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# *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

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## 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.**

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## 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 *p*athogenicity) and *hrc* (*hrp c*onserved) 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 Gijsegem, 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 pilus 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 $\alpha$-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. fluorescence* 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 Hrp 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 miniTn*5gμs* 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 Hrp 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-

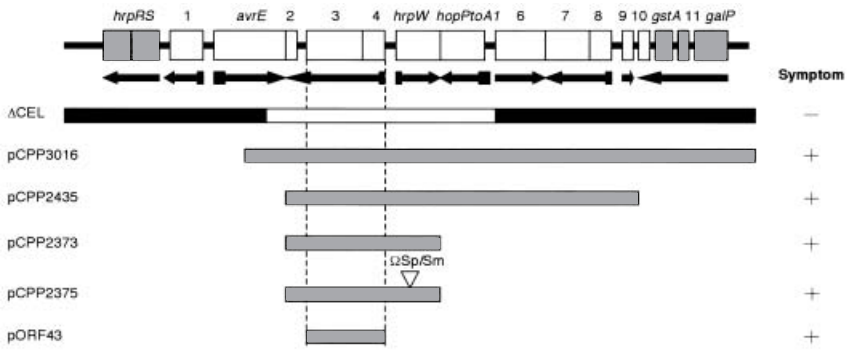


**Fig. 1.** Schematic representation of the complementation of the *P. syringae* pv. *tomato* ΔCEL mutant in tomato. Tomato plants were syringe infiltrated with $10^4$ cfu $ml^{-1}$ of ΔCEL mutant CUCPB5115 carrying different cloned regions of the CEL. Plants were scored for symptom development 4 days 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.

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

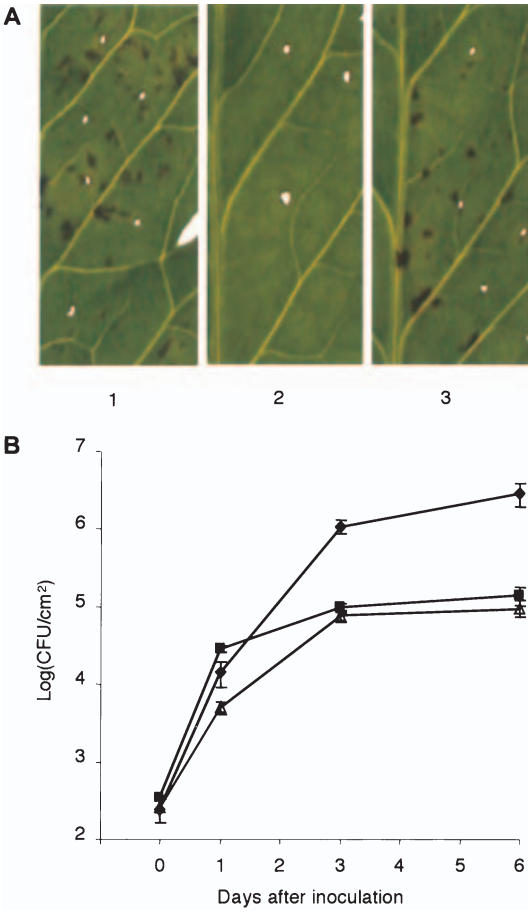


**Fig. 2.** Restoration of the ability to elicit disease symptoms in tomato to the *P. syringae* pv. *tomato* ΔCEL mutant by CEL ORF3 and ORF4.
A. Symptoms in tomato cv. Money Maker leaves 4 days after syringe infiltration with $10^4$ cfu $ml^{-1}$ of DC3000 (leaf 1), ΔCEL mutant CUCPB5115 (leaf 2) and 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.

