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Specific Bacterial Suppressors of MAMP Signaling Upstream of MAPKKK in *Arabidopsis* Innate Immunity

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SUMMARY

Plants and animals possess innate immune systems to prevent infections and are effectively "nonhosts" for most potential pathogens. The molecular mechanisms underlying nonhost immunity in plants remain obscure. In *Arabidopsis*, nonhost/nonpathogenic *Pseudomonas syringae* sustains but pathogenic *P. syringae* suppresses early MAMP (microbe-associated molecular pattern) marker-gene activation. We performed a cell-based genetic screen of virulence factors and identified AvrPto and AvrPtoB as potent and unique suppressors of early-defense gene transcription and MAP kinase (MAPK) signaling. Unlike effectors of mammalian pathogens, AvrPto and AvrPtoB intercept multiple MAMP-mediated signaling upstream of MAPKKK at the plasma membrane linked to the receptor. In transgenic *Arabidopsis*, AvrPto blocks early MAMP signaling and enables nonhost *P. syringae* growth. Deletions of *avrPto* and *avrPtoB* from pathogenic *P. syringae* reduce its virulence. The studies reveal a fundamental role of MAMP signaling in nonhost immunity, and a novel action of type III effectors from pathogenic bacteria.

INTRODUCTION

Molecular mechanisms that distinguish self and nonself are fundamental in innate immunity to prevent potential infections by microorganisms in plants and animals (Ausubel, 2005; Barton and Medzhitov, 2003; Nürnberger et al., 2004). In mammals, Toll-like receptors (TLRs) and nucleotide binding oligomerization domain (NOD) proteins with leucine-rich repeats (LRR) are involved in the recognition of evolutionarily conserved PAMPs or MAMPs (pathogen- or microbe-associated molecular patterns) and activate common signaling pathways involving MAPK cascades and nuclear factor-κB (NF-κB) to induce antimicrobial cytokine and peptide production (Barton and Medzhitov, 2003; Inohara et al., 2005). As in mammals, plants respond to an array of MAMPs from both nonpathogenic and pathogenic microbes (Ausubel, 2005; Nürnberger et al., 2004). However, because plants do not have specialized immune cells, all plant cells appear to have the ability to respond to MAMPs, including flagellin, harpin (HrpZ), lipopolysaccharide (LPS), chitin, and necrosis-inducing proteins (NPP), and activate defense gene transcription and MAPK signaling (Asai et al., 2002; Fellbrich et al., 2002; Lee et al., 2001; Navarro et al., 2004; Ramonell et al., 2005; Zeidler et al., 2004). The best-characterized plant MAMP receptor is the LRR receptor kinase FLS2 that perceives a conserved 22 amino acid peptide (flg22) from bacterial flagellin and activates MAPK cascades and WRKY transcription factors in *Arabidopsis* (Asai et al., 2002; Gomez-Gomez and Boller, 2002). Emerging evidence indicates the importance of MAMP and MAPK signaling in plant defense against pathogenic bacteria and fungi (Ramonell et al., 2005; Zeidler et al., 2004; Zipfel et al., 2004). For example, the *Arabidopsis fls2* mutant is more susceptible than wild-type (wt) to infection by the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000, and the treatment of wt but not the *fls2* mutant plants with flg22 enhances resistance to DC3000 (Zipfel et al., 2004). Activation of the flg22-mediated MAPK cascade confers resistance to both bacterial and fungal pathogens (Asai et al., 2002). A very recent study shows that the flagellin mutant of a nonhost bacterium *P.s. tabaci* causes disease symptoms in *Arabidopsis* (Li et al., 2005).

Many gram-negative bacterial pathogens have evolved type III secretion system (TTSS) to inject effector proteins

into plant and animal cells (Alfano and Collmer, 2004; Galan and Cossart, 2005). A key function of type III effectors in animal pathogens is to block immune responses (Galan and Cossart, 2005). In the case of plant bacterial pathogens, many type III effectors were originally identified as so-called avirulence (Avr) factors that turn virulent strains into avirulent ones. For instance, in the presence of disease resistance (*R*) genes in specific plant genotypes, Avr factors trigger potent gene-for-gene resistance and hypersensitive response (HR), a localized programmed cell death (PCD; Dangl and Jones, 2001; Staskawicz et al., 2001). Interestingly, one *P. syringae* type III effector, AvrPto, triggers disease resistance in tomato plants carrying the corresponding *R* gene *Pto*, that encodes a serine/threonine kinase (Pedley and Martin, 2003). Pto could also recognize another type III effector, AvrPtoB, which shares little sequence similarity with AvrPto (Kim et al., 2002). However, in the absence of Pto, AvrPto actually promotes pathogen growth and virulence (Shan et al., 2000a).

Recently, more type III effectors of plant bacterial pathogens have been observed to promote pathogenicity as the type III effectors of mammalian pathogens (Alfano and Collmer, 2004; Mudgett, 2005). Genetic and functional analyses have revealed that a large number of *P. syringae* type III effectors, including AvrPtoB and a tyrosine protein phosphatase HopPtoD2, suppress *R* gene-mediated PCD in plants (Abramovitch et al., 2003; Espinosa et al., 2003; Jamir et al., 2004). Moreover, many type III effectors, such as AvrPto, AvrRpt2, AvrRpm1, and HopAI1, suppress defense responses elicited either by TTSS-defective mutant bacteria or flg22 (Hauck et al., 2003; Kim et al., 2005; Li et al., 2005; Oh and Collmer, 2005). It remained unresolved how a large number of type III effectors shared similar but unknown molecular actions.

Nonhost immunity is the most prevalent form of plant defense against a broad spectrum of potential pathogens. Currently, the molecular mechanisms underlying nonhost immunity and pathogenicity are obscure. It is also unclear whether nonhost and gene-for-gene defense pathways share the same regulatory mechanisms (Mysore and Ryu, 2004; Thordal-Christensen, 2003). The conventional phenotypic tests that have been used to characterize the plant immune responses, including PCD, cell wall modification, pathogenesis-related (*PR*) gene activation and the inhibition of bacterial growth, measure relatively late outcomes in plant defense. To distinguish different defense pathways and to differentiate defense suppression mechanisms by type III effectors, we tested the idea of using molecular markers and biochemical assays to monitor specific and early-defense responses. We first investigated the activation of early MAMP-specific marker genes (Asai et al., 2002) in *Arabidopsis* leaves inoculated with a naturally nonhost/nonpathogenic bacterial strain, *P. syringae* pv. *phaseolicola* NPS3121 (*Psp* NPS3121). Although not a pathogen in *Arabidopsis*, *Psp* NPS3121 rapidly activated *FRK1* and other MAMP-specific early-defense genes. Interestingly, the same marker-gene activation was also observed in *Arabidopsis* leaves inoculated with DC3000 and a DC3000 TTSS mutant, but the expression appeared to be subsequently suppressed only by virulent DC3000. Using a cell-based genetic screen of type III effectors as virulence factors, we discovered that AvrPto and AvrPtoB from DC3000 were specific suppressors of the MAMP-mediated early-defense responses, including transcription activation and MAPK signaling occurring within minutes of elicitation. AvrPto and AvrPtoB specifically targeted the MAMP signaling pathways, which can be uncoupled from some gene-for-gene-mediated transcription and PCD in *Arabidopsis*. Distinct from type III effectors of mammalian bacterial pathogens that directly target MAPKK and MAPK (Galan and Cossart, 2005; Orth et al., 1999), AvrPto and AvrPtoB acted upstream of MAPKKK in MAMP signaling near the plasma membrane receptor. Mutagenesis analysis of AvrPto and AvrPtoB revealed that their virulence activity in *Arabidopsis* was different from that in tomato and tobacco, in which AvrPto and AvrPtoB can also activate gene-for-gene responses. The new findings provide strong evidence for dynamic coevolution of type III effector actions in individual plant-bacterium warfare. Importantly, AvrPto blocks early MAMP signaling and facilitates the growth of two nonhost *P. syringae* strains in plants, verifying the robustness of cell-based genetic screens. Our results suggest that plant MAMP signaling is essential in nonhost immunity and shed new light on the molecular action of type III virulence effectors.

