**Pseudomonas syringae** pv. *tomato* DC3000 HopPtoM (CEL ORF3) is important for lesion formation but not growth in tomato and is secreted and translocated by the Hrp type III secretion system in a chaperone-dependent manner

Jorge L. Badel, Kinya Nomura, Sruti Bandyopadhyay, Rena Shimizu, Alan Collmer, and Sheng Yang He

1Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA.
2Department of Energy Plant Research Laboratory and Department of Plant Biology and 3Molecular and Cellular Biology Graduate Program, Michigan State University, East Lansing, MI 48824, USA.

**Summary**

*Pseudomonas syringae* pv. *tomato* DC3000 is a pathogen of tomato and *Arabidopsis* that injects virulence effector proteins into host cells via a type III secretion system (TTSS). TTSS-deficient mutants have a Hrp phenotype, that is, they cannot elicit the hypersensitive response (HR) in non-host plants or pathogenesis in host plants. Mutations in effector genes typically have weak virulence phenotypes (apparently due to redundancy), but deletion of six open reading frames (ORF) in the DC3000 conserved effector locus (CEL) reduces parasitic growth and abolishes disease symptoms without affecting function of the TTSS. The inability of the ΔCEL mutant to cause disease symptoms in tomato was restored by a clone expressing two of the six ORF that had been deleted: CEL ORF3 (HopPtoM) and ORF4 (ShcM). A Δ*hopPtoM:nptII* mutant was constructed and found to grow like the wild type in tomato but to be strongly reduced in its production of necrotic lesion symptoms. HopPtoM expression in DC3000 was activated by the HrpL alternative sigma factor, and the protein was secreted by the Hrp TTSS in culture and translocated into *Arabidopsis* cells by the Hrp TTSS during infection. Secretion and translocation were dependent on ShcM, which was neither secreted nor translocated but, like typical TTSS chaperones, could be shown to interact with HopPtoM, its cognate effector, in yeast two-hybrid experiments. Thus, HopPtoM is a type III effector that, among known plant pathogen effectors, is unusual in making a major contribution to the elicitation of lesion symptoms but not growth in host tomato leaves.

**Introduction**

The ability of the plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* to grow in the intercellular spaces and to cause disease in its host plants, tomato and *Arabidopsis*, and to elicit the hypersensitive response (HR) in non-host plants, such as tobacco, is dependent on the function of *hrp* (HR and pathogenicity) and *hrc* (hrp conserved) genes that encode a type III secretion system (TTSS) (Alfano and Collmer, 1997; Lindgren, 1997; He, 1998; Jin et al., 2003). Genes encoding the TTSS have been found in many Gram-negative bacteria including animal pathogens of the genera *Yersinia*, *Shigella*, *Salmonella* and *Escherichia* as well as in plant pathogens of the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Pantoea* and *Ralstonia* (He, 1998; Hueck, 1998; Galán and Collmer, 1999; Cornelis and Van Gijssegem, 2000; Frederick et al., 2001; Buttner and Bonas, 2002). The proteins encoded by these genes form a secretion apparatus that bacteria use to translocate effectors directly into eukaryotic cells where they modulate the host defence response and/or modify host metabolism favouring parasitism and pathogenesis (Kjemtrup et al., 2000; Cornelis, 2002). However, in plant pathogens some effectors delivered by the system are recognized in a gene-for-gene dependent manner by resistance proteins present in non-host plants and in resistant cultivars of the host resulting in the elicitation of the HR (Keen, 1990). The HR is a defence-associated programmed cell death at the site of attempted infection. Because the recognition of some effectors results in an avirulence phenotype, these effectors are referred to as avirulence (Avr) proteins (Leach and White, 1996; White et al., 2000; Collmer et al., 2001). Other proteins that travel the Hrp pathway appear to be accessory...
proteins that help in effector delivery, these include the Hrp plus structural protein HrpA (Roine et al., 1997; Jin and He, 2001; Li et al., 2002; He and Jin, 2003) and possibly harpins (Wei et al., 1992; He et al., 1993; Preston et al., 1995; Kim and Beer, 1998; Charkowski et al., 1998). Mutations in hrp/hrc genes generally abolish virulence. In contrast, mutations in most P. syringae effector genes have only subtle effects that are difficult to observe macroscopically using traditional virulence assays or colony counting methods, suggesting effector functional subtlety and/or redundancy. Indeed, functional and bioinformatic analyses of the genome of P. syringae pv. tomato DC3000 (GenBank AE016853, AE016854, AE016855, http://pseudomonas-syringae.org) indicates that the bacterium produces over 30 effectors (Collmer et al., 2002; Fouts et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002; Greenberg and Vinatzer, 2003). Many of these effectors have been shown to be secreted by the Hrp pathway and, hence are referred to as Hops (Hrp outer proteins) (Alfano and Collmer, 1997).

