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The Majority of the Type III Effector Inventory of *Pseudomonas syringae* pv. *tomato* DC3000 Can Suppress Plant Immunity

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The *Pseudomonas syringae* type III protein secretion system (T3SS) and the type III effectors it injects into plant cells are required for plant pathogenicity and the ability to elicit a hypersensitive response (HR). The HR is a programmed cell death that is associated with effector-triggered immunity (ETI). A primary function of *P. syringae* type III effectors appears to be the suppression of ETI and pathogen-associated molecular pattern–triggered immunity (PTI), which is induced by conserved molecules on microorganisms. We reported that seven type III effectors from *P. syringae* pv. *tomato* DC3000 were capable of suppressing an HR induced by *P. fluorescens*(pHIR11) and have now tested 35 DC3000 type III effectors in this assay, finding that the majority of them can suppress the HR induced by HopA1. One newly identified type III effector with particularly strong HR suppression activity was HopS2. We used the pHIR11 derivative pLN1965, which lacks *hopA1,* in related assays and found that a subset of the type III effectors that suppressed HopA1-induced ETI also suppressed an ETI response induced by AvrRpm1 in *Arabidopsis thaliana*. *A. thaliana* plants expressing either HopAO1 or HopF2, two type III effectors that suppressed the HopA1-induced HR, were reduced in the flagellin-induced PTI response as well as PTI induced by other PAMPs and allowed enhanced in planta growth of *P. syringae*. Collectively, our results suggest that the majority of DC3000 type III effectors can suppress plant immunity. Additionally, the construct pLN1965 will likely be a useful tool in determining whether other type III effectors or effectors from other types of pathogens can suppress either ETI, PTI, or both.

Pseudomonas syringae is a gram-negative bacterial plant pathogen that requires a type III protein secretion system (T3SS) and the type III effectors that it injects into plant cells to be pathogenic. The T3SS in *P. syringae* is encoded by hypersensitive response and pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes in the Hrp pathogenicity island (Alfano et al. 2000; Collmer et al. 2000). The T3SS injects type III effectors into host cells in which they contribute to disease in susceptible plants, primarily by suppression of plant innate immunity (Abramovitch et al. 2006; Espinosa and Alfano 2004). In resistant plants however, type III effectors can be recognized by plant resistance (R) proteins inducing effector-triggered immunity (ETI), which includes the elicitation of the hypersensitive response (HR), a programmed cell death in plants. ETI makes up one of the two branches of the innate immune system of plants (Jones and Dangl 2006).

The other branch of the plant innate immune system is activated by recognition of pathogen-associated molecular patterns (PAMPs) and is referred to as PAMP-triggered immunity (PTI) (Nurnberger et al. 2004; Zipfel 2008). PAMPs are present in pathogenic and nonpathogenic microorganisms and are also referred to as microbe-associated molecular patterns (MAMPs) (Ausubel 2005). One of the PAMPs whose role in inducing PTI is best understood is bacterial flagellin, which is recognized by the *Arabidopsis thaliana* receptor kinase FLS2 (Gomez-Gomez and Boller 2000; Zipfel et al. 2004). FLS2 also recognizes a conserved peptide of flagellin, flg22, that is often used in assays to induce outputs of PTI, including callose (β-1,3 glucan) deposition in the plant cell wall (Gomez-Gomez et al. 1999).

In 1988, a paper reported a cosmid named pHIR11 that contained a cluster of *hrp/hrc* genes from *P. syringae* pv. *syringae* 61 that, when expressed in saprophytes such as *P. fluorescens*, conferred upon them the ability to elicit an HR in tobacco (Huang et al. 1988). This predated the discovery that *hrp/hrc* genes encoded a T3SS, but it proved to be a useful tool to explore the function of these genes in *P. syringae*. For example, pHIR11 was instrumental in identifying the first protein found to be secreted by the *P. syringae* T3SS (HrpZ1) (He et al. 1993). At that time, HrpZ1 was thought to be responsible for the HR elicited by *P. syringae* because it was encoded by pHIR11, it was a type III–secreted protein, and purified HrpZ1 infiltrated into tobacco leaves elicited an HR. However, it was subsequently determined that a nonpolar insertion in *hrpZ1* in pHIR11 retained the ability to elicit an HR when expressed in *P. fluorescens* (Alfano et al. 1996). Thus, even though purified HrpZ1 elicited an HR, it was unlikely to be the elicitor of the bacterial-induced HR. This realization led to the discovery that HopA1 (formerly HrmA and HopPsyA) was the type III effector encoded by pHIR11 that was responsible for the pHIR11-induced HR and that HopA1 needed to be inside plant cells to elicit the HR (Alfano et al. 1997).

We have used pHIR11 as a tool to elucidate different components of the T3SS. For example, we used pHIR11 to help show that HopA1 required the ShcA type III chaperone to be secreted via the T3SS (van Dijk et al. 2002). Importantly, for the research we describe here, we previously used *P. fluores-*

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 4 and 5 appear in color online.

cens(pHIR11) in an assay in which we also expressed newly discovered type III effectors from *P. syringae* pv. *tomato* DC3000, to determine whether they could suppress the pHIR11-dependent HR. Remarkably, we found that seven of the 19 DC3000 type III effectors tested could fully or partially suppress the pHIR11-dependent HR (Jamir et al. 2004). pHIR11 has also been used to show that *Xanthomonas campestris* pv. *vesicatoria* type III effectors could also suppress HopA1-induced ETI (Fujikawa et al. 2006). Thus, pHIR11 has proved to be a simple and effective tool to increase our understanding of the T3SS in bacterial plant pathogens.

We modified pHIR11 by substituting *hopA1* with a kanamycin-resistance cassette, resulting in construct pLN18 (Jamir et al. 2004). This construct allowed us to perform bacterial mixing experiments to show that a type III effector could suppress ETI, even when delivered by a different bacterial strain from the one injecting the recognized type III effector (Jamir et al. 2004). Because *P. fluorescens*(pLN18) does not inject any type III effector but does contain PAMPs and induces PTI, this strain was used to show that a number of DC3000 type III effectors could suppress a PTI output (Oh and Collmer 2005).

Here, we test 35 DC3000 type III effectors for their ability to suppress pHIR11-dependent ETI and find that the majority are capable of ETI suppression. We also show that the well-characterized type III effectors AvrRpm1 and AvrRpt2 are also capable of suppressing pHIR11-dependent ETI. We made a derivative of pHIR11 named pLN1965 that substituted *hopA1* with a spectinomycin-resistance cassette. *P. fluorescens* (pLN1965) separately expressing different DC3000 type III effectors also suppressed the AvrRpm1-dependent ETI as well as PTI in *Arabidopsis thaliana*. Taken together, our data indicate that the vast majority of the DC3000 type III effectors can suppress ETI and that many of them can also suppress PTI. This suggests that either common targets for type III effectors are utilized in pathways needed for both ETI and PTI, that many type III effectors have multiple activities, or both. Additionally, construct pLN1965 will likely be useful for testing whether other type III effectors and effectors from other types of plant pathogens can suppress plant immunity.

Table 1. DC3000 type III effectors tested for their ability to suppress a HopA1-dependent hypersensitive response (HR) in tobacco elicited by *Pseudomonas fluorescens*(pHIR11)

Type III effectors	Plasmid	Suppression class[a]
AvrE	pLN2423	II
AvrPto	pLN1327	II
AvrPtoB	pLN347	I
HopA1	pLN1326	II
HopB1	pLN271	IV
HopC1	pLN50	III
HopD1	pLN167	I
HopE1	pLN162	I
HopF2	pLN1420	I
HopG1	pLN460	II
HopH1	pLN150	II
HopI1		Not tested
HopK1	pCPP5100	I
HopM1	pLN1156	II
HopN1	pLN1324	II
HopO1-1	pLN1622	II
HopO1-2	pLN1623	II
HopQ1-1	pLN518	III
HopR1	pLN1154	II
HopS2	pLN1624	I
HopT1-1	pLN256	III
HopT1-2	pLN567	III
HopU1	pLN223	II
HopV1	pLN517	II
HopX1	pCPP5068	I
HopY1	pLN1528	II
HopAA1-1	pLN1326	III
HopAA1-2	pLN1419	II
HopAD1	pLN165	III
HopAF1	pLN164	II
HopAI1	pLN1011	IV
HopAM1	pCPP5063	I
HopAN1	pLN1329	II
HopAO1	pLN130	II
HopAQ1	pLN1331	II
HopAS1′	pLN1328	IV

[a] Class I suppressors are type III effectors that suppressed the HopA1-dependent HR at 1×10^8 cells/ml; class II completely suppressed the HR at 2×10^7 cells/ml; class III suppressors are type III effectors that were variable in their ability to suppress the HopA1-dependent HR at 2×10^7 cells/ml; and class IV contains type III effectors that were unable to suppress the HR at 2×10^7 cells/ml.