RESULTS

Type III Effector-Mediated Suppression of MAMP-Specific Early-Defense Gene Induction

Purified MAMPs have been shown to activate specific transcription programs in *Arabidopsis*, parsley, and tobacco (Asai et al., 2002; Lee et al., 2001; Navarro et al., 2004; Ramonell et al., 2005; Zeidler et al., 2004). For example, flg22 treatment of *Arabidopsis* protoplasts, leaves, and seedlings leads to the rapid induction of *FRK1* (Asai et al., 2002; see Table S1 in the Supplemental Data available with this article online). To investigate the expression of MAMP marker genes in natural plant-microbe interactions, we compared the activation of *FRK1* by various *P. syringae* strains, including the nonhost *Psp* NPS3121, the virulent strain DC3000, and a DC3000 TTSS mutant *hrcC* in *Arabidopsis* leaves. Quantitative RT-PCR analysis showed that all three bacterial strains strongly activated *FRK1* expression 2 hr postinoculation (hpi; Figure 1A). Significantly, *FRK1* activation was reduced by 6 hpi with the virulent strain but was maintained or enhanced further following infection with the nonhost or the TTSS mutant strain. Simultaneous inoculation with the virulent and the nonhost or the TTSS mutant strains did not diminish the apparent suppression of *FRK1* expression by DC3000 (Figure 1A). The data suggest that DC3000 likely secretes type III effectors to shut down early-defense signaling.

The expression kinetics of *FRK1* calculated from AtGen Express microarray database (http://Arabidopsis.org/info/expression/ATGenExpress.jsp) in *Arabidopsis* leaves

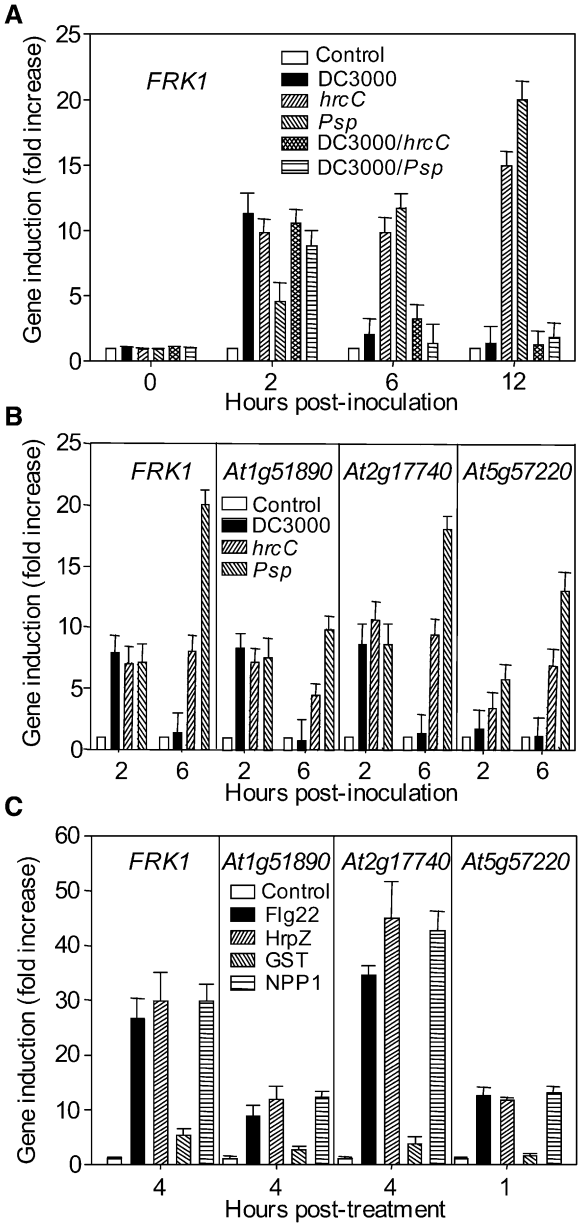

Figure 1. Virulent *Pseudomonas* Inhibits but Nonhost *Pseudomonas* Potentiates MAMP-Mediated Early Gene Expression

(A) Real-time RT-PCR analysis of *FRK1* expression in *Arabidopsis* leaves. Inoculation was performed with $MgCl_2$ (Control), DC3000, DC3000*hrcC*, *Psp* NPS3121 (*Psp*), and the combination of DC3000 with DC3000*hrcC* or *Psp* NPS3121 at 1×10^8 cfu/ml. The gene induction (fold change) by bacterial infiltration was compared to the expression level of $MgCl_2$ infiltration. The data are shown as means ± standard errors from three independent biological replicates.

(B) Expression patterns of *FRK1*, *At1g51890*, *At2g17740*, and *At5g57220* in *Arabidopsis* leaves inoculated with different *Pseudomonas* strains. The triplicated data were searched and presented as means ± standard errors from AtGenExpress microarray results published on TAIR (The *Arabidopsis* Information Resource) website.

was very similar to that quantified in our experiments by RT-PCR (Figure 1B). In addition, we collected data from AtGenExpress for three other genes (*At1g51890*, *At2g17740*, and *At5g57220*) that were highly activated by flg22 in *Arabidopsis* protoplasts, leaves, and seedlings (Table S1). All three genes showed similar expression patterns as *FRK1*, including a strong diminution following infection with DC3000 by 6 hpi (Figure 1B). It appears that the diminution of *FRK1*, *At1g51890, At2g17740*, and *At5g57220* expression during early DC3000-*Arabidopsis* interactions (2–12 hr) involves specific mechanisms for the suppression of these genes because no bacterial proliferation, cell death, or general repression of genes was observed (He et al., 2004; AtGenExpress; Table S2). Moreover, these early-defense marker genes were strongly activated by multiple MAMPs, including the bacterial elicitors flg22 and HrpZ and the fungal/oomycete elicitor NPP1 (Figure 1C), but not by other stress-related signals based on a survey of available global gene-expression profiles (AtGenExpress).

AvrPto and AvrPtoB Are Specific Suppressors of Early-Defense Signaling

To screen for type III effectors in suppressing the early-defense-related gene induction, we transiently expressed individual type III effectors in *Arabidopsis* mesophyll protoplasts and determined their effects on the activation of the *FRK1-LUC* reporter gene by flg22 (Asai et al., 2002). These bacterial effector proteins were well expressed in plant cells (Figure 2A). We examined HopPtoD2, HopPtoE, HopPtoK, AvrPto (DC), and AvrPtoB from DC3000, all of which displayed host defense-suppression activities by other assays (Abramovitch et al., 2003; Espinosa et al., 2003; Hauck et al., 2003; He et al., 2004; Jamir et al., 2004). Surprisingly, AvrPto (DC) and AvrPtoB, but not the other effectors tested, suppressed flg22 activation of *FRK1-LUC* (Figure 2B). The effect of AvrPto from DC3000 on suppressing MAMP signaling was as potent as AvrPto from *Pst* JL1065 (which differs in four amino acids; Figure 2B). Here, we designate AvrPto from *Pst* JL1065 as AvrPto and AvrPto from DC3000 as AvrPto (DC).

We extended our screen to several well-studied Avr effectors that have also been shown to have virulence activities, including AvrRpm1, AvrB, and AvrRpt2. Because the expression of AvrRpm1 and AvrB induced rapid PCD in wt *Arabidopsis* protoplasts (P.H. and L.S., unpublished data), we expressed AvrRpm1 and AvrB in the *rps3* mutant protoplasts, which lack functional RPM1 protein. Interestingly, although it has been reported that AvrRpm1 blocked flg22-elicited callose deposition (Kim et al., 2005), AvrRpm1 and AvrB did not suppress flg22 activation of the *FRK1* promoter (Figure 2C). Similarly, AvrRpt2 did not suppress

(C) Convergent gene activation by flg22, HrpZ, and NPP1 in *Arabidopsis* leaves. GST protein was used as a control for the NPP1-GST fusion protein. The peak induction level (means ± standard errors) from the AtGenExpress triplicated data is shown.