Secretion and/or translocation of some TTSS effectors in several animal pathogens is dependent on the function of customized chaperones. These type III chaperones appear to prevent the premature aggregation of effectors in the cytoplasm, to maintain effectors in a state competent for type III secretion, and to prevent the cytoplasmic proteolysis of effectors (Cornelis, 2002). Type III chaperones have most commonly been identified based on their small size, acidic isoelectric point, predicted α-helical secondary structure at the C-terminus, and the close linkage of their encoding gene with the effector gene (Cornelis, 2002). The involvement of chaperones in the Hrp TTSS of P. syringae has been demonstrated with the P. syringae pv. syringae 61 effector HopPsyA (van Dijk et al., 2002). Specifically, by heterologous expression in P. fluorescens of pHIR11 and a pHIR11 derivative lacking shcA, the P. syringae pv. syringae 61 ShcA chaperone was shown to be required for secretion of HopPsyA and for efficient elicitation of the HR in tobacco leaves. ShcA bound to a binding site located within the N-terminal 166 amino acids of HopPsyA (van Dijk et al., 2002).

Genes encoding the P. syringae Hrp secretion system are clustered on the core of a pathogenicity island (Pai) with a tripartite structure flanked by two loci that encode putative effector proteins (Alfano et al., 2000). An exchangeable effector locus (EEL), whose number of ORFs and their nucleotide sequences vary between closely related strains of the same pathovar, is located downstream of hrpK. A conserved effector locus (CEL) encoding at least seven open reading frames (ORF) that are conserved between the divergent strains P. syringae pv. syringae B728a and P. syringae pv. tomato DC3000 is located upstream of hrpR. Deletion of the P. syringae pv. tomato DC3000 EEL slightly reduces bacterial growth in tomato, whereas deletion of avrE, ORF2, ORF3, ORF4, hrpW and hopPtoA1 of the CEL strongly reduces bacterial multiplication and abolishes symptom production in tomato (Alfano et al., 2000). However, single mutations in avrE (Lorang and Keen, 1995), hrpW (Charkowski et al., 1998) and hopPtoA1 (Badel et al., 2002) do not abolish pathogenicity, consistent with the idea of functional subtlety and/or redundancy of effectors in P. syringae pv. tomato DC3000. Moreover, we recently showed that hopPtoA1 and its paralog hopPtoA2, located in an apparent pathogenicity island unlinked to the P. syringae DC3000 Hop Pai, contribute redundantly to the efficient formation of bacterial colonies in Arabidopsis, but their mutations do not result in a macroscopically observable reduction of disease symptoms or bacterial growth in planta (Badel et al., 2002). Boch et al. (2002) showed that expression of CEL ORF3 is induced in planta and in Escherichia coli cells expressing the alternative sigma factor HrpL, but neither induction in P. syringae pv. tomato DC3000 nor its role in virulence and bacterial growth in planta were tested. Intriguingly, CEL ORF3 was not detected in a separate study using genome-wide miniTn5sgus reporter mutagenesis and microarray analysis of HrpL-inducible genes (Fouts et al., 2002).

Here, we used complementation analysis of the P. syringae DC3000 CEL deletion and mutational analysis of the wild-type strain to demonstrate that CEL ORF3 is responsible for most of the loss of the disease symptoms in the P. syringae pv. tomato CEL mutant in tomato. We also demonstrate that expression of CEL ORF3 is HrpL-activated in P. syringae pv. tomato DC3000, that the CEL ORF3 protein is a Hop that is secreted in culture and translocated into the plant cell by the TTSS and that translocation is dependent on a chaperone encoded by CEL ORF4. Accordingly, we designated these proteins HopPtoM (CEL ORF3) and ShcM (CEL ORF4).

**Results**

The P. syringae pv. tomato DC3000 CEL deletion mutant can be restored to produce disease symptoms by a fragment containing ORF3 and ORF4

In our initial characterization of the P. syringae pv. tomato DC3000 Hop Pai, we generated a large deletion of the CEL locus and demonstrated its requirement for bacterial virulence (Alfano et al., 2000). Because the region deleted contains several putative effector genes, we conducted complementation analysis to identify key players in the CEL phenotype. We introduced plasmids carrying fragments of different sizes into the P. syringae pv. tomato CEL mutant and tested for restoration of disease symptoms. The smallest fragment tested that could comple-
ment the CEL mutation contained a functional copy of ORF43 (Fig. 1). Inoculation of tomato leaves with the CEL mutant complemented with ORF3 alone resulted in infrequent chlorosis (data not shown). Bacterial multiplication assays revealed that ORF43 restored bacterial speck lesions without a significant increase in the multiplication of the CEL mutant in tomato (Fig. 2).

