A *Pseudomonas syringae* pv. *tomato* *avrE1/hopM1* Mutant Is Severely Reduced in Growth and Lesion Formation in Tomato

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The model plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 grows and produces necrotic lesions in the leaves of its host, tomato. Both abilities are dependent upon the hypersensitive response and pathogenicity (Hrp) type III secretion system (TTSS), which translocates multiple effector proteins into plant cells. A previously constructed DC3000 mutant with a 9.3-kb deletion in the Hrp pathogenicity island conserved effector locus (CEL) was strongly reduced in growth and lesion formation in tomato leaves. The Δ CEL mutation affects three putative or known effector genes: *avrE1*, *hopM1*, and *hopAA1-1*. Comparison of genomic sequences of DC3000, *P. syringae* pv. *phaseolicola* 1448A, and *P. syringae* pv. *syringae* B728a revealed that these are the only effector genes present in the CEL of all three strains. AvrE1 was shown to carry functional TTSS translocation signals based on the performance of a fusion of the first 315 amino acids of AvrE1 to the Cya translocation reporter. A DC3000 Δ avrE1 mutant was reduced in its ability to produce lesions but not in its ability to grow in host tomato leaves. AvrE1 expressed from the 35S promoter elicited cell death in nonhost *Nicotiana tabacum* leaves and host tomato leaves in *Agrobacterium*-mediated transient expression experiments. Mutations involving combinations of *avrE1*, *hopM1*, and *hopAA1-1* revealed that deletion of both *avrE1* and *hopM1* reproduced the strongly reduced growth and lesion phenotype of the Δ CEL mutant. Furthermore, quantitative assays involving different levels of inoculum and electrolyte leakage revealed that the *avrE1/hopM1* and Δ CEL mutants both were partially impaired in their ability to elicit the hypersensitive response in nonhost *N. benthamiana* leaves. However, the *avrE1/hopM1* mutant was not impaired in its ability to deliver AvrPto1_(1–100)-Cya to nonhost *N. benthamiana* or host tomato leaves during the first 9 h after inoculation. These data suggest that AvrE1 acts within plant cells and promotes lesion formation and that the combined action of AvrE1 and HopM1 is particularly important in promoting bacterial growth in planta.

Additional keywords: basal resistance.

The plant-pathogenic bacterium *Pseudomonas syringae* pv. *tomato* can grow and produce necrotic “bacterial speck” lesions in the leaves of its host, tomato (Jones et al. 1991). *P. syringae* mutants deficient in the hypersensitive response (HR) and

pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes encoding the type III secretion system (TTSS) are unable to inject effector proteins into plant cells or to grow or cause disease symptoms in planta, which underscores the collective importance of TTSS effectors in *P. syringae* pathogenesis (Alfano and Collmer 2004). Functional analysis of the complete genome sequence of *P. syringae* pv. *tomato* DC3000, a pathogen of both tomato and *Arabidopsis*, has revealed that this one strain may harbor over 40 genes encoding proteins capable of traveling the TTSS (Buell et al. 2003; Lindeberg et al. 2005). Thus, it is not surprising that mutations affecting individual effector genes in *P. syringae* typically have little or no virulence phenotype.

However, a *P. syringae* pv. *tomato* DC3000 mutant with a deletion affecting 9.3 kb of the conserved effector locus (CEL) was strongly reduced in both growth and lesion formation in tomato (Alfano et al. 2000). The CEL is adjacent to the *hrp* and *hrc* genes in the Hrp pathogenicity island of DC3000, but the Δ CEL mutant did not appear deficient in the TTSS pathway, as indicated by its ability to secrete effector AvrPto1 in culture. *P. syringae* effectors are designated Avr (avirulence) proteins or Hrp outer proteins (Hop), depending on the phenotype by which they were discovered. Some effectors initially were observed to trigger hypersensitive defenses and avirulence in nonhost or resistant plants, whereas others were found to be Hops through observation of their TTSS-dependent secretion in culture or translocation into plant cells. A recently proposed unified nomenclature for *P. syringae* TTSS Avr and Hop proteins will be used here (Lindeberg et al. 2005).

The *P. syringae* pv. *tomato* DC3000 CEL deletion affected three effector genes: *avrE1*, *hopM1*, and *hopAA1-1*. HopAA1-1 and its paralog, HopAA1-2, contribute to the ability of *P. syringae* pv. *tomato* DC3000 to initiate the growth of colonies in host leaves, but a *hopAA1-1/hopAA1-2* mutant is not significantly reduced in its overall ability to grow or produce lesions in tomato leaves (Badel et al. 2002). In contrast, a *hopM1* mutant is reduced in its ability to produce lesions in tomato, although overall growth in planta is not affected (Badel et al. 2003). Recently, the Δ CEL deletion mutant was shown to be reduced in its ability to suppress basal resistance in *Arabidopsis*, and this defect could be complemented by either *avrE1* or *hopM1* expressed in trans on multicopy plasmids (DebRoy et al. 2004). This highlights the importance of the effectors in the CEL region.

To learn more about AvrE1 and its contribution to the Δ CEL phenotype, we tested AvrE1 TTSS-targeting signals, constructed *avrE1* mutations (alone and with CEL gene polymutant backgrounds), and subjected the mutants to multiple tests in host and nonhost plants. We report here that AvrE1 can be translocated by the DC3000 TTSS into plant cells, a Δ avrE1 mutant has a re-

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duced lesion phenotype in host tomato that is similar to that of a Δ *hopM1* mutant, transiently expressed *avrE1* can elicit necrosis in both host and nonhost plants, a series of CEL effector gene polymutants establishes that *avrE1* and *hopM1* are responsible for most of the Δ CEL phenotype in host tomato and nonhost *Nicotiana* spp., and the Δ *avrE1*/ Δ *hopM1* mutant shows no deficiency in its ability to translocate effectors into plant cells.

RESULTS

Comparison of the CEL regions in *P. syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. *phaseolicola* 1448A.

P. syringae strains DC3000, B728a, and 1448A represent the three major groups into which *P. syringae* pathovars have been shown to cluster in phylogenetic analyses (Sarkar and Guttman 2004; Sawada et al. 1999). A previous analysis based on DNA sequence of the complete DC3000 CEL and of fragments encompassing the B728a CEL between open reading frame (ORF)1 (lytic transglycosylase) and HopAA1-1 had suggested that similar genes were present in this region in these two strains (Alfano et al. 2000). Complete genome sequences are now available for B728a (Feil et al. 2005) and 1448A (Joardar et al. 2005). Here, we compared the arrangement and similarity of CEL genes and determined the right border (distal to *hrpR*) of the CEL in these three divergent strains of *P. syringae*. The results confirm the conserved nature of the CEL, which contains the same arrangement of genes and putative operons between ORF1 and ORF6 (Fig. 1A). Comparison of the nucleotide sequence reveals that the CEL ends just downstream of ORF6 (Fig. 1B). Interestingly, ORF2, which is a candidate chaperone for AvrE1, is encoded in the *shcM-hopM1* operon in all three strains. The 1448A CEL differs from the other two CELs in having a frameshift in the *hopM1* and *hopAA1-1* genes. Only the DC3000 CEL has the *shcN-hopN1* operon. In contrast, the CEL appears to end with ORF6 in B728a and 1448A. Thus, the genes beyond ORF6 are different in all three strains, and no obvious TTSS effector gene candidates are found immediately beyond ORF6 in B728a and 1448A. Comparison of the predicted CEL protein sequences of DC3000 with B728a and 1448A revealed that ORF2, ShcM, and ORF6 are the most conserved, and AvrE1 and HopM1 are the most divergent (Fig. 1C).