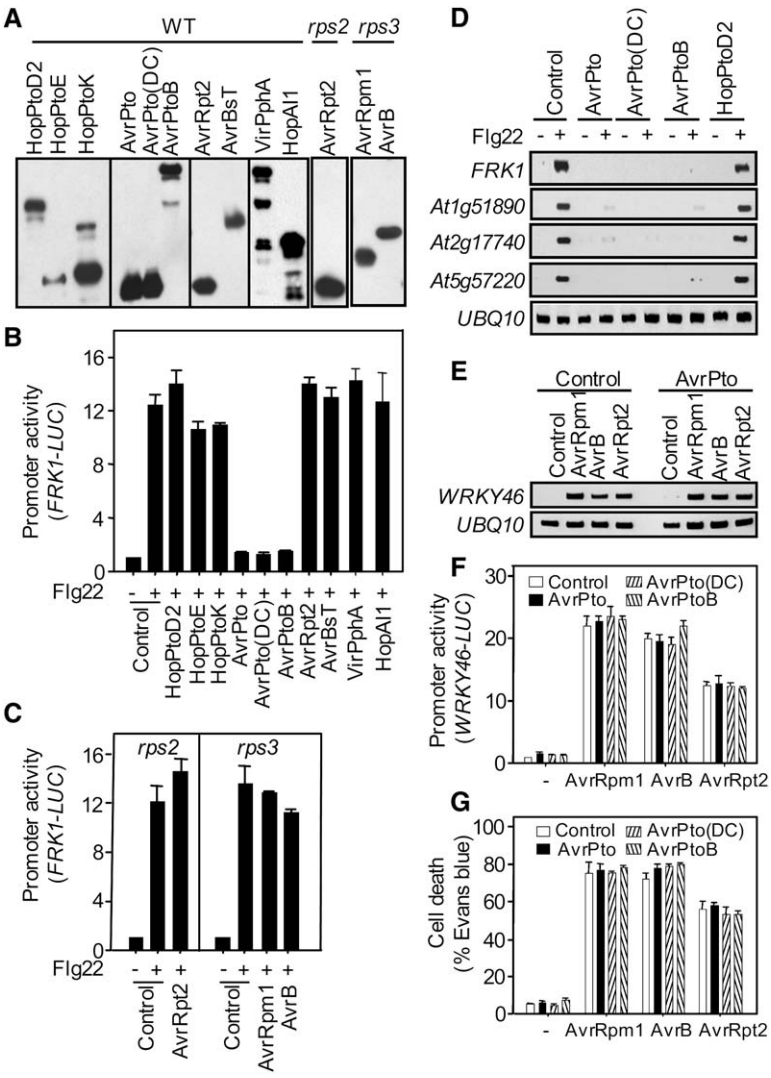

Figure 2. AvrPto and AvrPtoB Specifically Suppress Early MAMP Marker-Gene Activation by Flg22

(A) Protein expression of effectors in wt, *rps2*, and *rps3* mutant *Arabidopsis* protoplasts. Expression was detected by Western blot using an anti-HA antibody.

(B) Suppression of flg22-induced *FRK1* promoter activity by AvrPto and AvrPtoB. Protoplasts were cotransfected with an effector and a *LUC* reporter and incubated for 6 hr before treated with 100 nM flg22 for 3 hr.

(C) AvrRpt2, AvrRpm1, and AvrB do not suppress flg22-induced *FRK1* promoter activity in *rps2* or *rps3* protoplasts.

(D) RT-PCR analysis of AvrPto and AvrPtoB suppression. Transfected protoplasts were incubated for 6 hr before treated with 1 μM flg22 for 1 hr. *UBQ10* was used as an internal control.

(E) AvrPto does not affect endogenous *WRKY46* transcription activation by AvrRpm1, AvrB, and AvrRpt2.

(F) AvrPto and AvrPtoB do not affect *WRKY46* promoter activation by AvrRpm1, AvrB, and AvrRpt2.

(G) AvrPto and AvrPtoB do not interfere with cell death induced by AvrRpm1, AvrB, and AvrRpt2. Cell death was observed by Evans blue staining 16 hr after transfection.

All data are the representatives of four independent replicates and the pooled data are shown as means ± standard errors.

FRK1-LUC in either wt or *rps2* protoplasts, which lack functional RPS2 (Figures 2B and 2C). Recently, nine effectors were identified as the suppressors of flg22-induced *NONHOST1* (*NHO1*) expression (Li et al., 2005). However, HopAI1, one of the most potent suppressors of *NHO1*, did not affect flg22-induced early and specific MAMP reporter gene *FRK1-LUC* (Figure 2B). As reported, HopAI1 suppressed flg22-induced *NHO1* expression in the protoplasts (Figure S1). The results suggest that distinct mechanisms are utilized by type III effectors to suppress different defense responses occurring at different time points or steps during infection.

Although *avrPto*-like sequences exist only in a small subset of *P. syringae* strains, *avrPtoB*-like sequences are more widely distributed in *P. syringae* strains, including the nonhost *Psp* (Kim et al., 2002). Analysis of the whole-genome sequence of *Psp* strain 1448A did not identify *avrPto*-like sequences, and the *avrPtoB*-like sequence only produced a truncated protein (Figure S2). We also cloned and tested *virPphA*, an *avrPtoB* homolog from *Psp* NPS3121. Surprisingly, unlike AvrPtoB, VirPphA did not suppress flg22 induction of *FRK1-LUC* (Figure 2B). Similar to the reporter-gene assays, the activation of endogenous *FRK1* expression by flg22 was significantly blocked by AvrPto, AvrPto (DC), and AvrPtoB as shown by RT-PCR analysis (Figure 2D). Importantly, the flg22-induced expression of other MAMP marker genes, *At1g51890*, *At2g17740*, and *At5g57220*, was also substantially suppressed by AvrPto, AvrPto (DC), and AvrPtoB (Figure 2D).

AvrPto and AvrPtoB Do Not Interfere with Gene-for-Gene Defense in *Arabidopsis*

Many studies have suggested that several type III effectors, including AvrPtoB, can interfere with PCD induced by gene-for-gene interactions or other signals in *Arabidopsis*, tobacco, and tomato (Abramovitch et al., 2003; Jamir et al., 2004). We used the protoplast transient assay to test whether AvrPto or AvrPtoB could suppress Avr-R-mediated responses, including defense-gene activation and elicitation of cell death by AvrRpm1, AvrB, and AvrRpt2.

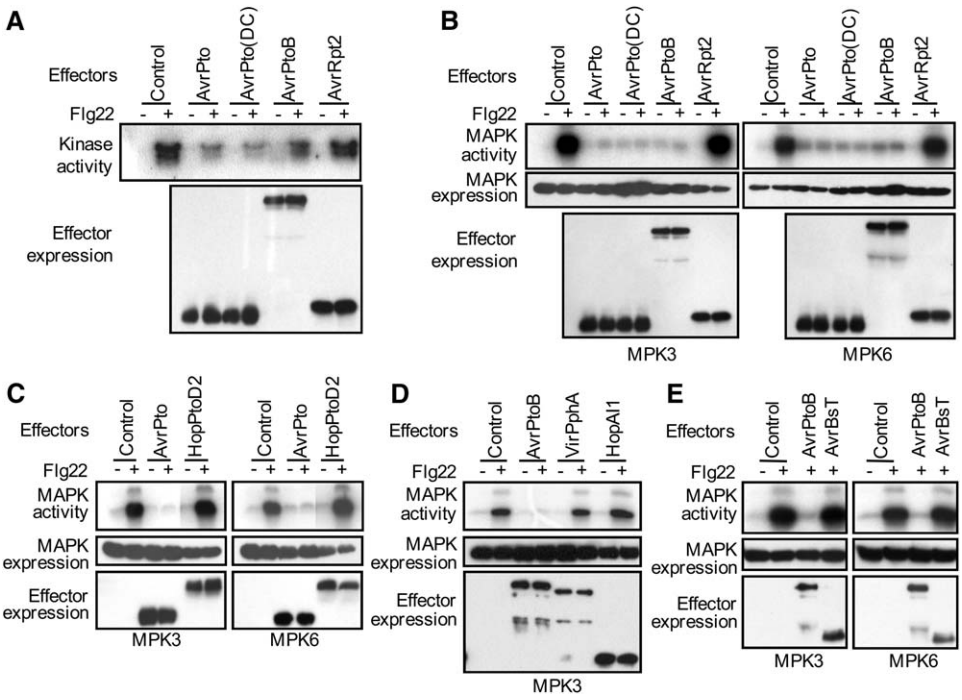

Figure 3. AvrPto and AvrPtoB Block MAPK Activation by Flg22

(A) AvrPto and AvrPtoB block endogenous MAPK activation by flg22. Transfected protoplasts were incubated for 6 hr before 1 μM flg22 treatment for 10 min. The kinase activity was detected by an in-gel kinase assay (top). Effector protein expression was examined by Western blot using an anti-HA antibody (bottom).