**A P. syringae pv. tomato DC3000 ORF3 (hopPtoM) deletion mutant is strongly reduced in its ability to elicit lesions in host tomato leaves**

The adjacent location of the large ORF3 (712 amino acids) and the small ORF4 (164 amino acids) in the same operon is suggestive of an effector-chaperone relationship. To test the hypothesis that ORF3 (hopPtoM) is an effector gene that plays an important role in the ability of *P. syringae* pv. *tomato* to elicit lesions in host tomato leaves, we deleted most of the hopPtoM coding sequence by marker exchange with a terminatorless *nptII* cassette and tested the mutant strain, CUCPB5368, for its ability to grow and produce symptoms in tomato leaves. The mutant strain caused a strong reduction in bacterial speck symptoms, as indicated by the size and frequency of the necrotic lesions, in tomato cv. Money Maker leaves 4 days after syringe infiltration with 10^4 cfu ml^-1 (Fig. 3A), but no reduction in growth of the hopPtoM mutant in planta was observed compared to the wild-type strain (Fig. 3B). The defect in causing disease symptoms in the hopPtoM mutant was restored by complementation of the mutation with the same plasmid carrying CEL ORF43 that restored the ability of the *P. syringae* pv. *tomato* CEL mutant CUCPB5115 to cause disease symptoms.

To determine whether the hopPtoM mutation would have a similar phenotype if the test bacteria were inoculated differently or the infection was monitored longer, we inoculated tomato cv. Money Maker by dipping and then monitored bacterial growth and the number and appearance of the lesions 4 days after inoculation. Plus signs indicate typical disease symptoms: extensive water-soaking followed by necrosis and chlorosis. Genes of the CEL locus are indicated with large white boxes. Arrows indicate direction of transcription, with small black boxes denoting Hrp boxes. The dotted fragment in the large black bar below the physical map indicates the region deleted in the ΔCEL mutant. Grey bars represent the length of the fragment used for complementation.

**Fig. 1.** Schematic representation of the complementation of the *P. syringae* pv. *tomato* ΔCEL mutant in tomato. Tomato plants were syringe infiltrated with 10^6 cfu ml^-1 of ΔCEL mutant CUCPB5115 carrying different cloned regions of the CEL. Plants were scored for symptom development 4 days after inoculation. Plus signs indicate typical disease symptoms: extensive water-soaking followed by necrosis and chlorosis. Genes of the CEL locus are indicated with large white boxes. Arrows indicate direction of transcription, with small black boxes denoting Hrp boxes. The dotted fragment in the large black bar below the physical map indicates the region deleted in the ΔCEL mutant. Grey bars represent the length of the fragment used for complementation.

**Fig. 2.** Restoration of the ability to elicit disease symptoms in tomato to the *P. syringae* pv. *tomato* ΔCEL mutant by CEL ORF3 and ORF4.

A. Symptoms in tomato cv. Money Maker leaves 4 days after syringe infiltration with 10^6 cfu ml^-1 of DC3000 (leaf 1), ΔCEL mutant CUCPB5115 (leaf 2) and CUCPB5115(pORF43) (leaf 3).

B. Bacterial multiplication of DC3000 (●), ΔCEL mutant CUCPB5115 (■) and CUCPB5115(pORF43) (▲). Each time point reflects the mean bacterial population recovered from three 0.5-cm-diameter leaf discs. Vertical lines indicate standard errors.
of lesions for 11–14 days (Fig. 4). We found that DC3000, CUCPB5368, and CUCPB5368(pORF43) populations increased for the first 3–6 days and then declined (Fig. 4A). By day 5 after inoculation, lesions ≥0.5 mm² were visible on leaves inoculated with DC3000 and CUCPB5368(pORF43), but in contrast, leaves inoculated with CUCPB5368 were virtually free of lesions (Fig. 4B–C). We also noted that pORF43 did not fully restore the lesion-eliciting activity of CUCPB5368 to the level of wild-type DC3000. The discrepancy increased over time, which would be consistent with the loss of pORF43 without antibiotic selection in planta (Fig. 4B–C). We also noted that pORF43 did not fully restore the lesion-eliciting activity of CUCPB5368 to the level of wild-type DC3000. The discrepancy increased over time, which would be consistent with the loss of pORF43 without antibiotic selection in planta (Fig. 4B). Furthermore, the lesions elicited by DC3000 grew larger and some coalesced, in contrast to those elicited by CUCPB5368(pORF43) (Fig. 4C). Finally, we observed that CUCPB5368 was similarly reduced in its ability to produce lesions in the leaves of hosts tomato cv. Rutgers, that increasing the copy number of hopPtoM through introduction of pORF43 into wild-type DC3000 did not result in enhanced growth or elicitation of lesions in tomato and that CUCPB5368 retained its ability to elicit the HR when infiltrated into leaves of non-host tobacco (data not shown).

