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The Downy Mildew Effector Proteins ATR1 and ATR13 Promote Disease Susceptibility in *Arabidopsis thaliana* [W]

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The downy mildew (*Hyaloperonospora parasitica*) effector proteins ATR1 and ATR13 trigger RPP1-Nd/WsB– and RPP13-Nd–dependent resistance, respectively, in *Arabidopsis thaliana*. To better understand the functions of these effectors during compatible and incompatible interactions of *H. parasitica* isolates on *Arabidopsis* accessions, we developed a novel delivery system using *Pseudomonas syringae* type III secretion via fusions of ATRs to the N terminus of the *P. syringae* effector protein, AvrRPS4. ATR1 and ATR13 both triggered the hypersensitive response (HR) and resistance to bacterial pathogens in *Arabidopsis* carrying *RPP1-Nd*/*WsB* or *RPP13-Nd*, respectively, when delivered from *P. syringae* pv *tomato* (*Pst*) DC3000. In addition, multiple alleles of ATR1 and ATR13 confer enhanced virulence to *Pst* DC3000 on susceptible *Arabidopsis* accessions. We conclude that ATR1 and ATR13 positively contribute to pathogen virulence inside host cells. Two ATR13 alleles suppressed bacterial PAMP (for Pathogen-Associated Molecular Patterns)-triggered callose deposition in susceptible *Arabidopsis* when delivered by DC3000 ΔCEL mutants. Furthermore, expression of another allele of ATR13 in plant cells suppressed PAMP-triggered reactive oxygen species production in addition to callose deposition. Intriguingly, although Wassilewskija (Ws-0) is highly susceptible to *H. parasitica* isolate Emco5, ATR13^{Emco5} when delivered by *Pst* DC3000 triggered localized immunity, including HR, on Ws-0. We suggest that an additional *H. parasitica* Emco5 effector might suppress ATR13-triggered immunity.

INTRODUCTION

Plant innate immunity provides defense against microbial attack and involves at least two components called PAMP (for pathogen-associated molecular pattern)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). The activation of PTI or ETI by direct or indirect recognition of extracellular or intracellular pathogen molecules, respectively, enhances plant disease resistance and restricts pathogen proliferation (Jia et al., 2000; Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Deslandes et al., 2003; Zipfel et al., 2004, 2006; Dodds et al., 2006; Ade et al., 2007). However, successful pathogens efficiently suppress plant immunity and cause disease (Grant et al., 2006; Jones and Dangl, 2006).

In *Pseudomonas syringae*, the type III secretion system (T3SS) has been found to direct the translocation of effectors to the host cell cytoplasm (Jin et al., 2003; Buttner and Bonas, 2006; Cornelis, 2006). The N termini of T3SS effector proteins contain signals required for their translocation (Mudgett and Staskawicz, 1999; Mudgett et al., 2000; Jin et al., 2003). An intact T3SS is required for bacterial virulence and survival in host cells, since T3SS-deficient mutant bacteria cannot grow or cause disease on normally susceptible host plants (Lindgren et al., 1986; Jin et al., 2003). Extensive efforts have been made to uncover in planta functions of T3SS effectors from *P. syringae*. Several effectors were initially identified as avirulence (Avr) proteins, such as AvrPto and AvrRpt2, which specify race-specific immunity (ETI) upon recognition by tomato (*Solanum lycopersicum*) Pto and *Arabidopsis thaliana* RPS2, respectively (Ronald et al., 1992; Innes et al., 1993). In recent years, several *P. syringae* effectors were shown to suppress *Arabidopsis* cell wall defense triggered by bacterial PAMPs. AvrPto, AvrE, and HopM1 were found to suppress PAMP-triggered callose deposition in the susceptible *Arabidopsis* accession Columbia (Col-0) (Hauck et al., 2003; DebRoy et al., 2004). It was further shown that HopM1 recruits At MIN7, a guanine nucleotide exchange factor, for its degradation to suppress PTI via callose deposition (Nomura et al., 2006). In addition, AvrRpm1 and AvrRpt2 were shown to suppress PAMP-triggered callose deposition in *Arabidopsis* plants lacking corresponding *R* genes (*RPM1* and *RPS2*, respectively) (Kim et al., 2005). Recently, HopAI1 was shown to be a phosphothreonine lyase that inactivates mitogen-activated protein kinases, disabling the oxidative burst and callose deposition (Zhang et al., 2007). In addition, *P. syringae* pv *tomato* (*Pst*) DC3000 HopU1 was found to ADP-ribosylate a RNA binding protein, GRP7, to suppress callose deposition and interfere with host defense, thus enhancing pathogen virulence (Fu et al., 2007).

In oomycete pathogens, such as *Phytophthora* spp and *Hyaloperonospora parasitica*, genes homologous with T3SS protein secretion components are absent. However, recent identification of several oomycete pathogen effectors that are presumably translocated to the host cell cytoplasm suggests that oomycete effectors may use an evolutionarily conserved

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mechanism to deliver effector proteins into plant cells (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). Four effector proteins from oomycete pathogens, ATR1 and ATR13 (*H. parasitica*), Avr3a (*P. infestans*), and Avr1b (*P. sojae*), contain an N-terminal signal peptide and an RxLR motif (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). The RxLR motif is similar to the RxLx motif that is required for the translocation of proteins from malaria parasites (*Plasmodium* spp) to host cells (Hiller et al., 2004; Haldar et al., 2006; Kamoun, 2006). An oomycete RxLR motif can confer host cell targeting in *Plasmodium* (Bhattacharjee et al., 2006). However, the mechanism of RxLR-mediated translocation of oomycete (and *Plasmodium*) effector proteins into host cells is still unknown.

Three tightly linked *Arabidopsis R* genes, *RPP1* (for Recognition of *Peronospora parasitica*)-*WsA*, *RPP1-WsB*, and *RPP1-WsC*, were cloned from accession Wassilewskija (Ws-0) (Botella et al., 1998). Each of these RPP1 genes specifies resistance to different *H. parasitica* isolates. In *Arabidopsis* accession Niederzenz (Nd), resistance is specified by a locus containing RPP1-Nd. The corresponding Avr gene, designated ATR1^{NdWsB} (for *Arabidopsis thaliana* Recognized1), recognized by RPP1-Nd was isolated by map-based cloning using a population derived from a cross between *H. parasitica* isolates Emoy2 and Maks9, which are avirulent and virulent, respectively, on Nd-0 (Rehmany et al., 2003, 2005). The *H. parasitica* Emoy2 allele of ATR1^{NdWsB} is recognized by both RPP1-Nd and RPP1-WsB, whereas the Maks9 allele is recognized only by RPP1-WsB (Rehmany et al., 2005). In addition, the *H. parasitica* Cala2 allele of ATR1^{NdWsB} is not recognized by either RPP1-WsB or RPP1-Nd (Rehmany et al., 2005). *Arabidopsis* accessions Col-0 and Nd-0 were found to be susceptible and resistant, respectively, to *H. parasitica* isolate Maks9, and resistance is conferred by RPP13-Nd (Bittner-Eddy et al., 1999). Col-5 (a glabrous derivative of Col-0) plants transformed with a cosmid clone containing RPP13-Nd are fully resistant to *H. parasitica* Maks9 (Bittner-Eddy et al., 2000). Subsequently, *ATR13* alleles were cloned from several isolates of *H. parasitica* (Allen et al., 2004). Using biolistic bombardment of Col-5 and Col-5 (*RPP13-Nd*), Allen et al. (2004) characterized several *ATR13* alleles. In addition, amino acid sequence alignments of avirulent and virulent alleles of *ATR13* suggested that *ATR13* has undergone diversifying selection, consistent with the polymorphism of the *RPP13* locus in different *Arabidopsis* accessions (Allen et al., 2004; Rose et al., 2004). However, the functions of these oomycete effectors during pathogenesis in susceptible host plants are unknown. Analysis of the functions of oomycete effectors will help reveal how oomycete pathogens overcome host recognition and promote plant disease susceptibility.

The focus of our current research has been to test the hypothesis that ATR1 and ATR13 are virulence determinants during *H. parasitica* pathogenesis in *Arabidopsis*. A bacterial T3SS-based effector delivery system was developed to overcome the difficulties of studying effector functions in *H. parasitica*. We show that chimeric proteins of bacterial (AvrRPS4) and oomycete (ATR1 and ATR13) effectors can be efficiently delivered to plant cells by *Pst* DC3000 and trigger allele-specific ETI in resistant *Arabidopsis* plants carrying the corresponding *RPP* gene. We also demonstrate that several alleles of ATR1 and ATR13 can positively contribute to pathogen virulence in susceptible *Arabidopsis* accession Col-0 when delivered by *Pst* DC3000, suggesting that these effectors may interfere with host mechanisms involved in resistance to both bacterial and oomycete pathogens. Furthermore, ATR13 suppresses PAMP-triggered callose deposition at *Arabidopsis* leaf cell walls and reactive oxygen species (ROS) production, also consistent with the idea that ATR13 suppresses host cell immunity. However, *ATR13* cannot fully restore the virulence of *Pst* DC3000 lacking a known bacterial suppressor of callose deposition, *HopM1*, indicating that ATR13 may suppress oomycete PTI by a mechanism that is distinct from HopM1. Finally, we show that even though *H. parasitica* Emco5 is virulent on Ws-0, ATR13^{Emco5} triggers race-specific immunity in Ws-0, and we propose that ATR13^{Emco5} recognition might be suppressed by an unknown effector(s) of *H. parasitica* Emco5.

