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The *HopPtoF* Locus of *Pseudomonas syringae* pv. *tomato* DC3000 Encodes a Type III Chaperone and a Cognate Effector

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Type III secretion systems are highly conserved among gram-negative plant and animal pathogenic bacteria. Through the type III secretion system, bacteria inject a number of virulence proteins into the host cells. Analysis of the whole genome sequence of *Pseudomonas syringae* pv. *tomato* DC3000 strain identified a locus, named *HopPtoF*, that is homologous to the avirulence gene locus *avrPphF* in *P. syringae* pv. *phaseolicola*. The *HopPtoF* locus harbors two genes, $ShcF_{Pto}$ and $HopF_{Pto}$, that are preceded by a single *hrp* box promoter. We present evidence here to show that $ShcF_{Pto}$ and $HopF_{Pto}$ encode a type III chaperone and a cognate effector, respectively. $ShcF_{Pto}$ interacts with and stabilizes the $HopF_{Pto}$ protein in the bacterial cell. Translation of $HopF_{Pto}$ starts at a rare initiation codon ATA that limits the synthesis of the $HopF_{Pto}$ protein to a low level in bacterial cells.

Many gram-negative bacterial pathogens of plants and animals harbor a specialized secretion system, termed type III secretion system (Galan and Collmer 1999). In plant bacterial pathogens, genes encoding the type III secretion system are called the hypersensitive response and pathogenicity (*hrp*) genes, because mutations in these genes abolish the induction of hypersensitive response (HR) on nonhost plants or resistant host plants and pathogenicity on host plants (He 1998; Lindgren 1997). Type III secretion system can secrete a group of proteins, termed Hrp-dependent outer (Hop) proteins, to the outside of the bacterial cell. Hop proteins bear the secretion signals at the N-terminus. The secretion signal sequences of different Hop proteins do not share significant homology but have unique biophysical properties that may lead the protein through the type III secretion pathway (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). Some Hop proteins are secreted into intercellular spaces that may act as the type III accessory proteins, whereas others are delivered inside the host cells (Galan and Collmer 1999). Hop proteins that are delivered into the host cells are designated type III effectors (Hueck 1998). Many type III effectors of plant bacterial pathogens were identified as avirulence (Avr) proteins because they induce HR and disease resistance on plants carrying the corresponding disease resistance (*R*) genes (Collmer 1998). The Avr proteins interact directly or indirectly with the corresponding R proteins in the host cells, leading to the activation of HR and disease resistance (Kim et al. 2002; Mackey et al. 2002; Scofield et al. 1996; Shao et al. 2003; Tang et al. 1996). A number of Avr proteins exhibit virulence functions on susceptible plants (White et al. 2000).

The function of certain type III effectors requires a partner protein termed type III chaperone (Bennett and Hughes 2000). Type III chaperones do not share sequence similarities, but they show common structural characteristics such as a small molecular mass, an acidic isoelectric point, and a predominantly helical secondary structure (Aldridge and Hughes 2001; Bennett and Hughes 2000). Most of the type III chaperones characterized to date are specific for a single effector protein, but some are associated with two or more effectors (Page and Parsot 2002). Chaperones often bind directly to the N-terminal portion of the secreted proteins. The loss of the chaperone often results in reduced stability of the partner protein in the bacterial cytoplasm (Fu and Galan 1998; Niebuhr et al. 2000) and sometimes aberrant secretion and translocation of the Hop proteins (Cambronne et al. 2000; van Dijk et al. 2002).

The complete genome sequence of *Pseudomonas syringae* pv. *tomato* DC3000 strain, a bacterial pathogen of tomato and *Arabidopsis thaliana*, enabled the identification of more than 30 putative type III effector genes (Collmer et al. 2002). Among them, the *HopPtoF* locus shows homology to the *avrPphF* locus of *P. syringae* pv. *phaseolicola*, a bean pathogen (Fouts et al. 2002; Zwiesler-Vollick et al. 2002). *avrPphF* is a plasmid-borne *avr* gene conferring specific resistance on bean cultivars carrying the *R1* disease resistance gene (Jackson et al. 1999; Tsiamis et al. 2000). *avrPphF* also confers cultivar-specific virulence on bean and soybean plants. In certain bean cultivars, *avrPphF* suppresses the HR caused by other *avr* gene. The *avrPphF* locus comprises two open reading frames (ORFs) that both are required for avirulence and virulence functions. The two ORFs are led by a single promoter containing the *hrp* box motif (Tsiamis et al. 2000). The proteins encoded by the *avrPphF* locus have not been characterized and, therefore, it is unclear how the two ORFs coordinate to condition the avirulence and virulence functions.

We report here that the DC3000 *HopPtoF* locus harbors two ORFs that are controlled by a single *hrp* box promoter. The first ORF encodes a molecular chaperone that interacts with the effector protein encoded by the second ORF and stabilizes the effector protein. We name the effector protein $HopF_{Pto}$ to reflect its nature as a Hop protein, homology to AvrPphF, and

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origin from *P. syringae* pv. *tomato*. The chaperone protein is designated $ShcF_{Pto}$ to indicate it as a specific Hop chaperone for $HopF_{Pto}$. The $HopF_{Pto}$ gene carries a rare initiation codon ATA that was reported to serve as a translation initiation site in mitochondria but not in bacterial cells (NCBI website). The use of ATA as the initiation codon limits the protein synthesis to a low level, and mutation of ATA to ATG of $HopF_{Pto}$ in the *HopPtoF* locus drastically increases the $HopF_{Pto}$ protein synthesis in bacterial cells.

RESULTS

Sequence characterization of the *HopPtoF* locus.

The *HopPtoF* locus (Fig. 1) of *P. syringae* pv. *tomato* DC3000 consists of two ORFs, $ShcF_{Pto}$ and $HopF_{Pto}$, that encode proteins homologous to ORF1 and ORF2 encoded by the *P. syringae* pv. *phaseolicola avrPphF* locus (Tsiamis et al. 2000). $ShcF_{Pto}$ is preceded by a Shine-Dalgarno (SD) sequence (–13GAGGAG–7) and a promoter carrying the *hrp* box motif. The sequence of $ShcF_{Pto}$ predicts a protein of 15 kDa with an isoelectric point 6.5. Secondary structure analysis using the PSIPRED program predicts a predominantly α-helical structure throughout the $ShcF_{Pto}$ protein and a C-terminal α-helical structure in particular (McGuffin et al. 2000). These characteristics often are observed in type III chaperones (Bennett and Hughes 2000). Compared with the *avrPphF ORF2* gene, a standard initiation codon ATG was not found in the $HopF_{Pto}$ gene. Instead, it has ATA at the predicted initiation site (Fig. 1). Gene annotation using the GLIMMER program (Salzberg et al. 1998) suggests that $HopF_{Pto}$ starts at GTG_{13}, 13 codons downstream of ATA_1. But GTG_{13} is not preceded by a SD sequence. In addition, homology between $HopF_{Pto}$ and the *avrPphF* ORF2 extends to the region between GTG_{13} and ATA_1. Seven bases upstream of ATA_1 is a typical SD sequence (-13AGAGGA-7). ATA can be used as the initiation codon in mitochondria but was not reported to act as a start codon in any bacteria. Downstream of GTG_{13}, there are eight in-frame ATGs.