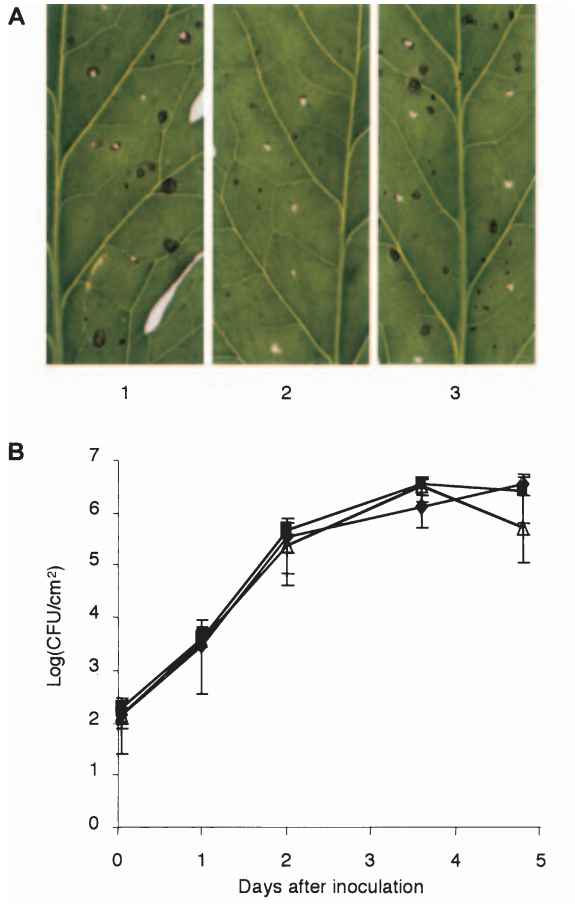


**Fig. 3.** Disease symptoms and growth *in planta* of *P. syringae* pv. *tomato* DC3000 wild-type and Δ*hopPtoM* strains after inoculation by syringe.
A. Symptoms in tomato cv. Money Maker leaves 4 days after syringe infiltration with $10^4$ cfu $ml^{-1}$ of DC3000 (leaf 1), Δ*hopPtoM* mutant CUCPB5368 (leaf 2), and CUCPB5368(pORF43) (leaf 3).
B. Bacterial multiplication in tomato cv. Money Maker of DC3000 (◆), Δ*hopPtoM* mutant CUCPB5368 (■) and CUCPB5368(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(pOFR43) populations increased for the first 3–6 days and then declined (Fig. 4A). By day 5 after inoculation, lesions ≥0.5 $mm^2$ 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). 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

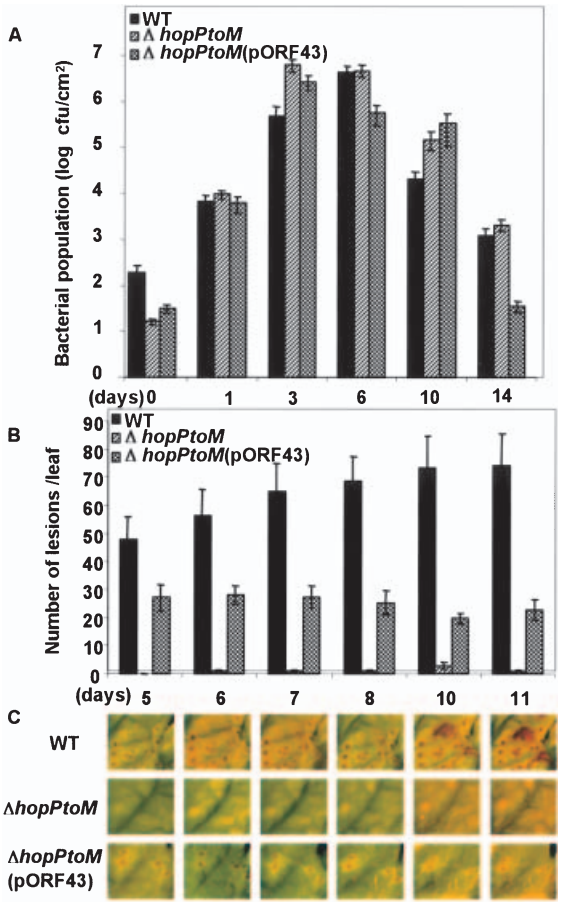


**Fig. 4.** Disease symptoms and growth *in planta* of *P. syringae* pv. *tomato* DC3000 wild-type and Δ*hopPtoM* strains after inoculation by dipping.
A. Bacterial multiplication in tomato cv. Money Maker of DC3000 (WT), CUCPB5368 (Δ*hopPtoM*) and CUCPB5368(pORF43).
B. Number of lesions per leaf appearing on plants beginning 5 days after inoculation with DC3000 (WT), CUCPB5368 (Δ*hopPtoM*) and CUCPB5368(pORF43). Bars indicate mean number and standard error for lesions ≥0.5 $mm^2$ on three whole leaves assayed for each treatment from days 5–11 after inoculation.
C. Development of symptoms on a single representative leaf after inoculation with DC3000 (WT), CUCPB5368 (Δ*hopPtoM*) and CUCPB5368(pORF43). The same area on each leaf was photographed on the indicated days post-inoculation.

