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Bacterial Effectors Target the Common Signaling Partner BAK1 to Disrupt Multiple MAMP Receptor-Signaling Complexes and Impede Plant Immunity

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SUMMARY

Successful pathogens have evolved strategies to interfere with host immune systems. For example, the ubiquitous plant pathogen *Pseudomonas syringae* injects two sequence-distinct effectors, AvrPto and AvrPtoB, to intercept convergent innate immune responses stimulated by multiple microbe-associated molecular patterns (MAMPs). However, the direct host targets and precise molecular mechanisms of bacterial effectors remain largely obscure. We show that AvrPto and AvrPtoB bind the *Arabidopsis* receptor-like kinase BAK1, a shared signaling partner of both the flagellin receptor FLS2 and the brassinosteroid receptor BRI1. This targeting interferes with ligand-dependent association of FLS2 with BAK1 during infection. It also impedes BAK1-dependent host immune responses to diverse other MAMPs and brassinosteroid signaling. Significantly, the structural basis of AvrPto-BAK1 interaction appears to be distinct from AvrPto-Pto association required for effector-triggered immunity. These findings uncover a unique strategy of bacterial pathogenesis where virulence effectors block signal transmission through a key common component of multiple MAMP-receptor complexes.

INTRODUCTION

Plants and animals use pattern-recognition receptors (PRRs) to detect pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) and activate the first line of innate-immune responses (Akira et al., 2006; Ausubel, 2005; Chisholm et al., 2006; Jones and Dangl, 2006). The mammalian cell-surface transmembrane PRRs consist of a limited number of Toll-like receptors that are critical for perceiving a diverse range of MAMPs derived from bacteria, fungi, parasites, and viruses (Akira et al., 2006). Plants appear to have evolved a large number of PRRs for recognition of a wide array of MAMPs from both pathogenic and nonpathogenic microbes (He et al., 2007b; Nürnberger et al., 2004; Shiu and Bleecker, 2003; Zipfel and Felix, 2005). There are hundreds of receptor-like kinases (RLKs) in plants (Shiu and Bleecker, 2003), some of which detect MAMPs and launch cascades of immune responses (Gómez-Gómez and Boller, 2000; Zipfel and Felix, 2005; Zipfel et al., 2006). The well-characterized MAMP receptors are the flagellin receptor (Flagellin Sensing 2, FLS2) and the elongation factor EF-Tu receptor (EFR) in *Arabidopsis* (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Affinity-crosslinking assays demonstrated direct binding of flagellin with FLS2 and EF-Tu with EFR, respectively (Chinchilla et al., 2006; Zipfel et al., 2006). In addition to their roles in plant immunity, RLKs are also implicated in plant growth and development, such as CLV1 (CLAVATA1) in controlling meristem size, BRI1 (brassinosteroid insensitive 1) in perceiving plant hormone brassinosteroids (BRs), and ERECTA-family receptors in stomatal patterning (Belkhadir et al., 2006; Clark et al., 1993; Shpak et al., 2005).

The recognition of different MAMPs by specific PRRs induces common-signaling pathways involving MAP-kinase activation and defense-gene transcription (Qutob et al., 2006; Zipfel et al., 2006). It remains unknown how distinct MAMP perception activates the convergent immune responses. It has been shown recently that flagellin could rapidly stimulate the association of FLS2 with another RLK, BAK1, which was originally identified as a BRI1-associated receptor kinase mediating BR signaling (Li et al., 2002; Nam and Li, 2002). However, *bak1*-mutant plants did not reduce flagellin binding, suggesting that BAK1 is not involved in flagellin perception (Chinchilla et al., 2007). Importantly, BAK1 is likely involved in multiple MAMP responses, including flagellin, EF-Tu, bacterial cold-shock protein, and oomycete elicitor INF1 in *Arabidopsis* and *Nicotiana benthamiana* (Chinchilla et al., 2007; Heese et al., 2007). The *bak1*-mutant plants also displayed enhanced susceptibility to some

necrotrophic fungi (Kemmerling et al., 2007). BAK1 appears to function in distinct receptor-signaling complexes to integrate multiple MAMP perception into downstream-signaling events.

Successful pathogens have evolved strategies to interfere with host immune systems. Many Gram-negative bacteria inject a battery of effector proteins through the type III secretion system to promote pathogenesis in plants and animals (Abramovitch et al., 2006; Alfano and Collmer, 2004; Galán, 2007; Grant et al., 2006). Some of these effectors function as enzymes or regulatory mimics to manipulate diverse host cellular activities essential for innate immunity. For instance, virulence effectors from plant and animal bacterial pathogens target evolutionarily conserved MAP-kinase cascade components with different enzymatic activities to impede host immunity (Shan et al., 2007). Two sequence-distinct effectors, AvrPto and AvrPtoB (HopAB2) from *Pseudomonas syringae*, have been found to intercept multiple MAMP-mediated signaling (de Torres et al., 2006; Hann and Rathjen, 2007; He et al., 2006). Significantly, expression of AvrPto or AvrPtoB suppresses defense responses and promotes bacterial proliferation in *Arabidopsis* and *N. benthamiana* (de Torres et al., 2006; Hann and Rathjen, 2007; He et al., 2006). Molecular analysis suggests that the potent suppression function of AvrPto and AvrPtoB occurs upstream of MAPKKK (MAP kinase kinase kinase) in MAP-kinase signaling cascades triggered by multiple MAMPs (He et al., 2006). However, the direct-host targets and precise molecular mechanisms underlying the suppression function of AvrPto and AvrPtoB remain obscure.

We have previously proposed that AvrPto and AvrPtoB likely target multiple RLKs involved in MAMP perception in *Arabidopsis* (He et al., 2006). Alternatively, AvrPto and AvrPtoB may target a convergent component upstream of MAPKKK in multiple-MAMP signaling. Our extensive analyses of *avrPto*-expressing transgenic plants revealed surprising AvrPto-associated growth defects not observed in the known MAMP-receptor mutants but resembling BR-deficient mutants. We discovered that AvrPto and AvrPtoB target BAK1, a signaling partner of multiple PRRs in plant immunity and in BR signaling. This targeting leads to the dissociation of ligand-induced MAMP-receptor complexes, thereby blocking the initiation of MAMP signaling. Remarkably, AvrPto and AvrPtoB delivered from pathogenic bacteria are sufficient to interfere with the bacterial-induced formation of FLS2-BAK1 receptor-signaling complex in plant leaves. Extensive mutagenesis and deletion analyses of AvrPto and AvrPtoB supported the biological significance of AvrPto/AvrPtoB-BAK1 interactions in their MAMP-suppression function. Our data also revealed that the AvrPto/AvrPtoB interaction with BAK1 for suppressing MAMP signaling in *Arabidopsis* is likely structurally distinct from AvrPto/AvrPtoB interaction with Pto kinase for activating effector-triggered immunity (ETI) in tomato.

RESULTS

The *avrPto* Transgenic Plants Display Brassinosteroid-Insensitive Phenotypes

An intriguing observation leading to our discovery of an AvrPto host target was obtained from the phenotype of transgenic *Arabidopsis* plants expressing *avrPto* under the control of a constitutive cauliflower mosaic virus *35S* promoter. Multiple transgenic plants constitutively expressing AvrPto displayed dwarfed stature with small, round, and thick leaves; short petioles; reduced apical dominance; and short inflorescences without viable seeds (Figures 1A and S1). The unexpected growth defects caused by AvrPto expression in transgenic *Arabidopsis* are not observed in known MAMP-receptor mutants, such as the *fls2* or *efr* mutants (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006), suggesting that the potential AvrPto host targets may not be limited to the proposed PRRs in plant innate immunity (He et al., 2006).

