The *Pseudomonas syringae* pv. *tomato* DC3000 Type III Effector HopF2 Has a Putative Myristoylation Site Required for Its Avirulence and Virulence Functions

Alexandre Robert-Seilaniantz, Libo Shan, Jian-Min Zhou, and Xiaoyan Tang

Department of Plant Pathology, Kansas State University, Manhattan 66506-5502 U.S.A.

Submitted 29 July 2005. Accepted 10 October 2005.

The *HopPtoF* locus in *Pseudomonas syringae* pv. *tomato* DC3000 harbors two genes, *ShcF* and *HopF2* (previously named *ShcF_{Pto}* and *HopF_{Pto}*), that encode a type III chaperone and a cognate effector protein, respectively. The *HopF2* gene has a rare initiation codon, ATA that was reported to be functional only in mitochondrial genes. Here, we report that the native *HopPtoF* locus of DC3000 confers an avirulence function in tobacco W38 plants, indicating that the ATA start codon directs the synthesis of a functional effector. However, disruption of *HopF2* in DC3000 genome did not alter the bacterial virulence in tomato plants. The *HopPtoF* locus displayed a measurable virulence activity in two strains of *P. syringae* pv. *tomato* when the ATA start codon was changed to ATG, and this change also elevated the avirulence function in W38 plants. *HopF2* contains a putative myristoylation site. Mutational analysis indicated that this site is required for plasma membrane localization and virulence and avirulence activities of *HopF2*.

Pseudomonas syringae pv. *tomato* DC3000 is the causal agent of bacterial speck diseases in tomato and *Arabidopsis* (Cuppels 1986; Whalen et al. 1991). It causes the hypersensitive response (HR) on nonhost plants such as tobacco and soybean and on tomato plants carrying the disease resistance gene *Pto* (Fouts et al. 2003; Kim et al. 2002; Lorang and Keen 1995). Like many other phytopathogenic bacteria, the ability of DC3000 to cause diseases and HR relies on the type III secretion system (TTSS) encoded by a cluster of *hrp* genes (Collmer et al. 2000). Bacteria inject a suite of type III effectors through the TTSS into the host cell (Cornelis and Van Gijsegem 2000; Galan and Collmer 1999). The complete genome sequence of DC3000 combined with various functional genomics studies enabled the identification of more than 40 type III effector genes in DC3000 (Buell et al. 2003; Chang et al. 2005; Fouts et al. 2002; Guttman et al. 2002; Lindeberg et al. 2005; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004; Zwiesler-Vollick et al. 2002).

In contrast to the large number of effector genes identified in DC3000, only a small number have been demonstrated through mutagenesis to have measurable effect on bacterial fitness such as bacterial multiplication, symptom development, or colony formation (Badel et al. 2002, 2003; Bretz et al. 2003; Espinosa et al. 2003; Lin and Martin 2005; Lopez-Solanilla et al. 2004), suggesting redundancy of effector proteins in DC3000 pathogenicity. Four effector mutant strains of DC3000 are reduced in

their ability to induce the expression of an *Arabidopsis* gene in susceptible interactions (He et al. 2004). A number of DC3000 effectors interfere with plant cell death when delivered individually into plant cells by *P. fluorescens* or directly expressed in plant via the *Agrobacterium*-mediated transient gene expression (Abramovitch et al. 2003; Jamir et al. 2004). Similarly, direct expression of several effectors was shown to repress the flagellin-induced expression of the *Arabidopsis* *NHO1* gene that is required for nonhost resistance (Li et al. 2005). The AvrPto protein has been shown to suppress the cell wall-based defense response and promote the growth of nonpathogenic bacteria when expressed in *Arabidopsis* transgenic plants (Hauck et al. 2003). In addition, HopM1 and AvrE suppress salicylic acid-dependent basal defense (DebRoy et al. 2004). The ability of DC3000 effectors to interfere with various plant defenses is crucial to bacterial parasitism.

In addition to the role in bacterial virulence, two effectors, AvrPto and AvrPtoB, trigger the HR and disease resistance in tomato plants carrying the disease resistance gene *Pto*. The activation of HR and disease resistance in tomato plants is mediated by physical interaction of AvrPto or AvrPtoB with the Pto protein (Kim et al. 2002; Tang et al. 1996). AvrE elicits HR on nonhost soybean plants carrying the corresponding *R* gene (Lorang and Keen 1995). DC3000 also induces HR on tobacco plants, but the effector proteins responsible for this phenotype have not been defined.

Here, we report the avirulence and virulence functions of the DC3000 type III effector HopF2. HopF2 is homologous to AvrPphF, a *P. syringae* pv. *phaseolicola* effector that specifies resistance in bean plants carrying the *R1* disease resistance gene but promotes virulence in the plant lacking the resistance gene (Tsiamis et al. 2000). The *HopF2* gene is preceded by *ShcF* encoding the chaperone of HopF2, and the two genes are organized into an operon (Shan et al. 2004). The *HopF2* gene carries an unusual translation initiation codon, ATA that was reported to be functional in mitochondrial genes of vertebrate, invertebrate, yeast, *Leishmania*, and *Paramecium*. ATA was proposed to act as start codon based on analyses of mitochondrial genomic DNA sequence, 5' sequence of mitochondrial RNAs, and alignment of RNA or DNA sequences with known mitochondrial proteins (Anderson et al. 1981; Boore and Brown 1994; Montoya et al. 1981). In this study, we found that the *HopF2* gene with the ATA start codon can induce an HR on tobacco W38 plants. When the ATA start codon was changed to ATG, the *ShcF-HopF2* operon displayed a virulence function in tomato plants. The HopF2 protein has a putative N-terminal myristoylation site that is required for the HopF2 protein localization to the plant plasma membrane and avirulence and virulence functions.

Corresponding author: Xiaoyan Tang; E-mail: xtang@ksu.edu

RESULTS

HopF2 has an avirulence function in tobacco W38 plants.

Because mutagenesis of individual effectors in DC3000 often fails to reveal the effector function, we sought to study the effector function using *Agrobacterium*-mediated transient gene expression. Eighteen effectors were expressed individually in tobacco W38 plants (Table 1). Among them, *HopF2* and *HopQ1-1* induced chlorosis and eventually death of the infiltrated leaf area (Fig. 1A). Other effectors did not induce a visible response. *HopF2* was further investigated in this study.

