGE.

FRK1 reporter assay

UBQ10-GUS, as an internal control, was co-transfected with *FRK1-LUC* and effector constructs, and the promoter activity was presented as the ratio of LUC/GUS. Protoplasts were collected 6 h after transfection for promoter activity assays. Protoplasts transfected with plasmid DNA without *HopF2* serve as controls.

CHAPTER IV

SUMMARY AND 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 PAMPs. Here we show that HopF2 directly interacts with plasma membrane-resident RLK BAK1-a signaling partner of multiple PAMP receptors. This conclusion was supported by our comprehensive Co-IP, BiFC and pull-down assays. The rapid heterodimerization of BAK1 with different PAMP receptors, including FLS2, EFR, and PEPR1/2, constitutes an initial step in PTI signaling. By targeting BAK1, our data explain the observations that HopF2 suppresses diverse early signaling events triggered by multiple PAMPs, including BIK1 phosphorylation, MAPK activation and immune gene expression. Our data are also consistent with that membrane localization is required for HopF2 virulence activity. Interestingly, HopF2 possesses distinct mechanisms in the suppression of two branches of PAMP-activated MAPK cascades. In contrast to direct blocking MKK5 in MEKK1-MKK4/5-MPK3/6 cascade, HopF2 functions at the plasma membrane and targets BAK1 upstream of MEKK1 in MEKK1-MKK1/2-MPK4 cascade, and BIK1 and its homologs. In this study, we also confirmed our previous finding that BAK1 interacts with AvrPto and AvrPtoB with Co-IP, BiFC and pull-down assays. Importantly, the severe lethality associated with *HopF2* transgene in WT *Arabidopsis* plants were largely alleviated in *bak1* mutant plants,

providing genetic and physiological evidence of BAK1 as a virulence target of HopF2. Thus, BAK1 is a virulence target of three sequence distinct bacterial effectors AvrPto, AvrPtoB and HopF2 (Fig. 7).

BIK1 is rapidly phosphorylated upon flg22 treatment and is directly phosphorylated by BAK1 (Lu et al., 2010; Zhang et al., 2010). Consistently, flg22-induced BIK1 phosphorylation depends on BAK1. Although it remains elusive how BIK1 regulates plant immune signaling, the current model suggests that BIK1 functions upstream or independent of MAPK cascades in flagellin signaling. It has been reported that *bik1pbl1* mutants did not affect flg22-induced MAPK activation, suggesting that BIK1 may function independent of MAPK cascade. However, the functional redundancy of BIK1 homologs may complicate this assay. Recently, it has been shown that a rice BIK1 homolog OsRLCK185 acts upstream of MAPK cascade in chitin- and peptidoglycan-induced plant immunity (Yamaguchi et al., 2013). Genetic analyses also indicate that RLCK SSP (Short suspensor) acts upstream of YDA (a MAPK kinase kinase)-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 BIK1 targets an immediate early step in flagellin signaling. Importantly, HopF2 virulence function is associated with its suppression of BIK1 phosphorylation. Interestingly, HopF2 did not interact with BIK1 or affect BIK1 *in vitro* kinase activity. All these observations are consistent with HopF2 targeting BAK1, which functions upstream of BIK1.

It has been reported that HopF2 targets MKK5 and likely other MKKs to block flg22-triggered signaling. The HopF2 homolog, HopF1 (AvrPphF) from *P. s.* pv *phaseolicola* has been shown to possess a marginal structural similarity to the catalytic domain of bacterial diphtheria toxin, an ADP-ribosyltransferase (Singer et al., 2004). Despite various efforts, there are no reports for any detectable ADP-ribosyltransferase activities assigned to HopF1. Wang et al., reported that HopF2 directly ADP-ribosylates MKK5 and blocks MKK5 kinase activity *in vitro* (Wang et al., 2010b). The complex MAPK signaling plays pivotal roles in transmitting PAMP signaling (Meng and Zhang, 2013; Shan et al., 2007). Two parallel MAPK cascades consisting of MKKK-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4 have been proposed to function downstream of PAMP receptor complex. Intriguingly, HopF2 did not directly interfere with MKK1 and MKK2 activity (Fig. 2B). Furthermore, HopF2 did not interfere with MEKK1-mediated MPK4 activation (Fig. 2C). Suppressing MKK5 but not MKK1/2 activity could not explain HopF2's suppression of flg22-induced MPK4 activation. Thus, HopF2 likely has additional target upstream of MEKK1-MKK1/2-MPK4 cascade. The identification of plasma membrane resident BAK1 as HopF2 target is consistent with these observations.