The *35S::avrPto* transgenic plants resembled weak *bri1* mutants that are insensitive to BRs, the plant hormone with roles in division, expansion, and differentiation of cells and reproductive development (Belkhadir et al., 2006). BRs have also been implicated to play a role in plant resistance to a wide range of pathogens (Nakashita et al., 2003). Pathogens may potentially manipulate BR biosynthesis or signaling to promote pathogenicity. Furthermore, three independent transgenic lines with dexamethasone (DEX)-inducible AvrPto expression displayed the open-cotyledon phenotype in dark-grown seedlings as found in BR biosynthesis and signaling mutants, such as the *det2* (*de-etiolated2*), *bri1*, and *bak1* (*bri1-associated receptor kinase1*) (Figures 1B and S2) (Belkhadir et al., 2006; Li et al., 2002; Nam and Li, 2002). Importantly, inducible AvrPto expression caused moderate but statistically significant alteration of BR-responsive gene activation (*SAUR-AC* and *IAA5*) and repression (*CPD*) controlled by brassinolide (BL) (Figure 1C). The control plants without DEX or BL treatment did not exhibit gene-expression changes (data not shown). AvrPto did not affect the plant response to another growth-promoting hormone auxin (Chen et al., 2007) (Figure S3), suggesting that AvrPto expression did not alter general hormone effects in plants. The results indicated that AvrPto specifically diminished BR signaling.

AvrPto and AvrPtoB Interact with BAK1

Plasma-membrane localization is essential for the AvrPto action in suppressing MAMP signaling in plants (He et al., 2006). We therefore hypothesized that AvrPto might directly interact and interfere with the functions of the BR receptor BRI1 and/or its associated receptor-like kinase BAK1. Using a yeast split-ubiquitin assay designed for membrane proteins (Obrdlik et al., 2004), we found that AvrPto interacted with BAK1 but not BRI1 in the yeast (Figure 1D). Furthermore, an AvrPto mutant (AvrPto^{S46P}) that is unable to suppress MAMP signaling (He et al., 2006) no longer interacted with BAK1 (Figure 1D). Although it has recently been shown that AvrPto associated with FLS2 in plant cells (Xiang et al., 2008), the interaction of AvrPto and FLS2 was not detected in the yeast split-ubiquitin assay, and the AvrPto-BAK1 interaction does not require FLS2 or other PRRs (Figure 1D).

We further examined the in vivo interaction of epitope-tagged AvrPto and BAK1 using coimmunoprecipitation (coIP) assay. AvrPto interacted strongly with BAK1 independent of the FLS2 ligand flg22 (a 22 amino acid synthetic-peptide elicitor derived from bacterial flagellin) (Figure 2A). AvrPto also interacted with BAK1 in the *fls2* mutant, indicating that this interaction is independent of FLS2 (data not shown). Consistent with the yeast split-ubiquitin assay results (Figure 1D), AvrPto^{S46P} and AvrPto^{Y89D}, which do not suppress MAMP signaling, displayed significantly reduced affinity to BAK1 in the CoIP assay (Figures 2A and S4A). AvrPto^{S147R}, which has suppression activity (He et al., 2006), still interacted with BAK1 (Figure S4A). AvrPtoB,

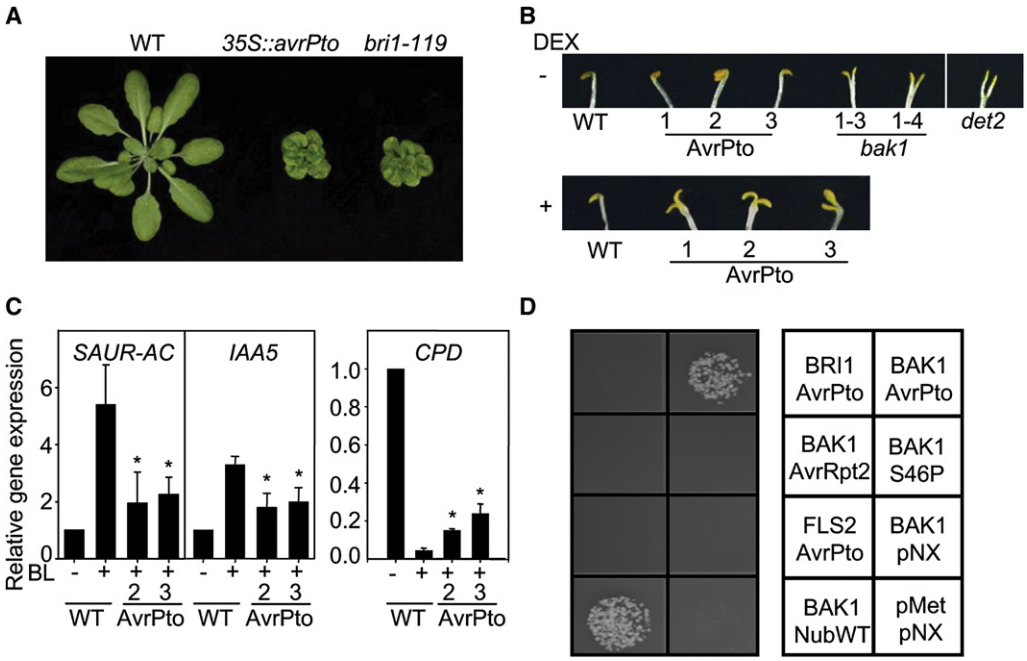

Figure 1. The *avrPto*-Transgenic Plants Display Brassinosteroid-Insensitive Phenotypes

(A) Transgenic *Arabidopsis* plants expressing *35S::avrPto* show similar growth phenotypes as the weak brassinosteroid-insensitive mutant *bri1-119*. The plants were grown in the soil under the 12 hr light cycle in a growth chamber. The 4-week-old plants are shown.

(B) The DEX-inducible *avrPto* transgenic (1, 2, and 3), *bak1*- (1–3 and 1–4), and *det2*-mutant seedlings exhibit open cotyledons in the dark. Seedlings were grown in the dark for 7 days with or without 10 μM DEX.

(C) Altered gene expression in *avrPto*-transgenic plants. Seedlings were pretreated with 10 μM DEX for 24 hr before treatment with 0.1 μM BL or control (DMSO) for 3 hr. The data are shown as means ± standard errors from three independent biological replicates. * indicates a significant difference with $p < 0.05$ when compared with data from wild-type (WT) based on the results of an unpaired Student's t test.

(D) AvrPto interacts with BAK1, but not FLS2 or BRI1, in a yeast split-ubiquitin assay. NubWT is wild-type N-terminal ubiquitin half (Nub) as a positive control. pNX and pMet are empty vectors with N- or C-terminal mutated-ubiquitin half as negative controls. S46P is a mutant of AvrPto without MAMP-suppression activity. The experiments were repeated three times with similar results.

a sequence-distinct effector exhibiting similar MAMP-suppression activity as AvrPto (He et al., 2006), also associated with BAK1 in vivo (Figure 2A). The N-terminal 387 amino acids of AvrPtoB are required and sufficient to block MAMP signaling (Xiao et al., 2007). Significantly, AvrPtoB$^{1–387}$—but not AvrPtoB$^{1–307}$ or AvrPtoB$^{308–553}$, which could not suppress flg22 signaling (Figure S6C)—associated with BAK1 in vivo (Figure 2B). AvrRpt2, an effector protein with a virulence function distinct from that of AvrPto and AvrPtoB (Chen et al., 2007; He et al., 2006), did not coimmunoprecipitate with BAK1, confirming the specificity and functional link of the BAK1 interaction with AvrPto and AvrPtoB.

