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The pathogenicity factor HrpF interacts with HrpA and HrpG to modulate type III secretion system (T3SS) function and *t3ss* expression in *Pseudomonas syringae* pv. *averrhoi*

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SUMMARY

To ensure the optimal infectivity on contact with host cells, pathogenic *Pseudomonas syringae* has evolved a complex mechanism to control the expression and construction of the functional type III secretion system (T3SS) that serves as a dominant pathogenicity factor. In this study, we showed that the *hrpF* gene of *P. syringae* pv. *averrhoi*, which is located upstream of *hrpG*, encodes a T3SS-dependent secreted/translocated protein. Mutation of *hrpF* leads to the loss of bacterial ability on elicitation of disease symptoms in the host and a hypersensitive response in non-host plants, and the secretion or translocation of the tested T3SS substrates into the bacterial milieu or plant cells. Moreover, overexpression of *hrpF* in the wild-type results in delayed HR and reduced *t3ss* expression. The results of protein–protein interactions demonstrate that HrpF interacts directly with HrpG and HrpA *in vitro* and *in vivo*, and protein stability assays reveal that HrpF assists HrpA stability in the bacterial cytoplasm, which is reduced by a single amino acid substitution at the 67th lysine residue of HrpF with alanine. Taken together, the data presented here suggest that HrpF has two roles in the assembly of a functional T3SS: one by acting as a negative regulator, possibly involved in the HrpSVG regulation circuit via binding to HrpG, and the other by stabilizing HrpA in the bacterial cytoplasm via HrpF–HrpA interaction prior to the secretion and formation of Hrp pilus on the bacterial surface.

Keywords: *hrpF*, *Pseudomonas syringae*, T3SS, *t3ss* regulation.

INTRODUCTION

Pseudomonas syringae, a plant-pathogenic bacterium that elicits necrotic spots and a hypersensitive response (HR) on the foliage of host and non-host plants, respectively, can be classified into numerous pathovars based on their specific interactions with different plant species (Hirano and Upper, 2000). The ability of *P. syringae* to infect its host plants depends on a cluster of genes, the *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity)/*hrc* (*h*ypersensitive *r*esponse and *c*onserved) genes, residing in a pathogenicity island (PAI) known as the Hrp PAI (Alfano *et al.*, 2000). The *hrp*/*hrc* genes coding for the type III secretion system (T3SS) are conserved in many Gram-negative plant- and animal-pathogenic bacteria (Galan and Collmer, 1999). In plant pathogens, the T3SS is a syringe-like structure and a protruding Hrp pilus that functions as a conduit to deliver virulence proteins, also known as type III effectors (T3Es), into host cells (Wei *et al.*, 2000). Under suitable conditions, the transcription of *P. syringae t3ss* is induced by multiple proteins, including HrpR, HrpS and HrpL, and the mode of regulation is regarded as group 1 Hrp T3SS (Alfano and Collmer, 1997), in which HrpL acts as an alternative sigma factor that recognizes the consensus '*hrp* box' in the promoter regions of *hrp* operons (Fouts *et al.*, 2002; Xiao *et al.*, 1994). The transcription of *hrpL* is induced by RpoN (σ^{54})-RNA polymerase holoenzyme (Hendrickson *et al.*, 2000) and the heterodimeric enhancer-binding proteins HrpR and HrpS (Hutcheson *et al.*, 2001; Jovanovic *et al.*, 2011). A mutation in the Hrp pilin-encoding *hrpA* gene of *P. syringae* pv. *tomato* (Pto) DC3000 has been reported to severely reduce the expression of *hrpRS*, *hrpL* and *t3ss* genes through an unknown mechanism (Wei *et al.*, 2000), suggesting that HrpA may have a positive regulatory effect on *t3ss* expression. In addition to the positive regulatory network, negative regulations involving Lon-HrpR (Bretz *et al.*, 2002) and HrpG–HrpV–HrpS (Wei *et al.*, 2005) have been reported to fine tune *t3ss* expression under different environmental conditions. The negative regulation is analogous to that found in the opportunistic animal pathogen *Pseudomonas aeruginosa*, whose *t3ss* is regulated by ExsADCE proteins (Dasgupta *et al.*, 2004; Frank and Iglewski, 1991; McCaw *et al.*, 2002; Thibault *et al.*, 2009). The involvement of HrpS, HrpV and HrpG in the regulation of *t3ss* expression is characteristically equivalent to ExsA (a transcriptional activator), ExsD (an anti-activator) and ExsC (an anti-anti-activator), respectively (Buttner, 2012; Dasgupta *et al.*, 2004; Frank and Iglewski,

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1991; McCaw *et al.*, 2002). However, to date, no *P. syringae* Hrp protein equivalent to *P. aeruginosa* ExsE has been identified.

The most prominent T3SS structure extending from bacterial surfaces is the needle filament of animal pathogens or the pilus of plant pathogens, which is polymerized by one small protein (e.g. PrgI in *Salmonella* sp., MxiH in *Shigella* sp., PscF in *P. aeruginosa*, YscF in *Yersinia pestis* and HrpA in *P. syringae*) (Blocker *et al.*, 2001; Hoiczyk and Blobel, 2001; Kimbrough and Miller, 2000; Pastor *et al.*, 2005; Roine *et al.*, 1997b). The T3SS needle-assembling protein of animal pathogens employs molecular chaperones, e.g. EscE-EscG of enteropathogenic *Escherichia coli* (Sal-Man *et al.*, 2013) and PscE-PscG cochaperones of *P. aeruginosa* (Quinaud *et al.*, 2005), as a general strategy to maintain the monomeric proteins in the cytoplasm (Quinaud *et al.*, 2007). The chaperone function for the prevention of polymerization of pilus protein prior to secretion might also exist in plant-pathogenic *P. syringae* or *Xanthomonas vesicatoria*; however, the identities of the pilus chaperones remain unknown.

A small secreted HrpF protein, encoded by the first gene adjacent to *hrpG* in the *hrpC* operon, has been demonstrated previously to be a pathogenicity factor in *P. syringae* pv. *syringae* 61 (Psy61) (Deng *et al.*, 1998; Ramos *et al.*, 2007), but its biochemical role in T3SS remains unclear. In this study, the coding sequence of *hrpF* was deleted in *P. syringae* pv. *averrhoi* strain HL1 (PavHL1) (a pathogen of starfruit; Wei *et al.*, 2012) to produce the unmarked mutant strain HL1-N1589. This mutant strain lost the ability to elicit HR in non-host plants and to multiply in host plants, as described for phenotypes of the Psy61 *hrpF* mutant (Deng *et al.*, 1998). We further analysed HrpF function in *t3ss* expression and its involvement in T3E secretion by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and assays of protein secretion and protein–protein interactions, and found that HrpF negatively regulates *t3ss* expression via binding to HrpG and is essential for T3SS function by preventing HrpA pilin from degradation in the bacterial cytoplasm. In addition, the biochemical activities of HrpF on HrpA were severely compromised by the substitution of the 67th lysine residue with alanine (Ala).

RESULTS

hrpF mutant of PavHL1 cannot elicit the HR on tobacco or cause disease on host plants

PavHL1, the causal agent of bacterial spot disease of carambola in Taiwan (Wei *et al.*, 2012), contains a functional T3SS encoded by the *hrp/hrc* gene cluster (GenBank accession number KJ534576), whose gene organization and nucleotide sequences are similar to those of the gene clusters reported in other *P. syringae* pathovars (Alfano *et al.*, 2000; Buell *et al.*, 2003; Feil *et al.*, 2005; Joardar *et al.*, 2005). Comparison of the amino acid sequence revealed that $HrpF_{Pav}$ is 80%–100% identical to HrpF of several *P. syringae* pathovars, but shares only 38% identity with HrpF of Pto (Fig. S1A, see Supporting Information). In this study, two homologous alleles of *hrpF*, one from PavHL1 ($hrpF_{Pav}$) and one from PtoDC3000 ($hrpF_{Pto}$), were chosen for biological analyses. The virulence functions of HrpF in PavHL1 and PtoDC3000 were first characterized by creating unmarked deletions of *hrpF*, followed by the infiltration of the mutant strains into plants for bioassays. As shown in Figs 1A–D and S1B,C, both mutants failed to elicit disease symptoms in host plants and the HR in non-host tobacco plants, and their multiplication in host plants was severely reduced in comparison with the wild-type (WT) strains. The starfruit leaves sprayed with PavHL1 and *hrpF*-complementing strain showed necrotic spots with surrounding chlorotic haloes (Fig. 1A) at 7 dpi (days post-inoculation). Interestingly, although *hrpF* orthologues from the pathovars *averrhoi* and *tomato* display low identity, heterologous expression of $HrpF_{Pto}$ in the Pav *hrpF* mutant HL1-N1589 partially restored the ability of the $hrpF_{Pav}$ mutant to multiply in starfruit leaves (Fig. 1B) and to trigger ion leakage at 7 hpi (hours post-inoculation) (Fig. 1D) and HR at 24 hpi in tobacco leaves (Fig. 1C). Pav HrpF could also partially complement the HR phenotype of the Pto *hrpF* mutant (see Fig. S1C), suggesting that the functions of HrpF are conserved among different *P. syringae* pathovars. We tested whether the *hrpF* mutant HL1-N1589 is defective in T3SS function by growing bacteria in *hrp*-inducing medium HrpMM (Hrp minimal medium; Huynh *et al.*, 1989) and then assaying T3E secretion using an immunoblotting method. Compared with the WT PavHL1, HopAK1, HrpZ and HrpA were not detectable in the supernatant fractions of HL1-N1589 or the T3SS-defective *hrcC* mutant, indicating that the mutation of $hrpF_{Pav}$ abolished the T3SS function to secrete proteins (Fig. 1E). In addition, the amount of HrpA in the cell pellet fraction of the *hrpF* mutant was consistently lower than that in the WT and *hrcC* mutant.

