Components of the *Pseudomonas syringae* Type III Secretion System Can Suppress and May Elicit Plant Innate Immunity

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Submitted 10 November 2009. Accepted 11 January 2010.

The type III secretion system (T3SS) of *Pseudomonas syringae* translocates into plant cells multiple effectors that suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). *P. syringae* pv. *tomato* DC3000 no longer delivers the T3SS translocation reporter AvrPto-Cya in *Nicotiana benthamiana* leaf tissue in which PTI was induced by prior inoculation with *P. fluorescens*(pLN18). Cosmid pLN18 expresses the T3SS system of *P. syringae* pv. *syringae* 61 but lacks the *hopA1_{Psy61}* effector gene. *P. fluorescens*(pLN18) expressing HrpH_{PtoDC3000} or HopP1_{PtoDC3000}, two T3SS-associated putative lytic transglycosylases, suppresses PTI, based on multiple assays involving DC3000 challenge inoculum (AvrPto-Cya translocation, hypersensitive response elicitation, and colony development in planta) or on plant responses (vascular dye uptake or callose deposition). Analysis of additional mutations in pHIR11 derivatives revealed that the pLN18-encoded T3SS elicits a higher level of reactive oxygen species (ROS) than does *P. fluorescens* without a T3SS, that enhanced ROS production is dependent on the HrpK1 translocator, and that HopA1_{Psy61} suppresses ROS elicitation attributable to both the *P. fluorescens* PAMPs and the presence of a functional T3SS.

Pseudomonas syringae is a plant pathogen that colonizes the intercellular spaces in leaves and other aerial organs and causes diseases in a wide variety of crops. *P. syringae* defeats plant innate immunity by injecting effector proteins into host cells via the type III secretion system (T3SS). A current model for *P. syringae*–plant interactions holds that bacterial pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs), such as flagellin, lipopolysaccharide, peptidoglycan, and elongation factor TU, elicit PAMP-triggered immunity (PTI) in plant cells, that an essential function of type III effectors is to suppress PTI, and that the activities of effectors inside plant cells can be recognized by R proteins in a second level of defense known as effector-triggered immunity (ETI) (Boller and Felix 2009; Jones and Dangl 2006). ETI commonly results in a localized, rapid, programmed cell death known as the hypersensitive response (HR). In a coevolutionary arms race with plants, pathogens

defeat ETI by mutating betraying effector genes or by injecting other effectors that suppress ETI (but which may fall under surveillance of new R proteins). According to this model, the outcome of an infection is determined largely by interactions of complex repertoires of pathogen effectors and plant R proteins, with the role of the T3SS being limited to the injection of effectors. That is, the T3SS machinery itself has no role in eliciting, suppressing, or being a target of plant defenses.

Progress has been made in understanding mechanisms of PAMP perception, in identifying global changes in gene expression, and in developing several assays for physiological and transcriptional markers (especially in *Arabidopsis*) for the PTI state (Boller and Felix 2009). PTI is readily induced experimentally by infiltrating high levels of a T3SS-deficient pathogen or a nonpathogen into plant leaf tissue; within 6 h, the infiltrated tissue no longer supports the growth of a virulent pathogen or HR elicitation by an avirulent pathogen (Klement et al. 2003; Oh and Collmer 2005). Other physiological markers for PTI include the formation of callose deposits (Hauck et al. 2003), reduced uptake of vascular dyes into minor veins (Oh and Collmer 2005), and the generation of reactive oxygen species (ROS) (Chinchilla et al. 2007; Torres et al. 2006). However, the changes in PTI-protected tissue that are responsible for this immunity and the corresponding basis for bacterial failure await elucidation.

Two model systems have proven particularly useful in studying the *P. syringae* T3SS and its effectors. The first is the reference strain *Pseudomonas syringae* pv. *tomato* DC3000, a pathogen of *Arabidopsis* and tomato whose genome sequence is continuously curated and whose type III effector repertoire has been comprehensively identified and largely disassembled through the construction of deletion polymutants (Kvitko et al. 2009; Lindeberg et al. 2006). The second model system involves cosmid pHIR11, which contains a functional cluster of *hrp/hrc* (HR and pathogenicity or conserved) T3SS genes that were cloned from the bean pathogen *P. syringae* pv. *syringae* 61 and enable nonpathogens like *P. fluorescens* and *Escherichia coli* to elicit the HR in tobacco (Huang et al. 1988). The *P. syringae* genomic region cloned in pHIR11 also includes genes encoding the HopA1_{Psy61} effector and its chaperone (Alfano et al. 1997; van Dijk et al. 2002). Nonpathogens carrying pHIR11 elicit the HR in tobacco because HopA1_{Psy61} acts as an avirulence determinant when injected into tobacco cells (Alfano et al. 1997). Importantly, *P. fluorescens* carrying derivatives of cosmid pHIR11 and its functional cluster of *P. syringae* pv. *syringae* 61 *hrp/hrc* genes enables individually expressed effectors and other substrates of the T3SS pathway to produce strong plant interaction phenotypes that are otherwise masked by redundancy when the

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Fig. 1. Pathogen-associated molecular pattern-triggered immunity (PTI) blocks type III secretion system (T3SS) effector translocation by challenge inoculation bacteria, but this defense response can be suppressed if the inducing bacteria deliver HopP1_{PtoDC3000}, HrpH_{PtoDC3000}, or AvrPto (positive control) via the T3SS. **A**, As depicted in the diagram, *Pseudomonas fluorescens*(pLN18) strains carrying a second plasmid (pX) expressing HrpH_{PtoDC3000} (HrpH), HopP1_{PtoDC3000} (HopP1), AvrPto, or pCPP3214 (vector) were infiltrated into *Nicotiana benthamiana* leaves at 2×10^8 CFU ml⁻¹ and were then challenged 6 h later with an overlapping inoculation of *P. fluorescens*(pLN18 + pCPP3221) expressing AvrPto-Cya at 2×10^7 CFU ml⁻¹. At 6 h after the challenge inoculation, 1.0-cm leaf disks were collected from areas of overlap between pretreatments and challenge inoculations. Cya translocation assays on disks from independent leaves were performed in triplicate, and the mean and standard deviation are shown. **B**, As depicted in the diagram, *P. fluorescens*(pLN18) cells carrying a second plasmid (pX) expressing HrpH_{PtoDC3000}-Cya (HrpH), HopP1_{PtoDC3000}-Cya (HopP1), AvrPto-Cya (AvrPto), or pCPP3214 (vector) were infiltrated into *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹ and were challenged 6 h later with an overlapping inoculation of *P. syringae* pv. *tomato* DC3000 at 2×10^7 CFU ml⁻¹. Leaves were photographed 24 h after the challenge inoculation. Confluent cell death was observed in the areas of overlapping inoculation marked with the continuous white line.

corresponding genes are mutated in *P. syringae* pv. *tomato* DC3000 or other source pathogens. pHIR11 has been described as a “workhorse” and its toolbox has been recently expanded by stable integration of a derivative into the genome of *P. fluorescens* Pf-0 (Thomas et al. 2009). DC3000 effectors that suppress ETI have been identified by their ability to block HR elicitation by *P. fluorescens*(pHIR11) in tobacco (Guo et al. 2009; Jamir et al. 2004). Similarly, *P. fluorescens* carrying pLN18 and similar HopA1_{Psy61}-deficient pHIR11 derivatives induce PTI in tobacco and *Nicotiana benthamiana*, unless expressing a PTI-suppressive effector, such as AvrPto (Guo et al. 2009; Oh and Collmer 2005).

In addition to effectors, substrates for the *P. syringae* T3SS include a variety of proteins that are thought to function in the pathway itself. These include the pilus subunit HrpA (Roine et al. 1997), putative lytic transglycosylases HrpH and HopP1 (Oh et al. 2007), harpin/translocators HrpZ1 (He et al. 1993), HrpW1 (Charkowski et al. 1998), and HopAK1 (Kvitko et al. 2007), conserved translocator HrpK1 (Petnicki-Ocwiega et al. 2005), and several proteins encoded in the core *hrp/hrc* cluster that appear to have structural or regulatory roles, or both, in assembly of the T3SS, including HrpB, HrpD, HrpF, HrpJ, and HrpP (Ramos et al. 2007).

