

The *Pseudomonas syringae* type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants

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Summary

The bacterial plant pathogen *Pseudomonas syringae* possesses a type III protein secretion system that delivers many virulence proteins into plant cells. A subset of these proteins (called Avr proteins) is recognized by the plant's innate immune system and triggers defences. One defence-associated response is the hypersensitive response (HR), a programmed cell death (PCD) of plant tissue. We have previously identified HopPtoD2 as a type III secreted protein from *P. s. pv. tomato* DC3000. Sequence analysis revealed that an N-terminal domain shared homology with AvrPphD and a C-terminal domain was similar to protein tyrosine phosphatases (PTPs). We demonstrated that purified HopPtoD2 possessed PTP activity and this activity required a conserved catalytic Cys residue (Cys₃₇₈). Interestingly, HopPtoD2 was capable of suppressing the HR elicited by an avirulent *P. syringae* strain on *Nicotiana benthamiana*. HopPtoD2 derivatives that lacked Cys₃₇₈ no longer suppressed the HR indicating that HR suppression required PTP activity. A constitutively active MAPK kinase, called NtMEK2^{DD}, is capable of eliciting an HR-like cell death when transiently expressed in tobacco. When NtMEK2^{DD} and HopPtoD2 were co-delivered into plant cells, the HR was suppressed indicating that HopPtoD2 acts downstream of NtMEK2^{DD}. DC3000 *hopPtoD2* mutants were slightly reduced in their ability to multiply *in planta* and displayed an enhanced ability to elicit an HR. The identification of HopPtoD2 as a PTP and a PCD suppressor suggests that the inactivation of MAPK pathways is a virulence strategy utilized by bacterial plant pathogens.

Introduction

The Gram-negative bacterial plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 has become an important model system in molecular plant pathology, in part, because it infects both tomato and the genetically amenable model plant *Arabidopsis* and because its type III protein secretion system (TTSS) and the effector proteins it delivers to plant cells are relatively well characterized (Alfano and Collmer, 1997; Galán and Collmer, 1999). The availability of the DC3000 draft nucleotide sequence has resulted in a substantial increase in the size of the Hrp regulon (Boch *et al.*, 2002; Fouts *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002) and has led to the identification of many type III effectors (Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002). The current inventory of DC3000 type III secreted proteins now stands at 36 with several other candidate effectors that await confirmation (Collmer *et al.*, 2002). Generally, mutants defective in individual effector proteins have subtle virulence phenotypes (Chen *et al.*, 2000; Shan *et al.*, 2000). However, the TTSS is essential for *P. syringae* pathogenesis because mutants defective in the TTSS are not pathogenic suggesting that collectively these effectors must be required and that many of them are likely functionally redundant.

Several type III effectors were originally named avirulence (Avr) proteins because they were recognized by components of the defence system of plants called resistance (R) proteins (Keen, 1990). In these cases, the effectors are not acting as virulence proteins, but instead betray the pathogen by triggering plant defence responses. These defence responses include an oxidative burst, production of pathogen-related proteins, phytoalexin production and elicitation of the hypersensitive response (HR) (Somssich and Hahlbrock, 1998; Cohn *et al.*, 2001). The HR is an example of a programmed cell death (PCD) pathway in plants and has long been associated with defence to viral, fungal and bacterial pathogens (Goodman and Novacky, 1994; Heath, 2000a). Because of its connection to the HR and pathogenicity, TTSSs in bacterial plant pathogens are also referred to as Hrp systems (for Hypersensitive Response and Pathogenicity).

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The actions and targets of *P. syringae* effectors that promote pathogenicity remain elusive (Staskawicz *et al.*, 2001). However, there were early indications that *P. syringae* could suppress the induction of defence related transcripts during infections on host plants (Jakobek *et al.*, 1993). Recent studies show that several *P. syringae* effectors contain biologically active cysteine protease catalytic triads similar to those found in YopJ and YopT from animal pathogenic *Yersinia* species (Orth *et al.*, 2000; Shao *et al.*, 2002). The plant targets for these proteins are currently unknown. The effector proteins AvrB and AvrRpm1 interact with the plant protein RIN4, which appears to act as a negative regulator of plant defences (Mackey *et al.*, 2002). In an important conceptual advance, two papers established genetically that the presence of the effectors VirPphA, AvrPphF and AvrPphC individually in *P. s. phaseolicola* were capable of blocking the HR on specific cultivars of bean (Jackson *et al.*, 1999; Tsiamis *et al.*, 2000). It was hypothesized that the HR blocking activity of the effectors could be due to the following: blockage of Avr secretion from bacterial cells and/or translocation into plant cells; modulation of signal transduction pathways within plant cells; or interference between Avr proteins once inside plant cells preventing their recognition by the surveillance system of the plant. Indeed, earlier reports seemed to support the last hypothesis because the presence of an Avr protein prevented the recognition of another Avr protein by the plant immune system (Reuber and Ausubel, 1996; Ritter and Dangl, 1996).

The DC3000 genome contains two ORFs whose predicted products share homology with AvrPphD, a well-distributed Avr protein originally identified from *P. s. phaseolicola* (Arnold *et al.*, 2001). Both of these ORFs, initially designated *avrPphD1_{Pto}* and *avrPphD2_{Pto}*, have Hrp promoters upstream and are expressed in a Hrp-dependent manner (Fouts *et al.*, 2002). Subsequently, both AvrPphD1_{Pto} and AvrPphD2_{Pto} were identified as type III secreted proteins and renamed HopPtoD1 and HopPtoD2 (Petnicki-Ocwieja *et al.*, 2002). *hopPtoD2* is located within the chromosome in a cluster of *hrp*-related genes in an apparent pathogenicity island (Pai) not linked to the Hrp Pai that encodes the TTSS apparatus (Alfano *et al.*, 2000; Badel *et al.*, 2002). In this report we show that HopPtoD2 possesses a C-terminal domain that shares similarity with protein tyrosine phosphatases (PTPs). We demonstrate that HopPtoD2 is an active PTP capable of suppressing the HR elicited by an avirulent *P. syringae* strain. Moreover, HopPtoD2 transiently expressed *in planta* was capable of suppressing the HR-like response elicited by a mitogen-activated protein kinase (MAPK) pathway. Our research suggests that inactivation of MAPK pathways are a pathogenic strategy employed by bacterial plant pathogens to circumvent the innate immune system of the plant.

