Plant Pathology Bulletin 15:171-185, 2006

Evidence for the interaction between HrpT and HrcC of *Pseudomonas syringae* pv. *syringae* 61

Yuan-Chuen Lin [1,2,3], Shih-Tien Hsu [1], and Hsiou-Chen Huang [2,4]

[1] Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan

[2] Graduate Institute of Biotechnology, National Chung Hsing University, Taichung 402, Taiwan

[3] Center of General Education, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

[4] Corresponding author, E-mail: hchuang@dragon.nchu.edu.tw; Tel: +886-4-22852155; Fax: +886-4-22853527

Accepted for publication: July 20, 2006

ABSTRACT

Lin, Y. C., Hsu, S. T., and Huang, H. C. 2006. Evidence for the interaction between HrpT and HrcC of *Pseudomonas syringae* pv. *syringae* 61. Plant Pathol. Bull. : 15: 171-185 .

The cloned *hrp/hrc* cluster of *Pseudomonas syringae* pv. *syringae* 61 (Psy61) encodes 28 proteins, many of which are assembled into a type III secretion system (T3SS). In a previous study, nonpolar mutagenesis of the *hrpT* gene (Psy61-N402) demonstrated that HrpT is required for production/secretion of harpin (encoded by *hrpZ*) and hypersensitive response (HR) eliciation on tobacco. In order to elucidate the role of HrpT in T3SS, a FLAG tag which can be easily detected by M2 monoclonal antibody in immunoblotting was constructed to fuse with *hrpT*, resulting pNCHU655. This recombinant pNCHU655 could restore *hrpT* mutant in the ability of HrpZ production/secretion and the HR elicitation on tobacco leaves, indicating that FLAG-tag did not interfere with the activity of HrpT. Therefore, HrpT-FLAG instead of the native HrpT was used for further biochemical assays. A western blot analysis probed with M2 antibody revealed that HrpT-FLAG is located in the outer membrane and coexisted with HrcC. Moreover, results of yeast two-hybrid assay and pull-down assay showed that HrpT and HrcC interacted with each other *in vivo* and *in vitro*. HrcC is one member of T3SS secretin family. Secretins, including YscC, InvG, MixD and PulD, require pilotins (as pilot proteins) to properly form a ring-like structure on the outer membrane. Pilotins are small outer membrane lipoproteins which share no or very limited sequence homology. The 7.5-kDa HrpT is predicted to contain an N-terminal signal sequence with 18 hydrophobic amino acid residues. Sequence analysis using the Prosite program indicates two cysteine residues C19 and C29 are the potential lipid attachment sites. Herein, we hypothesize that HrpT may function as a pilotin of HrcC in the biogenesis of T3SS.

INTRODUCTION

Pseudomonas syringae is an important plant pathogenic bacterium commonly used to study plant-microbe interactions. More than 50 pathovars have been identified based on host specificity. In general, *P. syringae* elicits leaf spots and foliar necrosis in host plants and the hypersensitive response (HR) in nonhost plants [24]. In host plants, disease symptoms are usually developed after several days of bacterial growth in the leaf apoplast. In nonhost, the defense-associated programmed cell death that characterizes the HR occurs within 24 h in the plant cells that are in contact with the bacteria [42]. The ability of *P. syringae* to cause diseases on their hosts and to elicit the

HR in nonhost plants is controlled by the *hrp* (hypersensitive response and pathogenicity) /*hrc* (hypersensitive response and conserved) genes residing in a pathogenicity island also known as the Hrp Pai [3,8,40]. The *hrp/hrc* genes are conserved among many Gram-negative plant pathog enic bacteria, including *P. syringae, Ralstonia solanacearum*, *Xanthomonas campestris*, *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, and *E. chrysanthemi* [4,7,9,10,19,40]. They can be classified into three categories according to their functions: (i) a regulatory system, (ii) a type III secretion system (T3SS), and (iii) the substrates of T3SS: harpin, Hop (Hrp outer proteins), and pilus proteins etc.[13,41]