Expression of hopPtoM can be activated by HrpL in P. syringae pv. tomato

Because the hopPtoM coding sequence is preceded by a Hrp box-like sequence, we decided to test the activity of...
its promoter region in response to the alternative sigma factor HrpL. We constructed a single-copy, transcriptional fusion of hopPtoM with uidA in the P. syringae pv. tomato DC3000 hrp/hrc mutant to obtain strain CUCPB5369 and expressed hrpL constitutively under the control of the nptII promoter in plasmid pCPP5032. We also introduced into CUPB5369 the plasmid pCPP5031, which carries only the nptII promoter in the same vector backbone and used the resulting strain as a HrpL– control (Fouts et al., 2002). We cultured CUCPB5369(pCPP5031) and CUCPB5369(pCPP5032) in liquid AB-citrate medium (Chilton et al., 1974) and measured GUS activity. The GUS activity of CUCPB5369, carrying a hopPtoM::uidA fusion, was threefold higher in the presence of pCPP5032 than in the presence of pCPP5031 (Fig. 5), demonstrating that expression of hopPtoM is activated by HrpL in a P. syringae pv. tomato genetic background.

HopPtoM, but not ShcM, is secreted in culture by the Hrp system

Our experiments showed that inoculation of tomato leaves with the CEL mutant complemented with a functional copy of hopPtoM resulted in infrequent chlorosis, whereas complementation with both hopPtoM and shcM resulted in disease symptoms in all cases. However, deletion of hopPtoM in P. syringae pv. tomato causes a strong reduction in disease symptoms. These observations prompted us to investigate whether HopPtoM and ShcM are secreted through the Hrp secretion system. We introduced pORF43 into the wild-type DC3000 and a hrcC mutant strain (formerly hrpH, defective in type III secretion; Yuan and He, 1996) and tested for secretion of HopPtoM and ShcM in both Luria–Bertani and hrp-inducing liquid media. Both HopPtoM and ShcM proteins were observed in the cell-bound fractions of all strains tested, except the CEL mutant, grown in hrp-inducing minimal medium but not when bacteria were grown in LB medium, suggesting that hopPtoM and shcM are induced under conditions that favour hrp gene expression (Fig. 6). As expected, the protein bands observed were stronger in all strains expressing in trans hopPtoM and shcM under the control of their native promoter. However, only HopPtoM was detected in the supernatant fractions of the wild-type grown in hrp-inducing minimal medium. HopPtoM was not detected in the supernatant fractions of the hrcC mutant grown in minimal medium, indicating that its secretion is Hrp-dependent. Furthermore, a periplasmic marker protein, β-lactamase, was found only in the cell fraction (Fig. 6B), suggesting no non-specific leakage.

To examine the possibility that the small ShcM protein

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**Fig. 5.** Induction of hopPtoM expression by HrpL. P. syringae pv. tomato Δhrp/hrc mutant CUCPB5114 and Δhrp/hrc hopPtoM::uidA1 mutant CUCPB5369 carrying pCPP5031 (HrpL−, black bars) or pCPP5032 (HrpL+, hatched bars) were grown at 30°C overnight in AB-citrate, adjusted to an optical density at 600 nm of 0.3 and incubated further for 6 h. GUS activity in the culture was determined by the MUG method. Bars indicate the mean of three measurements. Vertical lines indicate standard errors (not visible with black bar).

**Fig. 6.** A. Analysis of type III-dependent secretion of HopPtoM and ShcM. DC3000 (WT) or hrcC mutant derivatives carrying (+) or lacking (−) pORF43 were grown in rich media (LB) or hrp-inducing media (MM). Cultures were separated into supernatant (Sup) and cell (Cell) fractions by centrifugation and the presence of HopPtoM or ShcM in each fraction was detected by immunoblot analysis using an antibody against HopPtoM (α-HopPtoM) or ShcM (α-ShcM). WT: wild-type DC3000. pORF43 expresses hopPtoM and shcM under the native promoter to enhance the expression of both genes in hrp-inducing medium. In the cell fraction of the α-HopPtoM blot, a cross-reacting protein present in all lanes migrated slightly faster than HopPtoM. B. Dependence of HopPtoM secretion on ShcM. ΔCEL mutant and its derivative carrying pORF3, pORF4, or pORF43 were grown in hrp-inducing media (MM). Other conditions were the same as described in (A).

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functions as a chaperone for HopPtoM secretion, we conducted further in vitro secretion assays of HopPtoM in the presence or absence of the shcM gene supplied on a plasmid (Table 1). We found that secretion of HopPtoM required ShcM (Fig. 6B). We observed no significant difference in the level of HopPtoM with or without ShcM, suggesting that ShcM is not required for the stability of HopPtoM (Fig. 6B).

HopPtoM is translocated into plant cells by the Hrp system in a ShcM-dependent manner

We decided to investigate further the requirement of ShcM for the function of HopPtoM during plant interactions by determining the potential of the proteins to be translocated into plant cells. We used the AvrRpt2 reporter system published recently (Mudgett et al., 2000; Guttman and et al., 2000).

Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
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<td>Strains</td>
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<td>Alfano et al. (2000)</td>
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<td>Yuan and He (1996)</td>
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<td>Suicide vector with lacZa carrying sacB, Gm</td>
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Ap: ampicillin; Cm: chloramphenicol; Gm: gentamycin; Km: kanamycin, Nn: nalidixic acid; Tc: tetracycline; Sp: spectinomycin; Sm: streptomycin; Rif, rifampicin.
or in full-length HopPtoM (712 amino acids) and ShcM (164 amino acids) proteins were fused to a truncated AvrRpt2 protein (80–255 amino acids, AvrRpt280–255). Plasmids were introduced into ΔCEL mutant (CUCPB5115), Arabidopsis Col (rps2–) or rps2 mutant leaves were infiltrated with bacterial suspensions at OD600 = 0.2 and evaluated for HR elicitation. 1: CUCPB5115(pAVRRPT2), 2: CUCPB5115(pORF43), 3: CUCPB5115(pORF3::AVRRPT280–255), 4: CUCPB5115(pORF4::AVRRPT280–255), 5: CUCPB5115(pORF4 + pORF3::AVRRPT280–255), 6: CUCPB5115(pORF4::AVRRPT280–255 + pORF3). Col-0 leaves (1 and 5) showing HR collapse appear wrinkled (indicated by white arrows). Top: Number of leaves showing HR/number of leaves infiltrated for Col-0 and rps2 plants. Picture was taken 18 h after inoculation. Leaves representing the majority of each treatment are shown.

Greenberg, 2001. This system is based on the ability of an N-terminally truncated AvrRpt2 (AvrRpt280–255, type III secretion-incompetent but biologically active) to trigger an RPS2-dependent HR in Arabidopsis only when it is fused to a type III secretion/translocation signal present in the N-terminus of a type III effector. We fused full-length HopPtoM (712 amino acids) and ShcM (164 amino acids) proteins to a truncated AvrRpt2 protein (80–255 amino acids, AvrRpt280–255). Immunoblot analysis confirmed the production of HopPtoM::AvrRpt2 and ShcM::AvrRpt2 fusion proteins of expected sizes (data not shown). We then introduced various expression constructs (see Table 1) expressing wild-type AvrRpt2, HopPtoM + ShcM, ShcM::AvrRpt280–255, HopPtoM::AvrRpt280–255, ShcM + HopPtoM::AvrRpt280–255, ShcM::AvrRpt280–255 and HopPtoM into the ΔCEL mutant and inoculated both RPS2 and rps2 mutant (Kunkel et al., 1993) Arabidopsis. In general, no HR developed in rps2 leaves inoculated with any strain or in RPS2 leaves inoculated with bacteria expressing HopPtoM and ShcM. However, a robust AvrRpt2-dependent HR was observed at about 9 h after infiltration for the wild-type AvrRpt2 and 15 h for the HopPtoM::AvrRpt280–255 fusion protein expressed together with ShcM (Fig. 7). Leaves inoculated with HopPtoM::AvrRpt280–255 alone were reduced in turgidity, but no typical HR occurred. ShcM::AvrRpt280–255 alone or when expressed with HopPtoM did not give an HR.

ShcM physically interacts with HopPtoM in the yeast two-hybrid system

Our results from complementation, virulence, secretion and translocation analyses suggested that ShcM may act as a chaperone for HopPtoM. To obtain more conclusive evidence about this possibility, we fused the full-length HopPtoM protein (164 amino acids) to the DNA-binding domain (BD) and the full-length ShcM protein (712 amino acids) to the activation domain (AD) of the LexA-based two-hybrid system and tested for physical interactions in the yeast two-hybrid system. A blue colour developed in positive control yeast strains, in which BD was fused to the murine p53 gene and AD was fused to the SV40 large T-antigen (Fig. 8). No colour developed when yeast strains carried empty AD or BD, regardless of the nature of the other partner. A blue colour developed in strains carrying BD fused to HopPtoM and AD fused to ShcM, demonstrating physical interaction of these proteins in the yeast two-hybrid system.

To determine the portion of HopPtoM that interacts with ShcM, we constructed fusions of the LexA AD with various regions of HopPtoM (HopPtoM1–100, HopPtoM1–200, HopPtoM201–712, HopPtoM301–712 and HopPtoM401–712) and tested for interaction in yeast as described above. Yeast strains carrying HopPtoM1–200, HopPtoM201–712 and

![Fig. 7. Type III translocation analysis of HopPtoM and ShcM in Arabidopsis. Full-length HopPtoM (712 amino acids) and ShcM (164 amino acids) proteins were fused to a truncated AvrRpt2 protein (80–255 amino acids, AvrRpt280–255). Plasmids were introduced into ΔCEL mutant (CUCPB5115), Arabidopsis Col (rps2–) or rps2 mutant leaves were infiltrated with bacterial suspensions at OD600 = 0.2 and evaluated for HR elicitation. 1: CUCPB5115(pAVRRPT2), 2: CUCPB5115(pORF43), 3: CUCPB5115(pORF3::AVRRPT280–255), 4: CUCPB5115(pORF4::AVRRPT280–255), 5: CUCPB5115(pORF4 + pORF3::AVRRPT280–255), 6: CUCPB5115(pORF4::AVRRPT280–255 + pORF3). Col-0 leaves (1 and 5) showing HR collapse appear wrinkled (indicated by white arrows). Top: Number of leaves showing HR/number of leaves infiltrated for Col-0 and rps2 plants. Picture was taken 18 h after inoculation. Leaves representing the majority of each treatment are shown.](image)