To determine whether *HopF2* plays a role in the elicitation of HR on tobacco, the *HopF2*⁻ knockout mutant (Jamir et al., 2004) was compared with the wild-type DC3000 strain for the HR-inducing activity on tobacco W38 plants. The leaf area infiltrated with the wild-type DC3000 exhibited complete necrosis 12 h after inoculation, but the area infiltrated with the *HopF2*⁻ mutant displayed only a few small dead spots at this time point (Fig. 1B). The complete collapse of the *HopF2*⁻ infiltrated leaf area occurred 18 h after inoculation. To verify that the delayed HR was caused by mutation of *HopF2* rather than an unknown gene, a complementation experiment was performed using a plasmid carrying the native *SchF-HopF2* locus driven by the native promoter (*pLK-hrp-SchF-HopF2*^{ATA}-HA) (Shan et al. 2004). This plasmid restored the HR-inducing activity of the *HopF2*⁻ mutant strain to the wild-type level (Fig. 1B). These results indicated that the *HopF2* gene in DC3000 is actively involved in the induction of nonhost HR on tobacco W38 plants, and that the initiation codon ATA can direct the synthesis of a functional protein with an avirulence function.

To corroborate the avirulence function of *HopF2*, the plasmid *pLK-hrp-SchF-HopF2*^{ATA}-HA was introduced into *P. syringae* pv. *tabaci* 11528, and the resulting strain was tested for HR-inducing activity and growth in tobacco W38 plants. Strain 11528 is a virulent pathogen to tobacco W38 plants (Shan et al. 2000a). Expression of *SchF-HopF2*^{ATA}-HA endowed the bacterium with an HR-inducing activity on W38 plants (Fig. 1D). Consistent with the HR-inducing activity, this bacterium also displayed weaker disease symptoms (Fig. 1E) and less bacterial growth than did the original 11528 strain on tobacco W38 plants (Fig. 1F). The HR-inducing activity was not observed when this bacterium was infiltrated into tobacco

Xanthi plants. Replacement of the ATA start codon with ATG in the *pLK-hrp-SchF-HopF2*^{ATG}-HA plasmid accelerated the HR induction by 3 to 4 h (Fig. 1C and D). Consistently, 11528 carrying *pLK-hrp-SchF-HopF2*^{ATG}-HA also displayed weaker disease symptoms (Fig. 1E) and lower bacterial growth (Fig. 1F) than did 11528 carrying *pLK-hrp-SchF-HopF2*^{ATA}-HA. The results demonstrated that the *HopF2* gene encodes an avirulence protein that triggers the HR and disease resistance on tobacco W38 plants, and that the avirulence activity was further elevated by replacing the ATA start codon with ATG.

The *HopF2* protein has a virulence function in tomato.

To determine whether *HopF2* protein has a virulence function, we tested whether mutation of *HopF2* in DC3000 affects disease symptoms and bacterial growth on tomato plants. When inoculated into PtoS tomato plants, the *HopF2*⁻ mutant did not show a clear difference from the wild-type DC3000 strain on either bacterial growth (Fig. 2A) or disease symptoms (data not shown). Because the *HopF2* gene in DC3000 has the ATA initiation codon that gives rise to a very low amount of protein (Shan et al. 2004), this gene may not bear a significant virulence. Thus, we tested whether an elevated level of *HopF2* protein could enhance the bacterial virulence. We introduced the *pLK-hrp-SchF-HopF2*^{ATG}-HA plasmid into DC3000 because replacement of start codon ATA by ATG significantly increases the *HopF2* protein production in bacterial cells (Shan et al. 2004). As a control, we also introduced the *pLK-hrp-SchF-HopF2*^{ATA}-HA plasmid into DC3000. The two transformed DC3000 strains as well as the wild-type DC3000 strain were inoculated into tomato PtoS plants. DC3000 carrying the *pLK-hrp-SchF-HopF2*^{ATG}-HA plasmid displayed approximately fivefold more bacterial growth (Fig. 2B) and twofold more disease specks (Fig. 3A) than did DC3000 carrying *pLK-hrp-SchF-HopF2*^{ATA}-HA. We also introduced *pLK-hrp-SchF-HopF2*^{ATG}-HA and *pLK-hrp-SchF-HopF2*^{ATA}-HA into *P. syringae* pv. *tomato* T1 strain that does not have the *HopPtoF* locus (L. Shan and X. Tang, unpublished result). Like that in DC3000, the *pLK-hrp-SchF-HopF2*^{ATG}-HA plasmid in T1 strain also increased the bacterial growth (by eightfold) (Fig. 2C) and the number of disease specks (by 2.8-fold) (Fig. 3B). A *t* test indicated that the differences in bacterial growth and disease lesions were significant (*P* > 0.01). The enhanced virulence associated with the *pLK-hrp-SchF-HopF2*^{ATG}-HA plas-