RESULTS

AvrRPS4 Amino Acids 1 to 136 Can Direct AvrRpt2 Effector Protein into Plant Cells via the *Pst* DC3000 Type III Secretion Machinery

AvrRPS4 protein is secreted by the *Pst* DC3000 T3SS and is subsequently processed to a smaller form in plant cells (K.H. Sohn, Y. Zhang, and J.D.G. Jones, unpublished data). The in planta processing site of AvrRPS4 was identified as Gly-133–Gly-134 in *Nicotiana benthamiana* and *Arabidopsis* (K.H. Sohn, Y. Zhang, and J.D.G. Jones, unpublished data). Furthermore, it was shown that the C-terminal 88 amino acids of AvrRPS4 are sufficient for avirulence function. These findings led us to test whether AvrRPS4 N-terminal fusions to other proteins might be delivered into and processed in plant cells. A broad host range vector, pBBR 1MCS-5, carrying the nucleotide sequences encoding the N-terminal 136 amino acids of AvrRPS4 regulated by the AvrRPS4 promoter, was constructed and named pEDV3 (for Effector Detector Vector 3) (Figure 1A). *Pst* DC3000 carrying pBBR 1MCS-5 or pEDV3 grew to a similar level compared with the wild-type strain carrying no broad host range vector (see Supplemental Figure 1 online). To test the AvrRPS4-mediated delivery of effector proteins into plant cells and the in planta processing of AvrRPS4 fusion proteins using this vector, we used it to deliver amino acids 72 to 255 of AvrRpt2, which are required for its effector function (Mudgett and Staskawicz, 1999). First, we tested whether *Pst* DC3000 expressing AvrRPS4$_{1-136}$-AvrRpt2$_{72-255}$-HA (hence, AvrRPS4N-AvrRpt2-HA) can trigger RPS2-dependent localized immune responses in *Arabidopsis*. *Pst* DC3000 carrying pEDV3 (AvrRPS4N-AvrRpt2-HA) triggered a *hrp*-dependent hypersensitive response (HR) in accession Col-0, which carries functional *RPS2*, but not in *rps2-101c* (Figure 1B). Furthermore, when inoculated at low concentrations, *Pst* DC3000 carrying pEDV3 (AvrRPS4N-AvrRpt2-HA) caused disease on *rps2-101c* plants but not on wild-type plants (Figure 1C). In addition, *Pst* DC3000 carrying pEDV3 (AvrRPS4N-AvrRpt2-HA) showed significantly reduced growth in Col-0 (*RPS2*), whereas this strain grew comparably to *Pst* DC3000 carrying pEDV3 (AvrRPS4N-HA) in *rps2-101c* (see Supplemental Figure 2 online).

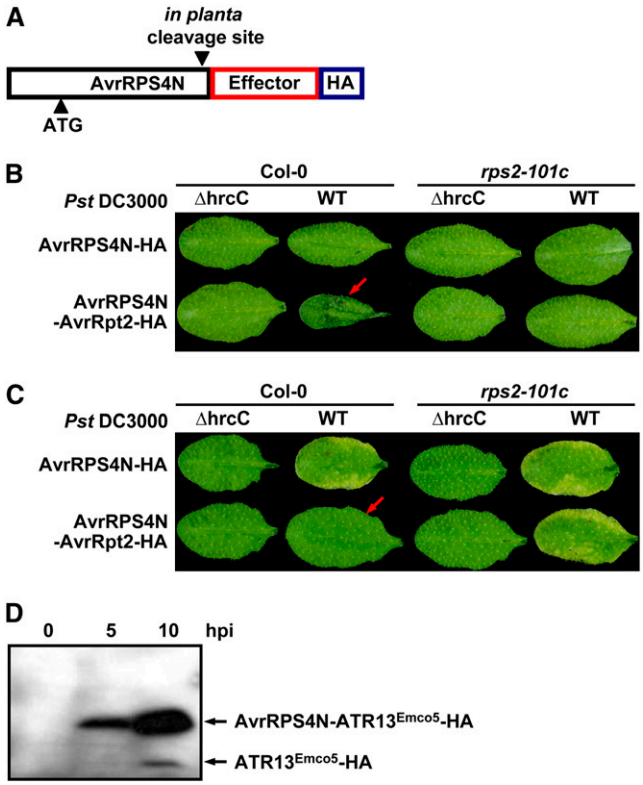

Figure 1. AvrRPS4 Can Direct the AvrRpt2 Effector Protein into Plant Cells via the *Pst* DC3000 Type 3 Secretion Machinery.

(A) Construction of pEDV3. The effectors AvrRpt2, ATR1, and ATR13 were cloned in-frame with the N-terminal 136 amino acids encoding T3S signal to express AvrRPS4-Effector-HA chimeric proteins in *Pst* DC3000. The expression of the chimeric effector proteins were designed to be driven by 128 bp of native AvrRPS4 promoter.

(B) $AvrRPS4_{1-136}$, designated AvrRPS4N, delivers $AvrRpt2_{72-255}$-HA by the *Pst* DC3000 T3SS and triggers RPS2-dependent HR in *Arabidopsis* accession Col-0. Leaves of wild-type Col-0 and *rps2-101c* were hand-inoculated using a 1-mL syringe with 5×10^7 colony-forming units (cfu)/mL *Pst* DC3000 wild type or Δ*hrcC* mutant carrying pEDV3 (AvrRPS4N) or pEDV3(AvrRPS4N-$AvrRpt2_{72-255}$). Labeling refers to the amino acid number in the respective protein. The red arrow indicates the leaf showing AvrRpt2-triggered HR. Photographs were taken at 22 h after infection. This experiment was repeated six times with similar results.

(C) *Pst* DC3000 expressing AvrRPS4N-AvrRpt2 is unable to cause disease symptoms on Col-0 (*RPS2*). Leaves of wild-type Col-0 and *rps2-101c* were hand-inoculated using a 1-mL syringe with 5×10^5 cfu/mL *Pst* DC3000 wild type or Δ*hrcC* mutant carrying pEDV3 (AvrRPS4N) or pEDV3(AvrRPS4N-AvrRpt2). The red arrow indicates the leaf showing no disease symptoms caused by AvrRpt2-triggered immunity. Photographs were taken at 5 d after inoculation. This experiment was repeated six times with similar results.

(D) AvrRPS4N-$ATR13^{Emco5}$ is processed in planta. Wild-type Col-0 leaves were hand-inoculated using a 1-mL syringe with 2×10^9 cfu/mL *Pst* DC3000 wild type expressing AvrRPS4N-$ATR13_{42-154}^{Emco5}$. Leaf samples were taken at 0, 5, and 10 h after infection (hpi) for protein extraction and immunoblot analysis using anti-HA antibody. The top bands show unprocessed AvrRPS4N-$ATR13^{Emco5}$ protein, and the bottom band represents processed $ATR13^{Emco5}$. This experiment was repeated twice with similar results.

$AvrRPS4_{1-136}$ Can Deliver Oomycete Effector ATR13 to *Arabidopsis* Leaf Cells

ATR13 is an *H. parasitica* effector protein that is recognized by a coiled coil–nucleotide binding–leucine-rich repeat protein, RPP13, in *Arabidopsis* accession Nd-0 (Allen et al., 2004). $ATR13^{Emoy2}$ and $ATR13^{Emco5}$ are virulent and avirulent alleles, respectively, on RPP13-Nd. The N-terminal signal peptide and RxLR motif of oomycete effectors are thought to be required for their translocation from haustorium to plant cell during pathogenesis (Bhattacharjee et al., 2006; Birch et al., 2006). Since the signal peptide and RxLR motif of ATR13 are not necessary for its avirulence function (Allen et al., 2004), we engineered AvrRPS4N-$ATR13_{42-154}^{Emoy2}$-HA and AvrRPS4N-$ATR13_{42-154}^{Emco5}$-HA (hence, AvrRPS4N-$ATR13^{Emoy2}$ and AvrRPS4N-$ATR13^{Emco5}$) fusion protein constructs without the signal peptide and RxLR motif of ATR13.

When *Pst* DC3000 pEDV3 (AvrRPS4N) and pEDV3 (AvrRPS4N-$ATR13^{Emco5}$) were grown in *hrp*-inducing minimal medium, the fusion proteins were detected as full-length protein without processing (see Supplemental Figure 3 online). However, we could detect processed $ATR13^{Emco5}$-HA protein from the protein extracts of leaves infiltrated with *Pst* DC3000 expressing AvrRPS4N-$ATR13^{Emco5}$ on *Arabidopsis* Col-0 leaves using anti-HA antibody (Figure 1D). We detected *hrp*-dependent expression of full-length AvrRPS4N-$ATR13^{Emco5}$ at 5 h after infection and processed $ATR13^{Emco5}$-HA protein at 10 h after infection (Figure 1D). These results demonstrated that AvrRPS4N can deliver C-terminally tagged effector proteins to plant cells, where they are subsequently processed. Therefore, we concluded that the AvrRPS4N-mediated effector delivery system can deliver oomycete effector proteins into plant cells.