It appears that multiple seemingly distinct effectors could target to the same host protein.We found here that AvrPto, AvrPtoB and HopF2 target BAK1. It is possible that pathogenic bacteria have evolved the strategy to secure the infection by targeting the key components in plant immunity with multiple virulence factors. This is also consistent with that only minute amount of individual effector is delivered into host cells and multiple effectors may function synergistically or in a specific hierarchy to exhibit

virulence activity. Notably, while appreciating the wealth information obtained from the study of novel enzymatic activities and host targets of pathogen effectors, we should interpret the results with caution since a large body of research on effector functions is based on effector overexpression and *in vitro* biochemical assays. It is likely that the secretion process and biological activities of different effectors are well coordinated and temporally regulated. The understanding of precise functions of individual effectors demands the future technology advance, such as single-molecule imaging of *in vivo* host-microbe infection process in the context of other effectors and host cellular dynamics.

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APPENDIX

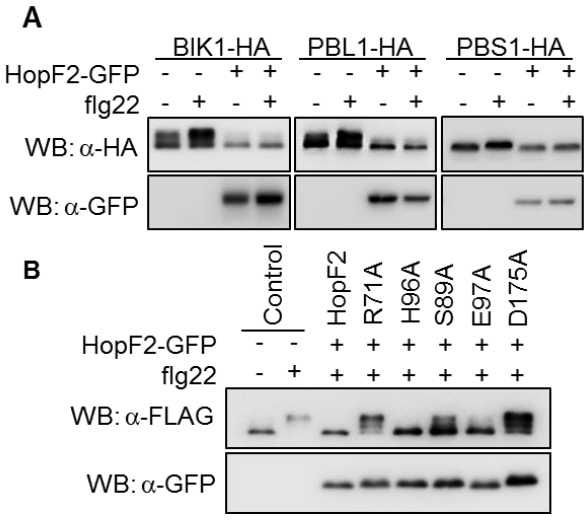

Figure 1. HopF2 suppresses flg22-induced phosphorylation of BIK1 and homologs.

(A) HopF2 blocks flg22-induced mobility shift of BIK1 and homologs. *Arabidopsis* protoplasts were co-transfected with hemagglutinin (HA)-tagged BIK1 or its homologs PBL1, PBS1 and green fluorescent protein (GFP)-tagged HopF2 for 10 hr and were treated with 1 μM flg22 for 10 min. **(B)** Conserved surface residues of HopF2 are required for its suppression function of flg22-induced BIK1 phosphorylation. Protoplasts were co-transfected with FLAG-tagged BIK1 and HA-tagged wild type HopF2 or its mutants R71A, H96A, S89A, E97A or D175A, and treated with flg22 as in (A).