The calmodulin-dependent adenylate cyclase (Cya) reporter system (Schechter *et al.*, 2004) was used to analyse the translocation of *P. syringae* T3Es into plant cells. The results shown in Table 1 confirm that the mutation in *hrpF* abolishes the translocation of T3Es [e.g. AvrPto1-cCya in Psy61 (Ramos *et al.*, 2007) and HopI1-cCya in Pav (this study)], and HrpF-cCya is translocated into plant cells in a T3SS-dependent manner. Taken together, it can be concluded that Pav HrpF is involved in T3SS function by assisting the secretion and translocation of T3Es, and HrpF itself can also be translocated into plant cells.

hrpF mutant HL1-N1589 expressing hemagglutinin (HA)-tagged $HrpF_{Pav}$ (HrpF-HA27) restores WT-like phenotypes

In order to analyse the functions of HrpF, the plasmid pNCHU1810 was constructed to express HA-tagged HrpF from the *lacZ*

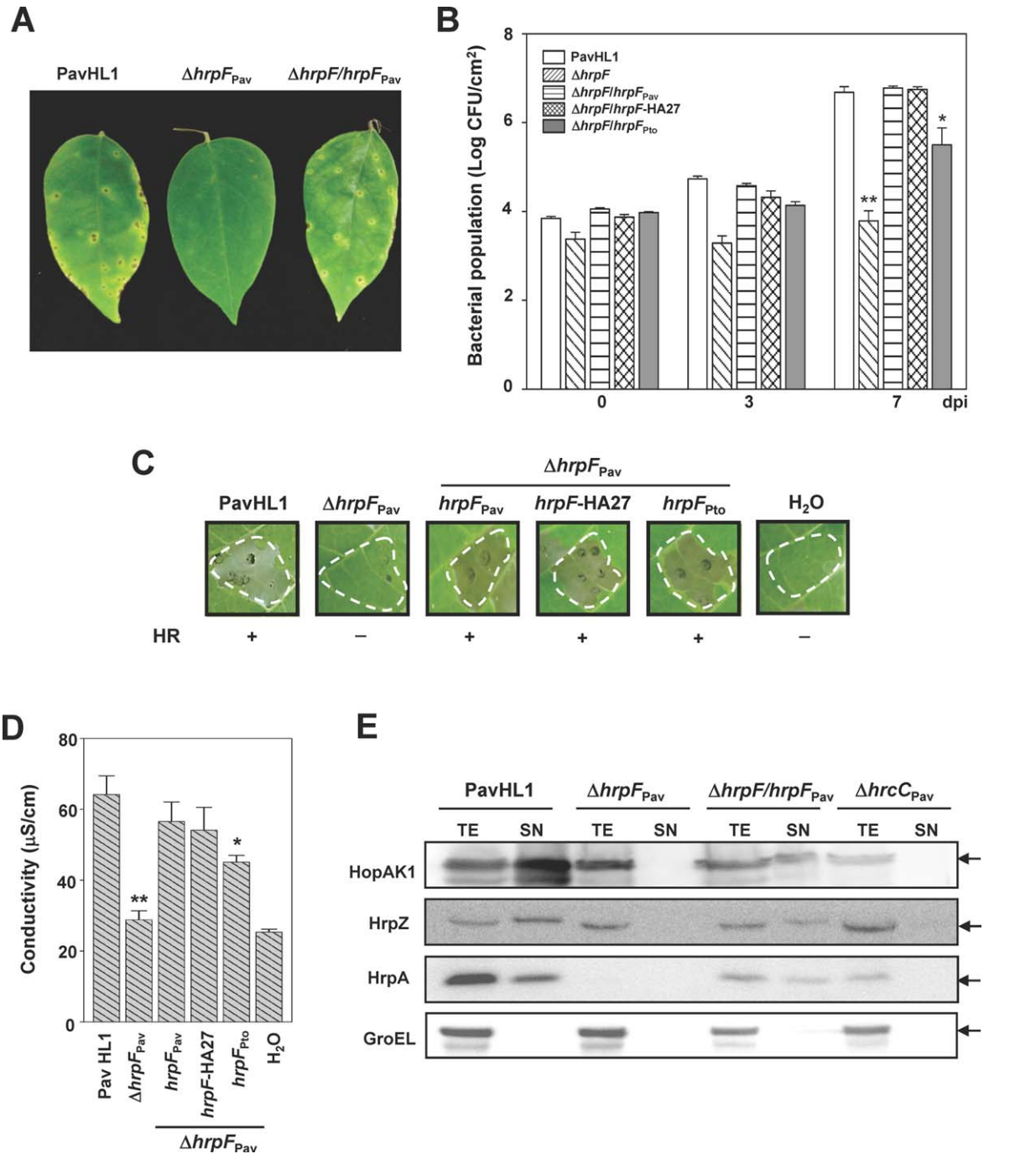

Fig. 1 Phenotypes of the *hrpF* mutant strains derived from *Pseudomonas syringae* pv. *averrhoi* HL1 (PavHL1). (A) Necrotic spot symptoms on host plants caused by PavHL1, *hrpF* mutant (Δ*hrpF*$_{Pav}$) and the complemented strain (Δ*hrpF/hrpF*$_{Pav}$). (B) Bacterial growth in starfruit leaves. Each bar represents the mean and standard deviation of triplicate samples. CFU, colony-forming units; dpi, days post-inoculation. Hypersensitive response (HR) elicitation (C) and conductivity assays (D) demonstrate incompatible reactions elicited by the test strains. +, HR positive; –, HR negative. The mean values of bacterial populations and conductivity between wild-type and test strains were significantly different according to Student's *t*-test (*$P < 0.05$; **$P < 0.01$). (E) Immunoblot analysis of type III effector (T3E) secretion in the test strains using antisera against HopAK1, HrpZ, HrpA and GroEL. Bacterial cultures in Hrp minimal medium (HrpMM) at 6 h post-inoculation (hpi) were separated into culture supernatant (SN) and total cell extract (TE) according to Experimental procedures. GroEL was detected as a reference for cellular integrity. PavHL1, wild-type harbouring pBBR1MCS-5; Δ*hrpF*$_{Pav}$, HL1-N1589; Δ*hrpF*/*hrpF*$_{Pav}$, HL1-N1589 harbouring pNCHU1591; Δ*hrpF*/*hrpF*-HA27, HL1-1589 harbouring pNCHU1810; Δ*hrpF*/*hrpF*$_{Pto}$, HL1-N1589 harbouring pNCHU1809; Δ*hrcC*$_{Pav}$, PavHL1-N1033.

Table 1 Translocation of HrpF-cCya or HopI1-cCya into *Nicotiana tabacum* cells by *Pseudomonas syringae* pv. *averrhoi*.

P. syringae pv. *averrhoi*	Genotype	Assay target*	Translocation† (pmol cAMP/μg protein)
HL1	Wild-type	HrpF-cCya (pNCHU1947)	32.22 ± 14.19
		HopI1-cCya (pNCHU1881)	355.18 ± 155.7
HL1-N1033	Δ*hrcC*::*nptII*	HrpF-cCya (pNCHU1947)	1.37 ± 0.23
		HopI1-cCya (pNCHU1881)	1.84 ± 1.9
HL1-N1589	Δ*hrpF*	HrpF-cCya (pNCHU1947)	2.47 ± 1.16
		HopI1-cCya (pNCHU1881)	2.33 ± 0.95
HL1-N1589/pNCHU1591	Δ*hrpF*/*hrpF*	HopI1-cCya (pNCHU1938)	29.07 ± 14.31
HL1-N1589/pNCHU1808	Δ*hrpF*/*hrpF*$_{K67A}$	HopI1-cCya (pNCHU1938)	2.16 ± 0.13
HL1/pBBR1MCS-5	Wild-type/EV‡	HopI1-cCya (pNCHU1938)	354.07 ± 81.55
HL1/pNCHU1591	Wild-type/*hrpF*	HopI1-cCya (pNCHU1938)	22.46 ± 16.76
HL1/pNCHU1593	Wild-type/*hrpG*	HopI1-cCya (pNCHU1938)	332.92 ± 55.01
HL1/pNCHU1866	Wild-type/*hrpFG*	HopI1-cCya (pNCHU1938)	101.40 ± 12.79

*Bacterial strains carried pNCHU1947, pNCHU1881 or pNCHU1938 (Table S1) which expresses HrpF-cCya or HopI1-cCya from a *lac* promoter of the plasmid.
†Levels of cyclic adenosine monophosphate (cAMP) in the leaf samples of *Nicotiana tabacum* were determined at 6 h post-infiltration with bacterial inoculum at an optical density at 600 nm (OD_{600}) of 0.3, and the values represent the mean and standard deviation ($n = 3$) in each treatment.
‡EV, empty vector.

promoter. The HA tag was inserted between the 27th glutamic acid (Glu) and 28th glycine (Gly) residues (E_{27}-GYPYDVPDYAG-G_{28}) to produce a HrpF-HA27 fusion protein, because the tag-fused HrpF proteins at the C-terminus (e.g. HrpF-cHA, HrpF-cFLAG or HrpF-cCya) failed to complement *hrpF* mutant phenotypes (data not shown). According to the predicted protein secondary structure at the Phyre Server (Kelley and Sternberg, 2009), the E27 and G28 residues reside in a loop region connecting two α-helices; therefore, the structural integrity and function of HrpF are probably maintained on insertion of the HA epitope. Inoculation assays were performed to test whether HrpF-HA27 was functional, and the results showed that HrpF-HA27 fully complemented the mutant phenotypes of HL1-N1589 in bacterial multiplication in starfruit leaves (Fig. 1B) and triggered the HR and ion leakage in tobacco leaves (Fig. 1C,D). In addition, HrpF-HA27 was secreted to the culture supernatant via T3SS (Fig. 5D) by culturing the HrpF-HA27-expressing strain HL1-N1589 (pNCHU1810) in HrpMM. Taken together, HrpF-HA27 is functionally equivalent to the native HrpF protein.