The putative lytic transglycosylases and harpin/translocators represent two classes of redundant factors that are important for effector translocation into host cells but not effector secretion into the bacterial milieu. HrpK1 is a member of a conserved family of T3SS translocator components and appears to be the most important translocator, although it functions redundantly with the HrpZ1, HrpW1, and HopAK1 harpins (Kvitko et al. 2007; Petnicki-Ocwiega et al. 2005). Harpins are glycine-rich, cysteine-lacking proteins that are unique to plant pathogens and can elicit plant defenses, including the HR, when purified and infiltrated into leaf intercellular spaces, but their role in defense elicitation during natural infections is less clear.

Specialized lytic transglycosylases associated with the T3SS of plant and animal pathogens are thought to act in the bacterial envelope to enable the peptidoglycan mesh to accommodate the T3SS machinery (Koraimann 2003). HrpH is typical of specialized lytic transglycosylases in having a soluble lytic transglycosylase superfamily 1 domain and being encoded in linkage with other T3SS genes. However, the *P. syringae* HrpH and HopP1 lytic transglycosylases are atypical because they are translocated into host cells (Oh et al. 2007). HrpH is also unlike the specialized lytic transglycosylases of animal and plant pathogens with Hrp2 T3SS (found in *Ralstonia* and *Xanthomonas* spp.) in being more than two times larger and possessing MLTD_N and Pfam-B domains and a novel, highly variable C-terminal region (Oh et al. 2007). This suggests that HrpH may have multiple functions.

Here, we report several novel findings. i) *P. syringae* pv. *tomato* DC3000 is unable to deliver into PTI-protected tissue the model effector AvrPto fused to the translocation reporter Cya. ii) HrpH_{PtoDC3000} and HopP1_{PtoDC3000} function as well as the reference effector AvrPto in suppressing PTI when delivered by *P. fluorescens*(pLN18), as based on multiple assays for PTI. iii) HopA1_{PtoDC3000} and HopA1_{Psy61} are also suppressors of PTI. iv) The PTI-suppressive activity of HopA1_{Psy61} masks a hidden capacity of the pHIR11 T3SS to strongly elicit plant generation of ROS, another PTI marker. v) HrpK1_{Psy61} contributes strongly to this elicitor activity.

As a note on nomenclature, effectors are designated Avr (avirulence) or Hop (Hrp outer protein). The Hop designation is also used for more recently found T3SS substrates, such as HopAK1, that appear to be T3SS helpers rather than injected

effectors but are encoded outside of the Hrp pathogenicity island. Because this report involves both *P. syringae* pv. *tomato* DC3000 and pHIR11, for several genes and their products, we will frequently use the extended notation of a unified nomenclature system, which includes a subscript denoting the source pathovar and strain (Lindeberg et al. 2005).

RESULTS

***P. fluorescens*(pLN18)-induced PTI prevents challenge-inoculated bacteria from translocating AvrPto-Cya or eliciting the HR in *N. benthamiana*, but this effect can be reversed if the inducing bacteria deliver AvrPto, HrpH_{PtoDC3000}, or HopP1_{PtoDC3000}**

PTI develops within 6 h after infiltration of the leaf tissue of *Nicotiana* spp. with *P. fluorescens* and other nonpathogens (Klement et al. 2003; Oh and Collmer 2005). One indicator of PTI is that challenge inoculation of the same tissue with a strain of *P. syringae* that normally elicits the HR produces no visible response (Klement et al. 2003; Oh and Collmer 2005). Because HR elicitation by *P. syringae* requires T3SS delivery of effectors into plant cells, this observation suggests that the *P. syringae* T3SS no longer functions in PTI-protected tissue. To directly test this notion, we infiltrated *N. benthamiana* leaf tissue with *P. fluorescens*(pLN18, pCPP3214) at 1×10^8 CFU ml⁻¹ and then made an overlapping infiltration 6 h later with *P. fluorescens*(pLN18) expressing *avrPto-cya* in trans. pLN18 is pHIR11 with *shcA_{P_{sy61}}-hopA1_{P_{sy61}}* replaced with *nptII* (Jamir et al. 2004), pCPP3214 is an empty vector control (Schechter et al. 2004), and Cya (adenylate cyclase) is a reporter for translocation into eukaryotic cells (Casper-Lindley et al. 2002; Sory and Cornelis 1994). As shown in Figure 1A, the challenge bacteria translocate AvrPto-Cya into tissue mock-inoculated with buffer but not into tissue previously inoculated with *P. fluorescens*(pLN18, pCPP3214). A similar effect was observed when the inducing inoculum involved nonpathogens not expressing a *P. syringae* T3SS, such as *Pseudomonas putida* (data not shown). Thus, bacteria in tissue expressing PTI are impaired in their delivery of type III effectors.

We have previously shown that *P. fluorescens*(pLN18) can translocate AvrPto-Cya into *N. benthamiana* cells (Schechter et al. 2004) and that T3SS-delivered AvrPto-Cya is sufficient to suppress PTI, based on the challenge HR assay (Oh and Collmer 2005). Here, we constructed a derivative of vector pCPP3234 that introduces a stop codon at the 3' terminus of a Gateway-cloned effector gene, thus preventing fusion with Cya. We used this plasmid to learn if *P. fluorescens*(pLN18) delivering AvrPto can restore the ability of a *P. fluorescens*(pLN18) challenge inoculum to translocate AvrPto-Cya. As shown in Figure 1A, AvrPto-Cya translocation by the challenge was observed. We then used the same stop-codon vector to produce HrpH_{PtoDC3000} and HopP1_{PtoDC3000} without a Cya fusion and tested their ability to prevent *P. fluorescens*(pLN18) from inducing PTI-associated blockage of AvrPto-Cya delivery. Again, we observed AvrPto-Cya translocation by the challenge.

As expected, prior inoculation with *P. fluorescens*(pLN18, pCPP3214) also blocked the macroscopic HR (Fig. 1B). However, prior inoculation with *P. fluorescens*(pLN18) additionally producing HrpH_{PtoDC3000}-Cya or HopP1_{PtoDC3000}-Cya permitted the challenge inoculation to elicit the HR. Thus, both the T3SS translocation and HR challenge assays indicate that the two DC3000 putative lytic transglycosylases that are produced in a HrpL-dependent manner and are T3SS substrates have PTI suppressive activity that is comparable to that of AvrPto.

***P. fluorescens*(pLN18) producing HrpH_{PtoDC3000} or HopP1_{PtoDC3000} promotes the formation of GFP-labeled colonies in *N. benthamiana* by challenge-inoculated *P. syringae* pv. *tomato* DC3000.**

An important aspect of PTI is that protected tissue poorly supports the growth of bacterial pathogens. Although DC3000 elicits the HR in *N. benthamiana*, it does so relatively slowly in a manner dependent upon avirulence determinant HopQ1-1, which permits an initial period of T3SS-dependent bacterial growth (Wei et al. 2007). Thus, we can use the same DC3000-*N. benthamiana* pathosystem to assess another aspect of PTI. Furthermore, by using confocal laser-scanning microscopy and green fluorescent protein (GFP)-labeled DC3000 challenge bacteria, we could observe the pattern of bacterial colony development in treated leaf tissues. As shown in a representative microscopy field in Figure 2A, we observed no significant development of DC3000 colonies beyond scattered individual cells in tissue pretreated with *P. fluorescens*(pLN18) carrying an empty vector. Thus, plant cells in the pretreated tissue appear to be uniformly protected by PTI. In contrast, numerous developing GFP-labeled DC3000 colonies are distributed within tissue that has not been pretreated and, therefore, is not expressing PTI. Interestingly, this protection appears to end within a few plant cells of the perimeter of the treated area (data not shown), and it is clearly lacking from the sample point B depicted in Figure 2, which is only a few millimeters from the pretreated area. In contrast, when the *P. fluorescens*(pLN18) inducing inoculum delivered AvrPto-Cya, GFP-labeled DC3000 colonies now developed in the challenged tissue. Importantly, GFP-labeled colonies also developed in tissue in which the inducer inoculum delivered HrpH_{PtoDC3000}-Cya and HopP1_{PtoDC3000}-Cya. This effect was not observed with HrpW1-Cya. Counts of relative numbers of detectable challenge bacteria or colonies of any size in treated and untreated tissues provided another measure of suppressor activity (Fig. 2C). Thus, HrpH_{PtoDC3000} and HopP1_{PtoDC3000} but not HrpW1_{PtoDC3000} can suppress the ability of PTI to block bacterial growth.