RESULTS

Screening the DC3000 type III effector inventory for suppressors of the HopA1 effector-triggered HR.

The cosmid pHIR11 encodes a functional T3SS and one type III effector HopA1 (Alfano and Collmer 1997; Lindeberg et al. 2005; van Dijk et al. 1999). We previously used this cosmid to screen DC3000 type III effectors for their ability to suppress the HopA1-induced HR when expressed in *P. fluorescens* (Jamir et al. 2004). Nineteen type III effectors were screened, and five, AvrPtoB (HopAB2), HopE1, HopF2, HopX1 (AvrPphE), and HopAM1 (AvrPpiB1), were capable of completely suppressing the HopA1-induced HR, while two others, HopD1 and HopK1, had an intermediate ability to suppress this HR (Jamir et al. 2004).

If the DC3000 type III effector inventory is limited to type III effectors that have been confirmed or to those for which there exists experimental evidence supporting that they are type III effectors, there are 36 type III effectors present in DC3000 (Lindeberg et al. 2005, 2006). We tested the remaining 29 DC3000 type III effectors in the *P. fluorescens*(pHIR11) assay with the exception of HopI1, due to difficulties in the isolation of its gene. Type III effector genes were cloned into pLN615 and were transformed into *P. fluorescens*(pHIR11). Each strain expressing individual type III effectors was infiltrated into tobacco leaves at a cell density of 1×10^8 cells/ml and was examined for its ability to suppress the HopA1-dependent HR by comparing each test strain with a control strain on the same leaf. Surprising, only one additional type III effector, HopS2, was capable of completely suppressing the HopA1-dependent HR at this cell density (Fig. 1). The suppression activity of HopS2 required the presence of the HopS2 cognate type III chaperone, which has been shown to be required for HopS2 to be secreted via the T3SS (Guo et al. 2005).

Based on the number of type III effectors that possessed suppressor activity in our earlier study (Jamir et al. 2004), we expected that many more of the remaining DC3000 type III effectors would be capable of suppressing the HopA1-dependent HR. We speculated that our assays may have failed to detect additional DC3000 type III effectors capable of suppressing the HR because the cell densities used far exceeded the threshold cell density needed for HR elicitation and this may have left unidentified type III effectors that possessed less-substantial suppressor activity. *P. fluorescens*(pHIR11) maintains its ability to elicit a consistent confluent HR at a cell density of 2×10^7 cells/ml and, therefore, we retested all of the type III effectors at this cell density. When DC3000 type III effectors were retested in the *P. fluorescens*(pHIR11) system at the lower cell density, the vast majority of them were able to suppress the HopA1-dependent HR (Table 1). To distinguish between the different levels of HR suppression activity displayed

by different type III effectors, we separated them into different suppressor classes: Class I suppressors were type III effectors that suppressed the HopA1-dependent HR at 1×10^8 cells/ml, class II completely suppressed the HR at 2×10^7 cells/ml, class III contained type III effectors that were variable in their ability to suppress the HopA1-dependent HR at 2×10^7 cells/ml, and class IV contained type III effectors that were unable to suppress the HR at 2×10^7 cells/ml. Class I contained eight type III effectors, class II contained 18 type III effectors, class III contained six type III effectors, and class IV contained three type III effectors.

Two well-studied type III effectors, AvrRpm1 and AvrRpt2, can also suppress the HR induced by HopA1.

Much is known about the activities and targets for the type III effectors AvrRpm1 and AvrRpt2 (Axtell and Staskawicz. 2003; Kim et al. 2005; Mackey et al. 2002, 2003). We tested whether these type III effectors were capable of suppressing the HR elicited by *P. fluorescens*(pHIR11) in experiments similar to the ones described above. The control strain elicited an HR at densities of 1×10^8 and 2×10^7 cells/ml, whereas strains harboring pVSP61::*avrRpm1* and pLN1906, which expressed AvrRpm1 and AvrRpt2, respectively, did not elicit an HR at these cell densities (Fig. 2). Thus, both AvrRpm1 and AvrRpt2 strongly suppressed the HopA1-dependent HR in tobacco and, by the classification used in Table 1, would both be considered class I suppressors. AvrRpm1 and AvrRpt2 have been reported to suppress PTI (Kim et al. 2005). Our results indicate that AvrRpm1 and AvrRpt2 can also suppress a HopA1-dependent ETI response, consistent with earlier reports indicating that these type III effectors could interfere with AvrRpm1-dependent and AvrRpt2-dependent ETI responses (Reuber and Ausubel 1996; Ritter and Dangl 1996).

Testing type III effector suppression of other ETI responses using a modified pHIR11 system.

In our earlier report, we modified pHIR11 by replacing *hopA1* with a kanamycin-resistance cassette resulting in pLN18, which encodes a functional T3SS and allows for the injection of other effectors into plant cells when expressed in *P. fluorescens*. However, *P. fluorescens*(pLN18) is limited because it uses kanamycin resistance to maintain pLN18. This is significant because *P. fluorescens* 55 is resistant to most of the other antibiotics commonly used to select for broad-host-range plasmids. To address this issue, we made a derivative of pLN18 in which the kanamycin-resistance cassette is replaced with a spectinomycin-resistance cassette resulting in construct pLN1965. *P. fluorescens*(pLN1965) allowed us to test whether other ETI responses were also suppressed by the type III effector that suppressed the HopA1-dependent HR. We introduced

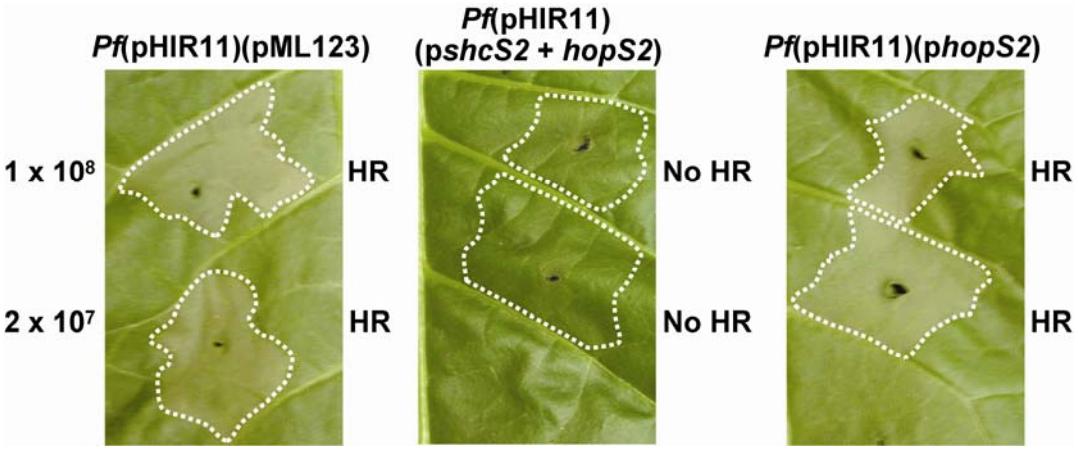

Fig. 1. The *Pseudomonas syringae* pv. *tomato* DC3000 type III effector HopS2 can suppress the HopA1-dependent hypersensitive response (HR) elicited by *P. fluorescens*(pHIR11). *P. fluorescens*(pHIR11) strains carrying a control vector, pLN1624 (p*shcS2* + *hopS2*) containing *hopS2* with its type III chaperone gene *shcS2*, or pLN452 (p*hopS2*) expressing *hopS2* alone were infiltrated into *Nicotiana tabacum* cv. Xanthi leaves at a cell density of 1×10^8 or 2×10^7 cells/ml. The leaves were evaluated for production of a HR and were photographed after 48 h. The ability of HopS2 to suppress the HopA1-dependent HR was dependent on the presence of its cognate type III chaperone ShcS2. This experiment was repeated 10 times with similar results.

