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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*ₚₚₒ and *HopF*ₚₚₒ that are preceded by a single *hrp* box promoter. We present evidence here to show that *ShcF*ₚₚₒ and *HopF*ₚₚₒ encode a type III chaperone and a cognate effector, respectively. *ShcF*ₚₚₒ interacts with and stabilizes the *HopF*ₚₚₒ protein in the bacterial cell. Translation of *HopF*ₚₚₒ starts at a rare initiation codon ATA that limits the synthesis of the *HopF*ₚₚₒ 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 (Alridge 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 (Cambron 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; Zwiegers-Vollick et al. 2002). *avrPphF* is a plasmid-borne *avr* gene conferring specific resistance on bean cultivars carrying the *Rl* 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*ₚₚₒ to reflect its nature as a Hop protein, homology to *AvrPphF*; and
origin from \textit{P. syringae pv. tomato}. The chaperone protein is designated ShcF\textsubscript{Pto} to indicate it as a specific Hop chaperone for HopF\textsubscript{Pto}. The HopF\textsubscript{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\textsubscript{Pto} in the HopPto\textsubscript{F} locus drastically increases the HopF\textsubscript{Pto} protein synthesis in bacterial cells.

**RESULTS**

**Sequence characterization of the HopPto\textsubscript{F} locus.**

The HopPto\textsubscript{F} locus (Fig. 1) of \textit{P. syringae pv. tomato} DC3000 consists of two ORFs, ShcF\textsubscript{Pto} and HopF\textsubscript{Pto} that encode proteins homologous to ORF1 and ORF2 encoded by the \textit{P. syringae pv. phaseolicola} \textit{avrPpH}\textsubscript{F} locus (Tsiamis et al. 2000). ShcF\textsubscript{Pto} is preceded by a Shine-Dalgarno (SD) sequence (-13GAGGAG-7) and a promoter carrying the hrp box motif. The sequence of ShcF\textsubscript{Pto} predicts a protein of 15 kDa with an isoelectric point 6.5. Secondary structure analysis using the PSIPRED program predicts a predominantly $\alpha$-helical structure throughout the ShcF\textsubscript{Pto} protein and a C-terminal $\alpha$-helical structure in particular (McGuffin et al. 2000). These characteristics often are observed in type III chaperones (Bennett and Hughes 2000). Compared with the \textit{avrPpH}\textsubscript{F} ORF2 gene, a standard initiation codon ATG was not found in the HopF\textsubscript{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\textsubscript{Pto} starts at GTG\textsubscript{13}, 13 codons downstream of ATA\textsubscript{1}. But GTG\textsubscript{13} is not preceded by a SD sequence. In addition, homology between HopF\textsubscript{Pto} and the \textit{avrPpH}\textsubscript{F} ORF2 extends to the region between GTG\textsubscript{13} and ATA\textsubscript{1}. Seven bases upstream of ATA\textsubscript{1} is a typical SD sequence (-13GAGGAG-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\textsubscript{13}, there are eight in-frame ATGs.

**ShcF\textsubscript{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 HopPto\textsubscript{F} and \textit{avrPpH} loci led us to test the secretion of the HopPto\textsubscript{F}-encoded proteins in culture. The ShcF\textsubscript{Pto} protein was tagged at the C-terminus with hemagglutinin (HA) epitope. The ShcF\textsubscript{Pto} protein was highly expressed in the \textit{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\textsubscript{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\textsubscript{Pto} is not secreted or the secretion of ShcF\textsubscript{Pto} is undetectable under the experimental conditions. We also tagged HopF\textsubscript{Pto} at its C-terminus with HA in the native context (i.e., \textit{hrp} box promoter, ShcF\textsubscript{Pto}, the spacer DNA between ShcF\textsubscript{Pto} and HopF\textsubscript{Pto}, and HopF\textsubscript{Pto}). However, we were unable to detect the HopF\textsubscript{Pto} protein in either bacterial cells or supernatant. We then replaced the \textit{hrp} box promoter with the strong constitutive \textit{nptII} promoter for the expression of the ShcF\textsubscript{Pto}/HopF\textsubscript{Pto}-HA genes. The \textit{nptII} promoter enabled the detection of a small amount of HopF\textsubscript{Pto}-HA protein in \textit{P. syringae pv. tomato} DC3000 cells but not in the supernatant (data not shown).