Results

HopPtoD2 has an N-terminal domain that possesses amino acid similarity with the avirulence protein AvrPphD and a C-terminal domain similar to protein tyrosine phosphatases

We reported that HopPtoD2 was secreted in culture by the TTSS of *P. s. tomato* strain DC3000 (Petnicki-Ocwieja *et al.*, 2002). HopPtoD2 is 468 amino acids in length with a predicted molecular mass of 51.4 kDa. Based on comparisons with proteins in the databases, HopPtoD2 appears to have a modular organization (Fig. S1A in *Supplementary material*). BLASTP searches (Altschul *et al.*, 1997) revealed that the amino terminal 200 amino acids share high similarity with several AvrPphD homologues present in other *P. syringae* pathovars as well as other plant pathogens (Fig. S1B, see *Supplementary material*). It is important to note that all of the other AvrPphD homologues share similarity throughout their entire sequence, whereas the similarity with HopPtoD2 is limited to the amino terminus of these proteins.

Our initial BLASTP searches showed that a C-terminal domain of HopPtoD2 shared sequence identity with an ORF (CAP0014) from *Clostridium acetobutylicum* (E-value 4e-26), which is predicted to encode a protein tyrosine phosphatase (PTP) as well as other PTPs including *avrBs1*-associated ORF1 from *X. c. vesicatoria* (Ronald and Staskawicz, 1988). The BLASTP searches also showed that the region of PTP similarity within HopPtoD2 contains a conserved catalytic domain of PTPs (pfam00102). PSI-BLAST searches further confirmed this similarity. Several of the PTPs showing similarity with HopPtoD2 are shown in Fig. S1C (see *Supplementary material*). HopPtoD2 has the invariant residues (HCxxGxxRS/T) present in the catalytic domains of functional PTPs (Denu *et al.*, 1996). Another indication that HopPtoD2 contained a PTP domain was that the threading program 3D-PSSM predicted that the C-terminal region of HopPtoD2 folds similar to several known PTP protein structures present in the structural classification of proteins database (Kelley *et al.*, 2000). Thus, HopPtoD2 appears to be a modular protein that contains an amino terminal domain homologous to AvrPphD and a carboxy terminal domain that has similarity with PTPs.

The AvrPphD domain of HopPtoD2 is widely distributed among P. syringae pathovars, whereas the PTP domain appears to be less common

Because of the modular nature of HopPtoD2 and considering that all of the other known homologues of AvrPphD do not contain a PTP domain, we sought to determine how prevalent this allele was in other *P. syringae* pathovars as well as other bacterial plant pathogens. DNA gel

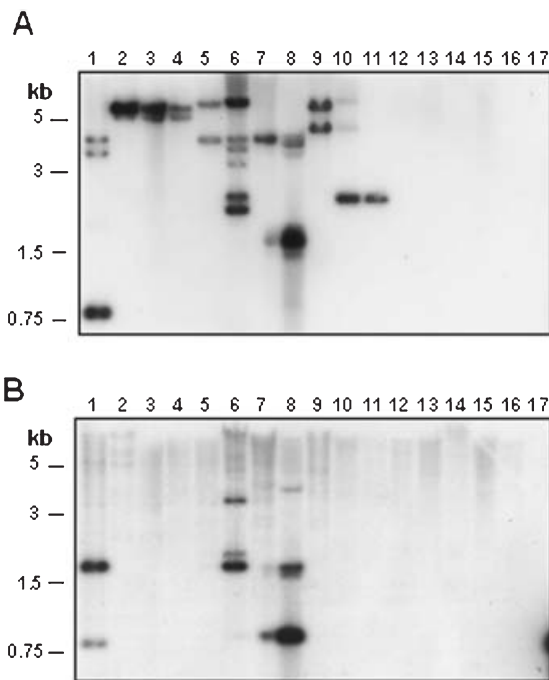


Fig. 1. DNA gel blot analysis of the distribution of the AvrPphD and PTP domains of HopPtoD2 in *P. syringae* strains and other plant pathogens. DNA from different bacteria was digested with *Bam*HI and *Bgl*II restriction enzymes and transferred to a nylon membrane and probed with either a fragment corresponding to the AvrPphD domain (A) or the PTP domain (B). Numbered lanes correspond to the following bacteria: 1, *P. s. tomato* DC3000; 2, *P. s. tomato* Pt23; 3, *P. s. tomato* 3357; 4, *P. s. tomato* 133; 5, *P. s. maculicola* 84–67; 6, *P. s. maculicola* 84–59; 7, *P. s. maculicola* NCPPB1886; 8, *P. s. maculicola* 438; 9, *P. s. glycinea* race 4; 10, *P. s. phaseolicola* HB10Y; 11, *P. s. phaseolicola* 343; 12, *P. s. pisi* 11; 13, *P. s. syringae* 61; 14, *P. s. syringae* B728A; 15, *P. s. tabaci* 11528; 16, *E. chrysanthemi* 3937; 17, *X. c. vesicatoria* 82–8. Molecular mass markers are indicated.

blots probed with a DNA fragment corresponding to the AvrPphD domain showed that this domain is widely present in other *P. syringae* strains (Fig. 1A). This is consistent with another report indicating that *avrPphD* alleles are distributed through many *P. syringae* strains (Arnold *et al.*, 2001). We did not detect any hybridization of this fragment with total DNA from the other bacterial plant pathogens tested (Fig. 1A). However, *avrPphD* homologues have been identified in several other bacterial plant pathogens by sequencing, but their limited similarity to *hopPtoD2* would prevent their detection with DNA gel blots (Noel *et al.*, 2001; Guo *et al.*, 2002; Salanoubat *et al.*, 2002). We found that fewer *P. syringae* strains contained DNA that could hybridize to a fragment corresponding to the PTP domain of HopPtoD2, and interestingly, other than DC3000 only *P. s. maculicola* strains hybridized (Fig. 1B). Thus, in our survey, the nucleotide sequence corresponding to the PTP domain was not broadly distributed in *P. syringae*.