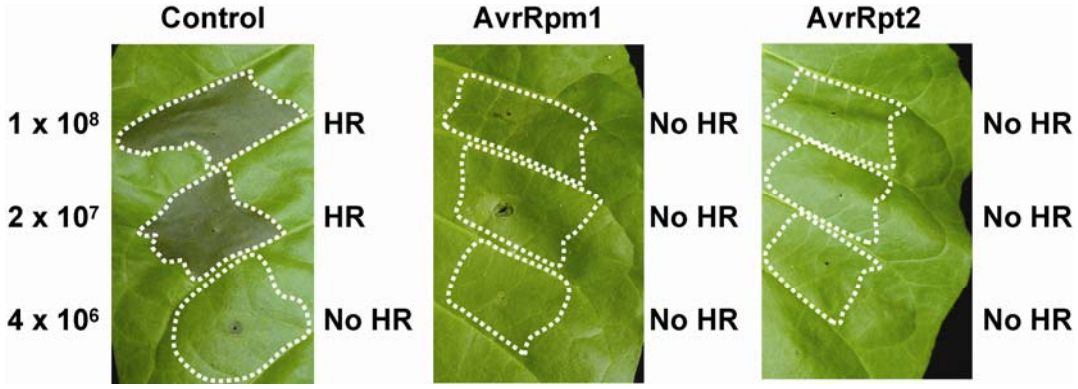

Fig. 2. *Pseudomonas syringae* type III effectors AvrRpm1 and AvrRpt2 can suppress the HopA1-dependent hypersensitive response (HR) in tobacco. *P. fluorescens*(pHIR11) strains carrying constructs pVSP61::*avrRpm1* (p*avrRpm1*), pLN1906 (p*avrRpt2*), or a vector control were infiltrated into *Nicotiana tabacum* cv. Xanthi leaves. *P. fluorescens*(pHIR11) carrying a vector control elicits a HR at the rate of 1×10^8 and 2×10^7 cells/ml, whereas the HR is suppressed in the *P. fluorescens*(pHIR11) strains expressing *avrRpm1* or *avrRpt2*, indicating that AvrRpm1 and AvrRpt2 can suppress the HopA1-dependent HR. Tobacco leaves were evaluated for the development of the HR and were photographed 48 h after infiltration.

avrRpm1 on a broad-host-range plasmid into *P. fluorescens* (pLN1965), also carrying compatible plasmids that encoded either HopE1, HopF2, AvrPtoB, HopAM1, or HopX1 and infiltrated each of these strains into *A. thaliana* Col-0. AvrRpm1 is recognized by R protein RPM1 present in this accession of *A. thaliana*, inducing ETI (Grant et al. 1995). All of these type III effectors were capable of suppressing the AvrRpm1-dependent HR (Fig. 3A). Callose deposition in the plant cell wall is another output of ETI. Using the same strains, we determined that each of these type III effectors was capable of reducing the amount of AvrRpm1-dependent callose deposition (Fig. 3B and C), indicating that these type III effectors can suppress multiple outputs of ETI.

Class I HR suppressors can also inhibit callose deposition induced by *P. fluorescens* PAMPs.

P. fluorescens(pLN1965) does not elicit an HR because it does not inject any type III effectors. However, it does contain flagellin and likely other PAMPs that are recognized by plants that lead to PTI. We found that when *P. fluorescens*(pLN1965) was infiltrated into *A. thaliana* Col-0 leaves, it induces callose deposition (Fig. 4), which is likely due to the recognition of *P. fluorescens* PAMPs. We infiltrated *P. fluorescens*(pLN1965) expressing either AvrPtoB, HopE1, HopF2, HopX1, or HopAM1 into *A. thaliana* Col-0 leaves, to test whether they were able to suppress callose deposition induced by *P. fluorescens*(pLN1965). Indeed, in each case, the callose deposition was reduced compared with that in a control strain (Fig. 4). Taken together, the ability of type III effectors to suppress callose deposition induced by both AvrRpm1 and *P. fluorescens* (pLN1965) suggests that either ETI and PTI responses overlap in their signaling pathways or that type III effectors can separately target unique components in each pathway, or both.

HopAO1 and HopF2 suppress flg22-induced innate immunity.

To further study the suppression of innate immunity by type III effectors, we chose to make transgenic *A. thaliana* Col-0

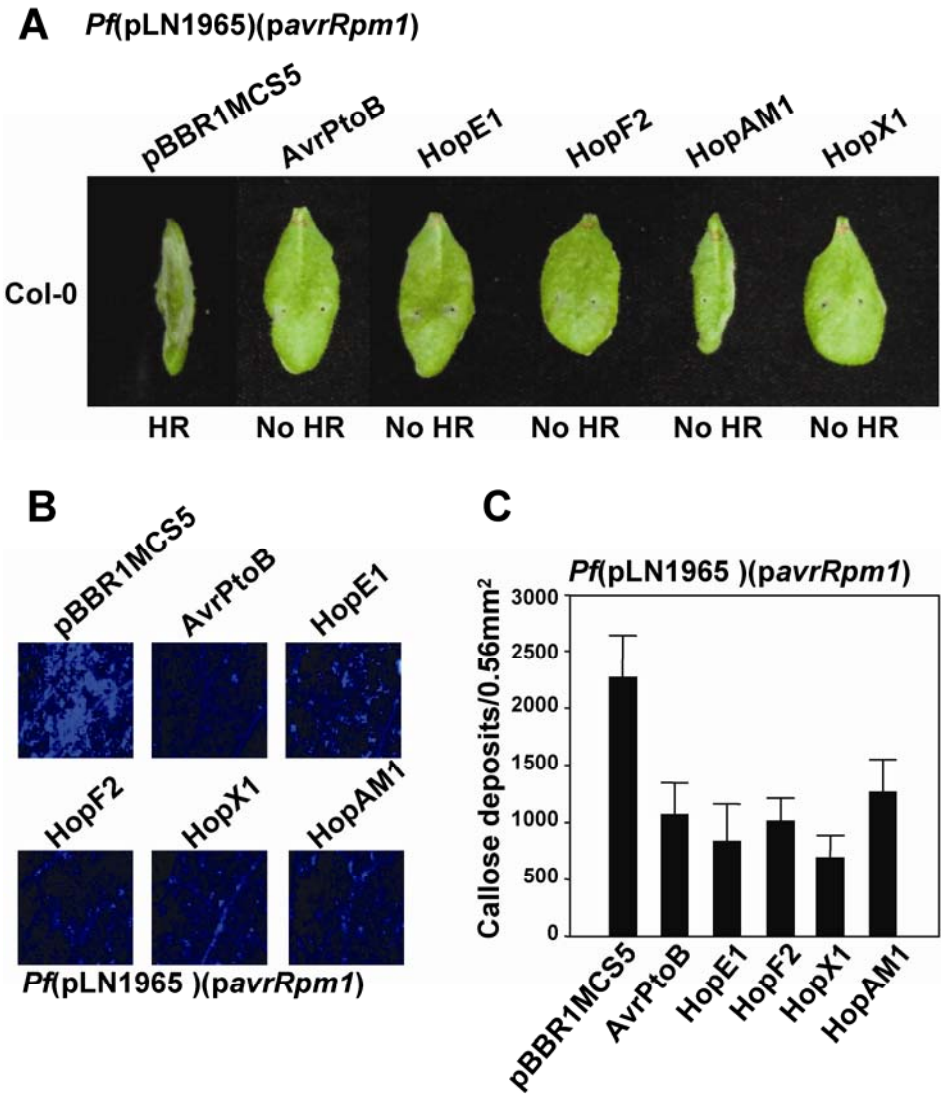

Fig. 3. Modification of the *Pseudomonas fluorescens*(pHIR11) system shows that a subset of type III effectors that are class I suppressors can also suppress the AvrRpm1-dependent effector-triggered immunity. **A,** *Arabidopsis thaliana* ecotype Col-0 leaves were infiltrated with *P. fluorescens*(pLN1965)(p*avrRpm1*) strains carrying an empty vector (pBBR1MCS5) or constructs that expressed different type III effectors known to suppress the HopA1-dependent hypersensitive response (HR). In each case, the type III effector was also capable of suppressing the AvrRpm1-dependent HR. **B,** *A. thaliana* ecotype Col-0 leaves were infiltrated with *P. fluorescens*(pLN1965)(p*avrRpm1*) strains carrying an empty vector (pBBR1MCS5) or constructs that expressed different type III effectors known to suppress the HopA1-dependent HR. Callose deposition induced by AvrRpm1 was suppressed in strains that expressed a type III effector known to suppress the HopA1-dependent HR. Leaves were microscopically viewed for evidence of callose deposition 16 h after infiltration.**C,** Callose deposits shown in B were quantified, and the average of 20 views of fields from five leaves and standard errors are shown.

plants that express either the class I suppressor HopF2 or the class II suppressor HopAO1. We have previously shown that HopAO1 (formally HopPtoD2) and HopF2 were capable of suppressing an HR response induced by an avirulent *P. syringae* strain or HopA1 (Espinosa et al. 2003; Jamir et al. 2004). We made several independent transgenic *A. thaliana* Col-0 lines that express HopAO1 or HopF2 fused to the hemagglutinin (HA) epitope under control of the constitutive *Cauliflower mosaic virus* 35S promoter. We confirmed that these lines expressed HopAO1-HA or HopF2-HA (Fig. 5A). When a conserved 22–amino acid peptide (flg22) from flagellin is infiltrated into *A. thaliana*, it induces PTI responses, which include callose deposition, alkalization, and an oxidative burst in a manner dependent on the FLS2 receptor kinase (Felix et al. 1999; Gomez-Gomez and Boller 2000; Gomez-Gomez et al. 1999). When 1 μM flg22 was infiltrated into transgenic plants expressing HopAO1-HA or HopF2-HA, they showed significantly reduced callose deposition compared with wild-type *A. thaliana* Col-0 plants (Fig. 5A and B). An inactive $flg22_{At}$ peptide derived from *Agrobacterium* flagellin was used as a control in these experiments, since this peptide is known not to induce high levels of callose deposition. Thus, both HopAO1 and HopF2 exhibited the ability to suppress flg22-induced callose deposition in *A. thaliana*.