**HopF\textsubscript{Pto} but not ShcF\textsubscript{Pto} is translocated into plant cells.**

The failure to detect the secretion of ShcF\textsubscript{Pto} in culture might be caused by experimental conditions that did not favor the secretion of this protein. Similar result was reported for the \textit{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\textsubscript{Pto} by fusing a partial AvrRpt2 protein (AvrRpt2 41-255) to the C-terminus of ShcF\textsubscript{Pto}. The ShcF\textsubscript{Pto} protein was tagged at the C-terminus with hemagglutinin (HA) epitope. The ShcF\textsubscript{Pto} protein was highly expressed in the \textit{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\textsubscript{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\textsubscript{Pto} is not secreted or the secretion of ShcF\textsubscript{Pto} is undetectable under the experimental conditions. We also tagged HopF\textsubscript{Pto} at its C-terminus with HA in the native context (i.e., \textit{hrp} box promoter, ShcF\textsubscript{Pto}, the spacer DNA between ShcF\textsubscript{Pto} and HopF\textsubscript{Pto}, and HopF\textsubscript{Pto}). However, we were unable to detect the HopF\textsubscript{Pto} protein in either bacterial cells or supernatant. We then replaced the \textit{hrp} box promoter with the strong constitutive \textit{nptII} promoter for the expression of the ShcF\textsubscript{Pto}/HopF\textsubscript{Pto}-HA genes. The \textit{nptII} promoter enabled the detection of a small amount of HopF\textsubscript{Pto}-HA protein in \textit{P. syringae pv. tomato} DC3000 cells but not in the supernatant (data not shown).

**Fig. 2.** ShcF\textsubscript{Pto} is not secreted in liquid culture. DC3000 (pLK-\textit{hrp-ShcF-hemagglutinin} [HA]) was cultured in King’s B broth (KB) and the hyper-sensitive response and pathogenicity (hrp)-inducing minimum medium (MM). The total protein (T) of the DC3000 (pLK-\textit{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-\textit{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\textsubscript{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\(^{241-255}\) 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, \(pLK-hrp-ShcF_{Pto}-AvrRpt2^{41-255}\) which gives rise to a fusion protein of ShcF\(_{Pto}\)-AvrRpt2\(^{241-255}\), did not cause HR on RPS2 plants (Fig. 3). This result further indicated that ShcF\(_{Pto}\) is not secreted. In contrast, \(pLK-hrp-ShcF_{Pto}-HopF_{Pto}-AvrRpt2^{41-255}\), which produces the ShcF\(_{Pto}\) protein and the HopF\(_{Pto}\)-AvrRpt2\(^{241-255}\) 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 \(pLK-hrp-ShcF_{Pto}-HopF_{Pto}-AvrRpt2^{41-255}\) 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 \(pLK-hrp-ShcF_{Pto}-HopF_{Pto}-AvrRpt2^{41-255}\) (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 \(avrRpt2\)\(^{41-255}\), and the resulting plasmids were introduced into P. syringae pv. phaseolicola for the translocation assay. All the deletion constructs, including the one \((pLK-hrp-ShcF_{Pto}-HopF_{Pto}^{151}-avrRpt2^{41-255})\) that has only the N-terminal...
portion of the HopF<sub>Po</sub> 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<sub>Po</sub> is further upstream of ATG<sub>54</sub>, the first ATG from the 5′ end.