An Avirulence Allele of ATR13 Triggers *RPP13-Nd*–Dependent HR in *Arabidopsis* When Delivered by *Pst* DC3000

We next tested RPP13-Nd–dependent HR elicitation of *Arabidopsis* plants in response to *Pst* DC3000 expressing AvrRPS4N, AvrRPS4N-$ATR13^{Emoy2}$, or AvrRPS4N-$ATR13^{Emco5}$. *Arabidopsis* Col-0, which carries an *RPP13* allele that recognizes neither ATR13 allele, did not manifest HR after infiltration of any of the strains (Figure 2). *Pst* DC3000 expressing AvrRPS4N-$ATR13^{Emco5}$ triggered strong HR in Col-5 plants carrying *RPP13-Nd* and wild-type Nd-0 plants, whereas AvrRPS4N and AvrRPS4N-$ATR13^{Emoy2}$ did not elicit HR symptoms at 24 h after infection in Col-5 (*RPP13-Nd*) and Nd-0. As expected, HR triggered by AvrRPS4N-$ATR13^{Emco5}$ required *Pst* DC3000 T3SS (Figure 2). These results indicate that T3SS-dependent expression and translocation of AvrRPS4N can deliver C-terminally tagged effector domains of ATR13 alleles to plant cells, resulting in allele-specific and *RPP13-Nd*–dependent HR.

$ATR13^{Emco5}$ Restricts the Growth of *Pst* DC3000 on *Arabidopsis* Lines Carrying *RPP13-Nd*

We next tested whether HR elicited in Col-5 (*RPP13-Nd*) and Nd-0 by $ATR13^{Emco5}$-HA is correlated with restricted growth and reduced disease symptoms after infiltration with low bacterial

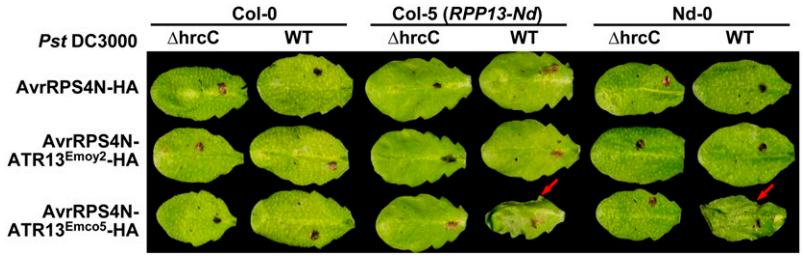

Figure 2. ATR13^{Emco5} Triggers RPP13-Nd–Dependent HR When Delivered by *Pst* DC3000.

Col-0, Col-5 (*RPP13-Nd*), and Nd-0 leaves were hand-inoculated using a 1-mL syringe with 5×10^7 cfu/mL *Pst* DC3000 wild type or Δ*hrcC* mutant carrying pEDV3 (AvrRPS4N), pEDV3 (AvrRPS4N-ATR13$_{42-187}^{Emoy2}$), or pEDV3 (AvrRPS4N-ATR13$_{42-154}^{Emco5}$). Labeling refers to the allele or amino acid number in the respective protein. Red arrows indicate the leaves showing ATR13^{Emco5}-triggered HR. Photographs were taken at 22 h after infection. The experiment was repeated five times with similar results.

inocula. Wild-type Col-0 leaves developed chlorotic and necrotic lesions at 4 to 5 d after inoculation of *Pst* DC3000 expressing AvrRPS4N, AvrRPS4N-ATR13^{Emoy2}, or AvrRPS4N-ATR13^{Emco5} (Figure 3A). Col-5 (*RPP13-Nd*) and Nd-0 also showed typical disease symptoms caused by *Pst* DC3000 after inoculation with *Pst* DC3000 expressing AvrRPS4N or AvrRPS4N-ATR13^{Emoy2}. However, Col-5 (*RPP13-Nd*) and Nd-0 did not show disease symptoms when infected by *Pst* DC3000 expressing AvrRPS4N-ATR13^{Emco5}, suggesting that attenuation of disease symptoms was due to the activation of *RPP13-Nd*–dependent resistance upon recognition of ATR13^{Emco5}. We measured the growth of *Pst* DC3000 expressing AvrRPS4N, AvrRPS4N-ATR13^{Emoy2}, or AvrRPS4N-ATR13^{Emco5} in infected Col-0, Col-5 (*RPP13-Nd*), and Nd-0 plants. Col-0, Col-5 (*RPP13-Nd*), and Nd-0 plants

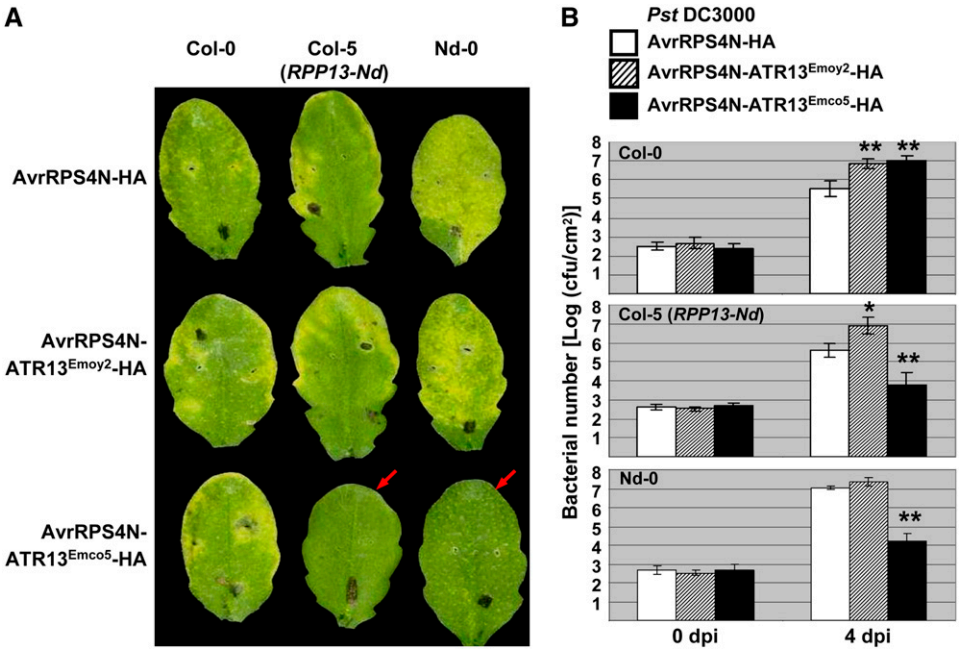

Figure 3. ATR13^{Emoy2} and ATR13^{Emco5} Confer Enhanced Virulence of *Pst* DC3000 in *Arabidopsis*.

(A) Disease symptoms of *Arabidopsis* leaves caused by *Pst* DC3000. Col-0, Col-5 (*RPP13-Nd*), and Nd-0 leaves were hand-inoculated using a 1-mL syringe with 5×10^5 cfu/mL *Pst* DC3000 wild type carrying pEDV3 (AvrRPS4N), pEDV3 (AvrRPS4N-ATR13^{Emoy2}), or pEDV3 (AvrRPS4N-ATR13^{Emco5}). Red arrows indicate the leaves showing no symptoms due to ATR13^{Emco5}-triggered immunity. Photographs were taken at 5 d after inoculation. This experiment was repeated four times with similar results.

(B) Leaf bacterial populations of *Arabidopsis* plants infected with *Pst* DC3000. Bacterial infection was done as described for **(A)**. Bacterial populations were measured at 0 and 4 d after inoculation (dpi). Each bar represents the mean number of bacterial colonies recovered on selective agar medium containing appropriate antibiotics from four independent replicates. This experiment was repeated three times with similar results. Single ($P < 0.05$) and double ($P < 0.01$) asterisks represent significant differences compared with *Pst* DC3000 (AvrRPS4N-HA).

allowed *Pst* DC3000 expressing AvrRPS4N to grow to high levels (Figure 3B). However, the growth of *Pst* DC3000 expressing AvrRPS4N-ATR13^{Emco5} was significantly reduced in Col-5 (*RPP13-Nd*) and Nd-0 plants, indicating that the recognition of ATR13^{Emco5} by RPP13-Nd in *Arabidopsis* caused restricted in planta bacterial growth when delivered from *Pst* DC3000.

Two Alleles of ATR13 Confer Enhanced Virulence of *Pst* DC3000 on Susceptible *Arabidopsis* Genotypes

ATR13 triggers localized immunity resulting in enhanced resistance to *Pst* DC3000 in *Arabidopsis* plants carrying *RPP13-Nd*. We also observed that *Pst* DC3000 expressing AvrRPS4N-ATR13^{Emoy2} or AvrRPS4N-ATR13^{Emco5} conferred enhanced growth compared with *Pst* DC3000 expressing AvrRPS4N in susceptible *Arabidopsis* (Col-0) plants (Figures 3A and 3B). The elevated growth of *Pst* DC3000 resulted in enhanced disease symptoms in Col-0 plants infected with *Pst* DC3000 expressing either AvrRPS4N-ATR13^{Emoy2} or AvrRPS4N-ATR13^{Emco5} (Figure 3A). In addition, AvrRPS4N-ATR13^{Emoy2} conferred enhanced growth of *Pst* DC3000 in Col-5 (*RPP13-Nd*), indicating that the virulence function of the ATR13^{Emoy2} allele is independent of *RPP13-Nd*. However, ATR13^{Emoy2} did not confer significantly enhanced growth of *Pst* DC3000 on Nd-0 at 3 d after inoculation. We obtained similar results by spraying bacteria on *Arabidopsis* leaves. Overall, these data indicate that the delivery of ATR13 alleles with distinct specificities by bacterial T3SS can confer enhanced virulence to *Pst* DC3000 growing on *Arabidopsis*.