Affinity-purified HopPtoD2 possesses tyrosine phosphatase activity

An affinity-purified GST-HopPtoD2 fusion protein encoded by pLN173 was used to determine if HopPtoD2 had PTP enzymatic activity. This fusion protein formed aggregates in *E. coli*. Thus, in order to extract GST-HopPtoD2 from inclusion bodies, it was solubilized with detergents and renatured (see *Experimental procedures*). GST-HopPtoD2 was subsequently tested for its ability to dephosphorylate phosphotyrosine-containing peptides derived from either the insulin receptor (residues 1142–1024) or EGF receptor (residues 1014–1024). GST-HopPtoD2 was active on both peptides and the results observed using the insulin receptor peptide are shown (Fig. 2). We also tested a GST-HopPtoD2 protein that contained a Ser in the place of Cys₃₇₈ (GST-HopPtoD2_{C378S}) encoded by pLN234. Several studies have shown that this conserved Cys is required for PTP activity (Zhang and Dixon, 1994). When GST-HopPtoD2_{C378S} was used in this assay, near background levels of PTP activity were detected indicating that HopPtoD2 had a similar catalytic mechanism as other characterized PTPs (Fig. 2). To determine whether HopPtoD2 was a dual-specificity phosphatase (DSP) that possessed Ser/Thr phosphatase activity we tested if GST-HopPtoD2 could dephosphorylate ³²P-labelled Ser/Thr residues of the myelin basic protein. Under the conditions used, we were unable to detect any liberated inorganic phosphate indicative of Ser/Thr phosphatase activity (data not shown). Thus, it appears that HopPtoD2 is a PTP not a DSP.

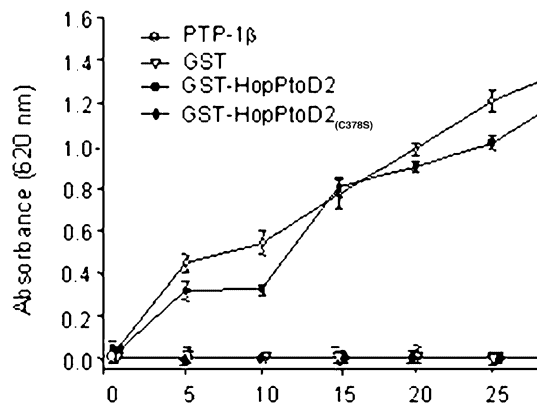


Fig. 2. Tyrosine phosphatase activity of purified GST-HopPtoD2. The PTP activity of purified fusion proteins GST-HopPtoD2 and GST-HopPtoD2_{C378S} was measured using a colorimetric PTP assay (see *Experimental procedures*). Results are shown from experiments that used a Tyr-phosphorylated peptide from the insulin receptor as the substrate. Reactions were performed at room temperature for 10 min with the indicated amounts of fusion proteins. The vector alone, which produced GST, was used as a negative control. Protein tyrosine phosphatase 1β (PTP-1β) was used as a positive control at a 1/20 dilution. These experiments were repeated at least three times with similar results.

HopPtoD2 suppresses the hypersensitive response elicited by an avirulent P. syringae pathovar

Ectopic expression of *hopPtoD2* in *P. s. phaseolicola* NPS3121 (*Pph*) did not lead to a detectable increase in virulence of *Pph* on *A. thaliana* (data not shown). We next tested whether *Pph*(pLN56) could alter the ability of this pathogen to elicit a defence-associated HR on *N. benthamiana*. To do so, we infiltrated *Pph*(pLN56) into leaves of *N. benthamiana* alongside *Pph* containing the control vector. *Pph* produced an HR on *N. benthamiana* within approximately 12 h. Interestingly, *Pph*(pLN56) expressing HopPtoD2 did not elicit an HR during this same time period (Fig. 3A). However, an HR was elicited by *Pph*(pLN56) about 24 h after the onset of the HR induced by *Pph* (data not shown). This delayed HR phenotype appeared to be dependent on which test plant was used because the delay in the onset of the HR was much shorter when *Pph*(pLN56) was infiltrated into tobacco (*N. tabacum* cv. Xanthi)(data not shown). To determine if the HR suppression phenotype that occurred when *hopPtoD2* was provided *in trans* was due to PTP activity, we cloned an inactive allele of *hopPtoD2* (*hopPtoD2*_{C378S}) into the same broad-host-range vector. Bacteria carrying this construct, pLN214, produced simi-

lar amounts of HopPtoD2 as produced by pLN56 (data not shown). When *Pph*(pLN214) was infiltrated into *N. benthamiana* an HR occurred within 12 h similar to the response observed with the *Pph* control (Fig. 3A). This indicated that HopPtoD2 was capable of HR suppression and this suppression was dependent on its PTP activity. We hypothesized that HR suppression was due to HopPtoD2 acting within the plant cell to suppress a signal transduction pathway activated by the avirulent *Pph*. However, these results could also be explained by over-expression of HopPtoD2, a known type III substrate, which may alter the ability of *Pph* to elicit an HR by simply affecting the flow of protein traffic through the TTSS. To eliminate this possibility, we performed bacterial mixing experiments of *Pph* and *Pph* producing HopPtoD2. When the HopPtoD2-containing *Pph* strain was infiltrated into *N. benthamiana* 4 h before the *Pph* strain lacking HopPtoD2, the HR was suppressed for 48 h after infiltration of the second strain (Fig. 3B). Because this HR suppression occurred when HopPtoD2 was produced by only a subset of infiltrated bacterial cells, HopPtoD2 can suppress an HR elicited by independent bacterial cells. Moreover, the HR suppression activity of HopPtoD2 was not due to blockage of the type III apparatus.