The 25-kb *hrp/hrc/hopPsyA* cluster of *P. syringae* pv. *syringae* 61 (Psy61) contains 28 open reading frames that are organized into 8 operons [3,16,26,28]. The majority of the gene products involved in assembly of the T3SS is encoded by genes organized in the *hrpJ*, *hrpU*, *hrpC* and *hrpZ* operons [17,28,38]. Among those genes, the nine highly conserved T3SS genes have been renamed *hrc* genes in plant pathogenic bacteria, with suffix letters corresponding to those of the *ysc* genes [8]. Thus, *hrcC* in plant pathogenic bacteria is equivalent to *yscC* in *Yersinia*. Except for *hrcC*, eight of the nine *hrc* genes also share significant sequence similarity with the components of the flagellar assembly machinery in Gram-negative bacteria [2,28]. Several of the *hrc* genes in the *hrpJ* (e.g *hrcV*) and *hrpU* (e.g. *hrcR, -S, -T, -U* genes) operons are candidates for the inner membrane proteins [8,28,38], and the *hrcC* gene in the *hrpC* operon encodes an outer membrane protein that has homologs found in type III (e.g. YscC, InvH, MxiD), type II (e.g. PulD), and filamentous phage (pIV) secretion systems [17,27]. These proteins belong to the family of secretins and they form stable oligomeric structures consisting of 12 to 14 subunits with a ring-like appearance, as shown by electron microscopy [14,33,39]. The *hrcJ* gene in the *hrpZ* operon encodes a lipoprotein, which spans the inner and outer membrane [17]. In addition, there are two cytoplasmic proteins, HrcN encoded by *hrpJ* operon and HrcQ encoded by *hrpU* operon. [28]. HrcN bears sequence similarity to the catalytic subunit of the mitochondrial F1 ATPase, and it may function as an ATPase-powered secretion pump at the base of T3S complex. In *P. syringae* pathovars, two proteins (HrcQa and HrcQb) substitute for the single HrcQ. HrcQa is homologous to the N-terminal and HrcQb to the C-terminal part of HrcQ of *R. solanacearum*. The cellular locations of the *hrc* gene products indicate that the *hrpJ-hrpU* and *hrpC-hrpZ* operons may be dedicated to protein translocation across the inner and outer membranes, respectively. The roles of *hrpJ-hrpU* in directing translocation across the inner membrane is consistent with the observation that the Hrc proteins encoded by these operons show similarity to proteins involved in the assembly of basal body in flagellum biogenesis [2,28,38].

Low-resolution electronic microscopy studies have revealed the morphology of T3SS apparatus of animal pathogens (*Salmonella typhimurium*, *Shigella flexneri*, and *E. coli*), while only the extracellular part of the apparatus has been visualized for plant pathogens [23,34,47,50]. This apparatus is a needle-like supermolecular structure and apparently composed of two major parts: the needle and the basal part which is markedly similar to the basal body of the flagellum [23,54]. In analogy with the flagellar filament, which is built by many copies of the flagellin protein, the T3SS extracellular appendages (needle part in animal pathogens, Hrp pilus in plant pathogens) are assembled through the stepwise polymerization of a major component, i.e. HrpA in *P. syringae* and *E. amylovora*, HrpY in *R. solanacearum*, MxiH in *Shigella*, and YscF in *Yersinia* [1,23]. The Hrp pilus and needle are both physically linked to the basal body and are assumed to play analogous functions. It is believed that via these structures the attachment to the host cell membrane is established and that they function as conduits for translocation/ secretion of substrates. Compared to flagellar biogenesis which has been extensively elucidated [1], little is known about the biogenesis of T3SS apparatus, especially the basal body.

In addition to the conserved Hrc proteins which are involved in the assembly of the basal part, there are some unique *hrp* genes only found in closely related strains which may also have functions in protein translocation/ secretion. In a previous study [16], the result of nonpolar mutagenesis on Psy61 *hrpT* gene (Psy61-N402) demonstrated that HrpT is required for production/secretion of harpin (encoded by *hrpZ*), HR eliciation on tobacco, lesion formation and bacterial multiplication on bean. *hrpT*, the forth gene in *hrpC* operon and next to *hrcC* gene, is predicted to encode a putative 7.5-kDa outer membrane lipoprotein and is highly conserved in *P. syringae* pathovars [16]. Recently, some studies reported that HrcC homologs, like PulD, InvG, YscC, and MixD, require pilot proteins (e.g. PulS, InvH, YscW or MixM, respectively) to promote their localization and stability on the outer membrane [12,14,15,20,36,46,54]. These pilot proteins are small outer membrane lipoproteins, which share no or very limited sequence homology with each other. Herein, we analyzed Psy61 HrpT sequence again and used a yeast two-hybrid system and a pull-down assay to investigate the interaction between HrpT and HrcC in this study. Our results suggest that HrpT is a potential candidate of pilot proteins for HrcC during the assembly of the basal part of T3SS.

MATERIALS AND METHODS

Bacterial strains, yeast strain and growth conditions

All bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) medium [43] at 37℃. *Pseudomonas* strains were grown on King's B medium (KB) [32] at 30℃. For induction of *hrp/hrc* genes, bacteria were grown in 5 ml of King's B (KB) broth at 30℃ to an OD_{600} of 0.6-0.8. Cells were collected by centrifugation, washed once with 5 ml of *hrp*-inducing minimal medium (HrpMM, 50 mM potassium phosphate, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM mannitol, and 10 mM fructose, pH 5.7) [29], resuspended in 5 ml of the same medium, and incubated with shaking at 22℃ for designated time. *Saccharomyces cerevisiae* strains were grown at 30℃ in YPDA medium or on yeast minimal synthetic dropout (SD) agar plates as described by manufacturer (Clontech). Antibiotics used in selective media were at the following final concentrations (micrograms per milliliter): ampicillin (Ap), 100; gentamycin (Gm), 10; kanamycin (Km), 50; nalidixic acid (Nx), 20; tetracycline (Tc), 20.