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GGGAACCTGATGCTGCTCAGTGACCACTCATAACCTTAAATTATGAGGATA
TGAGGAGATGCATTATGAAAAACGCATTTGACCTGCTTGTGGAAGGGCTGG
        ShcF  M  K  N  A  F  D  L  L  V  E  G  L  A
CTAAGGACTACAACATGCCGCCCTTGCCTGACAAGAAACATATCGATGAAG
  K  D  Y  N  M  P  P  L  P  D  K  K  H  I  D  E  V
TCTATTGCTTTGAGTTTCAAAGTGGTATGAACGTAAAAGTATACCAAGACG
  Y  C  F  E  F  Q  S  G  M  N  V  K  V  Y  Q  D  E
AATTTCGCTGGGTATATTTCACCGCTGACGTTGGGACATTTCAAGATAGCA
  F  R  W  V  Y  F  T  A  D  V  G  T  F  Q  D  S  S
GTATTGACACATTAAACTACGCGCTCCAGCTGAACAACTTTAGCCTTAGAA
  I  D  T  L  N  Y  A  L  Q  L  N  N  F  S  L  R  K
AACCTTTCCTGACCTTCGGAATGACGAAGGAGAAAAATGGTGTATTGCATA
  P  F  L  T  F  G  M  T  K  E  K  N  G  V  L  H  T
CACGCACCCCCTTGATTGAGGTAGACAACGTGCAAATGCGCAGGATATTTG
  R  T  P  L  I  E  V  D  N  V  Q  M  R  R  I  F  E
AGGAGCTTATAGGCGTGGCAGGTGAAATCAGAAAAACACTAAAACTCAAAT
  E  L  I  G  V  A  G  E  I  R  K  T  L  K  L  K  *
AGTAGCGAGGAAGTTTTTTAGCCTTGTGAACTCTGGTAATGTCACATAAAT
GCATCAATATTACATGGGCACTGTACATACACCAGAGCGTCGGGCATAAAA
GAAGCAATCTGCAGTTTTTAAAAAGAGGAAACCATTATAGGTAATATTTGC
                              HopF  M  G  N  I  C
GGCACCTCGGGCTCACGTCATGTGTATAGCCCATCCCATACACAACGAATA
G  T  S  G  S  R  H  V  Y  S  P  S  H  T  Q  R  I
ACTTCAGCTCCCTCTACATCCACTCATGTTGGTGGAGATACACTGACATCC
T  S  A  P  S  T  S  T  H  V  G  G  D  T  L  T  S
ATTCATCAGCTTTCGCATAGTCAGAGAGAGCAGTTTCTGAACATGCATGAT
I  H  Q  L  S  H  S  Q  R  E  Q  F  L  N  M  H  D
CCAATGAGAGTAATGGGACTTGACCATGATACCGAGCTTTTCAGAACGACG
P  M  R  V  M  G  L  D  H  D  T  E  L  F  R  T  T
GATAGTCGCTATATAAAAAACGATAAACTCGCGGGCAATCCACAATCCATG
D  S  R  Y  I  K  N  D  K  L  A  G  N  P  Q  S  M
GCGAGTATCCTTATGCATGAAGAACTGCGCCCCAATCGTTTTGCCAGCCAT
A  S  I  L  M  H  E  E  L  R  P  N  R  F  A  S  H
ACAGGTGCCCAACCACACGAAGCAAGGGCGTACGTTCCGAAAAGAATAAAA
T  G  A  Q  P  H  E  A  R  A  Y  V  P  K  R  I  K
GCCACCGATCTAGGAGTTCCATCACTGAACGTAATGACTGGCTCGCTAGCG
A  T  D  L  G  V  P  S  L  N  V  M  T  G  S  L  A
CGAGACGGAATTAGAGCTTATGATCACATGAGTGATAATCAGGTCTCTGTC
R  D  G  I  R  A  Y  D  H  M  S  D  N  Q  V  S  V
AAAATGCGACTGGGAGATTTTCTCGAAAGGGGTGGCAAGGTCTATGCCGAC
K  M  R  L  G  D  F  L  E  R  G  G  K  V  Y  A  D
GCTTCGTCTGTAGCTGACGATGGGGAAACATCACAAGCTCTGATTGTCACA
A  S  S  V  A  D  D  G  E  T  S  Q  A  L  I  V  T
TTGCCCAAAGGACAGAAAGTGCCGGTCGAAAGGGTCTGA
L  P  K  G  Q  K  V  P  V  E  R  V  *
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Fig. 1. Nucleotide sequence of the *HopPtoF* locus (AY321312) and deduced amino acid sequences of $ShcF_{Pto}$ and $HopF_{Pto}$. The amino acid sequence is presented in single-letter code bellow the nucleotide sequence. The *hrp* box sequence is in boldface, Shine-Dalgarno sequences in bold italics, termination codons in asterisks, and ATA_1 and GTG_{13} of $HopF_{Pto}$ are underlined.

$ShcF_{Pto}$ is not secreted in culture.

Many bacterial Avr proteins characterized to date are secreted through the type III secretion system (Galan and Collmer 1999). The similarity between the *HopPtoF* and *avrPphF* loci led us to test the secretion of the *HopPtoF*-encoded proteins in culture. The $ShcF_{Pto}$ protein was tagged at the C-terminus with hemagglutinin (HA) epitope. The $ShcF_{Pto}$ protein was highly expressed in the *hrp*-inducing minimum medium (MM) (Huynh et al. 1989) but not in King's B (KB) broth (King et al. 1954) (Fig. 2). In MM, $ShcF_{Pto}$ was detected only in the cell-bound fraction. In contrast, AvrPto, a type III effector, was detected in both bacterial cells and supernatant, indicating that $ShcF_{Pto}$ is not secreted or the secretion of $ShcF_{Pto}$ is undetectable under the experimental conditions. We also tagged $HopF_{Pto}$ at its C-terminus with HA in the native context (i.e., *hrp* box promoter, $ShcF_{Pto}$, the spacer DNA between $ShcF_{Pto}$ and $HopF_{Pto}$, and $HopF_{Pto}$). However, we were unable to detect the $HopF_{Pto}$ protein in either bacterial cells or supernatant. We then replaced the *hrp* box promoter with the strong constitutive *nptII* promoter for the expression of the $ShcF_{Pto}$-$HopF_{Pto}$-*HA* genes. The *nptII* promoter enabled the detection of a small amount of $HopF_{Pto}$-HA protein in *P. syringae* pv. *tomato* DC3000 cells but not in the supernatant (data not shown).

$HopF_{Pto}$ but not $ShcF_{Pto}$ is translocated into plant cells.

The failure to detect the secretion of $ShcF_{Pto}$ in culture might be caused by experimental conditions that did not favor the secretion of this protein. Similar result was reported for the *P. syringae* AvrB protein that showed poor secretion in culture (van Dijk et al. 1999). Therefore, we used an alternative approach to test the secretion of $ShcF_{Pto}$ by fusing a partial AvrRpt2 protein ($AvrRpt2^{41-255}$) to the C-terminus of $ShcF_{Pto}$ and testing the HR-inducing activity of the fusion protein on

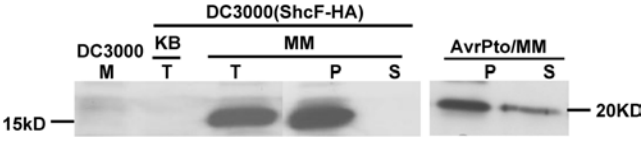

Fig. 2. $ShcF_{Pto}$ is not secreted in liquid culture. DC3000 (*pLK-hrp-ShcF-hemagglutinin* [*HA*]) was cultured in King's B broth (KB) and the hypersensitive response and pathogenicity (hrp)-inducing minimum medium (MM). The total protein (T) of the DC3000 (*pLK-hrp-ShcF-HA*) culture in MM was fractionated into pellet (P) and supernatant (S), separated by sodium dodecyl sulfate-gel electrophoresis, and probed with the anti-HA antibody. The pellet (P) and supernatant (S) fractions of DC3000 (*pLK-hrp-ShcF-HA*) in MM also were probed with anti-AvrPto antibodies as positive control of the type III secretion. Untransformed DC3000 was cultured in MM as negative control for $ShcF_{Pto}$-HA.

Arabidopsis plants carrying the *RPS2* gene. AvrRpt2 is an Avr protein that induces the HR on *Arabidopsis* plants carrying the *RPS2* gene (Bent et al. 1994). Upon secretion into the plant cells, AvrRpt2 is cleaved by a plant protease between the amino acid residues Gly71 and Gly72, exposing a potential myristoylation site for the C-terminal peptide that has the avirulence activity in *RPS2* plants (Mudgett and Staskawicz 1999). AvrRpt2$^{41\text{-}255}$ lacks the N-terminal type III secretion signal and, therefore, is unable to travel through the type III secretion system into the host cells. However, if the test protein carries the type III secretion signal, the fusion protein can be secreted into the host cells to induce the HR on plants carrying the *RPS2* gene. This strategy has been used successfully to demonstrate the secretion of type III effectors (Guttman and Greenberg 2001; Guttman et al. 2002; Mudgett et al. 2000; Petnicki-Ocwieja et al. 2002). In *P. syringae* pv. *phaseolicola,* *pLK-hrp-ShcF*$_{Pto}$-*avrRpt2*$^{41\text{-}255}$, which gives rise to a fusion protein of ShcF$_{Pto}$-AvrRpt2$^{41\text{-}255}$, did not cause HR on *RPS2* plants (Fig. 3). This result further indicated that ShcF$_{Pto}$ is not secreted. In contrast, *pLK-hrp-ShcF*$_{Pto}$-*HopF*$_{Pto}$-*avrRpt2*$^{41\text{-}255}$, which produces the ShcF$_{Pto}$ protein and the HopF$_{Pto}$-AvrRpt2$^{41\text{-}255}$ fusion protein, elicited an *RPS2*-dependent HR (Fig. 3A), indicating that the *HopF*$_{Pto}$ gene encodes a type III effector. Because ShcF$_{Pto}$ and HopF$_{Pto}$ are encoded by a single operon, ShcF$_{Pto}$ has the structural characteristics of a molecular chaperone, and the secretion of ShcF$_{Pto}$ was not detectable, we speculated that ShcF$_{Pto}$ serves as the type III chaperone for HopF$_{Pto}$.

We further tested whether the translocation of HopF$_{Pto}$ is dependent on type III secretion system. The HR-inducing activity of pLK-hrp-ShcFPto-HopFPto-AvrRpt241-255 was examined in a DC3000 type III secretion deficient mutant, the *hrcC* deletion strain (Wei et al. 2000). No HR was induced by the *hrcC* deletion strain carrying *pLK-hrp-ShcF*$_{Pto}$-*HopF*$_{Pto}$-*AvrRpt2*$^{41\text{-}255}$ (Fig. 3B), indicating that the translocation of HopF$_{Pto}$ relies on the type III secretion system.