Overexpression of $HrpF_{Pav}$ in PavHL1 reduces the level of HR elicitation, and co-expression of HrpFG counteracts the negative effect

Because the delayed HR phenotype was consistently observed when the *hrpF* complementing strain HL1-N1589 (pNCHU1591) was inoculated into tobacco leaves at 10^6 colony-forming units (CFU)/mL (data not shown), the negative effect of HrpF on T3SS function was tested by HR assays using serially titrated suspensions of the WT PavHL1 overexpressing *hrpF* as inocula. On infiltration of bacteria at a concentration of 2×10^6 CFU/mL into tobacco leaves, HL1 expressing *lac* promoter-driven HrpF or HrpF-HA27, in comparison with HL1 and HL1 expressing *hrpG*, displayed lower levels of ion leakage and delayed HR at 24 hpi. This phenomenon could be counteracted by the co-expression of *hrpFG* (Fig. 2A), whose expression was driven by the same *lac* promoter. There were no discernible differences between the above tested strains in the HR when the inoculum was higher than 2×10^6 CFU/mL (Fig. 2A, left panel). The negative effect on HR elicitation was further confirmed by the overexpression of *hrpF*-HA27 driven by a stronger *tac* promoter. The inoculation results showed that HL1 expressing the *tac*-driven HrpF-HA27 (pNCHU2164; Table S1, see Supporting Information) had a stronger negative effect than the *lac*-driven expression of HrpF-HA27 (pNCHU1810; Table S1) on HR elicitation by suppressing HR at 6×10^6 CFU/mL (Fig. S2 see Supporting Information), suggesting that the cumulative amount of HrpF in HL1 cells is negatively correlated with the ability to elicit HR, and the negative effect of HrpF on HR elicitation can be counteracted by the co-expression of HrpG.

HrpF interacts with HrpG and HrpA

The HrpF–HrpG and HrpF–HrpA interactions were examined on the basis of two findings: (i) the co-expression of HrpF and HrpG in the WT PavHL1 relieved the phenotypes, i.e. the suppression of HR elicitation by overexpression of HrpF only (Fig. 2A); and (ii) the cumulative HrpA protein in the whole cell fraction of the *hrpF* mutant was very low (Fig. 1E). A BacterioMatch II Two-Hybrid System (B2H) (Stratagene, Santa Clara, CA, USA) was used to determine protein interactions *in vivo*. The *Escherichia coli* transformants harbouring pTRG-HrpG and pBT bait vector showed about 95% interaction activity (data not shown), suggesting that the chimeric HrpG-RNA polymerase alone can activate the expression of the reporter cassette. Therefore, pTRG-HrpG was not

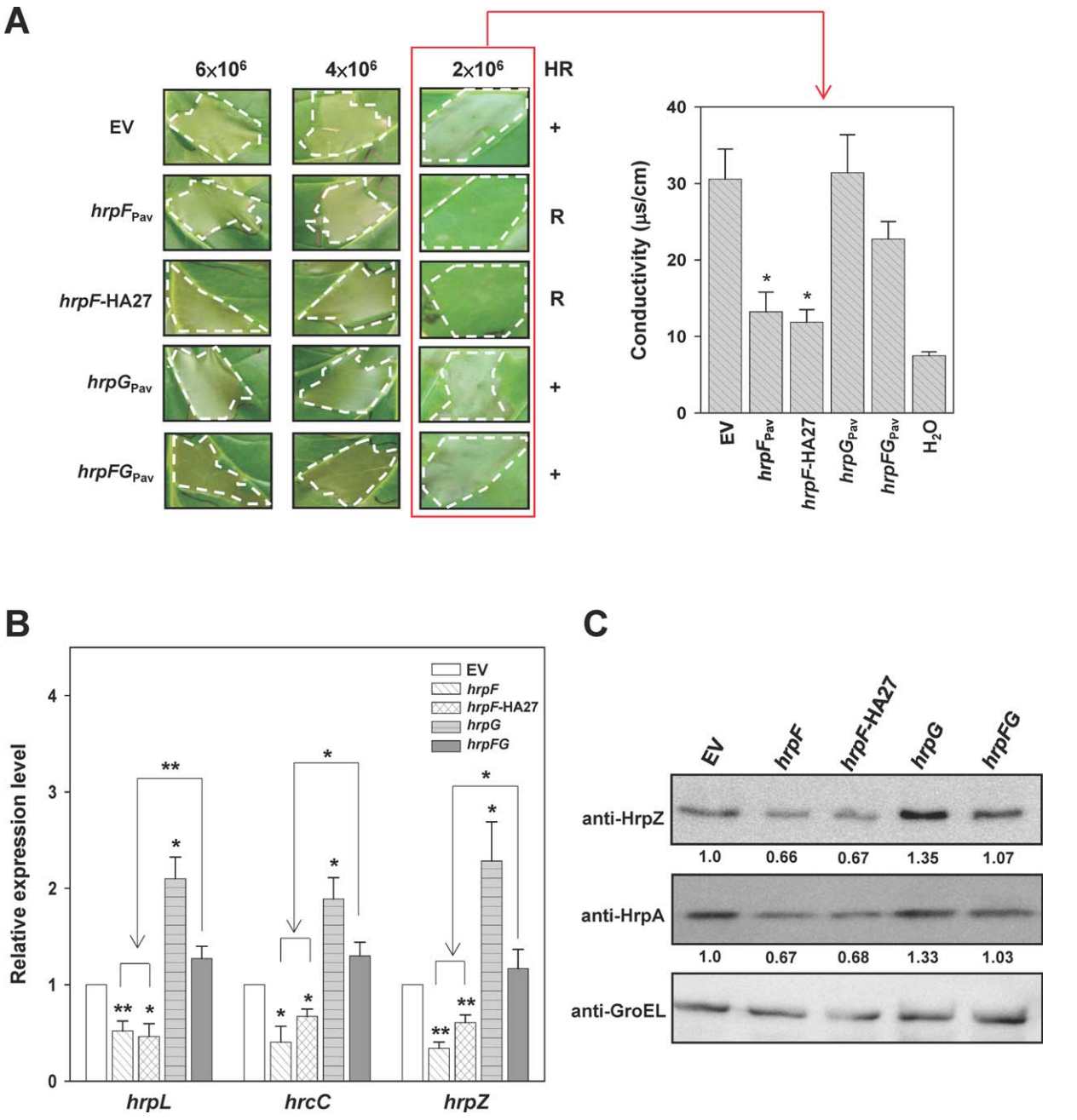

Fig. 2 Overexpression of HrpF showed negative effects on the hypersensitive response and Hrp expression. (A) Tobacco leaves were infiltrated at the indicated concentrations with *Pseudomonas syringae* pv. *averrhoi* HL1 (PavHL1) harbouring pBBR1MCS-5 (EV), pNCHU1591 ($hrpF_{Pav}$), pNCHU1810 (*hrpF*-HA27), pNCHU1593 ($hrpG_{Pav}$) or pNCHU1866 ($hrpFG_{Pav}$), and photographed at 24 h post-inoculation (hpi) for the hypersensitive response (HR, left panel). The ionic conductivity was assayed at 24 hpi (right panel) with the indicated inocula for quantitative analysis of cell death. +, wild-type HR; R, reduced HR. The conductivity values were significantly different between PavHL1 (EV) and the strains overexpressing $hrpF_{Pav}$ or *hrpF*-HA27 according to Student's *t*-test ($*P < 0.05$). (B) Relative transcription of *hrpL*, *hrcC* and *hrpZ* in PavHL1 overexpressing $hrpF_{Pav}$, *hrpF*-HA27, $hrpG_{Pav}$ and $hrpFG_{Pav}$ at 6 hpi. The 16S rRNA was quantified and used for normalization by the comparative CT (cycle threshold) method, and the relative expression level was expressed as the fold change using the quantitative polymerase chain reaction (qPCR) result of PavHL1 carrying the empty vector as the denominator and those of PavHL1 expressing the target gene as the numerators. The error bars denote standard deviation ($n = 3$). Statistical analysis by Student's *t*-test ($*P < 0.05$; $**P < 0.01$) was applied to compare the fold change of transcription between PavHL1 carrying the empty vector (EV) and various *hrp*-expressing plasmids. (C) The production of HrpZ and HrpA in PavHL1 overexpressing $hrpF_{Pav}$, *hrpF*-HA27, $hrpG_{Pav}$ and $hrpFG_{Pav}$ was determined by immunoblot analysis at 6 hpi. GroEL was detected as a protein loading control, and the relative translations of HrpZ and HrpA in PavHL1-derived strains are shown as fold changes in the banding intensity using the wild-type as the denominator.