Another way to test whether plants expressing HopAO1-HA and HopF2-HA were capable of suppressing PTI-dependent callose deposition is to infiltrate a *P. syringae* type III–defective mutant (Hauck et al. 2003). Since a type III–defective mutant is unable to inject any type III effectors, any PAMP recognized by the plant results in PTI. We infiltrated a *P. syringae* pv. *tomato* DC3000 *hrcC* mutant defective in type III secretion into transgenic plants expressing HopAO1-HA or HopF2-HA and examined callose deposition 16 h postinfiltration. The numbers of callose deposits were significantly reduced in the HopAO1-HA or HopF2-HA transgenic plants compared with wild-type plants (Fig. 5C). This result suggests that HopAO1 and HopF2 can suppress PAMP-induced callose deposition and is consistent with their ability to suppress flg22-induced PTI.

We determined how much of the callose deposition induced by the DC3000 *hrcC* mutant was due to recognition of flagellin by constructing a DC3000 *hrcC fliR* double mutant. The *fliR* gene encodes a protein in the flagellar basal body that is required for flagellar biogenesis (Fan et al. 1997; Kubori et al. 1997). We infiltrated the DC3000 *hrcC fliR* double mutant into wild-type *A. thaliana* and found that it induced less than half the number of callose foci than did the DC3000 *hrcC* single mutant (Fig. 5C), suggesting that flagellin is a major PAMP in *P. syringae*–plant interactions. We also infiltrated the DC3000 *hrcC fliR* mutant into *A. thaliana* Col-0 plants expressing either HopF2-HA or HopAO1-HA and found the residual callose deposition induced by the *hrcC fliR* mutant was suppressed in these plants (Fig. 5C), indicating that these type III effectors can suppress PTI induced by PAMPs other than flagellin.

DC3000 and a DC3000 type III-defective mutant grows to higher levels in *A. thaliana* plants expressing HopAO1-HA or HopF2-HA.

If plants expressing HopAO1-HA or HopF2-HA have a reduced PTI response, we thought they may be less able to defend themselves against *P. syringae*. To test this, we spray-inoculated DC3000 or the DC3000 *hrcC* mutant into wild type and *A. thaliana* plants expressing HopAO1-HA or HopF2-HA and determined how well each strain grew in these plants. DC3000 grew to higher levels in plants expressing either HopAO1-HA or HopF2-HA (Fig. 6A). Pretreatment with flg22 slightly reduced DC3000 growth in all plant lines when compared with the corresponding untreated plants (Fig. 6B). However, the expression of HopAO1-HA or HopF2-HA allowed increased DC3000 growth with or without flg22 pretreatment, suggesting that the PTI response is not completely suppressed by DC3000 type III effectors. Additionally, growth of DC3000 in type III effector–expressing plants pretreated with flg22 was reduced compared with untreated type III effector–expressing plants, which suggests that the PTI response was not completely suppressed by the in planta overexpression of these type III effectors.

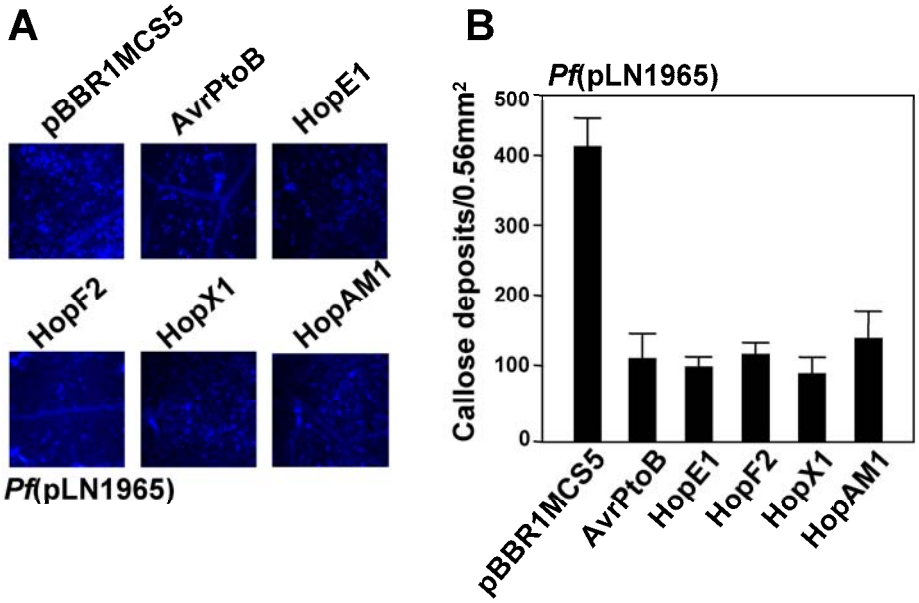

Fig. 4. Type III effectors belonging to the class I suppressor group can suppress callose deposition induced by pathogen-associated molecular pattern (PAMP)–triggered immunity. **A,** *Arabidopsis thaliana* ecotype Col-0 leaves were infiltrated with *Pseudomonas fluorescens*(pLN1965) strains carrying an empty vector (pBBR1MCS5) or constructs that expressed different type III effectors known to suppress the HopA1-dependent hypersensitive response. These type III effectors can suppress callose deposition triggered by PAMPs presented by *P. fluorescens*(pLN1965). Leaves were microscopically viewed for evidence of callose deposition 16 h after infiltration. **B,** Callose deposits shown in A were quantified, and the average of 20 views of fields from five leaves is shown. Each experiment was repeated three times with similar results, and the standard errors are indicated in the bar graphs.