Translation of HopF$_{Pto}$ starts at ATA.

Type III secretion signals are confined to the very N-terminal portion of the effector proteins. Thus, determination of the translation initiation site is necessary to understand the HopF$_{Pto}$ secretion signal. Compared with the *avrPphF ORF2* sequence that has the predicted start codon ATG, *HopF*$_{Pto}$ has ATA at position 1, a codon unknown for translation initiation in bacteria. Other potential start codons include GTG at position 13 and eight in-frame ATGs downstream of GTG$_{13}$ (Fig. 1). To unequivocally determine the translation initiation site of *HopF*$_{Pto}$, we first tested whether one of the in-frame ATGs serves as the initiation codon. Serial deletions from the 3′ end of *HopF*$_{Pto}$ were created that sequentially removed the ATGs, the remaining DNA fragments (including the native promoter, complete *ShcF*$_{Pto}$, the spacer DNA, and partial *HopF*$_{Pto}$) were fused to *avrRpt2*$^{41\text{-}255}$, and the resulting plasmids were introduced into *P. syringae* pv. *phaseolicola* for the translocation assay. All the deletion constructs, including the one (*pLK-hrp-ShcF*$_{Pto}$-*HopF*$_{Pto}$^*151-avrRpt2*$^{41\text{-}255}$) that has only the N-terminal

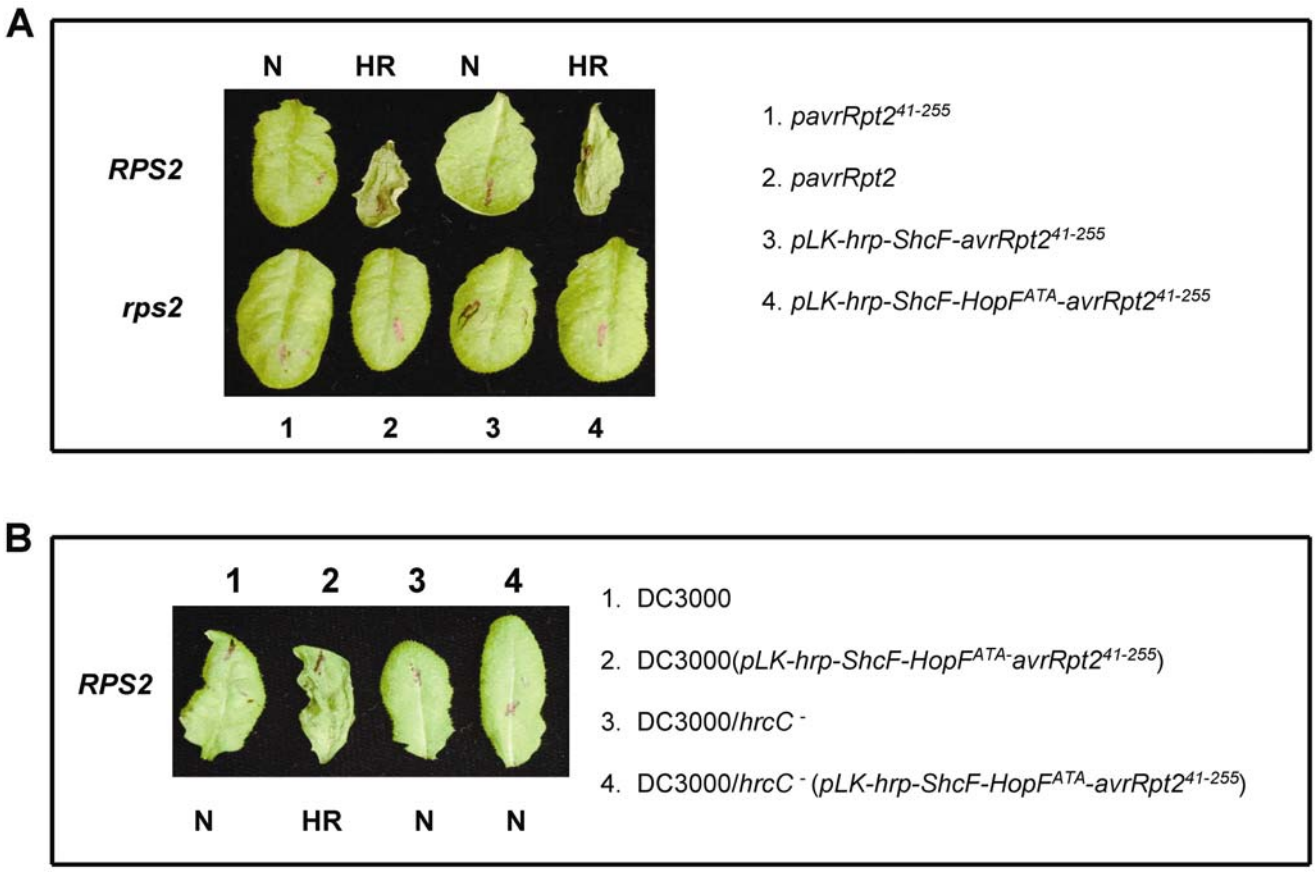

Fig. 3. HopF$_{Pto}$ but not ShcF$_{Pto}$ shows type III-dependent secretion in planta. **A,** Translocation assay of ShcF$_{Pto}$ and HopF$_{Pto}$. *ShcF*$_{Pto}$ and *HopF*$_{Pto}$ were fused in frame with the partial *avrRpt2*$^{41\text{-}255}$ gene in the plasmids *pLK-hrp-ShcF-avrRpt2*$^{41\text{-}255}$ and *pLK-hrp-ShcF-HopF*^{ATA}*-avrRpt2*$^{41\text{-}255}$, respectively. *Pseudomonas syringae* pv. *phaseolicola* NPS 3121 (*Psph*) was used as the carrier strain for the plasmids. Test strains were infiltrated into *Arabidopsis* Col-0 (*RPS2*) and Col-0 *rps2-201* (*rps2*) plants for the hypersensitive response (HR) assay. The HR was induced by Psph strains expressing the wild-type AvrRpt2 protein (*pavrRpt2*) and HopF$_{Pto}$-AvrRpt2$^{41\text{-}255}$ fusion protein (*hrp-ShcF-HopF*^{ATA}*-avrRpt2*$^{41\text{-}255}$). **B,** Type III-dependent secretion of HopF$_{Pto}$ in planta. *pLK-hrp-ShcF-HopF-avrRpt2*$^{41\text{-}255}$ was introduced into *P. syringae* pv. *tomato* DC3000 and DC3000 *hrcC*$^-$ mutant strains. Bacteria were infiltrated into *Arabidopsis* Col-0 plants (*RPS2*). The HR was induced by DC3000 carrying *pLK-hrp-ShcF-HopF-avrRpt2*$^{41\text{-}255}$. N = no visible response.

portion of the *HopF*$_{Pto}$ gene without any in frame ATGs, still retained the ability to cause HR on *RPS2* plants (Fig. 4A), suggesting that the translation initiation site of *HopF*$_{Pto}$ is further upstream of ATG_{54}, the first ATG from the 5′ end.

GTG_{13} was predicted by the GLIMMER program to be the translation start codon for *HopF*$_{Pto}$. To test this prediction, we constructed a plasmid, *pML-nptII-HopF*$_{Pto}^{GTG13}$*-HA*, to express the HopF$_{Pto}$-HA protein starting from GTG_{13}. This plasmid carries the *HopF*$_{Pto}$ gene starting at GTG_{13} and a synthetic SD sequence placed upstream of GTG_{13}. As control, we also constructed a plasmid, *pML-nptII-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA*, to express the HopF$_{Pto}$-HA protein encoded by the native *HopPtoF* locus. The gene transcription in both plasmids was controlled by the strong *nptII* promoter. We examined the HopF$_{Pto}$-HA protein