ATR1^{Emoy2} Triggers RPP1-Nd– and RPP1-WsB–Dependent HR in *Arabidopsis* and Confers Resistance When Delivered by *Pst* DC3000

To examine further whether the pEDV system can deliver other *H. parasitica* effectors, we tested *ATR1* (Rehmany et al., 2005). Like *ATR13*, *ATR1* is highly polymorphic and coevolves with its host *RPP* locus. While *ATR1^{Emoy2}* is recognized by both *RPP1-Nd* and *RPP1-WsB*, *ATR1^{Maks9}* is recognized by *RPP1-WsB* but not *RPP1-Nd*, and *ATR1^{Cala2}* is not recognized by *RPP1-WsB* or *RPP1-Nd* (Rehmany et al., 2005). We fused *AvrRPS4N* to the Emoy2 and Cala2 alleles of *ATR1*, starting at the RxLR motif, to construct *AvrRPS4N-ATR1*$_{105-368}^{Emoy2}$*-HA* and *AvrRPS4N-ATR1*$_{105-380}^{Cala2}$*-HA* (hence, *AvrRPS4N-ATR1^{Emoy2}* and *AvrRPS4N-ATR1^{Cala2}*), respectively. *Arabidopsis* Ws-0 and Nd-0, which carry *RPP1-WsB* and *RPP1-Nd*, showed HR in response to *Pst* DC3000 expressing AvrRPS4N-ATR1^{Emoy2} but not AvrRPS4N-ATR1^{Cala2} or the empty vector control (Figure 4). As expected, this allele-specific HR was not observed in Col-0, which contains neither RPP1-WsB nor RPP1-Nd. The same constructs for both alleles of ATR1 lacking the epitope HA tag were made and gave the same results (see Supplemental Figure 4 online).

ATR1 Enhances the Virulence of *Pst* DC3000 on *Arabidopsis*

We examined disease progression caused by *Pst* DC3000 expressing either AvrRPS4N-ATR1^{Emoy2} or AvrRPS4N-ATR1^{Cala2} in Col-0, Ws-0, and Nd-0. As expected, *Pst* DC3000 carrying pEDV3 (AvrRPS4N-ATR1^{Emoy2}) did not cause any chlorotic disease symptoms in Ws-0 or Nd-0, which correlated with the restricted growth of the bacteria (Figures 5A and 5B). However, *Pst* DC3000 carrying pEDV3 (AvrRPS4N-ATR1^{Cala2}) conferred enhanced disease symptoms on Col-0 and Ws-0 but not on Nd-0 (Figure 5A). The enhanced symptoms caused by ATR1^{Cala2} were correlated with elevated bacterial growth in the absence of any recognition event (Figure 5B), suggesting a contribution of ATR1^{Cala2} to virulence when delivered by *Pst* DC3000. In addition, ATR1^{Emoy2} also enhanced bacterial growth compared with *Pst* DC3000 carrying pEDV3 (AvrRPS4N) in the susceptible *Arabidopsis* accession Col-0, consistent with a virulence function of ATR1^{Emoy2} in the absence of *RPP1* (Figure 5B).

Two Alleles of ATR13 Suppress PAMP-Triggered Callose Deposition and Enhance Bacterial Virulence

ATR13 confers enhanced virulence on *Pst* DC3000 growing on *Arabidopsis* (Figure 3). We investigated how ATR13 confers enhanced virulence in susceptible *Arabidopsis* accessions. Since *H. parasitica* makes intimate contact with host cell walls during pathogenesis, we hypothesized that one important function of *H. parasitica* effectors might be the suppression of cell wall–based defense. Recently, three *Pst* DC3000 effectors, AvrPto, AvrE, and HopM1, were shown to suppress PAMP-triggered cell

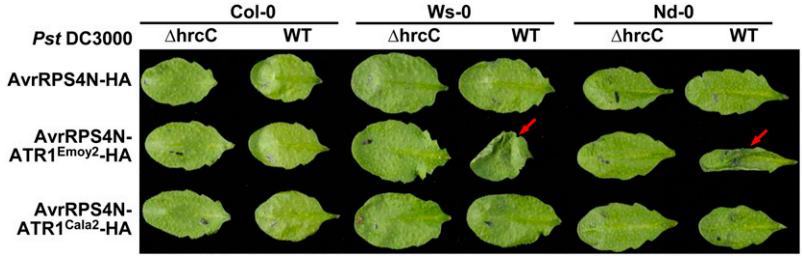

Figure 4. ATR1^{Emoy2} Triggers RPP1-Dependent HR When Delivered by *Pst* DC3000.

Col-0, Col-5 (*RPP13-Nd*), and Nd-0 leaves were hand-inoculated using a 1-mL syringe with 5×10^7 cfu/mL *Pst* DC3000 wild type or Δ*hrcC* mutant carrying pEDV3 (AvrRPS4N), pEDV3(AvrRPS4N-ATR1$_{105-368}^{Emoy2}$), or pEDV3 (AvrRPS4N-ATR1$_{105-380}^{Cala2}$). Labeling refers to the allele or amino acid number in the respective protein. Red arrows indicate the leaves showing ATR1^{Emoy2}-triggered HR. Photographs were taken at 22 h after infection.

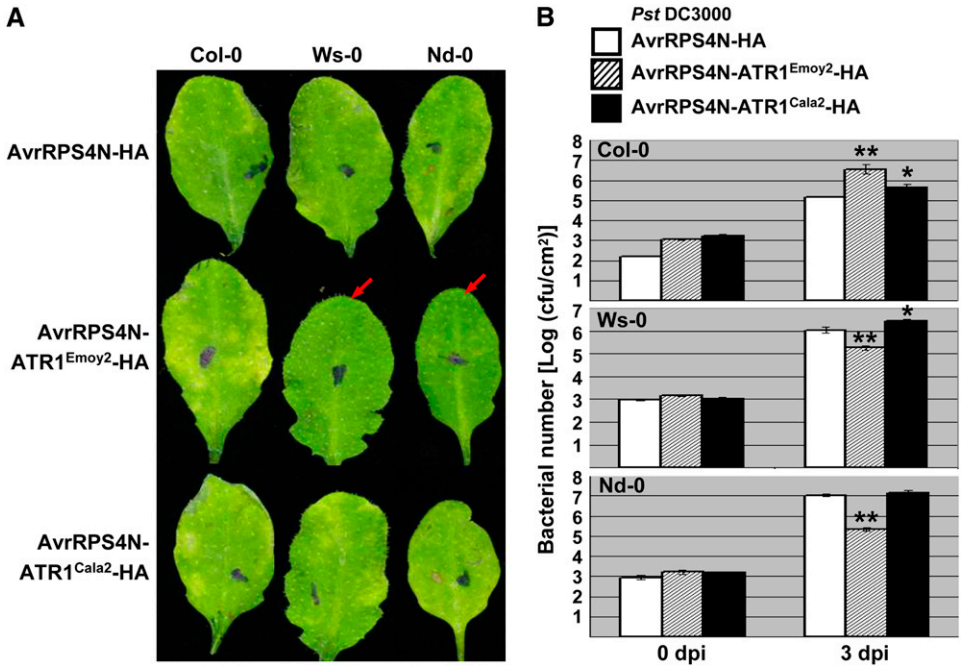

Figure 5. ATR1^{Emoy2} and ATR1^{Cala2} Confer Enhanced Virulence of *Pst* DC3000 in *Arabidopsis*.

(A) Disease symptoms of *Arabidopsis* leaves caused by *Pst* DC3000. Col-0, Col-5 (*RPP13-Nd*), and Nd-0 leaves were hand-inoculated using a 1-mL syringe with 5×10^5 cfu/mL *Pst* DC3000 wild type carrying pEDV3 (AvrRPS4N), pEDV3 (AvrRPS4N-ATR1^{Emoy2}), or pEDV3 (AvrRPS4N-ATR1^{Cala2}). Red arrows indicate the leaves showing no symptoms due to ATR1^{Emoy2}-triggered immunity. Photographs were taken at 5 d after inoculation.
(B) Leaf bacterial populations of *Arabidopsis* plants infected with *Pst* DC3000. Bacterial infection was done as described for **(A)**. Bacterial populations were measured at 0 and 3 d after inoculation (dpi). Each bar represents the mean number of bacterial colonies recovered on selective agar medium containing appropriate antibiotics from four independent replicates. Single ($P < 0.05$) and double ($P < 0.01$) asterisks represent significant differences compared with *Pst* DC3000 (AvrRPS4N-HA).

wall defense (callose deposition) when expressed in planta or delivered to *Arabidopsis* Col-0 leaf cells (Hauck et al., 2003; DebRoy et al., 2004; Nomura et al., 2006). HopM1 is located on conserved effector locus (CEL), and the *Pst* DC3000 ΔCEL strain can no longer suppress callose deposition (Alfano et al., 2000; DebRoy et al., 2004). Callose suppression of *Pst* DC3000 ΔCEL can be restored by introducing a plasmid (pORF43) expressing HopM1 and its chaperone, SchM (DebRoy et al., 2004). Therefore, we examined whether ATR13 can functionally complement AvrPto- or HopM1-mediated suppression of callose deposition using *Pst* DC3000 ΔAvrPto or in a ΔCEL mutant strain.