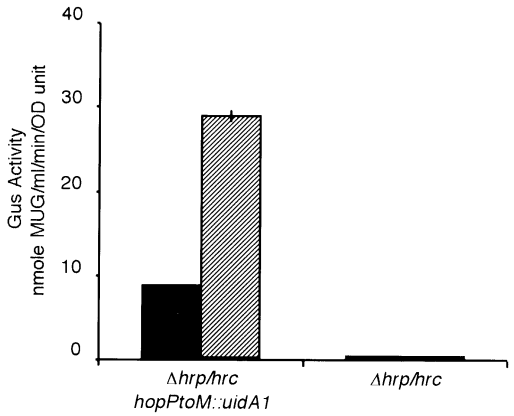


**Fig. 5.** Induction of *hopPtoM* expression by HrpL. *P. syringae* pv. *tomato* Δ*hrp/hrc* mutant CUCPB5114 and Δ*hrp/hrc hopPtoM*::*uidA1* mutant CUCPB5369 carrying pCPP5031 (HrpL$^-$, black bars) or pCPP5032 (HrpL$^+$, hatched bars) were grown at 30°C overnight in AB-citrate, adjusted to an optical 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).

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

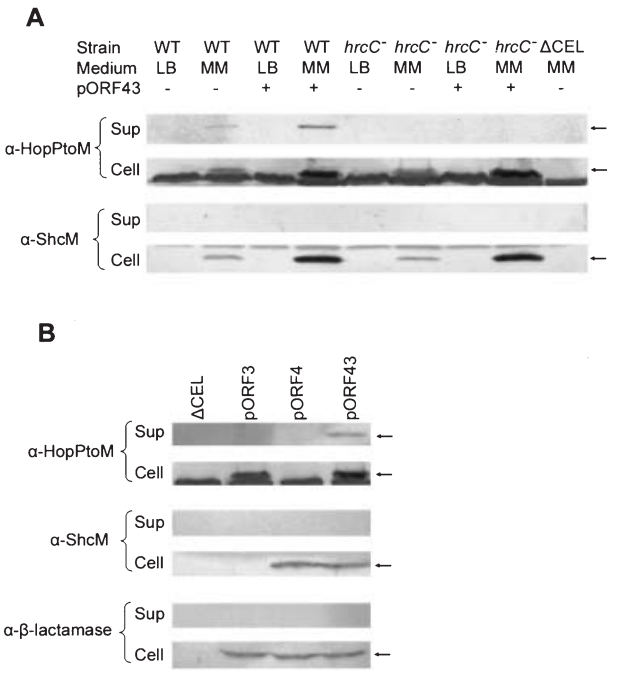


**Fig. 6.** A. Analysis of type III-dependent secretion of HopPtoM and ShcM. DC3000 (WT) or *hrcC* mutant derivatives carrying (+) or lacking (–) pORF43 were grown in rich media (LB) or *hrp*-inducing media (MM). Cultures were separated into supernatant (Sup) and cell (Cell) fractions by centrifugation 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).