Although we did not detect the interaction of AvrPto with FLS2 in the yeast assay (Figure 1D), AvrPto did associate with FLS2 independent of flg22 in vivo in the coIP assay (Figure S5A), perhaps due to higher protein expression. Significantly, the AvrPto^{S46P}, which lacks MAMP-suppression activity, still coimmunoprecipitated with FLS2 (Figure S6A). Moreover, two AvrPtoB-deletion mutants (AvrPtoB$^{1–307}$ and AvrPtoB$^{308–553}$) that do not suppress MAMP signaling (Xiao et al., 2007) (Figure S6C) did not associate with BAK1 (Figure 2B) but still associated with FLS2 (Figure S6B). These observations suggest that the association of AvrPto or AvrPtoB with FLS2 is distinct from that with BAK1 and may not be functionally relevant to the suppression of MAMP signaling.

To examine possible differential affinities and reveal possible artifacts arising from protein overexpression, we coexpressed BAK1 and FLS2 at similar amounts with reduced AvrPto-expression levels in the same plant cells. AvrPto specifically coimmunoprecipitated with BAK1 but not with FLS2 (Figure 2C), suggesting that AvrPto has higher affinity to BAK1 than FLS2 in vivo. Finally, we also observed that AvrPto could weakly coimmunoprecipitate with EFR and the highly overexpressed chitin receptor CERK1 (Figure S5B) (Miya et al., 2007; Zipfel et al., 2006), but not CLV1, BRI1, or a putative LysM receptor-like kinase At2g23770, a close homolog of CERK1 (Figures S5C and S6A). However, protein association does not necessarily correlate with functional significance as observed for AvrPto/AvrPtoB-FLS2 interactions (Figure S6).

AvrPto Disrupts Flagellin-Induced FLS2-BAK1 Complex Formation

Recent findings have shown that BAK1 associates with FLS2 only after flg22 perception and that BAK1 is critical for flagellin-induced signaling (Chinchilla et al., 2007; Heese et al., 2007). To investigate the biological significance of the AvrPto-BAK1 interaction, we tested flg22-induced FLS2-BAK1 association in the presence or absence of AvrPto. As shown in Figure 3A, AvrPto effectively diminished FLS2-BAK1 association activated

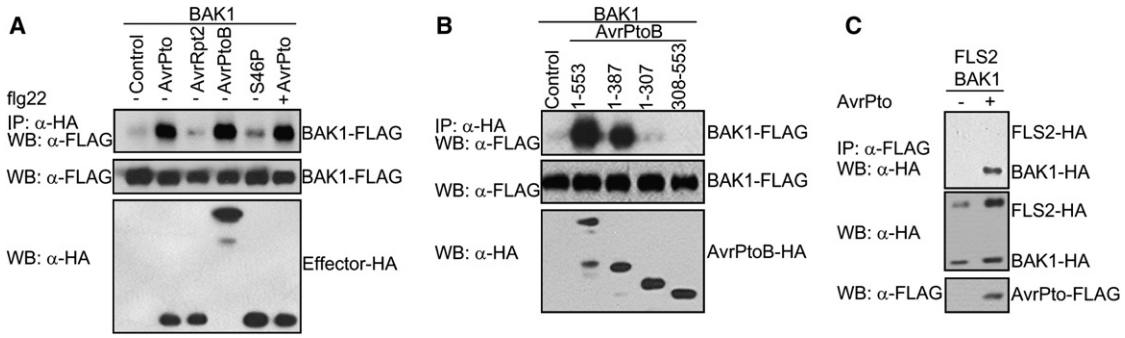

Figure 2. AvrPto and AvrPtoB Associate with BAK1 In Vivo

(A) AvrPto and AvrPtoB associate with BAK1 in vivo independent of flg22. CoIP was performed with protoplasts coexpressing BAK1-FLAG and different HA-tagged effector proteins. S46P is an AvrPto mutant. The coIP was carried out with anti-HA-agarose (IP: α-HA), and the proteins were analyzed using western blot analysis with an anti-FLAG antibody (WB: α-FLAG). The top panel shows coIP results, and the middle and bottom panels show protein expression. Protoplasts were treated with 1 μM flg22 for 5 min.

(B) AvrPtoB deletion mutants, which lack MAMP-suppression activity, do not associate with BAK1. The coIP was performed with protoplasts coexpressing BAK1-FLAG and HA-tagged full-length AvrPtoB$^{1-553}$ or AvrPtoB-deletion mutants.

(C) AvrPto has higher affinity to BAK1 than FLS2 in vivo. Moderate amounts of BAK1-HA, FLS2-HA, and AvrPto-FLAG were coexpressed in protoplasts. The above experiments were repeated three times with similar results.

by flg22. AvrPtoB also caused a similar effect, indicating at least one shared molecular mechanism for these two distinct virulence effectors in blocking MAMP signaling (Figure 3A). The interception of MAMP signaling by AvrPto likely occurred at the plasma membrane since the AvrPto^{G2A} protein, which no longer associates with the plasma membrane and lacks the suppression activity (He et al., 2006), lost the ability to interfere with the FLS2-BAK1 association induced by flg22 (Figure 3A). The AvrPto^{S46P} mutant with low-binding affinity for BAK1 also did not interfere with the FLS2-BAK1 association triggered by flg22 (Figure 3A), reinforcing the functional importance of the AvrPto-BAK1 interaction. As a negative control, a distinct virulence effector HopD2 (also known as HopPtoD2 and HopAO1) was not effective in interrupting the FLS2-BAK1 association triggered by flg22 (Figure 3A).

To demonstrate the physiological significance of AvrPto action in planta and to avoid protein overexpression, we generated transgenic plants expressing both functional BAK1-GFP at the endogenous level (Nam and Li, 2002) and DEX-inducible AvrPto-HA (He et al., 2006). Consistent with the cell-based assays, the association of endogenous FLS2 with BAK1-GFP was completely blocked by AvrPto (Figure 3B). To further control for possible effector protein overexpression artifacts, we examined the ability of bacterial-delivered type III effectors to disrupt the FLS2 and BAK1 complex formation activated by bacterial inoculation in *Arabidopsis* plants. The FLS2 and BAK1 association could be equally stimulated by *P. s. tomato* DC3000 and its type III secretion mutant *hrcC* 0.5 hr postinoculation (hpi) (Figure 3C). This is likely due to the presence of MAMP signals, especially flagellin in the bacteria. At 2 hpi, the FLS2 and BAK1