GTG<sub>13</sub> was predicted by the GLIMMER program to be the translation start codon for HopF<sub>Po</sub>. To test this prediction, we constructed a plasmid, pML-nptII-HopF<sub>Po</sub>-GTG<sub>13</sub>-HA, to express the HopF<sub>Po</sub>-HA protein starting from GTG<sub>13</sub>. This plasmid carries the HopF<sub>Po</sub> gene starting at GTG<sub>13</sub> and a synthetic SD sequence placed upstream of GTG<sub>13</sub>. As control, we also constructed a plasmid, pML-nptII-ShcF-HopF<sub>Po</sub>-HA, to express the HopF<sub>Po</sub>-HA protein encoded by the native HopF<sub>Po</sub> locus. The gene transcription in both plasmids was controlled by the strong nptII promoter. We examined the HopF<sub>Po</sub>-HA protein encoded by the native HopF<sub>Po</sub> gene in pML-nptII-ShcF-HopF<sub>Po</sub>-ATA-HA (lane 1) is ≈23 kDa and slightly larger than the protein translated from GTG<sub>13</sub> by the pML-nptII-HopF<sub>Po</sub>-GTG<sub>13</sub>-HA plasmids were cultured in minimal medium. Protein from the bacterial pellet was assayed with Western blot using the HA antibody. The HopF<sub>Po</sub> protein encoded by the native HopF<sub>Po</sub> gene in pML-nptII-ShcF-HopF<sub>Po</sub>-ATA-HA (lane 1) is ≈23 kDa and slightly larger than the protein translated from GTG<sub>13</sub> by the pML-nptII-HopF<sub>Po</sub>-GTG<sub>13</sub>-HA plasmid (lane 2). C, pLK-hrp-ShcF-HopF<sub>Po</sub>-avRpt2<sup>241-255</sup>, pLK-hrp-ShcF-HopF<sub>Po</sub>-avRpt2<sup>241-255</sup>, and pLK-hrp-ShcF-HopF<sub>Po</sub>-avRpt2<sup>241-255</sup> carry deletions of 35, 107, and 151 amino acid residues, respectively, at the C-terminus of HopF<sub>Po</sub>. 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<sup>ATA</sup>-hemagglutinin (HA) and pML-nptII-HopF<sub>TG13</sub>-HA plasmids were cultured in minimal medium. Protein from equal amount of bacterial cells was assayed with Western blot using the HA antibody.
produced by the two plasmids in DC3000 using Western blot analysis with the anti-HA antibodies. If GTG13 is the start codon, the two plasmids were expected to yield the HopFp0-hemagglutinin (HA) protein of the same size. However, the protein produced by pML-nptII-HopFp0-pTG13-HA (Fig. 4B, lane 2) was slightly smaller than the protein produced by pML-nptII-ShcFp0-HopFp0-HA (Fig. 4B, lane 1), indicating that GTG13 is not the start codon. To further test this possibility, we created point mutation in the Pseudomonas syringae pv. phaseolicola NPS 3121 (Psph)-HopFp0-chloramphenicol resistant (pML-shcFp0-HopFp0-avrRpt241-255) that converted GTG13 to GCG. GCG was not reported to act as a translational start codon in any organisms. Mutation of GTG13 to GCG did not affect the HR-inducing ability of pLK-hrp-ShcFp0-HopFp0-avrRpt241-255 (Fig. 4C), further supporting the theory that GTG13 is not the initiation codon for HopFp0. The authentic translation initiation site of HopFp0 must be further upstream of GTG13 because the HopFp0 protein encoded by the native HopFp0 locus is larger than the protein starting from GTG13.

We speculated that ATA1 functions as the start codon of HopFp0, because the homology between HopFp0 and AvrPphF ORF2 extends to the ATA1 codon, and the protein translated from ATA1 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 ATA1 to CTA and ATG in the plasmid pLK-hrp-ShcFp0-HopFp0-avrRpt241-255, (Fig. 4D), further supporting the theory that ATA1 is not the initiation codon for HopFp0. The authentic translation initiation site of HopFp0 must be further upstream of ATA1 because the HopFp0 protein encoded by the native HopFp0 locus is larger than the protein starting from ATA1.

Gene in the native HopFp0 locus showed the HR-inducing activity in the avrRpt241-255-mediated translocation assay and also could direct the synthesis of a small amount of protein in bacteria, we believed that ATA1 serves as the translation initiation codon of HopFp0.

ShcFp0 interacts with HopFp0 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 ShcFp0 indeed functions as the type III chaperone for HopFp0, we tested the interaction of ShcFp0 and HopFp0-HA in yeast and in protein pull-down assay.

Fig. 5. ShcFp0 interacts with HopFp0. A, Yeast two-hybrid analysis. ShcFp0 was cloned into the bait vector pEG202; the full-length HopFp0 gene and HopFp0 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-HopFp0 fusion proteins were purified with glutathione-conjugated cellulose beads. Washed beads carrying 50 µg of GST-AvrPto (lane 1) and GST-HopFp0 (the fusion proteins) (lane 2) were incubated with the bacterial lysate of Pseudomonas syringae pv. phaseolicola NPS 3121 strain containing ShcFp0-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.