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

**Table 1.** Strains and plasmids used in this study.

| Designation | Relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *Escherichia coli* DH5α | *SupE44 ΔlacU169* (f80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nx$^r$ | Life Technologies |
| *Pseudomonas syringae* pv. *tomato* | | |
| DC3000 | Wild type, Rif$^r$ | Cuppels (1986) |
| CUCPB5114 | *Δhrp/hrc*::ΩCm$^r$, Rif$^r$ Cm$^r$ | Fouts *et al*. (2002) |
| CUCPB5115 | *ΔCEL*::ΩSp/Sm$^r$, Rif$^r$ Sp$^r$ | Alfano *et al*. (2000) |
| hrcC mutant | hrcC::miniTn5Cm, Rif$^r$ Cm$^r$ | Yuan and He (1996) |
| CUCPB5368 | *ΔhopPtoM::nptII*, Rif$^r$ Km$^r$ | This study |
| CUCPB5369 | *ΔhopPtoM*::*uidA1*::*nptII*, Rif$^r$ Km$^r$ derivative of CUCPB5114 | This study |
| Plasmids | | |
| pBluescript-II SK or KS+ | ColE1 mcs-*lacZ*, Ap$^r$ | Stratagene |
| pRK415 | Broad-host-range vector unstable in the absence of selection, Tc$^r$ | Keen *et al*. (1988) |
| pUCP19 | *Escherichia–Pseudomonas* shuttle vector with *aacC1* and *lazα,* Gm$^r$ | Schweizer (1991) |
| pCPP54 | pCPP33 derivative carrying *sacB* and *sacR* from pUM24, Tc$^r$ | D. W. Bauer (Cornell University) |
| pJQ200SK | Suicide vector with *lacZα* carrying *sacB*, Gm$^r$ | Quandt and Hynes (1993) |
| pCPP2277 | pBluescript-II SK carrying a *uidA1::nptII* cassette | D. W. Bauer (Cornell University) |
| pCPP2988 | pBluescript-II SK(–) carrying 1.5 kb *Hin*dIII–*Sac*I fragment that contains *nptII* lacking terminator, cloned from pRZ102, Ap$^r$ Km$^r$ | Alfano *et al*. (1996) |
| pCPP3271 | pBluescript II SK carrying 2.2-kb *Kpn*I–*Xho*I fragment that contains the 3′ end of *avrE*, *avrF* and the 3′ –end of *hopPtoM*, Ap$^r$ | This study |
| pCPP3272 | pCPP3271 with *nptII* lacking terminator inserted into the *Hin*dIII–*Xho*I sites, Ap$^r$ Km$^r$ | This study |
| pCPP3273 | pCPP3272 carrying 2.2-kb *Hin*dIII–*Bam*HI fragment that contains the 5′ end of *hopPtoM*, *ShcM*, *hopPtoA1* and the 5′ end of *hrpW*, Ap$^r$ Km$^r$ | This study |
| pCPP3274 | pRK415 carrying *Bam*HI–*Kpn*I fragment from pCPP3273, Tc$^r$ Km$^r$ | This study |
| pCPP3275 | pCPP54 carrying *Bam*HI–*Sac*I fragment from pCPP3274, Tc$^r$ Km$^r$ | This study |
| pCPP3016 | Cosmid clone, pCPP47 carrying the 5′ end of *avrE* to the CEL edge of the *P.* s. *tomato* DC3000 Hrp pai, Tc$^r$ | Alfano *et al*. (2000) |
| pCPP2435 | pBlueScript II SK(+) carrying the 11 kb *Eco*RI fragment from pCPP3016; contains *avrE*, *hrpW*, and *hopPtoA1*, Ap$^r$ | Alfano *et al*. (1996) |
| pCPP5031 | pRK415 carrying *PnptII*, Tc$^r$ | Fouts *et al*. (2002) |
| pCPP5032 | pRK415 carrying *P. s*. pv. *tomato* DC3000 *hrpL* under control of *PnptII*, Tc$^r$ | Fouts *et al*. (2002) |
| pCPP3276 | pJQ200SK carrying *hopPtoM::uidA1::nptII* construct, Gm$^r$ Km$^r$ | This study |
| pORF3 | pUCP19 carrying the *hopPtoM* gene under the control of the *shcM* gene promoter, Ap$^r$ | This study |
| pORF4 | pUCP19 carrying the *shcM* gene under the control of its native promoter, Ap$^r$ | This study |
| pORF43 | pUCP19 carrying the *shcM-hopPtoM* genes with the native *shcM* gene promoter, Ap$^r$ | This study |
| pAVRRPT2 | pUCP19 carrying the *avrRpt2* gene under the control of its native promoter, Ap$^r$ | This study |
| pORF3::AVRRPT2$_{80–255}$ | pUCP19 carrying *hopPtoM*::*avrRpt2*$_{80–255}$; under the control of the *shcM* gene promoter, Ap$^r$ | This study |
| pORF4::AVRRPT2$_{80–255}$ | pUCP19 carrying *shcM*::*avrRpt2*$_{80–255}$; under the control of the *shcM* gene promoter, Ap$^r$ | This study |
| BD empty | pGILDA; yeast two-hybrid vector, Ap$^r$ | Origene Technologies, Inc. |
| AD empty | pB42AD; yeast two-hybrid vector, Ap$^r$ | Clontech Laboratories, Inc. |
| AD::p53 | pB42AD expressing p53; positive control, Ap$^r$ | Clontech Laboratories, Inc. |
| BD::SV40 | pGILDA carrying SV40; positive control, Ap$^r$ | Clontech Laboratories, Inc. |
| AD::ORF4 | pB42AD expressing full-length HopPtoM, Ap$^r$ | This study |
| BD::ORF3 | pGILDA expressing full-length HopPtoM, Ap$^r$ | This study |
| BD::ORF4 | pGILDA expressing full-length ShcM, Ap$^r$ | This study |
| AD::ORF3$_{1–100}$ | pB42AD expressing the first 100 amino acids of HopPtoM, Ap$^r$ | This study |
| AD::ORF3$_{1–200}$ | pB42AD expressing the first 200 amino acids of HopPtoM, Ap$^r$ | This study |
| AD::ORF3$_{201–712}$ | pB42AD expressing residues 201 through 712 of HopPtoM, Ap$^r$ | This study |
| AD::ORF3$_{301–712}$ | pB42AD expressing residues 301 through 712 of HopPtoM, Ap$^r$ | This study |
| AD::ORF3$_{401–712}$ | pB42AD expressing residues 401 through 712 of HopPtoM, Ap$^r$ | This study |

