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HopPtoN is a *Pseudomonas syringae* Hrp (type III secretion system) cysteine protease effector that suppresses pathogen-induced necrosis associated with both compatible and incompatible plant interactions

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Summary

***Pseudomonas syringae* pv. *tomato* DC3000 causes bacterial speck disease in tomato, and it elicits the hypersensitive response (HR) in non-host plants such as *Nicotiana tabacum* and *Nicotiana benthamiana*. The compatible and incompatible interactions of DC3000 with tomato and *Nicotiana spp.*, respectively, result in plant cell death, but the HR cell death occurs more rapidly and is associated with effective plant defense. Both interactions require the Hrp (HR and pathogenicity) type III secretion system (TTSS), which injects Hop (Hrp outer protein) effectors into plant cells. Here, we demonstrate that HopPtoN is translocated into tomato cells via the Hrp TTSS. A *hopPtoN* mutant produced eightfold more necrotic 'speck' lesions on tomato leaves than did DC3000, but the mutant and the wild-type strain grew to the same level in infected leaves. In non-host *N. tabacum* leaves, the *hopPtoN* mutant produced more cell death, whereas a DC3000 strain overexpressing HopPtoN produced less cell death and associated electrolyte leakage in comparison with wild-type DC3000. Transient expression of HopPtoN via infection with a PVX viral vector enabled tomato and *N. benthamiana* plants to tolerate, with reduced disease lesions, challenge infections with DC3000 and *P. syringae* pv. *tabaci* 11528, respectively. HopPtoN showed cysteine protease activity *in vitro*, and *hopPtoN* mutants altered in the predicted cysteine protease catalytic triad (C172S, H283A and D299A) lost HR suppression activity. These observations reveal that HopPtoN is a TTSS effector that can suppress plant cell death events in both compatible and incompatible interactions.**

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Introduction

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 is the causal agent of bacterial speck of tomato and Arabidopsis, and it multiplies to high levels and causes necrotic lesions on the leaves, stems, and fruits of infected plants. Like many strains of *P. syringae*, DC3000 also elicits the hypersensitive response (HR) in non-host plants such as tobacco (Klement *et al*., 1964; Fouts *et al*., 2003). The HR is a rapid, localized, defense-associated, programmed cell death triggered by the recognition of a plant pathogen. The relationship between the plant cell death events associated with the HR (incompatible interactions) and with disease (compatible interactions) is unclear at this point, but both plant responses require *Pst* to have a functional type III secretion system (TTSS), which is encoded by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes. Type III secretion systems are found in a wide variety of plant and animal pathogens, where they function to inject bacterial effector proteins into the cytosol of eukaryotic cells. Once inside the host cell, the effectors alter cellular processes to the advantage of the pathogen.

Pst DC3000 appears to carry at least 40 TTSS effector genes (Collmer *et al*., 2002; Buell *et al*., 2003; Greenberg and Vinatzer, 2003; Schechter *et al*., 2004). Only a few *P. syringae* effector genes have been shown to have a loss-of-function virulence phenotype (Ritter and Dangl, 1995; Badel *et al*., 2003; Bretz *et al*., 2003; Espinosa *et al*., 2003), apparently because of functional redundancy among the effectors. However, many effector genes have strong gain-of-function avirulence (Avr) phenotypes associated with gene-for-gene interactions that control the cultivar-specific resistance of some crop species against specific races of the pathogen. In these pathosystems, incompatible pathogen races produce an Avr (effector) protein that is recognized by a cognate *R* gene product in

the resistant cultivar, which results in elicitation of the HR and in avirulence (Keen, 1990).

P. syringae is divided into more than 50 groups known as pathovars, based on specificity for host species. For example, DC3000 is in pathovar tomato and is avirulent on soybean and many other crop species. This broader limitation in host range may result from the defensive recognition of multiple effectors by plant species that are non-hosts for a given pathovar, a hypothesis that is supported by the finding that various soybean cultivars can detect several *Pst* effectors, any one of which elicits the HR (Kobayashi *et al*., 1989). The role of TTSS effector proteins as Avr determinants in incompatible interactions has been particularly well studied, but we know much less about the function of these proteins in compatible interactions (Dangl and Jones, 2001; Martin *et al*., 2003; Chang *et al*., 2004).

Avirulence phenotypes provided the first means to identify TTSS effectors in *P. syringae* (Keen, 1990), but many recently identified *P. syringae* TTSS effectors have been found on the basis of their ability to travel the TTSS pathway (Badel *et al*., 2002; Collmer *et al*., 2002; Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002; Schechter *et al*., 2004). These effectors have accordingly been designated as Hops (Hrp outer proteins) (Alfano and Collmer, 1997). Many Hops have characteristic patterns in their N-terminal 50 amino acids. The patterns include an isoleucine, leucine, valine, alanine, or proline in the third or fourth amino acid position, a high percentage of serine in the first 50 amino acids, and the absence of acidic residues within the first 12 amino acids of the protein (Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002). These patterns are useful for identifying Hop candidates, but experimental verification is still required. The adenylate cyclase (Cya) assay, which is now routinely used to study translocation of phytopathogen effectors into plant cells, employs fusions of the test protein to the catalytic domain of Cya (*Bordetella pertussis* adenylate cyclase), which produces cAMP exclusively in the presence of eukaryotic calmodulin (Sory and Cornelis, 1994; Casper-Lindley *et al*., 2002; Schechter *et al*., 2004).

Effector genes are found throughout the *Pst* DC3000 genome, both individually and in clusters such as the Hrp pathogenicity island conserved effector locus (CEL) (Alfano *et al*., 2000; Buell *et al*., 2003). There are 10 genes in the CEL of DC3000: PSPTO1378, $avrE_{Pto}$, PSPTO1376, *hopPtoM*, *shcM*, *hrpW*, *hopPtoA1*, PSPTO1371, PSPTO1370 (designated CEL ORF7 previously and *hopPtoN* in this work), and PSPTO1369 (designated *shcN* in this work). Four of these genes encode known Avr/Hop proteins (*avrE*, *hopPtoM*, *hrpW* and *hopPtoA1*), another is predicted to encode a TTSS substrate (PSPTO1370), and three are known or predicted to encode TTSS chaperones (PSPTO1376, *shcM* and PSPTO1369). Because several effectors encoded in the CEL of the Hrp pathogenicity island appear conserved between *P. syringae* pathovars and have probably been horizontally acquired with the genes encoding the TTSS machinery (Alfano *et al*., 2000), they are thought to play a particularly important role in pathogenesis. In fact, there is good experimental evidence to support this hypothesis. For example, a deletion affecting *avrE*, *hopPtoM* and *hopPtoA1* strongly reduced the virulence of DC3000 in tomato (Alfano *et al*., 2000). Additionally, HopPtoM is important for the formation of disease lesions in tomato (Badel *et al*., 2003), and HopPtoA1 and its paralogue HopPtoA2 contribute to the initial development of individual bacterial colonies in tomato leaves (Badel *et al*., 2002). Another predicted TTSS substrate in this region is encoded by PSPTO1370, which has several characteristics of an effector gene: it is expressed *in planta*, activated by the HrpL alternative sigma factor, and possesses patterns in its predicted N-terminal 50 amino acids that are associated with TTSS substrates (Boch *et al*., 2002; Fouts *et al*., 2002; Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002).