Fig. 6. HopFp0 is unstable but can be secreted in the absence of ShcFp0. 1, Untransformed Pseudomonas syringae pv. phaseolicola NPS 3121 (Psph)-2, Psph carrying both pML-nptII-HopFp0-FLAG and pHM2-hrp-ShcFp0-hemagglutinin (HA) plasmids; and 3, Psph carrying pML-nptII-HopFp0-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 HopFp0-FLAG, ShcFp0-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 HopFp0-FLAG and ShcFp0-HA proteins. The weak bands of the same size as ShcFp0-HA in lanes 1/T and 3/P are from nonspecific hybridizations.
HopF<sub>Pso</sub> using the yeast two-hybrid assay. Expression of neither ShcF<sub>Pso</sub> nor HopF<sub>Pso</sub> alone in yeast activated the lacZ reporter gene, as indicated by the white yeast colonies (Fig. 5A). However, expression of ShcF<sub>Pso</sub> and HopF<sub>Pso</sub> together in the same yeast cell activated the lacZ reporter gene, indicating the interaction of the two proteins. HopF<sub>Pso</sub> carrying deletion of the N-terminal 13 amino acid residues still showed interaction with ShcF<sub>Pso</sub> (Fig. 5A). The interaction of HopF<sub>Pso</sub> with ShcF<sub>Pso</sub> was further confirmed by the protein pull-down assay. GST-HopF<sub>Pso</sub> fusion protein, but not GST-AvrPto, specifically pulled down the ShcF<sub>Pso</sub>-HA protein from the protein extract of <i>P. syringae pv. phaseolicola</i> expressing the ShcF<sub>Pso</sub>-HA gene (Fig. 5B).

**ShcF<sub>Pso</sub> stabilizes HopF<sub>Pso</sub> 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<sub>Pso</sub> affects HopF<sub>Pso</sub>, we tagged the C-terminus of HopF<sub>Pso</sub>-<i>ATG</i> with FLAG epitope and expressed the protein using the npII<sub>P</sub> promoter. The HopF<sub>Pso</sub> gene with ATG as start codon was used in this experiment to facilitate the protein detection. We investigated the stability and secretion of HopF<sub>Pso</sub>-FLAG in the presence or absence of ShcF<sub>Pso</sub> in <i>P. syringae pv. phaseolicola</i> strains. Upon induction in MM, over 50-fold more HopF<sub>Pso</sub>-FLAG protein was detected in <i>P. syringae pv. phaseolicola</i> carrying both pHM-hrp-ShcF<sub>Pso</sub>-HA and pML-HopF<sub>Pso</sub>-<i>ATG</i>-FLAG than in the strain carrying pML-HopF<sub>Pso</sub>-<i>ATG</i>-FLAG alone (Fig. 6). In both strains, the HopF<sub>Pso</sub> protein was detected in the supernatant as well as in the bacterial pellet. The HopF<sub>Pso</sub> protein in the supernatant was not caused by cell lysis because the ShcF<sub>Pso</sub> protein and the control NPTII protein were not detected in the supernatant (Fig. 6). These results indicated that ShcF<sub>Pso</sub> is required for the stability but dispensable for the secretion of the HopF<sub>Pso</sub> protein.

**DISCUSSION**

In this study, we showed that the HopPtoF locus of DC3000 encodes two proteins, ShcF<sub>Pso</sub> and HopF<sub>Pso</sub>. The HopF<sub>Pso</sub> gene carries a rare initiation codon ATA that limits the HopF<sub>Pso</sub> protein synthesis in <i>P. syringae</i> to a low level. ShcF<sub>Pso</sub> serves as the type III chaperone that interacts with and stabilizes the type III effector protein HopF<sub>Pso</sub>.