***P. fluorescens*(pLN18) producing HrpH_{PtoDC3000} or HopP1_{PtoDC3000} suppresses PTI-associated reduction of vascular dye uptake in inoculated *N. benthamiana* leaf areas.**

The previous three assays involved challenge inoculations to indicate development of the PTI-induced protected state in plant tissues. A physiological marker for PTI that can be directly assayed without a challenge inoculation involves the uptake of vascular dyes. This assay uses neutral red as a vascular dye to detect a reduction in water uptake into minor veins. This effect is possibly due to either stomatal closure, reduced xylem conductivity, or both and could be important in PTI, because local desiccation of leaf tissue is emerging as a major defense against bacteria (Freeman and Beattie 2009; Melotto et al. 2006; Oh and Collmer 2005). The PTI-associated reduction in dye uptake that can be observed by feeding neutral red through the base of excised petioles 6 h after inoculation with *P. fluorescens*(pLN18) can be documented both photographically and spectrophotometrically (Fig. 3). We have previously shown that *P. fluorescens*(pLN18) expressing AvrPto-Cya no longer elicits this response (Oh and Collmer 2005). Here, we observed that HrpH_{PtoDC3000}-Cya and HopP1_{PtoDC3000}-Cya have a similar ability to suppress this PTI-associated plant response that is otherwise induced by *P. fluorescens*(pLN18).

***P. fluorescens*(pLN18) producing HrpH_{PtoDC3000} or HopP1_{PtoDC3000} suppresses PTI-associated callose production in *N. benthamiana*.**

Another PTI-associated marker that can be directly assayed without a challenge inoculation involves the formation of cal-

lose deposits, which are considered to indicate cell wall-based defenses (Hauck et al. 2003). Here, we inoculated leaves with *P. fluorescens*(pLN18) carrying an empty vector or expressing HrpH_{PtoDC3000}-Cya, HopP1_{PtoDC3000}-Cya, or AvrPto-Cya at 1×10^8 CFU ml⁻¹ and, 6 h later, assayed for callose deposits using aniline blue and fluorescence microscopy. Typical microscopy fields and callose deposit counts from several experiments are presented in Figure 4, and they show that *P. fluorescens* (pLN18), in contrast to buffer control, induces callose formation. Importantly, HrpH_{PtoDC3000}-Cya, HopP1_{PtoDC3000}-Cya, and AvrPto-Cya all suppress this response.

Unlike HrpH_{PtoDC3000}, HrpH_{Psy61} expressed in trans in *P. fluorescens*(pLN18) appears unstable and does not suppress PTI-associated ROS production in *N. benthamiana* cells.

A puzzling aspect of the foregoing results is that, in multiple assays, HrpH_{PtoDC3000} is able to suppress the PTI that is induced by *P. fluorescens*(pLN18), despite the fact that the *hrp* gene cluster cloned in pLN18 includes the native *hrpH_{Psy61}*. Furthermore, the *hrpH_{Psy61}* gene appears to be functional, because deleting it strongly reduces the ability of pHIR11 derivatives to translocate HopA1_{Psy61}-Cya into plant cells (Oh et al. 2007).

Fig. 2. HrpH_{PtoDC3000} and HopP1_{PtoDC3000}, delivered by *P. fluorescens* expressing a *Pseudomonas syringae* type III secretion system (T3SS), are able to suppress pathogen-associated molecular pattern-triggered immunity (PTI) in *Nicotiana benthamiana*, as indicated by colonization of DC3000 expressing green fluorescent protein (GFP). As depicted in the diagram, *P. fluorescens*(pLN18) strains carrying a second plasmid (pX) expressing HrpH-Cya (HrpH), HopP1_{PtoDC3000}-Cya (HopP1), HrpW1_{PtoDC3000}-Cya (HrpW1), AvrPto-Cya (AvrPto), and pCPP3214 (vector) were infiltrated into *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹ and were challenged 6 h later with an overlapping inoculation of GFP-expressing *P. syringae* pv. *tomato* DC3000(pCPP3069) at 2×10^7 CFU ml⁻¹. At 6 h after the challenge inoculation, GFP-labeled bacteria were visualized by confocal microscopy. Representative microscopy fields from **A**, overlapping areas pretreated with *P. fluorescens* (pLN18) strains and **B**, areas treated with only challenge bacteria. Scale bar = 50 μ m. **C**, Relative numbers of detectable bacteria or colonies counted in three independent microscopy images obtained as in rows A and B. Data are expressed as the mean percentage of A relative to B and the standard deviation.

One possible explanation is that the ability of HrpH_{PtoDC3000} to suppress PTI in our assays is dependent upon its expression in trans as a Cya fusion driven by a vector tac promoter. Alternatively, HrpH_{PtoDC3000} and HrpH_{Psy61} may be intrinsically different in this ability. To address this issue, we inserted *hrpH*_{Psy61} into the same vector backbone used to express HrpH_{PtoDC3000}-Cya, and we assayed for the ability of the two proteins to be translocated into plant cells by *P. fluorescens*(pLN18) and to suppress defense-associated generation of ROS, a convenient and quantitative assay that has been used in *N. benthamiana* (Hann and Rathjen 2007). To permit further comparison of the performance of T3SS substrates produced by DC3000 and the

pHIR11 system in these assays, we analyzed HopA1_{PtoDC3000} and HopA1_{Psy61} as well as our positive control AvrPto. As expected, HrpH_{PtoDC3000}-Cya, AvrPto_{PtoDC3000}-Cya, and HopA1_{PtoDC3000}-Cya were translocated by *P. fluorescens*(pLN18), and HopA1_{Psy61}-Cya was translocated by pCPP5316 (a pHIR11 derivative with a *hopA1*_{Psy61}-cya translational fusion) (Supplementary Fig. S1A). Furthermore, all of these proteins suppressed ROS production in comparison with the vector control.

Fig. 3. HrpH_{PtoDC3000} and HopP1_{PtoDC3000} delivered by *Pseudomonas fluorescens* expressing a *P. syringae* type III secretion system (T3SS) are able to suppress pathogen-associated molecular pattern-triggered immunity (PTI) as indicated by the vascular-staining assay in *Nicotiana benthamiana*. *P. fluorescens*(pLN18) strains expressing HrpH-Cya (HrpH), HopP1_{PtoDC3000}-Cya (HopP1), AvrPto-Cya (AvrPto), or pCPP3214 (vector) were infiltrated into *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹. **A**, Differential accumulation of neutral red in minor veins of inoculated leaf areas (white circles) was observed. At 6 h after infiltration, leaves were detached at the base of the petiole using a razor blade, the petioles were immersed in 1% neutral red solution, and 12 h later, the leaves were photographed. **B**, For quantitative analysis of dye accumulation in treated leaf tissues, 0.5-cm leaf disks were excised 12 h after petioles were placed in neutral red solution and dye was extracted into ethanol and measured by spectrophotometry. The results are the mean and standard deviation of five samples.

Fig. 4. HrpH_{PtoDC3000} and HopP1_{PtoDC3000} delivered by *Pseudomonas fluorescens* expressing *P. syringae* type III secretion system (T3SS) are able to suppress pathogen-associated molecular pattern-triggered immunity (PTI), as indicated by callose deposition in *Nicotiana benthamiana*. *P. fluorescens*(pLN18) strains carrying a second plasmid expressing HopP1_{PtoDC3000}-Cya (HopP1), HrpH_{PtoDC3000}-Cya (HrpH), AvrPto-Cya (AvrPto), and pCPP3214 (vector) were infiltrated into *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹, and 24 h later, leaf disks from infiltrated areas were excised for callose staining. Callose deposits, stained with aniline blue, were visualized and counted by epifluorescence microscopy.