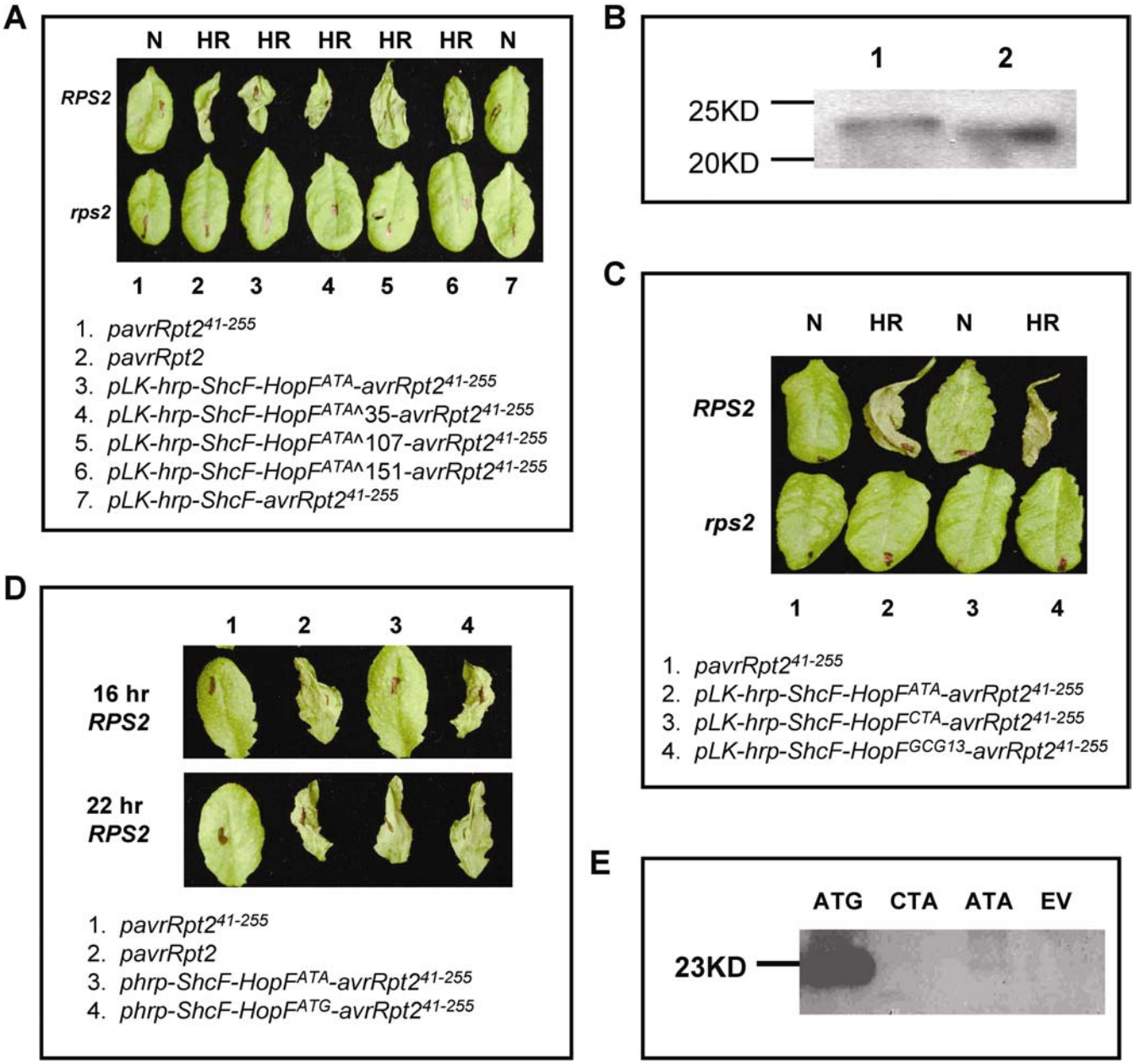

Fig. 4. ATA is the translation initiation codon of HopF$_{Pto}$. **A,** *pLK-hrp-ShcF-HopF^35-avrRpt2*$^{41-255}$, *pLK-hrp-ShcF-HopF^107-avrRpt2*$^{41-255}$, and *pLK-hrp-ShcF-HopF^151-avrRpt2*$^{41-255}$ carry deletions of 35, 107, and 151 amino acid residues, respectively, at the C-terminus of HopF$_{Pto}$. The plasmids were introduced into *Pseudomonas syringae* pv. *phaseolicola* NPS 3121 (*Psph*) and the resulting bacterial strains were infiltrated into *Arabidopsis* Col-0 plants (*RPS2*) and Col-0 *rps2-201* (*rps2*) plants. **B,** DC3000 strains carrying *pML-nptII-ShcF-HopF*^{ATA}*-hemagglutinin (HA)* and *pML-nptII-HopF*^{GTG13}*-HA* plasmids were cultured in minimal medium. Protein from the bacterial pellet was assayed with Western blot using the HA antibody. The HopF$_{Pto}$ protein encoded by the native *HopF*$_{Pto}$ gene in *pML-nptII-ShcF-HopF*^{ATA}*-HA* (lane 1) is ≈23 kDa and slightly larger than the protein translated from GTG_{13} by the *pML-nptII-HopF*^{GTG13}*-HA* plasmid (lane 2). **C**, *pLK-hrp-ShcF-HopF*^{ATA}*-avrRpt2*$^{41-255}$, *pLK-hrp-ShcF-HopF*^{CTA}*-avrRpt2*$^{41-255}$ (mutation of ATA_1 to CTA), and *pLK-hrp-ShcF-HopF*^{GCG}*-avrRpt2*$^{41-255}$ (mutation of GTG_{13} to GCG) were introduced into *Psph*, and the resulting bacterial strains were infiltrated into *Arabidopsis* Col-0 plants (*RPS2*) and Col-0 *rps2-201* (*rps2*) plants for the hypersensitive response (HR) assay. The HR was disrupted by mutation of ATA_1 to CTA but not affected by mutation of GTG_{13} to GCG. **D,** *RPS2 Arabidopsis* leaves were injected with *Psph* strains carrying *avrRpt2*$^{41-255}$, *pavrRpt2*, *pLK-hrp-ShcF-HopF*^{ATA}*-avrRpt2*$^{41-255}$, and *pLK-hrp-ShcF-HopF*^{ATG}*-avrRpt2*$^{41-255}$ (mutation of ATA_1 to ATG). The HR caused by *Psph*(*pLK-hrp-ShcF-HopF*^{ATG}*-avrRpt2*$^{41-255}$) was visible ≈16 h after inoculation, significantly earlier than the HR caused by *Psph*(*pLK-hrp-ShcF-HopF*^{ATA}*-avrRpt2*$^{41-255}$), which was visible ≈22 h after inoculation. **E,** *Psph* strains carrying *pML-nptII-ShcF-HopF*^{ATG}*-HA* (ATG), *pML-nptII-ShcF-HopF*^{CTA}*-HA* (CTA), *pML-nptII-ShcF-HopF*^{ATA}*-HA* (ATA), and *pML* empty vector (EV) were cultured in minimal medium. Protein from equal amount of bacterial cells was assayed with Western blot using the HA antibody. The inoculation experiments for **A, C,** and **D** were repeated numerous times with the same results.

produced by the two plasmids in DC3000 using Western blot analysis with the anti-HA antibodies. If GTG_{13} is the start codon, the two plasmids were expected to yield the $HopF_{Pto}$-HA protein of the same size. However, the protein produced by *pML-nptII-HopF*$_{Pto}^{GTG13}$*-HA* (Fig. 4B, lane 2) was slightly smaller than the protein produced by *pML-nptII-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* (Fig. 4B, lane 1), indicating that GTG_{13} is not the start codon. To further test this possibility, we created point mutation in the construct *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41-255}$ that converted GTG_{13} to GCG. GCG was not reported to act as a translational start codon in any organisms. Mutation of GTG_{13} to GCG did not affect the HR-inducing ability of *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41-255}$ (Fig. 4C), further supporting the theory that GTG_{13} is not the initiation codon for *HopF*$_{Pto}$. The authentic translation initiation site of *HopF*$_{Pto}$ must be further upstream of GTG_{13} because the $HopF_{Pto}$ protein encoded by the native *HopPtoF* locus is larger than the protein starting from GTG_{13}.

We speculated that ATA_1 functions as the start codon of *HopF*$_{Pto}$, because the homology between $HopF_{Pto}$ and AvrPphF ORF2 extends to the ATA_1 codon, and the protein translated from ATA_1 exhibits the features of type III secretion signal sequence at its N-terminus (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). We tested this possibility by converting ATA_1 to CTA and ATG in the plasmid *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41-255}$. CTA never was reported to act as an initiation codon in any organisms. Interestingly, mutation of ATA_1 to CTA in *HopF*$_{Pto}$ completely disrupted the HR-inducing activity (Fig. 4C), whereas mutation of ATA_1 to ATG accelerated the HR (Fig. 4D). In addition, mutation of ATA_1 to ATG in *HopF*$_{Pto}$ in the *pML-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* plasmid resulted in the detection of a large amount of the $HopF_{Pto}$-HA protein produced by *P. syringae* carrying the plasmid, while no protein was detected when ATA_1 was converted to CTA (Fig. 4E). The $HopF_{Pto}$-HA proteins produced by *pML-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-HA* (indicated by ATG in Fig. 4E) is the same size as that encoded by the native *HopPtoF* locus (indicated by ATA in Fig. 4E). Because the *HopF*$_{Pto}^{ATA}$ gene in the native *HopPtoF* locus showed the HR-inducing activity in the *avrRpt2*$^{41-255}$-mediated translocation assay and also could direct the synthesis of a small amount of protein in bacteria, we believed that ATA_1 serves as the translation initiation codon of $HopF_{Pto}$.

$ShcF_{Pto}$ interacts with $HopF_{Pto}$ in yeast and in protein pull-down assay.