![Fig. 8. Physical interaction between HopPtoM and ShcM in the LexA two-hybrid system. Full-length ShcM protein (164 amino acids) was fused to the DNA-binding domain (BD) in pGILDA and a series of truncated HopPtoM proteins were fused to the transcriptional activation domain (AD) in pB42AD. Yeast strains were grown at 30°C for 2 days on galactose X-gal complete minimal medium. A blue colour developed in strains carrying BD fused to HopPtoM and AD fused to ShcM, demonstrating physical interaction of these proteins in the yeast two-hybrid system. To determine the portion of HopPtoM that interacts with ShcM, we constructed fusions of the LexA AD with various regions of HopPtoM (HopPtoM1–100, HopPtoM1–200, HopPtoM201–712, HopPtoM301–712 and HopPtoM401–712) and tested for interaction in yeast as described above. Yeast strains carrying HopPtoM1–200, HopPtoM201–712 and](image)
HopPtoM301–712 turned blue after 2 days of growing in galactose Xgal minimal medium only when ShcM was also present (Fig. 8). In contrast, yeast strains carrying the N-terminal 100 amino acids of HopPtoM and HopPtoM401–712 remained white (Fig. 8). These results suggest that ShcM interacts with several independent regions between residues 100 and 400 of HopPtoM.

Discussion

Effector proteins injected into host cells by the Hrp TTSS are now thought to be key to the pathogenicity of _P. syringae_, but study of the functions of these proteins has been thwarted by the typically weak contributions they individually make to virulence – in contrast to the strong phenotypes typically associated with mutations in _hrp/hrc_ genes encoding the secretion pathway. The strong reduced-virulence phenotype reported by Alfano _et al._ (2000) for a _P. syringae pv. tomato_ DC3000 mutant lacking six ORFs in the Hrp pathogenicity island CEL is exceptional in that regard, and we have shown here that CEL ORF3 is largely responsible for the disease symptom phenotype in tomato. Moreover, we have found that ORF3 (now designated HopPtoM) is secreted in culture in a Hrp-dependent manner and is translocated into plant cells in a manner dependent on CEL ORF4 (ShcM), a chaperone with which HopPtoM physically interacts in yeast two-hybrid tests. Unexpectedly, assays in tomato of the CEL CEL and _Δ hoppingtoM_ mutants and their complemented derivatives consistently indicate that the role of HopPtoM in pathogenesis is in the production of necrotic lesions rather than in pathogen multiplication per se.

The demonstration of the requirement of ShcM for efficient translocation and function of HopPtoM in the plant cell is consistent with the presence of customized chaperones in plant pathogenic bacteria and supports recent findings with _P. syringae pv. syringae_ ShcA and _E. amylovora_ DspB/F (van Dijk _et al._, 2002; Gaudriault _et al._, 2000). Bacterial chaperones have been suggested to maintain their cognate effectors in a state competent for secretion and/or translocation of HopPtoM in the absence of the chaperone (Gaudriault _et al._, 2002). In addition, it has been shown that chaperones are not absolutely required for translocation of some effectors of animal pathogenic bacteria. For instance, deletion of the binding site for the chaperone Syce in the _Yersinia enterocolitica_ YopE effector does not prevent its translocation into the eukaryotic cell (Boyd _et al._, 2000).

The failure of mutations in typical _P. syringae_ effector genes to have a strong virulence phenotype is thought to be the major reason that such genes have not been reported for _P. syringae_ random mutants that have been screened for loss of virulence. The relatively few effectors shown to have virulence phenotypes were largely demonstrated to do so by heterologous expression in weakly virulent strains or pathovars. For example, expression of _avrPto_ in _P. syringae pv. tomato_ T1 enhances bacterial multiplication and bacterial speck symptoms in tomato PtoS or _prf-3_ plants (Chang _et al._, 2000; Shan _et al._, 2000). Similarly, expression of _avrRpt2_ in _P. syringae pv. maculicola_ ES4326 enhances bacterial growth and development of chlorosis in Arabidopsis _rps2_ plants (Chen _et al._, 2000; Gutman and Greenberg, 2001). However, expression of effectors, such as AvrPto, in more virulent strains, such as _P. syringae pv. tomato_ DC3000 or PT23 is not associated with changes in virulence phenotypes (Shan _et al._, 2000). Alternatively, VirPPhA and AvrPphF represent effectors whose contribution to virulence appears to result from their ability to block the HR-eliciting activity of other effectors in certain host plant genotypes (Jackson _et al._, 1999; Tsiamis _et al._, 2000). So far only _avrA_ and _avrE_ in _P. syringae pv. tomato_ PT23 (Lorang _et al._, 1994) and _avrRpm1_ in _P. syringae pv. maculicola_ Psm M2 (Ritter and Dangl, 1995) have been shown to contribute visibly to the symptoms and growth of native strains in the absence of other mutations.