Col-0 exhibited extensive callose deposition when inoculated with the *Pst* DC3000 Δ*hrcC* mutant, whereas essentially no callose deposition was observed in leaves infiltrated with the wild-type *Pst* DC3000 (Figure 6A) (Hauck et al., 2003). Infiltration of water did not induce any callose deposition. As expected, *Pst* DC3000 ΔCEL wild type or carrying pEDV3 (AvrRPS4N) was completely unable to suppress callose deposition in Col-0, whereas *Pst* DC3000 ΔCEL carrying pORF43 (HopM1/ShcM) could restore the callose suppression phenotype to the level of wild-type *Pst* DC3000 (Figure 6A) (DebRoy et al., 2004). We tested whether two alleles of ATR13 could suppress callose deposition induced by *Pst* DC3000 ΔCEL. Significantly, *Pst* DC3000 ΔCEL carrying pEDV3 (AvrRPS4N-ATR13^{Emoy2}) or pEDV3 (AvrRPS4N-ATR13^{Emco5}) suppressed callose deposition induced by *Pst* DC3000 ΔCEL to a level comparable to that of *Pst* DC3000 ΔCEL carrying pORF43 (HopM1/ShcM) (Figure 6A). We were unable to observe the accumulation of callose after inoculation of *Pst* DC3000 ΔAvrPto or ΔAvrPto/AvrPtoB in Col-0; therefore, we did not investigate ATR13-triggered callose suppression further with these mutants.

We tested whether the two alleles of ATR13 can also enhance *Pst* DC3000 ΔCEL growth on susceptible host plants. *Pst* DC3000 ΔCEL carrying pEDV3(AvrRPS4N-HA) grew ~100-fold less than *Pst* DC3000 wild type, and the decreased growth of the *Pst* DC3000 ΔCEL mutant was recovered to a level comparable to that of wild-type *Pst* DC3000 by delivering HopM1/SchM (pORF43) (Figure 6B). *Pst* DC3000 ΔCEL carrying pEDV3 (AvrRPS4N-ATR13^{Emoy2}-HA) or pEDV3 (AvrRPS4N-ATR13^{Emco5}-HA) grew ~10-fold more than *Pst* DC3000 ΔCEL carrying pEDV3 (AvrRPS4N-HA), suggesting that both alleles of ATR13 can enhance pathogen virulence (Figure 6B).

Transient Expression of ATR13 in *Arabidopsis* Leaf Cells Suppresses Two PTI Phenotypes

We tested whether ATR13 can suppress flg22-triggered defense responses in transgenic *Arabidopsis* (Col-5) plants conditionally expressing ATR13^{Maks9} from a dexamethasone-inducible promoter (Allen et al., 2004). Spraying 5-week-old Col-5 (Dex-*ATR13^{Maks9}*)

plants with dexamethasone solution induced the transcription of *ATR13*^{Maks9} (Figure 7C). Infiltration of flg22 triggered substantial callose deposition in wild-type Col-0 leaves with or without pretreatment of dexamethasone (Figure 7A). In Col-5 (Dex-*ATR13*^{Maks9}), flg22-triggered callose depositions were greatly suppressed when the leaves were sprayed with dexamethasone before flg22 treatment, confirming that ATR13 suppresses PAMP-triggered callose depositions in plant cell (Figure 7A). It has been shown that flg22 triggers the production of ROS (Felix et al., 1999). Therefore, we tested whether the expression of ATR13^{Maks9} suppressed the flg22-triggered ROS burst by approximately threefold, suggesting that ATR13 might act upstream of ROS production in the PTI signaling pathway (Figure 7B).

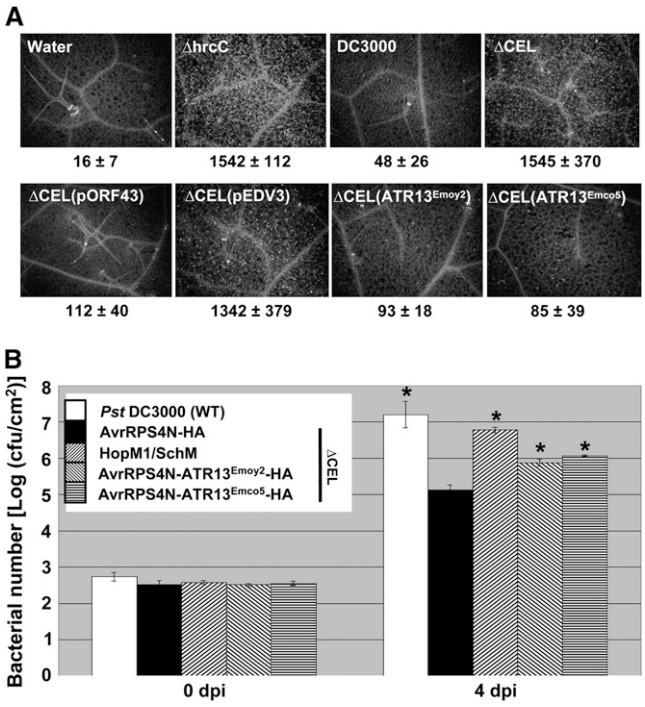

Figure 6. ATR13 Suppresses PAMP-Triggered Callose Deposition at Susceptible *Arabidopsis* Leaf Cell Walls.

(A) Five-week-old Col-0 leaves were hand-inoculated with sterilized water or 1 × 10^8 cfu/mL suspensions of *Pst* DC3000 Δ*hrcC* mutant or wild type, *Pst* DC3000 ΔCEL mutant, or *Pst* DC3000 ΔCEL mutant carrying pORF43 (HopM1/ShcM), pEDV3 (AvrRPS4N), or pEDV3 (AvrRPS4N-ATR13^{Emco5}). Leaf samples were taken at 14 h after infection and stained with aniline blue for visualization of callose (white dots). This experiment was repeated four times with similar results.

(B) Leaf bacterial populations of 5-week-old *Arabidopsis* Col-0 leaves infected with *Pst* DC3000 wild type or ΔCEL strains. Five-week-old *Arabidopsis* Col-0 leaves were hand-inoculated using a 1-mL syringe with 5 × 10^5 cfu/mL *Pst* DC3000 wild type or *Pst* DC3000 ΔCEL carrying pEDV3 (AvrRPS4N), pORF43 (HopM1/ShcM), pEDV3 (AvrRPS4N-ATR13^{Emoy2}), or pEDV3 (AvrRPS4N-ATR13$_{42-154}^{Emco5}$). This experiment was repeated three times with similar results. Asterisks ($P < 0.01$) represents significant differences compared with *Pst* DC3000 ΔCEL (AvrRPS4N-HA). dpi, d after inoculation.

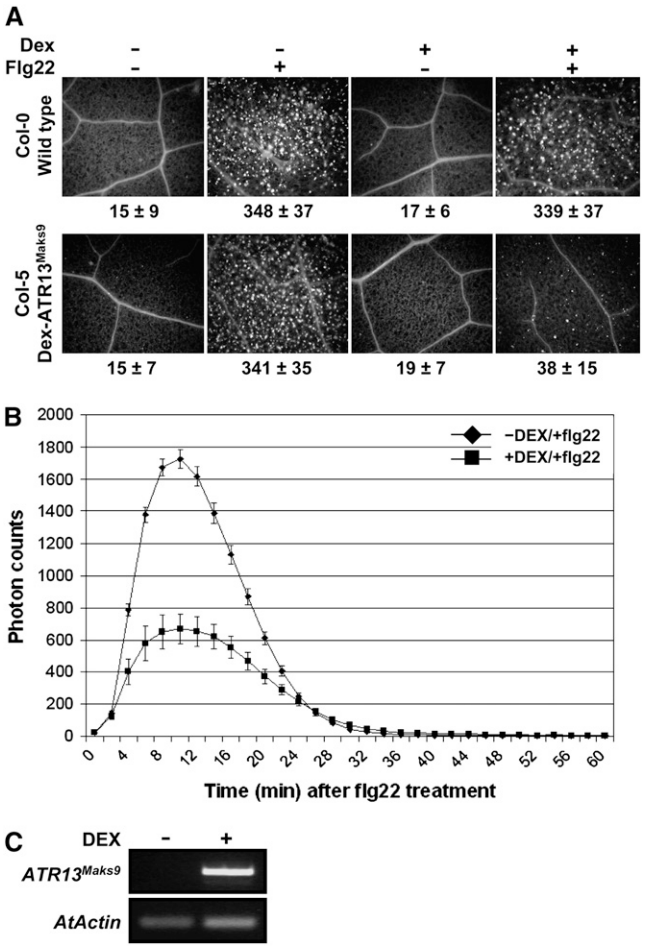

Figure 7. Expression of ATR13 in Plant Cells Suppresses PAMP-Triggered Immune Responses.

(A) Five-week-old *Arabidopsis* Col-0 and Col-5 (Dex-*ATR13*^{Maks9}) plants were sprayed with water or 20 μM dexamethasone (Dex; 0.02% Silwet-77). Twenty-four hours later, the leaves were infiltrated with water or 1 μM flg22. Leaf samples were taken at 8 h after flg22 infiltration and stained with aniline blue for visualization of callose (white dots). This experiment was repeated twice with similar results.

(B) Leaf discs from 5-week-old Col-5 (Dex-*ATR13*^{Maks9}) plants were taken and floated on water or 20 μM dexamethasone solution prior to flg22 treatment. Luminescence was measured every 2 min for at least 60 min after treatment with flg22 using the Photek camera system. Lines and error bars represent means and SE, respectively, of 48 independent samples tested at the same time. The same experiments were done twice with similar results.

(C) Expression analysis of ATR13^{Maks9} in Col-5 (Dex-*ATR13*^{Maks9}) plants. PCR products were amplified from cDNA derived from Col-5 (Dex-*ATR13*^{Maks9}) leaves sprayed with water or 20 μM dexamethasone (0.02% Silwet-77). PCR was performed using gene-specific primers and separated on a 1.2% agarose gel. At *Actin* (At5g09810) was also amplified from the same cDNA samples as a control.