Table 1. DC3000 type III secretion system effectors tested in tobacco^a

Effector	Forward primer	Reverse primer	Enzymes	Necrosis
HopAI1 (PSPT00906)	aactcgagcgtatagaaacagcagaaacaac	ttttcgaagcagctccaggcgggtggcctc	XhoI/Csp45I	No
HopAQ1 (PSPT04703)	aactcgagcgtataaatgagactgcag	ttttcgaatgcactgccaccagcaatcgag	XhoI/Csp45I	No
HopQ1-2 (PSPT04732)	aactcgagcgtgtatcagcacttctc	ttttcgaatccgggactgcgctgac	XhoI/Csp45I	No
HopT1-2 (PSPT04593)	ttgtcgacgataagtgccagctgatttc	ttttcgaagcctaccgacgagcggctgaat	SalI/Csp45I	No
HopS1 (PSPT04597)	ttctcgagtctctgatgcgataaaaagag	ttttcgaagacctcccaagctctg	XhoI/Csp45I	No
HopS2 (PSPT04588)	aactcgaggccttcataaaaaagctg	ttttcgaagccagatgagctgccacag	XhoI/Csp45I	No
HopAS1 (PSPT00474)	aactcgagccgatggagccgctcatgaccttaag	ttttcgaagaaaaactcggtttctgttcaacctg	XhoI/Csp45I	No
HopJ1 (PSPT01179)	aactcgagcaacagggcggttcgtcaaggaaag	aaatcgataaccttgcgcttgagtgctgccttcgaatg	XhoI/ClaI	No
HopAA1-1 (PSPT01372)	aagtcgactgttcaacttccgtagaaacgagag	aaatcgatcgaccgatagccgaaacggtatttc	SalI/ClaI	No
HopC1 (PSPT00589)	aactcgagtcagggaactgaaccgcttatg	ttttcgaagtgtattttgaagcgaataactgaac	XhoI/Csp45I	No
HopF2 (PSPT00502)	ttctcgaggaaaccattatgggtaattttgc	ttttcgaagacctttcgaccggcactttc	XhoI/Csp45I	Yes
HopAF1 (PSPT01568)	ccgctcgagcggatgggctatgtatttc	ccctcgaaattgtgcgaccagatgttt	XhoI/Csp45I	No
HopK1 (PSPT00044)	ccgctcgagcggatgaatcgatttcaaccagc	ccctcgaaagcagtagagcgtgtcgcgac	XhoI/Csp45I	No
HopL1 (PSPT02872)	aactcgagaaactacagctctacacatgttg	ttttcgaatctcgtttgaagcgtggatgac	XhoI/Csp45I	No
HopQ1-1 (PSPT00877)	ccgctcgagcggatgcactgtctatcacc	ccctcgaaatctggggctaccgtcga	XhoI/Csp45I	Yes
HopU1 (PSPT00501)	ccgctcgagcggatgaatataaatacacaactg	ccctcgaaatctgacttaatacaataaatg	XhoI/Csp45I	No
HopO1-2 (PSPT04594)	ttctcgagacgtgtgtatgaatc	aaatcgattctgttcaaatcgacgtg	XhoI/ClaI	No
HopT1-1 (PSPT0A0019)	aactcgagtgactcatatgatgaaaacag	ttttcgaatgactttgagccgctgcctgac	XhoI/Csp45I	No

^a Effector genes were amplified from DC3000 genomic DNA by polymerase chain reaction, digested with corresponding enzymes, and cloned into *pER8-Flag* plasmid. Nomenclature of effectors was described by Lindeberg and associates (2005). The start codons of *HopF2* and *HopS2* were changed from ATA and GTG, respectively, to ATG. Primers for HopJ1 were designed based on old annotation that misses the first 36 amino acid residues. All the cloned genes were introduced into *Agrobacterium tumefaciens* GV3101 for transient expression in tobacco W38 plants. Effectors that induced necrosis on W38 plants are indicated.

mid was observed in numerous inoculation experiments. The higher virulence activity associated with the *pLK-hrp-ShcF-HopF^{ATG}-HA* plasmid was consistent with the higher level of HopF2 protein encoded by this plasmid and indicated that the HopF2 protein at a higher dosage has a virulence function.

The putative myristoylation site is required for the plant plasma membrane localization of HopF2.

The HopF2 protein is predicted to have an N-terminal eukaryotic consensus sequence (MGNIC) for fatty acylations. The G₂ residue is the potential myristoylation site, and C₅

Fig. 1. Avirulence function of HopF2 in tobacco. **A**, Induction of cell death by HopF2. *Agrobacterium* strains carrying the *pER8-Flag* empty vector (EV) and *pER8-HopF2-Flag* were infiltrated into tobacco W38 leaves at a concentration of 4×10^8 CFU/ml. After 2 days, the expression of HopF2 was induced by daily application of estradiol at 30 μ M. Leaf was photographed 7 days after estradiol application. **B**, Requirement of HopF2 for induction of nonhost hypersensitive response (HR) by DC3000. The wild-type DC3000 strain, HopF2⁻ knockout mutant, and HopF2⁻ mutant complemented with plasmid *pLK-hrp-ShcF-HopF^{ATA}-HA* were syringe infiltrated into W38 leaves at a concentration of 10^8 CFU/ml. Leaf areas infiltrated with the wild-type DC3000 (WT) and the complemented strain (HopF2⁻ + ShcF-HopF2) exhibited complete necrosis 12 h after inoculation. No necrosis was induced by the HopF2⁻ mutant at this time point. **C**, **D**, **E**, and **F**, Effect of the start codon and myristoylation site on HopF2 avirulence activity. Leaf panels were infiltrated with *Pseudomonas syringae* pv. *tabaci* strain 11528 (11528) at 1×10^8 CFU/ml, and 11528 strains carrying *pLK-hrp-SchF-HopF2^{ATA}-HA* (ATA), *pLK-hrp-SchF-HopF2^{ATG}-HA* (ATG), and *pLK-SchF-HopF2^{ATG}(G2A)-HA* (G2A). Leaves were photographed at **C**, 9 h and **D**, 13 h after inoculation to show the enhanced HR by HopF2 with ATG as start codon and diminished HR by G2A mutation. The same bacterial strains at 1×10^5 CFU/ml were injected into tobacco leaf panels for disease symptoms and bacterial growth studies. **E**, Disease symptoms were photographed 3 days after inoculation. **F**, The growth of bacteria strains was measured at 0, 2, and 3 days after inoculation. Each value represents the average of bacterial numbers from four 1-cm² leaf discs. Error bars indicate standard error. All the experiments were repeated at least three times with similar results.

residue is the potential palmitoylation site (Resh 1999). The effector proteins AvrB, AvrRpm1, and AvrPto containing these motifs are anchored to the plant plasma membranes (Nimchuk et al. 2000; Shan et al. 2000b). To determine the localization of HopF2 in plant cells, we replaced the ATA start codon with ATG, fused the *HopF2* gene in frame to the N-terminus of the green fluorescence protein (GFP) gene, placed the fusion construct under the CaMV 35S promoter in *pGFP* plasmid, and bombarded the plasmid DNA into onion epidermal cells to express the fusion protein. The green fluorescence of HopF2-GFP fusion was associated exclusively with the plasma membrane (Fig. 4A) and displayed the same localization pattern as that of the AvrPto-GFP fusion protein (Fig. 4B). The plasma membrane localization of HopF2-GFP and AvrPto-GFP also was observed in *Arabidopsis* leaf cells bombarded with the same plasmids (data not shown). Together, the results indicated that HopF2 is localized to the plasma membrane.