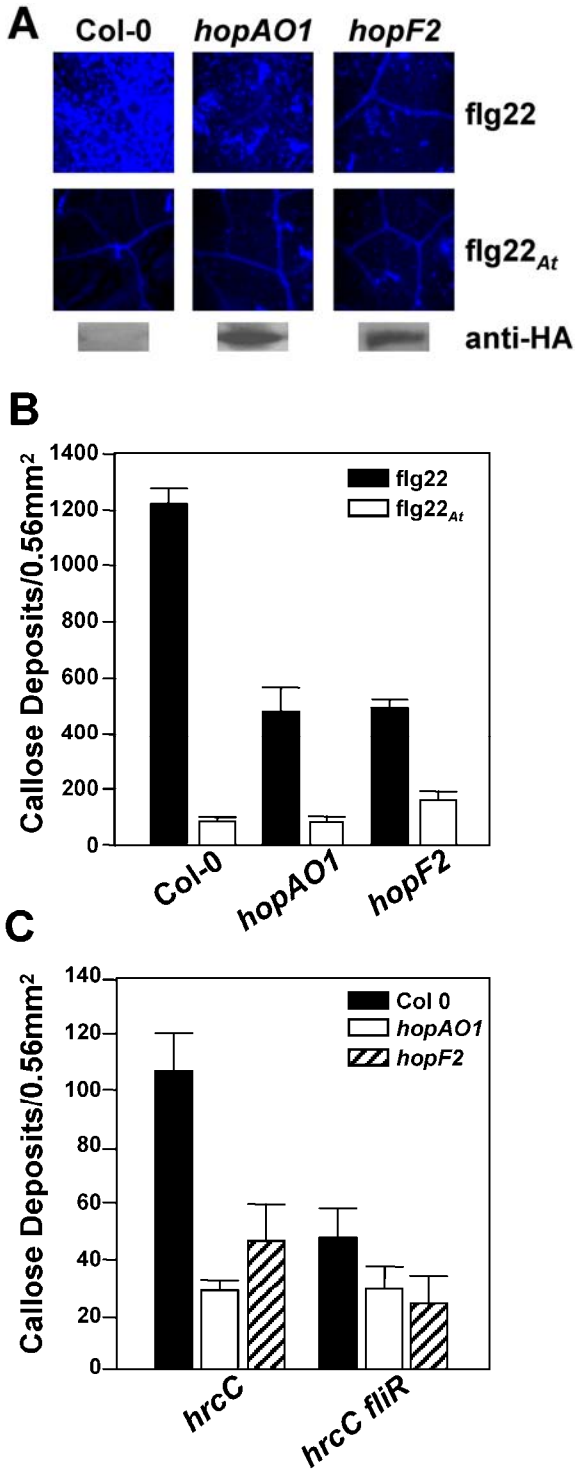

Fig. 5. Transgenic *Arabidopsis thaliana* plants expressing the HopAO1 or HopF2 type III effectors were reduced in pathogen-associated molecular pattern–triggered callose deposition. **A,** Callose deposition was visualized in wild-type *A. thaliana* Col-0 plants or in plants expressing HopAO1 hemagglutinin (HA) or HopF2-HA 16 h after treatment with flg22 or flg22$_{At}$, an inactive peptide from *Agrobacterium* flagellin. Immunoblots were carried out using anti-HA antibody on leaf tissue samples from plants used in the experiment, to confirm that HopAO1-HA and HopF2-HA were produced. **B,** Callose deposition in plants depicted in A was quantified by counting the number of callose foci per field of view. **C,** Callose deposition induced by a DC3000 *hrcC* mutant (defective in the ype III protein secretion system) or a DC3000 *hrcC fliR* (defective in flagellar biogensis) double mutant was quantified as noted in B. The numbers of callose foci in B and C are averages of 20 fields of view (four views per leaf samples) and standard errors are shown. All experiments were repeated three times with similar results.

The DC3000 *hrcC* mutant normally has restricted growth in planta, likely due to the induction of PTI and the inability of this mutant to suppress it by injecting type III effectors. Therefore, in planta growth assays using the *hrcC* mutant is another measure of PTI suppression (Hauck et al. 2003). In our experiments, the *hrcC* mutant accumulated to approximately 300-fold higher levels in *A. thaliana* plants expressing either HopAO1-HA or HopF2-HA compared with wild-type *A. thaliana* Col-0 plants (Fig. 6C). Taken together, these results suggest that both HopAO1 and HopF2 suppress PTI and ETI responses and this suppression allows the pathogen to grow better in plants.

DISCUSSION

In this report, we tested 35 DC3000 type III effectors for their ability to suppress the HopA1-dependent HR using *P. fluorescens* carrying cosmid pHIR11, which encodes a functional T3SS and one type III effector HopA1. The pHIR11 system was first used to assay for type III effectors that suppress the HR by Jamir and associates (2004). This earlier report tested 19 DC3000 type III effectors and identified seven that could suppress the HopA1-dependent HR. Here, we have extended this assay to include the complete DC3000 inventory of confirmed and likely type III effectors (Lindeberg et al. 2006), with the only exception being HopI1.

Based on our earlier results, we were expecting many more DC3000 type III effectors to completely suppress the HopA1-dependent HR when *P. fluorescens* (pHIR11) was infiltrated into tobacco at a cell density of 1×10^8 cells/ml. However, we identified only one additional DC3000 type III effector capable of HR suppression at this titer. This prompted us to infiltrate plants with bacteria at a cell density closer to the threshold cell density needed for production of a macroscopic HR and, when we did so, the majority of the DC3000 type III effectors possessed substantial HR suppression activity (Table 1). Each DC3000 type III effector was assigned to one of four different classes of HR suppressor, and there were only a few cases in which we were unable to detect any suppression of the HopA1-dependent HR.

The newly identified DC3000 type III effector as a class I suppressor was HopS2. Other than some similarity in its N-terminal secretion signal with HopS1', HopS2 has no obvious similarity with any proteins in the databases (Guo et al. 2005). We showed earlier that *hopS2* was expressed in conditions that induce expression of the T3SS and that HopS2 is injected into plant cells in a manner dependent on its type III chaperone ShcS2 (Guo et al. 2005). Based on a limited study, *hopS2* appeared to be well distributed in different *P. syringae* pathovars (Guo et al. 2005). The fact that this type III effector possesses particularly strong HR suppressor activity makes it an attractive type III effector to study further, to identify its plant targets. Our HR suppressor screen also included the well-characterized type III effectors AvrRpm1 and AvrRpt2 from other *P. syringae* strains, which were found also to be class I HR suppressors (Fig. 2). Both of these type III effectors are known to target RIN4 (Axtell and Staskawicz 2003; Mackey et al. 2003). RIN4 associates with RPM1 and RPS2, the R proteins that recognize AvrRpm1 and AvrRpt2, respectively, and modification of RIN4 appears to lead to induction of ETI. It would be of interest to know if the HopA1-dependent ETI response is also dependent on RIN4. Whatever the case, because of their similarly strong ability to suppress HopA1-induced ETI, it is possible that the type III effectors that make-up class I suppressors target related innate immunity components.

We modified cosmid pHIR11 to allow us to test whether a subset of the class I HR suppressors could also suppress the

HR induced by a different type III effector. This pHIR11 derivative, pLN1965, still encodes a functional T3SS but lacks *hopA1*. Using *P. fluorescens* (pLN1965), we showed that the type III effectors HopE1, HopF2, AvrPtoB, HopAM1, and HopX1, which were class I suppressors, all were capable of suppressing the AvrRpm1-dependent HR and callose deposition in *A. thaliana* Col-0 (Fig. 3). Because *P. fluorescens*(pLN1965) does not inject any type III effectors that induce ETI but does induce PTI, this system also allowed us to demonstrate that these five type III effectors also suppressed PTI-induced callose deposition (Fig. 4). In the majority of published reports, *P. syringae* type III effectors have been shown to suppress PTI or ETI, but both types of immunity generally are not tested. The most notable exceptions to this are the type III effectors AvrPtoB and HopAO1, which have been shown to suppress both ETI and PTI (Abramovitch and Martin 2005; Bretz et al. 2003; de Torres et al. 2006; Espinosa et al. 2003; Rosebrock et al. 2007; Underwood et al. 2007). The results from this study suggest that many *P. syringae* type III effectors likely suppress both ETI and PTI, possibly because type III effectors have multiple plant targets or their plant targets have roles in either one or both ETI and PTI.

We envision that *P. fluorescens*(pLN1965) will be useful for researchers studying other type III effectors. For example, they could use this system to screen whether the type III effectors they are interested in induce ETI or suppress innate immunity. Moreover, the system could be adapted for the identification of effectors from other bacterial, oomycete, fungal, or viral pathogens. Recently oomycete effector genes were identified by fusing candidate effector genes to nucleotide sequences corresponding to the N-terminal type III secretion signal of different *P. syringae* type III effectors (Rentel et al. 2008; Sohn et al. 2007). Expressing these fusion proteins in *P. syringae* allowed them to be injected into plant cells via the T3SS, facilitating the discovery of their in planta effects. However, studying these heterologous effectors in the wild-type *P. syringae* background may mask some of their effects in plant cells, due to the injection of many *P. syringae* type III effectors. We suggest that performing analogous experiments using *P. fluorescens* (pLN1965) may provide clearer plant phenotypes because this system does not inject any other type III effectors that could potentially mask interesting phenotypes. In addition, because this strain does not grow well in plant tissue determining whether a test effector contributes to in planta growth may be made more clear.

To investigate two of the type III effectors in planta phenotypes in more detail, we made transgenic *A. thaliana* plants that constitutively expressed a class I suppressor, HopF2, and a class II suppressor, HopAO1. We found that transgenic expression of both of these type III effectors suppressed flg22-induced PTI (Fig. 5). HopAO1 is a protein tyrosine phosphatase that has been previously shown to suppress a nonhost HR and other ETI responses (Bretz et al. 2003; Espinosa et al. 2003), and more recently, it has been shown to suppress PTI responses, using transgenic *A. thaliana* plants expressing HopAO1 (Underwood et al. 2007). Our experiments reported here with *A. thaliana* HopOA1-expressing plants essentially confirm that these plants are suppressed in their PTI responses. In our experiments, flg22 pretreatment still reduced the ability of DC3000 to multiply in HopAO1-expressing plants compared with untreated plants. Thus, HopAO1 did not completely suppress the PTI response in these plants, as it did in the earlier study.