The ShcF<sub>Pso</sub> protein exhibits several structural and functional characteristics supporting its nature as the chaperone of HopF<sub>Pso</sub>. Structurally, ShcF<sub>Pso</sub> 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<sub>Pso</sub> is required for the stability of HopF<sub>Pso</sub> in bacterial cytoplasm. The interaction of ShcF<sub>Pso</sub> with HopF<sub>Pso</sub> in the yeast two-hybrid system and in a protein pull-down assay provided further evidence that ShcF<sub>Pso</sub> is the chaperone of HopF<sub>Pso</sub>. Unlike ShcA and ShcM, two chaperones of <i>P. syringae pv. phaseolicola</i> that facilitate secretion but are dispensable for the stability of their partner effectors HopPsaA and HopPtoM, respectively (Badel et al. 2003; van Dijk et al. 2002), ShcF<sub>Pso</sub> is dispensable for the secretion but required for the stability of HopF<sub>Pso</sub>. The requirement of type III chaperone for effector protein stability also was reported for the <i>Erwinia amylovora</i> type III chaperone DspB/F which is required for the stability of its cognate effector DspAE (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<sub>Pso</sub> gene does not have a standard translation initiation site. However, this locus is able to direct the synthesis of the HopF<sub>Pso</sub> protein in both HA tagging and AvrRpt2241-255 tagging experiments. Gene annotation using the GLIMMER program (Salzberg et al. 1998) suggested that the translation of HopF<sub>Pso</sub> starts at GTG<sub>13</sub>, 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<sub>13</sub> to GCG (GGC does not serve as the start codon in any organisms) in pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-avrRpt2241-255 did not affect the HopF<sub>Pso</sub>-AvrRpt2241-255 protein synthesis, as indicated by the HR-inducing activity of this plasmid in RPS2 plants. Furthermore, the protein synthesized from GTG<sub>13</sub> was smaller than the protein encoded by the native gene. These results strongly argue against GTG<sub>13</sub> as the initiation codon of HopF<sub>Pso</sub>.

In contrast, several pieces of evidence support the theory that ATA<sub>1</sub> is the translation initiation site for HopF<sub>Pso</sub> although ATA was not reported to act as the start codon in bacteria. First, we could detect a small amount of the HopF<sub>Pso</sub> protein encoded by the native HopPtoF locus in the HA tagging experiment when the strong constitutive npII<sub>P</sub> promoter was used to drive the expression of the genes. Second, in the AvrRpt2-mediated translocation assay, the fusion of the HopF<sub>Pso</sub>-<i>ATG</i> gene in the native HopPtoF locus with avrRpt2241-255 (in the pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-<i>ATG</i>-avrRpt2241-255 plasmid) resulted in the induction of HR on RPS2 plants, suggesting that HopF<sub>Pso</sub>-<i>ATG</i> could direct the synthesis of the fusion protein. Additional evidence came from the mutagenesis studies of ATA<sub>1</sub> in the plasmids pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-avrRpt2241-255 and the pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-HA. We found that mutation of ATA<sub>1</sub> to CTA in the HopF<sub>Pso</sub>-<i>ATA</i> gene (in pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-<i>ATG</i>-avrRpt2241-255 plasmid) abolished the HR-inducing activity, whereas mutation of ATA<sub>1</sub> to ATG enhanced the HR-inducing activity of this plasmid. Consistently, we could not detect the HopF<sub>Pso</sub> protein when ATA<sub>1</sub> was changed to CTA, but mutation of ATA<sub>1</sub> to ATG in pLK-hrp-ShcF<sub>Pso</sub>-HopF<sub>Pso</sub>-HA dramatically increased the HopF<sub>Pso</sub> protein synthesis in <i>P. syringae</i> bacteria. Furthermore, mutation of the ATA<sub>1</sub> 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<sub>1</sub> is the start codon of HopF<sub>Pso</sub>-<i>ATA</i>. The direct evidence supporting ATA<sub>1</sub> as the start codon of HopF<sub>Pso</sub>-<i>ATG</i> can be provided by the protein sequence analysis. However, the low abundance of the HopF<sub>Pso</sub> 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 <i>Pseudomonas</i> bacteria. Among the three reported homologous genes, only the HopF<sub>Pso</sub> gene from DC3000 starts with ATG. The other two genes, one from <i>P. syringae pv. phaseolicola</i> and the other from <i>P. syringae pv. delphinii</i>, 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<sub>Pso</sub> also starts with ATG (Buell et al. 2003). This suggests that the ATA<sub>1</sub> codon of the DC3000 HopF<sub>Pso</sub> 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<sub>Pso</sub>-HA protein with the HopF<sub>Pso</sub>-HA gene carrying ATA<sub>1</sub> 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 pPT6 (Ronald et al. 1992) by removing the avrPto gene with a HindIII digestion. pHM2 plasmid was modified from pHM1 (Zhu et al. 1998) by replacing the BamHI fragment with DNA containing the cloning sites of BamHI, EcoRI, XhoI, KpnI, HindIII, and XbaI (X. Tang, unpublished data). Construction of pavrRpt241-255 was previously described (Petnicki-Ocwieja et al. 2002).