Type III chaperones often interact physically with the cognate Hop proteins (Aldridge and Hughes 2001; Bennett and Hughes 2000). To determine that $ShcF_{Pto}$ indeed functions as the type III chaperone for $HopF_{Pto}$, we tested the interaction of $ShcF_{Pto}$ and

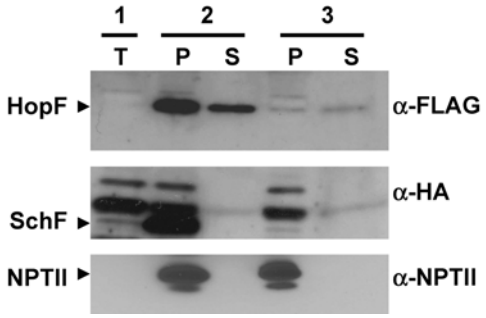

Fig. 6. $HopF_{Pto}$ is unstable but can be secreted in the absence of $ShcF_{Pto}$. 1, Untransformed *Pseudomonas syringae* pv. *phaseolicola* NPS 3121 (*Psph*); 2, *Psph* carrying both *pML-nptII-HopF*^{ATG}*-FLAG* and *pHM2-hrp-ShcF-hemagglutinin* (*HA*) plasmids; and 3, *Psph* carrying *pML-nptII-HopF*^{ATG}*-FLAG* alone were cultured in minimum medium and diluted to the same concentration with the same medium before protein analysis. Equal volumes of bacterial cultures were fractionated into pellet and supernatant and examined with antibodies against FLAG, HA, and NPTII for $HopF_{Pto}$-FLAG, $ShcF_{Pto}$-HA, and the control NPTII protein (for bacterial lysis). T, total bacterial protein in culture; P, pellet; S, supernatant. Arrows indicate the corresponding proteins. Equal loading of protein samples is shown by the nonspecific bands above the $HopF_{Pto}$-FLAG and $ShcF_{Pto}$-HA proteins. The weak bands of the same size as $ShcF_{Pto}$-HA in lanes 1/T and 3/P are from nonspecific hybridizations.

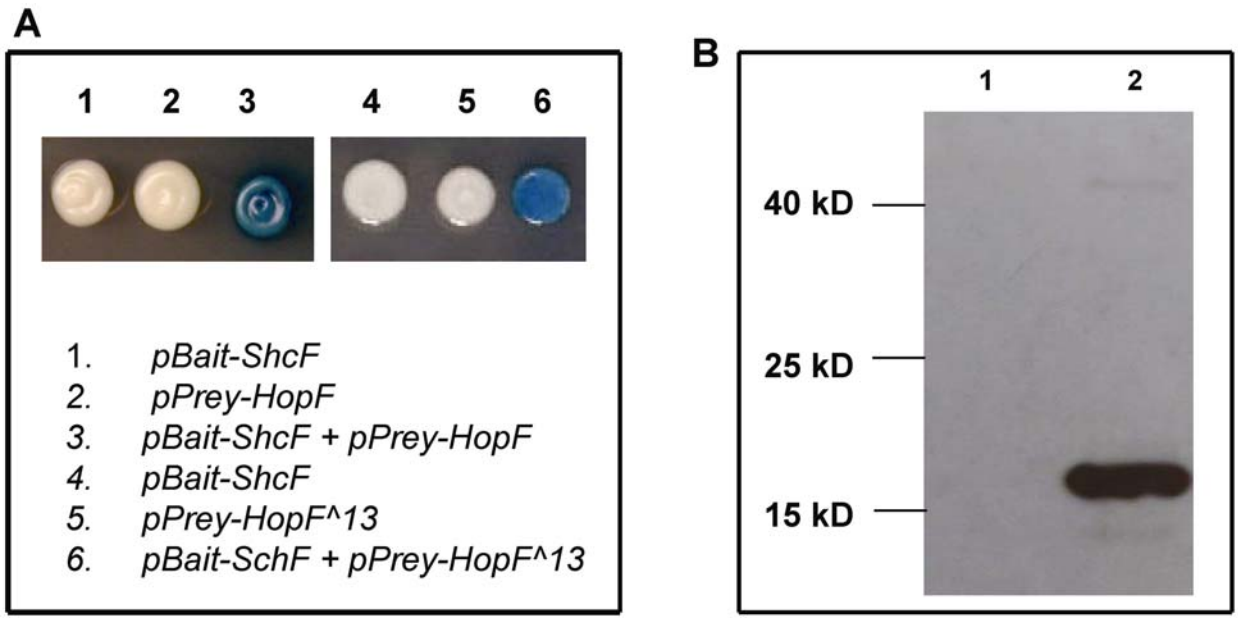

Fig. 5. $ShcF_{Pto}$ interacts with $HopF_{Pto}$. **A,** Yeast two-hybrid analysis. *ShcF*$_{Pto}$ was cloned into the bait vector pEG202; the full-length *HopF*$_{Pto}$ gene and *HopF*$_{Pto}$ lacking the first 13 codons were cloned into the prey vector pJG4-5. The LexA yeast two-hybrid system was used for protein interaction assay. **B,** Protein pull-down assay. The GST-AvrPto and GST-$HopF_{Pto}$ fusion proteins were purified with glutathione-conjugated cellulose beads. Washed beads carrying 50 μg of GST-AvrPto (lane 1) and GST-$HopF_{Pto}$ (the fusion proteins) (lane 2) were incubated with the bacterial lysate of *Pseudomonas syringae* pv. *phaseolicola* NPS 3121 strain containing $ShcF_{Pto}$-hemagglutinin (HA). The beads were washed with phosphate-buffered saline plus 0.1% Tween-20 and assayed with Western blot and the anti-HA antibody.

$HopF_{Pto}$ using the yeast two-hybrid assay. Expression of neither $ShcF_{Pto}$ nor $HopF_{Pto}$ alone in yeast activated the *lacZ* reporter gene, as indicated by the white yeast colonies (Fig. 5A). However, expression of $ShcF_{Pto}$ and $HopF_{Pto}$ together in the same yeast cell activated the *lacZ* reporter gene, indicating the interaction of the two proteins. $HopF_{Pto}$ carrying deletion of the N-terminal 13 amino acid residues still showed interaction with $ShcF_{Pto}$ (Fig. 5A). The interaction of $HopF_{Pto}$ with $ShcF_{Pto}$ was further confirmed by the protein pull-down assay. GST-$HopF_{Pto}$ fusion protein, but not GST-AvrPto, specifically pulled down the $ShcF_{Pto}$-HA protein from the protein extract of *P. syringae* pv. *phaseolicola* expressing the *$ShcF_{Pto}$-HA* gene (Fig. 5B).

$ShcF_{Pto}$ stabilizes $HopF_{Pto}$ in bacterial cytoplasm.

The interaction of a type III chaperone with the corresponding Hop protein often stabilizes the effector protein in bacterial cytoplasm or facilitates the secretion of the effector through the type III pathway. To determine how $ShcF_{Pto}$ affects $HopF_{Pto}$, we tagged the C-terminus of *$HopF_{Pto}$*^{ATG} with FLAG epitope and expressed the protein using the *nptII* promoter. The *$HopF_{Pto}$* gene with ATG as start codon was used in this experiment to facilitate the protein detection. We investigated the stability and secretion of $HopF_{Pto}$-FLAG in the presence or absence of $ShcF_{Pto}$ in *P. syringae* pv. *phaseolicola* strains. Upon induction in MM, over 50-fold more $HopF_{Pto}$-FLAG protein was detected in *P. syringae* pv. *phaseolicola* carrying both *pHM-hrp-$ShcF_{Pto}$-HA* and *pML-$HopF_{Pto}$*^{ATG}*-FLAG* than in the strain carrying *pML-$HopF_{Pto}$*^{ATG}*-FLAG* alone (Fig. 6). In both strains, the $HopF_{Pto}$ protein was detected in the supernatant as well as in the bacterial pellet. The $HopF_{Pto}$ protein in the supernatant was not caused by cell lysis because the $ShcF_{Pto}$ protein and the control NPTII protein were not detected in the supernatant (Fig. 6). These results indicated that $ShcF_{Pto}$ is required for the stability but dispensable for the secretion of the $HopF_{Pto}$ protein.

DISCUSSION

In this study, we showed that the *HopPtoF* locus of DC3000 encodes two proteins, $ShcF_{Pto}$ and $HopF_{Pto}$. The *$HopF_{Pto}$* gene carries a rare initiation codon ATA that limits the $HopF_{Pto}$ protein synthesis in *P. syringae* to a low level. $ShcF_{Pto}$ serves as the type III chaperone that interacts with and stabilizes the type III effector protein $HopF_{Pto}$.