To determine whether the putative myristoylation site of HopF2 is important for the plasma membrane localization as seen in AvrB, AvrRpm1, and AvrPto, the HopF2 Gly₂ residue was replaced by Ala, and the mutant protein HopF2(G2A) was fused with GFP. The HopF2(G2A)-GFP fusion protein displayed cytoplasmic fluorescence in onion epidermal cells (Fig. 4C), and was similar to that of the GFP protein (Fig. 4D). The requirement of the myristoylation site for the plasma membrane localization of HopF2 suggests that, similar to AvrB, AvrRpm1, and AvrPto, HopF2 is acylated upon delivery into plant cells.

Requirement of the putative myristoylation site for HopF2 avirulence and virulence functions.

To determine whether the putative myristoylation site of HopF2 is functionally important, the G2A mutation was introduced to the *HopF2* gene in the *pLK-hrp-ShcF-HopF2^{ATG}-HA* plasmid, and the resulting *pLK-hrp-ShcF-HopF2^{ATG}(G2A)-HA* plasmid was introduced into *P. syringae* pv. *tabaci* 11528 and *P. syringae* pv. *tomato* T1 strains. G2A mutation did not affect the TTSS-dependent secretion of HopF2 from these strains (data not shown). When inoculated into W38 plants, 11528 carrying *pLK-hrp-ShcF-HopF2^{ATG}(G2A)-HA* was unable to induce an HR (Fig. 1D) and displayed much more disease symptoms (Fig. 1E) and greater growth (Fig. 1F) than did the strain carrying *pLK-hrp-ShcF-HopF2^{ATG}-HA*, indicating that the myristoylation motif is required for the avirulence function of HopF2. However, strain 11528 carrying *pLK-hrp-ShcF-HopF2^{ATG}(G2A)-HA* displayed weaker disease symptoms (Fig. 1E) and less bacterial growth (Fig. 1F) than did the original 11528 strain. This residual resistance probably is related to the excessive accumulation of HopF2(G2A). When inoculated into tomato PtoS plants, T1 strain carrying the *pLK-hrp-ShcF-HopF2^{ATG}(G2A)-HA* plasmid and the original T1 strain displayed similar levels of bacterial growth (Fig. 2C) and disease symptoms (Fig. 3B). The results indicated that the putative myristoylation motif is required for the virulence function of the HopF2 protein.

DISCUSSION

The sequence of bacterial genomes and functional genomics studies rapidly increased the list of type III effectors of the plant bacterial pathogens (Alfano and Collmer 2004; Buttner et al. 2003; Cunnac et al., 2004; Mukaihara et al., 2004). However, functional redundancy and interactions among effectors in eliciting plant responses render it difficult to define the function of individual effectors and, thus, curtail further studies of effector proteins. Using *Agrobacterium*-mediated tran-

Fig. 2. Effect of HopF2 on growth of *Pseudomonas syringae* pv. *tomato* DC3000 and T1 strains in tomato. *pLK-hrp-ShcF-HopF2^{ATA}-HA*, *pLK-hrp-ShcF-HopF2^{ATG}-HA*, and *pLK-ShcF-HopF2^{ATG}(G2A)-HA* were introduced into strains DC3000 and T1. The resulting bacterial strains were vacuum infiltrated into tomato Rio-Grande PtoS plants at a concentration of 1×10^4 CFU/ml. Growth of bacteria was measured at the indicated times. **A**, Knock-out of *hopF2* in DC3000 does not affect bacterial growth. **B**, HopF2^{ATG} enhances DC3000 bacterial growth. **C**, Effects of HopF2^{ATG} and HopF2^{ATG}(G2A) mutations on T1 bacterial growth. Each value represents the average of bacterial numbers from six 1-cm² leaf discs. Error bars indicate standard error. The experiments were repeated two times with similar results.

sient gene expression, we identified the cell death-inducing function of HopF2 protein in tobacco W38 plants. Further studies confirmed that the cell death-inducing activity resulted from the avirulence function of this protein in W38 plants. The identification of the avirulence function allowed us to further characterize the *HopF2* gene and protein.

The *HopF2* gene has a unique start codon, ATA. This start codon is so poor in initiating protein translation that the HopF2 protein barely can be detected in *P. syringae* using Western blot analysis (Shan et al. 2004). The activity of ATA was inferred from the chimeric gene between *HopF2* and partial *avrRpt2* that was able to induce an HR in *Arabidopsis* plants carrying the disease resistance gene *RPS2* (Shan et al. 2004). Here, we observed that knockout of *HopF2* in DC3000 reduced its HR-inducing activity on tobacco W38 plants, suggesting that the native *HopF2* gene is, indeed, functional. Complementation of the HR-inducing activity in *HopF2*⁻ knockout mutant and *P. syringae* pv. *tabaci* 11528 by *ShcF-HopF2*^{ATA} further supported the functionality of ATA in translation of the HopF2 protein.

Although *ShcF-HopF2*^{ATA} in DC3000 and *P. syringae* pv. *tabaci* 11528 strains elicits an HR in W38 plants, it could not

elicit a measurable virulence reaction in tomato even when the locus was expressed in DC3000 and T1 strains using a low-copy plasmid. The virulence function was observed only when the *HopF2* start codon ATA was replaced by ATG. We showed previously that substitution of ATA by ATG drastically increased the HopF2 protein production in *P. syringae* cells (Shan et al. 2004). The enhanced virulence displayed by DC3000 and the T1 strain expressing *ShcF-HopF2*^{ATG} but not *ShcF-HopF2*^{ATA} suggested that the effector protein has to reach a certain threshold to induce the virulence reaction, and that the protein produced by *HopF2*^{ATA} in the low-copy plasmid cannot reach the threshold. The expression of *ShcF-HopF2*^{ATG} in T1 and DC3000 strains elevated the bacterial growth by five- to eightfold and disease lesions by 2- to 2.8-fold. This virulence activity is modest, but it is highly reproducible and statistically significant. The small virulence effect is common among *P. syringae* type III effectors (Espinosa et al. 2003; Lim and Kunkel 2005; Lin and Martin 2005; Lorang et al. 1995; Shan et al. 2000a).