AvrE1 is translocated into plant cells by the *P. syringae* Hrp TTSS system.

The *Erwinia amylovora* DspA/E protein is secreted in culture by the *E. amylovora* Hrp system, and this is dependent upon the DspB/F chaperone (Bogdanove et al. 1998a; Gaudriault et al. 1997, 2002). It had not been determined whether AvrE1 is secreted by the *P. syringae* Hrp system or whether any members of this effector family can be translocated into plant cells. However, CEL ORF2 is a homolog of DspB/F, and a plasmid carrying *avrE1*-ORF2 can partially complement an *E. amylovora* *dspE* mutant (Bogdanove et al. 1998b). Furthermore, the N-terminal 50 amino acids of AvrE1 show all of the patterns associated with TTSS substrates (Petnicki-Ocwieja et al. 2002). To determine whether AvrE1 can be translocated into plant cells, we constructed a translational fusion of the first 315 codons of *avrE1* with *cya*, which encodes the *Bordetella pertussis* adenylate cyclase. *Cya* is a reporter for the translocation of proteins into eucaryotic cells, where it produces cAMP in a calmodulin-dependent manner (Sory and Cornelis 1994). The *Cya* reporter works in plants (Casper-Lindley et al. 2002), and a cloned *P. syringae* pv. *syringae* 61 Hrp system heterologously expressed in *P. fluorescens* provides a convenient system for testing candidate *P. syringae* effectors

(Schechter et al. 2004). We used *P. fluorescens* carrying Hrp⁺ pLN18 and Δ *hrpC* derivative pCPP3297 to test the translocation of AvrE1₍₁₋₃₁₅₎-*Cya* and ORF2-*Cya*. We employed AvrE1₍₁₋₃₁₅₎ because full-length AvrE1-*Cya* constructs were toxic to *Escherichia coli* when induced by IPTG. We assayed for cAMP production 4 and 7 h after inoculation because we had found previously that AvrPto1-*Cya*-dependent cAMP production in *Nicotiana benthamiana* peaked within this period. We observed that AvrE1₍₁₋₃₁₅₎-*Cya* was translocated into *N. benthamiana* cells in an Hrp-dependent manner, whereas ORF2-*Cya* was not (Fig. 2). Furthermore, cAMP accumulation was equivalent in this semiquantitative assay for AvrE1₍₁₋₃₁₅₎-*Cya* and AvrPto1-*Cya*. Whether ORF2 is a chaperone for AvrE1 in *P. syringae* pv. *tomato* DC3000 remains to be tested, although it clearly is not required for translocation of AvrE1₍₁₋₃₁₅₎-*Cya* by *P. fluorescens* (pLN18), which lacks ORF2.

A *P. syringae* pv. *tomato* DC3000 *avrE1* mutant is reduced in its ability to elicit lesions but not to grow in host tomato leaves.

The *avrE1* gene was deleted from the DC3000 genome by recombination of polymerase chain reaction (PCR)-cloned flanking sequences and subsequent eviction of the *sacB*⁺ suicide vector pJQ200SK (Quandt and Hynes 1993). Tomato cv. Moneymaker plants were inoculated by dipping in bacterial suspensions containing wild-type DC3000, Δ *avrE1* mutant CUCPB5374, and CUCPB5374 carrying an *avrE1-cya* fusion expressed from plasmid pCPP5246. Replicate leaves were photographed 5 days after inoculation, when typical bacterial speck lesions had formed (Fig. 3A). The mutant produced visibly fewer lesions. Counting of lesions ≥ 0.25 mm² revealed that DC3000 produced approximately fourfold more lesions than the Δ *avrE1* mutant (Fig. 3B). Bacterial populations in tomato leaves were monitored for 5 days, but the Δ *avrE1* mutant was not impaired in its ability to grow in planta (Fig. 3C). Interestingly, the strain expressing AvrE1-*Cya* in trans grew less well in planta than wild-type DC3000.

AvrE1 induces cell death when transiently expressed in the leaves of nonhost *N. tabacum* and host tomato leaves.

Our observation that AvrE1 is translocated into plant cells and contributes to the ability of *P. syringae* pv. *tomato* to produce necrotic lesions in host tomato leaves led us to ask whether transient production of AvrE1 alone within plant cells was sufficient to promote cell death. We constructed derivatives of binary vector pLN314 that were designed to express HA-tagged HopQ1 and AvrE1 from the 35S promoter in planta. HopQ1, which elicits the HR in *N. benthamiana* (Schechter et al. 2004), was used as a positive control. *Agrobacterium tumefaciens* GV3101 carrying pLN314 was infiltrated with a blunt syringe into leaves of *N. tabacum* cv. Xanthi and tomato cv. Moneymaker at three different concentrations, and inoculated leaves were photographed 2 days later (Fig. 4). HopQ1 elicited tissue collapse typical of the HR in *N. tabacum*, which appeared within 24 h after inoculation, but it elicited no visible response in tomato. On the other hand, the cell death elicited by AvrE1 developed more slowly (approximately 36 h), and it was elicited in both *N. tabacum* and tomato. Identical results were observed with *N. benthamiana* (not shown).

A *P. syringae* pv. *tomato* DC3000 Δ *avrE1*/ Δ *hopM1* mutant is strongly impaired in its ability to produce lesions and grow in susceptible host tomato leaves.

P. syringae pv. *tomato* Δ CEL mutant CUCPB5115 is lacking genes encoding the effectors AvrE1, HopM1, and HopAA1-1, as well as the HrpW harpin (Alfano et al. 2000). CUCPB5115 is strongly impaired in both lesion formation and growth in tomato

A

B

C

	ORF1	AvrE1	ORF2	HopM1		ShcM	HrpW	HopAA1-1		ORF6
B728A	69.8 (75.0)	67.2 (77.0)	86.3 (91.6)	64.2 (75.1)		76.8 (87.2)	71.0 (78.2)	73.3 (81.3)		82.8 (87.9)
1448A	81.6 (75.4)	84.2 (87.3)	87.8 (93.1)	85.0 (92.5)	70.0 (73.0)	85.4 (92.1)	68.2 (74.1)	70.4 (75.1)	87.7 (91.7)	80.1 (85.7)

Fig. 1. Comparison of the conserved effector loci (CEL) of *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. *phaseolicola* 1448a. **A**, Overview of CEL regions and open reading frames (ORFs). The location of the CEL in the *P. syringae* Hrp pathogenicity island is shown in reference to the *hrp/hrc* cluster and the exchangeable effector locus (EEL). The CEL regions of the three *P. syringae* strains are shown aligned by their effector ORFs. Arrows indicate the direction of transcription, with small boxes denoting the presence of an Hrp box. Regions flanking the right side of the CEL are shaded (the *hrpR* gene flanking the left side is not shown). **B**, Nucleotide sequence alignment of the CEL regions of B728a and 1448a in reference to DC3000, as viewed with the Artemis Comparison Tool. Note that the orientation is opposite to that of panels A and C. The CEL region of DC3000 is extended to include *hopN1* and *shcN*. The numbers indicate the percentage nucleotide identity between DC3000 and B728a and between DC3000 and 1448a. For reference, the ORFs and transcription units in the CEL region of DC3000 are indicated by green boxes on the appropriate DNA strand. **C**, Comparison of the predicted amino acid sequences of proteins encoded by the CEL of DC3000 with those predicted for strains B728a and 1448a. The upper number shows percent identity and the number in parentheses shows percent similarity. The separate values for the 1448a HopM1 and HopAA1-1 ORFs reflect the sequences before and after the frameshift (with the frameshift removed) at codon 545 for HopM1 and 187 for HopAA1-1.