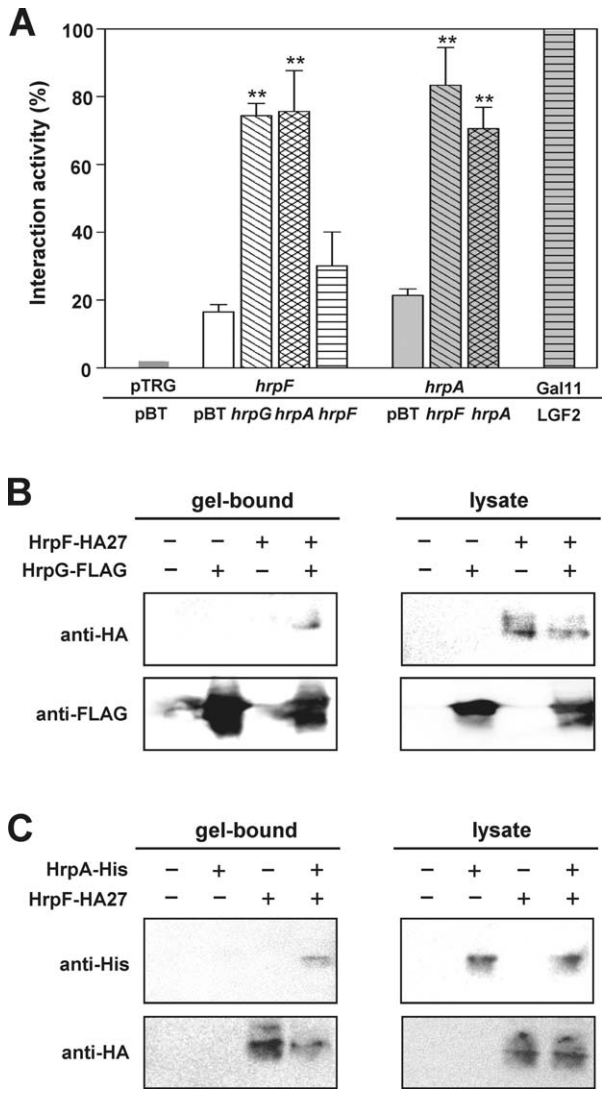

Fig. 3 HrpF interacts with HrpG and HrpA in bacterial two-hybrid and immunoprecipitation assays. (A) *In vivo* protein interactions were tested by bacterial two-hybrid assays. Statistical differences were assessed by Student's *t*-test (**$P < 0.01$) on the interaction between pBT vector and each pBT-expressing *hrp* gene when pTRG-*hrpF* or pTRG-*hrpA* was used as the bait. (B, C) Immunoblots of immunoprecipitated proteins probed with monoclonal anti-haemagglutinin (anti-HA) (B) and anti-histidine (anti-His) (C) antibodies to show *in vitro* protein interactions. *Escherichia coli* lysates expressing HrpG-FLAG, HrpA-His, HrpF-HA27 (indicated by '+') or vector alone (indicated by '−') were mixed with anti-FLAG M2 (B) or anti-HA (C) agarose beads for immunoprecipitation, as described in Experimental procedures. Gel-bound, immunoprecipitated proteins; lysate, total proteins.

included in this assay. As shown in Fig. 3A, compared with the positive control strain harbouring pBT-LGF2 and pTRG-Gal11 as 100% interaction activity, the transformants co-expressing pTRG-HrpF with pBT-HrpG or pBT-HrpA and the pairs of pTRG-HrpA with pBT-HrpF or pBT-HrpA exhibited 74.4 ± 2.1%, 75.6 ± 7.0%, 83.3 ± 6.4% and 70.6 ± 4.5% interaction activities, respectively. Because HrpA is the major component of Hrp pilus (Li *et al.*, 2002; Roine *et al.*, 1997b), the interaction of HrpA fusion proteins expressed from pTRG-HrpA and pBT-HrpA was also tested to show that HrpA indeed binds to itself in B2H. The interaction activity (*c.* 30%) of co-expression of pTRG-HrpF and pBT-HrpF was not significantly greater than that of co-expression of pTRG-HrpF and pBT, indicating that HrpF does not interact with itself in B2H. The results reveal that HrpF interacts with HrpG and HrpA, but does not self-associate, *in vivo*.

Next, an immunoprecipitation assay using anti-FLAG (binding with HrpG-FLAG) or anti-HA (binding with HrpF-HA27) affinity gel was applied to detect protein interactions *in vitro*. The FLAG- or HA agarose-bound proteins were analysed by immunoblotting using antibodies against HA or histidine (His) epitope, respectively. The results showed that HrpF-HA27 can be detected in the FLAG-gel-bound fraction (Fig. 3B) and HrpA-His (encoded by pNCHU1944, see Table S1 for plasmid descriptions) in the HA-bound fraction (Fig. 3C), revealing that HrpF binds to HrpG and HrpA *in vitro*. By contrast, HrpA-His and HrpG-FLAG could not be co-precipitated with the anti-FLAG or Ni-NTA resin (Fig. S3, see Supporting Information), suggesting that HrpA protein does not interact with HrpG protein *in vitro*.

HrpF negatively regulates the expression of *t3ss*

The results shown in Figs 2A and 3 indicate that HrpF may be involved in the negative feedback loop of *t3ss* expression via interaction with HrpG; therefore, we measured *t3ss* transcription in the *hrpF* mutant and WT overexpressing *hrpF* by qRT-PCR. For comparison of the *hrp/hrc* expression level between the WT and its derivative strains, the plasmids (Table S1) overexpressing *hrp* genes of interest driven by the *lac* promoter in the WT were consistently used in the following experiments. The transcription of four *hrp/hrc* genes, i.e. *hrpL*, *hrcC*, *hrpA* and *hrpZ*, which reside in different *hrp* operons and encode different components of T3SS, was monitored. After normalization with the 16S rRNA transcript, the relative quantities of the *hrp* genes were calculated as described in Experimental procedures. As shown in Fig. S4A (see Supporting Information), the levels of *hrpL*, *hrcC*, *hrpZ* and *hrpA* transcripts in WT increased gradually with increasing incubation time, and the transcription patterns of *hrpA* and *hrpZ* residing in the same transcription unit (Huang *et al.*, 1995) were the same (Fig. 4A). Similarly, the immunoblot results showed that HrpZ production in the *hrpF* mutant was about 1.5-fold that of the amount in WT, whereas the cumulative HrpA in the *hrpF* mutant was not positively correlated with the transcription level. At 6 hpi, the detectable HrpA in the *hrpF* mutant was about 3% of that produced in WT, suggesting that HrpA is post-translationally processed in the *hrpF* mutant (Fig. 4B). The fold changes of *hrpL*, *hrcC*, *hrpZ* and *hrpA* transcripts in the *hrpF* mutant strain HL1-N1589 compared with WT were greater than unity (Fig. 4A), and HL1-

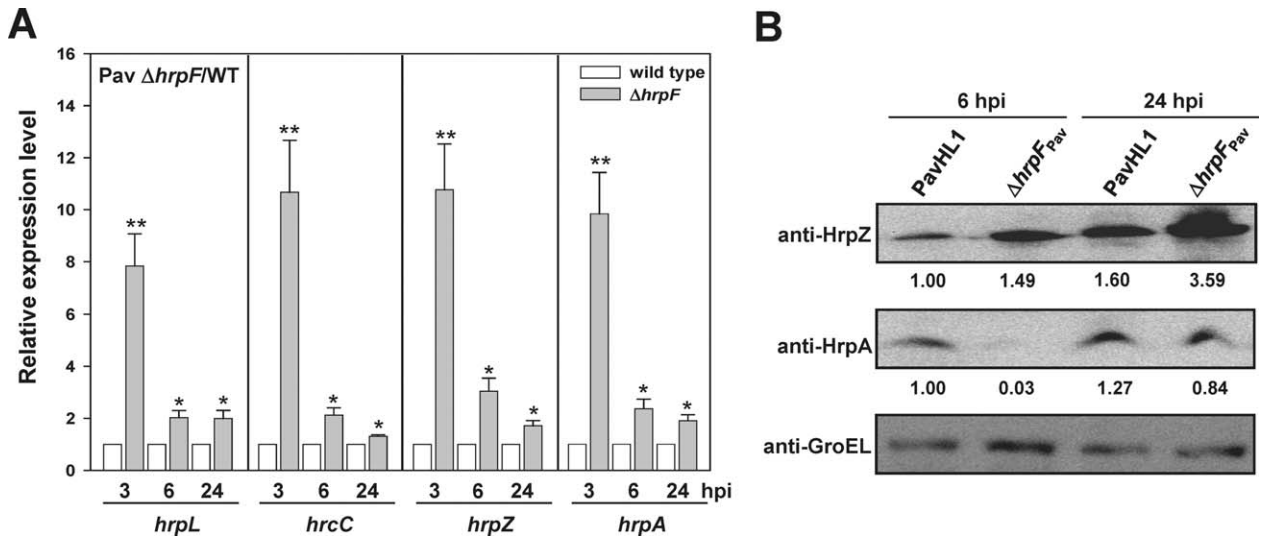

Fig. 4 Expression of *hrp* genes was elevated in the *hrpF* mutant. (A) Relative transcription of *hrpL*, *hrcC*, *hrpZ* and *hrpA* in the *hrpF* mutant and wild-type. Transcription of each *hrp* gene was normalized by the 16S rRNA transcript as described in Experimental procedures, and the relative expression levels of the *hrp* genes were determined at 3, 6 and 24 h post-inoculation (hpi) using the quantitative polymerase chain reaction (qPCR) results of the wild-type as the denominator. The relative expression levels of the *hrpL*, *hrcC*, *hrpZ* and *hrpA* transcripts were significantly different between the wild-type and *hrpF* mutant at 3 hpi. The error bars denote standard deviation ($n = 3$). Statistical analysis by Student's *t*-test ($*P < 0.05$; $**P < 0.01$) was applied to compare the fold change of transcription between the wild-type and *hrpF* mutant. (B) Immunoblot analysis of HrpZ and HrpA at 6 and 24 hpi in the wild-type and *hrpF* mutant. GroEL served as a protein loading control, and the relative production of HrpZ and HrpA is shown as the fold change in the banding intensity using the wild-type at 6 hpi as the denominator.