(B) AvrPto and AvrPtoB inhibit flg22 activation of MPK3 and MPK6. HA-tagged MPK3 or MPK6 was coexpressed with FLAG-tagged effectors. Transfected protoplasts were incubated for 6 hr before 1 μM flg22 treatment for 10 min. An anti-HA antibody was used for immunoprecipitation of MPK3 or MPK6. Kinase activity was detected by an in vitro kinase assay (top). Protein expression is shown for MAPKs (middle) and effectors (bottom).

(C) HopPtoD2 does not affect MPK3 and MPK6 activation by flg22.

(D) VirPphA and HopAI1 do not affect flg22 activation of MPK3.

(E) AvrBsT does not suppress flg22 activation of MPK3 and MPK6.

All of the above experiments were repeated three times with similar results.

Because these effectors do not appear to activate the MAMP-specific genes in *Arabidopsis*, we selected another marker gene, *WRKY46*, for these studies (P.H. and L.S., unpublished data). AvrPto or AvrPtoB was cotransfected with AvrRpm1, AvrB, or AvrRpt2 in wt protoplasts. AvrRpm1, AvrB, and AvrRpt2 induced endogenous *WRKY46* (Figure 2E), *WRKY46-LUC* (Figure 2F), and cell death (Figure 2G) equally well in the presence or absence of AvrPto or AvrPtoB, suggesting the occurrence of normal gene-for-gene-mediated defense signaling. Our results obtained using protoplast transient assays are consistent with the finding obtained in AvrPto-expressing plants, in which PCD (HR) occurred normally in response to DC3000 (*avrRpt2*) (Hauck et al., 2003). In addition, conjugated DC3000, carrying functional *avrPto* and *avrPtoB*, with plasmid expressing *avrRpm1*, *avrB*, or *avrRpt2* could trigger gene-for-gene responses in *Arabidopsis* ecotype Col-0. Thus, AvrPto and AvrPtoB appear to specifically inhibit MAMP-mediated but not the Avr-R defense signaling in *Arabidopsis*. Moreover, because AvrPto and AvrPtoB did not affect the expression of the endogenous *UBQ10* gene, the cotransfected *UBQ10-GUS* (Figures 2E and 2F), or a variety of other genes and promoters (data not shown), it does not seem likely that these effectors nonspecifically killed the protoplasts resulting in an apparent suppression of early-defense gene expression.

AvrPto and AvrPtoB Block MAPK Activation in Early Flg22 Signaling

MAPK activation is a convergent and evolutionarily conserved event in the earliest MAMP signaling in plants and animals (Asai et al., 2002; Barton and Medzhitov, 2003; Nürnberger et al., 2004). To elucidate the molecular mechanisms underlying AvrPto and AvrPtoB suppression of early MAMP signaling, we investigated the activation of endogenous MAPKs by flg22 in transfected protoplasts expressing AvrPto or AvrPtoB. Both AvrPto and AvrPtoB significantly blocked MAPK activation by flg22 (Figure 3A). The remaining low level of MAPK activation is most likely due to flg22 activation in untransfected cells since the protoplast transfection efficiency is about 90%. It has been shown that flg22 specifically activates MPK3 and MPK6 in *Arabidopsis* (Asai et al., 2002). To determine whether AvrPto and AvrPtoB suppress the activation of these same

MAPKs by flg22, a construct expressing epitope-tagged MPK3 or MPK6 was cotransfected into protoplasts. As shown in Figure 3B, the activation of MPK3 and MPK6 by flg22 was completely blocked by AvrPto and AvrPtoB but not by AvrRpt2.

HopPtoD2, a tyrosine protein phosphatase, blocks MAPK-associated cell death in plants (Espinosa et al., 2003). However, it was ineffective in blocking MPK3 and MPK6 activation by flg22 (Figure 3C). Consistent with the analysis of early gene activation, VirPphA from *Psp* NPS 3121 and HopAI1 from DC3000 did not block MPK3 and MPK6 activation by flg22 (Figure 3D and data not shown).

In mammalian cells, it has been shown that the *Yersinia* type III effector YopJ, a cysteine protease, can bind directly to MKKs and block both MAPK and IKK-NF-κB signaling pathways (Orth et al., 1999). However, AvrBsT, a YopJ-related protein from *Xanthomonas campestris* pv. *vesicatoria* (Mudgett, 2005), did not block the activation of MPK3 and MPK6 induced by flg22 (Figure 3E). Similarly, AvrBsT did not inhibit flg22 activation of *FRK1-LUC* (Figure 2B). Expressing YopJ in *Arabidopsis* protoplasts also did not affect MAPK activation by flg22 (data not shown). It appears unlikely from these results that AvrBsT targets plant MKKs, at least in *Arabidopsis*.

AvrPto and AvrPtoB Intercept the Early-Defense Signaling Activated by Several MAMPs

Plant innate immune responses are triggered by a variety of different MAMPs, and although different MAMPs are probably perceived by distinct receptors, convergent early-signaling events, including MAPK activation and specific defense-gene induction, have been observed in *Arabidopsis* plants and mesophyll protoplasts (Asai et al., 2002; Fellbrich et al., 2002; Lee et al., 2001; Navarro et al., 2004). To determine whether AvrPto interrupts the immune responses activated by MAMPs other than flg22, we treated AvrPto- or AvrPtoB-transfected protoplasts with HrpZ and NPP1. Similar to flg22, both HrpZ and NPP1 activated *FRK1* promoter in transfected *Arabidopsis* protoplasts, and the activation of this promoter was dramatically inhibited in the presence of AvrPto, AvrPto (DC), or AvrPtoB (Figure 4A). Moreover, AvrPto and AvrPtoB abolished MPK3 and MPK6 activation by HrpZ and NPP1 (Figure 4B). These results indicate that diverse MAMPs activate common *Arabidopsis* innate immunity-signaling pathways.

AvrPto and AvrPtoB Suppress MAMP Signaling Upstream of MAPKKK

To further elucidate the molecular mechanism of AvrPto and AvrPtoB action in suppressing MAMP signaling, we carried out epistasis analysis of the flg22 signaling pathway using gain-of-function components that activate either MPK3/MPK6 or *FRK1-LUC* in the absence of flg22 signal (Asai et al., 2002). As shown in Figure 5A, ectopic expression of WRKY22 or WRKY29 bypassed AvrPto suppression, suggesting that AvrPto inhibits flg22 signaling

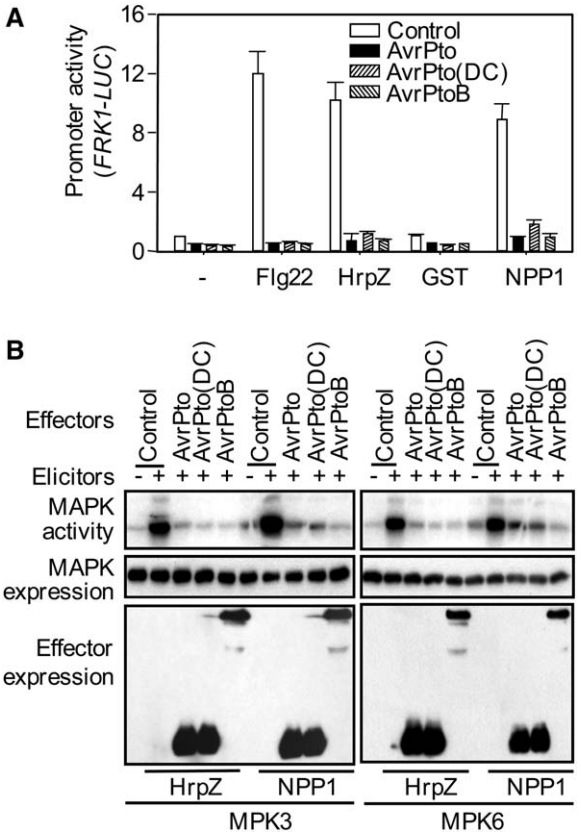

Figure 4. AvrPto and AvrPtoB Block Early-Defense Gene and MAPK Activation by HrpZ and NPP1

(A) AvrPto and AvrPtoB suppress *FRK1* promoter activation by multiple MAMPs. Transfected protoplasts were incubated for 6 hr and treated with 100 nM flg22, 1 μM HrpZ, 20 nM GST, or 20 nM NPP1-GST for 3 hr. The data are shown as means ± standard errors from four repeats.