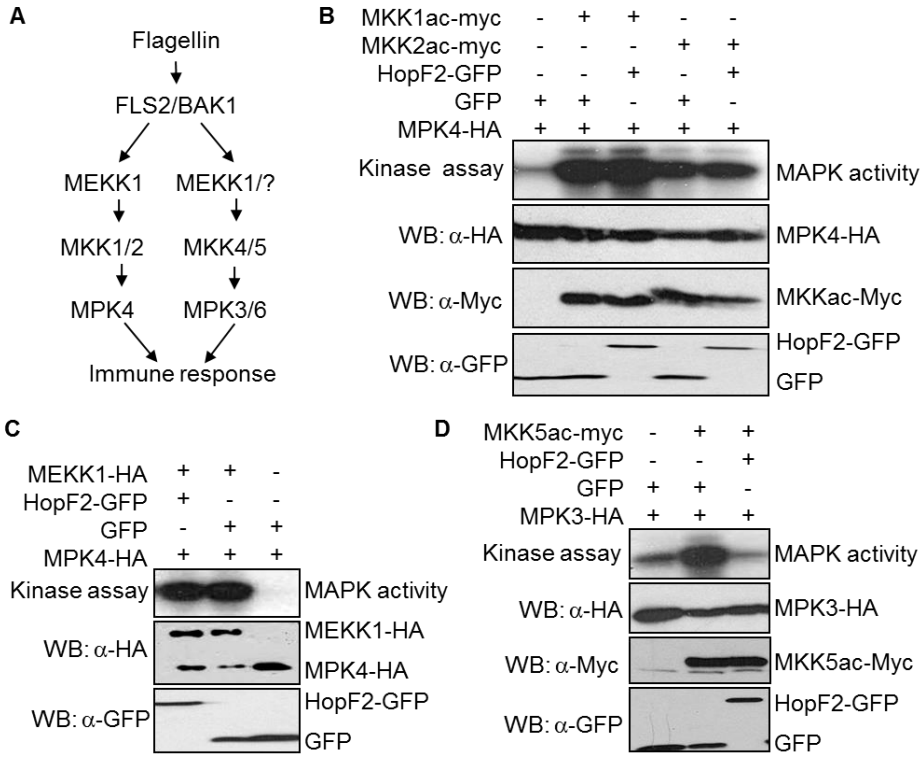

Figure 2. HopF2 targets additional component upstream of MAPK cascade.

(A) Flagellin activates two *Arabidopsis* MAPK cascades. **(B)** HopF2 does not suppress MKK1- or MKK2-mediated MPK4 activation. *Arabidopsis* protoplasts were co-transfected with 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 by α-HA antibody and subjected into an immunocomplex kinase assay using myelin basic protein as a substrate. **(C)** HopF2 does not suppress MEKK1-mediated MPK4 activation. HA-tagged MEKK1 (MEKK1-HA) was cotransfected with MPK4-HA and HopF2-GFP, and MPK4-HA kinase activity was detected in an immunocomplex kinase assay as in (B). **(D)** HopF2 suppresses MKK5-mediated MPK3 activation. Myc-tagged constitutively active form of MKK5 (MKK5ac-

myc) was cotransfected with MPK3-HA and HopF2-GFP, and MPK3-HA kinase activity was detected in an immunocomplex kinase assay as in (B).