N1589 showed the maximum transcription of these four genes at 3 hpi (Fig. 4A), whereas these transcripts reached the highest level at 6 hpi in the PtoDC3000 *hrpF* mutant (Fig. S4B, top panel), indicating that HrpF has a negative regulatory function in early *t3ss* expression. This result was different from that for the *nptII*-marked *hrpF* mutant of Psy61, which showed reduced *t3ss* expression (Fig. S4B, bottom panel) (Ramos *et al.*, 2007). The opposite patterns of *t3ss* expression in the marked and unmarked *hrpF* mutants are most probably a result of the *nptII* promoter-driven, constitutive expression of the downstream negative regulator *hrpV* (Wei *et al.*, 2005). Moreover, compared with WT, *in-trans* expression of *hrpF* or *hrpF*-HA27 in WT PavHL1 reduced the transcription of the three *hrp/hrc* genes at 6 hpi (Fig. 2B). The fold changes of the three *hrp/hrc* and *hrpF* genes in WT ectopically expressing *hrpG* were between two and four (Figs 2B and S4C), whereas co-expressed *hrpFG* genes counteracted the positive regulatory effect of HrpG on the transcription of the three *hrp/hrc* genes, reducing the fold changes to close to unity (Fig. 2B), indicating that HrpF and HrpG show inverse effects on *t3ss* expression. Overexpression of either *hrpF* or *hrpG*, which are organized in the same transcription unit (Huang *et al.*, 1995), may disrupt the stoichiometry of interacting proteins, leading to the change in *t3ss* expression. Immunoblot analyses using sera against HrpZ and HrpA and effector translocation assays using the Cya reporter system were also conducted to monitor T3SS expression and function. As shown in Fig. 2C, the cumulative levels of HrpZ and HrpA in WT harbouring the plasmids containing *hrpF*, *hrpG* or *hrpFG* were consistent with the qPCR results (Fig. 2B), and the expression patterns were positively correlated with the results of the HR phenotype and ionic conductivity (Fig. 2A). Cya translocation assay revealed that, in WT overexpressing *hrpF*, T3SS was functional and could secrete and translocate T3Es, but the translocated HopI1 was reduced to *c.* 6% relative to WT (Table 1). Taken together, the results reveal that the function of HrpF is characteristically equivalent to a negative regulator, and the presence of HrpG counteracts the negative effect of HrpF.

A single amino acid substitution (K67A) at the C-terminus of $HrpF_{Pav}$ affects the virulence and secretion/translocation of T3Es

As it was found that epitope tagging at the C-terminus of HrpF did not complement the *hrp*-minus phenotypes of the *hrpF* mutant (data not shown), the importance of the HrpF C-terminus in T3SS function was further analysed. The C-terminal three conserved amino acids of HrpF (denoted by asterisks in Fig. S1A) were replaced with Ala to produce $HrpF_{K67A}$, $HrpF_{D71A}$ and $HrpF_{Q74A}$. These HrpF variants were expressed in HL1-N1589 for complementation assays. The alleles encoding $HrpF_{D71A}$ and $HrpF_{Q74A}$ in HL1-N1589 restored bacterial multiplication to the WT level in starfruit leaves (data not shown), whereas the population of HL1-N1589 harbouring pNCHU1808 ($hrpF_{K67A}$) only reached 5.2 logs, not significantly different from the 4.9 logs of the *hrpF* mutant (CFU/cm^2 at 7 dpi) (Fig. 5A). By comparison with WT and HL1-

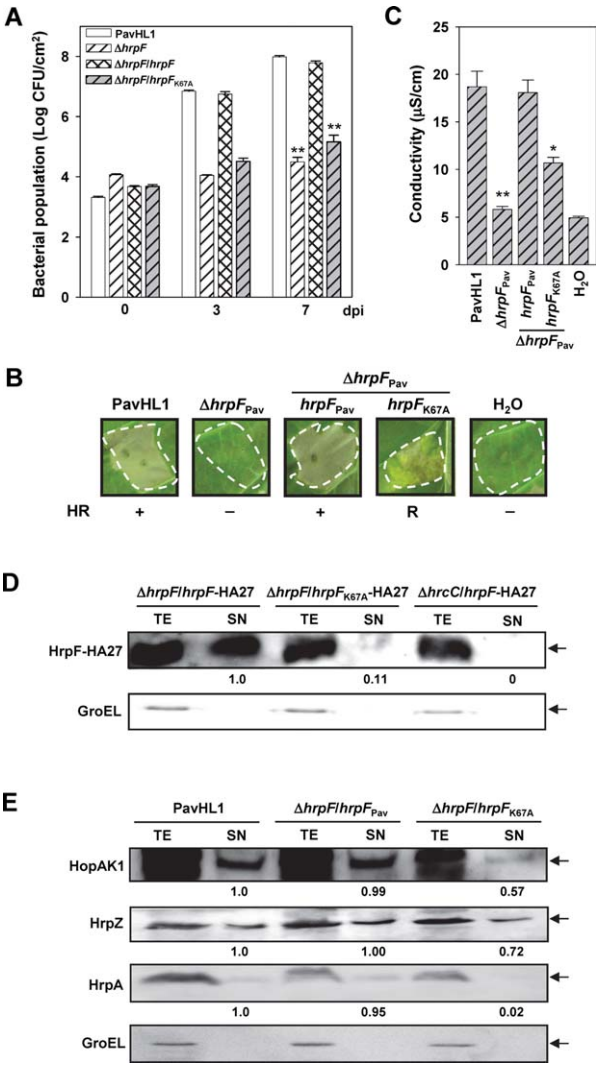

Fig. 5 The effects of *in-trans* expression of HrpF$_{K67A}$ on plant responses and type III effector (T3E) secretion. (A) Bacterial growth in starfruit leaves. (B) Elicitation of the hypersensitive response (HR) on tobacco leaves: +, HR positive; −, HR negative; R, reduced HR. (C) Conductivity on tobacco leaves. PavHL1, *Pseudomonas syringae* pv. *averrhoi* HL1 (pBBR1MCS-5); Δ*hrpF*$_{Pav}$, HL1-N1589 (pBBR1MCS-5); Δ*hrpF*/*hrpF*$_{Pav}$, HL1-N1589 (pNCHU1591); Δ*hrpF*/*hrpF*$_{K67A}$, HL1-N1589 (pNCHU1808). The mean values of the bacterial population (A) and conductivity (C) between the wild-type and individual strain were significantly different according to Student's *t*-test (*$P < 0.05$; **$P < 0.01$). (D) Differential secretion of HrpF-HA27 and HrpF$_{K67A}$-HA27 in the Pav *hrpF* mutant was detected by immunoblotting against anti-haemagglutinin (anti-HA) antibody and expressed as the fold change in the banding intensity using HrpF-HA27-expressing HL1-1589 as the denominator. (E) The secretion of HopAK1, HrpZ and HrpA in PavHL1 and HL1-1589 harbouring *hrpF* or *hrpF*$_{K67A}$ was detected by immunoblotting. Differential secretion of T3Es was indicated by the fold change using PavHL1 as the denominator. In (D) and (E), GroEL was detected as a marker of cellular integrity. SN, culture supernatant; TE, total cellular extract.

N1589 carrying the native *hrpF*, the *hrpF* mutant harbouring HrpF$_{K67A}$ also reduced the ability to induce the HR (Fig. 5B) and apoplastic ion leakage at 7 hpi (Fig. 5C) on tobacco leaves.

As HrpF is a secreted protein, we further investigated whether HrpF$_{K67A}$ is secreted into the bacterial milieu via T3SS. HL1-N1589 harbouring pNCHU1810 (HrpF-HA27) or pNCHU1945 (HrpF$_{K67A}$-HA27, HrpF$_{K67A}$ was internally tagged with HA as described for the preparation of HrpF-HA27) was cultured in HrpMM and subjected to a protein secretion assay using an immunoblotting method. The immunoblots showed that HrpF$_{K67A}$-HA27 can also be secreted to the culture supernatant, but the amount of HrpF$_{K67A}$-HA27 in the supernatant fraction is much lower than that of HrpF-HA27 (Fig. 5D), suggesting that the 67th lysine residue of HrpF is critical for its own secretion. Furthermore, the secretion of T3E substrates in HL1-N1589 harbouring *hrpF*$_{K67A}$ was quantitatively monitored. As shown in Fig. 5E, the immunoblot revealed that the amounts of secreted HopAK1 and HrpZ in HL1-N1589 harbouring *hrpF*$_{K76A}$ were lower than those in WT and WT expressing the native HrpF, and the secretion of HrpA in the complementing strain expressing *hrpF*$_{K76A}$ was severely reduced to approximately 2% of the amount secreted by WT. The efficiency of the HopI1-cCya translocation was also greatly reduced in HL-N1589 harbouring *hrpF*$_{K67A}$ (Table 1), indicating that the 67th lysine residue of HrpF is important for its function in assisting the secretion and translocation of T3E substrates.