In contrast, HrpH_{Psy61}-Cya was not translocated by *P. fluorescens*(pLN18) and did not suppress ROS production. Furthermore, immunoblot analysis of cell lysates of *P. fluorescens*(pLN18) expressing the proteins tested confirmed the accumulation of all of the proteins tested except for HrpH_{Psy61}-Cya. This observation was consistent with an apparent toxicity of HrpH_{Psy61}, as indicated by the comparatively slower growth of *P. fluorescens*(pLN18) carrying *hrpH_{Psy61}-cya* (data not shown) and by the observed inhibition of *E. coli* growth following isopropylβ-D-thiogalactopyranoside (IPTG)-induced expression of *hrpH_{Psy61}*. We previously reported the toxicity of HrpH_{PtoDC3000} when expressed in *E. coli* (Oh et al. 2007), but we have not observed HrpH_{PtoDC3000} to have any effect on the growth of *P. fluorescens*(pLN18). As previously noted, the *P. syringae* HrpH proteins are exceptional among T3SS-associated lytic transglycosylases in their large size and possession of the Pfam-B domain and C-terminal extension. Interestingly, comparison of HrpH_{PtoDC3000} and HrpH_{Psy61} using BLOSUM62 reveals that amino acids 351 through 480 are only 26.9% identical and 38.5% similar in this region, which occurs between the Pfam-B domain and the highly conserved C-terminal 35 amino acids. Whatever the basis for the instability and toxicity of HrpH_{Psy61}, we were unable to use gain-of-function tests in our *P. fluorescens*(pLN18) system to determine if HrpH_{Psy61} also has an ability to suppress PTI.

Analysis of pHIR11 mutants expressed in *P. fluorescens* reveals that the *P. syringae* pv. *syringae* 61 T3SS strongly elicits ROS production unless suppressed by HopA1_{Psy61}.

We had previously used the pHIR11 system to show that HrpH_{Psy61} was needed for the translocation of HopA1_{Psy61}-Cya into *N. benthamiana* and for HopA1_{Psy61}-dependent HR elicitation in tobacco (*N. tabacum* cv. Xanthi) (Oh et al. 2007). In the present work, we learned that HrpH_{PtoDC3000} and HopA1_{PtoDC3000} can suppress PTI-associated ROS production in *N. benthamiana*. These observations raised the possibility of complex interplay between HrpH_{Psy61} and HopA1_{Psy61} and other components of the pHIR11 T3SS in both eliciting and suppressing plant defenses. Accordingly, we assembled a series of pHIR11 mutants and analyzed them in *P. fluorescens*, using the ROS assay in *N. benthamiana*. The mutant plasmid designations and their genotypes are summarized in Figure 5, along with a physical map of the *hrp/hrc* genes. The mutations involved *hopA_{Psy61}* and *hrpH_{Psy61}* as well as genes encoding three other factors thought to control distinct steps in the T3SS pathway, HrcV_{Psy61}, a core component of the T3SS that is essential for secretion of T3SS substrates across the inner membrane (Charkowski et al. 1997) and HrpZ1_{Psy61} and HrpK1_{Psy61}, which are members of distinct protein families that redundantly contribute to the translocation of effectors across plant cell barriers (Kvitko et al. 2007; Petnicki-Ocwiega et al. 2005). Test bacteria were infiltrated into *N. benthamiana* leaf tissue at 2×10^8 CFU ml⁻¹ and were assayed for ROS production 15 h later (Fig. 5). Analysis of these results, from left to right in Figure 5A, reveals pHIR11 components that suppress and possibly elicit PTI, as summarized in the Figure 5B model.

Comparing the buffer and *P. fluorescens* treatments demonstrates that, as expected, *P. fluorescens* elicits ROS production. However, comparing *P. fluorescens* with *P. fluorescens* carrying either pHIR11 or pCPP5316 reveals that HopA1_{Psy61} must be able to suppress the ROS that *P. fluorescens* would otherwise elicit, and the Cya fusion does not diminish this ability. The latter observation is consistent with our observations with other T3SS substrates, such as HrpH_{PtoDC3000} and AvrPto, that a C-terminal Cya fusion (which enables confirmation that the

protein is translocated in assays for function) does not interfere with biological activity in planta.

The contrasting strong ROS elicitation by pLN18 reveals two more things. First, the T3SS itself contributes to strong ROS elicitation that is substantially above the level that would otherwise be elicited by *P. fluorescens* PAMPs (compare *P. fluorescens* without a T3SS plasmid with *P. fluorescens* carrying pLN18). Second, HopA1_{Psy61} is capable of suppressing the ROS that are elicited by both *P. fluorescens* PAMPs and T3SS factors (compare pHIR11, pLN18, and no T3SS plasmid).

The *hrcV_{Psy61}* mutant cannot secrete HopA1_{Psy61} but, nevertheless, elicits the same intermediate level of ROS as *P. fluorescens* without a T3SS plasmid and not the high level elicited by pLN18. This suggests that the T3SS-associated ROS elicitor is a T3SS substrate that must be secreted beyond the bacterial inner membrane to be active.

The next three mutants shown in Figure 5 are lacking *hopA1_{Psy61}* and either *hrpH_{Psy61}*, *hrpZ1_{Psy61}*, or *hrpK1_{Psy61}*. The *hopA1_{Psy61}/hrpH_{Psy61}* mutant is not significantly different from the pLN18. In contrast the *hopA1_{Psy61}/hrpK1_{Psy61}* mutant is the most reduced in ROS elicitation and is not significantly different from the *P. fluorescens* strains with no T3SS plasmid or the *hrcV_{Psy61}* mutant. The *hopA1_{Psy61}/hrpZ1_{Psy61}* mutant is intermediate among these three treatments in its ROS phenotype. Thus, HrpK1_{Psy61} appears to be particularly important in T3SS-associated ROS elicitation, although HrpZ1_{Psy61} may make a minor contribution.

DISCUSSION

Cosmid pHIR11 encodes the first T3SS from plant or animal pathogens to be shown to function heterologously in nonpathogens (Huang et al. 1988), and studies with pHIR11 provided early evidence that *hrp* genes direct effector translocation into plant cells (Gopalan et al. 1996; Pirhonen et al. 1996). In recent years, *P. fluorescens* strains carrying derivatives of pHIR11 have proven useful in studying the ability of individual *P. syringae* pv. *tomato* DC3000 effectors to perform important functions in bacterium-plant interactions, such as suppression of PTI and ETI or elicitation of ETI. Here, we have used pHIR11 derivatives to learn that components and functions of the *P. syringae* T3SS itself interact with plant defenses. Notably, the T3SS does not deliver effectors into PTI-protected tissue, T3SS-associated lytic transglycosylases can suppress PTI, and pHIR11 has a capacity to enhance elicitation of ROS, which is strongly dependent upon the HrpK1 translocator. Our discussion will focus on these findings, new questions they raise, and their relevance to the concept of host recognition of “patterns of pathogenesis” that is emerging from studies with animal pathogens (Vance et al. 2009).

An important feature of PTI-protected leaf tissue is that it supports neither growth of a virulent pathogen nor HR elicitation by an avirulent pathogen. The HR has been used as an assay readout for T3SS-mediated translocation of effectors in systems in which the tested effector or its fusion partner carries an avirulence determinant (Chang et al. 2005; Guttman and Greenberg 2001; Mudgett and Staskawicz 1999). Thus, the simplest explanation for the failure of bacteria to elicit the HR in PTI-protected tissue is that effectors are no longer being delivered. We have tested that notion here by using Cya rather than the HR as a reporter for translocation and found that a reference T3SS substrate, AvrPto-Cya, is indeed no longer translocated. However, we do not have any evidence that PTI acts directly against the T3SS per se, for example, by rendering the plant cell wall impenetrable to the Hrp pilus. On the contrary, our use of GFP-labeled DC3000 and confocal microscopy to monitor the development of bacterial colonies in