HopF2 displayed a virulence function not only in tomato but also in *Arabidopsis* and tobacco plants (Jamir et al. 2004; Li et al. 2005). In *Arabidopsis*, HopF2 suppresses the induc-

Fig. 3. Effect of HopF2 on disease symptoms on tomato. *pLK-hrp-SchF-HopF2*^{ATA}-HA, *pLK-hrp-SchF-HopF2*^{ATG}-HA, and *pLK-SchF-HopF2*^{ATG}(G2A)-HA were introduced into strains DC3000 and T1. The resulting bacterial strains were vacuum infiltrated into tomato Rio-Grande PtoS plants at a concentration of 1×10^4 CFU/ml. Disease symptoms were photographed 5 days after inoculation. **A**, Disease symptoms caused by DC3000, DC3000 carrying *pLK-hrp-SchF-HopF2*^{ATA}-HA, and DC3000 carrying *pLK-hrp-SchF-HopF2*^{ATG}-HA. **B**, Disease symptoms caused by T1, T1 carrying *pLK-hrp-SchF-HopF2*^{ATA}-HA, T1 carrying *pLK-hrp-SchF-HopF2*^{ATG}-HA, and T1 carrying *pLK-hrp-SchF-HopF2*^{ATG}(G2A)-HA.

tion of an innate immunity gene *NHOI* by flagellin when expressed directly in protoplast (Li et al. 2005) and blocks the HR elicited by type III effector HopPsyA when expressed in plant by *Agrobacterium*-mediated transient assay (Jamir et al. 2004). HopF2 also suppresses the HopPsyA-induced HR and Bax-induced program cell death in tobacco Xanthi plants (Jamir et al. 2004). The ability to suppress HR also was reported to the HopF2 homolog, AvrPphF, in *P. syringae* pv. *phaseolicola* strain (Tsiamis et al. 2000). AvrPphF triggers the HR and disease resistance on bean plants carrying the *R1* gene and suppresses HR and promotes virulence in certain bean cultivars that do not carry *R1* (Tsiamis et al. 2000). The mechanism by which AvrPphF acts as a virulence or avirulence factor in plant cells remains unknown. However, crystal structural analysis of AvrPphF identified four surface-exposed residues that are critical to both avirulence and virulence functions of AvrPphF (Singer et al. 2004), suggesting that the *R1* gene in bean is evolved to target the AvrPphF structure necessary for virulence. The four residues also are conserved in HopF2 protein. In addition, AvrPphF also carries a putative N-terminal myristoylation site. The high sequence similarity and conserved structural features between HopF2 and AvrPphF suggest that similar mechanisms in different plant species are involved in the virulence and avirulence functions of the two proteins.

The plant plasma membrane appears to be an important site targeted by bacterial type III effectors. To date, five type III effectors (AvrB, AvrRPM1, AvrPphB, AvrPto, and AvrRpt2)

were shown to be associated with the plant plasma membrane (Axtell and Staskawicz 2003; Nimchuk et al. 2000; Shan et al. 2000b). AvrRpt2 and AvrPphB probably carry an internal myristoylation site, and the rest carry an N-terminal myristoylation site (Mudgett and Staskawicz 1999; Nimchuk et al. 2000; Shan et al. 2000b). In addition to HopF2 and AvrPto, HopO1-1 of DC3000 also carries a putative N-terminal myristoylation site, and the fusion protein of HopO1-1 with GFP also is localized to the plant plasma membrane (L. Shan and X. Tang, *unpublished results*). The N-terminal myristoylation site is required for the plasma membrane localization and avirulence and virulence functions of AvrB, AvrRPM1, AvrPto, and HopF2 as well (Nimchuk et al. 2000; Shan et al. 2000b). The HopF2 mutant of the myristoylation site displayed residual avirulence activity in tobacco W38 plants. This residual activity probably is related to the overproduction of the HopF2(G2A) protein. A residual avirulence activity also was observed in AvrB and AvrRPM1 carrying the G2A mutation when the proteins are overexpressed (Nimchuk et al. 2000). It is possible that these mutant proteins can interact with the plasma membrane-associated targets by chance once they are overly accumulated in the plant cell cytoplasm. The virulence function, however, may require a higher threshold of HopF2 interaction with host target protein that was not met by the overproduction of HopF2(G2A) protein. Regardless, the results indicate that the putative myristoylation site plays an important role in both virulence and avirulence functions, likely through the association with the plasma membrane.

Fig. 4. Localization of HopF2 and HopF2(G2A) proteins in plant cells. *pHopF2*-green fluorescent protein (GFP), *pHopF2(G2A)*-GFP, *pavrPto*-GFP, and *pGFP* plasmids were bombarded into onion epidermal cells. The localization of **A**, HopF2-GFP, **B**, AvrPto-GFP, **C**, HopF2(G2A)-GFP, and **D**, GFP proteins was observed using a fluorescence microscope and documented with a charge-coupled device camera. The same image also was photographed under the light microscope. Arrows indicate cytoplasm and nucleus.

MATERIALS AND METHODS

Plant materials and bacterial strains.

Tomato Rio-Grande PtoS plants and tobacco W38 plants grown in a greenhouse were used for pathogenicity and HR assays. *Agrobacterium tumefaciens* GV3101 was used for transient expression of HopF2 in W38 plants. *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003), *P. syringae* pv. *tomato* T1, *P. syringae* pv. *tabaci* 11528 (Shan et al. 2000), and their derivatives were grown in King's B medium (KB) (King et al. 1954) at room temperature with appropriate antibiotics to approximately 2×10^9 CFU/ml before being harvested for pathogenicity and HR assays. Antibiotics were used at the following concentrations: rifampicin, 100 µg/ml; ampicillin, 100 µg/ml; spectinomycin, 50 µg/ml; and kanamycin, 50 µg/ml for *Escherichia coli* and 20 µg/ml for *P. syringae*.

Construction of plasmids.

Plasmids used in this study are listed in Table 2.

pER8-FLAG was modified from pER8 plasmid (Zuo et al. 2000) to facilitate cloning and FLAG-tagging of effector genes for *Agrobacterium*-mediated transient expression in tobacco. To generate the pER8-FLAG plasmid, a synthetic DNA fragment (5'-CTCGAGCCAGGGCCCTGGTTCGAAGACTACA AAGACCATGATGGAGACTATAAGGATCACGACATCG ATTACAAGGACGATGACGATAAGTGA~~ACTAGT~~-3') containing *XhoI*, *ApaI*, *BstXI*, *Csp45I*, *Clal*, and *SpeI* sites (underlined) and the 3xFLAG epitope sequence (bold) were cloned into the pER8 plasmid, replacing the *GFP* gene. Effector genes were amplified by polymerase chain reaction (PCR) from the DC3000 genomic DNA using primers in Table 1. The PCR products were digested with corresponding enzymes (Table 1) and cloned into pER8-FLAG plasmid. The sequence of effector genes was verified by sequencing analysis.

