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.

**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 PXV 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.

**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 (Collier *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*<sub>puno</sub>, 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 parologue 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_ conserved) 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.

**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, CUCPB5338. Tomato plants were inoculated with _HopPtoN_ and monitored for the formation of the necrotic lesions that are characteristic of bacterial speck. Unexpectedly, CUCPB5338 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.
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 CUCPB5388, 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 $8 \times 10^6$ cfu ml$^{-1}$ or higher, but at the threshold level of $4 \times 10^6$ 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
P. syringae HopPtoN cysteine protease TTSS effector

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 *Nicotiana 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$_2$O. The excised disks were shaken at room temperature for 1 h, and electrolyte leakage was measured by recording the conductivity of the H$_2$O 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 x 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 x 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.
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 Yop/T 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 carry 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 HopPtoN M 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-His6 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-His6 and papain were both able to cleave the resorufin-label from casein. However, we...
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

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**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$_2$O and shaken at room temperature for 1 h. Electrolyte leakage was measured by recording the conductivity of the H$_2$O after removal of the leaf disks.

**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, mutageneration 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 NtMEK220, 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. tumefaciens 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 polymerase 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 (Scheckter 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 His6-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 produce 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 MgCl₂ 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 MgCl₂. 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 resuspended in 30 ml of 10 mM MgCl₂, 150 μM acetylsyringone to a final OD600 of 0.3. The bacterial suspension was infiltrated into tomato 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 MgCl₂. 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).
treated 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-His₆, wild type or mutant, were grown at 25°C to an OD₆₀₀ 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, 0.1 M NaCl).

10 mM cysteine, 0.01% Brij-35 detergent) with 5 μM E64 (Sigma, St Louis, MO, USA) to a final volume of 80 μl. Pre-
liminary 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$_{754}$ of 200 μl of supernatant plus 300 μl
of 0.5 M Tris–HCl pH 8.8 was determined.

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