H. parasitica Race Emco5 Is Virulent on Ws-0, yet ATR13^{Emco5} Triggers HR and Restricts Bacterial Growth When Delivered from *Pst* DC3000

H. parasitica Emco5 is resisted in *Arabidopsis* plants carrying *RPP13-Nd* (Bittner-Eddy et al., 2000) but will infect accession Col-0 (Figure 8A). Consistent with this, Nd-0 showed HR symptoms when infiltrated with a 5 × 10^7 cfu/mL suspension of *Pst* DC3000 carrying pEDV3 (AvrRPS4N-ATR13^{Emco5}), whereas Col-0 did not (Figures 2 and 8A). Ws-0 was also highly susceptible to *H. parasitica* Emco5 (Figure 8A) (Botella et al., 1998). However, Ws-0 showed HR when inoculated with a 5 × 10^7 cfu/mL suspension of *Pst* DC3000 carrying pEDV3 (AvrRPS4N-ATR13^{Emco5}), whereas *Pst* DC3000 carrying pEDV3 (AvrRPS4N) did not trigger any visible symptoms. We examined whether Ws-0 exhibits restricted bacterial growth when ATR13^{Emco5} is delivered by *Pst* DC3000. In Ws-0, bacterial growth was reduced by at least 10-fold at 3 d after inoculation of *Pst* DC3000 carrying pEDV3 (AvrRPS4N-ATR13^{Emco5}) compared with pEDV3 (AvrRPS4N) (Figure 8C). Transcriptional expression of ATR13^{Emco5} was

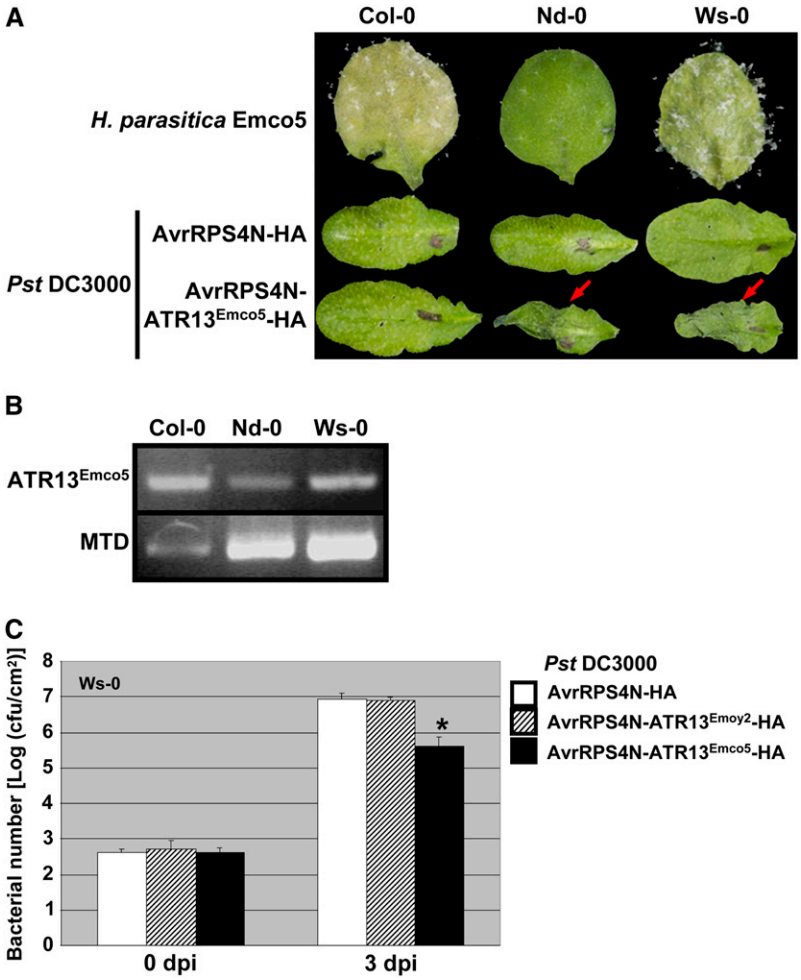

Figure 8. ATR13^{Emco5}-Triggered Immunity Is Suppressed by *H. parasitica* Isolate Emco5 in Ws-0.

(A) *Arabidopsis* accessions Col-0, Nd-0, and Ws-0 leaves were sprayed with 10^5 spores/mL *H. parasitica* isolate Emco5 (top panel; 2-week-old leaves) or hand-inoculated with 5 × 10^7 cfu/mL suspensions of *Pst* DC3000 carrying pEDV3 (AvrRPS4N) or pEDV3 (AvrRPS4N-ATR13^{Emco5}) (middle and bottom panels, respectively; 6-week-old leaves). Photographs were taken at 10 d after inoculation or 22 h after infection for *H. parasitica* Emco5– or *Pst* DC3000–infected leaves, respectively. Red arrows indicate ATR13^{Emco5}-triggered HR. This experiment was repeated three times with similar results.
(B) Expression analysis of ATR13^{Emco5} in infected *Arabidopsis* seedlings. PCR products were amplified from cDNA derived from *H. parasitica* Emco5–infected 2-week-old Col-0, Nd-0, or Ws-0 seedlings using gene-specific primers and separated on a 1.2% agarose gel. *H. parasitica* methylenetetrahydrofolate dehydrogenase (MTD) was also amplified from the same cDNA samples as a control.
(C) Bacterial populations of Ws-0 leaves infected with *Pst* DC3000. Leaves were hand-inoculated using a 1-mL syringe with 5 × 10^5 cfu/mL wild-type *Pst* DC3000 carrying pEDV3 (AvrRPS4N), pEDV3 (AvrRPS4N-ATR13^{Emoy2}), or pEDV3 (AvrRPS4N-ATR13^{Emco5}). Bacterial populations were measured at 0 and 3 d after inoculation (dpi). Each bar represents the mean number of bacterial colonies recovered on selective agar medium containing appropriate antibiotics from four independent replicates. This experiment was repeated twice with similar results. The asterisk ($P < 0.01$) represents a significant difference compared with *Pst* DC3000 (AvrRPS4N-HA).

confirmed in Col-0, Nd-0, and Ws-0 plants infected with *H. parasitica* Emco5 using RT-PCR with gene-specific primers (Figure 8B). The nucleotide sequence of the amplified ATR13^{Emco5} cDNA fragment was confirmed by sequencing after inoculation of Ws-0 with Emco5.

DISCUSSION

We demonstrate here that two effectors, ATR1 and ATR13, from the filamentous oomycete pathogen *H. parasitica* suppress host PTI during a compatible interaction and trigger ETI in resistant *Arabidopsis* accessions when delivered into *Arabidopsis* leaf cells by the *Pst* DC3000 T3SS. Furthermore, three alleles of ATR13 suppress PAMP-triggered callose deposition at *Arabidopsis* leaf cell walls. We also show that ATR13^{Maks9} can suppress the PAMP-triggered ROS burst on susceptible host plants. Additionally, we suggest that *H. parasitica* isolate Emco5 might carry additional effector(s) that suppress ATR13^{Emco5}-triggered immunity in *Arabidopsis* accession Ws-0.

Implications of T3SS-Mediated Delivery of Nonbacterial Proteins

Extensive efforts have been made to identify effector proteins from bacterial, fungal, and oomycete plant pathogens. There are several well-known strategies that have been used to screen bacterial effector proteins that are secreted to plant cells. For example, truncated forms of avirulence proteins, such as AvrRpt2 or AvrBs2, lacking domains required for T3SS have been introduced to bacterial chromosomes by engineered transposons to identify T3S effectors enabling secretion of the reporter proteins (Guttman et al., 2002; Roden et al., 2004). Moreover, bioinformatic analysis has been performed to predict T3S effectors from sequenced plant pathogenic bacteria (Petnicki-Ocwieja et al., 2002; Vinatzer et al., 2006).

Since bacterial effectors are likely to be secreted by T3SS in an unfolded state, it is remarkable that AvrRPS4-ATR chimeric proteins were delivered by *P. syringae* T3SS and elicited defense responses in host plants (Stebbins and Galan, 2001; Akeda and Galan, 2005). In some cases, secretion of T3S effectors is dependent on interaction with their chaperones, and often the chaperone binding domain resides immediately after the N-terminal effector secretion signal (Fu and Galan, 1998; Lee and Galan, 2004). Recent evidence suggests that unfolding of T3S effector proteins by an ATPase is required for secretion (Akeda and Galan, 2005). It is possible that 1 to 136 amino acids of AvrRPS4 carries a chaperone binding or ATPase-interacting domain that is required for efficient unfolding and secretion.