Biochemical activities have been associated with only a few *P. syringae* TTSS effectors (Innes, 2003; Chang *et al*., 2004). For example, AvrRpm1 induces the phosphorylation of RIN4, a protein implicated in both basal defenses and RPM1-mediated HR, and HopPtoD2 has protein tyrosine phosphatase activity (Mackey *et al*., 2002; Bretz *et al*., 2003; Espinosa *et al*., 2003). Furthermore, investigation of the Avr activities of AvrPphB and AvrRpt2 have revealed that AvrPphB has cysteine protease activity, and AvrRpt2 likely also has cysteine protease activity based on catalytic triad mutant phenotypes (Axtell *et al*., 2003; Shao *et al*., 2003). The *Pst* DC3000 PSPTO1370 also is predicted to be a cysteine protease because it possesses a potential catalytic triad (C172, H283, D299) that is characteristic of the YopT/AvrPphB family of TTSS effectors (Shao *et al*., 2002). Interestingly, members of this effector family are found in both plant and animal pathogens (Shao *et al*., 2002; Shao *et al*., 2003).

Here, we report that the PSPTO1370 protein, henceforth designated as HopPtoN, is translocated into plant cells in a TTSS-dependent manner, partially suppresses the cell death associated with both compatible and incompatible *P. syringae*–plant interactions, requires all three of the amino acids in the cysteine protease catalytic triad to suppress the HR, and displays cysteine protease activity *in vitro*.

Results

HopPtoN is translocated into plant cells by the P. syringae *Hrp TTSS*

The N-terminal 50 amino acids of HopPtoN (PSPTO1370)

has several characteristics of *P. syringae* TTSS substrates. It has a hydrophobic amino acid (isoleucine) in position three, no acidic amino acids in the first 12 positions, and a high serine content of 14%. Furthermore, *hopPtoN* is in a bicistronic operon, and the promoter-proximal ORF (PSPTO1369) is characteristic of a TTSS chaperone. Specifically, the predicted PSPTO1369 protein is small (18.2 kDa) and mostly α-helical (nnPredict). To determine whether or not HopPtoN is a type III substrate that can be translocated into plant cells and whether or not the putative PSPTO1369 chaperone has a role in the level of HopPtoN that is delivered to plant cells, we constructed two plasmids expressing a fusion of Cya to the C-terminus of full-length HopPtoN in vector pCPP3234 (Schechter *et al*., 2004). Plasmid pCPP3238 expressed HopPtoN-Cya alone, and plasmid pCPP3239 expressed both PSPTO1369 and HopPtoN-Cya. To demonstrate that HopPtoN translocation was dependent upon the Hrp TTSS, we used CUCPB5114, a derivative of DC3000 from which the *hrp*/*hrc* (*hrp c*onserved) genes have been deleted. As shown in Fig. 1A, DC3000(pCPP3238) translocated HopPtoN-Cya into tomato (*Lycopersicon esculentum* cultivar Money Maker) leaf cells, as indicated by Cya-dependent cAMP production. Infiltration with DC3000(pCPP3239) produced even more cAMP, suggesting that PSPTO1369, henceforth referred to as ShcN (specific Hrp chaperone), promoted the delivery of HopPtoN by the TTSS.

Because some chaperones are required for the stabilization of their client effectors within the bacterial cytoplasm (Feldman and Cornelis, 2003), we examined the accumulation of HopPtoN in DC3000(pCPP3238) and DC3000(pCPP3239) grown in King's medium B (Fig. 1B). In this medium, the native *shcN* and other genes in the Hrp regulon are not expressed (Fouts *et al*., 2002). Nevertheless, constitutively expressed HopPtoN accumulated to the same level in DC3000(pCPP3238) as in DC3000(pCPP3239) regardless of the expression of ShcN. Thus, ShcN is not needed to stabilize HopPtoN within DC3000. Finally, neither pCPP3238 nor pCPP3239 promoted any cAMP production when expressed in CUCPB5114, which indicates that translocation of HopPtoN-Cya is TTSS-dependent.

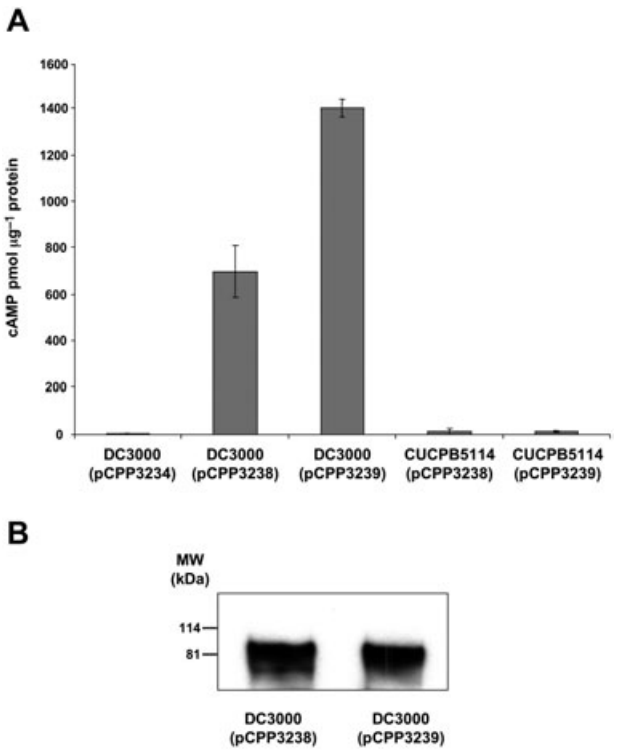

Fig. 1. HopPtoN-Cya fusions expressed in *Pst* DC3000 are translocated into tomato leaf cells by the *Pst* DC3000 Hrp TTSS.
A. Calmodulin- and Cya-dependent production of cAMP was used to measure translocation of HopPtoN-Cya fusions into plant cells. Tomato plants were infiltrated with 10^8 cfu ml^{-1} *Pst* DC3000 or CUCPB5114 (Δ*hrp*/*hrc*) harbouring pCPP3238 (HopPtoN-Cya) or pCPP3239 (ShcN-HopPtoN-Cya). Leaf samples were taken 8 h after inoculation with a 0.7-cm-diameter cork borer and assayed for cAMP. Values represent the mean and standard deviation for triplicate samples; similar results were obtained in multiple experiments.
B. To determine the effect of ShcN on HopPtoN stability, production of HopPtoN-Cya was induced with the addition of 100 μM IPTG to log-phase King's Medium B cultures of *Pst* DC3000 harbouring pCPP3238 (HopPtoN-Cya) or pCPP3239 (ShcN-HopPtoN-Cya). After 3 h, cell lysates were collected, and equal amounts of protein were resolved by SDS-PAGE. Immunoblot analysis with antibodies recognizing Cya was used to determine the amount of HopPtoN-Cya that had accumulated in each strain.

Mutating the Pst *DC3000* hopPtoN *worsens disease symptoms in tomato*

To test the role of HopPtoN in virulence in tomato, we used suicide plasmid pKnockout-Ω (Windgassen *et al*., 2000), carrying an internal fragment of *hopPtoN*, to construct a *hopPtoN* mutant derivative of DC3000, CUCPB5388. Tomato plants were inoculated with test bacteria and monitored for formation of the necrotic lesions that are characteristic of bacterial speck. Unexpectedly, CUCPB5388 produced more necrotic lesions than wild-type DC3000 (Fig. 2). The wild-type level of lesions was restored by expressing *hopPtoN in trans* following transformation with pCPP5071, which constitutively expresses *shcN-hopPtoN* from the vector *nptII* promoter, which has been shown to be a strong promoter in *P. syringae* (Gopalan *et al*., 1996).