Fig. 5. Analysis of key pHIR11 mutants expressed in *Pseudomonas fluorescens* suggests a bacterium-plant interaction model in which a functional *P. syringae* type III secretion system (T3SS) can elicit strong reactive oxygen species (ROS) production in *Nicotiana benthamiana*, but all ROS production is suppressed by HopA1_{Psy61}. **A**, *P. fluorescens* cells lacking pHIR11 or carrying pHIR11 or one of the indicated derivatives were infiltrated at 2×10^8 CFU ml⁻¹ into *N. benthamiana* leaves and were assayed for ROS production 15 h later. In the genotype table “+Cya” indicates that HopA1_{Psy61} is produced as a Cya fusion. ROS assay results are presented as the arithmetic mean and standard deviation based on three independent experiments of two samples from different plants in each experiment. Mean log(ROS) was determined to be not significantly different among experiments based on an analysis of variance randomized block analysis. The Tukey highly significant difference multiple comparisons procedure was performed on log-transformed values of the six assays for each pair of treatments; treatments with the same letter are not significantly different ($P = 0.05$). **B**, The model depicts *P. fluorescens* interacting with a plant cell and the factors involved in elicitation or suppression of ROS. Genes encoding proteins that are substrates for the T3SS are shaded; genes encoding regulators have diagonal hatching. *P. fluorescens* presents pathogen-associated molecular patterns (PAMPs), which are recognized by plant pattern-recognition receptors (PRR) leading to PAMP-triggered immunity and ROS production. *P. fluorescens* carrying pHIR11 derivatives that produce a T3SS capable of deploying the HrpK1 translocator elicit higher levels of ROS, but what plants recognize is unknown. All ROS production can be suppressed by the native HopA1_{Psy61} or, in its absence, HrpH_{PtoDC3000}, HopP1_{PtoDC3000}, or HopA1_{PtoDC3000} produced in trans from a plasmid. HrcV is necessary for T3SS export across the bacterial inner membrane. HrpK1 and secondarily HrpZ1 are thought to function as translocators in the host plasma membrane. HrpH is necessary for effector translocation, but its site of action has not been defined.

infected tissue revealed a uniform failure of bacteria to grow in PTI-protected tissue. Furthermore, the GFP signal from such bacteria was generally weaker than that from T3SS-deficient DC3000 mutants in naïve tissue (data not shown), which suggests that the T3SS may fail in PTI-protected tissue because of general stresses on the bacteria. Whatever its basis, the challenge inoculum Cya translocation assay provides a useful functional test for the PTI-protected state and its suppression.

Remarkably, HrpH_{PtoDC3000} and HopP1_{PtoDC3000} suppressed PTI, based on the challenge inoculum translocation and growth assays as well as on the vascular dye and callose assays, which involve altered water relations and cell-wall appositions implicated in plant defense. Although both of these proteins have soluble lytic transglycosylase domains, are produced in a HrpL-dependent manner, and are translocated by the T3SS, they have different structural features. HopP1 is typical of two-domain harpins, such as HrpW1_{PtoDC3000} and HopAK1_{PtoDC3000}. The N-terminal half of these proteins is harpin-like and the C-terminal half has an enzyme-like domain, with HrpW1_{PtoDC3000} and HopAK1_{PtoDC3000} carrying pectic enzyme domains (Kvitko et al. 2007). Notably, the pectic enzyme-like harpins appear to function redundantly with the HrpK1_{PtoDC3000} translocator, whereas HopP1_{PtoDC3000} does not (Kvitko et al. 2007). In this study, we included HrpW1_{PtoDC3000} in the challenge growth assay for PTI suppression and found no evidence that it shared with HopP1_{PtoDC3000} the ability to suppress PTI. We chose HrpW1_{PtoDC3000} for this test because it is widespread in plant pathogenic bacteria and the harpin domains of HopP1_{PtoDC3000} and HrpW1_{PtoDC3000} are similar (Fouts et al. 2002; Kvitko et al. 2007). Thus, it appears that proteins with the two-domain harpin structure can serve distinct functions. The *hopP1* gene appears to be of limited distribution in *P. syringae* strains. It is found in the sequenced genome of *P. syringae* pv. *tomato* T1 but not in genomes of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A, and it is not linked to the *hrpLhrc* gene cluster in DC3000 or T1 (Almeida et al. 2009; Lindeberg et al. 2006).

The *hrpH* gene, in contrast, appears universally associated with the *hrpLhrc* genes in *P. syringae* strains, and it makes the strongest contribution of the DC3000 HrpL-activated putative lytic transglycosylases (HrpH_{PtoDC3000}, HopP1_{PtoDC3000}, and HopAJ1_{PtoDC3000}) to effector translocation and associated phenotypes, based on studies with the pHIR11 system and DC3000 (Oh et al. 2007). Notably, *hrpH*_{PtoDC3000} was one of the DC3000 genes found to be upregulated in planta and to have a virulence defect when mutated (Boch et al. 2002).

Although HrpH_{PtoDC3000} has a capacity to suppress PTI, there are several reasons why it is difficult to assess the importance of that activity in either promoting effector translocation or virulence in DC3000. First, like many T3SS substrates, HrpH has multiple domains and, therefore, may have multiple functions (Cunnac et al. 2009). Thus, the promotion of effector translocation and suppression of PTI may be attributable to different domains and be mechanistically distinct. Second, we cannot study the potential of HrpH_{PtoDC3000} to suppress PTI in its native DC3000 background because of the confounding effects of the many PTI-suppressive effectors. Third, HrpH_{Psy61} differs from HrpH_{PtoDC3000} in having no demonstrable PTI-suppressive activity in gain-of-function tests, although the test is confounded by the greater toxicity and instability of heterologously expressed HrpH_{Psy61}. However, the native *hrpH*_{Psy61} appears to have no PTI-suppressive activity in pHIR11 derivatives lacking *hopA1*_{Psy61}. This observation is consistent with the hypothesis that the PTI-suppressive activity may be in the highly variable C-terminal region and that the two HrpH proteins are functionally different in this capacity. Fourth, we have no mechanistic explanation for why proteins with soluble

lytic transglycosylase domains, like HrpH_{PtoDC3000} and HopP1_{PtoDC3000}, should have the PTI-suppressive activity we observed. Although peptidoglycan has been shown to be a PAMP that is recognized by plants (Gust et al. 2007), our assays involve elicitation of PTI with living *P. fluorescens* cells that would be expected to present multiple PAMPs to *N. benthamiana* cells. This argues against HrpH_{PtoDC3000} and HopP1_{PtoDC3000} functioning by interfering specifically with peptidoglycan perception. One possibility is that the acquisition of a C-terminal PTI-suppressive domain by the universal T3SS-associated lytic transglycosylase is an innovation in *P. syringae* evolution that exploits the likelihood that HrpH is among the first T3SS substrates to enter the pathway and, therefore, among the first to enter the plant cell. In future work, we will explore the relationship between the different domains of HrpH_{PtoDC3000} and functions in translocation and PTI suppression as well as the basis for the different properties of HrpH_{PtoDC3000} and HrpH_{Psy61}. Finally, because either HrpH_{PtoDC3000} or HopP1_{PtoDC3000} can at least partially restore the capacity to translocate HopA1_{Psy61} to a *hrpH*_{Psy61} deletion derivative of pHIR11 (Oh et al. 2007), whereas AvrPto, a known PTI-suppressive effector, fails to do so (data not shown), we tentatively consider these putative lytic transglycosylases to be T3SS “helpers” rather than true effectors.

Our finding that HopA1_{Psy61} is a potent suppressor of PTI that masks a hidden ability of pHIR11 to strongly elicit ROS was unexpected. Previous studies had used either *P. fluorescens*(pHIR11), in tobacco to identify effectors that suppress ETI (exploiting the native HopA1_{Psy61} as an ETI elicitor), or *P. fluorescens*(pLN18) (lacking *hopA1*_{Psy61}), in *N. benthamiana* to identify effectors that suppress PTI. However, HopA1 itself had not been tested for its ability to suppress PTI. By using the quantitative ROS assay for defense elicitation in *N. benthamiana*, we were able to show that pLN18 elicits higher levels of ROS than *P. fluorescens* without any plasmid. Similarly, in a recent publication, *P. fluorescens* Pf-0 carrying pLN18 integrated into the genome is reported to elicit an occasional, spotty necrosis in tobacco leaves (Thomas et al. 2009). Importantly, we have shown that elicitation of this higher level of ROS requires that the T3SS is able to secrete proteins beyond the bacterial envelope and that, among genes encoding extracellular Hrp proteins that we tested, the *hrpK1*_{Psy61} mutant was most strongly reduced in ROS elicitation. It is also noteworthy that either HopA1_{PtoDC3000} or HopA1_{Psy61} can suppress ROS production attributable to both *P. fluorescens* PAMPs and the T3SS.