leaves, whereas mutations individually affecting AvrE1 and HopM1 are impaired only in lesion formation, and *hopAA1-1* mutants have only a subtle deficiency in colony initiation in planta (Alfano et al. 2000; Badel et al. 2002, 2003). We constructed polymutants involving the three effector genes to determine the minimal deficiency required for the Δ CEL phenotype. The mutants were Δ hopM1::*aadA1*/ Δ hopAA1-1::*nptII* (CUCPB5373), Δ avrE1/ Δ hopAA1-1::*nptII* (CUCPB5376), Δ avrE1/ Δ hopM1::*nptII* (CUCPB5377), and Δ avrE1/ Δ hopM1::*aadA1*/ Δ hopAA1-1::*nptII* (CUCPB5380). The mutants were assayed by infiltrating different concentrations of bacteria into tomato leaves using a blunt syringe (Fig. 5A). The Δ avrE1 mutant produced lesions indistinguishable from the wild type, but the numbers of lesions appeared slightly reduced (Fig. 5A). Note that these results, based on inoculation with a syringe, contrasted with those based on inoculation by dipping (Fig. 3). Polymutants with deletions affecting *hopAA1-1* and either *avrE1* or *hopM1* showed similar partial reductions in lesion frequency (data not shown). However, the Δ avrE1/ Δ hopM1 mutant produced no lesions at the levels of inoculum tested (Fig. 5A).

The effector polymutants also were tested for their ability to grow in tomato leaves following infiltration with a blunt syringe. Growth of the Δ avrE1/ Δ hopM1 and Δ avrE1/ Δ hopM1/ Δ hopAA1-1 mutants was impaired as much as that of Δ CEL mutant CUCPB5115 (Fig. 5B). The bacterial populations reached by these three mutants at 4 days after inoculation were significantly lower than that of the wild type according to the Tukey's Studentized Range Test. Growth of the Δ avrE, Δ hopM1, Δ avrE/ Δ hopAA1-1, and Δ hopM1/ Δ hopAA1-1 mutants was not significantly different from DC3000 upon repeated testing. To further test the relative ability of the Δ avrE1/ Δ hopM1 mutant to grow in host tomato leaves, we used the dipping inoculation method and followed bacterial growth over time. A TTSS-deficient Δ hrcQ-U mutant control (CUCPB5113) was most strongly

Fig. 2. AvrE1₍₁₋₃₁₅₎-Cya fusion is translocated into *Nicotiana benthamiana* cells by *Pseudomonas fluorescens* expressing a cloned *P. syringae* pv. *syringae* 61 Hrp system. The figure shows cAMP accumulation indicative of Cya translocation in *N. benthamiana* leaves after inoculation with *P. fluorescens* expressing pLN18 (*P. syringae* pv. *syringae* 61 *hrp*/*hrc* cluster) or pCPP3297 (pLN18 Δ hrcC) plus plasmids expressing AvrE1₍₁₋₃₁₅₎-Cya, open reading frame (ORF)2-Cya, or AvrPto1-Cya from the vector pCPP3234 tac promoter. These constructs are denoted simply as AvrE1, ORF2, and AvrPto1 in the figure. Leaves were inoculated by syringe infiltration with *P. fluorescens* transformants at 4×10^8 CFU/ml. Samples were collected with a 0.8-cm-diameter cork borer 4 and 7 h post inoculation and assayed for cAMP production. The values represent the mean and standard error from three samples.

impaired in growth, whereas the Δ avrE1/ Δ hopM1 and Δ CEL mutants showed an intermediate level of growth relative to wild-type DC3000 (Fig. 5C). Thus, the deficiency in the ability of the Δ CEL mutant to produce lesions and grow in host tomato leaves can be attributed to the combined effects of AvrE1 and HopM1.

Fig. 3. *Pseudomonas syringae* pv. *tomato* DC3000 Δ avrE1 mutant is diminished in its ability to produce normally developed disease lesions but not in its ability to grow in host tomato. **A**, Disease symptoms on tomato leaves 6 days after inoculation by dipping with 1×10^7 CFU/ml. In the Δ avrE1(*avrE1*) strain, the mutation is complemented with an AvrE1-Cya hybrid expressed without IPTG induction from the tac promoter in pCPP5246. **B**, Number of well-developed lesions per leaf appearing on plants 5 days after inoculation with DC3000, the Δ avrE1 mutant, and the Δ avrE1(*avrE1*) complemented mutant strain. Values indicate mean and standard error for the number of lesions ≥ 0.25 mm² on three whole leaves assayed for each treatment. **C**, Bacterial growth in tomato leaves of DC3000, the Δ avrE1 mutant, and the Δ avrE1(*avrE1*) complemented mutant strain. Each value represents the mean and standard error for the number of bacterial CFU/cm² of leaf recovered at the indicated time from three 0.5-cm-diameter disks from different leaves.

***A. P. syringae* pv. *tomato* DC3000 Δ avrE1/ Δ hopM1 mutant is impaired in its ability to elicit the HR in nonhost *N. benthamiana* leaves.**

We previously had reported that Δ CEL mutant CUCPB5115 produced a delayed HR in nonhost tobacco leaves when infiltrated at a concentration of 1×10^7 CFU/ml (Alfano et al. 2000). Here, we used a lower inoculum of 1.25×10^6 CFU/ml, which is close to the threshold needed for DC3000 to elicit the HR in *N. benthamiana*, to compare CUCPB5115 with the mutants constructed in this study. Wild-type DC3000, the Δ avrE1 mutant, and the Δ hopM1 mutant elicited HR collapse of infiltrated tissue 24 h after inoculation (Fig. 6A). In contrast, the Δ avrE1/ Δ hopM1, Δ CEL, and Δ hrcQ-U mutants failed to elicit the HR at this level of inoculum. To further document altered ability to elicit cell death in *N. benthamiana*, we measured electrolyte leakage from inoculated areas 24 h after inoculation. The wild-type, Δ avrE1, and Δ hopM1 strains induced strong leakage of electrolytes, whereas the Δ hrcQ-U, Δ CEL, and Δ avrE1/ Δ hopM1 mutants did not (Fig. 6B). We also tested these strains at the higher inoculum level of 2×10^7 CFU/ml. Only the Δ hrcQ-U mutant was significantly impaired in HR elicitation, based on both visible symptoms (Fig. 6C) and electrolyte leakage (Fig. 6D). Thus, the quantitative deficiency in the ability of the Δ CEL mutant to elicit the HR in nonhost *N. benthamiana* leaves can be attributed to the combined effects of AvrE1 and HopM1.