To construct pLK-hrp-ShcFp0-HA, a DNA fragment spanning 200 bp upstream of the hrp box promoter and the complete ShcFp0 (excluding the stop codon) was polymerase chain reaction (PCR)-amplified from DC3000 using an upstream primer 5′-TGAATTCCGACCTTTCGTATCCGAC-3′ (EcoRI site in boldface) and a downstream primer 5′-TTGTCGACTCAGCGTCTGAGGAGATTCGTAGATT CCTTGGAGTTTGAGT-3′ (preceding the ShcFp0 3′ end sequence) and a Sall site [bold), the HA sequence plus a stop codon [underlined], and a BamHI site [italics]). The PCR products were cloned into the EcoRI and Sali sites of pMODTM-2<MC> plasmid (Epicentre, Madison, WI, U.S.A.), confirmed by sequence analysis, and further cloned into the EcoRI and SalI sites of the pLK plasmid, resulting in pLK-hrp-ShcFp0-HA. The DNA fragment also was cloned into the EcoRI and SalI sites of the pBluescript SK(−) plasmid (Stratagene, La Jolla, CA, U.S.A.). The resulting pBS-hrp-ShcFp0-HA plasmid was digested further by EcoRI and KpnI and cloned into the same sites in pHM2 vector, resulting in pHM2-hrp-ShcFp0-HA. The DNA fragment spanning the hrp box promoter and ShcFp0 in pLK-hrp-ShcFp0-HA was released by EcoRI and BamHI digestion and cloned into the EcoRI and BamHI sites of pavrRpt241-255, resulting in pLK-hrp-ShcFp0-avrRpt241-255.

To generate pLK-hrp-ShcFp0-HopFp0-HA and pLK-hrp-ShcFp0-HopFp0-avrRpt241-255 plasmids, the same upstream primer for pLK-hrp-ShcFp0-HA and the downstream primer 5′-AAAGATCCGACCTTTCGTACGGA-3′, corresponding to the 3′ end of HopFp0 plus the BamHI site (bold), were used to PCR amplify the DNA fragment encompassing the hrp box promoter, ShcFp0 spacer between ShcFp0 and HopFp0, and the complete HopFp0 from DC3000. The PCR product was cloned into the EcoRI and BamHI sites of pMODTM-2<MC> plasmid for sequence analysis. The confirmed sequence was further cloned into the EcoRI and BamHI sites of pLK-hrp-ShcFp0-HA and pLK-hrp-ShcFp0-avrRpt241-255, respectively.

The serial deletions of the HopFp0 gene in plasmids pLK-hrp-ShcFp0-HopFp0-avrRpt241-255 were generated by PCR using the same upstream primer for pLK-hrp-ShcFp0-HA and one of the following downstream primers containing the BamHI site (bold): 5′-GAGGTATCCGACCTTTCGTACGGA-3′ for deletion of amino acid (aa) residues, 5′-GAGGTATCCGACCTTTCGTACGGA-3′ for deletion of 107 aa residues, and 5′-GAGGTATCCGACCTTTCGTACGGA-3′ for deletion of 151 aa residues from the HopFp0 C-terminus. The PCR products were digested with EcoRI and BamHI and cloned into pMODTM-2<MC> for sequence analysis. The confirmed sequences were cloned into the EcoRI and BamHI sites of phrpr-SHcFp0-avrRpt241-255, replacing ShcFp0.