DNA manipulations

DNA manipulations were performed essentially according to standard procedures [43]. The plasmids used are listed in Table 1. Plasmids were introduced into *Pseudomonas* strains by triparental mating [18]. Polymerase chain reactions (PCR) was performed with 1x *Taq* reaction buffer, 100 ng DNA, 0.5 μM of each primer, 0.4 mM

Table 1. Bacterial and yeast strains, plasmids and primers used in this study

Strain, plasmid, and primer	Relevant characteristic and sequence	Source or reference
Escherichia coli		
DH10B	*endA1 hsdR17 recA1 relA* Δ(*argF-lacZYA*)*U169* Φ80d *lacZ* Δ*M15*	Life Sciences Technologies (Gaithersburg, MD)
HB101	*F´ hsd20 recA13 thr leu thi pro*	(5)
BL21(DE3)	B strain F⁻ *omp*T $r_b^-m_b^-$ *hsdS gal* (λDE3 cIts857 int1 sam7 *nin5 lac*UV5-T7 gene1)	(48)
Pseudomonas syringae pv. *syringae*		
61	Wild type isolated from wheat, Nx^r	(25)
61-N402	Strain 61 derivative carrying Δ*hrpT* :: *npt*II non-polar mutation	(16)
Saccharomyces cerevisiae		
Y187	*MAT α, ura3-25, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4* Δ*, met, gal80* Δ*, URA3 :: GAL1*$_{UAS}$*-Gal1*$_{TATA}$*-lacZ*	Clontech
Plasmids		
pRK415	Broad host range vector, mob^+, Tc^r	(31)
pRK2013	Helper plasmid, IncP Tra $RK2^+$ Δ*repRK2 repE1*$^+$*, Km*^{r}	(18)
pFLAG-CTC	FLAG expression vector, Ap^r	IBI/Kodak (New Haven, CT)
pT7-7	T7 dependent expression vector, Ap^r	(49)
pET29a	Expression vector, Km^r	Novagen
pML122	Broad host range vector for expression from *npt*II promoter, IncQ, Gm^r	(35)
pML123	Broad host range vector for expression from *npt*II promoter, IncQ, Gmr	(35)
pGADT7	*GAL4*$_{(768-881)}$ AD/library, *LEU2*, HA-tag, Ap^r	Clontech
pGBKT7	GAL4$_{(1-147)}$ DNA-BD/bait, TRP1, c-Myc-tag, Km^r	Clontech
pGADT7-T	SV40 large T$_{(84-708)}$-antigen in pGADT7, *LEU2*, Ap^r	Clontech
pGBKT7-53	Murine p53$_{(72-390)}$ in pGBKT7, *TRP1*, Km^r	Clontech
pGBKT7-Lam	Human lamin C$_{(66-230)}$ in pGBKT7, *TRP1*, Km^r	Clontech

Table 1. Bacterial and yeast strains, plasmids and primers used in this study (continued)

Strain plasmid, and primer	Relevant characteristic and sequence	Source or reference
pNCHU316	2.1 kb *Bam*HI-*Hin*dIII fragment containing S-tag fused *hrcC* cloned in pET29a	(16)
pNCHU451	250 bp *Eco*RV-*Hin*dIII fragment containing *hrpT* cloned in pRK415	(16)
pNCHU639	prT1/prT3-generated 0.2 kb fragment containing *hrpT* cloned in pFLAG-CTC	This work
pNCHU653	0.7 kb *Nde*I-*Ssp*I fragment containing FLAG fused *hrpT* from pNCHU639 cloned in pT7-7	This work
pNCHU655	0.7 kb *Xba*I-*Bam*HI fragment containing FLAG fused *hrpT* from pNCHU653 cloned in pML123	This work
pNCHU692	0.7 kb *Xba*I-*Bam*HI fragment containing FLAG fused *hrpT* from pNCHU653 cloned in pML122	This work
pNCHU715	0.7 kb *Nde*I-*Bgl*II fragment containing *hrcQa* cloned in GADT7	This work
pNCHU718	0.2 kb *Nde*I-*Sal*I fragment containing *hrpT* cloned in pGBKT7	This work
pNCHU721	0.4 kb *Nde*I-*Sal*I fragment containing *hrcQb* cloned in GBKT7	This work
pNCHU725	2.1 kb *Nde*I-*Sal*I fragment containing *hrcC* cloned in pGADT7	This work This work
pNCHU726	2.1 kb *Nde*I-*Sal*I fragment containing *hrcC* cloned in pGBKT7	This work
Primer		
prT-1	5' -GGCAAACATATGAAGATCAGCAGC-3' (*Nde*I)	This work
prT-F	5' -GATCATGTCGACGTCCCTTAAC-3' (*Sal*I)	This work
prQa-1	5' -TCACCATATGAGCGCCCTGCG-3' (*Nde*I)	This work
prQa-2	5' -TCCAAGCTTAGATCTTAAGTACTC-3' (*Hin*dIII, *Bgl*II)	This work