The strong reduction in symptoms caused by CUCPB5368 in tomato is thus notable, and the lack of an associated reduction in growth argues against a model for...
P. syringae pathogenesis in which the Hrp system and effectors primarily promote parasitic growth, while toxins and other factors are largely responsible for visible symptoms. Such a model had been suggested by three sets of observations. First, most P. syringae strains produce phytoxins, which elicit an array of symptoms, but toxin-deficient mutants generally retain basic pathogenicity (Bender et al., 1999). Second, the gacS and salA regulatory gene mutations in P. syringae pv. syringae B728a strongly reduce lesion formation without a commensurate reduction in pathogen growth in bean (Willis et al., 1999). Third, compatible interactions of P. syringae and plants, which lead to disease, are characterized by the lack of any host necrosis during a prolonged period of bacterial multiplication (Klement et al., 1964).

Despite indications that the Hrp system has a primary role in promoting bacterial parasitic growth, experiments involving transient or transgenic expression in planta suggest that many effectors may have toxic effects on host plants (that lack known cognate R genes) (Kjemtrup et al., 2000), and our results show that HopPtoM is a major factor in the elicitation of the necrotic lesions that are characteristic of bacterial speck disease in tomato. Interestingly, preliminary analysis suggests that transgenic expression of hopPtoM in Arabidopsis plants causes necrosis (S.B. and S.Y. H., unpubl. results). Thus, expression of

Recombinant DNA techniques

DNA manipulations and polymerase chain reaction (PCR) were performed using standard protocols (Sambrook et al., 1989; Innis et al., 1990). Oligonucleotide primers for sequencing or PCR were purchased from Integrated DNA Technology. PCR was performed with Vent or Deep Vent polymerases (New England Biolabs) or HiFi polymerase (Stratagene, Madison, WI). DNA sequencing was done at the Cornell Biotechnology Center or the Michigan State University Genomic Technology Support Facility with Automated DNA sequencers, model 373 A (Applied Biosystems). DNA sequences were analysed with the DNASTAR software package. Database searches were performed using gapped BLASTN, BLASTP, and BLASTX (Altschul et al., 1997) (internet address: http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of complementation plasmids

Various fragments within the CEL were either subcloned from pCPP3016 (for pCPP2435, pCPP2373 and pCPP2375) or amplified by PCR (for pORF43, pORF3 and pORF4) and cloned into appropriate vectors as described in Table 1. The following primers were used to amplify ORF43 (for construction of pORF43): sense primer, 5'-GTGAATTCCGATGCACTCGAAGGCACGAGCCTTTCGG-3' (EcoRI site underlined) and antisense primer, 5'-CGAGATCCTTTAAGTTTAAACAGCATGTTG-3' (BanHI site underlined); ORF4 (for construction of pORF4): sense primer, 5'-GTGAATTCTGCTAAGTGGGCACGAGCCTTTCGG-3' (EcoRI site underlined) and antisense primer, 5'-CGGATCCGATGCACTCGAAGGCACGAGCCTTTCGG-3' (BanHI site underlined); and ORF3 (for construction of pORF3): sense primer, 5'-CGGATCCGATGCACTCGAAGGCACGAGCCTTTCGG-3' (BanHI site underlined) and antisense primer, 5'-CTTCTAGATTAAACAGCATGAAAGCATGACGACGAGCCTTTCGG-3' (XbaI underlined). pORF3 also contains the ORF4 promoter upstream of ORF3. The ORF4 promoter was amplified using sense primer 5'-GTGAATTCTGCTAAGTGGGCACGAGCCTTTCGG-3' (EcoRI underlined) and antisense primer 5'-CGGATCCGATGCACTCGAAGGCACGAGCCTTTCGG-3' (XbaI underlined). Plasmids were introduced into bacteria by electroporation.

Construction of the P. syringae pv. tomato hopPtoM marker-exchange mutation

A 2.2 kb fragment containing the 3' end of avrE, avrF and the 3' end of hopPtoM (Fig. 1) was PCR amplified from pCPP3016 with Vent polymerase using primers p0609 (5'-GGCGCAGGAGGTACAGCCCAAC-3'; KpnI site underlined) and p0610 (5'-ACGCGTACGATCCATCGT-3'; XhoI site underlined), and ligated into pBluescript-II SK to make pCPP3271. A HindIII–XhoI fragment carrying a terminatorless nptII cassette from pCPP2988 was ligated into pCPP3271 to obtain pCPP3272. A 2.2 kb fragment carrying the 5' end of hopPtoM, shcM, hopPtoA1 and the 5' end of hrpW was amplified from pCPP2357 using primers p0611 (5'-ACGATCTAACGCTTCTGTTA-3'; HindIII site underlined) and p0612 (5'-GGCGCAGGAGGTACAGCCCAAC-3'; KpnI site underlined). Plasmids were introduced into bacteria by electroporation.