***A. P. syringae* pv. *tomato* DC3000 Δ avrE1/ Δ hopM1 mutant is not impaired in its ability to deliver AvrPto1₍₁₋₁₀₀₎-Cya to nonhost *N. benthamiana* or host tomato leaves early in the interaction and is still able to elicit a Pto-dependent HR in host tomato.**

Given the importance of the TTSS to *P. syringae* interactions with plants, one possible explanation for the effects of the Δ avrE1/ Δ hopM1 mutation on both compatible and incompatible interactions is that the mutant is reduced in its ability to deliver effectors into plant cells. To test this, we used AvrPto1₍₁₋₁₀₀₎-Cya to monitor the performance of the TTSS in both compatible and incompatible interactions. We chose AvrPto1 because it is a particularly well-studied *P. syringae* pv. *tomato* effector (Lin and Martin 2005; Salmeron and Staskawicz 1993; Wulf et al. 2004), and we used truncated AvrPto1₍₁₋₁₀₀₎ because native AvrPto1 has been reported to be a suppressor of basal resistance (Hauck et al. 2003); however, the first 100 codons of AvrPto1 are sufficient to direct robust translocation of the Cya reporter (Schechter et al. 2004).

It should be noted here that the high levels of inoculum used in the Cya assay are sufficient to produce a rapid HR collapse in resistant leaves and an accelerated disease necrosis in susceptible leaves. DC3000 causes tissue collapse by 19 h in susceptible PtoS leaves at 2×10^7 CFU/ml and, at this level of inoculum, the Δ avrE1/ Δ hopM1 mutant also causes necrosis 5 h later (data not shown). Because plant cell death interferes with the production of cAMP in this assay, we optimized conditions to permit the latest sampling time before tissue collapse.

The translocation of AvrPto1₍₁₋₁₀₀₎-Cya was abolished in the Δ hrcQ-U mutant in all plants tested (Fig. 7A and B). However, neither the Δ avrE1 mutation, nor the Δ avrE1/ Δ hopM1 mutation diminished translocation, even in PtoS tomato when inoculum levels were reduced and the sampling time was extended to 9 h post inoculation. Thus, we could detect no deficiency in the ability of Δ avrE1/ Δ hopM1 mutant CUCPB5374 to deliver a test effector-Cya hybrid protein into plant cells in either compatible or incompatible interactions.

Because the experiments described above used a truncated AvrPto1 expressed from a vector promoter, we sought additional evidence that native effectors are still translocated into

plant cells by the Δ avrE1/ Δ hopM1 mutant. Elicitation of the HR in PtoR tomato by DC3000 results from the translocation of AvrPto1, AvrPtoB (HopAB2), or both, and genes encoding both effectors must be ablated to abolish Pto-dependent HR elicitation (Lin and Martin 2005). Here, we examined the effect of the newly constructed Δ avrE1 and Δ avrE1/ Δ hopM1 mutations on the development of the Pto-dependent HR by following the time course of visible tissue collapse and electrolyte leakage in PtoR Rio Grande plants that were inoculated at 2×10^7 CFU/ml with the mutants and with DC3000 and Δ hrcQ-U controls (Fig. 7C and D). The Δ hrcQ-U mutant produced no visible response. In contrast, DC3000 and the Δ avrE1 and Δ avrE1/ Δ hopM1 mutants produced HR collapse that began approximately 9 h after inoculation and was fully developed by 12 h. To further test that the collapse we observed with test strains at 12 h was Pto dependent, we also inoculated PtoS Moneymaker plants with the same bacteria. Despite the high

Fig. 4. AvrE1 induces cell death in nonhost *Nicotiana tabacum* and host tomato leaves. **A**, Assay for visible cell death in *N. tabacum* (tobacco) leaves resulting from transient expression of HopQ1 and AvrE1 following syringe inoculation with *Agrobacterium tumefaciens* carrying binary vectors encoding the test effectors. Leaves were inoculated at the indicated optical density at 600 nm (OD₆₀₀) and photographed 2 days later. **B**, The ability of transiently expressed HopQ1 and AvrE1 to elicit visible cell death was tested similarly in tomato cv. Moneymaker leaves. Inoculated leaves were photographed 2 days after inoculation.

level of inoculum, no response was visible at 12 h, as expected for a compatible interaction. To further document the HR collapse in PtoR leaves, we performed electrolyte leakage assays. At 0, 6, and 12 h after inoculation, only the $\Delta hrcQ-U$ mutant caused no electrolyte leakage, whereas the other strains induced increased electrolyte leakage within 6 h (Fig. 7D). These observations provide further evidence that the delivery of AvrPto1 is not impaired by the $\Delta avrE1/\Delta hopM1$ mutation and, therefore, suggest that the phenotypes of the mutant can be attributed to the effects of AvrE1 and HopM1 in plant cells rather than impaired delivery of other effectors.

DISCUSSION

We have shown that AvrE carries signals for TTSS-dependent translocation into plant cells and, like HopM1, contributes to the ability of DC3000 to produce lesions in the later stages of pathogenesis in tomato without a commensurate reduction in bacterial growth. Interestingly, when both *avrE1* and *hopM1* were mutated, bacterial growth was also strongly reduced. Although DC3000 is likely to translocate many effectors into host cells during infection, AvrE1 may play a direct role in lesion development because *Agrobacterium*-mediated transient expression of AvrE1 alone resulted in necrosis in both host tomato and nonhost *N. tabacum*. Furthermore, the $\Delta avrE1/\Delta hopM1$ mutant showed no reduction in its ability to translocate an AvrPto1₍₁₋₁₀₀₎-Cya reporter, which suggests that the strong virulence phenotype of the mutant can be attributed to AvrE1 and HopM1 rather than to a diminished ability to deliver other effectors. As discussed below, these findings have important implications regarding interrelations between type III effector activities in defense suppression and symptom expression.

The first member of the *avrE* family was cloned from *P. syringae* pv. *tomato* PT23 on the basis of its avirulence phenotype when expressed in *P. syringae* pv. *glycinea* (Kobayashi et al. 1989). AvrE1_{PtoPT23} causes *P. syringae* pv. *glycinea* race 4 to elicit the HR on all cultivars of soybean tested (Lorang et al. 1994). A PT23 *avrE1* mutant was reduced in both symptom production and growth in tomato (Lorang et al. 1994), but a DC3000 *avrE1* mutant showed no virulence phenotype when inoculated by syringe infiltration (Lorang and Keen 1995). Here, we have confirmed that observation with a DC3000 $\Delta avrE1$ mutant in assays involving inoculation with a syringe, but we also observed that, when inoculated by dipping, a DC3000 $\Delta avrE1$ mutant is significantly reduced in develop-

Fig. 5. Ability of various *Pseudomonas syringae* pv. *tomato* DC3000 conserved effector loci (CEL) effector mutants to produce disease lesions and to grow in host tomato cv. Moneymaker leaves. **A**, Visible symptoms in tomato leaves 7 days after inoculation by syringe infiltration with the indicated strains at the indicated levels of inoculum. **B**, Bacterial populations in tomato cv. Moneymaker leaves at 0 and 4 days following inoculation by syringe infiltration with the indicated strains at 5×10^4 CFU/ml. Each value represents the mean and standard error for the number of bacteria/cm² of leaf recovered from three 0.5-cm-diameter leaf disks from different leaves. No significant difference was observed among values at 0 days. At 4 days after inoculation, values denoted with the same letter are not statistically different (analysis of variance, $P < 0.0001$, Tukey's Studentized Range Test). These experiments were repeated at least three times with similar results, except that a significant reduction in growth of the $\Delta avrE1/\Delta hopAA1-1$ mutant was not observed consistently. **C**, Kinetics of bacterial growth in tomato cv. Moneymaker leaves following dipping in inoculum containing 1×10^7 CFU/ml of the indicated strains. Each time point reflects the bacterial population recovered from five 0.7-cm-diameter disks from different leaves, and the values presented are the mean and standard error. Leaves inoculated with the three mutants showed no symptoms.

ment of normal-sized lesions. DC3000 coronatine-deficient mutants similarly were observed to be reduced in virulence when inoculated by dipping but not by infiltration with a syringe (Mittal and Davis 1995).