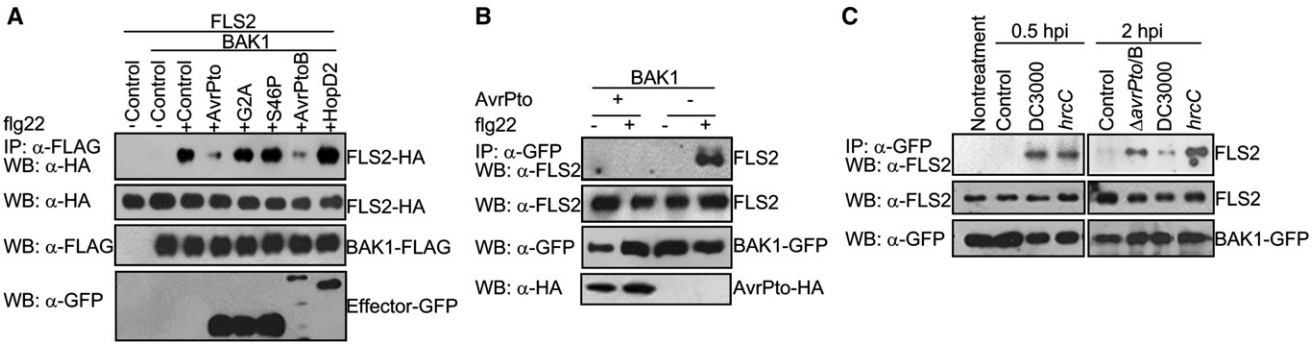

Figure 3. AvrPto Interferes with FLS2-BAK1 Association

(A) AvrPto and AvrPtoB suppress FLS2-BAK1 association stimulated by flg22 in protoplasts. CoIP was performed with protoplasts coexpressing BAK1-FLAG, FLS2-HA, and different GFP-tagged effector proteins. Protoplasts were treated with 1 μM flg22 for 5 min. G2A and S46P are two mutants of AvrPto.

(B) AvrPto abolishes FLS2-BAK1 association triggered by flg22 in seedlings. *BAK1::BAK1-GFP*-transgenic seedlings with or without the DEX inducible *avrPto-HA* transgene were treated with 10 μM DEX for 24 hr and stimulated with 1 μM flg22 for 5 min. The coIP was carried out with an anti-GFP antibody (IP: α-GFP), and the proteins were collected with protein G-agarose and analyzed using western blot with an anti-FLS2 antibody (WB: α-FLS2).

(C) AvrPto and AvrPtoB delivered by DC3000 interfere with FLS2-BAK1 association. *BAK1::BAK1-GFP*- transgenic plants were inoculated with different DC3000 strains for coIP assay. The experiments were repeated three times with similar results.

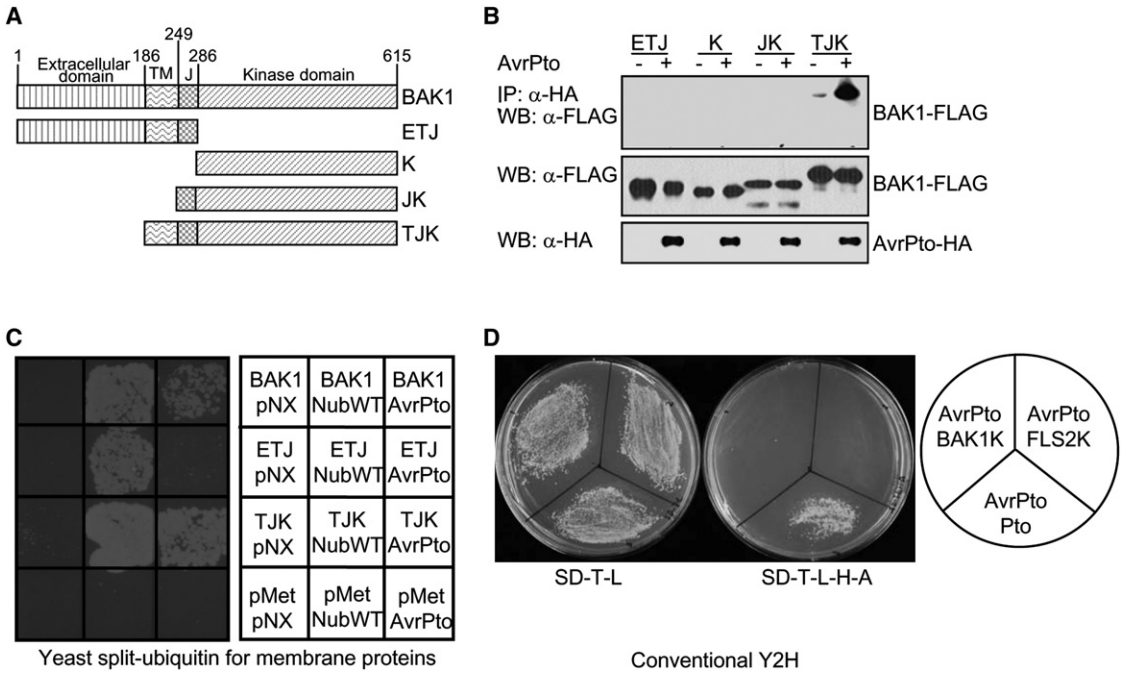

Figure 4. Interaction of BAK1-Deletion Mutants with AvrPto

(A) Schematic diagram of BAK1 and its deletion mutants. BAK1 contains an extracellular domain, a transmembrane domain (TM), an intracellular-juxtamembrane domain (J), and a kinase domain. The amino acid positions of each domain are indicated.

(B) TM, J, and kinase domain of BAK1 are required for association with AvrPto in vivo. The coIP was performed with protoplasts coexpressing FLAG-tagged BAK1-deletion mutants with or without AvrPto-HA.

(C) AvrPto interacts with BAK1TJK in a yeast split-ubiquitin assay. NubWT is a positive control. pNX and pMet are empty vectors.

(D) BAK1 kinase domain does not interact with AvrPto in a yeast two-hybrid assay. All the experiments were repeated three times with similar results.

association was reduced in plants inoculated with the virulent strain DC3000, but was enhanced further by *hrcC*, suggesting that effectors secreted from DC3000 have the ability to interfere with FLS2 and BAK1 association (Figure 3C). Most significantly, plants inoculated with a Δ*avrPto*Δ*avrPtoB* double mutant showed increased association between FLS2 and BAK1 as compared with DC3000-inoculated plants (Figure 3C). These data indicate that, when delivered by *P. s. tomato* at natural levels, AvrPto and AvrPtoB are able to suppress bacterial-induced FLS2 and BAK1 association and downstream signaling in intact plants.

The interaction between AvrPto and BAK1 also reduced the association between BRI1 and BAK1 in the presence or absence of BL (Figure S7A), consistent with the BR-insensitive phenotypes observed in *avrPto*-transgenic plants (Figures 1, S1, and S2). In this case, it appears the AvrPto interaction with BAK1 was sufficient to disrupt the BRI1-BAK1 complex since AvrPto did not directly interact with the receptor BRI1 (Figures 1D and S6A). These results suggested that AvrPto physically interferes with the formation of stable receptor complexes, FLS2-BAK1 and BRI1-BAK1, critical for distinct signaling pathways triggered by specific ligands, thereby contributing to its virulence function and to its effect on BR signaling in plant growth and development.

BAK1's Transmembrane and Kinase Domains Are Essential for Its Interaction with AvrPto

BAK1 encodes an RLK with a putative extracellular domain, a single transmembrane domain, an intracellular-juxtamembrane domain, and a kinase domain (Li et al., 2002; Nam and Li, 2002) (Figure 4A). To determine which domains of BAK1 interact with AvrPto, we generated different deletions of BAK1 and tested their interaction with AvrPto in plant cells and in yeast. BAK1 without kinase domain (ETJ) did not coimmunoprecipitate with AvrPto from plant cells (Figure 4B) and did not interact with AvrPto in the yeast split-ubiquitin assay (Figure 4C). However, BAK1-kinase domain alone is not sufficient to interact with AvrPto in the coIP assay (Figure 4B) or in a conventional yeast two-hybrid assay (Figure 4D). BAK1-kinase domain with juxtamembrane and transmembrane domains strongly coimmunoprecipitated with AvrPto in vivo (Figure 4B) and interacted with AvrPto in a yeast split-ubiquitin assay (Figure 4C), suggesting that BAK1-transmembrane domain is essential for its interaction with AvrPto. The data are consistent with the observation that AvrPto functions inside plant cells, and its plasma membrane localization is critical to suppress MAMP signaling.