HrpK1_{PtoDC3000} shows weak similarity to the HrpF/PopF family of T3SS translocators of *Xanthomonas* and *Ralstonia* spp., and it has a predicted C-proximal transmembrane domain like other T3SS translocators (Petnicki-Ocwiega et al. 2005). *hrpK1* mutants of *P. syringae* pv. *phaseolicola*, DC3000, and pHIR11 are only partially reduced in HR elicitation activity, which is consistent with the observation that HrpZ1 and other harpins can function redundantly (Kvitko et al. 2007; Mansfield et al. 1994; Petnicki-Ocwiega et al. 2005). We cannot say that HrpK1 is the T3SS-associated ROS elicitor, because of the possibility that it promotes the translocation of another factor. In this regard, it is noteworthy that transgenic expression of HrpK1 in tobacco had no effect on the moderate ability of *P. fluorescens* carrying a Δ *hrpK1* pHIR11 derivative to elicit the HR (Petnicki-Ocwiega et al. 2005). This argues against HrpK1 itself being the elicitor, although its elicitor activity may require insertion by a T3SS tip complex into the host plasma membrane (Blocker et al. 2008).

The HrpZ1_{Psy61} harpin was high on the list of candidate elicitors of T3SS-dependent ROS production, based on the ability of exogenously applied HrpZ1 proteins to elicit the HR in so-

lanaceous plants (He et al. 1993; Preston et al. 1995). Surprisingly, deleting *hrpZ1_{Psy61}* had little effect on the ability of pLN18 to elicit ROS in *N. benthamiana*. It is also important to note that deleting *hrpH_{Psy61}* did not significantly reduce ROS elicitation. Since HrpH appears to be essential for effector translocation, this argues that the fragment of the gene at one end of the pHIR11 insert DNA that encodes the first one third of AvrE is unlikely to be responsible for ROS elicitation.

P. syringae pv. *syringae* 61 Hrp proteins that are also T3SS substrates, based on Cya reporter fusions, have been comprehensively identified, and their contribution to diagnostic T3SS functions has been studied with nonpolar mutations (Ramos et al. 2007). Any one of these could potentially be perceived by plants and thereby elicit ROS. Unfortunately, mutations affecting the corresponding genes (other than *hrpJ*) disrupt the T3SS pathway, as indicated by the failure to secrete or translocate AvrPto-Cya. Similarly, mutation of the *hrpA* pilus subunit gene in *P. syringae* disrupts both secretion and translocation of T3SS substrates (Wei et al. 2000). Thus, the pleiotropic nature of these mutations confounds their use in identifying another elicitor of ROS. Transient expression in *N. benthamiana*, using *Agrobacterium tumefaciens*, is one potential approach for future efforts to identify the T3SS-associated ROS elicitor.

Another unresolved issue is whether *P. fluorescens*(pLN18) induces PTI or ETI. For several reasons, we postulate that the defenses induced by *P. fluorescens*(pLN18) are fundamentally PTI. First, pLN18 encodes no intact effectors. Second, heterologously expressed DC3000 effectors that fail to suppress the ETI induced by pHIR11-encoded HopA1_{Psy61} in tobacco, such as AvrPto and HopG1 (Jamir et al. 2004), are, nevertheless, able to suppress the defenses induced by pLN18 (Oh and Collmer 2005). This is particularly significant, because AvrPto has been shown by independent approaches to be a suppressor of PTI (Hauck et al. 2003). Third, *P. fluorescens* without any *P. syringae* T3SS is also able to induce defenses against HR and disease-associated plant cell death in overlapping challenge inoculation assays identical to those employed here (Chakravarthy et al. 2010). Furthermore, virus-induced gene silencing revealed that silencing *FLS2* in *N. benthamiana* prevented *P. fluorescens* from inducing PTI based on these challenge-inoculum cell assays (Chakravarthy et al. 2010). Thus, the PTI induced by *P. fluorescens* PAMPs, particularly flagellin, appears sufficient to protect plant tissue against susceptibility to T3SS-dependent HR elicitation. HrpH_{PtoDC3000} and HrpP_{PtoDC3000} are clearly able to suppress that basal PTI as well as any additional defenses induced by the pLN18-encoded T3SS.

A new concept emerging from studies primarily involving animal pathogens is that hosts discriminate pathogens from nonpathogens by patterns of pathogenesis (Vance et al. 2009). Such patterns could include presentation of PAMPs in a manner characteristic of pathogens or indicators that a microbe has access to the host cytosol. A functional T3SS could be involved in both types of signaling, for example, by permitting PAMPs such as peptidoglycan fragments and flagellin to be delivered into host cells or by creating pores in the host plasma membrane that are sensed through ion fluxes or damage products (Lightfield et al. 2008; Shin and Cornelis 2007; Sun et al. 2007; Vance et al. 2009). As described above, a substantial toolkit has been assembled for further exploring the basis for plant ROS elicitation by the *P. syringae* pv. *syringae* 61 T3SS functioning in *P. fluorescens*, and ongoing progress in deleting all active effector genes from DC3000 promises to provide a test for this phenomenon in a native *P. syringae* background. In summary, just as many effectors have been shown to function as double agents in both eliciting and suppressing plant defenses, our data indicate that the T3SS itself also has this property.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C. Strains of *P. fluorescens*(pLN18 and effector clones) and *P. syringae* pv. *tomato* DC3000 cultures were grown on King's B (KB) medium (King et al. 1954) at 28°C. *E. coli* DH5α was used for general cloning procedure. Additionally, *E. coli* TOP10 and DB3.1 cells were used for deletion in pHIR11 and propagation of pCPP3214 containing the lethal *ccdB* gene. Antibiotics were used at the following concentrations (μg ml⁻¹): kanamycin, 50; gentamicin, 40; tetracycline, 10; streptomycin, 100; ampicillin, 100; and rifampicin, 50. For marker exchange, gentamicin and kanamycin were used at half concentration.

DNA manipulations.

Plasmid DNA was isolated and manipulated using standard methods (Sambrook and Russel 2001). All constructs were created by polymerase chain reaction (PCR) using ExTaq polymerase (Takara Bio, Inc. Otsu, Shiga, Japan) and specific primers for each effector and the genomic DNA of *P. syringae* pv. *tomato* DC3000 as a template (Table 2). The PCR products were cloned into a pENTR/SD-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) and were sequenced at the Cornell Bio-Resource Center (Ithaca, NY, U.S.A.) with an ABI 3700 DNA analyzer. Entry clones were transferred into appropriate destination vectors, as indicated in Table 1. Each resulting construct was conjugated into *P. syringae* or *P. fluorescens* strains by tri-parental mating, using pRK2013.

Construction of pCPP6209, pCPP6211, and pCPP6212.

In order to make Δ *hopA1_{Psy61}* derivatives of pHIR11, pCPP5703, and pCPP2274, a 704-bp fragment carrying the left flanking region and a 954-bp fragment carrying the right flanking region of *hopA1_{Psy61}* were amplified by PCR using primer pair *hopA1_{Psy61}*Left-F and Left-R and the primer pair *hopA1_{Psy61}*Right-F and Right-R, respectively. Each fragment of left and right regions was ligated at the *KpnI* site and was then cloned into the *HindIII* and *XbaI* sites of pBluescript II SK(+) (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.), using terminal primer-introduced restriction sites. pBluescript II SK(+) harboring left and right flanking regions of *hopA1_{Psy61}* was electro-transformed into *E. coli* C2110 carrying pHIR11 (Gopalan et al. 1996), pCPP5703, and pCPP2274 to produce pHIR11Δ*hopA1_{Psy61}*, pCPP6209, and pCPP6211, respectively. Transformants were plated on LB agar medium supplemented with ampicillin and tetracycline at three times the normal level and incubated at 42°C. Integrant strains were cured of pBluescript II SK(+) by incubation at 30°C and daily 1:400 subinoculations over 4 days, and then, were plated on LB agar medium supplemented with 30 μg of tetracycline per milliliter. Recovered single colonies were screened for ampicillin sensitivity and were analyzed by colony PCR to confirm the deletion of *hopA1_{Psy61}*.