Ap: ampicilin; Cm: chloramphenicol; Gm: gentamycin; Km: kanamycin, Nx: nalidixic acid; Tc: tetracycline; Sp: spectinomycin; Sm: streptomycin; Rif, rifampicin.

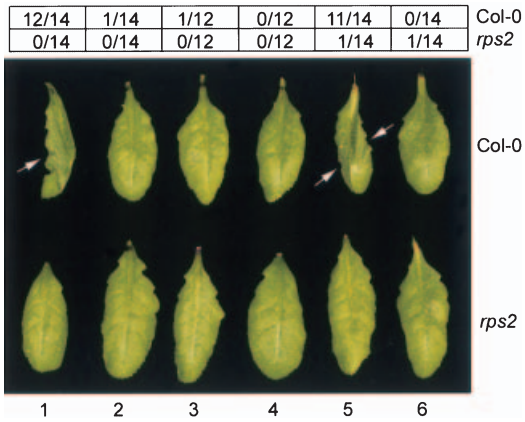


**Fig. 7.** Type III translocation analysis of HopPtoM and ShcM in Arabidopsis. Full-length HopPtoM (712 amino acids) and ShcM (164 amino acids) proteins were fused to a truncated AvrRpt2 protein (80–255 amino acids, AvrRpt2$_{80–255}$). Plasmids were introduced into ΔCEL mutant (CUCPB5115). Arabidopsis Col (*RPS2*+) or *rps2* mutant leaves were infiltrated with bacterial suspensions at $OD_{600}$ = 0.2 and evaluated for HR elicitation. 1: CUCPB5115(pAVRRPT2), 2: CUCPB5115(pORF43), 3: CUCPB5115(pORF3::AVRRPT2$_{80–255}$), 4: CUCPB5115(pORF4::AVRRPT2$_{80–255}$), 5: CUCPB5115(pORF4 + pORF3::AVRRPT2$_{80–255}$), 6: CUCPB5115(pORF4::AVRRPT2$_{80–255}$ + 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 (AvrRpt2$_{80–255}$, 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, AvrRpt2$_{80–255}$). 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::AvrRpt2$_{80–255}$, HopPtoM::AvrRpt2$_{80–255}$, ShcM + HopPtoM::AvrRpt2$_{80–255}$, or ShcM::AvrRpt2$_{80–255}$ + 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::AvrRpt2$_{80–255}$ fusion protein expressed together with ShcM (Fig. 7). Leaves inoculated with HopPtoM:: AvrRpt2$_{80–255}$ alone were reduced in turgidity, but no typical HR occurred. ShcM::AvrRpt2$_{80–255}$ 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 (HopPtoM$_{1–100}$, HopPtoM$_{1–200}$, HopPtoM$_{201–712}$, HopPtoM$_{301–712}$ and HopPtoM$_{401–712}$) and tested for interaction in yeast as described above. Yeast strains carrying HopPtoM$_{1–200}$, HopPtoM$_{201–712}$ and