The *pLK-hrp-ShcF-HopF2^{ATA}-HA* and *pLK-hrp-ShcF-HopF2^{ATG}-HA* plasmids were generated in our previous studies (Shan et al. 2004). The QuickChange site-directed mutagenesis kit (Stratgene, La Jolla, CA, U.S.A.) and the complementary primers (mutation site in boldface) 5'-GGAAACCATATGG CTAATATTTGCGGC-3' and 5'-GCCGCAAATATTAGCCA TAATGGTTTCC-3' were used to generate the G2A mutation in *pLK-hrp-ShcF-HopF2^{ATG}-HA*. The mutation was confirmed by sequencing analysis.

The *pGFP* plasmid were constructed by modifying the *pGFPS65C* vector (Reichel et al. 1996) to facilitate the cloning and expression of effector-GFP fusion proteins in plant cells. The following modifications were made to the *pGFPS65C* vector. First, the *EcoRI* and *XhoI* sites were removed by *EcoRI* and *XhoI* digestion, the ends were filled in by Klenow, and the

plasmid was religated. The *BamHI* and *XbaI* sites then were removed similarly. The resulting plasmid was digested by *NcoI*, partially filled in with Klenow and dATP and dCTP, and ligated with a polylinker DNA containing multiple cloning sites formed by the two oligonucleotides 5'-TGTGAATTCTC TAGACTCGAGGGATC-3' and 5'-TGGATCCCTCGAGTCT AGAGAATTCA-3'. The resulting plasmid carrying *EcoRI*, *XbaI*, *XhoI*, *BamHI*, and *NcoI* sites at the N-terminus of GFP gene was confirmed by sequencing analysis and named *pGFP*.

To generate *pHopF2-GFP*, the coding region of *HopF2* was amplified using an upstream primer 5'-GCTCTAGAATGGGT AATATTTGCGGCACCTCG-3' carrying the *XbaI* site (bold) and the start codon ATG (underlined) and a downstream primer 5'-AAGGATCCGACCCTTTTCGACCGCA-3' carrying a *BamHI* site (bold). The PCR product was digested with *XbaI* and *BamHI* and cloned upstream of *GFP* in the *pGFP* plasmid. The QuickChange site-directed mutagenesis kit (Stratgene) and the complementary primers (5'-CTCTAGAA TGGCTAATATTTGCGGC-3' and 5'-GCCGCAAATATTAG CCATTCTAGAG-3') were used to generate the G2A mutation in the *pHopF2-GFP* plasmid, resulting in *pHopF2(G2A)-GFP* plasmid.

To express the AvrPto-GFP fusion protein, the coding region of *avrPto* was PCR amplified using an upstream primer 5'-GTCCATGGGAAATATATGTGTGCGCG-3' and a downstream primer 5'-ACCCATGGATTGCCAGTTACGGTACGG GC-3' (*NcoI* site in bold for both primers) from pPtE6 (Ronald et al. 1992). The PCR product was digested with *NcoI* and inserted into the *NcoI* site of the *pGFPS65C* vector (Reichel et al. 1996). Plasmid containing the *avrPto* gene in the desired orientation, named *pavrPto-GFP*, was identified by PCR and verified by sequencing.

Agrobacterium-mediated transient expression of type III effectors.

For transient expression of type III effectors, *Agrobacterium* strains carrying the corresponding effector constructs were grown overnight in Luria Bertani medium containing spectinomycin at 30 mg/liter. The bacteria then were transferred into the inducing medium (NH₄Cl at 1 g/liter, MgSO₄·7H₂O at 0.3 g/liter, KCl at 0.15 g/liter, CaCl₂ at 0.01 g/liter, FeSO₄·7H₂O at 2.5 mg/liter, 5 mM MES (morpholineethanesulfonic acid), glucose at 2.5 g/liter, and NaH₂PO₄ at 0.12 g/liter) containing 200 µM acetosyringone and grown overnight. The resulting culture was washed twice with 10 mM MES, resuspended in 10 mM MES containing 200 µM acetosyringone to a final concentration of 4×10^8 CFU/ml, and infiltrated into fully expanded tobacco leaves. Two days later, the infiltrated area was painted daily with 30 µM estradiol for 7 days to induce the expression

Table 2. Plasmids

Designation	Characteristics and application	Reference
pER8	Binary vector containing an estradiol inducible promoter from which the <i>pER8-Flag</i> plasmid was derived.	Zuo et al. 2000
<i>pER8-Flag</i>	Base vector for expression of FLAG-tagged effector proteins by <i>Agrobacterium</i> -mediated expression.	This study
<i>pER8-HopF2-Flag</i>	<i>pER8-Flag</i> containing the <i>HopF2</i> gene with ATG start codon for <i>Agrobacterium</i> -mediated expression of HopF2-FLAG.	This study
<i>pLK-hrp-ShcF-HopF2^{ATA}-HA</i>	pLK containing the native <i>schF-HopF2</i> operon with ATA as the start codon.	Shan et al. 2004
<i>pLK-hrp-ShcF-HopF2^{ATG}-HA</i>	Mutation of the start codon ATA to ATG in pLK-hrp-ShcF-HopF2 ^{ATA} -HA.	Shan et al. 2004
<i>pLK-hrp-ShcF-HopF2^{ATG}(G2A)-HA</i>	Mutation of Gly2 to Ala in pLK-hrp-ShcF-HopF2 ^{ATG} -HA.	This study
<i>pGFPS65C</i>	Plasmid containing the enhanced green fluorescent protein (<i>GFP</i>) gene under the 35S promoter from which the <i>pGFP</i> plasmid was derived.	Reichel et al. 1996
<i>pGFP</i>	Base vector for cloning and expressing effector-GFP fusion proteins in plant cells via bombardment.	This study
<i>pHopF2-GFP</i>	For transient expression of HopF2-GFP in plant cells. <i>HopF2</i> has ATG as start codon.	This study
<i>pHopF2(G2A)-GFP</i>	For transient expression of HopF2(G2A)-GFP in plant cells. <i>HopF2</i> has ATG as start codon.	This study
<i>pavrPto-GFP</i>	For transient expression of AvrPto-GFP fusion protein in plant cells.	This study

of the effector proteins. Leaf response was monitored daily until 7 days after the first estradiol application.