dNTP, 2.5 mM $MgCl_2$, 10 % DMSO, and a mixture of 2.5 U *Taq* (Promega) plus 0.5 U *Pfu* (Stratagene) per 50 μl reaction mixture following the listed program: 94℃, 10 min for denaturation, 94℃ 1 min, 60℃ 1 min, 72℃ 1 min, thirty cycles for amplification, and 72℃ 10 min for extension. All primers used are listed in Table 1.

Construction of recombinant plasmid pNCHU655

The nonpolar mutation of *hrpT* (Psy 61-N402) and the complementation was described by Deng *et al.*[16] For protein localization and interaction assays, a C-terminal FLAG-fused *hrpT* gene is constructed and depicted in Fig. 1. In brief, ca 0.2 kb prT1-prT3-generated *hrpT* gene PCR fragment was cloned into the pFLAG-CTC (IBI/Kodak) at *Nde*I-*Sal*I site, resulting in pNCHU639 where FLAG sequence was fused at the C-terminus of HrpT. A 0.7 kb *Nde*I-*Ssp*I fragment containing the FLAG-fused *hrpT* gene was then subcloned into pT7-7 at *Nde*I-*Sma*I site, named pNCHU653 and subsequently the 0.7 kb *XbaI-Bam*HI fragment isolated from this plasmid was subcloned into pML123 and pML122 to produce pNCHU655 and pNCHU692, respectively.

HR assay in tobacco plants

Tobacco (*Nicotiana tabacum* L. cv. Van Hicks) plants were grown under greenhouse conditions and transferred to laboratory for the HR assays. Bacteria were grown overnight on KB agar supplemented with appropriate antibiotics and resuspended in distilled water at a cell density of 10^8 cfu/ml. Inoculations were performed by pricking leaves with a dissecting needle and then pressing the blunt end of the tuberculin syringe filled with bacterial suspension against the leaf surface while supporting the leaf with a finger[6]. The development of HR was observed within 24 hours at room temperature.