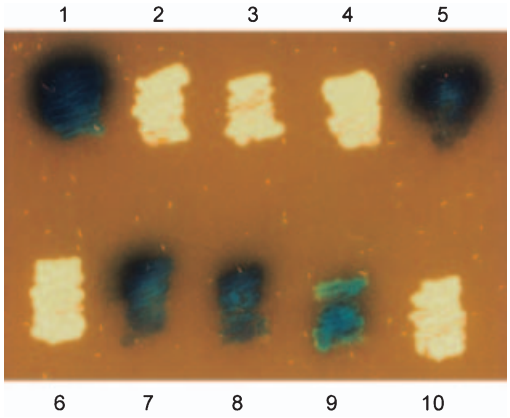


**Fig. 8.** Physical interaction between HopPtoM and ShcM in the LexA two-hybrid system. Full-length ShcM protein (164 amino acids) was fused to the DNA-binding domain (BD) in pGILDA and a series of truncated HopPtoM proteins were fused to the transcriptional activation domain (AD) in pB42AD. Yeast strains were grown at 30°C for 2 days on galactose X-gal complete minimal medium. A blue colour indicates interaction, whereas a white colour indicates no interaction. 1 = BD::SV40/AD::p53, 2 = BD-empty/AD-empty, 3 = BD-empty/AD::ORF4, 4 = BD::ORF3 (full-length, 712 amino acid)/AD-empty, 5 = BD::ORF3 (full length)/AD::ORF4, 6 = BD::ORF4/AD::ORF3$_{1–100}$ (1–100 amino acids), 7 = BD::ORF4/AD::ORF3$_{1–200}$ (1–200 amino acids), 8 = BD::ORF4/AD::ORF3$_{201–712}$ (201–712 amino acids), 9 = BD::ORF4/AD::ORF3$_{301–712}$ (301–712 amino acids), 10 = BD::ORF4/AD::ORF3$_{401–712}$ (401–712 amino acids). Yeast colonies containing AD::ORF3$_{1–200}$ alone, AD::ORF3$_{201–712}$ alone, and AD::ORF3$_{301–712}$ alone were white (data not shown).

$HopPtoM_{301–712}$ 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 $HopPtoM_{401–712}$ 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 ΔCEL and Δ*hopPtoM* 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*., 2002). Bacterial chaperones have been suggested to maintain their cognate effectors in a state competent for secretion conferring a competitive advantage over other non-chaperoned effectors for traffic through the TTSS (Boyd *et al*., 2000). According to this model, chaperoned effectors may be secreted by the bacterium either more efficiently or early during the interaction with the eukaryotic cell. HopPtoM is the only effector of the *P. syringae* CEL locus explored thus far whose function is chaperone dependent. Thus, it is possible that HopPtoM is translocated early into the plant cell where it alters host metabolism and provides favourable conditions for the action of other effectors delivered later in the secretion hierarchy. Alternatively, HopPtoM could function inside plant cells independently of other effectors to directly lead to the development of disease symptoms in tomato.

Although HopPtoM can be translocated efficiently in the presence of ShcM, our results suggest that some HopPtoM can still be injected into the plant cell in the absence of its chaperone. This conclusion is supported by two observations. First, expression of *hopPtoM* alone (i.e. without *shcM*) in the CEL mutant restores (although infrequently) the ability to cause disease symptoms. Second, ΔCEL mutant CUCPB5115 carrying the HopPtoM::$AvrRpt2_{80–255}$ fusion without simultaneous expression of ShcM is able to cause loss of turgidity in Arabidopsis leaves. Both complementation and translocation analyses suggest that secretion and/or translocation of HopPtoM in the absence of its chaperone is an inefficient process. Consistent with our interpretation, an *E. amylovora dspB/F* mutant retains some virulence to pear seedlings, suggesting that some DspA/E still travels the TTSS 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; Guttman 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 phytotoxins, 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*., 1990; Kitten *et al*., 1998), whereas B728a *hrp* mutants are strongly impaired in growth in planta but retain an ability to cause symptoms at a low frequency in the field (Hirano *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, *P. syringae* pv. *syringae* B728a *gacS* and *P. syringae* pv. *tomato* DC3000 *hopPtoM* mutants reveal independent mechanisms for the gratuitous induction of disease lesions that are without obvious benefit to the growth or survival of the pathogen in laboratory-grown plants. However, it is important to note that *gacS* does contribute significantly to the fitness of *P. syringae* pv. *syringae* B728 in the bean field (Hirano *et al*., 1997), and it is possible that the elicitation of lesions by HopPtoM may similarly confer some benefit to *P. syringae* pv. *tomato* in the tomato field, for example, in promoting dissemination of inoculum.

## 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 LM (Hanahan, 1985) 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 (Huynh *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.