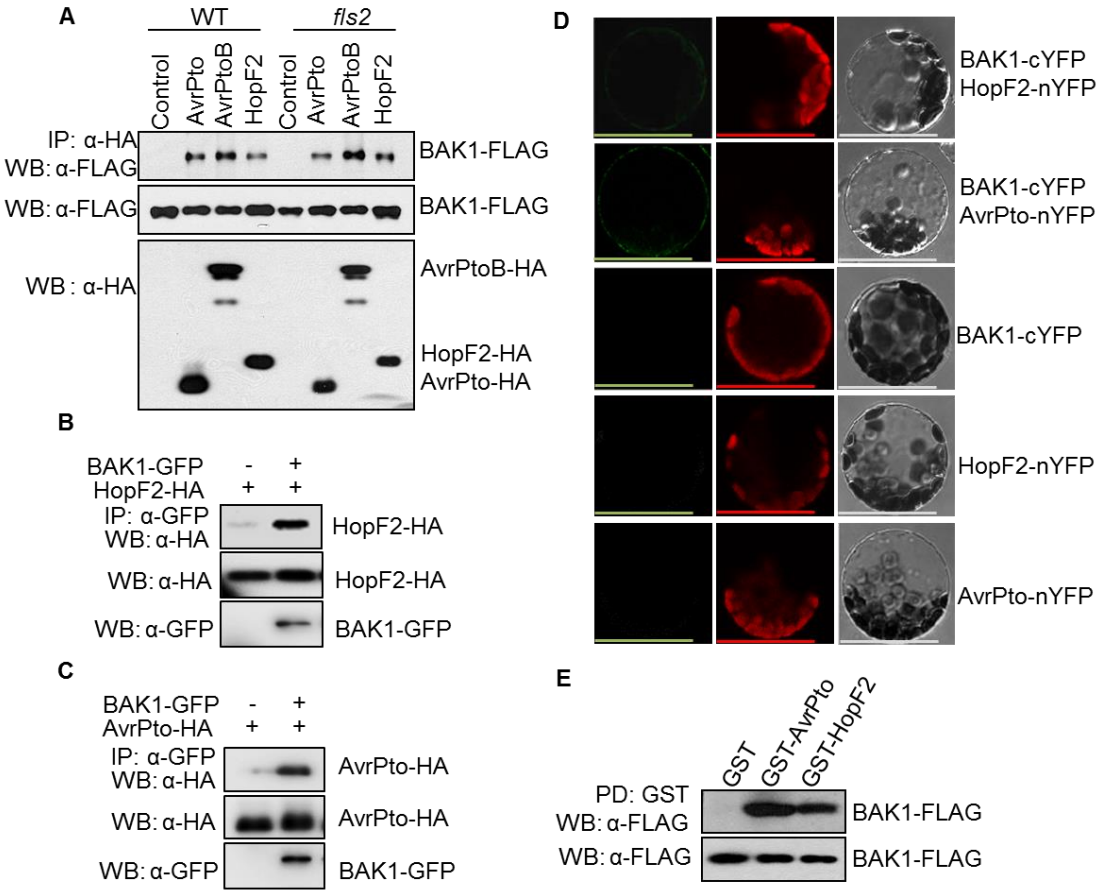

Figure 3. HopF2 and AvrPto interact with BAK1.

(A) HopF2 and AvrPto interact with BAK1 in *Arabidopsis* protoplasts. An α-HA co-IP was performed with protoplasts co-expressing FLAG-tagged BAK1 and HA-tagged AvrPto, AvrPtoB or HopF2, and the immunoprecipitated proteins were analyzed in a western blot with an α-FLAG antibody. **(B)** and **(C)** HopF2 or AvrPto interacts with BAK1 in *Arabidopsis* plants. pBAK1::BAK1-GFP transgenic seedlings with or without DEX-inducible effector transgene were treated with 5 μM DEX for 12 hr and subjected into an α-GFP co-IP assay, and the immunoprecipitated proteins were analyzed in a

western blot with an α-HA antibody. **(D)** The BiFC assays for HopF2-BAK1or AvrPto-BAK1 interactions in *Arabidopsis* protoplasts. The various BiFC constructs were transfected into protoplasts and the fluorescence were visualized under a confocal microscope. Bar=50 μm. **(E)** The pull-down assays for HopF2-BAK1 or AvrPto-BAK1 interactions. GST, GST-AvrPto and GST-HopF2 were expressed individually in *Escherichia coli,* purified with glutathione agarose, and used to pull-down the total proteins from protoplasts expressing FLAG-tagged BAK1. The pull-downed proteins were analyzed in a western blot with an α-FLAG antibody.

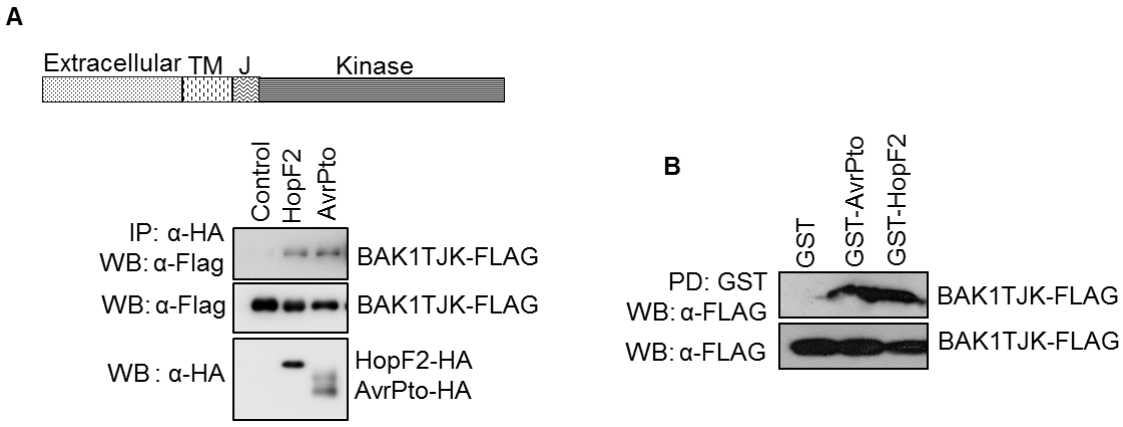

Figure 4. Transmembrane, juxtamembrane and kinase domains of BAK1(BAK1TJK) are enough for the BAK1-HopF2 or BAK1-AvrPto interactions.

(A) An α-HA co-IP was performed with protoplasts co-expressing FLAG-tagged BAK1TJK with or without AvrPto-HA or HopF2-HA, and the immunoprecipitated proteins were analyzed in a western blot with an α-FLAG antibody. **(B)** The pull-down assay was performed by using GST, GST-AvrPto and GST-HopF2 proteins expressed in *E. coli,* purified with glutathione agarose to bind the total proteins from protoplasts expressing BAK1TJK-FLAG. The pull-downed proteins were analyzed in a western blot with an α-FLAG antibody.