AvrPto/AvrPtoB Interaction with BAK1 Is Distinct from that with Pto

In certain tomato genotypes, Pto-protein kinase recognizes AvrPto to initiate effector-triggered immunity (ETI) mediated by the NB-LRR (nucleotide-binding-leucine-rich-repeat) protein Prf (Pedley and Martin, 2003). Recent crystal-structure analysis has identified key contact residues in two interfaces of Pto, H49/V51/F52 and T204, for AvrPto interaction (Xing et al., 2007). We aligned the Pto sequence with the kinase domains

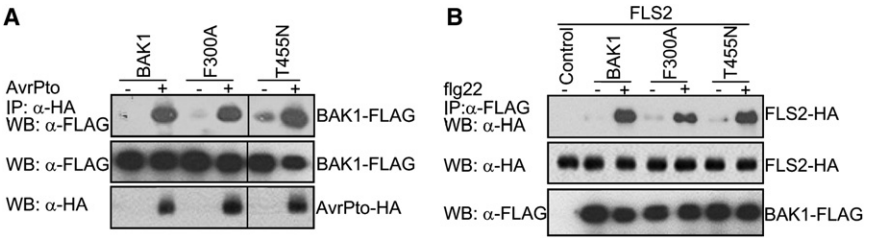

Figure 5. Association of BAK1 Mutants with AvrPto and FLS2

(A) Association of BAK1 mutants with AvrPto. F300A and T455N are two mutants of BAK1. BAK1(F300) is the equivalent of Pto(F52) in the first interaction interface for AvrPto, and BAK1(T455) is the equivalent of Pto(T204) in the second interface. (B) Association of BAK1 mutants with FLS2 activated by flg22. The experiments were repeated three times with similar results.

of BAK1 and other receptor kinases, including SERK4, SERK5, FLS2, EFR, BRI1, CLV1, ERECTA, CERK1, and At2g23770 (Figure S8). The overall sequence similarity of these receptor kinases is not correlated with their differential binding affinity to AvrPto (Figure S8B).

There is no significant sequence conservation in the first interface of AvrPto-Pto interaction among these receptor kinases as the critical residues, Pto(H49) and Pto(V51), are both absent (Xing et al., 2007). Pto(F52), which makes van der Waals contact with AvrPto (Xing et al., 2007), is relatively conserved among them (Figure S8A). However, the BAK1^{F300A} mutation did not affect its interaction with AvrPto in the coIP assay (Figure 5A) and the yeast split-ubiquitin assay (data not shown). The second interface for AvrPto-Pto interaction is relatively conserved. The residue T204 in Pto that determines the specific recognition of AvrPto (Xing et al., 2007) is present in most receptor kinases except CLV1 (Figure S8A). However, unlike Pto^{T204N}, the BAK1^{T455N} mutation did not compromise its interaction with AvrPto (Figure 5A). The same BAK1^{F300A} and BAK1^{T455N} mutations also did not affect the association of BAK1 with FLS2 induced by flg22 (Figure 5B). Finally, AvrPtoB$^{1–307}$, which is sufficient to interact with Pto and trigger ETI in tomato but lacks the MAMP-suppression activity (Xiao et al., 2007), did not interact with BAK1 in *Arabidopsis* (Figures 2B and S6C). Our results therefore suggest that structural aspects of the AvrPto/AvrPtoB interaction with BAK1 for suppressing MAMP signaling are different from those involved in the interaction of these effectors with Pto for activating ETI.

The crystal structure of the AvrPto-Pto complex and an in vitro kinase assay suggested that AvrPto may function as a kinase inhibitor (Xing et al., 2007). However, AvrPto activation of ETI signaling is independent of its kinase-inhibition activity on the Pto kinase (Xing et al., 2007). AvrPto did not affect BL-enhanced BAK1 and BRI1 phosphorylation at least as detected by an anti-Thr-P antibody in plant cells (Figure S7B). It remains possible that AvrPto interferes with other specific phosphorylated residues (e.g., Ser) on BAK1. Limited by the availability of anti-Ser/Thr-P antibody and a lack of information on flg22-mediated FLS2 phosphorylation, we were unable to detect in vivo phosphorylated FLS2 or BAK1 triggered by flg22 using the same assay despite extensive efforts. An in vitro kinase assay of FLS2 and BAK1 was not feasible since FLS2 and BAK1 association cannot be triggered by flg22 in vitro (Chinchilla et al., 2007).

BAK1 Is Involved in Multiple MAMP Signaling

We have shown that AvrPto is able to block immune signaling triggered by individual MAMPs and by the *hrcC* mutant without a functional type III secretion system (He et al., 2006). To further connect the physiological function of a host target to the virulence function of AvrPto, we examined the importance of BAK1 in MAMP signaling in intact plants inoculated with *hrcC*-mutant bacteria. Three independent loss-of-function *bak1* mutants in the Col-0 (*bak1-3* and *bak1-4*) and Ws background (*bak1-1*) displayed reduced early marker gene response activated by the *hrcC* mutant (Figure 6A). The induction of early MAMP marker genes by the *hrcC* mutant was likely due to the presence of multiple MAMPs from the bacteria as the lack of the FLS2 receptor in the Col-0 *fls2* mutant and Ws (also an *fls2* mutant) did not significantly affect the responses (Figure 6A). The reduced response in *bak1* mutants suggested that BAK1 could be a shared-signaling partner for other MAMPs besides flagellin.

Analyses of responses to more individual MAMPs in isolated leaf cells and in whole seedlings supported the important role of BAK1 in flg22, elf18, HrpZ, peptidoglycan (PGN), and lipopolysaccharide (LPS) but not in chitin or NPP1 signaling (Figures 6B and S9). These results suggest that BAK1 functions in many but not all MAMP-signaling responses. Interestingly, AvrPto could be equally effective in suppressing the immune responses triggered by all these MAMPs (Figure 6C) (He et al., 2006). Thus, AvrPto might target other receptor-signaling complexes in addition to the ones involving BAK1 or other unknown MAMP-signaling components. In the *bak1* mutant, the reduction of flg22, elf18, HrpZ, PGN, and LPS responses was partial but statistically significant (Figures 6B and S9), suggesting the presence of additional host targets for AvrPto providing redundant functions to BAK1.

CoIP analysis indicated that the closest BAK1 homologs, BKK1 (SERK4) and SERK5, could also form complexes with AvrPto and provide partially overlapping activity in the MAMP- and BR-signaling pathways (Figure S10) (He et al., 2007a). A recent study has showed that the *bak1 bkk1* double mutant is seedling lethal, and BAK1 and BKK1 play independent roles in BR signaling and in the suppression of cell death (He et al., 2007a). The seedling lethality of the double mutant precluded the examination of its responses to MAMPs and pathogen infections. We carried out quantitative pathogen-infection assays in the *bak1*-mutant plants pretreated with flg22. Correlated with their partial reduction of the early marker gene activation in *bak1* (Figures 6A, 6B, and S9), the resistance to DC3000 infection induced by flg22 was reduced in the *bak1*-mutant lines (Figure S11). Together, our data suggest that BAK1 is involved in diverse MAMP signaling and that a single effector protein can target BAK1 in multiple host receptor-signaling complexes to maximize its virulence functions.