This increased-lesion phenotype was detected when bacteria were inoculated with a blunt syringe, but it was observed most clearly when plants were inoculated by dipping, as shown in Fig. 2A. Close inspection of many inoculated plants revealed that the primary effect of mutating *hopPtoN* was to increase the number, rather than the size, of lesions. This can be seen in the representative panels shown in Fig. 2A, and it is further documented with the lesion counts on tomato leaflets presented in Fig. 2B,

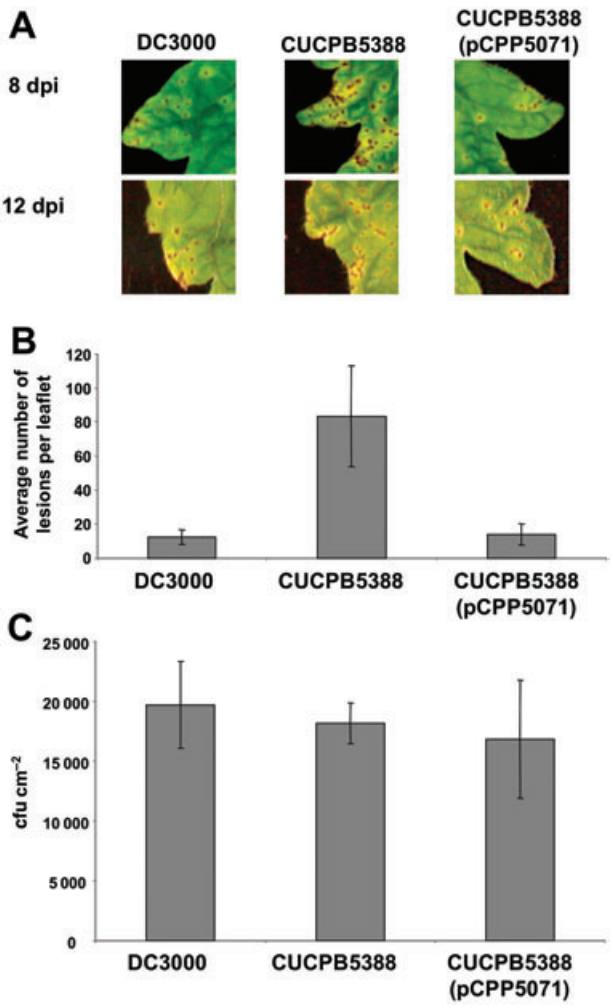

Fig. 2. A *Pst* DC3000 *hopPtoN* mutant produces more necrotic lesions in infected tomato leaves.
A. Disease symptoms were observed on tomato cultivar Money Maker plants 8 and 12 days after inoculation by dipping in suspensions of 1×10^6 cfu ml^{-1} *Pst* DC3000, CUCPB5388 (*hopPtoN*::pKnockout) and CUCPB5388 complemented with pCPP5071, which expresses *shcN-hopPtoN*.
B. The numbers of lesions on equivalent tomato leaflets (with regard to age and location on plants), following inoculation with different bacteria as indicated, were counted 8 days post inoculation. The values shown represent the mean and standard error from five leaflets.
C. The numbers of bacteria per unit area in tomato leaflets 8 days post inoculation were determined by taking material from the same leaflets that were analysed above, homogenizing the tissue, and serially diluting and plating on medium that selects for *Pst* DC3000. Similar numbers were seen in multiple experiments.

which show that CUCPB5388 produced *c.* eightfold more lesions than DC3000. Lesions appeared 4 days post inoculation, expanded to near full size by 8 days, and were surrounded by extensive chlorosis at 12 days (Fig. 2A). No new lesions were observed beyond 8 days post inoculation (data not shown). Assays of bacterial populations in leaves 8 days post inoculation revealed no significant differences in the populations achieved by the DC3000 or *hopPtoN* mutant CUCPB5388 (Fig. 2C). These data indicate that HopPtoN suppresses the ability of *Pst* to elicit necrotic disease lesions in host tomato leaves, but this change in lesion phenotype does not have an apparent effect on bacterial growth *in planta*.

Altered production of HopPtoN affects the ability of Pst *DC3000 to elicit the HR in non-host tobacco leaves*

Tobacco (*Nicotiana tabacum*) has been extensively used to study the HR. *P. syringae* pathovars that have hosts other than tobacco typically elicit the HR in tobacco leaves, which is a useful diagnostic feature (Palleroni, 1984). We inoculated tobacco leaves via a blunt syringe with DC3000 and the *hopPtoN* mutant strain CUCPB5388. We used a dilution series of inoculum, and 24 h after inoculation, we observed that CUCB5388, relative to DC3000, caused a slight but reproducible increase in confluent tissue collapse at the threshold level of inoculum needed for elicitation of the HR (Fig. 3A).

To determine if we could suppress the HR by overexpressing HopPtoN in *Pst* DC3000, we inoculated tobacco leaves with DC3000 and DC3000(pCPP5071). Tobacco leaves were inoculated by blunt syringe infiltration of both strains, at decreasing levels of inoculum, and the threshold level for eliciting the HR was observed 24 h later (Fig. 3B). Both DC3000 and DC3000(pCPP5071) elicited confluent tissue death, indicative of the HR in infiltrated areas when inoculated at 8×10^6 cfu ml^{-1} or higher, but at the threshold level of 4×10^6 only DC3000 elicited tissue death. Similar results were observed in multiple experiments.

Although death of the infiltrated tissue is the signature of the HR, the HR is also accompanied by loss of electrolytes from the infiltrated tissue, which provides a more quantitative measure of the response (Cook and Stall, 1968; Goodman, 1968; Torres *et al.*, 2002). Accordingly, we sampled leaf sections that had been inoculated with DC3000 and DC3000(pCPP5071) at inoculum levels near the threshold needed for elicitation of confluent tissue collapse, and we measured the leakage of electrolytes, as indicated by the increased conductivity of water in which leaf samples were bathed (Fig. 4). Electrolyte leakage from inoculated leaf tissue was substantially lower with DC3000(pCPP5071) than with empty vector control DC3000(pCPP5040) (Fig. 4).

It is important to note that the threshold inoculum level needed by *Pst* DC3000 to elicit the visible HR and electrolyte leakage from tobacco varies with the age of the leaves, the season, and other factors affecting the hypersensitivity of the plants. However, we have repeatedly observed that at the empirically determined threshold for

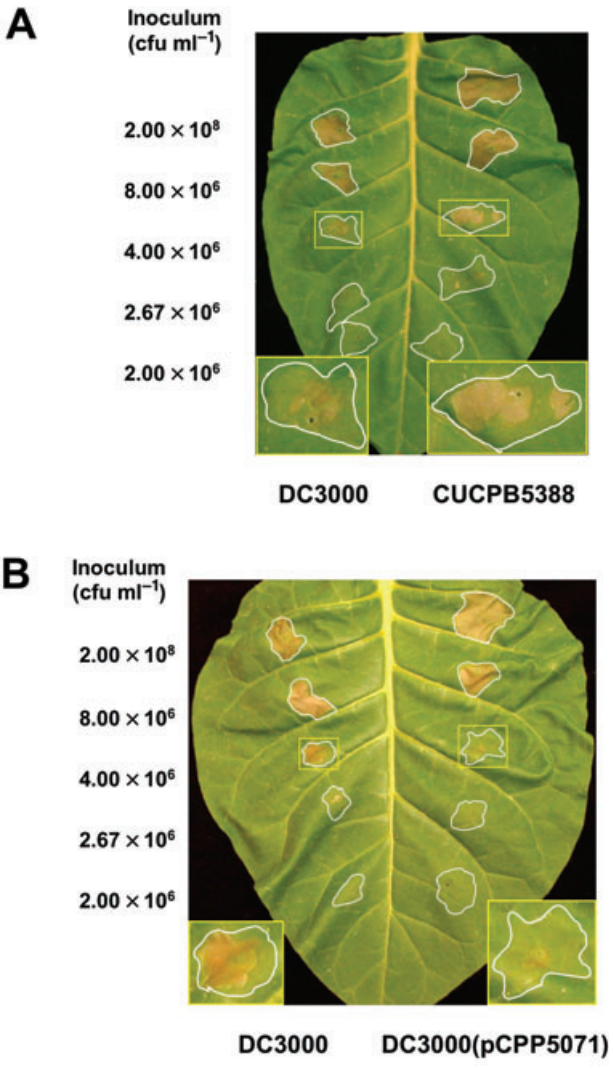

Fig. 3. Altered production of HopPtoN affects the ability of *Pst* DC3000 to elicit the HR in non-host tobacco leaves.
A. Leaves of *Nicotiana tabacum* cultivar Xanthi were infiltrated with a blunt syringe containing inoculum at the indicated levels, progressing through a dilution series. Bacteria were *Pst* DC3000 and mutant CUCPB5388 (*hopPtoN*::pKnockout). Leaves were photographed 24 h after inoculation, and the white outlines indicate the areas inoculated. The insets show the differential responses at the threshold level of inoculum for elicitation of the HR.
B. Leaves of *N. tabacum* cultivar Xanthi were syringe-infiltrated as above with *Pst* DC3000 (Wild-type) or *Pst* DC3000(pCPP5072), which overexpresses *hopPtoN*, and photographed 24 h later. The insets show the differential responses at the threshold level of inoculum for elicitation of the HR. Panels A and B show representative leaves from four replicate experiments.