To create a Δ *hrpK1_{Psy61}* derivative of pHIR11Δ*hopA1_{Psy61}*, 1,319- and 1,226-bp fragments of left and right regions of *hrpK1_{Psy61}* were PCR amplified, using primer pairs *hopA1_{Psy61}*Left-F and *hrpK1_{Psy61}*Left-R and *hrpK1_{Psy61}*Right-F and Right-R, respectively, and were cloned into pBluescript II SK(+) and used for mutagenesis with *E. coli* C2110 carrying pHIR11Δ*hopA1_{Psy61}*, as described above to produce pCPP6212.

To transfer pCPP6209, pCPP6211, and pCPP6212 into *P. fluorescens* 55, tri-parental mating was performed with pRK2013 as a helper. All transconjugants were confirmed by colony PCR using *hopA1_{Psy61}* and *hrpK1_{Psy61}* primer pairs. Additionally, colony PCR was repeatedly conducted with a *hrpB* primer pair to confirm maintenance of intact derivatives of

pHIR11. All *P. fluorescens* 55 strains harboring pHIR11 derivative lacking *hopA1*_{Psy61} or *hrpK1*_{Psy61} were maintained at room temperature during experiments.

Plant growth and bacterial inoculations.

Fully expanded leaves from 6-week-old *N. benthamiana* plants were used for assays. Plants were grown under greenhouse conditions and were transferred to the laboratory one day before inoculation. The tested plants were maintained in the laboratory with a light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 25°C. To infiltrate *P. fluorescens* (pLN18 and effector clones) strains into plants, bacteria cells were first grown on KB plates at 28°C overnight, then scraped from the agar, and resuspended in 5 mM MES (pH 5.5) at 2×10^8 CFU ml⁻¹. To induce the expression of effectors, each bacterial suspension was supplemented with 200 μM IPTG. For inoculation of DC3000, bacteria were grown on a KB plate at 28°C overnight and were resuspended in 10 mM MgCl₂. Bacterial infiltration was performed with a blunt syringe.

Challenge-inoculation Cya translocation reporter assays.

Strains of *P. fluorescens*(pLN18) expressing different T3SS substrates and a negative control strain were infiltrated at equi-

distant spots on 6-week-old fully expanded leaves of *N. benthamiana* plants. The area of infiltration was marked with a felt-tipped pen to ensure that the second challenge inoculation overlapped the pretreated area. Each leaf had at least one nega-

Table 2. Primers used to construct and analyze mutations

Primers	Sequences 5' to -3'	Sites ^a
<i>hopA1</i> _{Psy61} Left-F	TAATAAGCTTGGATCCAATCCACGC TCT	<i>HindIII</i>
<i>hopA1</i> _{Psy61} Left-R	TAATGGGTACCTAAAAGCTCCTTAC GCTA	<i>KpnI</i>
<i>hopA1</i> _{Psy61} Right-F	TAATGGGTACCAATCGGGGCTAAA TAGCT	<i>KpnI</i>
<i>hopA1</i> _{Psy61} Right-R	TAATCTAGAAATCGACAGCTCCAG GTTG	<i>XbaI</i>
<i>hrpK1</i> _{Psy61} Left-R	TAATGGGTACCTAGCAGGGAAGAG GTTGA	<i>KpnI</i>
<i>hrpK1</i> _{Psy61} Right-F	TAATGGGTACCGACAGATAACCAC TCAGG	<i>KpnI</i>
<i>hrpK1</i> _{Psy61} Right-R	TAATCTAGACCATCGTCTTCTGCT CTGT	<i>XbaI</i>
<i>hopA1</i> _{PtoDC3000} -F	CACCATGAACCCCATTCAGT	...
<i>hopA1</i> _{PtoDC3000} -R	GCATTTCGTGTTTCAAGG	...

^a Restriction sites.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>Escherichia coli</i> DH5 α	<i>SupE44 lacU169 (f80 lacZAM15) hsdR17 recA1 endA1, gyrA96 thi-1 relA1</i>	Life Technologies, Gaithersburg, MD, U.S.A.
TOP10	<i>F⁻mcrA Δmrr-hsdRMS-mcrBC) ϕ80lacZAM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7679 galU galK rpsL endA1 nup</i>	Invitrogen, Carlsbad, CA, U.S.A.
DB3.1	<i>F⁻gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5λ-leu mtl-1</i>	Invitrogen
BL21(DE3)	<i>F⁻ompT hsdS_B(r_B⁻m_B⁻) gal dcm (DE3)</i>	Invitrogen
<i>Pseudomonas fluorescens</i> 55	Wild type, Nx ^r	Huang et al. 1988
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Wild type, Rif ^r	Cuppels 1986
Plasmids		
pENTR/SD-TOPO	Entry vector of Gateway cloning, Km ^r Cm ^r	Invitrogen
pET-DEST42	pET Gateway compatible vector allowing for T7-regulated expression of a protein with a C-terminal His ₆ -V5 tag; Amp	Invitrogen
pRK2013	Helper plasmid; ColE1 replicon TraRK ⁺ Mob ⁺ ; Km ^r	Figurski and Helinski 1979
pHIR11	pLAFR3 containing a 25-kb <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrc</i> - <i>hrp</i> gene cluster	Huang et al. 1988
pLN18	pHIR11 derivative with <i>shcA</i> and <i>hopA1</i> replaced by an <i>nptII</i> cassette; Tc ^r Km ^r	Jamir et al. 2004
pCPP2078	pHIR11 containing <i>hrcV</i> ::TnphoA; Tc ^r , Km ^r	Huang et al. 1991
pCPP2274	pHIR11 containing an unmarked deletion <i>hrpZ1</i> ; Tc ^r	Gopalan et al. 1996
pCPP3069	pCPP45 carrying a red-shift <i>gfp</i> (S65T) driven by a trp promoter (1.1-kb <i>PstI</i> - <i>KpnI</i> fragment subcloned from pTB93F); Tc ^r	W.-L. Deng
pCPP3214	pCPP3234 containing codons 2 to 406 of <i>cya</i> , Sp ^r /Sm ^r ; used as empty vector control	Schechter et al. 2004
pCPP3221	pCPP3234 expressing AvrPto-Cya; Sp ^r /Sm ^r	Schechter et al. 2004
pCPP3234	pVLT35 containing Gateway reading frame B cassette and codons 2 to 406 of <i>cya</i> ; Sp ^r /Sm ^r Cm ^r	Schechter et al. 2004
pCPP3256	pCPP3234 expressing HopP1 _{PtoDC3000} -Cya; Sp ^r /Sm ^r	Kvitko et al. 2007
pCPP3258	pCPP3234 expressing HrpW1-Cya; Sp ^r /Sm ^r	Kvitko et al. 2007
pCPP3297	pLN18 containing an unmarked <i>hrcC</i> deletion; Tc ^r	Schechter et al. 2004
pCPP3489	pCPP3234 carrying <i>avrPto</i> -stop- <i>cya</i> ; Sp ^r /Sm ^r	Oh et al. 2007
pCPP5154	pENTR/SD-TOPO containing <i>avrPto</i> ; Km ^r	Munkvold et al. 2008
pCPP5316	pHIR11 containing <i>hopA1</i> - <i>cya</i> translational fusion; Tc ^r	Oh et al. 2007
pCPP5708	pCPP3234 expressing HrpH _{PtoDC3000} -Cya; Sp ^r /Sm ^r	Oh et al. 2007
pCPP5712	pCPP3234 carrying <i>hrpH</i> _{PtoDC3000} -stop- <i>cya</i> ; Sp ^r /Sm ^r	Oh et al. 2007
pCPP5713	pCPP3234 carrying <i>hopP1</i> _{PtoDC3000} -stop- <i>cya</i> ; Sp ^r /Sm ^r	Oh et al. 2007
pCPP5721	pENTR/SD-TOPO containing <i>hrpH</i> _{PtoDC3000} ; Km ^r	Oh et al. 2007
pCPP5725	pET-DEST42 expressing HrpH _{PtoDC3000} ; Amp ^r	Oh et al. 2007
pCPP5743	pENTR/SD-TOPO containing <i>hopP1</i> _{PtoDC3000} ; Km ^r	Kvitko et al. 2007
pCPP5789	pENTR/SD-TOPO containing <i>hrpH</i> _{PtoDC3000} -stop; Km ^r	Oh et al. 2007
pCPP5790	pENTR/SD-TOPO containing <i>hopP1</i> _{PtoDC3000} -stop; Km ^r	Oh et al. 2007
pCPP5792	pENTR/SD-TOPO containing <i>avrPto</i> -stop; Km ^r	Oh et al. 2007
pCPP5871	pET-DEST42 expressing HrpH _{Psy61} ; Amp ^r	Oh et al. 2007
pCPP6207	pCPP3234 expressing HrpH _{Psy61} -Cya; Sp ^r /Sm ^r	This study
pCPP6208	pCPP3234 expressing HopA1 _{PtoDC3000} -Cya; Sp ^r /Sm ^r	This study
pCPP6209	pCPP5703 (pCPP5316 Δ <i>hrpH</i> ::FRTGm ^r) containing an unmarked deletion of <i>hopA1</i> _{Psy61} ; Tc ^r	This work
pCPP6211	pCPP2274 containing an unmarked deletion of <i>hopA1</i> _{Psy61} ; Tc ^r	This work
pCPP6212	pHIR11 containing unmarked deletions of <i>hopA1</i> _{Psy61} and <i>hrpK1</i> _{Psy61} ; Tc ^r	This work