(B) AvrPto and AvrPtoB intercept activation of MPK3 and MPK6 by HrpZ and NPP1. Transfected protoplasts were incubated for 6 hr before treatment with 1 μM HrpZ, 20 nM GST, or 20 nM NPP1-GST for 10 min. The experiments were repeated three times with similar results.

upstream of WRKY transcription factors. Similar results were obtained for AvrPto (DC) and AvrPtoB (data not shown). Moreover, constitutively active MKK4/5 or MEKK1, which activates MPK3/6 in the absence of flg22 (Asai et al., 2002), overrode AvrPto and AvrPtoB suppression (Figures 5B and 5C and data not shown), indicating that AvrPto and AvrPtoB block MAMP signaling very early, probably immediately after signal perception at or upstream of MAPKKK.

Mutational Analysis of AvrPto and AvrPtoB as Novel Suppressors of MAMP Signaling

To further investigate the activity requirement of AvrPto as a virulence effector in *Arabidopsis* cells, we analyzed a set of well-defined AvrPto mutants (Table S3 and Figure 6B) that structurally separate distinct avirulence and virulence functions in tomato and tobacco (Shan et al., 2000a,

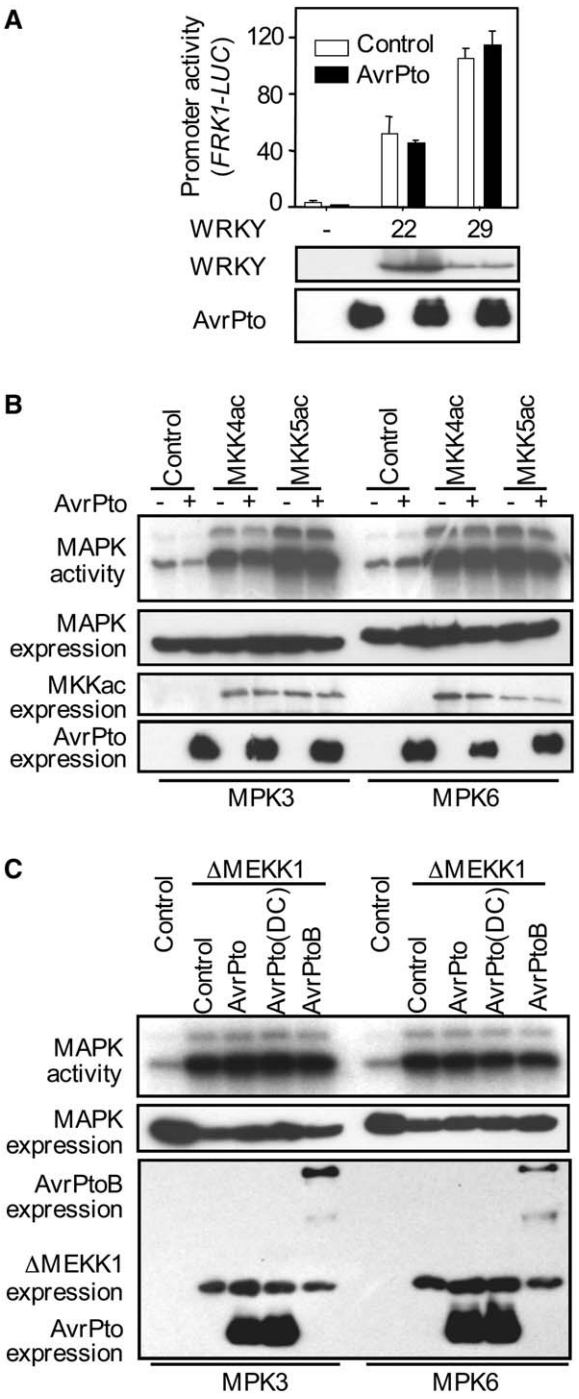

Figure 5. AvrPto and AvrPtoB Suppressors Act Upstream of MAPKKK

(A) AvrPto functions upstream of WRKY transcription factors. WRKY transcription factors were coexpressed with *FRK1-LUC*, *UBQ10-GUS*, and AvrPto. The expression of WRKYs and AvrPto is shown. The data are shown as means ± standard errors.

(B) AvrPto does not inhibit MKK4/5 activation of MPK3/6. Protoplasts were transfected with HA-tagged MAPK, MYC-tagged constitutively active MKK (MKKac), and FLAG-tagged AvrPto.

(C) AvrPto and AvrPtoB do not block MEKK1 activation of MPK3/6. Protoplasts were transfected with HA-tagged MAPK, FLAG-tagged constitutively active MEKK1 (ΔMEKK1), and FLAG-tagged effectors. All of the above experiments were repeated four times with similar results.

2000b). As in transgenic tobacco (Shan et al., 2000b), AvrPto-GFP was properly targeted to the plasma membrane in *Arabidopsis* protoplasts (Figure 6A). The G2A mutation of the myristoylation site in AvrPto disrupted its membrane localization (Figure 6A) as well as its functions in suppressing *FRK1-LUC* (Figure 6C) and MPK3/6 (Figure 6D) activation by flg22. Three other point mutations, S46P, S94P, and I96T, but not P146L and S147R, also abolished the AvrPto suppressor functions in *Arabidopsis* (Figures 6C and 6D). S94 and I96 were only required for AvrPto interaction with Pto and the avirulence function in tomato, but not the virulence function in tomato or the avirulence function in tobacco. P146L and S147R mutants specifically blocked avirulence function in tobacco but still kept the interaction with tomato Pto (Shan et al., 2000b). It is intriguing that residues required for the specific interaction between AvrPto and tomato Pto were also important for AvrPto in blocking MAMP-mediated defense in *Arabidopsis* (Table S3). To further examine this observation, we tested whether expression of tomato Pto in *Arabidopsis* could inhibit the AvrPto suppressor function in MAMP signaling. As shown in Figure 6E, expression of Pto partially relieved the AvrPto-mediated suppression of *FRK1-LUC* induction by flg22. However, it is likely that AvrPto is very effective with multiple targets and cannot be completely sequestered by the ectopic expression of tomato Pto in *Arabidopsis* cells. The mutational analysis suggests that plasma membrane localization and protein-protein interaction via S46, S94, and I96 are essential for the suppressor activity of AvrPto.

Because AvrPto acts at the plasma membrane and blocks MAPK activation upstream of MAPKKK within minutes of MAMP elicitation, and the flg22 receptor FLS2 shares a Pto-like kinase domain (Shiu and Bleecker, 2003), we tested the ability of FLS2 to interfere with the AvrPto suppressor activity in early signaling initiated by multiple MAMPs in *Arabidopsis*. Significantly, FLS2 could partially interfere with the suppressor activity of AvrPto, although expression of FLS2 alone did not enhance the flg22 response (Figure 6E). The interference apparently required the membrane localization of FLS2 because overexpression of ΔFLS2 with only the kinase domain did not affect the AvrPto function (Figure 6E). The steady-state level of the FLS2 protein was too low to carry out coimmunoprecipitation experiments with AvrPto. However, the low level of FLS2 expression was also sufficient to partially relieve the suppression of HrpZ and NPP1 signaling by AvrPto (Figure 6F).