wild-type DC3000 with a given batch of plants, CUCPB5388 produces a stronger HR, and DC3000 (pCPP5071) produces a reduced HR and reduced electrolyte leakage. Thus, either mutation or overexpression of HopPtoN can affect the ability of *Pst* to elicit the HR in non-host tobacco, and HopPtoN appears to suppress the HR.

Transient expression of HopPtoN via a PVX viral vector in tomato and Nicotiana benthamiana *reduces symptoms elicited by virulent* P. syringae *strains*

The previous experiments suggested that HopPtoN can operate inside plant cells to reduce the cell death associated with *P. syringae* interactions in both host tomato and non-host tobacco, but they leave open the possibility that altered production of HopPtoN affects the deployment of other effectors. To address this concern, we used a viral vector to express HopPtoN in plant cells. We cloned *hopPtoN* into PVX expression vector pQuiet2, such that the bacterial gene would be expressed strongly in plant cells following systemic infection by the viral vector. We then introduced the PVX constructs into PVX-susceptible tomato and *N. benthamiana* plants by *Agrobacterium tumefaciens*-mediated infection. Four weeks later, typical leaf-crinkling symptoms of systemic PVX infection were evident in *N. benthamiana* (Fig. 5). At this time we inoculated the PVX-infected tomato and *N. benthamiana* plants with *Pst* DC3000 and the tobacco wildfire pathogen *P. syringae* pv. *tabaci* 11528, respectively. In both cases, we introduced the inoculum by syringe infiltration so that we could observe multiple, identical inoculations on a single leaf, and we used the low levels of inoculum that are typically employed to assay the compatible interactions of virulent strains with their susceptible hosts. The representative leaves in Fig. 5 reveal that the confluent disease necrosis observed 7 days after infiltration of the *P. syringae* strains was markedly reduced in the PVX::*hopPtoN*-infected leaves that were expressing *hopPtoN*. The PVX::*hopPtoN*-infected tomato leaves showed relatively more chlorosis than necrosis, and some inoculation zones in PVX::*hopPtoN*-infected *N. benthamiana* leaves produced no necrosis. However, we saw no effect of *hopPtoN* expression on bacterial population levels *in planta* 4 days after inoculation, when bacterial populations were at their maximum (data not shown). These observations provide further evidence that HopPtoN acts inside plant cells to reduce the necrosis associated with *P. syringae* infection of susceptible hosts.

Delivery of HopPtoN by a cloned P. syringae *Hrp system expressed in non-pathogen* Pseudomonas fluorescens *can suppress the HR normally elicited in* Nicotiana benthamiana *by a* Pst *DC3000 challenge inoculation*

Several *Pst* DC3000 TTSS effectors have been shown able to suppress the HR when delivered by *Pseudomonas. fluorescens* 55 carrying pHIR11, which expresses the *hrp*/*hrc* genes from *P. syringae* pv. *syringae* 61 (Jamir *et al.*, 2004). In order to rapidly observe the effects of HopPtoN on plant cells in the absence of other type III effectors we expressed the protein in *P. fluorescens*

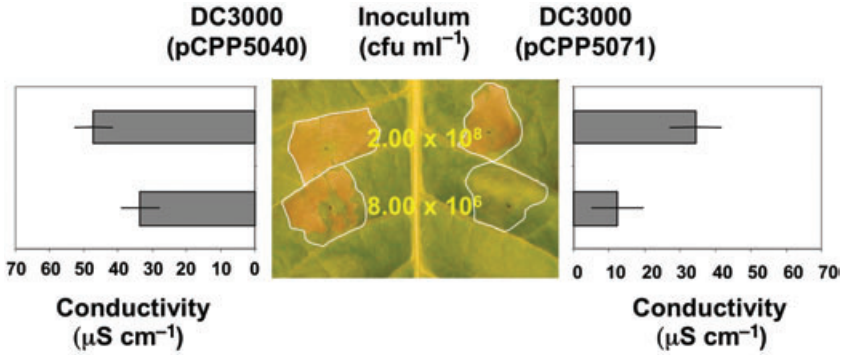

Fig. 4. Overexpression of HopPtoN in *Pst* DC3000 reduces electrolyte leakage from inoculated non-host tobacco leaves. Tobacco leaves were inoculated with a dilution series of inoculum, as described for Fig. 3, using DC3000(pCPP5040) as a vector control and DC3000(pCPP5071), which overexpresses *hopPtoN*. Leaves were photographed 24 h later, and then equal amounts of the infiltrated areas at the threshold level of inoculum for HR elicitation were excised from the leaves and vacuum infiltrated in 10 ml of H_2O. The excised disks were shaken at room temperature for 1 h, and electrolyte leakage was measured by recording the conductivity of the H_2O after removal of the leaf disks. The conductivity values represent the mean and standard deviation from four leaves, and a leaf with typical differential responses at the threshold level of inoculum for elicitation of the HR is shown. The experiment was repeated several times with similar results.