The PTI response in *A. thaliana* plants expressing HopF2 was also suppressed based on the suppression of flg22-induced callose deposition, and the increased growth of the DC3000 type III–defective *hrcC* mutant. Other reports have suggested that HopF2 was capable of suppressing both ETI in tobacco

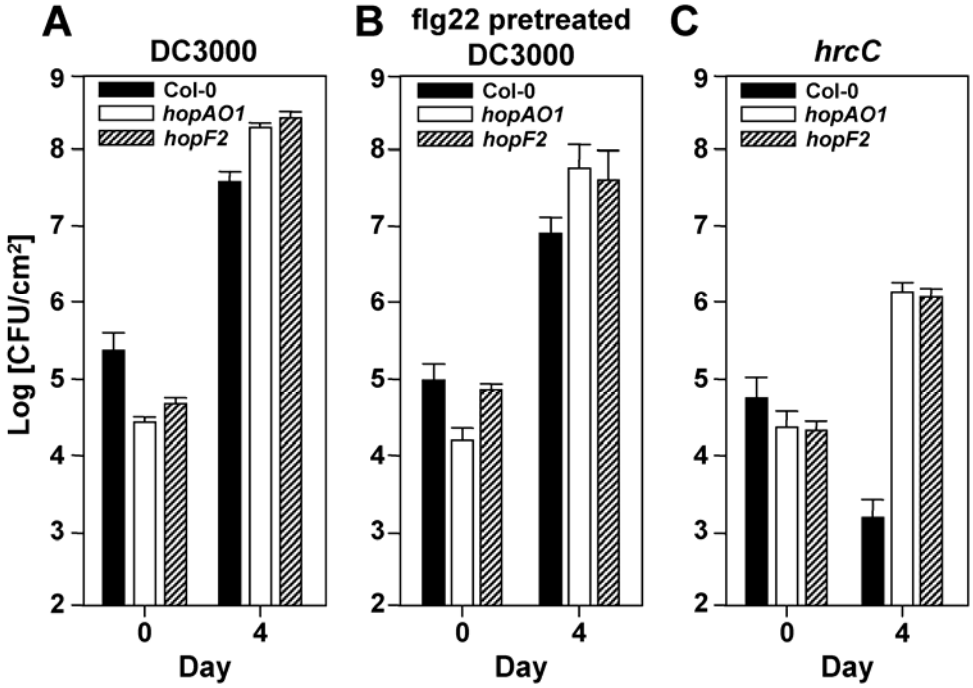

Fig. 6. Transgenic *Arabidopsis thaliana* plants expressing HopAO1 or HopF2 are more susceptible to *Pseudomonas syringae* and a type III–defective mutant than is wild-type *Arabidopsis*. **A,** *A. thaliana* Col-0 plants were spray-inoculated with *P. syringae* pv. *tomato* DC3000. DC3000 was able to grow to higher levels in plants expressing HopAO1 or HopF2 than in *A. thaliana* Col-0. **B,** *A. thaliana* Col-0 plants were pretreated with 1 μM flg22, and after 16 h, were spray-inoculated with DC3000. Overall, the pretreatment with flg22 reduced bacterial growth comp plants ared with non-pretreated plants in A. DC3000 grew to higher levels on plants expressing HopAO1 and HopF2 than it did on *A. thaliana* Col-0. **C,** *A. thaliana* were spray-inoculated with a DC3000 *hrcC*. The growth of the DC3000 *hrcC* mutant was enhanced in transgenic plants expressing HopAO1 or HopF2 as compared with wild-type Col-0. In all experiments, plants were inoculated at a cell density of 2×10^8 cells/ml, and bacteria were enumerated at days 0 and 4.

and PTI in *Nicotiana benthamiana* when delivered by bacteria (Jamir et al. 2004; Oh and Collmer 2005). Another report indicated that HopF2 and a number of other *P. syringae* type III effectors were capable of suppressing flagellin-induced gene expression in *A. thaliana* protoplasts (Li et al. 2005) consistent with HopF2 suppressing PTI. The enzymatic activity of HopF2 and its plant targets are currently unknown. However, its molecular structure has been determined, and one portion of it shared limited structural similarity with ADP-ribosyltransferases, but this activity could not be demonstrated (Singer et al. 2004). The results described here further establish that HopF2 can suppress both ETI and PTI.

A DC3000 *hrcC fliR* double mutant was capable of inducing callose deposition, although it was greatly reduced, indicating that flagellin is a primary PAMP in *A. thaliana* Col-0. Transgenic *A. thaliana* plants expressing HopAO1 or HopF2 were suppressed in their callose deposition in response to the DC3000 *hrcC fliR* double mutant, indicating that these type III effectors suppressed PTI responses induced by PAMPs other than flagellin. This is consistent with the induction of PTI in *A. thaliana* plants defective in FLS2, the receptor kinase that recognizes flagellin, which suggests that additional bacterial PAMPs are recognized by *A. thaliana* Col-0 (Zipfel et al. 2004). We now know that one of these additional bacterial PAMPs is the translation elongation factor EF-Tu (Zipfel et al. 2006), but likely other bacterial PAMPs and PAMP receptors await discovery.

Evolutionary models suggest that bacterial pathogens first acquired type III effectors that suppressed only PTI (Chisholm et al. 2006; Espinosa and Alfano 2004). The rationale behind this is that the existence of PTI likely predated ETI and that PTI probably was the primary selection for pathogens to initially acquire a T3SS and a minimal set of type III effectors. The most logical place to look for genes encoding type III effectors that target PTI but not ETI is in the conserved effector locus (CEL) within the Hrp pathogenicity island (Alfano et al. 2000) because these type III effectors genes are conserved in all *P. syringae* strains tested and because acquiring an apparatus without type III effector genes would serve little benefit to the pathogen. Interestingly, two conserved type III effector genes within the CEL are *avrE* and *hopM1* and both are class II suppressors in HopA1-induced HR suppression assays, indicating that both were capable of suppressing ETI. These results suggest that these type III effectors either act on host targets needed for both PTI and ETI, or they contain multiple activities, or both. Moreover, these results imply that the archetypal type III effectors may have also had the ability to suppress both PTI and ETI, which suggests that HopM1 and AvrE1 target components used in both ETI and PTI.

Collectively our results indicate that the lion's share of *P. syringae* type III effectors suppress plant innate immunity. However, they also illustrate the need for assays that report more specifically the responses affected in the host as well as the

Table 2. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference or source
E. coli		
DH5α	*supE44 ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nal^r	Hanahan 1983; Life Technologies, Gaithersburg, MD, U.S.A.
DB3.1	*F$^-$ gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 (r_B^-, m_B^-) supE44 ara-14 galK2 lacY1 proA2 rpsL20* (Sm^r) *xyl-5 λ$^-$ leu mtl-1*	Invitrogen, Carlsbad, CA, U.S.A.
C2110	PolA^{TS} Nal^r	Kahn and Hanawalt 1979
Pseudomonas fluorescens 55	Nal^r	M. Sasser
DC3000	Wild type; spontaneous Rif^r	Cuppels 1986
DC3000 *hrcC*	*hrcC* mutant defective in T3SS, Cm^r	Yuan and He 1996
UNL143	DC3000 *fliR hrcC* double mutant, Cm^r Sp^r	This work
pBluescript-II KS+	Cloning vector, Ap^r	Stratagene, La Jolla, CA, U.S.A.
pBBR1MCS5	Broad-host-range vector, Gm^r	Kovach et al. 1995
pCPP5040	pML123-hemagglutinin (HA) derivative gateway destination vector, Gm^r Cm^r	
pCPP5063	pCPP5040 derivative carrying *hopAM1*, Gm^r	Jamir et al. 2004
pCPP5068	pCPP5040 derivative carrying *hopX1*, Gm^r	Jamir et al. 2004
pCPP5100	pCPP5040 derivative carrying *hopK1*, Gm^r	Jamir et al. 2004
pENTR/D-TOPO	Gateway system entry vector, Km^r	Invitrogen. Carlsbad, CA, U.S.A.
pHIR11	Cosmid pLAFR3 derivative carring genomic DNA of *P. syringae* pv. *syringae* 61, Tc^r	Huang et al. 1988
pML123	Broad-host-range cloning vector, Gm^r Km^r	Labes et al. 1990
pRK415	Broad-host-range vector, unstable in absence of selection, Tc^r	Keen et al. 1988
pVSP61::*avrRpm1*	pVSP61 derivative carrying *avrRpm1*, Km^r	Mackey et al. 2002
pLN50	pML123 derivative carrying *hopC1-flag*, Gm^r	Jamir et al. 2004
pLN130	pML123 derivative carrying *hopAO1-ha*, Gm^r	Espinosa et al. 2003
pLN150	pML123 derivative carrying *hopH1-flag*, Gm^r	Jamir et al. 2004
pLN162	pML123 derivative carrying *hopE1- flag*, Gm^r	Jamir et al. 2004
pLN164	pML123 derivative carrying *hopAF1-flag*, Gm^r	Jamir et al. 2004
pLN165	pML123 derivative carrying *hopAD1-flag*, Gm^r	Jamir et al. 2004
pLN167	pML123 derivative carrying *hopD1-flag*, Gm^r	Jamir et al. 2004
pLN223	pML123 derivative carrying *hopU1-flag*, Gm^r	Petnicki-Ocwieja et al. 2002
pLN256	pML123 derivative carrying *hopT1-1-flag*, Gm^r	Guo et al. 2005
pLN271	pML123 derivative carrying *hopB1-flag*, Gm^r	Jamir et al. 2004
pLN347	pML123 derivative containing *avrPtoB-ha*, Gm^r	Jamir et al. 2004
pLN452	pLN615 derivative containing *hopS2*, Gm^r	Guo et al.
pLN460	pML123 derivative carrying *hopG1-flag*, Gm^r	Jamir et al. 2004
pLN462	pPZP212 derivative gateway destination binary vector containing 35S promoter and a HA tag for C-terminal fusions, Sp^r	Jamir et al. 2004
pLN517	pML123 derivative carrying *shcV/hopV1-ha*, Gm^r	Jamir et al. 2004
pLN518	pML123 derivative carrying *hopQ1-1-flag*, Gm^r	This work
pLN525	pLN462 derivative carrying *hopF2*, Sp^r	Jamir et al. 2004