Recent studies have discovered that AvrPtoB carries an E3 ubiquitin ligase activity, and its conserved E2 binding residues are required to suppress PCD activated by a gene-for-gene response in tomato (Janjusevic et al.,

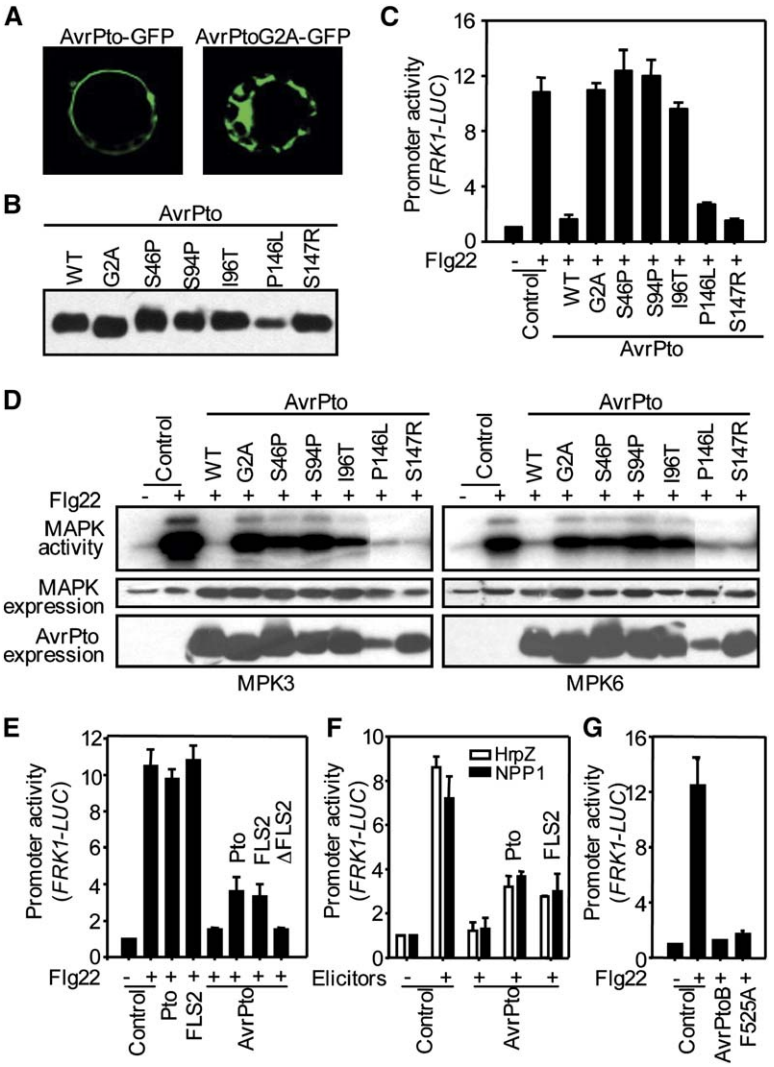

Figure 6. Mutational Analysis of AvrPto and AvrPtoB Suppressor Function

(A) Membrane localization of AvrPto in protoplasts. The pictures were taken 12 hr after transfection with AvrPto-GFP or AvrPtoG2A-GFP mutant in the protoplasts using confocal microscope.

(B) Expression of different AvrPto mutants.

(C) Effect of flg22-activated *FRK1* promoter by different AvrPto mutants.

(D) Effect of flg22-activated MPK3/6 by different AvrPto mutants.

(E) Pto and FLS2 partially interfere with AvrPto suppressor function in flg22 signaling. A construct expressing the tomato Pto or the *Arabidopsis* FLS2 was cotransfected with a plasmid expressing AvrPto under the control of the dexamethasone-inducible promoter. Transfected protoplasts were incubated for 4 hr to express Pto or FLS2 first before treated with 20 μM dexamethasone (DEX) to induce AvrPto for 3 hr and then treated with 100 nM flg22 for 3 hr. Expression of Pto or FLS2 alone did not affect *FRK1-LUC* activation.

(F) Pto and FLS2 partially interfere with AvrPto suppressor function in HrpZ and NPP1 signaling.

(G) A mutation in E2 binding residue of AvrPtoB does not affect its suppressor function in flg22 signaling.

The experiments were repeated at least three times and the pooled data are shown as means ± standard errors.

2006). Since AvrPto does not carry the E3 ligase sequence, the suppressor function shared by AvrPto and AvrPtoB in *Arabidopsis* is likely uncoupled from the E3 ligase activity of AvrPtoB. Consistent with this hypothesis, a key mutation in one of the E2 binding residues F525 abolished AvrPtoB E3 ligase and anti-PCD activities in tomato (Janjusevic et al., 2006) but did not affect its suppressor function in flg22 signaling in *Arabidopsis* (Figure 6G).

AvrPto Impaired Nonhost Immunity and Enabled Nonhost Bacteria Growth in Transgenic Plants

To determine the importance of MAMP signaling in nonhost defense, we tested whether inducible expression of AvrPto could inhibit nonhost immunity in intact plants. We generated *avrPto* transgenic plants under the control of the dexamethasone-inducible promoter (McNellis et al., 1998). As shown in protoplasts, AvrPto was localized to the plasma membrane in transgenic plants (Figure S3). Significantly, AvrPto expression enabled the growth of two nonhost strains *Psp* NPS3121 and *P.s. tabaci* (Figures 7A and S4). Consistent with transient expression of AvrPto in protoplasts, the expression of AvrPto in stable transgenic plants suppressed flg22-activated endogenous MAPKs (Figure 7B), and the induction of *FRK1*, *At1g51890*, *At2g17740*, and *At5g57220* by flg22, DC3000 *hrcC*, and *Psp* NPS3121 (Figure 7C). In addition, expression of AvrPto in transgenic plants also suppressed MPK3/6 activation by flg22 (Figure S5). The suppression appeared to act upstream of MAPKKK because preexpression of AvrPto at a high level could not block MEKK1 activity (Figure S6). The transgenic plant analyses validated the protoplast transient assays and demonstrated that expression of a single type III effector in plant cells can suppress nonhost immunity.

Both AvrPto and AvrPtoB Contribute to DC3000 Virulence in *Arabidopsis*

To determine the effect of *avrPto* and *avrPtoB* on DC3000 virulence in *Arabidopsis*, we dip-inoculated Col-0 plants with the DC3000 *avrPto* or *avrPtoB* deletion mutant, or

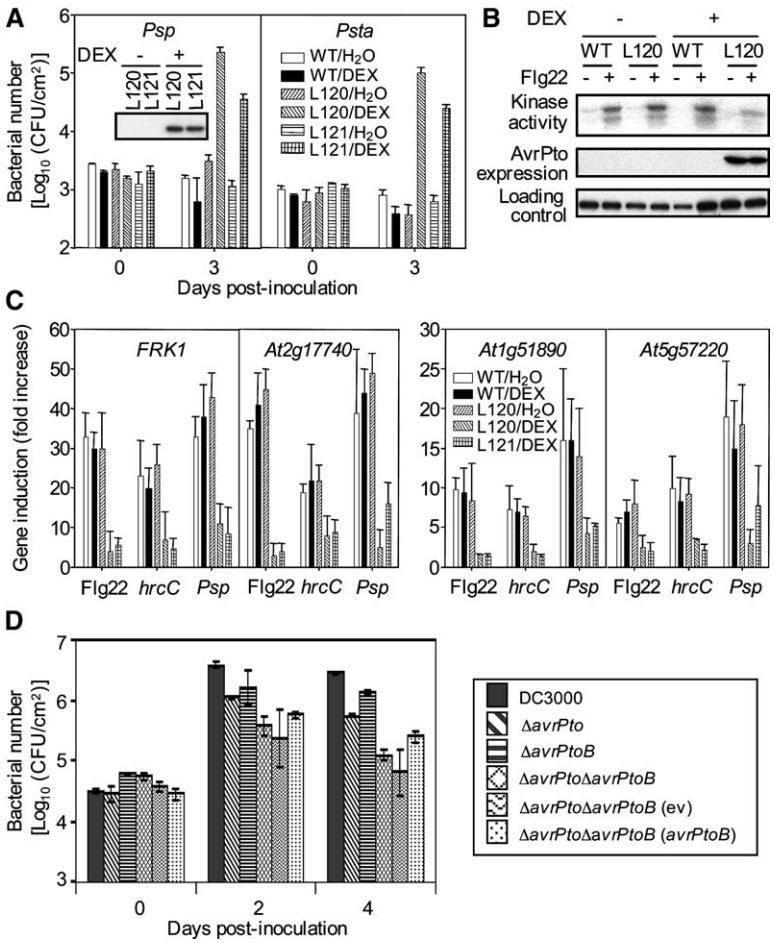

Figure 7. Analysis of *avrPto* Transgenic Plants and DC3000 Deletion Mutants

(A) AvrPto transgenic plants support nonhost bacteria growth. *Arabidopsis* wt or *avrPto* transgenic plant (L120 and L121) leaves were inoculated with *Psp* NPS3121 (*Psp*), or *P.s. tabaci* (*Psta*) at 5 × 10^5 cfu/ml. The experiment was repeated three times with similar results. AvrPto protein expression after DEX treatment is shown.