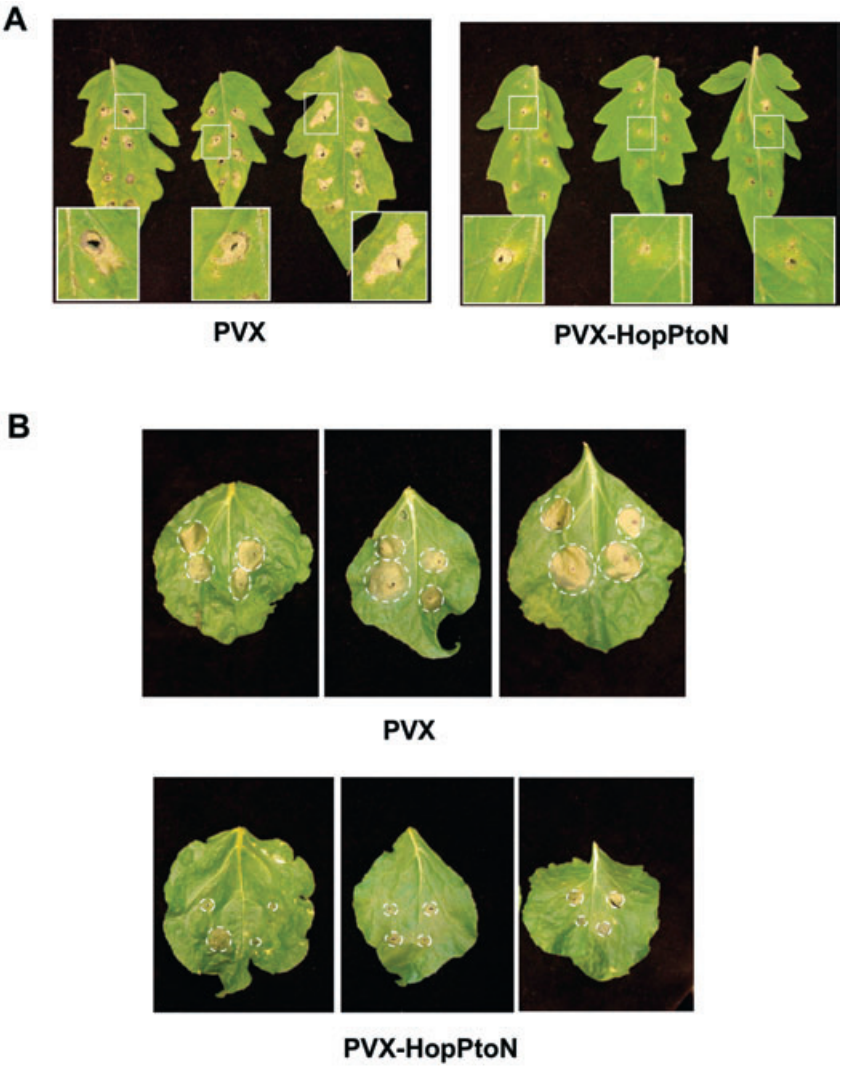

Fig. 5. Transient expression of HopPtoN with a PVX viral vector in tomato and *N. benthamiana* reduces symptoms elicited by virulent *P. syringae* strains.
A. Symptoms were observed in tomato cultivar Money Maker plants that were infected four weeks previously with an empty PVX expression vector or with PVX-HopPtoN and then challenge-inoculated via a blunt syringe with 1×10^4 cfu ml^{-1} *Pst* DC3000. Leaves were photographed 1 week after challenge inoculation.
B. Symptoms were similarly observed in *N. benthamiana* plants that were infected four weeks previously with an empty PVX expression vector or with PVX-HopPtoN and then challenge inoculated via a blunt syringe with 1×10^5 cfu ml^{-1} *P. syringae* pv. *tabaci* 11528. Leaves were photographed 1 week after challenge inoculation. The leaves shown in panels A and B are representative of nine replicate plants. Dashed lines indicate approximate area of necrotic lesions.

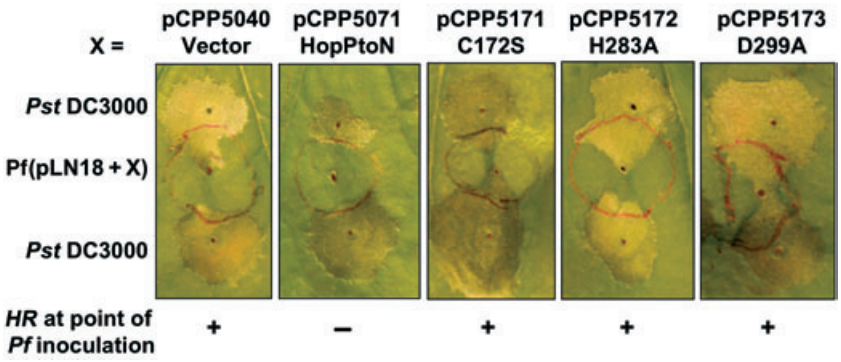

Fig. 6. Native HopPtoN delivered by a cloned *P. syringae* Hrp system expressed in non-pathogen *P. fluorescens* suppresses the HR that is normally elicited in *N. benthamiana* by a *Pst* DC3000 challenge inoculation, but HopPtoN cysteine protease catalytic triad mutants lose suppressor activity. Lines mark the areas that were inoculated with a suspension (6×10^8 cfu ml^{-1}) of *P. fluorescens* 55 harbouring two plasmids: pLN18, which expresses the *hrp/hrc* cluster from *P. s. syringae* 61, and pCPP5040 derivatives expressing the indicated *hopPtoN* alleles. Two hours after inoculation with the candidate suppressor strains, indicated as Pf(pLN18 + X), the leaves were challenge inoculated with 1×10^7 cfu ml^{-1} of *Pst* DC3000. In each case, the infiltration zone for the challenge inoculum extended to the hole through which the Pf(pLN18 + X) inoculum had been delivered, thus producing uniform, partially overlapping inoculation areas. Leaves were photographed 18 h after challenge inoculation with *Pst* DC3000. HopPtoN mutants C172S, H283A, and D299A are altered in each of the amino acids in the cysteine protease catalytic triad. The scores at the bottom of the figure indicate whether any visible collapse is present at the point of Pf(pLN18 + X) inoculation; a minus denotes the absence of the HR and therefore indicates that the test effector has suppressor activity.

containing pLN18, a derivative of pHIR11 that lacks the functional effector protein *hopPsyA* (Jamir *et al*., 2004). By using overlapping zones of infiltration in non-host *N. benthamiana* leaves, in which *P. fluorescens*(pLN18, pCPP5040) or *P. fluorescens*(pLN18, pCPP5071), which expresses *shcN-hopPtoN*, were delivered 2 h before challenge inoculation of *Pst* DC3000, we found that HopPtoN was capable of strongly suppressing the HR (Fig. 6). This test provided a clear gain-of-function phenotype for exploring the potential biochemical activity of HopPtoN *in planta*.

Mutagenesis of the predicted cysteine protease catalytic triad in HopPtoN abolishes its ability to suppress the HR

HopPtoN has a predicted cysteine protease catalytic triad (C172, H283 and D299), which it shares with other members of the YopT/AvrPphB family of TTSS effectors (Shao *et al*., 2002). To test the role of the catalytic triad in the ability of HopPtoN to suppress the HR, we constructed site-directed mutations affecting each of these residues in a plasmid encoding both *shcN* and *hopPtoN*, pCPP5060. These constructs were moved via Gateway cloning methods into pCPP5040 and then transformed into *P. fluorescens*(pLN18) and *Pst* DC3000 for biological testing. We first tested the ability of these HopPtoN mutants to suppress the HR that is normally elicited by DC3000 in *N. benthamiana*, using the method described above involving inoculation with *P. fluorescens*(pLN18) expressing HopPtoN variants followed by an overlapping DC3000 challenge inoculation. In contrast to native HopPtoN, the C172S, H283A, and D299A mutants showed no HR-suppressive activity (Fig. 6). Immunoblot analysis revealed no difference between the native protein and the three mutants in their levels in *P. fluorescens*(pLN18) (data not shown), and these mutations are beyond the N-terminal region in *P. syringae* effectors that carries TTSS targeting signals and are therefore unlikely to be affected in delivery to plant cells (Petnicki-Ocwieja *et al*., 2002; Schechter *et al*., 2004).

We then tested the ability of the HopPtoN catalytic triad mutants to suppress the HR elicitation activity of DC3000 in tobacco when overexpressed directly in DC3000 (Fig. 7). Whereas DC3000 overexpressing the native HopPtoN *in trans* suppressed HR elicitation and associated electrolyte leakage in tobacco leaves at the threshold level of inoculum for wild-type DC3000, DC3000 overexpressing the HopPtoM C172S, H283A, and D299A variants at the same inoculum level failed to do so (Fig. 7). These observations suggest that HopPtoN has cysteine protease activity which is essential for HR suppressor activity.