The difference in the relative importance of AvrE1 in *P. syringae* pv. *tomato* strains PT23 and DC3000 is noteworthy. The PT23 *avrE1* mutant is like a DC3000 *avrE1/hopM1* mutant in being strongly deficient in both lesion formation and growth. This could be attributed to differences intrinsic to the AvrE1 orthologs or to differences in the effector arsenals of the two strains. The observation that the *P. syringae* pv. *phaseolicola* 1448A *hopM1* gene carries a frameshift suggests the possibility that PT23 also may have a *hopM1* deficiency. Interestingly, a HopM1 homolog has not been reported in *Erwinia amylovora*, and mutations affecting *dspE* have a strong virulence phenotype that can be partially complemented with *avrE1*_{PtoDC3000} (Bogdanove et al. 1998b).

AvrE1 appears to be a typical effector in that its expression is HrpL dependent (Lorang and Keen 1995), the protein possesses N-terminal amino acid patterns associated with TTSS substrates (Petnicki-Ocwieja et al. 2002), and the targeting region is capable of mobilizing the TTSS-dependent translocation of a Cya fusion into plant cells. Our conclusion that AvrE1 is targeted primarily to the interior of plant cells must be tentative at this point because the Cya fusion contained only the first 315 of the 1,794 amino acids in the native protein. In a recent study, fusion of an alternative translocation reporter, $\Delta 79\text{AvrRpt2}$, to an N-terminal portion of AvrE1_{PtoDC3000} expressed from its native promoter on a multicopy plasmid was reported to produce translocation results permitting AvrE1 to be cautiously listed as an effector (Chang et al. 2005). Our strategy here was to use an IPTG-inducible vector tac promoter to produce enough of the AvrE1₁₋₃₁₅-Cya hybrid to enable clear testing of the ability of AvrE1₁₋₃₁₅ to be translocated

Fig. 6. ΔCEL and $\Delta\text{avrE1}/\Delta\text{hopM1}$ mutants are similarly reduced in their ability to elicit a hypersensitive response (HR) and associated electrolyte leakage in nonhost *Nicotiana benthamiana*. **A**, Visible HR collapse elicited by test strains following inoculation at 1.25×10^6 CFU/ml. The representative leaf shown was inoculated by syringe infiltration with the indicated strains and photographed 24 h later. At this threshold level of inoculum for the visible HR, the ΔCEL and $\Delta\text{avrE1}/\Delta\text{hopM1}$ mutants failed to cause tissue collapse. **B**, Electrolyte leakage from leaf areas inoculated at 1.25×10^6 CFU/ml. Leaf disks were removed from areas inoculated with the indicated strains using a 0.7-cm-diameter cork borer 24 h after inoculation. The excised disks were shaken at room temperature for 1 h after vacuum infiltration in 10 ml of H_2O , and electrolyte leakage was measured by recording the conductivity of the H_2O after removal of the leaf disks. The conductivity values represent the mean and standard error from four leaves. Values denoted with the same letter are not statistically different (analysis of variance, $P < 0.0001$, Tukey's Studentized Range Test). **C**, Visible HR collapse elicited by test strains following inoculation at 2×10^7 CFU/ml and photography as described above. **D**, Electrolyte leakage from leaf areas inoculated with test strains at 2×10^7 CFU/ml as described above. All experiments were repeated at least three times with similar results.

by the TTSS. Because the activities of the $\Delta 79\text{AvrRpt2}$ and Cya reporters have no known relationship with the levels needed for the native function of AvrE1, we gained alternative evidence for function. Specifically, *avrE1* mutants have a reduced lesion phenotype, and *avrE1* transiently expressed in planta has a matching gain-of-function phenotype. These observations strongly suggest that AvrE1 acts within plant cells to contribute significantly to DC3000 interactions with tomato.

Important progress has been made in exploring the virulence functions of *P. syringae* effectors by expressing individual effector genes in heterologous strains or in homologous strains with large deletions (Chen et al. 2000; DebRoy et al. 2004). This approach has been useful because mutations in typical effector genes often have little or no phenotype. Alternatively, mutagenesis of sets of effectors suspected of performing a similar function provides a potential means to uncover and study phenotypes in native backgrounds. For example, mutagenesis of the *hopAA1-1* and *hopAA1-2* paralogs in DC3000 revealed a reduced colony initiation phenotype (Badel et al. 2002). Similarly, mutations in DC3000 affecting AvrPto1 and AvrPtoB, which share an ability to interact with the tomato Pto protein, resulted in a significant reduction in lesion formation and growth in tomato (Lin and Martin 2005). Although AvrE1 and HopM1 show no similarity and are not known to interact with any proteins in planta, there are several indications that have a related function. They are encoded by closely linked genes; mutations in each have a similar phenotype; the putative AvrE1 chaperone is encoded in the HopM1 operon; and, most importantly, either gene can restore to the DC3000 ΔCEL mutation an ability to suppress basal resistance in *Arabidopsis* when expressed in trans on a multicopy plasmid (DebRoy et al. 2004).

These observations raise fundamental questions regarding the relationship among basal resistance, parasitic growth, and plant cell death in *P. syringae*-plant interactions. Foremost, is there a relationship between the basal resistance suppression and cell death-promoting activities of AvrE1 and HopM1? It is possible that these proteins have multiple domains, as was observed with AvrPtoB (Abramovitch et al. 2003), and that separate domains independently direct the two activities. However, we think that it is more likely that a process associated with the suppression of basal defense by AvrE1 and HopM1 early in pathogenesis leads to enhanced cell death later in the interaction. Two observations are worth highlighting here. We have found that AvrE1 and HopM1 collectively contribute to the ability of DC3000 to elicit cell death in both compatible (host) and incompatible (nonhost) interactions. In addition, there is increasing evidence that the cell deaths that occur rapidly during incompatible interactions and more slowly during compatible interactions are fundamentally similar responses (Klement 1982; Richael and Gilchrist 1999; Tao et al. 2003). These ob-

servations, coupled with the ability of AvrE1 and HopM1 to restore basal resistance suppression to the ΔCEL mutant (DebRoy et al. 2004) and to produce a delayed necrosis when transiently expressed in host tomato leaves, strongly support a link between suppression of basal resistance and elicitation of cell death.