HrpF is required for HrpA stability in the bacterial cytoplasm

As shown by the results illustrated in Figs 1E, 4B and 5E, the cumulative amount of HrpA in the cell pellets was severely reduced in the *hrpF* mutant, and the plasmid-borne *hrpF*$_{K67A}$ cannot fully complement the mutant phenotypes. Therefore, the involvement of the 67th lysine residue of HrpF in T3SS function was further analysed by testing the interactions between HrpF$_{K67A}$-HA27 and HrpA. The immunoprecipitation assay (Fig. 6A) showed that the amount of HrpA pulled down by HrpF$_{K67A}$-HA27 was about 35% of that by HrpF-HA27. Far western analysis confirmed that HrpF-HA27, but not HrpF$_{K67A}$-HA27 (barely dectectable), binds to denatured HrpA (Fig. 6B), indicating that the 67th lysine residue of HrpF is critical for its interaction with HrpA. The biochemical activity of HrpF on HrpA was tested by the culture of PavHL1 and its derivatives harbouring pNCHU2046 (co-expressing HrpA and HrpZ from the constitutive *lac* promoter on the vector) in HrpMM to induce *t3ss* expression. The translation of T3SS was stopped by the addition of 100 ppm of chloramphenicol to the culture medium at 2 hpi. Total cell cultures containing culture medium and bacterial cells were harvested at 0, 2 and 4 h post-chloramphenicol treatment for immunoblot analyses using sera against HrpA and HrpZ. As shown in Fig. 6C, the level of HrpZ remained constant in PavHL1 and its derivatives at different

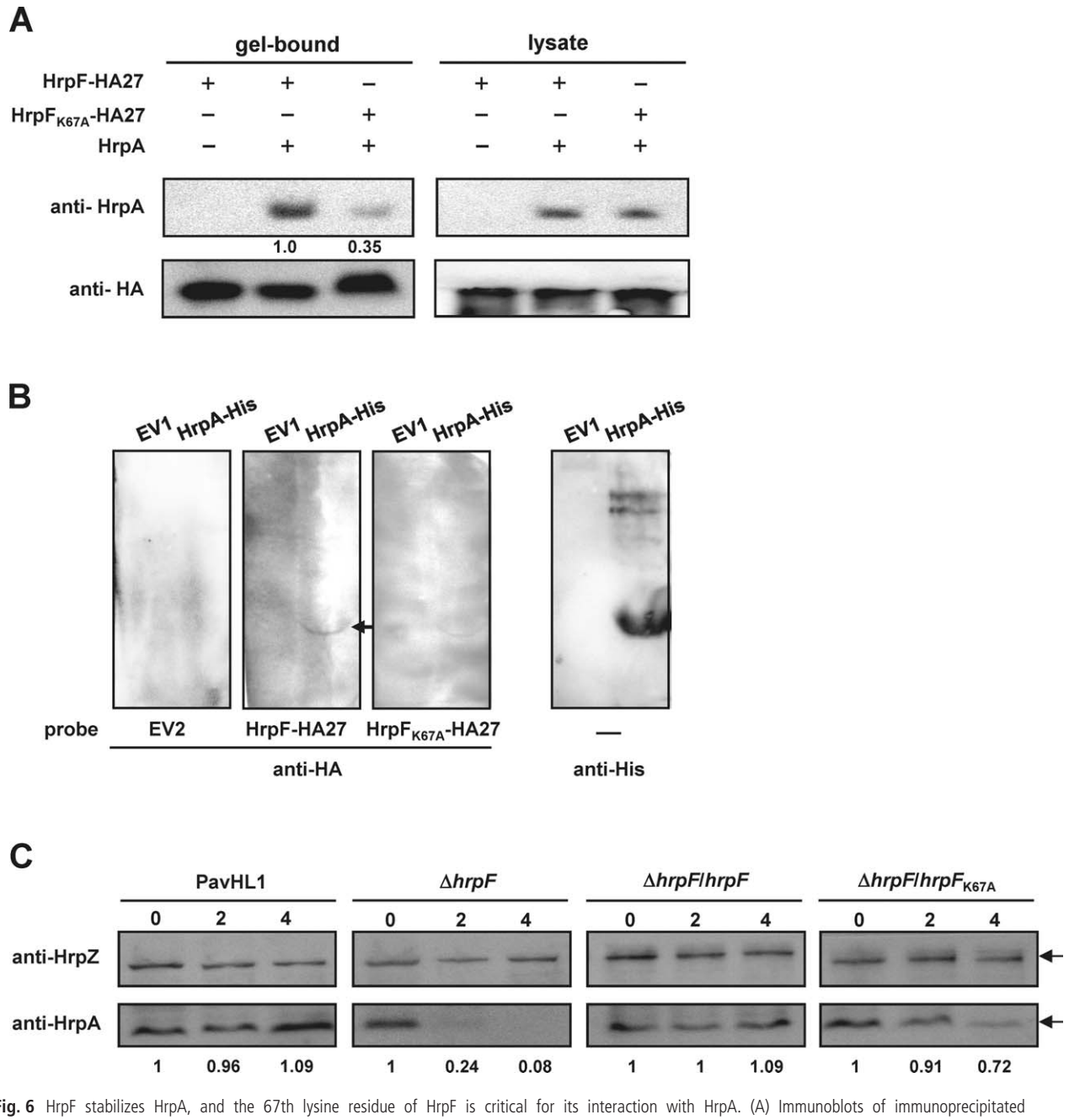

Fig. 6 HrpF stabilizes HrpA, and the 67th lysine residue of HrpF is critical for its interaction with HrpA. (A) Immunoblots of immunoprecipitated proteins probed with anti-HrpA (top) and anti-haemagglutinin (anti-HA) (bottom) antibodies to show *in vitro* protein interactions. *Escherichia coli* lysates expressing HrpF-HA27 (pNCHU1867), HrpF$_{K67A}$-HA27 (pNCHU1868), HrpA (pNCHU1869) ('+') or vector alone ('−') were mixed with anti-HA agarose beads for immunoprecipitation and detected by anti-HrpA serum as described in Experimental procedures. Gel-bound, immunoprecipitated proteins; lysate, total proteins as a control to detect the expression of recombinant proteins. (B) Far western analysis shows that HrpF-HA27 and HrpF$_{K67A}$-HA27 bind to HrpA-His *in vitro*. *Escherichia coli*-expressed HrpA-His (pNCHU1944) or EV1 (pETite) was probed with bacterial lysates expressing EV2 (pET29a), HrpF-HA27 (pNCHU1867) or HrpF$_{K67A}$-HA27 (pNCHU1868) as described in Experimental procedures. HrpA-His-bound HrpF-HA was further detected with HA monoclonal antibody. (C) Immunodetection of HrpA stability in the *Pseudomonas syringae* pv. *averrhoi* (Pav) *hrpF* mutant. PavHL1, wild-type harbouring pRK415 and pNCHU2046; Δ*hrpF*, HL1-N1589 harbouring pRK415 and pNCHU2046; Δ*hrpF/hrpF*, HL1-N1589 harbouring pNCHU1590 and pNCHU2046, Δ*hrpF/hrpF*$_{K67A}$, HL1-N1589 harbouring pNCHU2068 and pNCHU2046. Total cell cultures were harvested at 0, 2 and 4 h post-chloramphenicol treatment for immunodetection with anti-HrpZ and anti-HrpA sera. The stability of HrpA is shown by the fold changes in HrpA banding intensity, which was first normalized by the HrpZ bands and calculated using the value at 0 h post-chloramphenicol treatment as the denominator.

time points, whereas the cumulative amount of HrpA decreased significantly in the *hrpF* mutant on chloramphenicol treatment, which can be greatly improved by complementing the mutant strain with a plasmid-borne *hrpF*, or moderately by $hrpF_{K67A}$. The data, together with the protein–protein interaction assays in Fig. 3, indicate that HrpF binds to HrpA to prevent HrpA degradation, and the 67th lysine residue of HrpF is important for its binding to HrpA and the maintenance of HrpA stability. In comparison with HrpF-HA27, the binding of $HrpF_{K67A}$-HA27 to HrpG-FLAG was reduced to approximately 70% (Fig. S5A, see Supporting Information). Therefore, the effect of $HrpF_{K67A}$ on *t3ss* expression was measured by *in-trans* expression of *lac* promoter-driven $HrpF_{K67A}$ in PavHL1 to show that there were no apparent differences between HrpF- and $HrpF_{K67A}$-expressing strains in HR elicitation (see Fig. S5B,C) or HrpZ/HrpA production in the cytoplasm (see Fig. S5D). Hence, the 67th lysine residue of HrpF is important for binding with HrpA, but has little effect on *t3ss* expression.

DISCUSSION

The *hrp/hrc* genes coding for T3SS in plant bacterial pathogens are organized into several operons and reside in a PAI that is located on a chromosome or a large plasmid of the pathogenic bacteria (Arnold *et al.*, 2003). In this study, we characterized the role of Pav HrpF, whose coding gene is immediately upstream of *hrpG*, in T3SS function. The results shown here demonstrate that: (i) the *hrpF* mutant displays *hrp*-minus phenotypes and defects in the secretion/translocation of T3Es, indicating that Pav HrpF is essential for T3SS function; (ii) the expression of *t3ss* is increased in the unmarked *hrpF* mutants of PavHL1 and PtoDC3000, and ectopic expression of *hrpF* in WT PavHL1 reduces *t3ss* expression, whereas co-expression of HrpFG from a plasmid derepresses the negative impact of high-dosage HrpF on *t3ss* transcription, suggesting that HrpF acts together with HrpG to modulate *t3ss* expression; (iii) HrpF binds to HrpA to prevent HrpA degradation in the bacterial cytosol; and (iv) the 67th lysine of HrpF is a critical residue for HrpF–HrpA interaction.