During interactions of filamentous oomycete pathogens (such as *P. infestans* and *H. parasitica*) with host plants, a set of effectors are thought to be translocated to plant cells (Birch et al., 2006; Kamoun, 2006). However, it is not clear how these pathogen molecules are delivered. *P. infestans* and *H. parasitica* effectors usually carry a signal peptide and an RxLR amino acid motif (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Kamoun, 2006). The RxLR motif of a *P. infestans* effector, Avr3a, can functionally complement the RxLx motif that is required for the translocation of *Plasmodium falciparum* effector proteins into hosts (Hiller et al., 2004; Bhattacharjee et al., 2006). Recently, it was shown that the RxLR motif is required for the translocation of Avr3a into the plant cell from *P. infestans* (Whisson et al., 2007). Additionally, no high-throughput method to transform filamentous oomycete pathogens has been developed to date. Therefore, the well-characterized bacterial T3SS could provide an efficient assay system to deliver oomycete effector proteins to study their cellular functions. It will be fascinating to try to use the T3SS-based effector detector system for studies of fungal as well as oomycete effectors, since there are several advantages of using bacterial T3SS. These include the following facts: (1) a wide range of phytopathogenic bacteria exist, (2) these bacteria are relatively easy to manipulate, and (3) mutant strains of bacterial pathogens such as *Pst* DC3000 lacking one or more T3S effectors are available. Recently, several shotgun genome sequencing projects of oomycete plant pathogens, such as *P. infestans*, *P. ramorum*, *P. sojae*, *P. capsici* (genome information of *P. capsici* will be released soon), and *H. parasitica*, have been completed (Tyler et al., 2006; http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html; http://genome.wustl.edu/pub/organism/Fungi/Hyaloperonospora_parasitica/assembly/Hyaloperonospora_parasitica-2.0; http://genome.jgi-psf.org/Physo1_1/Physo1_1.home.html; http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html; http://www.jgi.doe.gov/sequencing/why/CSP2006/Pcapsici.html). Bioinformatic analysis of genome sequences of several *Phytophthora* spp and *H. parasitica* revealed several hundred signal peptide and RxLR motif-containing candidate effectors (Tyler et al., 2006; Win et al., 2007). Hitherto, there has been no efficient tool to experimentally confirm true effectors from these oomycete pathogens, apart from generating transgenic plants constitutively or conditionally expressing candidate effectors. Heterologous expression and delivery of the oomycete candidate effectors by bacterial pathogens to test their avirulence as well as virulence functions in host cells would accelerate such analysis. Some of the oomycete or fungal effectors may share common functions with bacterial effectors and therefore cannot be assayed properly using a bacterial strain that carries an effector(s) with a similar function. However, this problem can be solved using a reference strain, such as *Pst* DC3000, in which many single or multiple effector knockout strains are available. In addition, various approaches to reveal the diversity and functions of bacterial effectors will increase our chances to precisely test oomycete or fungal effectors using the EDV system.

Possible Roles of ATR1 and ATR13 in *H. parasitica* Pathogenesis in *Arabidopsis*

Oomycete-derived Nep1 (for necrosis and ethylene-inducing peptide1)-like proteins (NLPs) have been found to trigger various immune responses, including the activation of mitogen-activated protein kinase, callose deposition, and production of nitric oxide and ethylene, in *Arabidopsis* (Qutob et al., 2006). Two fungal effectors, Avra10 and AvrK1, from the obligate biotrophic fungal pathogen of barley (*Hordeum vulgare*), *Blumeria graminis* f sp *hordei*, have been cloned and found to enhance infection rates of the pathogen in susceptible host plants lacking corresponding R genes (Ridout et al., 2006). For *H. parasitica*, due to limited accessibility for genetic manipulation of the pathogen, it has not

been demonstrated that secreted proteins contribute to the pathogen virulence by suppressing host immunity. Since avirulent and virulent alleles of both ATR1 and ATR13 enhance the virulence of *Pst* DC3000 on susceptible hosts, it is reasonable to propose that ATR1 and ATR13 may promote *Arabidopsis* susceptibility by targeting host proteins that are involved in resistance to both bacterial and oomycete pathogens. It seems likely that effector functions of ATR1 and ATR13 are not identical to those of *Pst* DC3000 effectors, since both enhance the in planta growth of wild-type bacteria in susceptible *Arabidopsis* accessions. These results were surprising because *Pst* DC3000, which carries >30 effector proteins, is already a highly virulent bacterial pathogen (Collmer et al., 2002; Buell et al., 2003). Moreover, ATR13-mediated suppression of callose deposition at *Arabidopsis* cell walls suggests that ATR13 may play a role in suppressing PTI activated by oomycete elicitors such as Nep1-like proteins or CBEL (Gaulin et al., 2006; Qutob et al., 2006). ATR13-mediated callose suppression is the first indication to date of the suppression of host PTI by an oomycete effector, and this suggests that the molecular functions of bacterial and oomycete effectors can overlap. Qutob et al. (2006) showed that there is a significant overlap between *Arabidopsis* genes that are upregulated by treatments of synthetic peptide derived from bacterial flagellin, flg22, and purified Nep1-like protein from *Phytophthora parasitica*, indicating that downstream signaling networks activated by bacterial and oomycete PAMPs at least partially overlap. In addition, *Arabidopsis* lines carrying mutations at plant innate immune signaling components, such as enhanced disease susceptibility1, show enhanced susceptibility to both *Pst* DC3000 and *H. parasitica* (Aarts et al., 1998). Together, these data suggest that bacterial and oomycete effectors may suppress host immune networks by targeting plant proteins positioned in defense signaling pathways that are activated by both bacterial and oomycete pathogens. A *Pst* DC3000 effector, HopM1, suppresses callose deposition and enhances bacterial virulence by degrading At MIN7 protein via the *Arabidopsis* proteasome (Nomura et al., 2006). Although ATR13 has a suppressor function of PAMP-induced callose deposition similar to HopM1, it seems likely that ATR13 and HopM1 have distinct modes of action in *Arabidopsis*, because the delivery of ATR13 by *Pst* DC3000 still enhances the growth of the bacteria carrying functional HopM1. Therefore, ATR13 may target defense components other than At MIN7 that are involved in cell wall–based defense in *Arabidopsis*. In addition, transient expression of ATR13^{Maks9} suppressed flg22-triggered ROS production within 20 min, suggesting that the virulence function of ATR13 might be the suppression of early steps in PTI signaling (Figure 7B). These findings suggest that multiple alleles of ATR13 contain common virulence functions that have evolved during interaction with host plants.

Molecular Evolution of *H. parasitica* Virulence to Suppress Host Immunity

It is now apparent that pathogens have evolved to overcome plant innate immunity triggered by direct or indirect recognition of pathogen molecules by plant extracellular or intracellular receptors (Jones and Dangl, 2006). In bacterial pathogens, especially *P. syringae* strains, proteins that are secreted to host plant cells, so-called T3SS effectors, are key factors for suppressing host immunity and bacterial survival (Jin et al., 2003). Our T3SS-based analysis of effector functions of ATR13 and phenotypic analysis of *H. parasitica* isolates in *Arabidopsis* accessions Col-0, Nd-0, and Ws-0 enabled us to learn more about the molecular evolution of *H. parasitica* virulence, particularly effectors, during its coevolution with *Arabidopsis*. ATR13 has undergone diversifying selection processes during *H. parasitica*–*Arabidopsis* coevolution (Allen et al., 2004). There are several hypotheses that could explain our paradoxical observations that *H. parasitica* Emco5 causes disease on Ws-0, yet ATR13^{Emco5} is recognized by Ws-0. Conceivably, ATR13 protein is not expressed during the infection process on Ws-0. However, this is unlikely, because at least *ATR13^{Emco5}* mRNA was expressed (Figure 8B). Another possibility is that not enough ATR13^{Emco5} protein is delivered to trigger immunity. This is possible if translocation of *H. parasitica* effectors to plant cell cytoplasm requires plant extracellular components, which can vary among *Arabidopsis* accessions. Nevertheless, this hypothesis is also inconsistent with our observation that Ws-0 is highly susceptible to *H. parasitica* Emco5, because it would be very difficult for the pathogen to successfully colonize host plant cells without delivering its effectors. Since the compatibility of pathogens on host plants is the outcome of a complex interaction between pathogen and host factors, the susceptible phenotype of a host plant to a given pathogen can be due not only to the absence of an avirulence effector protein(s) but also to the presence of an ETI suppressor(s), a hypothesis that is supported in the literature. The presence of inhibitor genes was initially suggested in studies on flax rust fungus (*Melampsora lini*) on flax (*Linum usitatissimum*) (Jones, 1988). In *P. syringae* pv *phaseolicola*, it was shown that the presence of AvrPphC converts a normally avirulent strain to virulence by interfering with AvrPphF-triggered immunity (Tsiamis et al., 2000). Moreover, VirPphA and AvrPtoB have been shown to have suppressor functions on several Avr-triggered HRs (Jackson et al., 1999; Abramovitch et al., 2003). It was also shown that the ETI suppressor function of AvrPtoB requires E3-ligase activity (Abramovitch et al., 2006; Janjusevic et al., 2006). Suppression of AvrRpm1-triggered immunity by another *Pseudomonas* effector, AvrRpt2 (Ritter and Dangl, 1996), was later shown to be due to direct proteolysis of RIN4 by AvrRpt2 (Mackey et al., 2003). Previously, it was found that the Emco5 allele of ATR1^{NdWsB} is recognized by *Arabidopsis* accession Ws-0, which is susceptible to *H. parasitica* Emco5, indicating the presence of an ETI suppressor in *H. parasitica* Emco5 (Rehmany et al., 2005). Therefore, we favor the hypothesis that *H. parasitica* Emco5 carries one or more suppressors that are able to convert normally avirulent *H. parasitica* carrying the Emco5 allele of ATR1 or ATR13 to virulence on Ws-0. It will be interesting to test whether such putative suppressors can be identified among the *H. parasitica* Emco5 effector complement.