The $ShcF_{Pto}$ protein exhibits several structural and functional characteristics supporting its nature as the chaperone of $HopF_{Pto}$. Structurally, $ShcF_{Pto}$ is predicted to be a small cytoplasmic protein (molecular mass = 15 kDa) with a relatively low isoelectric point (pI = 6.5) and a secondary structure predominantly helical. These characteristics are shared by other type III chaperones (Aldridge and Hughes 2001; Bennett and Hughes 2000; Page and Parsot et al. 2002). Functionally, $ShcF_{Pto}$ is required for the stability of $HopF_{Pto}$ in bacterial cytoplasm. The interaction of $ShcF_{Pto}$ with $HopF_{Pto}$ in the yeast two-hybrid system and in a protein pull-down assay provided further evidence that $ShcF_{Pto}$ is the chaperone of $HopF_{Pto}$. Unlike ShcA and ShcM, two chaperones of *P. syringae* pv. *syringae* that facilitate secretion but are dispensable for the stability of their partner effectors HopPsyA and HopPtoM, respectively (Badel et al. 2003; van Dijk et al. 2002), $ShcF_{Pto}$ is dispensable for the secretion but required for the stability of $HopF_{Pto}$. The requirement of type III chaperone for effector protein stability also was reported for the *Erwinia amylovora* type III chaperone DspB/F which is required for the stability of its cognate effector DspA/E (Gaudriault et al. 2002). In addition, several animal bacterial type III chaperones also protect their corresponding effectors from proteolysis but are not required for the effector secretion (Parsot et al. 2003).

Unlike the *avrPphF ORF2* that has an in-frame ATG downstream of the SD sequence, the *$HopF_{Pto}$* gene does not have a standard translation initiation site. However, this locus is able to direct the synthesis of the $HopF_{Pto}$ protein in both HA tagging and AvrRpt2$^{41\text{-}255}$ tagging experiments. Gene annotation using the GLIMMER program (Salzberg et al. 1998) suggested that the translation of $HopF_{Pto}$ starts at GTG_{13}, a codon that often is used as an alternative initiation codon in bacteria (Osawa et al. 1992). However, this codon is not preceded by a typical SD sequence for ribosomal binding. In addition, mutagenesis of GTG_{13} to GCG (GCG does not serve as the start codon in any organisms) in *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$-avrRpt2*$^{41\text{-}255}$ did not affect the $HopF_{Pto}$-AvrRpt2$^{41\text{-}255}$ protein synthesis, as indicated by the HR-inducing activity of this plasmid in *RPS2* plants. Furthermore, the protein synthesized from GTG_{13} was smaller than the protein encoded by the native gene. These results strongly argue against GTG_{13} as the initiation codon of $HopF_{Pto}$.

In contrast, several pieces of evidence support the theory that ATA_1 is the translation initiation site for $HopF_{Pto}$, although ATA was not reported to act as the start codon in bacteria. First, we could detect a small amount of the $HopF_{Pto}$ protein encoded by the native *HopPtoF* locus in the HA tagging experiment when the strong constitutive *nptII* promoter was used to drive the expression of the genes. Second, in the AvrRpt2-mediated translocation assay, the fusion of the *$HopF_{Pto}$*^{ATA} gene in the native *HopPtoF* locus with *avrRpt2*$^{41\text{-}255}$ (in the *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$*^{ATA}*-avrRpt2*$^{41\text{-}255}$ plasmid) resulted in the induction of HR on *RPS2* plants, suggesting that *$HopF_{Pto}$*^{ATA} could direct the synthesis of the fusion protein. Additional evidence came from the mutagenesis studies of ATA_1 in the plasmids *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$-avrRpt2*$^{41\text{-}255}$ and the *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$-HA*. We found that mutation of ATA_1 to CTA in the *$HopF_{Pto}$*^{ATA} gene (in *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$*^{ATA}*-avrRpt2*$^{41\text{-}255}$ plasmid) abolished the HR-inducing activity, whereas mutation of ATA_1 to ATG enhanced the HR-inducing activity of this plasmid. Consistently, we could not detect the $HopF_{Pto}$ protein when ATA_1 was changed to CTA, but mutation of ATA_1 to ATG in *pLK-hrp-$ShcF_{Pto}$-$HopF_{Pto}$-HA* dramatically increased the $HopF_{Pto}$ protein synthesis in *P. syringae* bacteria. Furthermore, mutation of the ATA_1 to ATG resulted in a protein the same size as that encoded by the native gene. Taken together, these results strongly supported the theory that ATA_1 is the start codon of *$HopF_{Pto}$*^{ATA}. The direct evidence supporting ATA_1 as the start codon of *$HopF_{Pto}$*^{ATA} can be provided by the protein sequence analysis. However, the low abundance of the $HopF_{Pto}$ protein encoded by the native *HopPtoF* locus deterred our effort to obtain enough protein for sequence analysis.

The use of ATA as the start codon does not appear to be general for the *HopF* loci in *Pseudomonas* bacteria. Among the three reported homologous genes, only the *$HopF_{Pto}$* gene from DC3000 starts with ATA. The other two genes, one from *P. syringae* pv. *phaseolicola* and the other from *P. syringae* pv. *delphinii*, both started with ATG (Deng et al. 2003; Tsiamis et al. 2000). The *HopPtoO* gene of DC3000, which encodes a protein with the N-terminal 10 amino acid residues identical to those of $HopF_{Pto}$, also starts with ATG (Buell et al. 2003). This suggests that the ATA_1 codon of the DC3000 *$HopF_{Pto}$* gene might be derived from mutation of ATG.

The use of a nonstandard codon for translation initiation is not uncommon in bacteria and other organisms. When a non-ATG codon is used, it often results in low translation efficiency (Osawa et al. 1992). Consistently, we were able to detect only a small amount of the $HopF_{Pto}$-HA protein with the *$HopF_{Pto}$-HA* gene carrying ATA_1 as the start codon. The finding that ATA can act as a translation initiation codon is significant to bacterial genomics in prediction of putative ORFs.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Escherichia coli strain XL1-Blue was used for gene cloning. *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* NPS 3121 were used for secretion and translocation assays. Antibiotics were used at the following concentrations: rifampcin, 100 μg/ml; ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml for *E. coli* and 20 μg/ml for *P. syringae*; spectinomycin, 50 μg/ml.

Construction of plasmids.

Three broad host range plasmids, pLK, pML123 (Petnicki-Ocwieja et al. 2002), and pHM2 vector were used to construct plasmids listed in Table 1. pLK plasmid was derived from pPTE6 (Ronald et al. 1992) by removing the *avrPto* gene with a *Hin*dIII digestion. pHM2 plasmid was modified from pHM1 (Zhu et al. 1998) by replacing the *Bam*HI fragment with DNA containing the cloning sites of *Bam*HI, *Eco*RI, *Xho*I, *Kpn*I, *Hin*dIII, and *Xba*I (X. Tang, *unpublished data*). Construction of *pavrRpt2*$^{41\text{-}255}$ was previously described (Petnicki-Ocwieja et al. 2002).

To construct *pLK-hrp-ShcF*$_{Pto}$*-HA*, a DNA fragment spanning 200 bp upstream of the *hrp* box promoter and the complete *ShcF*$_{Pto}$ (excluding the stop codon) was polymerase chain reaction (PCR)-amplified from DC3000 using an upstream primer 5′-TT**GAATTC**GCCCCTTCGTTACCTTCCAGC-3′, (*Eco*RI site in boldface) and a downstream primer 5′-TT**GTC GAC**TCAGACGTAGTCTGGGACGTCGTATGGGTA*GGAT CC*TTTGAGTTTTAGTGTTTTTCTG-3′ (preceding the *ShcF*$_{Pto}$ 3′ end sequence are a *Sal*I site [bold], the HA sequence plus a stop codon [underlined], and a *Bam*HI site [italics]). The PCR products were cloned into the *Eco*RI and *Sal*I sites of pMOD^{TM}-2<MCS> plasmid (Epicentre, Madison, WI, U.S.A.), confirmed by sequence analysis, and further cloned into the *Eco*RI and *Sal*I sites of the pLK plasmid, resulting in *pLK-hrp-ShcF*$_{Pto}$*-HA*. The DNA fragment also was cloned into the *Eco*RI and *Sal*I sites of the pBluescript SK(–) plasmid (Stratagene, La Jolla, CA, U.S.A.). The resulting pBS-*hrp-ShcF*$_{Pto}$*-HA* plasmid was digested further by *Eco*RI and *Kpn*I and cloned into the same sites in pHM2 vector, resulting *pHM2-hrp-ShcF*$_{Pto}$*-HA*. The DNA fragment spanning the *hrp* box promoter and *ShcF*$_{Pto}$ in *pLK-hrp-ShcF*$_{Pto}$*-HA* was released by *Eco*RI and *Bam*HI digestion and cloned into the *Eco*RI and *Bam*HI sites of *pavrRpt2*$^{41\text{-}255}$, resulting in *pLK-hrp-ShcF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$.

To generate *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* and *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$ plasmids, the same upstream primer for *pLK-hrp-ShcF*$_{Pto}$*-HA* and the downstream primer 5′-AA**GGATCC**GACCCTTTCGACCGGCA-3′, corresponding to the 3′ end of *HopF*$_{Pto}$ plus the *Bam*HI site (bold), were used to PCR amplify the DNA fragment encompassing the *hrp* box promoter, *ShcF*$_{Pto}$, spacer between *ShcF*$_{Pto}$ and *HopF*$_{Pto}$, and the complete *HopF*$_{Pto}$ from DC3000. The PCR product was cloned into the *Eco*RI and *Bam*HI sites of pMOD^{TM}-2<MCS> plasmid for sequence analysis. The confirmed sequence was further cloned into the *Eco*RI and *Bam*HI sites of *pLK-hrp-ShcF*$_{Pto}$*-HA* and *pLK-hrp-ShcF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$, replacing *ShcF*$_{Pto}$ and resulting in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* and *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$, respectively.