One model for explaining this linkage is an extension of the "guard hypothesis" that effectors elicit the HR in resistant plant hosts because the activity of the effector on a guarded susceptibility target (rather than the presence of the effector per se) has been detected by a corresponding R protein (Dangl and Jones 2001; Van der Biezen and Jones 1998). A pertinent example is the action of AvrRpt2 on the *Arabidopsis* RIN4 protein, which is a regulator of basal resistance that is "guarded" by the RPS2 R protein (Kim et al. 2005). However, AvrE1 and HopM1 contribute to cell death in the compatible host tomato in the absence of a corresponding R protein. Nevertheless, the general concept of the guard hypothesis would apply if plant cells were able to guard basal resistance pathways by sensing perturbations in pathway flux in addition to modifications of guarded components (Van der Biezen and Jones 1998). According to this model, tomato leaves producing AvrE1 at high levels through *Agrobacterium*-mediated transient expression would sense strong suppression of the basal resistance system and, consequently, trigger cell death as we observed here. On the other hand, AvrE1 delivered at much lower levels by DC3000 during infection would perturb basal resistance insufficiently unless another suppressor, such as HopM1, also was delivered. The ability of strains with mutations in either *avrE1* or *hopM1* to grow to high populations in planta without eliciting plant cell death is consistent with the notion of a general guarding of basal resistance pathways that is quantitative rather than absolute in nature. It also is important to note here that plants impaired in their ability to activate programmed cell death are less susceptible to virulent strains of *P. syringae* (Lincoln et al. 2002; Richael et al. 2001; Stone et al. 2000), and any model also must accommodate these observations. Our results suggest that AvrE1 and HopM1 will be particularly useful research targets for unraveling the relationship among basal resistance suppression, parasitic growth, and plant cell death in *P. syringae*-plant interactions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains DH5 α , TOP10, and DB3.1 (Invitrogen, Carlsbad, CA, U.S.A.) were used for general cloning and Gateway technology manipulations, respectively. The $\Delta\text{hrcQbRSTU}::\text{Sp}^r$ ($\Delta\text{hrcQ-U}$) DC3000 mutant CUCPB5113 was used as a TTSS-deficient control rather than

Fig. 7. Analysis of the ability of the *Pseudomonas syringae* pv. *tomato* DC3000 *avrE1*/*hopM1* mutant to deliver AvrPto₍₁₋₁₀₀₎-Cya into nonhost *Nicotiana benthamiana* and host tomato and to elicit a Pto-dependent hypersensitive response (HR) in tomato. **A**, AvrPto₍₁₋₁₀₀₎-Cya translocation by DC3000 wild-type and mutant strains into *N. benthamiana*. Leaves were syringe infiltrated with the indicated strains at 1×10^8 CFU/ml and assayed 6 h later for cAMP accumulation indicative of translocation of AvrPto₍₁₋₁₀₀₎-Cya for samples taken with a 0.8-cm-diameter cork borer. The values presented are the mean and standard error for three leaves. **B**, AvrPto₍₁₋₁₀₀₎-Cya translocation by DC3000 wild-type and mutant strains into PtoS tomato cv. MoneyMaker. Leaves were syringe infiltrated with the indicated DC3000 wild-type and mutant strains at 1×10^8 CFU/ml and 2×10^7 CFU/ml and assayed for cAMP accumulation 6 and 9 h later, respectively. The values represent the mean and standard error for three leaves. **C**, HR elicitation in PtoR tomato leaves by DC3000 wild-type and mutant strains. Leaves were inoculated as in B with the indicated strains at 2×10^7 CFU/ml and photographed at the indicated hours after inoculation. The inoculated areas are marked with a black line if no tissue collapse was observed, a dotted red line if partial collapse was observed, and a solid red line if complete collapse was observed. **D**, Electrolyte leakage from tomato PtoR tissue inoculated with DC3000 wild-type and mutant strains. Leaf disks were excised from infiltrated areas at the indicated time and assayed for electrolyte leakage with a 0.7-cm-diameter cork borer. The conductivity values represent the mean and standard error from three different leaves, and all experiments were repeated at least three times with similar results. Values denoted with the same letter are not statistically different (analysis of variance, $P < 0.0001$, Tukey's Studentized Range Test). No significant difference was observed among values at 0 days.

an *hrcC* mutant because of the potential confounding effects of *hrcC* mutations on Hrp regulation through changes in expression of the *hrpV* regulator downstream in the same operon (Preston et al. 1998). *E. coli* and *A. tumefaciens* were grown in Luria-Bertani (LB) (Hanahan 1985) at 37 and 30°C, respectively. *P. syringae* cells were grown in King's B (KB) (King et al. 1954) at 30°C. For *E. coli* and *P. syringae*, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 20 µg/ml; kanamycin, 50 µg/ml; rifampicin, 50 µg/ml; spectinomycin, 50 µg/ml; and tetracycline, 10 µg/ml. Because *P. fluorescens* 55 is resistant to spectinomycin and low levels of tetracycline, plasmids were maintained in this strain by using streptomycin at 100 µg/ml and tetracycline

at 40 µg/ml. For *A. tumefaciens* GV3101, antibiotics were used at the following concentrations: rifampicin, 50 µg/ml and gentamicin, 15 µg/ml, to select for the Ti plasmid (Weigel and Glazebrook 2002). The binary vector pLN314 was maintained in this strain by using 40 µg/ml of chloramphenicol.

Recombinant DNA techniques.

DNA manipulations and PCR were performed according to standard procedures (Innis et al. 1990; Sambrook et al. 1989). Oligonucleotide primers for sequencing or PCR were purchased from Integrated DNA Technology (Coralville, IA, U.S.A.). PCR was performed with either ExTaq (Takara Bio, Inc., Otsu, Shiga, Japan) or Deep Vent polymerase (New England Bio-