DISCUSSION

Lacking specialized immune cells and adaptive immunity, plants have evolved large numbers of potential PRRs to recognize

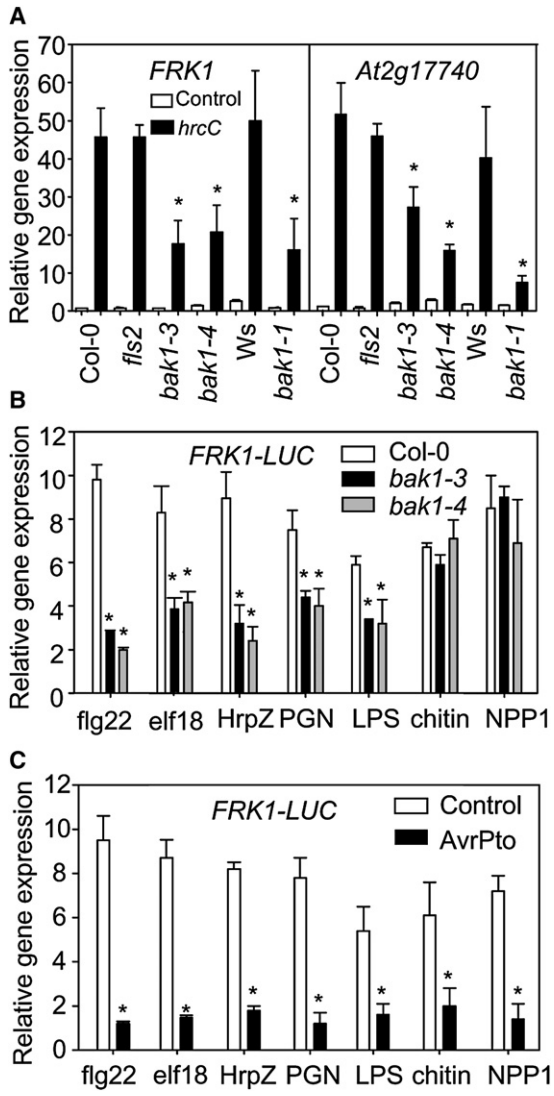

Figure 6. BAK1 Is Important in Multiple MAMP-Signaling Pathways

(A) Three *bak1* mutants are impaired in the early marker gene activation by *hrcC* infiltration. The leaves from Col-0, Ws, *fls2*, and *bak1* mutants (*bak1-3*, *bak1-4*, and *bak1-1*) were inoculated with *hrcC* at 10^8 cfu/ml or with water control. Leaves were collected 6 hr after inoculation for RNA isolation and quantitative RT-PCR analysis.

(B) The *bak1* mutants display reduced *FRK1-LUC* induction by many but not all MAMPs. Protoplasts were transfected with the *FRK1-LUC* reporter and treated with different MAMPs for 3 hr.

(C) AvrPto blocks *FRK1-LUC* induction by multiple MAMPs. Protoplasts were transfected with *FRK1-LUC* with or without AvrPto. Transfected protoplasts were incubated for 3 hr to express AvrPto before treatment with different MAMPs for 3 hr. Different MAMPs in (B) and (C) are flg22, 10 nM; elf18, 10 nM; HrpZ, 100 nM; PGN, 50 μg/ml; chitin, 50 μg/ml; LPS, 50 μg/ml; and NPP1, 20 nM. The data are shown as means ± standard errors from three independent biological replicates. * indicates a significant difference with $p < 0.05$ when compared with data from control plants or treatment based on the results of an unpaired Student's t test.

diverse MAMPs and trigger innate immunity. BAK1 was recently discovered as a key component in MAMP signaling in *Arabidopsis* and tobacco (Chinchilla et al., 2007; Heese et al., 2007). We report here that bacterial virulence effectors, AvrPto and AvrPtoB, target BAK1 and block the ligand-induced formation of MAMP-receptor complexes, thereby effectively impeding multiple MAMP-signaling initiation. Significantly, natural levels of AvrPto and AvrPtoB delivered by pathogenic bacteria are sufficient to interfere with the endogenous MAMP-receptor complex formation in plants. AvrPto and AvrPtoB have been long studied as triggers to elicit potent immunity in tomato upon recognition by a serine/threonine kinase, Pto, in concert with the NB-LRR protein Prf (Pedley and Martin, 2003). BAK1 as a newly identified AvrPto/AvrPtoB target for bacterial virulence also contains a cytosolic serine/threonine-kinase domain. It has been suggested that effector targets in host cells for mediating virulence or immunity might share similar molecular mechanisms (Jones and Dangl, 2006). Surprisingly, our extensive comparative mutagenesis and functional analysis reveal that the interactions of BAK1 and Pto with AvrPto/AvrPtoB exhibit distinct molecular features, indicating dynamic evolution in response to pathogen challenges.

Multiple Roles of BAK1 in Plant Development, Innate immunity, and Cell Death

BAK1 was first identified as a signaling partner of the BR receptor BRI1, and it plays important roles in BR-mediated plant development (Belkhadir et al., 2006; Li et al., 2002; Nam and Li, 2002). Recently, BAK1 has emerged as an important player in MAMP signaling where it associates with flagellin receptor FLS2 upon flagellin treatment (Chinchilla et al., 2007; Heese et al., 2007). There are several lines of evidence suggesting that BAK1 may associate with multiple MAMP receptors. First, *bak1* mutants show reduced activation of early MAMP marker genes triggered by nonpathogenic bacteria, DC3000 *hrcC* (Figure 6A). The immune response induced by *hrcC* is likely determined by multiple MAMPs. Second, BAK1 is required for host responses resulting from recognition of several MAMPs, including flagellin, EF-Tu, HrpZ, PGN, and LPS (Figure 6B). Third, *bak1* mutants display altered disease susceptibility to several pathogens, including bacteria, necrotrophic fungi, and oomycetes (Heese et al., 2007; Kemmerling et al., 2007). However, *bak1* mutants have been shown to have normal binding capacity for BR to BRI1 and flagellin to FLS2, respectively (Chinchilla et al., 2007; Kinoshita et al., 2005). Although BAK1 is a partner of both BRI1 and FLS2, no overlapping responses are induced by BR and flagellin. All these suggest that BAK1 is not involved in signal perception but rather functions as an adaptor or partner of diverse RLK complexes to regulate different downstream responses.