The serial deletions of the *HopF*$_{Pto}$ gene in plasmids *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$ were generated by PCR using the same upstream primer for *pLK-hrp-ShcF*$_{Pto}$*-HA* and one of the following downstream primers containing the *Bam*HI site (bold): 5′-CA**GGATCC**ACCCCTTTCGAGAAAATC-3′ for deletion of 35 amino acid (aa) residues, 5′-CA**GGATCC**TT CATGCATAAGGATACT-3′ for deletion of 107 aa residues, and 5′-CA**GGATCC**GTTCAGAAACTGCTCTCT-3′ for deletion of 151 aa residues from the HopF$_{Pto}$ C-terminus. The PCR products were digested with *Eco*RI and *Bam*HI and cloned into pMOD^{TM}-2<MCS> for sequence analysis. The confirmed sequences were cloned into the *Eco*RI and *Bam*HI sites of *phrp-ShcF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$, replacing *ShcF*$_{Pto}$.

A QuickChange site-directed mutagenesis kit (Stratagene) was used to generate point mutations in the *HopF*$_{Pto}$ gene in pMOD::*hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$ and pMOD::*hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* with the following three pairs of complementary primers: 5′-GAGGAAACCATT**CTA**GGTAATATTT GCGGC-3′ and 5′-GCCGCAAATATTACC**TAG**AATGGTTT CCTC-3′ (ATA$_1$ to CTA); 5′-CGGGCTCACGTCAT**GCG**TAT AGCCCATCCC-3′ and 5′-GGGATGGGCTATA**CGC**ATGAC GTGAGCCCG-3′ (GTG$_{13}$ to GCG); and 5′-GAGGAAACCA TT**ATG**GGTAATATTTGCGGC-3′ and 5′-GCCGCAAATATT ACC**CTA**AATGGTTTCCTC-3′ (ATA$_1$ to ATG). The mutants were confirmed by sequence analysis and subsequently cloned into the *Eco*RI and *Bam*HI sites of *pLK-hrp-ShcF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$ and *pLK-hrp-ShcF*$_{Pto}$*-HA* plasmids, resulting in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{CTA}$*-avrRpt2*$^{41\text{-}255}$, *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{GCG}$*-avrRpt2*$^{41\text{-}255}$, and *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-avrRpt2*$^{41\text{-}255}$, *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{CTA}$*-HA*, and *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-HA*.

To generate *pML-nptII-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA* and *pML-nptII-HopF*$_{Pto}^{GTG13}$*-HA* plasmids, we used the forward primers 5′-AAAAAGCAGGCT*TCGAAGGAGATAGAACC*ATGA

Table 1. Plasmids

Designation	Characteristics
pavrRpt2$^{41\text{-}255}$	Broad host plasmid pLK carrying partial *avrRpt2* gene with the N-terminal 40 codons deleted
pavrRpt2	Full length *avrRpt2* gene in broad host plasmid
pLK-hrp-ShcF$_{Pto}$*-avrRpt2*$^{41\text{-}255}$	*ShcF*$_{Pto}$ under native promoter and fused in frame with partial *avrRpt2*, in *pLK*
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*-avrRpt2*$^{41\text{-}255}$	*HopPtoF* operon under the native promoter fused with partial *avrRpt2*, in *pLK*
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*^35-avrRpt2*$^{41\text{-}255}$	Deletion of 35 amino acids (aa) from the C-terminus of HopF$_{Pto}$
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*^107-avrRpt2*$^{41\text{-}255}$	Deletion of 107 aa from the C-terminus of HopF$_{Pto}$
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*^151-avrRpt2*$^{41\text{-}255}$	Deletion of 151 aa from the C-terminus of HopF$_{Pto}$
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{CTA}$*-avrRpt2*$^{41\text{-}255}$	Mutation of ATA$_1$ of *HopF*$_{Pto}$ to CTA in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{GCG13}$*-avrRpt2*$^{41\text{-}255}$	Mutation of GTG$_{13}$ of *HopF*$_{Pto}$ to GCG in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-avrRpt2*$^{41\text{-}255}$	Mutation of ATA$_1$ of *HopF*$_{Pto}$ to ATG in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-avrRpt2*$^{41\text{-}255}$
pLK-hrp-ShcF$_{Pto}$*-HA*	Hemagglutinin (HA)-tagged *ShcF*$_{Pto}$ under native promoter, in *pLK*
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*-HA*	*HopF*$_{Pto}$ in the *HopPtoF* operon tagged by HA, in *pLK*
pLK-hrp-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*-HA*	ATA$_1$ of *HopF*$_{Pto}$ replaced by ATG in *pLK-hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}$*-HA*
pML-nptII-HopF$_{Pto}^{GTG}$*-HA*	HA-tagged *HopF*$_{Pto}$ starting at GTG$_{13}$ in *pML123* plasmid
pML-nptII-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*-HA*	*HopPtoF* locus driven by *nptII* promoter, *HopF*$_{Pto}$ is HA-tagged, in *pML123*
pML-nptII-ShcF$_{Pto}$*-HopF*$_{Pto}^{ATA}$*-HA*	ATA$_1$ of *HopF*$_{Pto}$ replaced by ATG in *pML-nptII-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-HA*
pML-nptII-ShcF$_{Pto}$*-HopF*$_{Pto}^{CTA}$*-HA*	ATA$_1$ of *HopF*$_{Pto}$ replaced by CTA in *pML-nptII-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-HA*
pHM2-hrp-ShcF$_{Pto}$*-HA*	HA-tagged *ShcF*$_{Pto}$ under native promoter, in broad host plasmid pHM2
pML-nptII-HopF$_{Pto}^{ATG}$*-Flag*	Flag-tagged *HopF*$_{Pto}^{ATG}$ driven by *nptII* promoter, in *pML123*

AAAACGCATTT-3′ and 5′-AAAAAGCAGGCT*TCGAAGG AGATAGAACCT*GTGTATAGCCCATCC-3′ containing the attB2 sequence (underlined) and SD sequence (italic) and the same reverse primer 5′-AGAAAGCTGGGTAACAGACCCT TTCGAC-3′ containing the attB1 sequence (underlined) in PCR with the DC3000 genomic DNA as template. The PCR products were cloned into pDONR201 (Invitrogen, Carlsbad, CA, U.S.A.) using a BP reaction of Gateway system (Invitrogen). The genes then were cloned into pCPP5040, the pML123-based destination vector containing the *nptII* promoter, a gateway site, and the HA tag (Petnicki-Ocwieja et al. 2002).

To generate *pML-nptII-HopF*$_{Pto}^{ATG}$*-Flag*, the *HopF*$_{Pto}^{ATG}$ gene was amplified by PCR using the forward primer 5′-CG**CTCGAG***AAGCTT*AAAAAGAGGAAACCATTATGG-3′ (*Xho*I, bold; *Hin*dIII, italic; ATG, underlined) and the reverse primer 5′-GC**TTCGAA**GACCCTTTCGACCGGCAC-3′ (*Csp*45I, bold) and pMOD::*hrp-ShcF*$_{Pto}$*-HopF*$_{Pto}^{ATG}$*-avrRpt2*$^{41-255}$ as template. The PCR products were digested by *Xho*I and *Csp*45I and cloned into *pBS-Flag* (J. Chen and X. Tang, *unpublished*), resulting in *pBS-HopF*$_{Pto}^{ATG}$*-Flag.* This plasmid then was digested with *Hin*dIII and *Xba*I, and the insert was cloned into the same sites in pML123, resulting in *pML-nptII-HopF*$_{Pto}^{ATG}$*-Flag.*

Secretion assays.

P. syringae bacteria were grown in KB medium (King et al. 1954) containing appropriate antibiotics. The bacterial cells were spun down at 4,000 rpm, washed twice with the *hrp*-inducing minimal medium (pH 5.8) (Huynh et al. 1989), diluted in MM to an optical density at 600 nm = 0.2, and grown at room temperature for another 16 h. The bacterial culture was centrifuged at 4,000 rpm for 5 min, and the supernatant was transferred into a clean microcentrifuge tube and spun at 14,000 rpm for 5 min to remove all residual bacteria. The protein in bacterial pellet and supernatant was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and examined by immunoblotting using anti-HA, anti-FLAG, and anti-AvrPto antibodies.

Translocation assays.

The *avrRpt2*$^{41-255}$ fusion plasmids were introduced into *P. syringae* pv. *phaseolicola* NPS 3121 or *P. syringae* pv. *tomato* DC3000 and the DC3000 *hrcC* mutant strains by electroporation. Bacteria were grown overnight in liquid KB medium with appropriate antibiotics, washed twice with 10 mM $MgCl_2$, and resuspended in the same solution. Bacteria at the concentration of 2×10^8 cells/ml were infiltrated into *Arabidopsis* Col-0 and Col-0 *rps2-201* plants with a needleless syringe.