Table 1. Strains and plasmids used in this study

Designation	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i> strains		
DH5α	<i>SupE44 ΔlacU169 (f80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> , Nx ^r	Life Technologies
TOP10	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen
DB3.1	F ⁻ <i>gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 λ⁻ leu mtl-1</i>	Invitrogen
<i>Agrobacterium tumefaciens</i>		
GV3101	Wild type, Rif ^r Gm ^r	Weigel and Glazebrook 2002
<i>P. fluorescens</i> 55	Wild type, Nx ^r	Huang, H.-C. 1988
<i>P. syringae</i> pv. <i>tomato</i> strains		
DC3000	Wild type, Rif ^r	Cuppels 1986
CUCPB5106	<i>ΔhopAA1-1::nptII</i> , Rif ^r Km ^r	Badel et al. 2002
CUCPB5113	<i>ΔhrcQbRSTU::Sp^r</i>	W.-L. Deng
CUCPB5115	<i>ΔCEL::ΩSp/Sm^r</i> , Rif ^r Sp ^r	Alfano et al. 2000
CUCPB5368	<i>ΔhopM1::nptII</i> , Rif ^r Km ^r	Badel et al. 2003
CUCPB5373	<i>ΔhopAA1-1::nptII ΔhopM1::aadA1</i> , Rif ^r Km ^r Sp ^r	This study
CUCPB5374	<i>ΔavrE</i> , Rif ^r	This study
CUCPB5376	<i>ΔhopAA1-1::nptII ΔavrE</i> , Rif ^r Km ^r	This study
CUCPB5377	<i>ΔhopM1::nptII, ΔavrE</i> , Rif ^r Km ^r	This study
CUCPB5380	<i>ΔhopAA1-1::nptII ΔhopM1::aadA1 ΔavrE</i> , Rif ^r Km ^r Sp ^r	This study
Plasmids		
pLN18	<i>ΔhopAI-shcA</i> derivative of pHIR11, Tc ^r Km ^r	Jamir et al. 2004
pCPP3221	pVLT35 expressing AvrPto1 ₍₁₋₁₆₄₎ -Cya, Sp ^r /Str ^r	Schechter et al. 2004
pCPP3234	pVLT35 containing Gateway Reading Frame B cassette and codons 2-406 of <i>cya</i> , Sp ^r /Str ^r , Cm ^r	Schechter et al. 2004
pCPP3297	pLN18 carrying an unmarked deletion in <i>hrcC</i>	Schechter et al. 2004
pCPP5244	pCPP3234 expressing AvrE ₍₁₋₃₁₅₎ -Cya	This study
pCPP5245	pCPP3234 expressing open reading frame (ORF)2-Cya	This study
pCPP5246	pCPP3234 expressing AvrE1-Cya	This study
pBluescriptII SK or KS	ColE1 <i>mcs-lacZ</i> , Ap ^r	Stratagene
pRK415	Broad-host-range vector unstable in the absence of selection, Tc ^r	Keen et al. 1988
pCPP54	pCPP33 derivative carrying <i>SacB</i> and <i>SacR</i> from pUM24, Tc ^r	D. W. Bauer, Cornell University
pJQ200SK	Suicide vector with <i>lacZα</i> carrying <i>sacB</i> , Gm ^r	Quandt and Hynes 1993
pLN314	pCAMBIA1201 containing Gateway cassette, Cm ^r	James R. Alfano, University of Nebraska
pCPP5247	pLN314 expressing HopQ1	This study
pCPP5248	pLN314 expressing AvrE1	This study
pCPP2357	Cosmid clone, pCPP47 carrying <i>hrcJ</i> to <i>hrpW1</i> of the DC3000 Hrp pathogenicity island, Tc ^r	Alfano et al. 2000
pCPP3016	Cosmid clone, pCPP47 carrying 5' end of <i>avrE</i> to conserved effector locus (CEL) edge of the <i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 Hrp pathogenicity island, Tc ^r	Alfano et al. 2000
pCPP3271	pBluescript II SK carrying 2.2-kb <i>KpnI-XhoI</i> fragment that contains 3' end <i>avrE1</i> , ORF2 and the 3' end <i>hopM1</i> , Ap ^r	This study
pCPP3278	pRK415 carrying a 2.2-kb fragment containing 5' end <i>hopM1</i> , <i>shcM</i> , <i>hopAA1-1</i> and 5' end <i>hrpW1</i> , Tc ^r	This study
pCPP3279	pCPP3278 with <i>aadA1</i> terminatorless cassette inserted into <i>HindIII</i> site, Tc ^r Sp ^r	This study
pCPP3280	pCPP3271 carrying <i>BamHI-HindIII</i> fragment from pCPP3279, Ap ^r Sp ^r	This study
pCPP3281	pRK415 carrying <i>BamHI-KpnI</i> fragment from pCPP3280, Tc ^r Sp ^r	This study
pCPP3282	pCPP54 carrying <i>BamHI-SacI</i> fragment from pCPP3281, Tc ^r Sp ^r	This study
pCPP2436	5.5-kb <i>EcoRI</i> -fragment containing <i>P. syringae</i> pv. <i>tomato</i> DC3000 Hrp pathogenicity island region 3' end of <i>hrpS</i> to 5' end of <i>avrE1</i> in pBluescript SK+, Ap ^r	This study
pCPP3283	pJQ200SK carrying 1.8-kb fragment containing 5' end <i>avrE</i> and upstream sequence and 2.2-kb fragment containing 3' end <i>avrE</i> and downstream sequence, Gm ^r	This study
pCPP5044	pBluescriptII SK carrying <i>aadA1</i> from pH45ΩSp ^r /Str ^r , Ap ^r	Badel et al. 2002

^a Nx^r, Rif^r, Gm^r, Km^r, Sp^r, Tc^r, Str^r, Cm^r, and Ap^r = nalidixic acid, rifampicin, gentamicin, kanamycin, spectinomycin, tetracycline, streptomycin, chloramphenicol, and ampicillin resistant, respectively.

labs, Beverly, MA, U.S.A.). All DNA sequencing was done at the Cornell Bioresource Center with an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). DNA sequences were analyzed with the Vector NTI software package (Infomax; Invitrogen). Database searches were performed using gapped BLASTN, BLASTP, and BLASTX (Altschul et al. 1997).

Construction of plasmids.

All gene expression plasmids in this study were created by using Gateway cloning technology (Invitrogen). To make the plasmid-expressing Cya-fusion proteins for the adenylate cyclase assay, full-length ORF2 and the first 945 nucleotides of *avrE1* were PCR amplified from the DC3000 genomic DNA with Deep Vent polymerase (New England Biolabs) by using the upstream primers 5'-CACCATGACGATGAAAACCTTCGC AAC-3' and 5'-CACCGTGCAGTCACCATCGATCCA-3' and the downstream primers 5'-AGCCTGTAAAAAGCACGC GC-3' and 5'-CATTTTCACGGCCGGTGGTTTCTTTCC-3'. PCR products were cloned into the pENTR/SD/D-TOPO vector (Invitrogen) according to the manufacturer's directions. The site-specific recombination reaction (or LR reaction) transferred entry clones into pCPP3234, which is derived from the broad-host-range vector pVLT35 and contains a tac promoter to express insert genes and generates hybrid proteins with a C-terminal Cya fusion (Schechter et al. 2004). Based on semiquantitative translocation assays involving AvrPto-Cya, the tac promoter is expressed in infected plants without IPTG induction at a level approximating the AvrPto native promoter (data not shown). For the complementation test of *avrE1*, full-length *avrE1* was amplified from DC3000 genomic DNA using the same upstream primers described above and the downstream primers 5'-GCTCTTCAGTTCGAACCCCTC-3' and, again, the PCR product was cloned into the pENTR/SD/D-TOPO vector (Invitrogen) to enable recombining the *avrE1* fragment into pCPP3234. For the transient gene expression experiment in plant cells, *avrE1* and *hopQ1* (pCPP3373) (Schechter et al. 2004) were recombined into pLN314, which was modified to be a Gateway destination vector based on pCAMBIA1201 and has a C-terminal HA epitope tag (kindly provided by James R. Alfano), to obtain pCPP5248 and pCPP5247, respectively. All expression constructs were confirmed by sequence analysis.

Agrobacterium-mediated transient expression.

A. tumefaciens strain GV3101 carrying pCPP5247, pCPP5248, and pLN314 was grown at 30°C in LB supplemented with chloramphenicol at 40 µg/ml, gentamicin at 15 µg/ml, and rifampicin at 50 µg/ml to stationary phase. Bacteria were sedimented by centrifugation at 4,000 rpm for 10 min at room temperature and washed and resuspended in 10 mM morpholinoethanesulfonic acid (MES) (pH 5.5) and 100 µM acetosyringone to a final optical density at 600 nm (OD₆₀₀) of 0.1, 0.2, and 0.5. Cells were infiltrated into abaxial air spaces of *N. tabacum* cv. Xanthi and tomato cv. Moneymaker leaves using a 1-ml blunt syringe. Plants inoculated with bacteria were incubated at 25°C with 12 h of daily illumination.

Construction of *P. syringae* pv. *tomato avrE1* and CEL effector polymutants.

