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 Avr-PphD 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 NtMEK2DD, is capable of eliciting an HR-like cell death when transiently expressed in tobacco. When NtMEK2DD and HopPtoD2 were co-delivered into plant cells, the HR was suppressed indicating that HopPtoD2 acts downstream of NtMEK2DD. 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).
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. 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 et al., 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 *avrPphD*1*po* and *avrPphD*2*po*, have Hrp promoters upstream and are expressed in a Hrp-dependent manner (Fouts et al., 2002). Subsequently, both AvrPphD1*po* and AvrPphD2*po* 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 search also showed that the region of PTP similarity within HopPtoD2 contains a conserved catalytic domain of PTPs (pfam00102). PSI-BLASTP 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 (H/CxxGxxRS/T) present in the catalytic domains of functional PTPs (Denu et al., 1996). Another indication that HopPtoD2 contains 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
The HopPtoD2 protein tyrosine phosphatase

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.

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-HopPtoD2C378S) encoded by pLN234. Several studies have shown that this conserved Cys is required for PTP activity (Zhang and Dixon, 1994). When GST-HopPtoD2C378S 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. 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 BamHI and BglII 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.

**Fig. 2.** Tyrosine phosphatase activity of purified GST-HopPtoD2. The PTP activity of purified fusion proteins GST-HopPtoD2 and GST-HopPtoD2C378S 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 1b (PTP-1b) was used as a positive control at 1/20 dilution. These experiments were repeated at least three times with similar results.
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 (hopPtoD2C378S) into the same broad-host-range vector. Bacteria carrying this construct, pLN214, produced similar 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 (hopPtoD2), a HopPtoD2 encoding construct; or pLN214 (hopPtoD2C378S). The middle panel shows that HopPtoD2 suppressed the HR, whereas HopPtoD2C378S had no effect on HR elicitation (right panel). B. In bacterial mixing experiments, Pph carrying either pML122 (vector control), pLN56 (hopPtoD2), or pLN214 (hopPtoD2C378S) 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 pZP212 (empty vector), pLN592, a vector expressing HopPtoD2, or pLN628, a vector expressing the inactive HopPtoD2C378S. Leaf panels were infiltrated with MOG101(pTA7002::nptmek2G), which expressed active NtMEK2G. Also, included was an agroinfiltration with MOG101(pTA7002::nptmek2C), which expressed an inactive NtMEK2C. The NtMEK2G-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 ntmek2DD allele that encodes an altered NtMEK2 protein, NtMEK2DD, 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 ntmek2DD. Approximately 24 h after induction, a visible HR was observed in plant tissue infiltrated with ntmek2DD-containing Agrobacterium and a control strain, whereas, in leaf panels that expressed both HopPtoD2 and NtMEK2DD, the HR-like response caused by NtMEK2 alone. To determine the requirement of activation of NtMEK2, we used an ntmek2DD construct indicating that the PTP domain of HopPtoD2 was required in trans as shown for UNL112 (Fig. 4A). The reduced growth of UNL112 in planta was not complemented by the hopPtoD2C378S 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 10^-7 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, hopPtoD2C378S, 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 10^8 cells ml\(^{-1}\). The last dilution (10^3 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 10^3 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 similar 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.
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 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. Moreover, 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 effectors proteins VirPpH, AavrPpH and AavrPpH 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 VirPpH homologue in DC3000, AavrPtoB, was recently confirmed to act as a PCD suppressor (Abramovic 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 DH5α 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 (μg 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 HindIII and BamHI 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) and also containing a 35S promoter and a haemagglutinin epitope sequence flanking the PTP nucleotide sequence was PCR amplified with 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 3SS 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 x 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 Pharma 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. P07805; 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 assayed 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\(^{200}\) 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.

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**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.

**References**


Somssich, I.E., and Hahlbrock, K. (1998) Pathogen defence...