In the early stage of *P. syringae*–plant interactions, a functional T3SS is essential for the suppression of plant defence and subsequent bacterial colonization (Abramovitch and Martin, 2004; Abramovitch *et al.*, 2006). *hrpF* mutant strains of *P. syringae* fail to elicit disease symptoms or programmed cell death in their host or non-host plant species, and the secretion or translocation of T3SS substrates is abolished under *t3ss*-inducing conditions (Deng *et al.*, 1998; Figs 1 and S1). Transmission electron micrographs also showed the lack of the T3SS pilus structure on the surface of the *hrpF* mutant (Fig. S6, see Supporting Information). These results indicate that HrpF is essential for T3SS function. HrpF is a small, acidic protein (pI values ranging from 3.8 to 4.6 among HrpF homologues), and is conserved in all *P. syringae* pathovars, but shares low sequence similarity with other proteins, e.g. HrpA pilin protein (Deng *et al.*, 1998). The threading protein structure at the Phyre Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi) (Kelley and Sternberg, 2009) predicted that HrpF shares low structural similarity with the T3SS needle proteins of *Shigella flexneri* MxiH (*E*-value: 8.7), *Y. pestis* YscF (*E*-value: 20) and *Salmonella enterica* PrgI (*E*-value: 23) in the region rich in α-helical coiled-coil structures. The coiled-coil structure is a versatile protein domain commonly found in molecular chaperones and proteins with various biological functions (Lupas and Gruber, 2005; Mason and Arndt, 2004), and polymerization of the T3SS needle filaments is also dependent on the coiled-coil interactions between monomeric needle proteins (Blocker *et al.*, 2008). Prior to secretion, the prevention of premature polymerization of the needle proteins is accomplished by T3SS chaperone functions (Cornelis, 2006). Many T3SS needle proteins, including *P. aeruginosa* PscF, *Y. pestis* YscF and *Aeromonas hydrophilia* AscF, have been found to employ a bimolecular chaperone, i.e. PscE–PscG (Quinaud *et al.*, 2005), YscE–YscG (Sun *et al.*, 2008) and AscE–AscG (Chatterjee *et al.*, 2011), for the maintenance of the monomeric state and protein stability within the bacterial cytoplasm. The *pscE*- and *pscG*-deleted strains of *P. aeruginosa* cannot accumulate PscF protein in the bacterial cytoplasm or secrete T3Es into the bacterial milieu under Ca^{2+}-depleted conditions, nor can they induce T3SS-mediated cytotoxicity in a macrophage cell line, indicating that the needle chaperones are absolutely required for T3SS function (Quinaud *et al.*, 2005). In this study, the biochemical assays of protein–protein interactions and HrpA stability suggest that HrpF may function like a T3SS pilus stabilizer to prevent HrpA degradation in the bacterial cytoplasm, which is important in the early *t3ss*-inducing phase (e.g. 6 hpi in Fig. 4B) to facilitate the assembly of the Hrp pilus. Thus, bacteria lacking HrpF cannot form the Hrp pilus, nor can they secrete or translocate T3Es to the bacterial milieu or plant cells, resulting in the accumulation of HrpA in the bacterial cytosol in the late *t3ss*-inducing phase (e.g. 24 hpi in Fig. 4B). Interestingly, differential secretome analysis of PtoDC3000 (Schumacher *et al.*, 2014) revealed that extracellular HrpA was exclusively processed at the N-terminus at 6 h post-*t3ss* induction, and the secreted form was switched to full-length HrpA at 48 hpi. Similar results, showing that the extracellular HrpA contains both full-length and N-terminally truncated forms at 2 dpi, have been reported by Roine *et al.* (1997a). Considering the interaction between HrpA and HrpF in the bacterial cytoplasm, HrpF may play a role in the temporal regulation of HrpA cleavage by direct binding. In the early stage of *t3ss* expression, HrpA bound with HrpF remains intact in the bacterial cytoplasm; on completion of the T3SS, HrpA is dissociated from HrpF and processed at or after secretion by an unidentified protease, and assembles into the Hrp pilus to facilitate the subsequent secretion and translocation of HrpF and T3Es. In later stages, HrpA, HrpF and T3Es may be secreted together

into the bacterial milieu *in vitro*, resulting in the high abundance of HrpA in the culture supernatant and the alteration of the truncated form to the intact form. As HrpF does not contain any protease domain, the role of HrpF in the post-translational cleavage of HrpA remains to be addressed.

On induction, a negative feedback regulatory circuit acts together with the positive feed-forward loop to create an oscillation motif in the *t3ss* regulatory network. In *P. syringae*, HrpV negatively regulates *t3ss* expression via binding to the positive regulator HrpS (Buttner, 2012; Jovanovic *et al.*, 2014; Preston *et al.*, 1998; Tang *et al.*, 2006), whereas HrpG acts as a suppressor of HrpV to release HrpS for the up-regulatory state (Jovanovic *et al.*, 2011; Wei *et al.*, 2005). So far, this regulatory mode has only been found in the group 1 Hrp T3SSs (Wei *et al.*, 2005). HrpG and HrpV of *P. syringae* and *Erwinia amylovora* share 32% and 40.8% sequence similarity, respectively, and the two proteins have been found to form a stable heterodimer in *E. amylovora* (Gazi *et al.*, 2015). However, the function of HrpF in *E. amylovora* has not been studied. Although there is no apparent sequence similarity in the *t3ss* regulatory proteins of *P. aeruginosa* and *P. syringae*, the HrpSVG negative feedback regulatory system shares common features with the ExsADCE system of *P. aeruginosa*, in which the ExsA-dependent *t3ss* activation is coupled with ExsE secretion (Buttner, 2012; Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). The results presented in this study reveal that the expression of the *hrp* regulon is transiently induced in the *hrpF* mutant and repressed in WT overexpressing the *hrpF* gene, and the negative effect of ectopically expressed HrpF on *t3ss* expression can be suppressed by the co-expression of *hrpF* and *hrpG* genes. Moreover, the chaperone-like HrpG interacts directly with HrpV (Wei *et al.*, 2005) and HrpF (Fig. 3), suggesting that HrpF might act as the ExsE counterpart in *P. syringae*.

Although we cannot rule out the possibility that *in-trans* expression of HrpF leads to the accumulation of misfolded proteins as inclusion bodies in the cytoplasm, circumstantial evidence obtained from the ecotopic expression of HrpF-HA27 in the *hrpF* mutant (Fig. 5D,E) shows normal secretion of Hrp outer proteins (HrpF-HA27, HopAK1, HrpA and HrpZ), indicating that T3SS is functional, and the negative effect of overexpressed HrpF on the elicitation of HR is probably caused by the suppression of *t3ss*. Alternatively, the negative effect of HrpF on *t3ss* expression may result partially from its interaction with HrpA, which has been found to be involved in the positive regulation of *t3ss* via an unknown mechanism (Wei *et al.*, 2000). As the truncated and intact HrpA can be secreted in a temporal manner (Schumacher *et al.*, 2014), it can be postulated that the truncated HrpA forms the T3SS pilus, whereas the intact HrpA may act as a secretion-competent regulator that activates *t3ss* in the cytoplasm, and its positive effect on *t3ss* expression can be reduced on secretion. Nevertheless, the positive regulation of HrpA is probably monitored by its chaperone-like protein HrpF, and the mutation of *hrpF* reduces the stability of HrpA, which greatly affects the construction of the T3SS conduit, leading to the transient activation of *t3ss* expression [Figs 4A and S4B (top panel)]. The actual role of HrpA in *t3ss* expression needs to be investigated. This hypothetical *t3ss* regulatory mechanism involving HrpA–HrpF–HrpG–HrpV–HrpS in *P. syringae* is not found in the other *t3ss* regulatory systems; however, the model describes how HrpA regulates *t3ss* expression without direct binding to any known regulatory components (Wei *et al.*, 2000).

A comparison of HrpF orthologues from *P. syringae* pathovars reveals that $HrpF_{Pav}$ and $HrpF_{Pto}$ share 62% similarity in amino acid sequences (Fig. S1A). The three amino acid residues at the C-terminus, K67, D71 and Q74, which are conserved in all HrpF orthologues, were selected for preliminary characterization. Single amino acid substitution of the three residues with Ala showed that HrpF harbouring the K67A substitution only partially restored the ability of the Pav *hrpF* mutant HL1-N1589 to elicit plant responses *in planta* (Fig. 5B,C). *In vitro* secretion assays of three representative Hrp outer proteins, i.e. HopAK1, HrpZ and HrpA, showed that $HrpF_{K67A}$ had a minor effect on the secretion of HopAK1 and HrpZ; however, the cumulative HrpA levels in both the cell-bound and secreted fractions were low (Fig. 5E), revealing that the 67th lysine residue is critical for HrpA stability (Fig. 6C). The 67th lysine residue of HrpF is a negatively charged amino acid and may be involved in protein–protein interactions with other Hrp proteins. *In vitro* immunoprecipitation assays and far western analysis revealed that single amino acid substitution attenuates the binding of $HrpF_{K67A}$ to HrpA (Fig. 6A,B). The secretion of HrpZ and HopAK1 is dependent on the HrpA pilus (Jin and He, 2001); therefore, we predict that bacteria expressing the variant $HrpF_{K67A}$ can transiently assemble a functional T3SS to aid in T3E secretion, thus partially compensating for the HrpF function in HR elicitation (Fig. 5B,C), but not bacterial growth (Fig. 5A).

In conclusion, we found that HrpF has a chaperone-like activity for the stabilization of HrpA in the bacterial cytoplasm, and is a secreted *t3ss* regulator in plant-pathogenic *P. syringae*. *t3ss* regulation in *P. syringae* involves a complex protein–protein coupling interaction, which presumably builds up an oscillating regulatory circuit by sensing small changes in the cellular concentrations of the key regulators to quickly respond to the surroundings (Tian *et al.*, 2009). Based on the results of this study, a hypothetical model is proposed, in which small amounts of HrpR and HrpS at the early stages of *t3ss* expression result in a low concentration of type III secretion proteins, and the negative regulator HrpV binds to HrpS to suppress *t3ss* expression. Meanwhile, HrpG interacts with HrpF, and HrpF binds to and stabilizes HrpA in the cytoplasm to form a stable protein complex. On contact with plant cells, the assembly of T3SS on the cell envelop facilitates the secretion of HrpA and HrpF, shifting the equilibrium of protein interaction from

HrpF–HrpG to HrpG–HrpV to release HrpS for subsequent up-regulation of *t3ss*. The binding affinity between these proteins is unclear and worthy of further investigation. The findings in this study denote the stealthy life style of pathogenic *P. syringae*, which uses interlinked regulatory networks to up- and down-regulate *t3ss* expression and to modulate pilus protein stability prior to the assembly of extracellular filaments.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, culture conditions and DNA manipulations

The bacterial strains, plasmids and primers used in this study are described in Tables S1 and S2 (see Supporting Information). The cultural conditions of *E. coli* and *P. syringae*, and DNA manipulation, are described in Methods S1 (see Supporting Information).