Expression of FLAG-tagged *hrpT* or *hrcC* gene in *E. coli* and immunodetection

E. coli BL21(DE3) harboring pNCHU639 (pFLAG-CTC::*hrpT*) or pNCHU316 (*hrcC*) was induced with 0.5

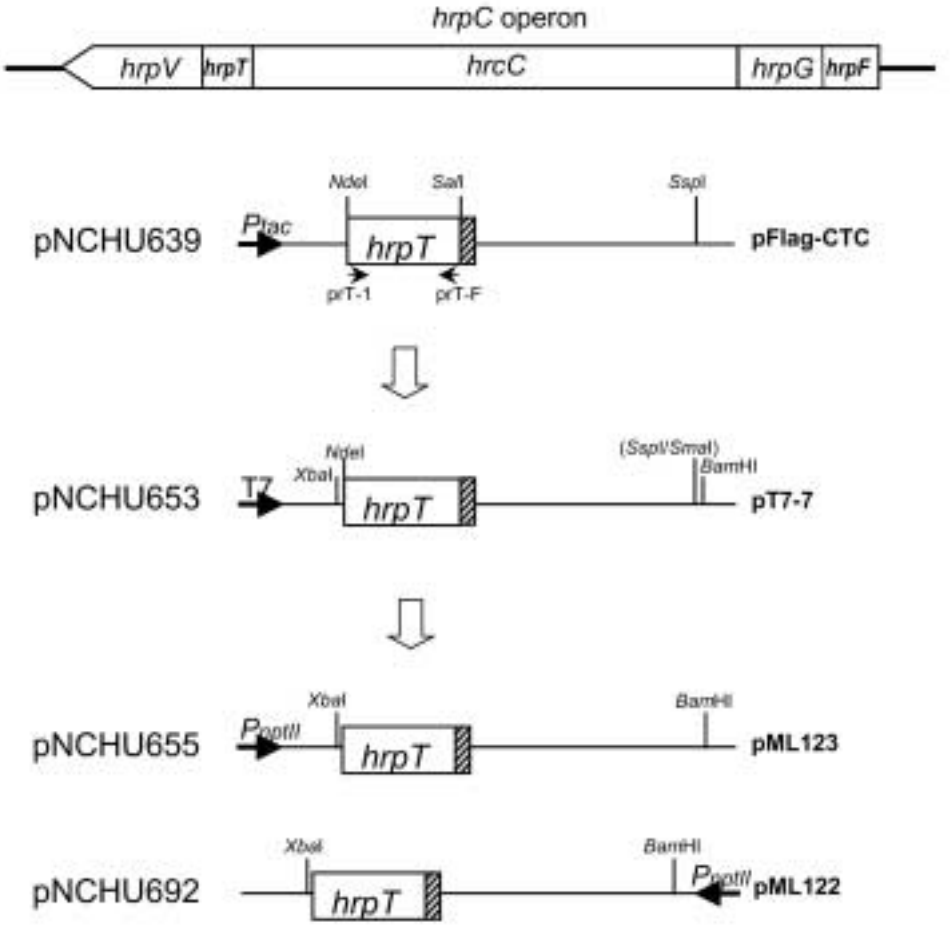

Fig.1. Construction of the *hrpT*-FLAG fusion clone. Primers prT-1 and prT-F used in the PCR-amplified DNA fragments are indicated under the corresponding gene. Shadow boxes refer to FLAG sequence (Asp-Tyr-Lye-Asp-Asp-Asp-Asp-Lye). Parenthesis refers to joining DNA fragments with two indicated restriction enzymes, resulting in loss of their cutting sites. These recombinant DNAs were constructed in pFLAG-CTC, pT7-7, pML123 and pML122, resulting in pNCHU639, pNCHU653, pNCHU655 and pNCHU692 respectively. The promoter permits the FLAG-fused *hrpT* expression was indicated by the heavy arrow.

mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h followed by addition of rifampicin (200 μg/ml final concentration) and incubation for an additional 2 h to inhibit *E. coli* RNA polymerase activity. Bacterial cells were collected by centrifugation, resuspended in 2× loading buffer, sonicated (Sonicator XL-2020; Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.) for one minute, and heated to 100℃ for 5 min before being subjected to electrophoresis. Proteins resolved by SDS-15% polyacrylamide gel electrophoresis (PAGE) were visualized with Coomassie brilliant blue R250 staining or immunostaining. Prestained molecular size standards (Broad range, New England Bio-Labs) were used to estimate the molecular weights of proteins. Resolved proteins were electrotransferred to Immobilon-P membranes (Millipore) in a semi-dry transfer unit TE-70 (Hoefer) following the manufacturer's instructions. The membranes were probed with the mouse monoclonal antibodies of anti-FLAG M2 (Sigma), NptII antibody (5'→3' Prime) at a ratio of 1 to 5000, preabsorbed HrpZ [22], or HrcC antibodies [17] at a ratio of 1 to 500 followed by alkaline phosphatase-conjugated anti-mouse IgG (Boehringer Mannheim) or anti-rabbit antibody, respectively, and stained with 0.25 mM CDP-Star (disodium 2-chloro-5 (4 -methoxyspiro {1,2-inoxetane-3, 2'-(5'-chloro) tricyclo [3.3.1.1] decan}-4-yl) phenyl phosphate, Boehringer Mannheim) as described by the manufacturer.

Cellular fractionation

After overnight induction of *hrp/hrc* expression in 5 ml HrpMM, bacteria were separated into cells and supernatant by centrifugation at 2,000 g for 10 min. Extracellular proteins were precipitated from the

supernatant by addition of 5% (w/v) cold trichloroacetic acid (TCA) and incubated on ice for 1 h. The precipitated proteins were resuspended in 30 μl extraction buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20 (v/v), 10% glycerol (v/v), 1 mM phenylmethylsulphonyl fluoride (PMSF)] and designated as the supernatant fraction. For Triton extraction of membrane proteins[44,45], bacteria were grown and harvested after Hrp/Hrc proteins induction as described above and resuspended in 10 mM HEPES (pH 7.4) containing 0.25 M sucrose and 1 mM PMSF for brief sonication. Unbroken cells were removed by centrifugation at 1000 *g* for 10 min at 4℃, and supernatants were ultracentrifuged for 1 hour at 100,000 *g*, 4℃. Ultracentrifuged supernatants were cytoplasmic and periplasmic fractions, and pellets were designated as crude membranes. The membrane fraction was dissolved in 1.0 ml of Triton-Mg solution (1% Triton X-100, 10 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.4], 1 mM PMSF), and mixed vigorously for 30 min at room temperature. Separation of inner and outer membrane proteins was achieved by centrifugation at 15,600 *g* for 30 min. The supernatants containing inner membrane proteins were precipitated with 5% (w/v) TCA, and the pellets were further fractionated into outer membrane and nonextracted portions with Triton-EDTA solution (50 mM Tris-HCl [pH 7.4], 10 mM EDTA, 1% Triton X-100, 1 mM PMSF) following a 30-min centrifugation at 12,000 *g*. The supernatants containing the outer membrane proteins were precipitated by TCA and resuspended in 2× loading buffer, as was done for the pellets (nonextracted portion). All samples were subjected to immunoblot analysis as described above.