### *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′-GTGAATTCGCTAAGTGGGCAATTGGAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CAGGATCCTTTAAGGTTAAAACAGCAT-3′ (*Bam*HI underlined); *ORF4 (*for construction of pORF4): sense primer, 5′-GTGAATTCGCTAAGTGGGCAATTGGAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CGGGATCCGATCATTGGAATCTCCCAG-3′ (*Bam*HI site underlined); and *ORF3* (for construction of pORF3): sense primer, 5′-CAGGATCCAAACGCGAGAGCCTTTCGG-3′ (*Bam*HI site underlined) and antisense primer, 5′-CTTCTAGATTAAAACAGCATGAAGCATGC-3′ (*Xba*I underlined). pORF3 also contains the *ORF4* promoter upstream of *ORF3*. The *ORF4* promoter was amplified using sense primer 5′-GTGAATTCGCTAAGTGGGCAATTGGAC-3′ (*Eco*RI underlined) and antisense primer 5′-CAGGATCCGT TGATAAGGGTGTGGTAC-3′ (*Bam*HI 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′-GCGCCGGGGTACCCTTCAATGTTC-3′; *Kpn*I site underlined) and p0610 (5′-ACGGGCTCGAGGTCCTATCTG-3′; *Xho*I site underlined), and ligated into pBluescript-II SK to make pCPP3271. A *Hin*dIII–*Xho*I 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′-ACAGTATCAAGCTTCTGGTTTA-3′; *Hin*dIII site under-

lined) and p0612 (5′-GCGGTCGGGATCCATCTGTGAG-3′; *Bam*HI site underlined), and cloned into pCPP3272 to obtain pCPP3273. The *Bam*HI–*Kpn*I 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 *Bam*HI–*Sac*I fragment into pCPP54 to obtain pCPP3275. The mutated *hopPtoM* in pCPP3275 was marker 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::uid*A 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′-GTCGCTGCTGGTCTAGAGAATCGTGTC-3′; *Xba*I site underlined) and p0890 (5′-GTGTCCTGCGAGCTCTTG GAGTTGGT-3′; *Sac*I 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′-ATCACCGTGGGCCCGATGACCTACA-3′; *Apa*I site underlined) and p0892 (TACAGCAAGGCTCGAGCGGG TATTCAG-3′; *Xho*I site underlined). The digested fragments were ligated to an *uid*A::*nptII* cassette obtained from pCPP2277 digested with *Xba*I and *Xho*I and to pJQ200SK digested with *Apa*I and *Sac*I to obtain pCPP3276. pCPP3276 was maker exchanged into the *P. syringae* pv. *tomato hrp/hrc* mutant CUCPB5114 to obtain strain CUCPB5369. CUCPB5369 was transformed with pCPP5032, which expresses *hrpL* 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 *hrp*-inducing fructose minimal medium or *hrp*-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 membrane (Millipore Corp.). Immunoblot analyses were performed using rabbit and chicken antibodies raised against *E. coli*-expressed HopPtoM and ShcM, respectively, at Cocalico Biologicals, Inc.

### *Type III translocation analysis*

The truncated $avrRpt2_{80–255}$ gene, which encodes type III secretion/translocation-incompetent, but biologically active, AvrRpt2 (Mudgett *et al.*, 2000) was cloned into the *Xba*I–*Hin*dIII sites of pUCP19 (Schweizer, 1991). Full-length *hopPtoM* (*ORF3*) or *shcM* (*ORF4*) genes were amplified by PCR and fused to the 5′ end of $avrRpt2_{80–255}$. The recombinant plasmids were introduced into ΔCEL mutant by electroporation. The transformants were grown in low-salt LB to $OD_{600} = 0.6$. Bacteria were collected by centrifugation and resuspended in sterile water to $OD_{600} = 0.2$. The bacterial suspensions were infiltrated into leaves of 6-week-old *RPS2*$^+$ Arabidopsis ecotype Col or *rps2* mutant (Kunkel *et al.*, 1993) plants. Tissue collapse was monitored over a 48 h period at room temperature. The HR elicited by AvrRpt2 and the HopPtoM-AvrRpt2 fusion appeared in about 9 and 15 h respectively.