Interestingly, BAK1 also plays a negative role in the control of plant programmed cell death (PCD) (He et al., 2007a; Kemmerling et al., 2007), consistent with eventual lethality of *avrPto*-expressing transgenic plants and the transition of DC3000 to a necrotrophic pathogen at the late-infection stage (Glazebrook, 2005). PCD is associated with both immunity and susceptibility in plant-microbe interactions. Although *bak1* mutants do not exhibit spontaneous cell death, they develop spreading necrosis upon pathogen infection, suggesting the role of BAK1 in the control of microbial-induced PCD (Kemmerling et al., 2007). This is further supported by *bak1 bkk1* double mutants, which display spontaneous cell death, seedling lethality, and constitutive-defense responses (He et al., 2007a). The BAK1-controlled cell death appears to be BR independent since other BR

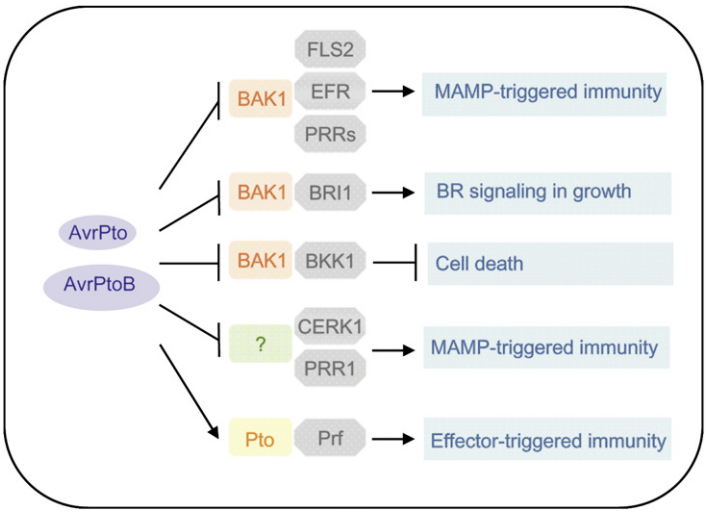

Figure 7. A Model of Distinct AvrPto and AvrPtoB Actions via Their Target Proteins in Controlling Plant Immunity, Development, and Cell Death

AvrPto and AvrPtoB directly target *Arabidopsis* BAK1, a signaling partner of multiple MAMP receptors and BR receptor BRI1, and prevent stable receptor-signaling complex formation and diverse downstream signaling triggered by different MAMPs and BR. BAK1, as well as its closest homolog BKK1, also plays a negative role in the control of plant cell death. AvrPto and AvrPtoB may also target BAK1-independent receptor complexes or other unknown components to intercept MAMP-signaling pathways. In tomato, AvrPto and AvrPtoB are recognized by Pto kinase in concert with the NB-LRR protein Prf to initiate effector-triggered immunity.

biosynthesis or signaling mutants do not exhibit cell death-related phenotype, and the application of BR in *Arabidopsis bak1* mutants did not affect plant-cell death and disease resistance (Kemmerling et al., 2007). However, BR has been implicated in immunity in certain plant species as treatment of tobacco and rice with BR induced a broad range of resistance to bacteria, fungi, and virus (Nakashita et al., 2003). Thus, targeting BAK1 can serve multiple purposes to simultaneously modulate MAMP-receptor complexes, BR signaling, and cell death for the benefit of bacterial infection, life style, and fitness (Figure 7).

Complex Relationship between Bacterial Effectors and Host Targets

Pathogenic bacteria deliver type III effectors into host cells to promote pathogenicity. We have discovered that AvrPto and AvrPtoB directly target BAK1—a shared-signaling partner but not a PRR—and prevent stable-receptor signaling complex formation and diverse downstream-signaling pathways during infection. By targeting BAK1, AvrPto and AvrPtoB block multiple MAMP-signaling and BR-signaling pathways, presumably a more effective strategy than targeting individual receptors. This is consistent with the BR-insensitive phenotype observed in AvrPto-transgenic plants. From an evolutionary point of view, it may be parsimonious for a pathogen effector to target BAK1.

Recently, it has been proposed that AvrPto targets FLS2 and EFR and blocks flg22 signaling (Xiang et al., 2008). However, our extensive functional and protein-interaction analyses of AvrPto and AvrPtoB mutants and deletions demonstrated that the association of AvrPto/AvrPtoB with FLS2 is not correlated with their suppression activity in MAMP signaling and only occurs when all proteins are overexpressed. Importantly, the same set of comprehensive analyses support the functional significance of the interactions between AvrPto/AvrPtoB and BAK1 in vivo (Figures 1D, 2A, 2B, 3A, S4, S5, and S6). The association of AvrPto with FLS2 or EFR is consistently much weaker than that with BAK1, and AvrPto preferentially binds to BAK1 over FLS2 in yeast and plant cells (Figures 1D and 2C). Our diverse assays also define multiple BAK1 domains for the AvrPto-BAK1 interaction while the kinase domain alone is not sufficient (Figure 4). It is possible that AvrPto may target multiple RLKs when it is expressed at a high level. However, during the natural plant-microbe interaction, the amount of AvrPto delivered into host cells by bacteria would not appear to be sufficient to interact with many RLKs at relatively low affinity to suppress signaling triggered by multiple MAMPs.

Emerging evidence indicates that bacterial effectors can have multiple targets in their hosts. Besides BAK1, AvrPto and AvrPtoB may also target BAK1-independent receptor complexes (Figure 7) or other unknown components to intercept multiple MAMP-signaling pathways. Apparently, AvrPto and AvrPtoB interact with proteins closely related to BAK1, BKK1/SERK4, and SERK5 (Figure S10), which may provide redundant functions with BAK1 in MAMP signaling and BR signaling. Several type III effectors possess enzymatic activities and could potentially target many host proteins (Chisholm et al., 2005; Shan et al., 2007). It is likely that bacterial effectors can target distinct host pathways to promote pathogenicity, whereas plants may use multiple host components to subdue their virulence.

Distinct Host Targets in Elicitation and Suppression of Immunity

In tomato, the protein kinase Pto recognizes bacterial effector AvrPto or AvrPtoB to initiate ETI in concert with the NB-LRR protein Prf (Pedley and Martin, 2003). It was postulated that in tomato plants lacking Prf, Pto might be a virulence target of AvrPto/AvrPtoB. However, these effectors are now known to exert their virulence activities in the absence of Pto in tomato and *Arabidopsis* (He et al., 2006; Xiao et al., 2007). Analyses of BAK1 and AvrPto mutations and AvrPtoB deletions in this study further suggest that, at least for AvrPto and AvrPtoB, their dual but opposite activities as virulence factors in disrupting MAMP signaling and as determinants for ETI are mediated through distinct host targets and mechanisms, e.g., interfering with the FLS2-BAK1 receptor-signaling complex and recognizing the Pto-Prf immune sensor complex, respectively. Furthermore, the molecular basis of AvrPto-BAK1 and AvrPto-Pto (Xing et al., 2007) interactions appears to be distinct.

Based on modeling with the mammalian PKA inhibitor (PKI), AvrPto was proposed to function as a kinase inhibitor of tomato Pto kinase with a high affinity (Ki = 1 nM) (Grove et al., 1987; Xing et al., 2007). Although AvrPto binds to Pto with a relatively high affinity in vitro (Kd = 0.11 μM), 100-fold higher concentration of AvrPto (11 μM) was required to inhibit 50% Pto-kinase activities

(Xing et al., 2007). AvrPto as a kinase inhibitor is 10,000× less potent than the established PKI (Grove et al., 1987). It is not clear whether AvrPto delivered by bacteria into plant cells reaches such high protein concentrations that were used for the in vitro inhibitor assays. In our in vivo phosphorylation assay, AvrPto did not affect the phosphorylation of its *Arabidopsis* virulence target BAK1 (Figure S7B). As AvrPto delivered from bacteria during infection is sufficient to interfere with MAMP signaling (Figure 3C), the detailed molecular and biochemical action of AvrPto and AvrPtoB on targeting receptor kinases requires further characterization in vivo in a physiological context.