Assays to determine the interactions between HrcC and HrpT

A. Yeast two-hybrid assay

Yeast two-hybrid assay was performed using MATCHMAKER GAL4 Two-Hybrid System3 kit (Clontech). The 0.2 kb *hrpT*, 2.2 kb *hrcC*, 0.7 kb *hrcQa* or 0.4 kb *hrcQb* was fused in frame to the transcriptional activation domain or to the GAL4 DNA binding domain to produce pNCHU725 (pGADT7::*hrcC*), pNCHU715 (pGADT7::*hrcQa*), pNCHU718 (pGBKT7::*hrpT*), pNCHU726 (pGBKT7::*hrcC*), and pNCHU721 (pGBKT7::*hrcQb*), respectively. pGADT7 and pGBKT7 derivatives were co-transformed into yeast Y187 carrying a β-galactosidase reporter gene. Enzymatic activity of β-galactosidase in Y187 was monitored by colony lift and quantitative enzymatic assays as describe by the manufacturer.

B. Pull-down assay with an anti-FLAG M2 gel

E. coli BL21 (DE3) carrying pNCHU316 (pT29a::S-*hrcC*), pNCHU639 (*hrpT*-FLAG), or pFLAG-CTC were grown in LB broth to an OD_{600} of 0.5-0.6 at 37℃. After adding IPTG to a final concentration of 0.5 mM, the cells were grown for an additional hour at 30℃. Bacteria were collected by centrifugation, and then resuspended in 1 ml of sonication buffer (20 mM Tris-HCl, 100 mM NaCl, 10% glycerol, pH 8.0). Cell suspensions were sonicated for 2 min. and the cell lysates were centrifuged at 15,000 *g* for 15 min at 4℃ to remove cell debris. The clarified lysate of *E. coli* BL21 (pNCHU316) and BL21 (pNCHU639) or (pFLAG-CTC) were mixed together for 16 hr at 4℃. To pull down FLAG-fused HrpT protein, a 50 μl anti-FLAG M2 gel was prepared according to the manufacturer's instruction (Sigma) and mixed with 1 ml of each cell lysate by rotation for 16 h at 4℃ followed by a brief spin at 10,600 *g* for 5 s. The pellet was washed three times with 1 ml of TBS buffer[53] and the bound proteins were eluted with 200 μl of 0.1 M glycine-HCl (pH 3.5), neutralized with 20 μl of 0.5 M Tris HCl (pH7.4) plus 1.5 M NaCl, precipitated with 5% TCA, and subjected to immunoblotting analysis as described above.

RESULTS AND DISCUSSION

FLAG-fused HrpT complements the abilities of HR elicitation and harpin secretion in *hrpT* mutant Psy61-N402.

In a previous study, the result of nonpolar mutagenesis on *hrpT* gene (Psy61-N402) demonstrated that HrpT is required for production/secretion of harpin (encoded by *hrpZ*), HR eliciation on tobacco, lesion formation and bacterial multiplication on bean[16]. Here, a FLAG tag which can be easily detected by M2 monoclonal antibody in immunoblotting was constructed to fuse with *hrpT* (Fig. 1), which was then applied to further investigate the function of HrpT in T3SS. First, the expressed HrpT-FLAG was confirmed by immunoblotting. *E. coli* carrying pNCHU639 (FLAG-fused *hrpT*) was induced with IPTG and then a 7.5-kDa inducible protein was detected by anti-FLAG M2 antibody in a western blot (compared to *E. coli* carrying an empty vector pFLAG-CTC) (Fig. 2). This FLAG-fused *hrpT* fragment was further subcloned in a broad host range vector pML123, resulting pNCHU655 in which the transcription of FLAG-fused *hrpT* gene is driven by the *nptII* promoter. The same fragment was also cloned into pML122, resulting in pNCHU692 as a negative control with the gene orientation opposite to the *nptII* promoter (Fig.1).

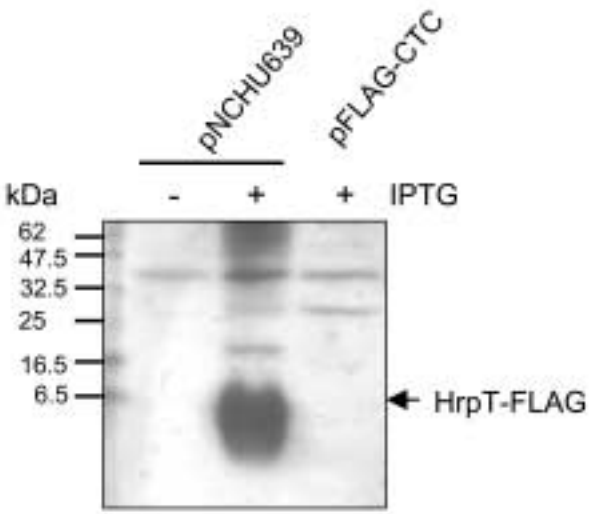

Fig. 2. The overexpressed product of plasmid-borne *hrpT*-FLAG gene (pNCHU639) in *E. coli* BL21 (DE3) detected in immunoblotting with FLAG M2 monoclonal antibody. The vector pFLAG-CTC was used as a negative control.