HR and bacterial growth assays.

P. syringae bacterial strains were cultured in liquid KB medium containing appropriate antibiotics overnight at room temperature with constant shaking. To prepare the inoculum, the bacterial cells were centrifuged at $4,000 \times g$, washed twice with sterile water, and resuspended in 10 mM $MgCl_2$. For HR assay, bacteria at 1×10^8 CFU/ml in 10 mM $MgCl_2$ were syringe infiltrated into fully expanded tobacco W38 leaves. The necrosis was examined visually every 2 h after inoculation till the experiment was finished. For assays of disease symptoms and bacterial growth in tobacco, *P. syringae* pv. *tabaci* 11528 and its derivatives at 1×10^5 CFU/ml were syringe infiltrated into fully expanded tobacco W38 leaves. For bacterial growth and disease symptom assays on tomato, DC3000, T1, and their derivative strains at 2×10^4 CFU/ml in 10 mM $MgCl_2$ plus Silwet L-77 at 30 μ l/liter were vacuum infiltrated into PtoS plants. Bacterial numbers were determined at the indicated times. Leaf discs 1 cm^2 in size were ground in sterile water, and the diluted bacteria were plated onto tryptic soy agar medium (Zhu et al. 1999) containing appropriate antibiotics to determine the colony forming units.

Protein localization assay in plant cell.

Localization of HopF2, HopF2(G2A), and AvrPto proteins in plant cells was determined by tagging the effector proteins to the N-terminus of GFP protein. Inner epidermal peels of white onion (*Allium cepa*) were placed inside-up on a 0.5x MS (Murashige and Skoog 1962) plate and bombarded with *pHopF2-GFP*, *pHopF2(G2A)-GFP*, *pavrPto-GFP*, or *pGFP* plasmid DNA-coated gold particles using a custom-made particle inflow gun (Finer et al. 1992). After bombardment, the plates were sealed with Parafilm and placed at room temperature in the dark for approximately 18 h to allow the expression of fusion proteins. Onion peels were mounted in water and viewed with the fluorescence microscope. Images were obtained using a charge-coupled device camera coupled to the microscope.

ACKNOWLEDGMENTS

A. Robert-Seilaniantz and L. Shan contributed equally to this article. This work was supported by the NSF Plant Genome Program through Cooperative Agreement DBI-0077622 to X. Tang. The Kansas State University Agricultural Research Experimental Station contribution number is 06-24-J. We thank J. Alfano for providing the HopF2 mutant strains, B. Friebe for the use of fluorescence microscope in protein localization assay, and J. Zuo and N.-H. Chua for providing the pER8 plasmid.

LITERATURE CITED

Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B., and Martin, G. B. 2003. *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO (Eur. Mol. Biol. Organ.) J. 22:60-69.

Alfano, J. R., and Collmer, A. 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. 42:385-414.

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. and Young, G. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Axtell, M. J., and Staskawicz, B. J. 2003. Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell 112:369-377.

Badel, J. L., Charkowski, A. O., Deng, W. L., and Collmer, A. 2002. A gene in the *Pseudomonas syringae* pv. *tomato* Hrp pathogenicity island conserved effector locus, hopPtoA1, contributes to efficient formation of bacterial colonies in planta and is duplicated elsewhere in the ge-

nome. Mol. Plant-Microbe Interact. 15:1014-1024.

Badel, J. L., Nomura, K., Bandyopadhyay, S., Shimizu, R., Collmer, A., and He, S. Y. 2003. *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. Mol. Microbiol. 49:1239-1251.

Boore, J. L., and Brown, W. M. 1994. Complete DNA sequence of the mitochondrial genome of the black chiton, *Katharina tunicata*. Genetics 138:423-443.

Bretz, J. R., Mock, N. M., Charity, J. C., Zeyad, S., Baker, C. J., and Hutcheson, S. W. 2003. A translocated protein tyrosine phosphatase of *Pseudomonas syringae* pv. *tomato* DC3000 modulates plant defence response to infection. Mol. Microbiol. 49:389-400.

Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M., and Collmer, A. 2003. The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Proc. Natl. Acad. Sci. U.S.A. 100:10181-10186.

Buttner, D., Noel, L., Thieme, F., and Bonas, U. 2003. Genomic approaches in *Xanthomonas campestris* pv. *vesicatoria* allow fishing for virulence genes. J. Biotechnol. 106:203-214.

Chang, J. H., Urbach, J. M., Law, T. F., Arnold, L. W., Hu, A., Gombar, S., Grant, S. R., Ausubel, F. M., and Dangel, J. L. 2005. A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. Proc. Natl. Acad. Sci. U.S.A. 102:2549-2554.

Collmer, A., Badel, J. L., Charkowski, A. O., Deng, W. L., Fouts, D. E., Ramos, A. R., Rehm, A. H., Anderson, D. M., Schneewind, O., van Dijk, K., and Alfano, J. R. 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. Proc. Natl. Acad. Sci. U.S.A. 97:8770-8777.

Cornelis, G. R., and Van Gijsegem, F. 2000. Assembly and function of type III secretory systems. Annu. Rev. Microbiol. 54:735-774.

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., and Genin, S. 2004. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: Identification of novel effector proteins translocated to plant host cells through the type III secretion system. Mol. Microbiol. 53:115-128.

Cuppels, D. A. 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. 51:323-327.

DebRoy, S., Thilmony, R., Kwack, Y. B., Nomuram, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proc. Natl. Acad. Sci. U.S.A. 101:9927-9932.

Espinosa, A., Guo, M., Tam, V. C., Fu, Z. Q., and Alfano, J. R. 2003. The *Pseudomonas syringae* type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. Mol. Microbiol. 49:377-387.

Finer, J. J., Vain, P., Jones, M. W., and McMullen, M. D. 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11:323-328.

Fouts, D. E., Abramovitch, R. B., Alfano, J. R., Baldo, A. M., Buell, C. R., Cartinhour, S., Chatterjee, A. K., D'Ascenzo, M., Gwinn, M. L., Lazarowitz, S. G., Lin, N. C., Martin, G. B., Rehm, A. H., Schneider, D. J., van Dijk, K., Tang, X., and Collmer, A. 2002. Genomewide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. Proc. Natl. Acad. Sci. U.S.A. 99:2275-2280.