Our studies support the notion that distinct effector proteins can share the same host targets, and also each effector protein can manipulate multiple host factors. Potent and versatile type III suppressors such as AvrPto and AvrPtoB could be used as valuable molecular probes to search for new receptors, receptor partners, and signaling regulators for multiple MAMPs and to elucidate the mechanisms important for plant-innate immunity and bacterial pathogenicity. As exemplified by the AvrPto-BAK1 interaction and a study on viral effector (Fontes et al., 2004), it is possible that some animal pathogens have also evolved effectors to target PRRs or their immediate-signaling complexes to effectively intercept innate-immune signaling near the MAMP perception step to promote pathogenicity. Future studies will uncover the complex cellular networks involving a plethora of host proteins and effectors derived from various pathogens contributing to the dynamic and intimate relationships in host and microbe interactions.

EXPERIMENTAL PROCEDURES

Plant Growth, Pathogen Assay, and Generation of Transgenic Plants

Wild-type (Col-0 and Ws) *fls2* and *bak1* mutant *Arabidopsis* plants were grown in a growth chamber at 23°C, 65% relative humidity, 75 μE light with a 12 hr photoperiod for 30 days before protoplast isolation or bacterial inoculation. To examine the open-cotyledon phenotype, DEX-inducible *avrPto* transgenic *bak1-3, bak1-4*, and *det2* mutant seedlings were grown at 23°C for 7 days in the dark with or without 10 μM DEX on 1/2 MS plates with 1% sucrose and 0.9% agar. The *fls2* (*Salk_141277*) mutant is in the Col-0 background. The *bak1-1* mutant is in the Ws background, which does not have a functional FLS2 (Li et al., 2002), and *bak1-3* (*Salk_034523*) and *bak1-4* (*Salk_116202*) mutants are in the Col-0 background (Chinchilla et al., 2007; He et al., 2007a; Heese et al., 2007; Kemmerling et al., 2007).

P. syringae tomato DC3000, *hrcC*, and Δ*avrPto*Δ*avrPtoB* strains were grown overnight at 28°C in the KB medium with appropriate antibiotics. Bacteria were collected, washed, and diluted to the desired density with H_2O. *Arabidopsis* leaves were infiltrated with bacteria using a needleless syringe. The *avrPto*-transgenic plants were generated by *Agrobacterium*-mediated transformation with the *avrPto* construct under the control of a constitutive cauliflower mosaic virus *35S* promoter with an HA-epitope tag. DEX-inducible *avrPto*-transgenic plants were reported previously (He et al., 2006). The DEX-inducible *avrPto*-transgenic plants were crossed to the *BAK1::BAK1-GFP* transgenic plants (Nam and Li, 2002). The transgenic plants carrying both *avrPto-HA* and *BAK1-GFP* were confirmed by western blot.

MAMP Preparation

flg22 was synthesized according to the published sequence (Felix et al., 1999). HrpZ and NPP1 were prepared as described (Fellbrich et al., 2002; Lee et al., 2001). GST was used as a control of NPP1. PGN from *Staphylococcus aureus* (Fluka Cat # 77140), chitin from crab shells (Cat # C9752), and LPS from *Pseudomonas aeruginosa* (Cat # L2012) were purchased from Sigma.

Plasmid Constructs and Protoplast-Transient Assays

Bacterial or plant genes were amplified by PCR and introduced into a plant-expression vector with a HA-, FLAG-, or GFP-epitope tag at the C terminus. All effector constructs were reported previously (He et al., 2006). *Arabidopsis* genes except EFR were PCR amplified from Col-0 cDNA and confirmed by DNA sequencing. Protoplast-transient assay was carried out as described (He et al., 2006). For reporter assays, 50 μl protoplasts at a density of 2×10^5 /ml were transfected with 10 μg plasmid DNA-expressing effectors and reporters. For immunoprecipitation assays, 1 ml protoplasts were transfected with 200 μg plasmid DNA-expressing receptor-like kinases and/or type III effectors.

RT-PCR Analysis

Total RNA was isolated from leaves or seedlings with TRIzol Reagent (Invitrogen). First strand cDNA was synthesized from 1 μg of total RNA with reverse transcriptase. Real-time RT-PCR analysis was carried out with an iCycler iQ real-time PCR-detection system using iQ SYBR green supermix (BIO-RAD). *UBQ10* was used as a control gene, and the expression of individual genes was normalized to the expression of *UBQ10*.

Immunoprecipitation Assay

Proteins were prepared from 1 ml transfected protoplasts 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 a protease inhibitor cocktail from Roche). After being vortexed vigorously for 30 s, the samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was incubated with prewashed anti-HA-agarose or anti-FLAG-agarose beads for 3 hr at 4°C with gentle shaking. The beads were collected and washed four times with washing buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, and a protease inhibitor cocktail) and once with 50 mM Tris-HCl (pH 7.5). Coimmunoprecipitated proteins were analyzed by western blot with an anti-HA or anti-FLAG antibody. The protein bands with appropriate molecular weights were shown.

To perform immunoprecipitation assay with seedlings, 12-day-old seedlings carrying both the DEX-inducible *avrPto* and *BAK1::BAK1-GFP* transgenes were treated with or without 1 μM flg22 for 5 min. Proteins from five seedlings were extracted with 1 ml of extraction buffer by grinding. To detect the BAK1 and FLS2 association induced by bacteria, bacteria were first cultured at 28°C in the KB medium with appropriate antibiotics for overnight. Then, the bacteria were transferred into the minimal medium containing 10 mM fructose (pH 6.0) and cultured at room temperature for another 4 hr (Huynh et al., 1989). The bacteria were collected and diluted at 5×10^8 cfu/ml with water, and hand infiltrated into 4-week-old *BAK1::BAK1-GFP*-transgenic plant leaves. Proteins from eight leaves were extracted with 1.5 ml of extraction buffer. The samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant of each sample was adjusted to the same concentration of protein and incubated with an anti-GFP antibody for 2 hr at 4°C with gentle shaking. The samples were further incubated with protein-G-agarose for 2 hr and washed four times with the washing buffer and once with 50 mM Tris-HCl (pH 7.5). Coimmunoprecipitated proteins were analyzed by western blot with an anti-FLS2 antibody (Heese et al., 2007).

Yeast Assays

Mating-based split-ubiquitin system (mbSUS) was described by Obrdlik et al., 2004. PCR products of *BAK1*, *BRI1*, and *FLS2* were introduced into p*MetYCgate* vector by in vivo cloning and transformed into the yeast AP4 strain. PCR products of *avrPto, avrPtoS46P*, and *avrRpt2* were introduced into p*NXgate32-3HA* vector by in vivo cloning and transformed into the yeast AP5 strain. Yeast-diploid colonies were obtained after mating and selected on the selection medium, and the specific protein-protein interaction was tested by growing yeast on the synthetic minimal medium with or without 200 μM methionine. The results were recorded 4 days after yeast grew at 28°C on the synthetic minimal medium with 200 μM methionine for stringent selection. Yeast two-hybrid assay was performed with MATCHMAKER Two-Hybrid System 3 from Clontech according to manufacturer's handbook. AvrPto was cloned into pGADT7 vector. Pto and the kinase domains of BAK1 (BAK1K) and FLS2 (FLS2K) were cloned into pGBKT7 vector.

The primer sequences of constructs in yeast assays and protoplast transient assays are listed in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental data include 11 figures, Supplemental Experimental Procedures, and Supplemental References and can be found online at http://www.cellhostandmicrobe.com/cgi/content/full/4/1/17/DC1/.

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