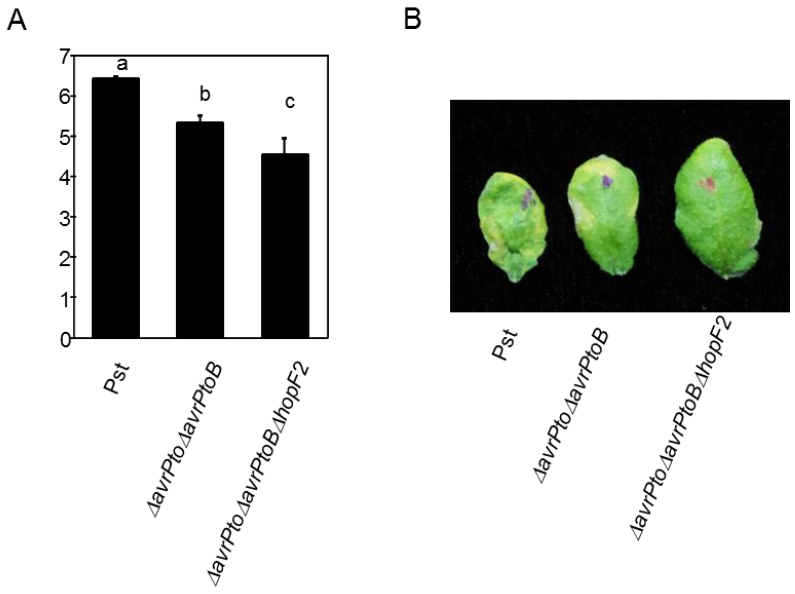

Figure 5. Additive virulence of HopF2, AvrPto and AvrPtoB.

Arabidopsis leaves were inoculated with wild-type or mutant *P.s.* pv tomato DC3000 at 5 x 10^5 CFU/ml. **(A)** The bacterial counting was performed 3 days after inoculation. **(B)** Disease symptoms of 4-week old plants were recorded after 4days of *P.syringae* infection.

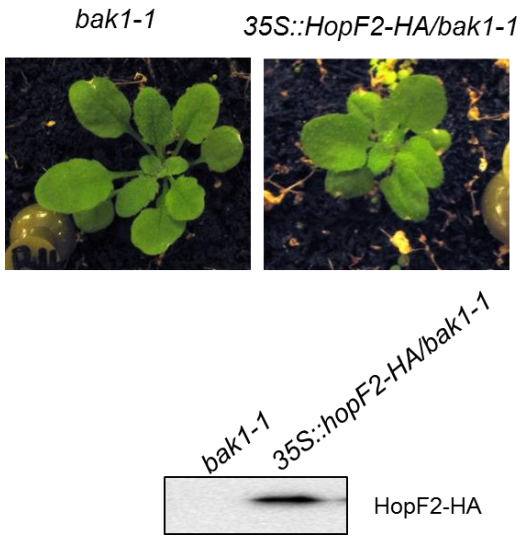

Figure 6. HopF2 virulence depends on BAK1 in transgenic plants.

4-week-old transgenic *Arabidopsis* plants constitutively expressing HA-tagged HopF2 protein under 35S promoter in *bak1-1* (BAK1 null mutant in WS-wild type background) background (*35S::HopF2-HA/bak1-1)*. The expression of HopF2 protein was shown in an α-HA western blot.