Fouts, D. E., Badel, J. L., Ramos, A. R., Rapp, R. A., and Collmer, A. 2003. A *Pseudomonas syringae* pv. *tomato* DC3000 Hrp (type III secretion) deletion mutant expressing the Hrp system of bean pathogen *P. syringae* pv. *syringae* 61 retains normal host specificity for tomato. Mol. Plant-Microbe Interact. 16:43-52.

Galan, J. E., and Collmer, A. 1999. Type III secretion machines: Bacterial devices for protein delivery into host cells. Science 284:1322-1328.

Guttman, D. S., Vinatzer, B. A., Sarkar, S. F., Ranall, M. V., Kettler, G., and Greenberg, J. T. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. Science 295:1722-1726.

Hauck, P., Thilmony, R., and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc. Natl. Acad. Sci. U.S.A. 100:8577-8582.

- He, P., Chintamanani, S., Chen, Z., Zhu, L., Kunkel, B. N., Alfano, J. R., Tang, X., and Zhou, J. M. 2004. Activation of a COI1-dependent pathway in *Arabidopsis* by *Pseudomonas syringae* type III effectors and coronatine. *Plant J.* 37:589-602.
- Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and Alfano, J. R. 2004. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. *Plant J.* 37:554-565.
- Kim, Y. J., Lin, N. C., and Martin, G. B. 2002. Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. *Cell* 109:589-598.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Li, X., Lin, H., Zhang, W., Zuo, Y., Zhang, J., Tang, X., and Zhou, J.-M. 2005. Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc. Natl. Acad. Sci. U.S.A.* 102:12990-12995.
- Lim, M. T., and Kunkel, B. N. 2005. The *Pseudomonas syringae* *avrRpt2* gene contributes to virulence on tomato. *Mol. Plant-Microbe Interact.* 18:626-633.
- Lin, N. C., and Martin, G. B. 2005. An *avrPto/avrPtoB* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 does not elicit Pto-mediated resistance and is less virulent on tomato. *Mol. Plant-Microbe Interact.* 18:43-51.
- Lindeberg, M., Stavrinides, J., Chang, J. H., Alfano, J. R., Collmer, A., Dangel, J. L., Greenberg, J. T., Mansfield, J. W., and Guttman, D. S. 2005. Proposed guidelines for a unified nomenclature and phylogenetic analysis of type III Hop effector proteins in the plant pathogen *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 18:275-282.
- Lopez-Solanilla, E., Bronstein, P. A., Schneider, A. R., and Collmer, A. 2004. HopPtoN is a *Pseudomonas syringae* Hrp (type III secretion system) cysteine protease effector that suppresses pathogen-induced necrosis associated with both compatible and incompatible plant interactions. *Mol. Microbiol.* 54:353-365.
- Lorang, J. M., and Keen, N. T. 1995. Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato*: A hrp-linked avirulence locus consisting of at least two transcriptional units. *Mol. Plant-Microbe Interact.* 8:49-57.
- Montoya, J., Ojala, D., and Attardi, G. 1981. Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs. *Nature* 290:465-470.
- Mudgett, M. B., and Staskawicz, B. J. 1999. Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: Demonstration of secretion and processing during bacterial pathogenesis. *Mol. Microbiol.* 32:927-941.
- Mukaihara, T., Tamura, N., Murata, Y., and Iwabuchi, M. 2004. Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*. *Mol. Microbiol.* 54:863-875.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R. T., Katagiri, F., and Dangel, J. L. 2000. Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. *Cell* 101:353-363.
- Petnicki-Ocwieja, T., Schneider, D. J., Tam, V. C., Chancey, S. T., Shan, L., Jamir, Y., Schechter, L. M., Janes, M. D., Buell, C. R., Tang, X., Collmer, A., and Alfano, J. R. 2002. Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U.S.A.* 99:7652-7657.
- Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Koncz, C., Schell, J., Reiss, B., and Maas, C. 1996. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 93:5888-5893.
- Resh, M. D. 1999. Fatty acylation of proteins: New insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta* 1451:1-16.
- Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. 1992. The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J. Bacteriol.* 174:1604-1611.
- Schechter, L. M., Roberts, K. A., Jamir, Y., Alfano, J. R., and Collmer, A. 2004. *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. *J. Bacteriol.* 186:543-555.
- Shan, L., He, P., Zhou, J. M., and Tang, X. 2000a. A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol. Plant-Microbe Interact.* 13:592-598.
- Shan, L., Oh, H. S., Chen, J., Guo, M., Zhou, J., Alfano, J. R., Collmer, A., Jia, X., and Tang, X. 2004. The *HopPtoF* locus of *Pseudomonas syringae* pv. *tomato* DC3000 encodes a type III chaperone and a cognate effector. *Mol. Plant-Microbe Interact.* 17:447-455.
- Shan, L., Thara, V. K., Martin, G. B., Zhou, J. M., and Tang, X. 2000b. The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell* 12:2323-2338.
- Singer, A. U., Desveaux, D., Betts, L., Chang, J. H., Nimchuk, Z., Grant, S. R., Dangel, J. L., and Sondek, J. 2004. Crystal structures of the type III effector protein AvrPphF and its chaperone reveal residues required for plant pathogenesis. *Structure* 12:1669-1681.
- Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* 274:2060-2063.
- Tsiamis, G., Mansfield, J. W., Hockenhull, R., Jackson, R. W., Sesma, A., Athanassopoulos, E., Bennett, M. A., Stevens, C., Vivian, A., Taylor, J. D., and Murillo, J. 2000. Cultivar-specific avirulence and virulence functions assigned to *avrPphF* in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:3204-3214.
- Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3:49-59.
- Zhu, W., Yang, B., Wills, N., Johnson, L. B., and White, F. F. 1999. The C terminus of AvrXa10 can be replaced by the transcriptional activation domain of VP16 from the herpes simplex virus. *Plant Cell* 11:1665-1674.
- Zuo, J., Niu, Q. W., and Chua, N. H. 2000. Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* 24:265-273.
- Zwiesler-Vollick, J., Plovianich-Jones, A. E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B. N., and He, S. Y. 2002. Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. *Mol. Microbiol.* 45:1207-1218.

AUTHOR-RECOMMENDED INTERNET RESOURCE

NCBI Taxonomy Browser, genetic code webpage:
<http://www3.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c>