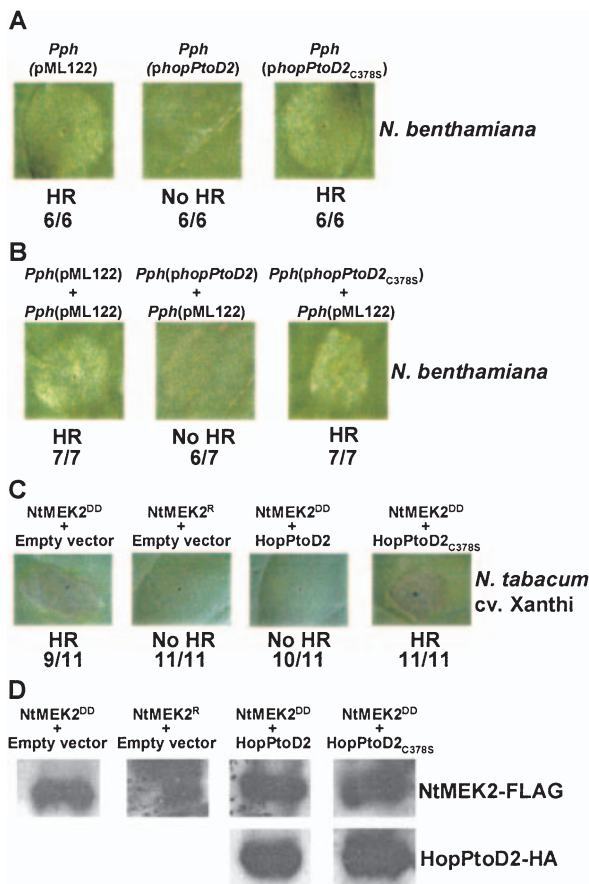


Fig. 3. Programmed cell death elicited by an avirulent *P. syringae* or an activated MAPKK can be suppressed by HopPtoD2 in a PTP-dependent manner.

A. *N. benthamiana* leaves were infiltrated with *P. s. phaseolicola* (*Pph*) carrying either pML122 (vector control); pLN56 (*phopPtoD2*), a HopPtoD2 encoding construct; or pLN214 (*phopPtoD2*_{C378S}). The middle panel shows that HopPtoD2 suppressed the HR, whereas HopPtoD2_{C378S} had no effect on HR elicitation (right panel).

B. In bacterial mixing experiments, *Pph* carrying either pML122 (vector control), pLN56 (*phopPtoD2*), or pLN214 (*phopPtoD2*_{C378S}) were co-infiltrated into *N. benthamiana* leaf tissue with *Pph*(pML122). Each infiltrated strain was at an OD₆₀₀ of 0.1, which is a sufficient cell density to elicit an HR. Only in treatments that produced HopPtoD2 was the HR suppressed (middle panel).

C. *Agrobacterium*-mediated transient expression of HopPtoD2 suppresses the cell death induced by the NtMEK2 MAPKK. *N. tabacum* cv. Xanthi leaves were infiltrated with *A. tumefaciens* MOG101 carrying either pPZP212 (empty vector), pLN592, a vector expressing HopPtoD2, or pLN628, a vector expressing the inactive HopPtoD2_{C378S}. Leaf panels were infiltrated with MOG101(pTA7002::*ntmek2*^{DD}), which expressed active NtMEK2^{DD}. Also, included was an agroinfiltration with MOG101(pTA7002::*ntmek2*^R), which expressed an inactive NtMEK2^R. The NtMEK2^{DD}-HR-like response was only suppressed when HopPtoD2 was present. The fraction underneath each picture in (A–C) indicates the number of times the results shown were observed over the number of times the experiment was performed.

D. Immunoblot of tobacco tissue infiltrated with the agroinfiltrations in (C). One cm diameter leaf disks were sampled from infiltrated tissue, crushed in liquid nitrogen, resuspended in 100 µl 1× SDS tracking buffer, and 20 µl of each sample was separated and analysed with SDS-PAGE and immunoblots. The NtMEK2 proteins were fused to a FLAG epitope and the HopPtoD2 proteins were fused to the haemagglutinin epitope (HA), which allowed detection in tissue with commercially available antibodies.

HopPtoD2 suppresses the NtMEK2-dependent HR-like response indicating that its substrate is downstream of this MAPK kinase

It seemed likely that HopPtoD2 was acting on a signal transduction pathway inside plant cells to suppress the HR. A good candidate pathway to test was the MAPK pathway in tobacco that utilizes the SIPK and WIPK MAPKs, which are known to activate plant defences in response to abiotic and biotic stresses (Nurnberger and Scheel, 2001; Zhang and Klessig, 2001). The upstream MAPK kinase (MAPKK) for both of these MAPKs is NtMEK2 (Yang *et al.*, 2001). We tested whether co-expression of NtMEK2 and HopPtoD2 in plant cells would suppress the HR-like response induced by NtMEK2 alone. To eliminate the requirement of activation of NtMEK2, we used an *ntmek2* allele that encodes an altered NtMEK2 protein, NtMEK2^{DD}, which has its Ser and Thr residues within the phosphorylation motif replaced with Asp residues making the protein constitutively active (Yang *et al.*, 2001). We infiltrated into tobacco (*N. tabacum* cv. Xanthi) an *Agrobacterium* strain carrying *hopPtoD2* on a binary vector 4 h before infiltration of a second *Agrobacterium* strain carrying *ntmek2^{DD}*. Approximately 24 h after induction, a visible HR was observed in plant tissue infiltrated with *ntmek2^{DD}*-containing *Agrobacterium* and a control strain, whereas, in leaf panels that expressed both HopPtoD2 and NtMEK2^{DD}, the HR-like response caused by NtMEK2^{DD} was suppressed (Fig. 3C). Control agroinfiltrations lacking either HopPtoD2 or an inactive NtMEK2 confirmed that the HR-like response was dependent on NtMEK2 and that the HR suppression required HopPtoD2 (Fig. 3C). Based on immunoblots, both HopPtoD2 and NtMEK2 proteins were made *in planta* in similar amounts (Fig. 3D). Similar results were observed when identical experiments were carried out with *N. benthamiana* (Fig. S2 in *Supplementary material*). Moreover, we co-infiltrated purified HopPtoD2 into tobacco with *ntmek2^{DD}*-containing *Agrobacterium* strains and these retained the ability to elicit an HR-like response suggesting that HopPtoD2 needs to be inside plant cells to function (data not shown). The HR suppression activity of HopPtoD2 was dependent on an active PTP domain indicated by the fact that HopPtoD2_{C378S} was unable to suppress the HR (Fig. 3C). Similar results were obtained with agroinfiltration co-delivery experiments using *N. benthamiana* (data not shown). Given that MAPKs are activated, in part, by Tyr phosphorylation (Luan, 2000), SIPK and/or WIPK represent potential HopPtoD2 targets. However, thus far, we have been unable to show a direct interaction between HopPtoD2 and either SIPK or WIPK in standard yeast two hybrid interaction assays (data not shown).