^a Nx^r, Rif^r, Km^r, Cm^r, Tc^r, Sp^r, Sm^r, and Amp^r indicate resistance to nalidixic acid, rifampicin, kanamycin, chloramphenicol, tetracycline, spectinomycin, streptomycin, and ampicillin, respectively.

tive control. The treated plants were maintained at 25°C under illumination for 6 h. Translocation assays were performed as described previously (Schechter et al. 2004). Bacterial strains carrying plasmids expressing Cya fusions were infiltrated into *N. benthamiana* at 1×10^8 CFU ml⁻¹. Leaf disks were collected 6 h after infiltration with 1.0-cm-diameter cork border and were ground in 250 µl of 0.1 M HCl in a 1.5-ml microfuge tube using a plastic pestle driven by a Dremel tool. The cyclic AMP (cAMP) levels were determined using the Correlate-EIA cAMP immunoassay kit according to the manufacturer's instructions (Assay Designs Inc, Ann Arbor, MI, U.S.A.).

Challenge-inoculation HR assays.

To test the ability of T3SS substrates heterologously expressed in *P. fluorescens*(pLN18) to suppress PTI based on the associated inhibition of the HR and electrolyte leakage, *P. syringae* pv. *tomato* DC3000 was challenge-inoculated 6 h later at 2×10^{-7} CFU ml⁻¹, so as to partially overlap with the pre-treated area. After 24 h, leaves were photographed using a Nikon E-995 digital camera.

Challenge-inoculation bacterial colony development assay.

To observe the suppression of PTI-associated inhibition of the growth of pathogen challenge inoculation, *P. syringae* pv. *tomato* DC3000 was transformed with pCPP3069, which expresses green fluorescent protein (GFP) from a vector trp promoter. At 6 h after infiltration of *P. fluorescens*(pLN18) cells expressing different effectors or carrying empty vector, suspensions of GFP-expressing strains in 10 mM MgCl₂ at 2×10^7 CFU ml⁻¹ were challenge-inoculated on the edge of the preinfiltrated area so as to produce an overlapped area and a nonoverlapped area. At 6 h after challenge, which is prior to the start of macroscopic necrosis, leaf strips were cut with a razor to include both inoculation areas. The development of bacterial colonies was observed using the 16× objective of a Bio-Rad MRC600 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) confocal laser-scanning microscope.

Vascular dye-accumulation assay.

P. fluorescens(pLN18) cells expressing different effectors or carrying empty vector at 2×10^8 CFU ml⁻¹ were infiltrated at four spots on 6-week-old fully expanded upper leaves of *N. benthamiana* plants. Each leaf was detached with a razor at the base of the petiole, and the petiole was directly placed in a 1% (wt/vol) neutral red water solution for 12 h. Images were obtained using a Nikon E-995 digital camera.

To quantify the relative amount of transported neutral red dye in each treated area, leaf disks were collected with a 0.5-cm-diameter cork borer from each inoculated area. Leaf disks were placed separately in wells of a 96-well microtiter plate with 200 µl of 100% ethanol. Each well of the plate was tightly sealed with Parafilm to prevent any evaporation of solution, and the samples were incubated at room temperature for 1 h on a plate shaker at 200 rpm. To measure relative levels of dye extracted from each sample, 50 µl of extracted ethanol solution from each well was diluted with 150 µl of 5 mM MES (pH 5.5). The absorbance of each well was read at 562 nm, using a MicroStation Reader (Biolog, Hayward, CA, U.S.A.). The experiment was repeated three times with similar results.

Callose-deposition assay.

P. fluorescens(pLN18) strains expressing different effectors were infiltrated into 5-week-old *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹. At 24 h postinoculation, leaf disks of *N. benthamiana* samples measuring 1.0 cm in diameter were collected. The collected leaves were cleared by being separately placed in a six-well plate containing 2 ml of 95% ethanol. The sam-

ples were incubated at 37°C until the leaf was cleared of chlorophyll. Cleared leaves were rinsed twice with 70% ethanol and, then, twice with water. The leaf disks were immersed in 150 mM K₂HPO₄ (pH 9.5) containing 0.1% aniline blue for 2 h in the dark. The stained samples were washed with distilled water and were mounted in 60% glycerol. Callose was observed by epifluorescence microscopy with an Olympus BX60F microscope and MNU filter tube (360 to 370 nm excitation filter, DM400 dichroic mirror, and BA420 long-pass filter; Olympus, Tokyo).

ROS assay.

For ROS measurements, *P. fluorescens*(pLN18) strains expressing different effectors and a negative control (10 mM MgCl₂) were infiltrated into 5-week-old *N. benthamiana* leaves at 2×10^8 CFU ml⁻¹. At 15 h postinoculation, leaf disks (0.5 cm in diameter) were excised and placed into wells of 96-well plates, and then, 100 µl of 0.5 mM L-012 (Wako, Kyoto, Japan) in 10 mM morpholinepropanesulfonic acid morpholinepropanesulfonic acid-KOH buffer (pH 7.4) was added. The intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence as previously described (Asai et al. 2008). Chemiluminescence was monitored using a Veritas luminometer (Promega, Fitchburg, WI, U.S.A.) and was quantified by the BrightGlo Promega program.

Immunoblot analysis.

Protein samples were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and were transferred to Immobilon-P membranes (Millipore, Billerica, MA, U.S.A.) by using a Semiphor semidry transfer system (Hoefer, Holliston, MA, U.S.A.). Immunoblot analysis was carried out by using a Western-Light Plus kit (Applied Biosystems, Bedford, MA, U.S.A.) according to the manufacturer's instructions. Cya fusion proteins were detected by using primary anti-Cya (3D1) mouse monoclonal immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a dilution of 1:5,000 and secondary anti-mouse IgG-alkaline phosphatase conjugate antibodies (Sigma-Aldrich, St. Louis) at a dilution of 1:30,000.

Bacterial toxicity assay.

E. coli BL21(DE3) harboring native *hrpH*_{P_{sy61}} or *hrpH*_{P_{toDC3000}} expressed from pET-DEST42 were grown in 5 ml of LB broth supplemented with ampicillin at 37°C overnight, inoculated to new media, and allowed to grow until the optical density at 600 nm (OD₆₀₀) had reached 0.10. The *T7*/*lacO* promoter of the vector, driving expression of the test proteins, was activated by addition of 0.5 mM IPTG, and the OD₆₀₀ was further monitored. The experiment was repeated three times with similar results.

ACKNOWLEDGMENTS

We thank K. E. Loeffler for photography, C.-S. Oh for guidance with the ROS assays, W.-L. Deng for construction of pCPP3069, and G. Hooker for statistical analysis. This work was supported by National Science Foundation grants MCB-0544066 and DBI-0605059.

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