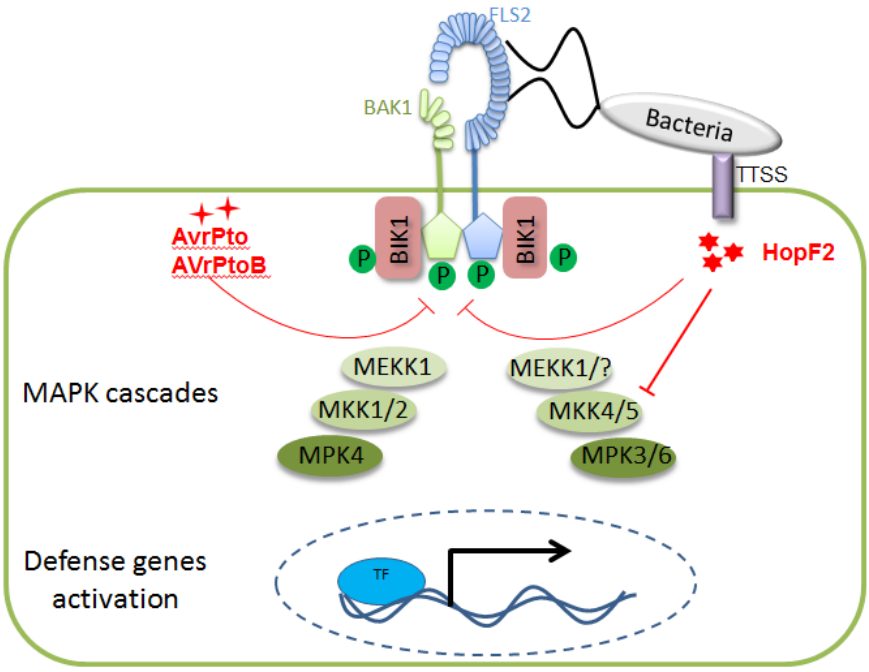

Figure 7. A model of plant targets of effector HopF2 and AvrPto.

Outside of the plasma membrane, recognition of conserved bacterial flagellin by FLS2 triggers immune responses, including activation of mitogen-activated protein kinase (MAPK) cascade. Inside of the plasma membrane, recognition of effector proteins AvrB/AvrRpm1 by RPM1 causes RIN4 phosphorylation leading to activation of RPM1, and recognition of effector protein ArvRpt2 by RPS2 causes RIN4 degradation leading to activation of RPS2. Activated RPM1 and RPS2 trigger effector-triggered immunity (ETI). Bacterial has evolved strategies to attenuate both PTI and ETI. On one side, effector protein AvrPto targets BAK1, effector protein HopF2 targets BAK1 and MKK5 to suppress PTI, on the other side, HopF2 targets RIN4 to suppress ETI.

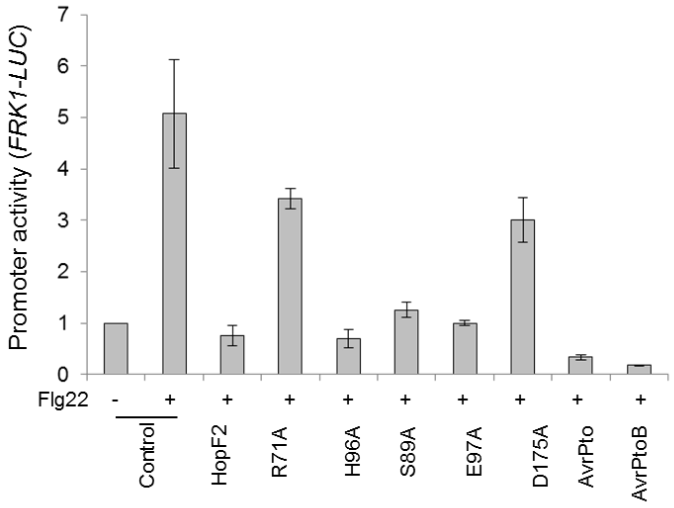

Figure S1. Residues R71 and D175 are essential for HopF2 suppression function of flg22-induced FRK1 promoter activation.

Protoplasts were co-transfected with FRK1-Luciferase reporter and HA-tagged HopF2 or its mutants R71A, H96A, S89A, E97A or D175A for 3 hr and treated with flg22 for another 3 hr.

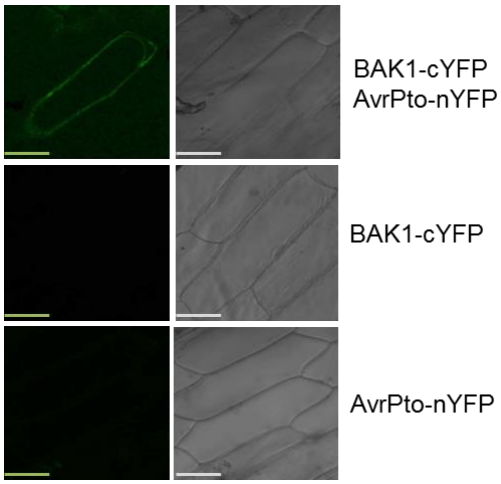

Figure S2. BiFC assay for BAK1-AvrPto interaction.

Onion epidermal cells transiently expressing BAK1-cYFP and AvrPto-nYFP were visualized under a confocal microscope. Bar=50 μm.