METHODS

Construction of pEDV3

Standard PCR techniques were used to create pEDV3. The multiple cloning site (MCS) of a broad host range vector, pBBR 1MCS-5 (Kovach

et al., 1995), was modified by inserting two annealed oligonucleotides, 5′-GAAGCTTATCGATGAATTCCAGCT-3′ and 5′-GGAATTCATCGATAAGCTTCGTAC-3′, at *Kpn*I and *Sst*I sites to create mpBBR 1MCS-5 (MCS was replaced by *Hin*dIII-*Cla*I-*Eco*RI). A total of 128 bp (from ATG) of the AvrRPS4 promoter and the first 408 bp of the AvrRPS4 open reading frame was amplified from pV316-1A (Hinsch and Staskawicz, 1996) by standard PCR using KS_96 (5′-CCCAAGCTTTTTCCCCGAAGATTAGGAACTGTC-3′) and KS_107 (5′-GGAATTCTTATGCGTAGTCTGGTACGTCGTACGGATAGGATCCGTCGACTCGTTTACCTCCACCCAATAGG-3′) primers to create a DNA fragment containing 5′-*Hin*dIII-*AvrRPS4N*-*Sal*I-*Bam*HI-HA-*Eco*RI-3′. The amplified DNA fragment was digested with *Hin*dIII and *Eco*RI and purified from a 1.0% agarose gel to ligate with *Hin*dIII- and *Eco*RI-treated mpBBR 1MCS-5. The resulting plasmid was named pEDV3. The sequence of the inserted DNA fragment was confirmed by sequencing with M13F and M13R primers.

AvrRpt2 and ATR13 Plasmid Constructions

The DNA fragment encoding $AvrRpt2_{72\text{-}255}$ was amplified from plasmid pVSP61 (own promoter:AvrRpt2; a gift from B.J. Staskawicz) using KS_115 (5′-ACGCGTCGACGGGTGGTTCAAAAAGAAATCATCTA-3′) and KS_116 (5′-CGGGATCCGCGGTAGAGCATTGCGTGTG-3′) primers to have *Sal*I and *Bam*HI at the 5′ and 3′ ends, respectively. DNA fragments encoding $ATR13_{42\text{-}187}^{Emoy2}$, $ATR13_{42\text{-}154}^{Emco5}$, $ATR13_{42\text{-}147}^{Emco5}$, $ATR13_{42\text{-}112}^{Emco5}$, $ATR13_{76\text{-}154}^{Emco5}$, and $ATR13_{105\text{-}154}^{Emco5}$ were amplified from DNA of *Hyaloperonospora parasitica* Emoy2 or Emco5 spores as templates using standard PCR conditions to have *Sal*I and *Bam*HI at the 5′ and 3′ ends, respectively. Amplified *AvrRpt2* and *ATR13* fragments were digested with *Sal*I and *Bam*HI and cloned in *Sal*I- and *Bam*HI-treated pEDV3 to create $AvrRpt2_{72\text{-}255}$-pEDV3, $ATR13_{42\text{-}187}^{Emoy2}$-pEDV3 (KS_108 [5′-ACGCGTCGACGCAGCCGCCAGCGAAGT-3′] and KS_110 [5′-CGGGATCCCTGACTGGCAACGGCAGTCT-3′]), and $ATR13_{42\text{-}154}^{Emco5}$-pEDV3 (KS_108 and KS_109 [5′-CGGGATCCCTGTCTGTCAAGAGCATCCCGA-3′]). DNA sequences of these constructs were verified by sequencing inserted DNA fragments using M13F and M13R primers.

Bacterial Strains and Plasmid Mobilizations

Bacterial strains used in this study were as follows: *Escherichia coli* DH5α, *Pseudomonas syringae* pv *tomato* DC3000 wild type, Δ*hrcC*, and ΔCEL (Yuan and He, 1996; Alfano et al., 2000). *E. coli* DH5α and *Pst* DC3000 strains were grown in low-salt Luria-Bertani broth at 37 and 28°C, respectively. *Pst* DC3000 cultures were centrifuged to recover bacteria, washed briefly, and resuspended in sterile water for infection assays. Plasmids were mobilized from *E. coli* DH5α to wild-type or mutant *Pst* DC3000 strains by standard triparental matings using *E. coli* HB101 (pRK2013) as a helper strain.

RNA Expression Analysis

Total RNA was extracted from *H. parasitica* Emco5–infected 2-week-old *Arabidopsis thaliana* seedlings from accessions Col-0, Nd-0, and Ws-0 at 48 h after inoculation or from transgenic Col-5 (Dex-$ATR13^{Maks9}$) plants after 24 h of dexamethasone treatment (20 μM) using TRIZOL reagent (Invitrogen) following the instructions from the manufacturer. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT) primer. PCR (39 cycles for $ATR13^{Emco5}$ and *MTD*, 25 cycles for $ATR13^{Maks9}$ and At *Actin*) was performed using Taq DNA polymerase (Qiagen). Primers used for PCR were KS_271 (5′-GCAGCCGCCAGCGAAGTAT-3′) and KS_272 (5′-CTGTCTGTCAAGAGCATCCCGAAG-3′) primers for $ATR13^{Emco5}$, MTD1 (5′-GACCCGGCTGCGAAGAAGTATGC-3′) and MTD2 (5′-CCAGCGGCCGACCAACAATG-3′) for *MTD*, KS_271 (5′-GCAGCCGCCAGCGAAGTAT-3′) and KS_168 (5′-CGGGATCCTACACTGGCATACTGGATGGGGG-3′) for $ATR13^{Maks9}$, and AC1 (5′-ATGGCAGACGGTGAGGATATTCA-3′) and AC2 (5′-GCCTTTGCAATCCACATCTGTTTG-3′) for At *Actin* (At5g09810) amplifications.

Protein Analysis

Frozen plant tissue was ground and mixed with an equal volume of cold protein isolation buffer (20 mm Tris-HCl, pH 7.5, 1 mm EDTA, pH 8.0, 5 mm DTT, 150 mm NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol, and 1× Protease Inhibitor Cocktail [Sigma-Aldrich]). The mixture was spun down, the supernatant was transferred to a new tube, and SDS loading buffer (300 mm Tris-HCl, pH 6.8, 8.7% SDS, 5% β-mercaptoethanol, 30% glycerol, and 0.12 mg/mL bromophenol blue) was added. Proteins were separated by SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad), and probed with horseradish peroxidase–conjugated anti-HA antibody (Sigma-Aldrich). Bands were visualized using ECL+ (Amersham).

Pst DC3000 and *H. parasitica* Pathology Experiments

Five-week-old *Arabidopsis* plants were hand-infiltrated with 5×10^7 or 5×10^5 cfu/mL *Pst* DC3000 suspensions for HR or in planta growth assays, respectively. HR symptoms were scored at 22 h after infection, and in planta bacterial growth was measured at 0 and 3 (or 4) d after infiltration by extracting bacteria from leaf discs and plating a series of dilutions on the medium supplemented with appropriate antibiotics. Disease symptoms were observed until 7 d after infiltration. For *H. parasitica* Emco5 infection, 2-week-old plants were sprayed with a spore suspension at a concentration of 10^5 spores/mL. Plants were kept in a growth cabinet at 17°C for 7 to 10 d with a 10-h/14-h day/night cycle.

Callose Staining and Microscopic Analysis

Leaves of 5-week-old *Arabidopsis* accession Col-0 plants were hand-infiltrated with 1×10^8 cfu/mL *Pst* DC3000 suspensions, and leaf samples were taken at 12 to 14 h after inoculation. For transgenic Col-5 (Dex-$ATR13^{Maks9}$) plants, 4-week-old leaves were sprayed with dexamethasone (20 μM) and subsequently hand-infiltrated with flg22 (1 μM) after 24 h. Samples were taken at 8 h after flg22 treatment. A total of eight leaf discs were taken from eight leaves for callose staining. Harvested leaf samples were cleared three times (overnight incubation at room temperature) with 100% methanol, washed three times (2 h for each washing) with sterilized water, and stained with aniline blue (0.05% in phosphate buffer, pH 8.0) for 24 h. Leaf samples stained with aniline blue were examined with a Zeiss Axiophot photomicroscope using an A3 fluorescence cube, and the images were analyzed using Image-Pro Plus software (Media Cybernetics).

Measurement of ROS Production

A total of 96 leaf discs from 5-week-old Col-5 (Dex-$ATR13^{Maks9}$) plants were taken and floated on water or 20 μM dexamethasone solution for 19 h prior to flg22 treatment. ROS production was measured by adding luminol (final concentration, 20 μM; Sigma-Aldrich), 1 μg of horseradish peroxidase (Sigma-Aldrich), and flg22 (final concentration, 100 nM) to a 96-well plate containing leaf discs (48 samples for each pretreatment). Luminescence was measured every 2 min for at least 60 min after treatment with flg22 using the Photek camera system.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AJ870977 (*avrRpt2*), L43559 (*avrRps4*), AY842877 ($ATR1^{Emoy2}$), AY842883 ($ATR1^{Cala2}$), AY785302 ($ATR13^{Emoy2}$), and AY785305 ($ATR13^{Emco5}$).

Supplemental Data

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

Supplemental Figure 1. Test of pBBR 1MCS-5 and pEDV3 on Bacterial Growth.

Supplemental Figure 2. Delivery of AvrRPS4N-AvrRpt2-HA Confers RPS2-Dependent Resistance against *Pst* DC3000 in *Arabidopsis* Accession Col-0.

Supplemental Figure 3. AvrRPS4N-ATR13^{Emco5}-HA Is Not Processed When Expressed in *Pst* DC3000.

Supplemental Figure 4. The C-Terminal HA Epitope Tag Does Not Interfere with ATR1^{Emoy2}-Triggered HR When Delivered by *Pst* DC3000.

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NOTE ADDED IN PROOF

Rentel et al. (2008) have also shown that ATR13 proteins can be delivered to plant cells via type III secretions, in their case after fusion to the AvrRpm1 N terminus, where they also confer reduced bacterial virulence depending on the host genotype.

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