In the HR test, *hrpT* mutant and their corresponding complemented strains (Psy61-N402 carrying *hrpT*-containing pNCHU451, pNCHU655 or pNCHU692) were infiltrated into tobacco leaves, and their HR elicitation activities were recorded at 12 h post inoculation. As shown in Fig. 3, the leaf panel infiltrated with Psy61-N402 and Psy61-N402 (pNCHU692) showed no response (sometimes spotty necrosis, data not shown), whereas those with Psy61-N402 (pNCHU451), Psy61-N402 (pNCHU655), and wild-type Psy61 all showed confluent necrosis. Furthermore, a western blotting analysis was performed to determine if the loss of HR phenotype in the *hrpT* mutant Psy61-N402 is due to an incompetent T3SS. Bacteria were incubated in HrpMM for 5 h at 22℃ and then separated into cell pellet and supernatant fractions by centrifugation. The immunoblotting of protein extracts from both fractions using a serum against HrpZ (harpin) was conducted and an immunodetection of NptII was used as a control for equal loading and cytoplasmic marker. Results showed that both accumulation and secretion of HrpZ protein were dramatically reduced in Psy61-N402, and the signals were very weak as seen in lane 3 and 4 of Fig. 4, suggesting that the HR-deficient phenotype observed in *hrpT* mutant is partially due to the reduction of effector secretion. This mutant phenotype was restored to the wild type level by complementing with HrpT or with HrpT-FLAG (Fig. 4 lane 5-8). Complementation results showed that both HrpT (encoded in pNCHU451) and HrpT-FLAG (encoded in pNCHU655) were capable of restoring the HR elicitation and harpin secretion phenotype to the wild type level in Psy61-N402, suggesting the FLAG tag did not interfere with the HrpT activity. Therefore, the plasmid pNCHU655 was used in the following biochemical assays.

HrpT is associated with the outer membrane

In a previous study [16], sequence data show that HrpT is a putative outer membrane lipoprotein. Due to more HrpT homologs cloned from pathogenic bacteria recently, homology search again was performed with BLAST program in NCBI web site (http://www.ncbi. nlm.nih.gov). According to multiple alignments with CLUSTAL W program [52], HrpT is highly similar to that from strains of *P. syringae* and less similar to that from *Erwinia*, and it has very low homology to the N-termini of YscW, InvH, MxiM and PulS proteins from *Yersinia enterocolitica*, *Salmonella enterica*, *Shigella flexneri* and *Klebsiella oxytoca*, respectively (Fig. 5). YscW, InvH, MxiM and PulS have been identified to be outer membrane proteins and to play as pilot proteins in promoting the localization and stability of YscC, InvG or MxiD of T3SS and the secretin PulD of the type II secretion system, respectively [12,14,15,20,46]. It is very interesting that HrpT in plant pathogenic bacteria is much smaller than their orthologs in animal pathogens. Also, data show that HrpT protein is predicted to contain an N-terminal signal sequence with 18 hydrophobic amino acid residues. Sequence analysis using the Prosite program (http://www.expasy.ch/prosite/) indicates that two consensus sequences for lipid attachment (#9-#19, VLVVFATLTGC; #19-#29, CATHGCSGTAC) were found and C19 and C29 residues are the potential lipid attachment sites [21]. However, only C19 is conserved in all piolot proteins shown in Fig. 5. Herein, to investigate the cellular localization of HrpT protein in Psy61, total cell extract from bacteria grown in induction media was applied to cell fractionation using Triton X-100 extraction. The collected fractions were analyzed by immunoblotting using anti-FLAG M2 antibody and anti-HrcC serum. HrcC of Psy61 has been determined to be indeed an outer membrane protein by Triton solubilization, sucrose-gradient isopycnic centrifugation, and immunogold labeling of the bacterial cell surface [17], and it was used as an outer membrane marker in this study. As shown in Fig. 6, HrpT-FLAG and HrcC were detected predominantly in the outer membrane, indicating that HrpT is also an outer membrane protein and coexists with HrcC protein. Taken together, sequence comparison and cellular localization assay imply that HrpT may interact with HrcC during the assembly of T3SS.