HopPtoN has cysteine protease activity in vitro

HopPtoN is a member of the CA clan of papain-like cysteine proteases (Barrett and Rawlings, 2001; Shao *et al*., 2002).To determine if HopPtoN has protease activity *in vitro*, we first constructed HopPtoN-His_6 fusions involving the native protein and the three catalytic triad mutants and purified the proteins from *Escherichia coli* extracts by affinity chromatography using non-denaturing buffers. We then assayed protease activity of the proteins using resorufin-labeled casein as a substrate (Twining, 1984). The unaltered HopPtoN-His_6 and papain were both able to cleave the resorufin-label from casein. However, we

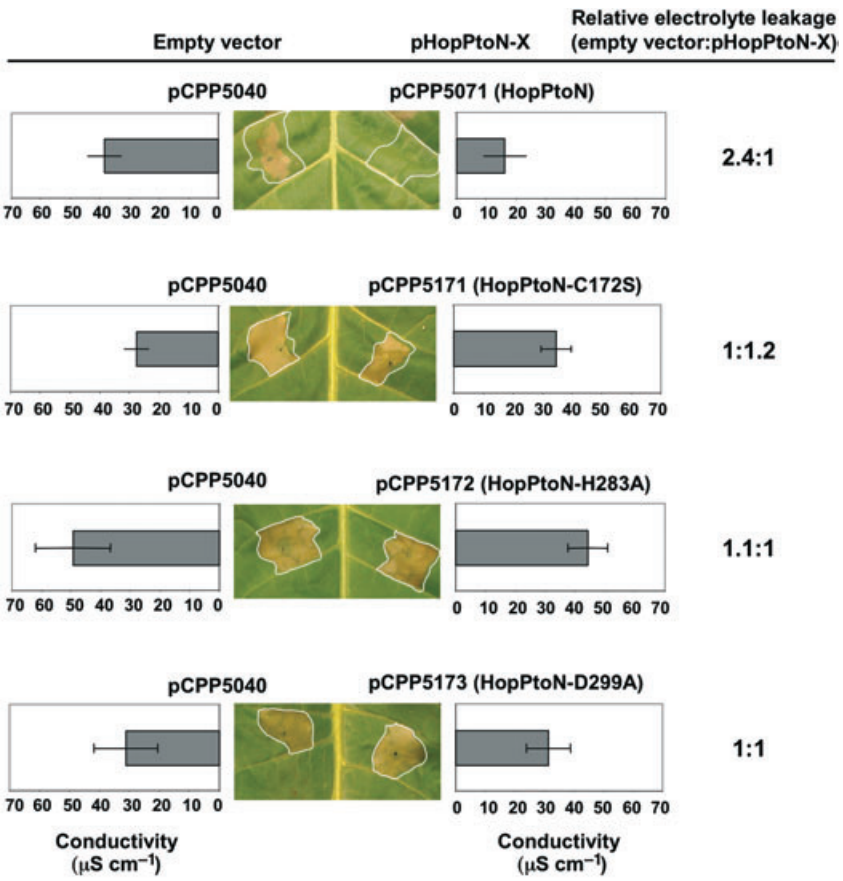

Fig. 7. The ability of overexpressed HopPtoN in *Pst* CUCPB5388 (*hopPtoN*::pKnockout) to suppress the HR and the associated electrolyte leakage in inoculated non-host tobacco leaves is dependent on the cysteine protease catalytic triad. Tobacco leaves were inoculated, with infiltrated areas designated by a white outline as in Fig. 3, with CUCPB5388 (*hopPtoN*::pKnockout) or CUCPB5388 overexpressing *hopPtoN* variants in derivatives of vector pCPP5040 and photographed 24 h later. Representative leaf sections at the threshold level of inoculum needed for the vector control to elicit a visible HR were photographed, then equal amounts of the infiltrated area were excised from the leaf immediately after photography. The leaf disks were placed in 10 ml of H_2O and shaken at room temperature for 1 h. Electrolyte leakage was measured by recording the conductivity of the H_2O after removal of the leaf disks.

saw no significant cleavage with the three altered HopPtoN-His_6 proteins or when we added E64, a specific inhibitor of the CA clan of papain-like cysteine proteases, to the native HopPtoN or papain (Fig. 8). From these data, we conclude that HopPtoN is a cysteine protease in the CA clan that requires all three of the catalytic residues, C172, H283 and D299.

Discussion

TTSS effectors are now seen to be collectively required for the pathogenicity of *P. syringae*, and a better understanding of pathogenesis will require determination of the virulence phenotypes and biochemical activities of individual effectors. To that end, we have shown that the *Pst* DC3000 effector HopPtoN is delivered into plant cells via the Hrp TTSS, suppresses cell death in both compatible and incompatible interactions, and possesses cysteine protease activity that is essential for suppressor activity. A particularly striking finding is that a *Pst hopPtoN* mutant is increased in its ability to produce disease lesions on its host tomato.

The *Pst hopPtoN* gene is located downstream of *shcN* (Alfano *et al.*, 2000), and a strain of DC3000 overexpressing SchN and HopPtoN delivered HopPtoN into plant cells

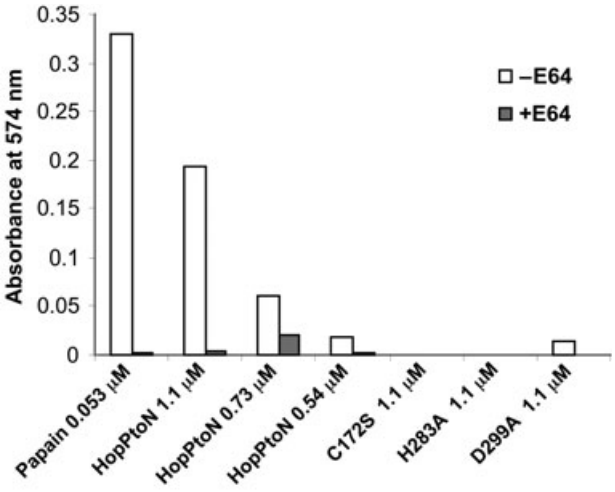

Fig. 8. HopPtoN has cysteine protease activity *in vitro*. Papain or HopPtoN purified under non-denaturing conditions (wild-type and mutant proteins) were combined with resorufin-labeled casein in the presence or absence of the cysteine protease inhibitor E64. The assay mixture was incubated for 1 h at 37°C, then the reaction was stopped by adding TCA to a final concentration of 10%. The samples were centrifuged, the supernatants were collected, and the A_{574} was recorded.

twice as efficiently as when ShcN was not similarly expressed. The location of the *shcN* gene, the physical properties of the predicted ShcN protein, and the stimulation of HopPtoN translocation all suggest that ShcN is a chaperone for HopPtoN (Feldman and Cornelis, 2003). Several other effector genes in the *Pst* DC3000 genome are linked with apparent chaperone genes, and *shcN* joins a growing list of these chaperone candidates for which there is experimental evidence of a role in effector delivery (Alfano and Guo, 2003; Badel *et al*., 2003; Buell *et al*., 2003).

Chaperones can affect the secretion (into the medium) and translocation (into host cells) of TTSS effectors by multiple mechanisms, including stabilization, maintenance in a secretion/translocation-competent state, piloting to the TTSS pathway, and establishment of a hierarchy for secretion/translocation (Feldman and Cornelis, 2003). ShcN does not appear necessary to stabilize HopPtoN within *Pst* DC3000. However, our focus in this report is on the biological activity of HopPtoN, and future experiments are needed to determine the mechanism by which ShcN promotes delivery of HopPtoN into plant cells. A particular important question is whether chaperoned effectors are delivered preferentially over other effectors by DC3000 (Feldman and Cornelis, 2003).

There is growing evidence that some effectors are able to suppress the defense-associated HR. First, the *P. syringae* pv. *phaseolicola* VirPphA was observed to block the HR elicited by other effectors, whose Avr activity is apparently masked by VirPphA (Jackson *et al*., 1999). Then, AvrPtoB, a *Pst* DC3000 effector showing 52% amino acid identity to VirPphA, was demonstrated to operate inside plant cells to block *R* gene-dependent programmed cell death and also to block the Avr activity of a domain within AvrPtoB itself (Abramovitch *et al*., 2003). Most recently, a survey of multiple DC3000 effectors using a *P. fluorescens*(pHIR11) system revealed that a third of the effectors can act inside plant cells to block the HR elicited in tobacco by the pHIR11-encoded effector HopPsyA (Jamir *et al*., 2004). Furthermore, mutagenesis of several of these effectors enables *Pst* DC3000 to elicit an enhanced HR in tobacco, as was similarly observed here with *hopPtoN* mutant CUCPB5388.