(B) Expression of AvrPto suppresses flg22 activation of endogenous MAPKs. *Arabidopsis* wt or *avrPto* transgenic plant (L120) leaves were collected 30 min after infiltration with 10 μM flg22. The experiment was repeated twice with similar results.

(C) Real-time RT-PCR analysis of MAMP marker gene induction. *Arabidopsis* wt or *avrPto* transgenic plant (L120 and L121) leaves were collected 6 hpi with H_2O, flg22 (10 μM), or bacteria, DC3000*hrcC* or *Psp* NPS3121 at 1 × 10^8 cfu/ml. The gene induction (fold change) by bacterial infiltration was compared to the expression level of control infiltration. The data are the average of three independent replicates.

(D) Bacterial growth assay of DC3000 deletion mutants. *Arabidopsis* plants were dipped with DC3000 or *avrPto* or/and *avrPtoB* mutant bacterial strains for 30 s at the density of 1 × 10^7 cfu/ml. (ev): empty vector; (*avrPtoB*): complementation with *avrPtoB*.

The data are shown as means ± standard errors.

Δ*avrPto*Δ*avrPtoB* double mutant (Lin and Martin, 2005). Two days after inoculation, populations of Δ*avrPto* and Δ*avrPtoB* were 3- and 2-fold, respectively, lower than that of DC3000, whereas the bacterial number of the double mutant was reduced by more than 10-fold (Figure 7D). Ectopic expression of *avrPtoB* partially restored bacterial growth of Δ*avrPto*Δ*avrPtoB*. Thus, both AvrPto and AvrPtoB appear to contribute to the pathogenicity of DC3000 in *Arabidopsis*. The Δ*avrPto*Δ*avrPtoB* double mutant partially reduced the suppression activity of DC3000 on *FRK1* expression (data not shown).

DISCUSSION

Although plant cells have long been known to respond to diverse MAMPs, there has been limited molecular and genetic evidence supporting a major role of the conserved and convergent MAMP signaling pathways in natural plant-microbe encounters (Gomez-Gomez and Boller, 2002; Nürnberger et al., 2004). Using MAMP-specific early-defense marker genes, our data show that MAMP signaling was rapidly activated by both pathogenic and nonpathogenic *Pseudomonas* strains in *Arabidopsis* leaves. However, in the interactions with a virulent strain, but not a nonhost strain or a TTSS-deficient mutant, MAMP signaling was subsequently repressed. To investigate the underlying molecular mechanisms, we established a cell-based genetic screen focusing on the earliest defense responses and identified AvrPto and AvrPtoB as specific suppressors of early MAMP signaling. Distinct from the *Yersinia* type III effector YopJ, which blocks MKK and IKK signaling in mammalian innate immunity, AvrPto and AvrPtoB most likely exert their suppressor activities upstream of MAPKKK near the plasma membrane MAMP receptors. Mutagenesis analysis of AvrPto reveals the importance of plasma membrane localization and protein-protein interaction for its suppressor action. Importantly, AvrPto suppresses early MAMP signaling and impairs nonhost immunity in plants, and the deletions of *avrPto* and *avrPtoB* from DC3000 reduce its virulence. Previous studies already indicate the importance of MAMP and MAPK signaling in plant defense against infections (Asai et al., 2002; Zipfel et al., 2004). Together, our results provide molecular evidence that the convergent and sustained MAMP signaling may prevent most microorganisms from infection in most plants while successful virulent bacteria inject specific type III effectors targeting the early MAMP signaling pathways.

Rapid Activation of MAMP Signaling in Plant Immunity

Direct exposure to diverse purified MAMPs has been shown to rapidly activate MAPK signaling and alter transcription programs in *Arabidopsis*, parsley, and tobacco (Asai et al., 2002; Fellbrich et al., 2002; Lee et al., 2001; Ligterink et al., 1997; Navarro et al., 2004; Ramonell et al., 2005; Zeidler et al., 2004; Zhang and Klessig, 2001). Moreover, bacterial extracts suppress DC3000 growth in the absence of the flg22 receptor FLS2 in *Arabidopsis* (Zipfel et al., 2004), indicating that other MAMPs are likely recognized during plant-microbe interactions to activate rapid changes in transcription profiles.

Real-time RT-PCR analysis of the early-defense genes (Figures 1 and 7) shows that specific and convergent MAMP-mediated defense responses occur rapidly after inoculation with different bacteria. The data are consistent with the results from triplicated microarray experiments from the AtGenExpress project (Figure 1B). Similar early and robust *FRK1* activation by DC3000, DC3000 *hrpA*, and DC3000 (*avrRpm1*) has been observed in *Arabidopsis* leaves (de Torres et al., 2003). However, infiltration with virulent bacteria carrying different effector genes in leaf cells showed very complex gene-expression patterns, including genes induced by wounding, flooding, and cell death (de Torres et al., 2003; Tao et al., 2003). It has been suggested that nonhost, basal, MAMP, and gene-for-gene defense responses are very similar and only differ in quantitative nature and timing (Mysore and Ryu, 2004; Navarro et al., 2004; Tao et al., 2003). Our data reveal distinct defense gene regulation in early gene-for-gene and MAMP signaling pathways (Figure 2; P.H., L.S., and J.S., unpublished data). Further comparison of *Arabidopsis* gene-expression profiles induced by purified MAMPs, individual type III effectors, and different bacterial strains using higher stringency (e.g., low p value and high log2 ratio) at different time points (earlier than 3 hr) will more precisely define genes that are either specific or common to MAMP-mediated and gene-for-gene defense pathways or induced by abiotic stresses.

Stability and robustness of inducible nonhost defense is the likely consequence of activation of multiple signaling pathways against a broad range of microorganisms (Mysore and Ryu, 2004; Thordal-Christensen, 2003). Recent new findings in the genetic dissection of nonhost immunity include the isolation of the *Arabidopsis nho1* and *pen* (*penetration*) mutants that are more susceptible to *Psp* NPS3121 and *Blumeria graminis* f. sp. *Hordei*, respectively (Kang et al., 2003; Lipka et al., 2005). However, the regulation and function of *NHO1*, encoding a glycerol kinase, is complex (Eastmond, 2004). It is not only activated by flg22 and other MAMPs but is also strongly activated by leaf senescence and various stresses, such as heat, cold, wounding, and UV-B, and different hormones, including gibberellin and ABA (AtGenExpress, Eastmond, 2004). Few mutants in the convergent MAMP-mediated defense signaling pathways downstream of the receptors have been identified (Gomez-Gomez and Boller, 2002; Nürnberger et al., 2004). As one of the key mechanisms to protect plants from infection by a broad spectrum of potential pathogens, it is not surprising that the conserved MAMP signaling components, such as the MAPK cascades and WRKY transcription factors, are encoded by functionally redundant genes in *Arabidopsis* (Asai et al., 2002; Zhang and Klessig, 2001). The discovery of potent suppressors such as AvrPto and AvrPtoB offers a powerful tool for dissecting the role of MAMP signaling in the plant nonhost immunity.