The following primers were used in the construction of $avrRpt2_{80–255}$ gene fusions. *ORF4*::$avrRpt2_{80–255}$: sense primer, 5′-GTGAATTCGCTAAGTGGGCAATTGGAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-ACTCTAGATTGGAA TCTCCCAGGAG-3′ (*Xba*I site underlined); *ORF4*+*ORF3*::$avrRpt2_{80–255}$: sense primer, 5′-GTGAATTCGCTAAGT GGGCAATTGGAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-GTTCTAGAAAGCGTCTCGGTACGGTCC-3′ (*Xba*I site underlined), using genomic DNA as a template; *ORF3*::$avrRpt2_{80–255}$: sense primer, 5′-GTGAATTCGCTAAG TGGGCAATTGGAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-GTTCTAGAAAGCGTCTCGG TACGGTCC-3′ (*Xba*I site underlined), using pORF3 as a template.

### *Yeast two-hybrid analysis*

The LexA-based yeast two-hybrid system (Clontech Laboratories Inc.) was used. *shcM* and *hopPtoM* fragments were amplified by PCR and cloned into pB42AD or pGILDA. The following primers were used to amplify full-length *shcM*: sense primer, 5′-CGAATTCATGACCAACAATGACCAGTAC-3′ (*Eco*RI site underlined) and antisense primer, 5′-GATC CTCGAGCTGATCATTGGAATCTCC-3′ (*Xho*I site underlined); full-length *hopPtoM*: sense primer, 5′-GGAATTC ATGATCAGTTCGCGGATCGGC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CCTGCTCGAGTGACGGATGTTAT TCAAAG-3′ (*Xho*I site underlined); sequence corresponding to the first 100 amino acids of HopPtoM: sense primer, 5′-GGAATTCATGATCAGTTCGCGGATCGGC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CCTGCTCGAGAC TAACCGATCAACAACGC-3′ (*Xho*I site underlined); sequence corresponding to the first 200 amino acids of HopPtoM: sense primer, 5′-GGAATTCATGATCAGTTCGCG GATCGGC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CTTGCTC GAGCGGCCTATTCGCCAAGGGC-3′ (*Xho*I site underlined); sequence corresponding to amino acids 201–712 of HopPtoM: sense primer, 5′-CGAATTCGCCGGTCGT GCAAGCAAGGC -3′ (*Eco*RI site underlined) and antisense primer, 5′-CCT GCTCGAGTGACGGATGTTAT TCAAAG-3′ (*Xho*I site underlined); sequence corresponding to amino acids 301–712 of HopPtoM: sense primer, 5′-AGAATTCGGGCCGATTGTCG CGGCTGC-3′ (*Eco*RI site underlined) and antisense primer, 5′-CCTGCTCGAGTGACGGATGTTATTCAAAG-3′ (*Xho*I site underlined); and sequence corresponding to amino acids 401–712 of HopPtoM: sense primer, 5′-CGAATTCAAAAGC GAACACGGTGAGCT-3′ (*Eco*RI site underlined) and antisense primer, 5′-CCTGCTCGAGTGACGGATGTTATTCAAA G-3′ (*Xho*I site underlined). The constructs were transformed into the EGY48 strain carrying the *lacZ* reporter plasmid. Activation of the LacZ reporter was determined colorimetrically using Xgal as the substrate.

### *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 $10^4$ cfu $ml^{-1}$ in 10 mM MES (morpholinoethanesulfonic acid) pH 5.5 were infiltrated into leaves using a blunt syringe or by dipping of plants for 30 s in bacterial suspensions containing $10^6$ cfu $ml^{-1}$ in 10 mM MES (morpholinoethanesulfonic acid) pH 5.5 and 0.02% (v/v) Silwet L-77 (Lehle Seeds). Plants were monitored daily over a 5 to 14 day period for symptom development and bacterial multiplication. To determine whether the *P. syringae* pv. *tomato hopPtoM* mutant can elicit the HR in tobacco, bacterial suspensions at optical density at 600 nm of 0.8, 0.3 and 0.003 were infiltrated into individual leaf panels as indicated for tomato. Plant assay experiments were repeated at least three times with similar results.

### *DNA gel blots*

To confirm replacement of *hopPtoM* with the *nptII* cassette, total DNA of wild-type and mutant strains was digested with *Hind*III and analysed on DNA gel blots according to standard procedures (Sambrook *et al*., 1989). A 1.6 kb *Asc*I–*Bsr*GI *hopPtoM* fragment or the *nptII* cassette was labelled with $^{32}P$ using the Prime-It II kit (Stratagene) and then used as probe. Immobilon-N membrane (Millipore Corp.) was used, and blots were hybridized at 50°X overnight in HYB-9 DNA hybridization solution (GENTRA Systems).

## Acknowledgements

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