(*continued on following page*)

[a] Nal^r, Rif^r, Cm^r, Sp^r, Ap^r, Gm^r, Km^r, and Tc^r = resistant to nalidixic acid, rifampicin, chloramphenicol, spectinomycin, ampicillin, gentamycin, kanamycin, and tetracycline.

urgency to identify specific plant targets and sites of action for type III effectors. We are pursuing a line of experimentation to do this for several of the type III effectors demonstrated to suppress plant immunity. The identification of plant targets for type III effectors promises to reveal key strategies for bacterial pathogenesis and important undiscovered components of innate immunity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and DNA constructs are listed in Table 2. *E. coli* and *Agrobacterium* strains were grown in Luria-Bertani (LB) medium at 37 and 30°C, respectively, with the appropriate antibiotics. *Pseudomonas* strains were cultured in King's B (KB) medium at 30°C with antibiotics. The concentrations (μg ml^{-1}) of antibiotics used in the experiments are as follows: ampicillin, 100; rifampicin, 100; kanamycin, 50; tetracycline, 20; spectinomycin, 50; gentamicin, 10; and nalidixic acid, 100.

DNA manipulation.

Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from New England Biolabs (Beverly, MA, U.S.A.). Thermostable *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) was used to amplify DNA fragments for cloning. Primers listed in Table 3 were ordered from Integrated DNA Technologies (Coralville, IA, U.S.A.). For Gateway cloning, target DNA fragments were amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase and were cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. pENTR constructs were recombined with Gateway Destination vectors by LR reactions using LR Clonase purchased from Invitrogen.

For construction of pLN1965, which lacks the *shcA*/*hopA1* operon, a 2.3-kb DNA fragment upstream of *shcA* was amplified by PCR from pHIR11, using primers P1892 and P0023, and was cloned into the *Xba*I and *Eco*RI sites of pBluescript KS^+, resulting in construct pLN1949. Primers P0227 and P0228 were used to PCR-amplify a 1.5-kb DNA region downstream of *hopA1* from pHIR11, and the amplified product was cloned into the *Xho*I and *Kpn*I sites of pLN1949 resulting in construct pLN1952. An omega cassette containing a spectinomycin-resistant gene was isolated from pHP45 by digestion with *Eco*RI and was cloned into pLN1952 at the *Eco*RI site within pLN1952, resulting in pLN1954, which was electroporated into *E. coli* C2110(pHIR11). The resulting strain was grown in LB medium containing tetracycline and spectinomycin at 30°C, which was then switched to 37°C for selection of recombination events. The pHIR11 derivatives that had undergone marker exchange were selected for based on spectinomycin resistance and ampicillin sensitivity and were confirmed

Table 2. (*continued from previous page*)

Strain or plasmid	Characteristics[a]	Reference or source
pLN567	pML123 derivative carrying *hopT1-2-flag*, Gm^r	Guo et al. 2005
pLN592	pLN462 derivative carrying *hopAO1*, Sp^r	Espinosa et al. 2003
pLN615	pML123 derivative gateway destination vector containing a HA tag for C-terminal fusions, Gm^r Cm^r	This work
pLN677	pBBR1MCS5 derivatived gateway destination vector containing a HA tag for C-terminal fusions, Gm^r Cm^r	Petnicki-Ocwieja et al. 2005
pLN682	pLN677 derivative carrying *hopE1*, Gm^r	This work
pLN683	pLN677 derivative carrying *shcF/hopF2* operon, Gm^r	This work
pLN685	pLN677 derivative carrying *avrPtoB*, Gm^r	This work
pLN686	pLN677 derivative carrying *hopAM1*, Gm^r	This work
pLN687	pLN677 derivative carrying *hopX1*, Gm^r	This work
pLN734	pLN1636 derivative containing a 2.0 kb downstream region of *fliR*. Ap^r Sp^r	This work
pLN745	pLN734 derivative containing a 1.5 kb upstream region of *fliR*. Ap^r Sp^r	This work
pLN746	pRK415 derivative containing a 1.5 kb upstream region, a Sp^r Ω cassette, and a 2.0-kb downstream of *fliR,* Tc^r Sp^r	This work
pLN1154	pLN615 derivative carrying *hopR1*, Gm^r	This work
pLN1156	pLN615 derivative carrying *shcM*/*hopM1*, Gm^r	This work
pLN1323	pLN615 derivative carrying *shcA/hopA1* from DC3000, Gm^r	This work
pLN1324	pLN615 derivative carrying *shcN/hopN1*, Gm^r	This work
pLN1326	pLN615 derivative carrying *hopAA1-1*, Gm^r	This work
pLN1327	pLN615 derivative carrying *avrPto1*, Gm^r	This work
pLN1328	pLN615 derivative carrying *hopAS1′*, Gm^r	This work
pLN1329	pLN615 derivative carrying *hopAN1*, Gm^r	This work
pLN1331	pLN615 derivative carrying *hopAQ1*, Gm^r	This work
pLN1419	pLN615 derivative carrying *hopAA1-2*, Gm^r	This work
pLN1420	pLN615 derivative carrying *shcF/hopF2*, Gm^r	This work
pLN1528	pLN615 derivative carrying *hopY1*, Gm^r	This work
pLN1622	pLN615 derivative carrying *shcO*/*hopO1-1*, Gm^r	This work
pLN1623	pLN615 derivative carrying *shcO*/*hopO1-2*, Gm^r	This work
pLN1624	pLN615 derivative carrying *shcS2*/*hopS2*, Gm^r	This work
pLN1636	pBluescript KS- containing Sp resistance Ω cassette at *Hin*d III site. Ap^r Sp^r	This work
pLN1906	pDSK509 derivative carrying *avrRpt2,* Km^r	This work
pLN1949	pBluescript II KS+ derivative carrying a 2.3-kb fragment upstream of *shcA* in pHIR11, Ap^r	
pLN1952	pBluescript II KS+ derivative carrying both a 2.3-kb fragment upstream of *shcA* and 1.5 kb fragment downstream of *hopA1* in pHIR11, Ap^r	This work
pLN1954	pBluescript-II KS+ derivative carrying both a 2.3-kb fragment upstream of *shcA*, an omega Sp resistant gene cassette, and 1.5-kb fragment downstream of *hopA1* in pHIR11, Ap^r Sp^r	This work
pLN1965	pHIR11 derivative containing a deletion of *shcA/hopA1* operon that is replaced with Sp resistant gene cassette, Tc^r Sp^r	This work
pLN2423	pLN615 derivative carrying *avrE1*, Gm^r	This work

using PCR and digestion with restriction enzymes. One of these derivatives that lacked *shcA/hopA1* and contained the spectinomycin omega cassette was designated pLN1965. This construct was introduced into *P. fluorescens* 55 by triparental mating.

Construction of a DC3000 *fliR hrcC* double mutant.

We used homologous recombination to delete the *fliR* gene in the DC3000 *hrcC* mutant. To do this, a 2.0-kb downstream region of *fliR* was amplified with primers P1070 and P1071 and was cloned into pLN1636 in the *Xho*I and *Kpn*I restriction enzyme sites resulting in pLN734. A 1.5-kb upstream region of *fliR* was amplified with primers P1080 and P1081 and was cloned into pLN734 in the *Bam*HI and *Eco*RV sites resulting in construct pLN745. A fragment containing the *fliR* flanking regions with an Ω fragment between them was cut out using *Xho*I and *Eco*RV and was cloned into pRK415, resulting in construct pLN746. pLN746 was transformed into the DC3000 *hrcC* mutant by electroporation. Homologous recombination in the *fliR* region was selected using spectinomycin and screening for loss of tetracycline resistance by growing a DC3000 *hrcC*(pLN746) culture for 5 days. Each day a new culture was inoculated using the fresh overnight culture in KB medium containing spectinomycin. The DC3000 *fliR hrcC* mutant, designated UNL142, was confirmed using PCR.

Protein manipulation and immunoblots.