Generation of non-polar, unmarked mutants of HL1-N1589 and DC3000-N1865, and non-polar *nptII* insertion of *hrcC* mutant (HL1-N1033) and complementation

The unmarked mutation of *hrpF* in PavHL1 was constructed using the procedures described by Wei *et al.* (2007). To generate the unmarked *hrpF*-deleted mutant (HL1-N1589) of PavHL1, the flanking fragments of *hrpF* were cloned into the suicide vector pK18*mobsacB* (Schafer *et al.*, 1994) to produce the respective pNCHU1589 (details in Methods S1), followed by transformant selection and counter-selection for mutants, as described previously (Wei *et al.*, 2007). For the generation of a non-polar mutation of *hrcC* in PavHL1, the partial sequence of *hrcC* was replaced with an *nptII* marker (Alfano *et al.*, 1996) (Table S1 and Methods S1). For the expression of an HA-tagged HrpF, a chimeric $hrpF_{Pav}$-HA gene, coding for internally tagged HA between the 27th and 28th codons of $HrpF_{Pav}$, was generated by crossover PCR (Sukdeo and Charles, 2003) with primers prhrpF-1F/prhrpF$_{nr\text{-}HA}$ and prhrpF$_{cf\text{-}HA}$/prhrpF-2R to produce pNCHU1810, and the Pav $hrpF_{K67A}$ allele was synthesized by PCR using the primers prhrpF-1F/prhrpF$_{K67A}$-R to generate pNCHU1808 (Table S1 and S2). These recombinant plasmids were conjugated into $hrpF_{Pav}$ mutant HL1-N1589 for virulence assays on host plants and HR elicitation and conductivity on non-host plants.

Plant bioassays

Tobacco, tomato and starfruit plants were grown under glasshouse conditions as described in Methods S1. For disease symptom development in the leaves of starfruit and tomato, the bacterial suspension was adjusted to a cell density of 1×10^8 CFU/mL for spray or dipping inoculation, and inoculated plants were kept humid by wrapping in plastic bags at 28–30 °C. For bacterial multiplication assays, a cell density of 1×10^5 CFU/mL was infiltrated into the leaves of starfruit and tomato, and three leaf discs (6 mm in diameter) were harvested from three infiltrated leaves for bacterial enumeration, as described previously (Wei *et al.*, 2007, 2012). Tobacco leaves were infiltrated with bacteria at 1×10^8 CFU/mL or by serial titration for HR elicitation and ion leakage experiments, and the ionic conductivity was measured as an indicator of membrane damage during the onset of HR (Goodman, 1968) at 7 or 24 hpi by a conductivity meter (Radiometer analytical IONcheck 30, Lyon, France), as described by Wei *et al.* (2012).

Immunodetection of Hrc/Hrp proteins

After induction of *hrp*/*hrc* expression in HrpMM (Huynh *et al.*, 1989) for 6 h at 22 °C, extracellular and cytoplasmic proteins of cultured bacteria were separated and collected by centrifugation. The total protein concentration of the cell lysates and supernatants was quantified, and equal amounts of the total proteins were subjected to immunodetection (details in Methods S1). The intensity of chemiluminescence shown in immunoblots was quantified by an LAS-4000mini and analysed by Multi Gauge 3.0 software (Fujifilm Co., Tokyo, Japan).

Isolation of total bacterial RNA and quantitative determination of *t3ss* expression

The High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used to prepare total RNA from PavHL1 and its derivatives. Bacterial *t3ss* expression was induced in HrpMM for 3, 6 and 24 h at 22 °C. Bacterial cells were collected, and the extracted RNAs were subjected to qPCR experiments as described in Methods S1. For the detection of the transcripts of *hrpL*, *hrcC*, *hrpZ* and *hrpA* in PavHL1 and its derivative strains, the following primer pairs (Table S2), i.e. prhrpL-3F/4R, prhrcC-1F/2R, prhrpZ-5F/6R and prhrpA-17F/18R, respectively, specific for PavHL1, were employed. The amplification of the 16S rRNA gene using the primers pr16S-3F/4R (Table S2) was applied to normalize the relative expression of *t3ss* by the comparative CT (cycle threshold) method using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Cya translocation reporter assays

PavHL1, *hrcC* mutant HL1-N1033 and *hrpF* mutant HL1-N1589, carrying the plasmids expressing HrpF-cCya or HopI1-cCya fusion proteins, were employed for Cya activity assays in tobacco plants at 6 h after infiltration with bacterial concentrations at an optical density at 600 nm (OD_{600}) of 0.3 using the procedures described by Wei *et al.* (2005).

Assays to determine protein–protein interactions of HrpF with HrpG and HrpA

Bacterial two-hybrid assay

The assay was performed using a BacterioMatch® II Two-Hybrid System Library Construction Kit (Stratagene). The coding sequences of $hrpF_{Pav}$, $hrpG_{Pav}$ and $hrpA_{Pav}$ were translationally fused to reporter genes in the target vector pTRG or bait vector pBT. The derivatives of pTRG and pBT (corresponding plasmids described in Table S1) were simultaneously transformed into *E. coli* XL1 blue MRF′ Km strain carrying *HIS3* and *aadA* reporter genes. Protein–protein interaction was selected for the growth of transformants harbouring the bait and target plasmids on a selective medium containing 5 mM 3-amino-1,2,4-triazole (3-AT). The strength of the interaction was calculated (%) by comparing the number of bacterial colonies grown on the selective medium with those grown on the non-

selective medium that does not contain 3-AT, as described by the manufacturer.

Immunoprecipitation assay with an anti-HA agarose or anti-FLAG agarose

The recombinant proteins were induced and collected from *E. coli* C41 (DE3) carrying NCHU1867 (pET29a::*hrpF*$_{Pav}$-HA27) or pNCHU1868 (pET29a::*hrpF*$_{K67A}$-HA27), or BL21 (DE3) carrying pNCHU1869 (pET29a::*hrpA*$_{Pav}$), pNCHU1944 (pETite::*hrpA*$_{Pav}$-His) (Table S1) or pNCHU668 (pT7-7::*hrpG*$_{Psy}$-FLAG) (Wei *et al.*, 2005), as described in Methods S1. HrpG-FLAG protein was filtered through a 0.45-μm membrane (Millipore, Bedford, MA, USA) to remove inclusion bodies, and the concentration of total proteins was adjusted to 1 mg/mL. To pull down FLAG-bound HrpF-HA27, HA-bound HrpA protein or HA-bound HrpA-His protein, the anti-FLAG M2 agarose (Sigma-Aldrich, St. Louis, MO, USA) or polyclonal anti-HA agarose (Immunology Consultants Laboratory, Inc., Newberg, OR, USA) was prepared according to the manufacturer's instructions. The gel-bound proteins were eluted and subjected to immunoblotting analysis as described in Methods S1.

Far western blotting analysis

Escherichia coli C41 (DE3) carrying pNCHU1867 (pET29a:: *hrpF*$_{Pav}$-HA27) or pNCHU1868 (pET29a::*hrpF*$_{K67A}$- HA27), and *E. coli* BL21 (DE3) harbouring pET29a, pETite or pNCHU1944 (pETite::*hrpA*$_{Pav}$-His), were used to express the target proteins (see Methods S1 for details). The HrpF-HA27 or HrpF$_{K67A}$-HA27 protein extract served as the protein probe for the detection of the Ni-NTA resin-purified HrpA-His protein, which was prepared as described previously (Steen *et al.*, 1986; Studier *et al.*, 1990). HrpA-His protein and control proteins expressed from the empty vector were separated by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore). The membranes were probed with the sonicated cell lysate containing HrpF-HA27 or HrpF$_{K67A}$-HA27, as reported previously (Wei *et al.*, 2005). Immunodetection was performed using monoclonal anti-HA antibody (Sigma).

HrpA stability analysis

For the analysis of HrpA stability, pNCHU2046 (pBBR1MCS-2::*hrpAZ*$_{Pav}$) constitutively expressing *hrpAZ* was transformed into PavHL1 (WT), HL1-N1589 (*hrpF* mutant) and HL1-N1589 harbouring pNCHU1590 (pRK415::*hrpF*) or pNCHU2068 (pRK415::*hrpF*$_{K67A}$). The transformants were cultured in King's medium B (KB) broth to an OD_{600} of 0.3, changed to HrpMM and incubated for 2 h to induce *t3ss* expression. To stop translation, chloramphenicol was added at a final concentration of 100 ppm, and the total cell cultures (culture medium plus cell mass) were harvested at 0, 2 and 4 h post-chloramphenicol treatment, sonicated and precipitated by 5% cold trichloroacetic acid (TCA) for immunodetection as described above.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Phenotypes of the *hrpF* mutant strains derived from *Pseudomonas syringae* pv. *tomato* DC3000.
Fig. S2 The hypersensitive response (HR) on tobacco leaves elicited by PavHL1 and its derived strains.
Fig. S3 Immunoprecipitation assays of HrpA and HrpG.
Fig. S4 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses of *hrp* transcripts in *Pseudomonas syringae* strains grown in Hrp minimal medium (HrpMM).
Fig. S5 The effects of $HrpF_{K67A}$ on *t3ss* expression.
Fig. S6 Immunogold labelling of Hrp pilus on bacterial surfaces.
Table S1 List of bacterial strains and plasmids used in this study.
Table S2 Primers used in this study.
Methods S1 Experimental procedures.