A QuickChange site-directed mutagenesis kit (Stratagene) was used to generate point mutations in the HopFp0 gene in pMOD::hrp-ShcFp0-HopFp0-avrRpt241-255 and pMOD::hrp-ShcFp0-HopFp0-HA with the following three pairs of complementary primers: 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′ and 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′ and 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′; 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′ and 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′; 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′; and 5′-GAGGGGAACCACTCTTACTAATATTTT GGCCG-3′. The PCR products were digested with EcoRI and BamHI and further cloned into pMODTM-2<MC> for sequence analysis. The confirmed sequences were cloned into the EcoRI and BamHI sites of phrpr-SHcFp0-avrRpt241-255, replacing ShcFp0.

To generate pNL-nptII-ShcFp0-HopFp0-HA and pNL-nptII-HopFp0 (GTG13)-HA plasmids, we used the forward primers 5′-AAAAGGCAAGCTTCAAGGGAAGATAGAACCAGA

<table>
<thead>
<tr>
<th>Designation</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>pavrRpt2</td>
<td>Broad host plasmid pLK carrying partial avrRpt2 gene with the N-terminal 40 codons deleted</td>
</tr>
<tr>
<td>pavrRpt2</td>
<td>Full length avrRpt2 gene in broad host plasmid</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>ShcFp0 under native promoter and fused in frame with partial avrRpt2, in pLK</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>HopPtoF operon under the native promoter fused with partial avrRpt2, in pLK</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>Deletion of 35 amino acids (aa) from the C-terminus of HopFp0</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>Deletion of 107 aa from the C-terminus of HopFp0</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>Deletion of 151 aa from the C-terminus of HopFp0</td>
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<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>Hemagglutinin (HA)-tagged ShcFp0 under native promoter, in pLK</td>
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<tr>
<td>pLK-hrp-ShcFp0</td>
<td>HopFp0 in the HopPtoF operon tagged by HA, in pLK</td>
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<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>HA-tagged HopFp0 starting at ATG in pML123 plasmid</td>
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<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>HopPtoF locus driven by nptII promoter, in pNL</td>
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<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>ATG of HopFp0 replaced by ATG in pNL-nptII-ShcFp0-HopFp0-avrRpt2-241-255</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>ATG of HopFp0 replaced by ATG in pNL-nptII-ShcFp0-HopFp0-avrRpt2-241-255</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>HA-tagged ShcFp0 under native promoter, in broad host plasmid pHM2</td>
</tr>
<tr>
<td>pLK-hrp-ShcFp0-avrRpt2</td>
<td>Flag-tagged HopFp0 driven by nptII promoter, in pML123</td>
</tr>
</tbody>
</table>
AAAACGCGATTT-3' and 5'-AAAAAGCAAGGGCTTCGAAAGG AGATGGAAACCTGGTGTATGACCCCACCC-3' containing the attB2 sequence (underlined) and SD sequence (italic) and the same reverse primer 5'-AGAAAGCAGGCTTTACACAGACCCCT TTTCGAC-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-HopFpotoATG-Flag, the HopFpotoATG gene was amplified by PCR using the forward primer 5'-CCGCCAGAACCTTTAAGGAAAAGCATTATTGG-3' (Xhol, bold; HindIII, italic; ATG, underlined) and the reverse primer 5'-GCTTCGAGACCCCTCGACCCGAC-3' (Csp451, bold) and pMOD::hrp-ShcFpoto/HopFpotoATG-avrRpt241-255 as template. The PCR products were digested by Xhol and Csp451 and cloned into pBS-Flag (J. Chen and X. Tang, unpublished), resulting in pBS-HopFpotoATG-Flag. This plasmid then was digested with HindIII and XbaI, and the insert was cloned into the same sites in pML123, resulting in pML-nptII-HopFpotoATG-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) (Hyunh 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 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-HopFpoto 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.

**In vitro protein pull-down assay.**

HopFpoto was released from pJG4-5::HopFpoto by EcoRI and XhoI digestion and inserted into pGST-AvrPto plasmid (Shan et al. 2000) for production of the glutathione-S-transferase (GST)-HopFpoto recombinant protein. Procedures described by Guan and Dixon (1991) were followed for purification of the GST-HopFpoto 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 ShcFpoto tagged with HA.

*P. syringae* pv. *phaseolicola* carrying pLK-hrp-ShcFpoto/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-HopFpoto 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.

**ACKNOWLEDGMENTS**

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**AUTHOR-RECOMMENDED INTERNET RESOURCE**


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