DC3000 hopPtoD2 mutants display an enhanced ability to elicit the HR on non-host plants consistent with HopPtoD2 acting as a PCD suppressor

We sought to determine if mutants defective in HopPtoD2 production were altered in their ability to cause disease in host plants. We hypothesized that interactions with a host plant may not result in disease if the pathogen no longer possessed the HopPtoD2 PCD suppressor. To test this, we constructed two different types of DC3000 *hopPtoD2* mutants: UNL105 contains a marked insertion mutation and UNL112 contains an unmarked *hopPtoD2* deletion. We tested both of these *hopPtoD2* mutants for their ability to grow *in planta* and to cause disease symptoms on the host plant tomato (*Lycopersicon esculentum* cv. money-maker). Both mutants produced disease symptoms similar to those caused by wild-type DC3000 (data not shown). However, each mutant was slightly reduced in their ability to grow in tomato and this reduction in *in planta* growth was complemented when *hopPtoD2* was supplied *in trans* as shown for UNL112 (Fig. 4A). The reduced growth of UNL112 *in planta* was not complemented by the *hopPtoD2_{C378S}* construct indicating that the PTP domain was required (Fig. 4A). Similar results were seen when pathogenicity assays with UNL112 and UNL105 were done using *A. thaliana* Col-0 as the host plant (Fig. S3 in the *Supplementary material*). These results suggest that HopPtoD2 is a virulence factor.

Based on our findings we were cognizant of the possibility that a pathogen may encode multiple PCD suppressors, each contributing, perhaps incrementally, to the suppression of plant defences. To test if we could observe a difference between DC3000 and the *hopPtoD2* mutants in their ability to induce defence responses on non-host plants we infiltrated into tobacco leaves different dilutions of each and assayed for their ability to elicit an HR. Interestingly, the *hopPtoD2* mutants were consistently capable of eliciting the HR at a 10-fold higher dilution (Fig. 4B). Similar results were observed when these strains were infiltrated into *N. benthamiana* (data not shown). It is important to note that DC3000 produced a typical HR at dilutions of 10⁷ cells ml⁻¹ or higher. This enhanced HR phenotype produced by the *hopPtoD2* mutants was due to the absence of HopPtoD2 because when *hopPtoD2* was supplied *in trans* the HR-eliciting ability was comparable to an HR caused by the wild-type strain (Fig. 4B). Moreover, the PTP domain of HopPtoD2 was required because constructs that produced the inactive PTP, HopPtoD2_{C378S}, were unable to complement UNL105 (Fig. 4B). Thus, the phenotype of *hopPtoD2* mutants on non-host plants was consistent with HopPtoD2 acting as a PCD suppressor and the HR titration assays used here may be useful in the identification of other PCD suppressors in bacterial plant pathogens.

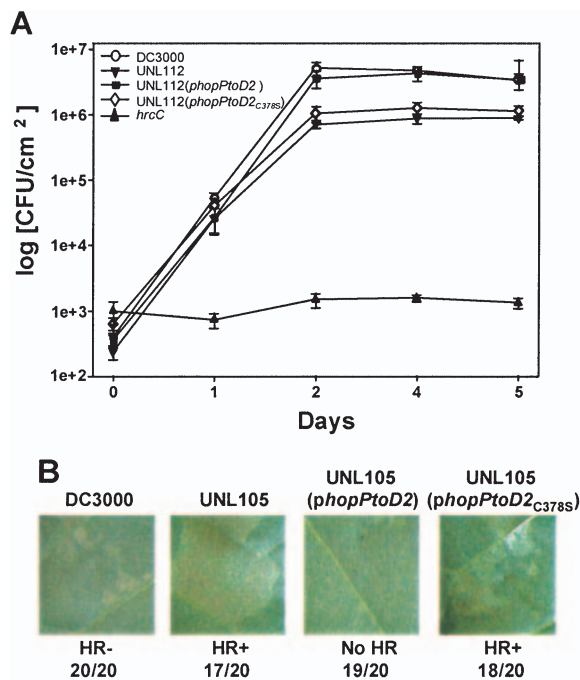


Fig. 4. DC3000 *hopPtoD2* mutants are reduced in bacterial growth in host tomato and have an enhanced ability to elicit an HR on non-host tobacco.

A and B. DC3000, a DC3000 *hrcC* mutant defective in type III secretion, and the *hopPtoD2* mutant UNL112 were dip-inoculated into tomato (A). Samples were taken over a 5-day period and bacteria were enumerated. Bacterial growth was reduced for UNL112 in tomato and the reduction was complemented when *hopPtoD2* was supplied *in trans* with pLN56, but not when pLN214 was used, which carries *hopPtoD2*_{C378S}. Each assay was done at least three times and error bars indicate standard deviations.