Type III Effectors as Suppressors of Plant Defense System

Animal bacterial pathogens secrete a few type III effectors with well-characterized enzymatic activities (Galan and Cossart, 2005). Plant bacterial pathogens, on the other hand, produce a relatively large number of putative type III effectors (Alfano and Collmer, 2004). Recently, several *Pseudomonas* type III effectors have been shown to suppress different types of PCD in plants and in yeast (Abramovitch et al., 2003; Jamir et al., 2004). For example, AvrPtoB blocks HopPsyA-mediated PCD in tobacco and *Arabidopsis* Ws-0 (Jamir et al., 2004). However, AvrPto and AvrPtoB do not interfere with PCD and transcription activation triggered by AvrRpt2, AvrRpm1, or AvrB in *Arabidopsis* (Figures 2F and 2G). It is possible that distinct mechanisms are utilized by different *R* genes to trigger PCD. Our results uncouple MAMP-mediated defense from some specific gene-for-gene defense in *Arabidopsis*. Although AvrPto, AvrRpt2, AvrRpm1, and HopAI1 all support the growth of DC3000 TTSS mutants or inhibit callose formation in transgenic *Arabidopsis* (Hauck et al., 2003; Kim et al., 2005; Li et al., 2005; Figure S4), our analyses show that AvrPto supports nonhost bacteria growth in plants (Figure 7A). It is clear from our studies that AvrPto and AvrPtoB act uniquely at a very early stage immediately after MAMP signal perception (Figures 5 and 6). In contrast, AvrRpt2, AvrRpm1, and HopAI1 do not interfere with early MAMP-specific gene activation and MAPK signaling (Figures 2 and 3). Thus, there appears to be diverse mechanisms by which type III effectors block the plant defense responses. Recent studies have uncovered MAPK-independent pathways acting downstream of the FLS2 receptor in flg22 signaling (S. Ramu and J.S., unpublished data; M. Willmann and J.S., unpublished data). AvrPto and AvrPtoB could suppress the flg22-mediated induction of *PAL1* (Figure S7), which is activated by both MAPK-dependent and MAPK-independent pathways in flg22 signaling (J.S., unpublished data). It is likely that AvrPto and AvrPtoB block multiple signaling pathways initiated from the receptor complex. Future research will unravel the detailed molecular mechanisms blocking the convergent signaling pathways in response to diverse MAMPs.

Although AvrPto and AvrPtoB function similarly as suppressors of MAMP-mediated defense responses in *Arabidopsis*, they share very limited sequence homology (Kim et al., 2002). It is intriguing that AvrPto and AvrPtoB were originally identified as Avr factors displaying the same

recognition specificity in their interactions with different variants of Pto resistance protein (Kim et al., 2002). Surprisingly, mutational analysis of AvrPto identified the same residues essential for the avirulence function in tomato and the virulence MAMP suppressor activity in *Arabidopsis*. Furthermore, expression of tomato Pto in *Arabidopsis* protoplasts partially interfered with the AvrPto suppressor function in MAMP signaling. It is possible that AvrPto and AvrPtoB could target Pto-like kinases or Pto-like receptor kinases involved in MAMP perception in *Arabidopsis*. Apparently, expression of a small amount of the flg22 receptor FLS2 can interfere with the AvrPto suppressor activity. To further elucidate the molecular actions of AvrPto and AvrPtoB in blocking early MAMP signaling, it will be of great interest to identify more target(s) in *Arabidopsis*.

Dynamic Coevolution in Plant-Bacterium Interactions

Although plants lack the elaborate adaptive immune system found in mammals, they have expanded the innate immune system through the evolution of a large set of pattern-recognition receptors (PRRs; Meyers et al., 2003). It appears that plant bacterial pathogens evolved numerous type III effectors that can suppress plant immunity (Alfano and Collmer, 2004; Mudgett, 2005). To survive, individual plants further evolved highly specific resistance genes to counteract specific virulence effectors in gene-for-gene defense (Dangl and Jones, 2001; Pedley and Martin, 2003; Staskawicz et al., 2001). Thus, dynamic coevolution in individual plant-bacterium interactions drives the resistance/susceptibility or nonhost/host relationship.

DC3000 appears to be a highly evolved virulent pathogen in plants and evolved or acquired AvrPto and AvrPtoB as potent suppressors of MAMP-mediated immune responses. To survive infection by this sophisticated pathogen, tomato but not *Arabidopsis* evolved the unique *Pto* and *Prf* gene products to recognize AvrPto and AvrPtoB and trigger specific gene-for-gene defense (Pedley and Martin, 2003). Analogous evolution also occurred in an unknown R protein recognizing different part of the AvrPto protein in tobacco (Shan et al., 2000b). Apparently, AvrPto can either activate MAPK signaling in Pto-mediated gene-for-gene defense in tomato (Pedley and Martin, 2004) or suppress MAPK signaling in MAMP-mediated defense in *Arabidopsis*. The manifestation of the distinct virulence and avirulence actions of AvrPto from *Pseudomonas* in different plants provides a fascinating example for how type III effectors and genes involved in plant defense continue to dynamically evolve in the plant-bacterium battlefield.

EXPERIMENTAL PROCEDURES

Plant Growth, Bacterial Inoculation, and Generation of Transgenic Plants

Wild-type (wt) Col-0 and mutant *rps2-101C* or *rps3-3 Arabidopsis* plants were grown in a growth chamber at 23°C with a 13 hr photoperiod for 30 days before bacterial inoculation or protoplast isolation. Different *P. syringae* strains were grown overnight at 28°C in the KB medium with appropriate antibiotics. Bacteria were pelleted by centrifugation, washed, and diluted to the desired density with 10 mM $MgCl_2$. *Arabidopsis* leaves were either infiltrated with bacteria using a needleless syringe or inoculated by dipping for 30 s containing 0.025% silwet L-77. Plant leaves were collected at the indicated time for RNA isolation and bacterial counting. To measure bacterial growth, two leaf discs were ground in 100 μl H_2O and serial dilutions were plated on KB medium with appropriate antibiotics. Bacterial colony forming units (cfu) were counted 2 days after incubation at 28°C. Each data point is shown as triplicates.

AvrPto transgenic plants were generated by *Agrobacterium*-mediated transformation with the *avrPto* construct under the control of the dexamethasone-inducible promoter with an HA epitope tag (McNellis et al., 1998). To induce AvrPto expression, transgenic plants were sprayed with 20 μM dexamethasone containing 0.025% silwet L-77 1 day before infiltration with flg22 or bacteria for RT-PCR and disease assays.

Type III Effector and Reporter Constructs

All effector constructs were made by cloning PCR fragments into a plant expression vector with an HA or FLAG epitope tag at the C terminus (Asai et al., 2002). *avrRpm1* was amplified from DC3000 (*avrRpm1*); *avrB* was amplified from DC3000 (*avrB*); *avrRpt2* was amplified from *P.s.* pv. *maculicola* ES4326 (*avrRpt2*); *avrPto* was amplified from DC3000 and *Pst* JL1065; *avrPtoB*, *hopAI1*, *hopPtoD2*, *hopPtoE*, and *hopPtoK* were amplified from DC3000; *virPphA* was amplified from *Psp* NPS3121; *avrBsT* was amplified from *Xcv* 75-3.

The *WRKY46* (*At2g46400*) promoter was amplified from *Arabidopsis* Col-0 genomic DNA and fused with a luciferase reporter gene to generate *WRKY46-LUC* (Asai et al., 2002). The reporter-gene construct *FRK1-LUC*, the MAPK constructs, MPK3 and MPK6, the active MKK4, MKK5, and MEKK1 constructs, and the FLS2 construct were reported previously (Asai et al., 2002). Pto was PCR amplified from pEG202::Pto (Kim et al., 2002).

Arabidopsis Protoplast Transient Expression and MAPK Assays

Protoplast transient expression and MAPK in-gel and in vitro assays were carried out as reported previously (Asai et al., 2002) and the detailed procedures are described in Supplemental Experimental Procedures. *UBQ10-GUS* was always cotransfected with *FRK1-LUC* as an internal control, and the promoter activity was presented as LUC/GUS ratio. Protoplasts were collected 6 hr after transfection for protein expression, kinase activity, and promoter activity assays. Protoplasts transfected with plasmid DNA without effectors were used as controls.

RT-PCR Analysis

Total RNA was isolated from leaves or protoplasts after treatment by using TRIzol Reagent (Invitrogen). Complementary DNA was synthesized from 1 μg of total RNA using 0.1 μg oligo (dT) primer and reverse transcriptase (Invitrogen). RT-PCR was run for 30 cycles. Real-time RT-PCR analysis was carried out with an iCycler iQ real-time PCR detection system with iQ SYBR green supermix (BIO-RAD). The expression of *FRK1*, *At1g51890*, *At2g17740*, or *At5g57220* was normalized to the expression of *UBQ10*. The primer sequences for different effectors and RT-PCR are listed in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include seven figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/125/3/563/DC1/.

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