All of these observations are focused on regulation of the programmed cell death associated with incompatible interactions, and they do not address the nature of the cell death associated with the necrotic lesions that develop in the later stages of a compatible interaction. What is the relationship between the cell death events accompanying incompatible and compatible interactions? A review of physiological observations of *P. syringae*–plant interactions made before 1982 led to the conclusion that 'the development of hypersensitive and normosensitive necrosis must be considered as being similar processes; their induction however, requires different numbers of bacterial cells and their development proceeds at different rates (Klement, 1982). A subsequent review of pioneering studies on defense gene expression in compatible and incompatible interactions also highlighted the similarity of the responses despite the differences in timing (Lamb *et al*., 1992). A recent large-scale mRNA expression profile analysis of Arabidopsis responses during compatible and incompatible interactions with *P. syringae* comprehensively documented the overall similarity of the responses and further proposed that a common signal transduction pathway underlies both (Tao *et al*., 2003). This report also noted the higher variability (reduced system robustness) in the response of Arabidopsis to compatible *P. syringae* strains. Several observations with HopPtoN and other *Pst* DC3000 effectors support this model.

The concept that a common signal transduction pathway leads to cell death and other responses in both compatible and incompatible *P. syringae*–plant interactions is supported by our finding that a single TTSS effector, HopPtoN, suppresses cell death in both interactions. The further concept of lower system robustness (higher variability) of the compatible response is consistent with the variable effects on lesion formation of three recently studied *Pst* DC3000 effectors. Mutations in *hopPtoN*, *hopPtoM*, and *hopPtoD2*, enhance, reduce, or do not affect, respectively, the ability of *Pst* DC3000 to cause bacterial speck disease lesions in tomato (Badel *et al*., 2003; Bretz *et al*., 2003; Espinosa *et al*., 2003). It is noteworthy that *hopPtoD2* nevertheless is able to suppress HR cell death (Espinosa *et al*., 2003). The ability of other effectors with HR suppressor activity to alter the necrosis associated with disease now demands close scrutiny.

The ability of HopPtoN and HopPtoD2 to suppress the HR warrants further discussion because it is dependent on different biochemical activities. HopPtoD2 is a protein tyrosine phosphatase that appears to act downstream of $NtMEK2^{DD}$, a constitutively active mitogen-activated protein kinase that can activate an HR-like programmed cell death (Espinosa *et al*., 2003). In contrast, as shown here, HopPtoN is a cysteine protease. The target(s) of HopPtoD2 phosphatase activity and HopPtoN protease activity are unknown, but the plant response model described above would suggest that they are components of a single signal transduction pathway that controls programmed cell death. Indeed, the Arabidopsis RIN4 protein, which mediates HR responses involving the R genes *RPM1* and *RPS2*, appears to be phosphorylated by AvrRpm1 and proteolytically degraded by AvrRpt2 (Mackey *et al*., 2002; 2003; Axtell and Staskawicz, 2003). This is an example of a single plant protein that is modified in different ways by two *P. syringae* effectors, although in this case, the documented consequence of these activities is R gene-dependent elicitation of the HR.

HopPtoM and HopPtoN appear to have opposite effects on lesion formation in host tomato leaves. Both of the encoding genes are located in the Hrp pathogenicity island CEL, and given the observation that bacterial genes with related function are commonly clustered (Lawrence and Roth, 1996), it is tempting to speculate that these proteins function in some coordinated way to modulate lesion formation during pathogenesis. It is also noteworthy that the altered lesion phenotypes of *hopPtoM* and *hopPtoN* mutants are not accompanied by a commensurate change in bacterial growth *in planta* (Badel *et al*., 2003). Early observations of *P. syringae*–plant interactions have highlighted the cessation of bacterial growth that attends cell death during both incompatible and compatible interactions (Klement *et al*., 1964). Whether plant cell death is causal or merely coincidental with the inhibition of bacterial growth is unclear. However, our observations argue against a causal role in compatible interactions and suggest that the characteristic speck, spot, and blight symptoms that are diagnostic of various *P. syringae* diseases are orchestrated by the TTSS effector system (in concert with diffusible, low molecular weight toxins in some cases (Bender *et al*., 1999)).

We have shown here that the loss of a single effector can produce an increase in tomato bacterial speck symptoms that appears, at least in laboratory assays, to have no parasitic benefit. Questions for the future include the proteolytic targets of HopPtoN action, the nature of any selection pressures encountered by *P. syringae* in the field that would calibrate the degree of death elicited in hosts, and the potential use of *hopPtoN* transgenes to produce crops that can tolerate *P. syringae* infections through reduced symptom expression.

Experimental procedures

Plasmids, strains and DNA

The strains and plasmids used in this study are described in Table 1. All *Pseudomonas* strains were grown in King's B broth (KB) at 30°C and all *A. tumefacians* and *E. coli* strains were grown in Luria broth (LB) at 37°C. All *hopPtoN* and *shcN-hopPtoN* expression plasmids were created using Gateway™ cloning technology (Invitrogen, Carlsbad, CA). In short, the gene or genes of interest were amplified by ploly-merase chain reaction (PCR) from the DC3000 genome with ExTaq (TaKaRa) using primers that introduced an attB1 sequence and a Shine-Dalgarno sequence upstream and an attB2 sequence downstream. These PCR products were combined with pDONR201 along with BP clonase enzyme per manufacturer's protocol (Invitrogen). All pDONR clones were checked by sequencing, and expression clones were made by combining the pDONR plasmids with the desired expression vector along with LR clonase. The recombination reactions were carried out as suggested by the manufacturer (Invitrogen). All expression constructs were confirmed by sequence analysis. Four Gateway compatible expression vectors were used: (i) pCPP3234, a derivative of the broad-host-range vector pVLT35 that employs a tac promoter to express insert genes and generates hybrid proteins with a C-terminal Cya fusion for TTSS translocation studies (Schechter *et al*., 2004); (ii) pCPP5040, a derivative of the broad-host-range vector pML123 (Labes *et al*., 1990), which expresses insert genes from the *nptII* promoter, and generates protein products with a C-terminal HA tag for expression in *Pseudomonas* spp.; (iii) pQuiet2, a PVX amplicon with PVX in the min-binary vector pCB302-3 for transient expression of transgenes in plants (He *et al*., 2004); and (iv) pET-DEST42, which allows for T7-regulated expression of insert genes and production of proteins with a C-terminal His_6-V5 epitope tag for purification from *E. coli* (Invitrogen).

Construction of the *Pst* DC3000 *hopPtoN* mutant was carried out by PCR amplifying an internal 660-bp sequence of *hopPtoN*, corresponding to nucleotides 100–760 of the coding sequence, from the DC3000 genome and cloning into pKnockout-Ω (Windgassen *et al*., 2000). The resultant plasmid was transferred into DC3000 via conjugation. As pKnockout cannot replicate in DC3000, single crossover integrants were selected by resistance to spectinomycin. Orientation of integration was determined by Southern blot analysis. The site-directed mutants of *hopPtoN* were made in pCPP5060 using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) and then transferred via Gateway cloning reactions into expression vectors pCPP5040 and pET-DEST42 to produced the plasmids listed in Table 1. All plasmids were confirmed by sequencing.