B. *N. tabacum* cv. Xanthi leaves were infiltrated with *P. syringae* strains that were 10-fold serially diluted from 10⁸ cells ml⁻¹. The last dilution (10⁶ cells ml⁻¹) that resulted in an HR is shown. The following strains were infiltrated: DC3000; the *hopPtoD2* mutant, UNL105; UNL105 complemented with pLN56, which encodes a functional HopPtoD2; UNL105 complemented with pLN214 (*hopPtoD2*_{C378S}). The HR was scored for each sample. At 10⁶ cells ml⁻¹, DC3000 produced a spotty HR (HR-), UNL105 and UNL105(*hopPtoD2*_{C378S}) produced a strong HR (HR+), and UNL105 (*hopPtoD2*) produced no visible HR (No HR). The fraction underneath each picture indicates the number of times the results shown were observed over the number of times the experiment was performed. The results indicated that UNL105 elicited an enhanced HR and complementation required an active HopPtoD2 PTP.

Discussion

We have shown that HopPtoD2 is a modular protein that contains an N-terminal domain from AvrPphD, a well-distributed Avr protein, and a C-terminal region that possesses PTP activity. The PTP domain appears to have a more limited distribution in *P. syringae* strains (Fig. 1). The modular nature of HopPtoD2 is reminiscent of several type III effectors from animal pathogens (Kaniga *et al.*, 1996). One of these is the *Salmonella* effector SptP (Kaniga *et al.*, 1996), which has an N-terminal domain that possesses GTPase-activating protein activity and is sim-

ilar to N-terminal domains in the *Yersinia* spp. YopE effector (Cornelis, 2002) and the *P. aeruginosa* ExoS effector (Pederson *et al.*, 1999). Like HopPtoD2, the SptP C-terminal domain is an active PTP as is the C-terminal domain of the *Yersinia* YopH effector. (Kaniga *et al.*, 1996). Based on this precedent, it is plausible that the N-terminal domain of HopPtoD2 includes another enzymatic activity shared by the other AvrPphD homologues, whereas the C-terminal domains of the AvrPphD homologues, which are unrelated to HopPtoD2, may contain a different activity. Alternatively, the shared N-terminal domain of these proteins may carry only information required for secretion, translocation and targeting within host cells. Several animal cell targets for the SptP and YopH PTPs have been identified and both effectors modulate actin polymerization and pathogen uptake into host cells (Black and Bliska, 1997; Murli *et al.*, 2001). Thus, the effects of these proteins on host cells will likely be different from the effects of HopPtoD2 on plants. However, the specific targets that these proteins act on may share significant similarities. For example, one function of SptP is to down-modulate the MAPK Erk (Murli *et al.*, 2001). It will be important to compare and contrast these PTPs with HopPtoD2 to better understand HopPtoD2's role in plants.

We have demonstrated that the PTP activity of HopPtoD2 is required to suppress an HR that would normally occur in *N. benthamiana* in response to an avirulent *P. syringae* pathovar (Fig. 3A). Bacterial mixing experiments showed that this HR suppression phenotype was not simply a consequence of blocking or altering secretion of type III effectors from the bacterial cell as HR suppression still occurred when HopPtoD2 was present in only a subpopulation of the cells infiltrated (Fig. 3B). The HR suppression phenotype was also not an effect of interference between Avr proteins as HopPtoD2 transiently expressed in tobacco suppressed an HR-like response induced by an activated MAPK pathway (Fig. 3C). Indeed, these results suggest that HopPtoD2 functions inside plant cells to cause HR suppression through down-modulation of a MAPK pathway. We also demonstrated that DC3000 mutants defective in HopPtoD2 were reduced in their ability to grow *in planta* (Fig. 4A) and exhibited an enhanced ability to elicit an HR in HR titration assays when compared to wild-type DC3000 (Fig. 4B). Taken together, these phenotypes are also consistent with HopPtoD2 acting as an HR suppressor.

How does the PCD suppression activity contribute to *P. syringae* pathogenicity? It is possible that the role of HopPtoD2 is to suppress general defence responses that are activated when a resistant plant recognizes another type III effector as an Avr protein. Indeed, independently, another group has identified HopPtoD2 as a PTP and found that it also suppresses the defence-associated oxidative burst and induction of a pathogen-related protein

(S. Hutcheson, pers. comm.). These results are consistent with HopPtoD2 acting as a suppressor of Avr-induced immunity.

However, another possibility is that HopPtoD2 is a more general defence suppressor that inhibits other defence responses induced by Avr proteins and pathogen-associated molecular patterns (PAMPs). Pathogen-associated molecular patterns are molecules, such as LPS or flagellin, which represent conserved molecular patterns that are unique to microorganisms. Insects, mammals and plants have receptor-based systems to recognize PAMPs (Boller, 1995; Medzhitov and Janeway, 2000). MAPK cascades appear to be involved in each of these recognition systems (Aderem and Ulevitch, 2000; Asai *et al.*, 2002; Khush *et al.*, 2002). In insects and mammals, the Toll or Toll-like receptors that are utilized in their innate immune systems have leucine rich repeat (LRR) domains. In plants, the flagellin PAMP is recognized by the LRR-containing receptor kinase FLS2, which resembles an R protein, Xa21, that recognizes a *Xanthomonas* Avr signal (Song *et al.*, 1995; Gomez-Gomez *et al.*, 2001). Thus, the innate immune systems that recognize PAMPs in plants share similarities to the R proteins, which recognize pathogen Avr proteins (Dangl and Jones, 2001). One difference between PAMP surveillance and Avr surveillance is that the latter also induces the HR. It will be interesting to determine whether HopPtoD2 can suppress the defence response initiated in response to flagellin. If so, it may indicate that HopPtoD2 can suppress defence responses that plant pathologists have typically referred to as non-host resistance (Heath, 2000b).