Leaf disks of transgenic and wild-type plants were harvested with a 0.64-cm^2 cork borer and were ground in liquid nitrogen. The ground tissues were resuspended with 100 µl of 1.5× sodium dodecyl sulfate (SDS) sample buffer, followed by centrifugation for 5 min at 14,000 rpm. The decanted supernatant was subjected to SDS–polyacrylamide gel electrophoresis. The proteins were transferred to PVDF (polyvinylidene difluoride) membranes (Milipore, Billerica, MA, U.S.A.). Anti-HA primary antibodies (Roche, Basal, Switzerland) were used to detect HA fusion proteins bound on PVDF membranes. Secondary antibodies used in immunoblots were anti–rat immunoglobulin G alkaline-phosphatase conjugates (Sigma Chemical Co., St. Louis). Proteins on immunoblots were visualized using the CDP-Star chemiluminescence detection kit (Tropix, Bedford, MA, U.S.A.) followed by autoradiography.

Transgenic *Arabidopsis* plants.

The *Agrobacterium* binary constructs pLN592 and pLN525, which constitutively express *hopAO1-ha* and *hopF2-ha*, respectively, were electroporated into *Agrobacterium tumefaciens* C58C1. The resulting strains were used for *Arabidopsis* floral-dipping transformation. Transgenic lines were selected on Murashige-Skoog plates for kanamycin resistance. The expression of transgenes was detected by immunoblot using anti-HA antibodies. Transgenic plant lines with high and stable expression of HopAO1-HA or HopF2-HA were used in experiments.

Plant HR assay.

The DC3000 type III effector genes to be tested in *P. fluorescens* HR assays were cloned into pENTR/D-TOPO and were then recombined with pLN615, a pML123 destination vector that expresses C-terminal HA-tagged proteins (Table 2). The resulting constructs were electroporated into *P. fluorescens* (pHIR11). Bacterial strains grown on KB plates were resuspended at 1×10^8 cells/ml in 5 mM MES (morpholineethane-

Table 3. Primers for plasmid constructs

Gene name	Construct	Primers	Sequence
avrE1	pLN1098	P1127	5′-CACCACAGAGAGATTAACAGTGCAGTCACCATC-3′
		P1131	5′-CTCTTCAGTTCGAACCCCTCTTTCTTCAAG-3′
avrPto1	pLN307	P689	5′-CACCTGTACTAAAGAGGGTATAAGAATGGGA-3′
		P690	5′-TTGCCAGTTACGGTACGGGCT-3′
shcA/hopA1	pLN1028	P755	5′-CACCGTTGATGAGCAGAACAATGAGCAAC-3′
		P758	5′-TTTCGTGTTTCGAAGGGCCGG-3′
shcA/hopA1	pLN1965	P0023	5′-ATGAGAATTCGCATCTCCATGCATCTT-3′
		P0227	5′-CGGACTCGAGCTCAGGGCGCGAAACTGA-3′
		P0228	5′-GTATGGTACCCCGACCTGGCAACCGCAG-3′
		P1892	5′-AGTCTCTAGAGGTGCTATCCACGCAGCG-3′
shcF/hopF2	pLN536	P680	5′-CACCACCTTAAATTATGAGGATATGAGG-3′
		P682	5′-GACCCTTTCGACCGGCACTTT-3′
shcM/hopM1	pLN1464	P1108	5′-CACCCTCAGAGGCCTAACAATGACCAACAATGACCAG-3′
		P1128	5′-ACGCGGGTCAAGCAAGCCCTC-3′
shcN/hopN1	pLN1070	P1110	5′-CACCAATGAGCGGGGAATAACAATGCGGCCTGTCGAGGCA-3′
		P1129	5′-TCGCAAGTGAAAGTCTGCTCTGGGCAC-3′
hopR1	pLN1133	P1415	5′-CACCAGGAGTTTAATAATAACGATGGTCAAGG -3′
		P634	5′-CACGTTATCGAGTTCGCCCCA-3′
hopY1	pLN1491	P1713	5′-CACCGCGATAAAGGGAGATGAAACAATGAAC-3′
		P1714	5′-CTGGTAGTTGATGCCCGTGGCG-3′
hopAA1-1	pLN887	P1230	5′-CACCGAACGAGAGGGAATAACAATGCACATCAAC-3′
		P1231	5′-CGACCGCATAGGCCGAAACGG-3′
HopAA1-2	pLN888	P1202	5′-CACCGACCGAGATAGAATAACAATGCACATCAACCAATC-3′
		P1203	5′-CAAACGCCTGAGCTGAAACGG-3′
hopAI1	pLN1011	P1322	5′-CACCCCCATAAACAAAACAATGCTCGC-3′
		P1323	5′-GCGAGTCCAGGGCGGTGGCATCAGC-3′
hopAN1	pLN1007	P1314	5′-CACCGCGACCGACCTGGCAACAATGCTGGTGC-3′
		P1315	5′-GGCTTCTCCCGCCAATTGCTTGAG-3′
hopAQ1	pLN1015	P1310	5′-CACCCAAAGAGGTTTAACAATGAATCGAATTTC-3′
		P1311	5′-TGCACTGCCACCAGCAATCGAGCG-3′
hopAS1	pLN1009	P1318	5′-CACCCGATGGAGCCCAACAATGACCTTAAG-3′
		P1319	5′-AGAAAACTCGGCTTTCTGTTCAACC-3′
fliR	pLN746	P1070	5′-ATGACTCGAGGCTCGTTGAGATGGCAG A-3′
		P1071	5′-ATGAGGTACCTGCTGAAAGATACCGACC-3′
		P1080	5′-ATGAGGATCCCACATGCGCCCTGGCGATG-3′
		P1081	5′-ATGAGATATCGGTACTGATCTGGATATCGGTCAG-3′

sulfonic acid), pH 5.6; three fivefold serial dilutions were made, and each dilution was infiltrated into *Nicotiana tabacum* cv. Xanthi leaves side by side with the vector control strain *P. fluorescens*(pHIR11 + pML123). HR responses were recorded 24 to 48 h after infiltration.

Callose deposition assay.

To induce callose deposition wild-type *A. thaliana* Col-0 or transgenic plants were infiltrated with 1 μM flg22 or flg22$_{A_t}$ (flg22 from *Agrobacterium,* which is not recognized by FLS2 in Col-0). Alternatively, they were infiltrated with either the *hrcC* mutant or the *hrcC fliR* double mutant. In an additional strategy, we expressed AvrPtoB (pLN685), HopE1 (pLN682), HopF2 (pLN683), HopX1 (pLN687), HopAM1 (pLN686), and pBBR1MCS-5 (vector) in *P. fluorescens*(pLN1965). The suspensions of the strains were infiltrated into Col-0 at 1×10^7 cells/ml. For the experiments to determine if the type III effectors suppress callose deposition induced by AvrRpm1, pLN685 (AvrPtoB), pLN682 (HopE1), pLN683 (HopF2), pLN686 (HopAM1), pLN687 (HopX1), and pBBR1MCS-5 were electroporated into *P. fluorescens*(pLN1965) harboring pVSP61::*avrRpm1*. The resulting strains were infiltrated into *A. thaliana* Col-0 at a rate of 1×10^6 cells/ml. To determine the levels of callose deposition 16 h after infiltration, leaf samples were cleared with an alcoholic lactophenol solution, followed by a rinse in 50% ethanol and a rinse in water as described (Adam and Somerville 1996). The completely cleared leaves were stained with 0.01% (wt/vol) aniline blue in a solution of 150 mM K_2HPO_4, pH 9.5, for 30 min. The callose deposits were visualized with a fluorescence microscope (Zeiss Axionplan 2, Carl Zeiss, Oberkochen, Germany), and the number of callose deposits was determined using Quantity One software (Bio-Rad, Hercules, CA, U.S.A.).

In planta growth and pathogenicity assays.

Wild-type *A. thaliana* Col-0 or transgenic plants were grown in a growth chamber at 24°C with 10 h of light per day. Bacterial growth in Col-0 and transgenic plants was determined by spray-inoculating *A. thaliana* leaves with DC3000 or the DC3000 type III–defective *hrcC* mutant. In some instances, plants were pretreated with flg22 by infiltration of either 1 μM flg22 or 1 μM flg22$_{At}$. Plants were spray-inoculated with bacterial suspensions 12 h after pretreatment. The ability of bacteria to grow in planta was determined as described previously (Espinosa et al. 2003). Briefly, four leaf disks were harvested with a 0.4-cm^2 cork borer 0 and 4 days after inoculation. The samples were macerated in 250 μl of sterile water for 1 min and were serially diluted. Each dilution (20 μl) was plated on KB medium containing the appropriate antibiotics. The colonies recovered after 48 h on plates were enumerated.

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