Bacterial inoculations

All tomato (*L. esculentum* cv. Money Maker) plants used in this study were between 3 and 4 weeks old, and all *N. benthamiana* and *N. tabacum* cultivar Xanthi were between 4 and 6 weeks old and were grown in greenhouse conditions Some disease assays with tomato plants were performed by dipping the plants for 30 s into a 10 mM $MgCl_2$ solution containing 0.02% Silwet and the bacterial inoculum. All other plant assays were performed by infiltrating a bacterial suspension into plant leaves using a blunt syringe. The areas of infiltration were marked lightly on the back of leaves. Levels of bacterial inoculum differed between experiments and are noted in the figures or legends. Bacterial levels *in planta* were determined by cutting leaf disks with a boring tool (inner diameter 0.7 cm) and placing the plant material in 500 μl of 10 mM $MgCl_2$. The disks were completely homogenized and the resulting suspension, containing the bacteria, was diluted and plated on KB plates with rifampicin (50 μg ml^{-1}) and cycloheximide (2 μg ml^{-1}).

PVX inoculations

A. tumefaciens strain GV3101 carrying pQuiet2, a PVX amplicon with the Gateway™ cassette (kindly provided by Gregory B. Martin) and pQuiet2::*hopPtoN* were grown overnight in 250 ml of LB with appropriate antibiotics. Cells were harvested by centrifugation at 7000 *g* for 10 min and re-suspended in 30 ml of 10 mM $MgCl_2$, 150 μM acetosyringone to a final OD600 of 0.3. The bacterial suspension was infil-

Table 1. Strains and plasmids.

Strain or plasmid	Genotype or relevant phenotype	Source or reference
E. coli		
DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ*lacZYA-argF*)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻	Invitrogen
P. syringae pv. *tomato*		
DC3000	Wild type, Rif^r	Cuppels, 1986
CUCPB5114	DC3000 Δ*hrpK-hrpR*::ΩCm, Rif^r Cm^r	Fouts *et al*., 2003
CUCPB5388	DC3000 *hopPtoN*::pKnockout, Sp^r Str^r	This study
P. fluorescens 55	Wild type, Nx^r	Huang *et al*., 1988
P. syringae pv. *tabaci* 11528	Wild type, Nx^r	American Type Culture Collection
Agrobacterium tumefaciens GV3101 (pMP90)	Carries Vir plasmid encoding T-DNA transfer machinery, Rif^r, Gm^r	American Type Culture Collection 33970
Plasmids		
pDONR201	Entry vector of Gateway™ cloning, Km^r,Cm^r	Invitrogen
pCPP5060	pDONR201 containing *shcN-hopPtoN*, Km^r	This study
pCPP5061	pDONR201 containing *hopPtoN*, Km^r	This study
pCPP3234	pVLT35::Gateway™ cassette-Cya fusion, broad-host-range vector containing *tac* promoter and *lacI*^{q}, Sp^r, Str^r, Cm^r	Schechter *et al*., 2004
pCPP3238	pCPP3234 expressing HopPtoN-Cya, Sp^r,Str^r	This study
pCPP3239	pCPP3234 expressing ShcN-HopPtoN-Cya, Sp^r,Str^r	This study
pKnockout-Ω	Sm^r Sp^r mob in pBluescript IISK(–) with modified MCS	Windgassen *et al*., 2000
pCPP5040	pML123::Gateway™ cassette, broad-host-range vector allowing for constitutive expression from *nptII* promoter and C-terminal HA tag, Gm^r, Cm^r	This study
pCPP5071	pCPP5040 expressing ShcN-HopPtoN-HA tag, Gm^r	This study
pQuiet2	PVX amplicon with Gateway™ cassette, Km^r	He *et al*., 2004
pCPP3216	pQuiet2 expressing HopPtoN	This study
pLN18	pLAFR3 derivative containing 25 kb *P. syringae* pv. *syringae* 61 *hrc/hrp* cluster with *shcA* and *hopPsyA* replaced by an *nptII* cassette, Tc^r Km^r	Jamir *et al*., 2004
pCPP5142	pCPP5060 derivative with *shcN-hopPtoN* C172S, Km^r	This study
pCPP5143	pCPP5060 derivative with *shcN-hopPtoN* H283A, Km^r	This study
pCPP5144	pCPP5060 derivative with *shcN-hopPtoN* D299A, Km^r	This study
pCPP5171	pCPP5040 derivative with *shcN-hopPtoN* C172S and C-terminal HA tag, Gm^r	This study
pCPP5172	pCPP5040 derivative with *shcN-hopPtoN* H283A and C-terminal HA tag, Gm^r	This study
pCPP5173	pCPP5040 derivative with *shcN-hopPtoN* D299A and C-terminal HA tag, Gm^r	This study
pET-DEST42	pET Gateway compatible vector allowing for T7-regulated expression of a protein with a C-terminal His_6-V5 tag, Amp^r	Invitrogen
pCPP3390	pET-DEST42 expressing ShcN-HopPtoN-His_6-V5 tag. Amp^r	This study
pCPP3391	pET-DEST42 expressing ShcN-HopPtoN C172S-His_6-V5 tag. Amp^r	This study
pCPP3392	pET-DEST42 expressing ShcN-HopPtoN H283A-His_6-V5 tag. Amp^r	This study
pCPP3393	pET-DEST42 expressing ShcN-HopPtoN D299A-His_6-V5 tag. Amp^r	This study

trated with a blunt syringe into two lower leaves of two-week-old tomato or four-week-old *N. benthamiana* plants. Challenge inoculations with *P. syringae* strains were carried out four weeks later, when typical leaf-crinkling symptoms of PVX infection were seen systemically in *N. benthamiana*.

Plant cAMP and electrolyte leakage assays

Cya assays were performed as previously described (Schechter *et al*., 2004). Briefly, tomato plants were inoculated via a blunt syringe with *Pst* strains containing plasmids expressing Cya-fusions. Plant tissue was sampled 8 h later using a boring tool with a 0.7 cm inner diameter, placed in 300 μl of 0.1 M HCl, and ground into a fine suspension. Samples were incubated at –20°C overnight, and cAMP levels were determined using the Correlate-EIA cAMP immunoassay kit according to the manufacturer's directions (Assay Designs). Electrolyte leakage assays were initiated by removing four leaf samples using a boring tool with an inner diameter of 0.7 cm. Samples were placed into a glass test tube with 10 ml of distilled water and a vacuum was pulled 5 times using a 30-ml syringe connected to the tube by means of a rubber stopper with a hole for the tip of the syringe. The tubes were then shaken for 1 h (200 r.p.m) at room temperature. Plant material was removed and conductivity was measured using a YSI Model 3100 Conductivity Instrument with a YSI 3252 probe (YSI Incorporated, Yellow Springs, OH).

Cysteine protease assays

E. coli cultures containing plasmids expressing HopPtoN-His_6, wild type or mutant, were grown at 25°C to an OD_{600} of 0.6 and induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h. Cells were harvested by centrifugation at 4000 *g* for 20 min, and the pellets were frozen overnight at –80°C. Native HopPtoN was purified per protocols 9 and 10 in the QIAexpressionist handbook (Qiagen). Protease activity was assayed as described (Twining, 1984), with minor modifications using resorufin-labeled casein (Roche) as a substrate. Briefly, lyophilized samples of papain, HopPtoN, or the mutant versions of HopPtoN, were dissolved in assay buffer (0.1 M sodium phosphate pH 7.0, 10 mM EDTA,

10 mM cysteine, 0.01% Brij-35 detergent) with 5 μM E64 (Sigma, St Louis, MO, USA) to a final volume of 80 μl. (Preliminary tests in the pH range of 6.5–9.0 indicated that pH 7.0 was optimal.) The assay mixture was incubated for 1 h at 37°C, then the reaction was stopped by treatment with 150 μl of 10% trichloroacetic acid for 10 min at 37°C. Samples were centrifuged, and the A_{574} of 200 μl of supernatant plus 300 μl of 0.5 M Tris-HCl pH 8.8 was determined.

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