From an evolutionary perspective, it would be logical for the pathogen to evolve suppressors that act at convergence points of many defence pathways. By doing this, the pathogen would increase its likelihood of disabling a pathway that could potentially result in a successful defence response. Based on our findings that HopPtoD2 acts downstream of NtMEK2, this is a plausible mechanism of action. NtMEK2 activates the MAPKs SIPK and WIPK (Yang *et al.*, 2001), which participate in the responses to many different abiotic and biotic plant stresses (Zhang and Klessig, 2001). It is not clear whether the HR elicited by avirulent bacteria on plants is equivalent to the HR-like response observed when NtMEK2^{DD} is expressed in tobacco. However, because HopPtoD2 suppresses both, our results support that they are related. Orthologues of NtMEK2, SIPK and WIPK were recently reported to be involved in the *Arabidopsis* FLS2-dependent defence response to flagellin (Asai *et al.*, 2002). We are currently testing whether HopPtoD2 can act similarly on these MAPK cascades. To our knowledge, with the exception of MAPK activation, tyrosine kinases do not play a central role in immunity in plants. Thus, SIPK and WIPK constitute good candidate targets for HopPtoD2. More-

over, there have been reports implicating PTPs or DSPs in the negative regulation of *Arabidopsis* MAPKs (Gupta *et al.*, 1998; Xu *et al.*, 1998; Ulm *et al.*, 2002). However, it is possible that other hitherto unknown targets exist that can be inactivated by PTPs. Future studies with HopPtoD2 may elucidate the defence-related MAPK pathways that function in plant defences.

The pioneering studies by Jackson *et al.* (1999) and Tsiamis *et al.* (2000) revealed that the *P. s. phaseolicola* effector proteins VirPphA, AvrPphC and AvrPphF were capable of blocking the HR. From our vantage point, it appears likely that these proteins are functioning in plants as suppressors of defence responses. Indeed, the VirPphA homologue in DC3000, AvrPtoB, was recently confirmed to act as a PCD suppressor (Abramovitch *et al.*, 2003). How many of the *P. syringae* type III effectors are acting as PCD and/or general defence suppressors? The availability of the DC3000 genome should help us answer this question by facilitating the identification of PCD suppressors using genome-wide approaches. HopPtoD2 and other effectors that behave similarly will help us understand how plant pathogenic bacteria evade host defences and are likely to be important tools to dissect signal transduction pathways that control plant innate immunity.

Experimental procedures

Bacterial strains and media

Escherichia coli strain DH5 α was used for general cloning and DNA manipulations. *Pseudomonas* s. pv. *tomato* DC3000 and *P. s. pv. phaseolicola* NPS3121 strains were grown in King's B (KB) broth at 30°C (King *et al.*, 1954). *Escherichia coli* and *Agrobacterium tumefaciens* MOG101 (Hood *et al.*, 1993) were grown in LB broth at 37°C or 30°C respectively. Antibiotics were used at the following concentrations ($\mu\text{g ml}^{-1}$): rifampicin, 100; ampicillin, 100; gentamicin, 10; kanamycin, 50; and spectinomycin 50. The *hopPtoD2* nucleotide sequence has been deposited in the GenBank database under accession no. AY198373.

Construction of plasmids

hopPtoD2 was cloned into the *Hind*III and *Bam*HI sites of pML122 (Labes *et al.*, 1990) using PCR with the primers P423 and P374 resulting in pLN56. These primer sequences and all others used in this manuscript are provided in Table S1 in the *Supplementary material*. To construct pML122 derivatives that contained *hopPtoD2* with a mutation that altered the catalytic Cys₃₇₈ to Ser, we cloned *hopPtoD2* into pBluescript KS using the above primers and site-directed mutagenesis was performed on this construct, pLN349, using the Quik™ Change Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers P538 and P539 according to the manufacturer's instructions. The *hopPtoD2* allele containing the mutation was cloned from pBluescript-KS into pML122 generating pLN214. pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ) derivatives containing *hopPtoD2* in frame

with glutathione S-transferase (GST) were constructed as follows. The coding region of *hopPtoD2* was amplified by PCR from DC3000 using primers P490 and P460. The amplified fragment was digested with *EcoRI* and *XhoI* and cloned into pGEX-5X-1 resulting in pLN173. Site-directed mutagenesis was performed on pLN173 using the Quik™ Change Kit (Stratagene) using the same primers as described above resulting in pLN234. *hopPtoD2* was PCR-cloned into the pENTR/D-TOPO vector (Invitrogen) using the primers P957 and P958 resulting in pLN560. The *hopPtoD2* insert in pLN560 was transferred to pLN462 using Gateway Technology (Invitrogen) resulting in pLN592. pLN462 is a modified binary pPZP212 vector (Hajdukiewicz *et al.*, 1994) compatible with Gateway Technology, and also containing a 35S promoter and a haemagglutinin epitope sequence flanking one of the *attR* sites. A *hopPtoD2* allele that possesses the catalytic Cys₃₇₈ to a Ser mutation was cloned into pLN462 using a similar strategy as described above resulting in pLN628.

Construction of DC3000 *hopPtoD2* mutants

We made two independent mutants defective in *hopPtoD2* using different strategies. In the first, we cloned an internal fragment of *hopPtoD2* into *XcmI* digested pKnockout-Ω (Windgassen *et al.*, 2000) using the primer P162 and P163. The resulting construct, pLN5, was conjugated into DC3000 by triparental mating using spectinomycin as selection for the plasmid marker. The *hopPtoD2* mutant UNL105 was confirmed with primers that flank the *hopPtoD2* coding region. In the second strategy, we constructed an unmarked *hopPtoD2* deletion mutation by using the suicide vector pKNG101 (Kaniga *et al.*, 1991), which contains the *sacB* gene and allows selection for plasmid loss. Polymerase chain reaction fragments representing upstream and downstream regions of the *hopPtoD2* coding region were amplified using the following primer sets: P280 and P281, with *SalI* and *SmaI* sites, respectively; and P282 and P283, with *SmaI* and *XbaI* sites respectively. These fragments were separately cloned into pKNG101 resulting in construct pLN357. pLN357 was mobilized into DC3000 by triparental mating and recombination of the plasmid into the DC3000 genome was selected for with streptomycin. Transconjugates were picked onto KB plates containing 5% sucrose to select for plasmid eviction. To identify the *hopPtoD2* mutant UNL112, we used primers that flank the *hopPtoD2* coding region.