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains, cosmid clones and plasmids used in this study are described in Table 1. E. coli cells were grown in LB (Hirano, 1997), or low-salt (5 g l⁻¹) Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37°C, and P. syringae cells were grown in King's B (King et al., 1954), low-salt LB, or hrp-inducing fructose minimal medium (Hyunh et al., 1989) at 30°C. E. coli DH5α (Life Technologies) was used as host for DNA clones and the pBluescript-II SK plasmid (Stratagene) was used as the vector. Antibiotics were used at the following concentrations (μg ml⁻¹): ampicillin, 100; kanamycin, 50; rifampicin, 50; gentamycin, 10; tetracycline, 10; spectinomycin, 50.
lined) and p0612 (5'-CGGTCGATCATGTCGAG-3'; BamHI site underlined), and cloned into pCPP3272 to obtain pCPP3273. The BamHI–KpnI fragment containing the nptII cassette plus P. syringae pv. tomato DC3000 flanking DNA from pCPP3273 was inserted into pRK415 to construct pCPP3274, and then cloned as a BamHI–SacI fragment into pCPP54 to obtain pCPP3275. The mutated hopPtoM in pCPP3275 was marked exchanged into P. syringae pv. tomato DC3000 to construct strain CUCPB5368. The mutation was confirmed with DNA gel blot analysis as indicated below. For complementation of the hopPtoM mutant, pORF43 was conjugated into CUCPB5368.

Construction of a genomic uidA transcriptional fusion of hopPtoM

A hopPtoM::uidA transcriptional fusion was constructed by amplifying a 2.1 kb fragment containing the first 1016 bp of the hopPtoM coding region and upstream sequences (Fig. 1) from pCPP3016 with Deep Vent polymerase using primers p0889 (5'-GTGCTGCTGTCATGAGATCGGCTCAG-3'; XbaI site underlined) and p0890 (5'-GTGACTCTGAGGACTCTTTG GAGTGGTG-3'; SacI site underlined). A second 2.1 kb fragment including the last 257 bp of the hopPtoM coding region and downstream sequence was amplified using primers p0891 (5'-ATACCGGTGGGCATGAGCTCTACA-3'; Apal site underlined) and p0892 (TACAGAGGCTGACCGGATTATCAG-3'; Xhol site underlined). The fragments were ligated into an uidA::nptII cassette obtained from pCPP2277 digested with Xbal and Xhol and to pJQ200SK digested with Apal and SacI to obtain pCPP3276. pCPP3276 was maker exchanged into the P. syringae pv. tomato hrl/hrp mutant CUCPB5114 to obtain strain CUCPB5369. CUCPB5369 was transformed with pCPP5032, which expresses hrl under the control of the nptII promoter, and with pCPP5031, which is the vector carrying only the nptII promoter.

Secretion assays

Bacteria were grown in low-salt LB broth until OD_{600} = 0.6. Bacteria were collected by centrifugation and resuspended in hrl-inducing fructose minimal medium or hrl-repressing LB and incubated with shaking at 20°C for 12 h. Cultures were separated into cell and supernatant fractions by centrifugation at 15 000 g. The cell and supernatant fractions were concentrated 5 and 50 times respectively. Proteins were separated on SDS–PAGE gels and transferred to Immobilon-P. The cell and supernatant fractions were separated into cell and supernatant fractions by centrifugation. The cell and supernatant fractions were separated into cell and supernatant fractions by centrifugation. The cell and supernatant fractions were separated into cell and supernatant fractions by centrifugation. The cell and supernatant fractions were separated into cell and supernatant fractions by centrifugation.
GUS assays
CUCPB5114(pCPP5031), CUCPB5114(pCPP5032), CUCPB5369(pCPP5031) and CUCPB5369(pCPP5032) were grown in liquid AB-citrate (Chilton et al., 1974) at 30°C overnight. Cultures were diluted to an optical density at 600 nm of 0.3 and grown further for 6 h. Next, 100 μl of liquid culture were used to determine GUS activity by the MUG (4-methylumbelliferyl β-D-glucuronic acid) method (Jefferson, 1987) and a fluorometer Hoefer DyNA Quant 200 (Amersham Pharmacia Biotech Inc.).

Plant assays
Nicotiana tobacco cv. Xanthi, Lycopersicum esculentum cv. Money Maker, and Arabidopsis thaliana plants were grown and inoculated with bacteria as described previously (Gopalan et al., 1996). For virulence assays in tomato, bacterial suspensions containing 10^6 cfu ml^-1 in 10 mM MES (morpholinoethanesulfonic acid) pH 5.5 were infiltrated into plant assmbs containing 10^4 cfu ml^-1 (Gopalan et al., 1996). For virulence assays in tomato, bacteria were grown further for 6 h. Next, 100 μl of liquid culture were used to determine GUS activity by the MUG (4-methylumbelliferyl β-D-glucuronic acid) method (Jefferson, 1987) and a fluorometer Hoefer DyNA Quant 200 (Amersham Pharmacia Biotech Inc.).

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