To create the Δ *hopM1*/ Δ *hopAA1-1* mutant, a 2.2-kb fragment carrying the 5' end of *hopM1*, *shcM*, and *hopAA1-1* and the 5' end of *hrpW* was PCR amplified from pCPP2357 using primers 5'-ACAGTATCAAGCTTCTGGTTTA-3' and 5'-GCGGTCGGGATCCATCTGTGAG-3' with restriction sites for *Hind*III and *Bam*HI (underlined), respectively, and cloned into the same sites of pRK415 to obtain pCPP3278. The

aadA1 cassette lacking transcriptional termination signals from pCPP5044 was ligated into the *Hind*III site of pCPP3278 to make pCPP3279. The approximately 3.5-kb *Bam*HI-*Hind*III fragment obtained from pCPP3279 after complete digestion with *Bam*HI and partial digestion with *Hind*III was subcloned into the same sites of pCPP3271 to make pCPP3280. The *Bam*HI-*Kpn*I fragment carrying the antibiotic resistance cassette flanked by *P. syringae* pv. *tomato* DNA from pCPP3280 was inserted into pRK415 to obtain pCPP3281 and then cloned as a *Bam*HI-*Sac*I fragment into pCPP54 to obtain pCPP3282. The mutated *hopM1* in pCPP3282 was marker-exchanged into the *P. syringae* pv. *tomato* Δ *hopAA1-1* mutant CUCPB5106 to obtain CUCPB5373.

To create *avrE1* deletions in wild-type DC3000, the single mutants Δ *hopAA1-1* and Δ *hopM1*, and the polymutant Δ *hopM1*/ Δ *hopAA1-1*, a 1.8-kb fragment carrying 59 bp of the 5' end of *avrE1* and upstream sequence, was PCR amplified from pCPP2436 using primers 5'-AGGGGTGAAGCTTGGC TGAATGATGC-3' and 5'-GTGCGCTTTTCAGGGCCCTT CAATAG-3' with restriction sites for *Hind*III and *Apa*I (underlined), respectively. A second 2.2-kb fragment carrying 958 bp of the 3' end of *avrE1* and downstream sequence was PCR amplified from pCPP3016 using primers 5'-CAGGCCCACT CAAGCTTCACAAACA-3' and 5'-AGCGACAAGCTGAGC TCCACGATTCC-3' with restriction sites for *Hind*III and *Sac*I (underlined), respectively. The digested fragments were ligated to pJQ200SK previously digested with *Apa*I and *Sac*I to create pCPP3283, and the deletion was introduced by marker exchange-*eviction* into *P. syringae* pv. *tomato* DC3000 wild type, CUCPB5106, CUCPB5368, and CUCPB5373 to obtain strains CUCPB5374, CUCPB5376, CUCPB5377, and CUCPB5380, respectively.

DNA gel blots.

Replacement of *hopM1* with the *aadA1* cassette in CUCPB5373 was confirmed by probing *Hind*III-digested total DNA with the *Asc*I-*Bsr*GI *hopM1* fragment and *Eco*RI-digested total DNA with the PCR-amplified *aadA1* cassette. To confirm the unmarked *avrE1* deletions, total DNA of CUCPB5374, CUCPB5376, CUCPB5377, and CUCPB5380 was digested with *Xho*I and probed with a 2.0-kb *Pst*I fragment internal to *avrE1*. Digested DNA (4 µg) was transferred to Immobilon-N membrane (Millipore Co., Bedford, MA, U.S.A.) and blots were hybridized at 50°C overnight in HYB-9 DNA hybridization solution (GENTRA Systems, Research Triangle Park, NC, U.S.A.) with the indicated probes labeled with ³²P by using the Prime-It II kit (Stratagene). The membranes were washed two times in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) followed by three washes in 2× SSC. Membranes were exposed to OMAT X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) for 2 to 3 h.

Plant materials and bacterial inoculations.

Tomato (*Lycopersicon esculentum* cv. Moneymaker and *L. esculentum* cv. Rio Grande-PtoR), *N. benthamiana*, and *N. tabacum* cv. Xanthi plants were grown in Cornell soil mix under greenhouse conditions. Plants were moved to the laboratory approximately 24 h before inoculation with bacterial suspensions. For virulence and HR assays in tomato and *N. benthamiana*, bacteria grown on KB plates for 2 days at 30°C were prepared for inoculation by suspension in 10 mM MgCl₂ or 10 mM MES, pH 5.5. Bacterial cells in 10 mM MgCl₂ or 10 mM MES typically were infiltrated into tomato leaves using a blunt syringe as described previously (Gopalan et al. 1996). Inoculated plants were maintained under high humidity conditions in an incubator at 25°C with 12 h of light and 12 h of darkness and monitored daily for symptom development, bacterial mul-

tiplication, and HR reactions. An alternative bacterial virulence and growth assay was done by dipping the plants for 30 s into a 10-mM MgCl₂ solution containing 0.02% Silwet and the bacterial inoculum. Sampling time points and the level of bacterial inoculum differed between experiments and are noted in the figures or legends. For adenylate cyclase assays, bacteria typically were adjusted to an OD₆₀₀ of 0.3 (1 × 10⁸ CFU/ml) in 5 mM MES, pH 5.5, and 100 μM IPTG to induce expression of *cya* fusions. The areas of infiltration were marked lightly on the underside of leaves. During these experiments, plants were maintained in the lab at approximately 24°C with 12 h of illumination.

Electrolyte leakage assay.

Electrolyte leakage assays were performed as described previously (López-Solanilla et al. 2004). Three or four leaf samples of infiltrated *N. benthamiana* or tomato PtoR leaf tissue were removed using a boring tool with an inner diameter of 0.7 cm at indicated times. Samples were put into a glass test tube with 10 ml of distilled water and infiltrated with water by pulling a vacuum five times using a 30-ml syringe connected to the tube via a rubber stopper with a hole for the tip of the syringe. Plant material was removed after shaking for 1 h at 200 rpm at room temperature, and the sample conductivity was measured using a YSI model 3100 Conductivity Instrument with a YSI 3252 probe (YSI Incorporated, Yellow Spring, OH, U.S.A.).

Adenylate cyclase assay.

Cya assays were performed as described previously (Schechter et al. 2004). Bacterial strains carrying plasmids expressing Cya fusions were infiltrated into tomato or *N. benthamiana* leaves using a blunt syringe. Leaf disks were collected between 4 and 9 h later with a 1.0-cm-diameter cork borer, ground to a powder using a plastic pestle, and placed in 250 μl of 0.1 M HCl. cAMP levels were determined using the Correlate-EIA cAMP immunoassay kit according to the manufacturer's directions (Assay Designs Inc, Ann Arbor, MI, U.S.A.).

Statistical analysis.

The statistical comparisons of bacterial populations recovered from inoculated tissues were performed by applying the options PROC GLM and TUKEY to the log₁₀ transformation of CFU/cm² using SAS (version 8.2 for Windows; SAS Institute Inc., Cary, NC, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Pseudomonas-Plant Interaction *Pseudomonas syringae* website:
pseudomonas-syringae.org
 NCBI BLAST website: www.ncbi.nlm.nih.gov/BLAST/
 CAMBIA website: www.cambia.org
 The Sanger Institute's Artemis comparison tool:
www.sanger.ac.uk/Software/ACT/