DNA gel blots

Total DNA was digested with restriction enzymes and separated by electrophoresis and transferred to Immobilon-Ny+ membrane (Millipore, Bedford, MA). Hybridizations were carried out at 55°C in hybridization solution [7% sodium dodecyl sulphate (SDS), 2 mM EDTA and 0.5 M Na₂HPO₄]. The probe corresponding to the AvrPphD domain was PCR amplified using the primers P437 and P425. The fragment containing the PTP nucleotide sequence was PCR amplified with the primers P406 and P438. Membranes were washed twice in a solution containing 0.1% SDS and 1 × SSC. Membranes were exposed to film for approximately 12 h.

Affinity purification of recombinant *HopPtoD2*

To purify HopPtoD2 we made a construct that fused the 5' end of *hopPtoD2* with the glutathione S-transferase (GST) gene. Bacterial cultures expressing this construct were grown to mid-log phase and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside at 37°C overnight. Bacterial cells were pelleted and resuspended in ice cold lysis buffer containing 50 mM Tris-HCl pH 8.0, 1 mM EDTA, a protease inhibitor cocktail tablet (Roche, Indianapolis, IN) and 100 mM NaCl. To renature GST-HopPtoD2 from inclusion bodies, we followed a protocol described by Berndt and Cohen (1990). The dialysate was aliquoted and stored at -20°C until used. To purify GST-HopPtoD2 from lysates by affinity chromatography, we used Microspin GST Purification Module following the instructions from the manufacturer (Amersham Pharmacia Biotech).

Phosphatase assays

To study the PTP activity of HopPtoD2, a non-radioactive PTP assay kit was used (Cat. No. PTP101; Sigma, St Louis, MO), following the manufacturer's instructions. Briefly, the assay is based on the liberation of phosphate from Tyr-phosphorylated peptide substrates from either the insulin receptor or EGF receptor. The generated inorganic phosphate can be quantified using spectrophotometry at 620 nm. As a positive control, one unit of purified protein tyrosine phosphatase 1β (Cat. No. P-7365; Sigma, St Louis, MO) was used. To determine whether GST-HopPtoD2 possessed Ser/Thr phosphatase activity, we used an assay kit from New England Biolabs (Cat. No. P0780S; Beverly, MA). This assay is based on the dephosphorylation of ³²P-labelled myelin basic protein (MBP), which is phosphorylated at multiple Ser and Thr residues. Affinity-purified GST-HopPtoD2 samples were tested for Ser/Thr phosphatase activity by mixing with ³²P-MBP in phosphatase buffer. Liberated ³²P in the TCA supernatants was measured using a liquid scintillation counter.

Plant bioassays

DC3000 strains were assessed for their ability to cause disease symptoms and multiply *in planta* by dipping tomato (*Lycopersicon esculentum* cv. moneymaker) plants into bacterial suspensions that were adjusted to an OD₆₀₀ of 0.2 in 10 mM MgCl₂ containing 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) and enumerated as previously described (Alfano *et al.*, 2000). DC3000 strains were tested for their ability to elicit an HR on *Nicotiana tabacum* cv. Xanthi by infiltrating strains adjusted to an OD₆₀₀ of 0.2 along with 10-fold serially diluted samples with needleless syringe. Avirulent *P. s. phaseolicola* NPS3121 (*Pph*) carrying either *hopPtoD2* alleles or vector controls were adjusted to an OD₆₀₀ of 0.2 in 10 mM MgCl₂ and infiltrated into *N. benthamiana* leaves. For mixing experiments involving two different *Pph* strains, *Pph* carrying *hopPtoD2* *in trans* was infiltrated 4 h before the *Pph* carrying the vector control. The leaves were assessed for the development of an HR after 24 h. *Agrobacterium*-mediated transient expression experiments were done by infiltrating *A. tumefaciens* MOG101 at an OD₆₀₀ of 0.4 into *N. benthamiana* and *N. tabacum* cv. Xanthi plants

using a needleless syringe as described (Van den Ackerveken *et al.*, 1996). *hopPtoD2* alleles were carried on the binary vector pPZP212 (Hajdukiewicz *et al.*, 1994) and expressed from a constitutive 35S promoter. The *ntmek2* alleles encoding the constitutively active NtMEK2^{2D} were expressed from a dexamethasone (DEX) inducible promoter on pTA7002 (Yang *et al.*, 2001) and were induced with DEX as previously described (Aoyama and Chua, 1997). For co-expression experiments, *Agrobacterium* strains carrying *hopPtoD2* were infiltrated 4 h before infiltration of the NtMEK2 carrying strains.

Acknowledgements

We thank K. Jaeger for the pKnockout-Ω plasmid, S. Zhang for providing us with the NtMEK2 constructs and D. Klessig for providing us with SIPK and WIPK constructs. We also thank J. Stone and R. Chollett for helpful suggestions concerning HopPtoD2 purification and K. van Dijk, T. Petnicki-Ocwieja and S. T. Chancey for reviewing the manuscript. This work was supported by the National Science Foundation (NSF) Plant Genome Research Program Cooperative Agreement DBI-0077622 and NSF Grant IBN-0096348.

Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3588/mmi3588sm.htm>

Fig. S1. Structure and sequence alignment of HopPtoD2.

Fig. S2. *Agrobacterium*-mediated transient expression of HopPtoD2 suppresses the cell death induced by the NtMEK2 MAPKK.

Fig. S3. DC3000 *hopPtoD2* mutants are reduced in bacterial growth in host *A. thaliana* Col-0.

Table S1. PCR primer sequences used in experimental procedures.

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