Interaction assay in yeast two-hybrid system.

ShcF$_{Pto}$, *HopF*$_{Pto}^{ATG}$, and *HopF*$_{Pto}^{GTG13}$ (*HopF*$_{Pto}$ with the N-terminal 13 codons deleted) were PCR amplified using the following three pairs of primers: 5′-GC**GAATTC**ATGAAAAA CGCATTTGAC-3′ and 5′-GC**CTCGAG**CTATTTGAGTTTT AGTGT-3′ for *ShcF*$_{Pto}$; 5′-GC**GAATTC**ATGGGTAATATT TGCGGCACCTCG-3′ and 5′-TA**CTCGAG**TCAGACCCTTT CGACCGG-3′ for *HopF*$_{Pto}$; and 5′-GC**GAATTC**ATGTATAG CCCATCCCAT-3′ and 5′-TA**CTCGAG**TCAGACCCTTTCG ACCGG-3′ for *HopF*$_{Pto}^{GTG13}$ (*Eco*RI and *Xho*I sites are in boldface). The PCR products were digested with *Eco*RI and *Xho*I and cloned into *pEG202* and *pJG4-5* plasmids (Golemis et al. 1997), resulting in *pEG202::ShcF*$_{Pto}$, *pJG4-5::HopF*$_{Pto}$, and *pJG4-5::HopF*$_{Pto}^{GTG13}$. Procedures described by Golemis and associates (1997) were followed to test the interaction of ShcF$_{Pto}$/HopF$_{Pto}$ and ShcF$_{Pto}$/HopF$_{Pto}^{GTG13}$ in yeast.

In vitro protein pull-down assay.

HopF$_{Pto}$ was released from *pJG4-5:: HopF*$_{Pto}$ by *Eco*RI and *Xho*I digestion and inserted into *pGST-AvrPto* plasmid (Shan et al. 2000) for production of the glutathione-S-transferase (GST)-HopF$_{Pto}$ recombinant protein. Procedures described by Guan and Dixon (1991) were followed for purification of the GST-HopF$_{Pto}$ protein with the glutathione-conjugated cellulose beads (Sigma, St. Louis). The GST-AvrPto fusion protein was produced for control using the same procedure. The GST fusion proteins coupled with the cellulose beads were used in protein pull-down assay with ShcF$_{Pto}$ tagged with HA.

P. syringae pv. *phaseolicola* carrying *pLK-hrp-ShcF*$_{Pto}$*-HA* was grown in 2 ml of KB medium containing kanamycin and rifampcin for overnight and then transferred into 10 ml of hrp-inducing MM. Bacteria cells were harvested 16 h after culture in MM, resuspended in 5 ml of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), and sonicated on ice. The cell lysate was centrifuged at 4°C, and the supernatant was incubated with beads coupled with 50 μg of GST-HopF$_{Pto}$ and GST-AvrPto, respectively, overnight at 4°C with constant shaking. The beads were washed five times with phosphate-buffered saline plus 0.1% Tween-20, boiled in 1× SDS sample buffer, and analyzed with Western blot and the anti-HA antibody.

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LITERATURE CITED

Aldridge, P., and Hughes, K. T. 2001. How and when are substrates selected for type III secretion? Trends Microbiol. 9:209-214.

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.

Bennett, J. C. Q., and Hughes, C. 2000. From flagellum assembly to virulence: The extended family of type III export chaperones. Trends Microbiol. 8:202-204.

Bent, A. F., Kunkel, B. N., Dahlbeck, D., Brown, K. L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B. J. 1994. *RPS2* of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265:1856-60.

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.

Cambronne, E. D., Cheng, L. W., and Schneewind, O. 2000. LcrQ/YscM1, regulators of the Yersinia yop virulon, are injected into host cells by a chaperone-dependent mechanism. Mol. Microbiol. 37:263-73.

Collmer, A. 1998. Determinants of pathogenicity and avirulence in plant pathogenic bacteria. Curr. Opin. Plant Biol. 1:329-335.

Collmer, A., Lindeberg, M., Petnicki-Ocwieja, T., Schneider, D., and Alfano, J. 2002. Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. Trends Microbiol. 10:462-467.

Deng, W. L., Rehm, A. H., Charkowski, A. O., Rojas, C. M., and Collmer, A. 2003. *Pseudomonas syringae* exchangeable effector loci: sequence

diversity in representative pathovars and virulence function in *P. syringae* pv. *syringae* B728a. J. Bacteriol. 185:2592-2602.
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.
Fu, Y., and Galan, J. E. 1998. Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of *Salmonella typhimurium*. J. Bacteriol. 180:3393-3399.
Galan, J. E., and Collmer, A. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284:1322-1328.
Gaudriault, S., Paulin, J.-P., and Barny, M.-A. 2002. The DspB/F protein of *Erwinia amylovora* is a type III secretion chaperone ensuring efficient intrabacterial production of the Hrp-secreted DspA/E pathogenicity factor. Mol. Plant Pathol. 3:313-320.
Golemis, E. A., Serebriiskii, I., Gyuris, J., and Brent, R. 1997. Interaction trap/two-hybrid system to identify interacting proteins. Pages 20.1.1–20.1.35 in: Current Protocols in Molecular Biology. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. Greene Publishing Associates/John Wiley and Sons, New York.
Guan, K. L., and Dixon, J. E. 1991. Eukaryotic proteins expressed in *Escherichia coli:* An improved thrombin cleavage and purification of fusion proteins with glutathione S-transferase. Anal. Biochem. 192:262-267.
Guttman, D. S, and Greenberg, J. T. 2001. Functional analysis of the type III effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the use of a single-copy genomic integration system. Mol. Plant-Microbe Interact. 14:145-155.
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.
He, S. Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. Annu. Rev. Phytopathol. 36:363-392.
Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379-433.
Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
Jackson, R. W., Athanassopoulos, E., Tsiamis, G., Mansfield, J. W., Sesma, A., Arnold, D. L., Gibbon, M. J., Murillo, J., Taylor, J. D., and Vivian, A. 1999. Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar phaseolicola. Proc. Natl. Acad. Sci. U.S.A. 96:10875-10880.
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 109:589-98.
King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 22:301-307.
Lindgren, P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. 35:129-152.
Mackey, D., Holt, B. F, Wiig, A., and Dangl, J. L. 2002. RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell 108:743-754.
McGuffin, L. J., Bryson, K., and Jones, D. T. 2000. The PSIPRED protein structure prediction server. Bioinformatics 16:404-405.
Mudgett, M. B, Chesnokova, O., Dahlbeck, D., Clark, E. T, Rossier, O., Bonas, U., and Staskawicz, B. J. 2000. Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. Proc. Natl. Acad. Sci. U.S.A. 97:13324-13329.
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.
Niebuhr, K., Jouihri, N., Allaoui, A., Gounon, P., Sansonetti, P. J., Parsot, C. 2000. IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. Mol. Microbiol. 38:8-19.
Osawa, S., Jukes, T. H., Watanabe, K., and Muto, A. 1992. Recent evidence for evolution of the genetic code. Microbiol. Rev. 56:229-264.
Page, A. L., and Parsot, C. 2002. Chaperones of the type III secretion pathway: jacks of all trades. Mol. Microbiol. 46:1-11.
Parsot, C., Hamiaux, C., and Page, A. L. 2003. The various and varying roles of specific chaperones in type III secretion systems. Curr. Opin. Microbiol. 6:7-14.
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.
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.
Salzberg, S. L., Delcher, A. L., Kasif, S., and White, O. 1998. Microbial gene identification using interpolated Markov models. Nucleic Acids Res. 26:544-548.
Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. Science 274:2063-2065.
Shan, L., Thara, V. K., Martin, G. B., Zhou, J. M., and Tang, X. 2000. The Pseudomonas AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. Plant Cell 12:2323-2338.
Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J. E., and Innes, R. W. 2003. Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science 301:1230-1233.
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.
van Dijk, K., Fouts, D. E., Rehm, A. H., Hill, A. R., Collmer, A., and Alfano, J. R. 1999. The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. J. Bacteriol. 181:4790-4797.
van Dijk, K., Tam, V. C., Records, A. R., Petnicki-Ocwieia, T., and Alfano, J. R. 2002. The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of *Pseudomonas syringae*. Mol. Microbiol. 44:1469-1481.
Wei, W., Plovanich-Jones, A., Deng, W. L., Jin, Q. L., Collmer, A., Huang, H. C., and He, S.-Y. 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*. Proc. Natl. Acad. Sci. U.S.A. 97:2247-2252.
White, F. F., Yang, B., and Johnson, L. B. 2000. Prospects for understanding avirulence gene function. Curr. Opin. Plant Biol. 3:291-298.
Zhu, W., Yang, B., Chittoor, J. M., Johnson, L. B., and White, F. F. 1998. AvrXa10 contains an acidic transcriptional activation domain in the functionally conserved C terminus. Mol. Plant-Microbe Interact. 11:824-832.
Zwiesler-Vollick, J., Plovanich-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.

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