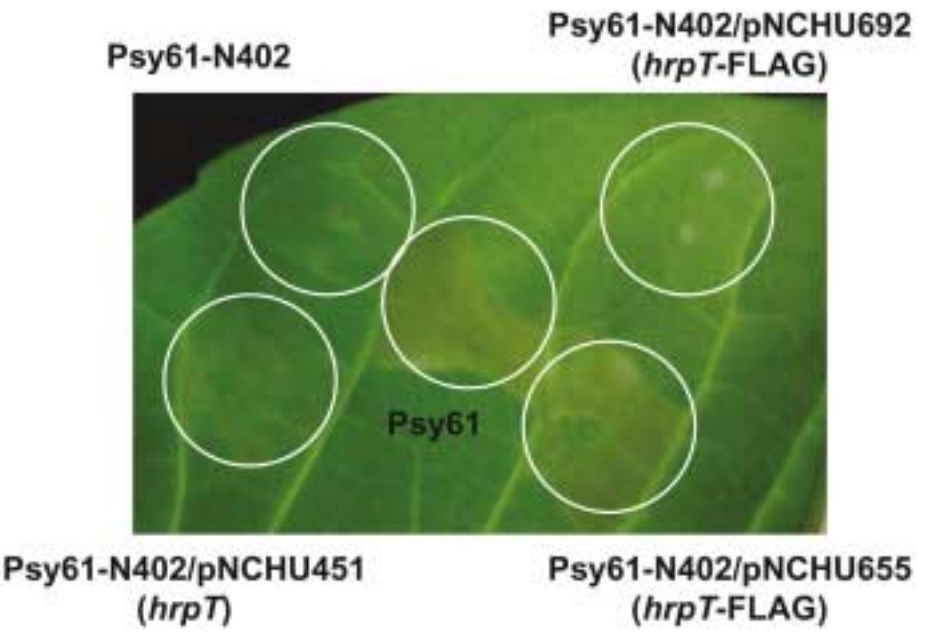

Fig. 3. The hypersensitive response of tobacco leaf elicited by wild type (Psy61), *hrpT* mutant (Psy61-N402), and complemented strains Psy61-N402 (pNCHU451 or pNCHU655) and Psy61-N402 (pNCHU692). The ability to elicit HR was abolished in Psy61-N402 and was restored by plasmid-borne *hrpT*-FLAG (pNCHU655) in complementation. A leaf of *Nicotiana tabacum* L. cultivar VanHicks was infiltrated with a blunt syringe containing bacteria at the concentration of 10^8 cfu/ml and photographed at 12 h post inoculation. Plasmid pNCHU451, the construct carrying *hrpT* ; pNCHU655, the construct carrying *hrpT* - FLAG.

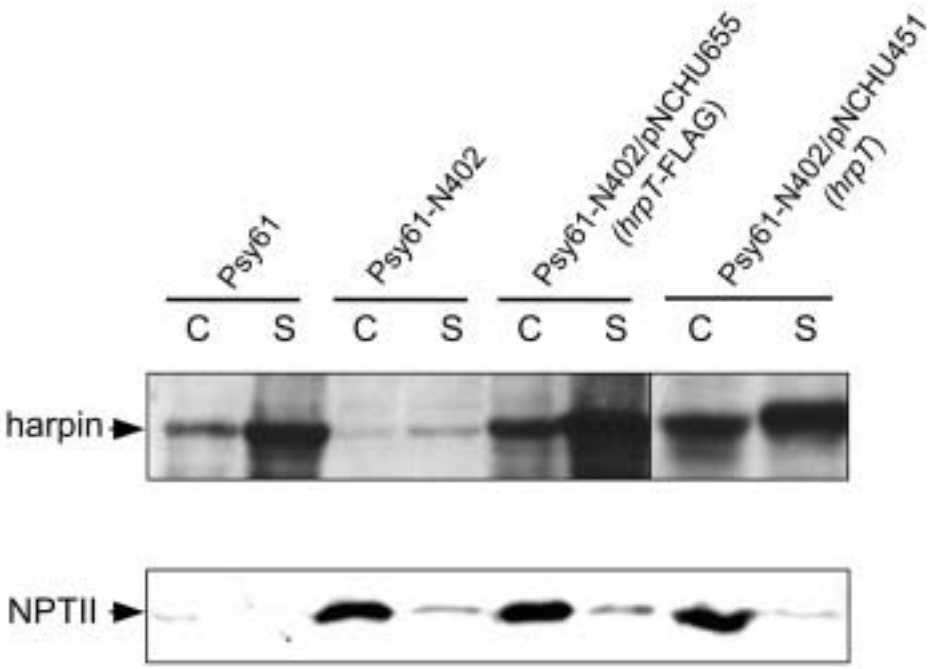

Fig. 4. The production and secretion of HrpZ (harpin) were reduced in *hrpT* mutant Psy61-N402 and were restored by plasmid-borne *hrpT*-FLAG (pNCHU655) or *hrpT* (pNCHU451) in complementation. Proteins prepared from the cell-bound fractions (C) and culture supernatant fractions (S) were described in the experimental procedures and subjected to SDS-PAGE and immunoblot analyses. The upper panel was probed with harpin antiserum and the lower panel was probed with NptII antiserum to serve as an internal control.

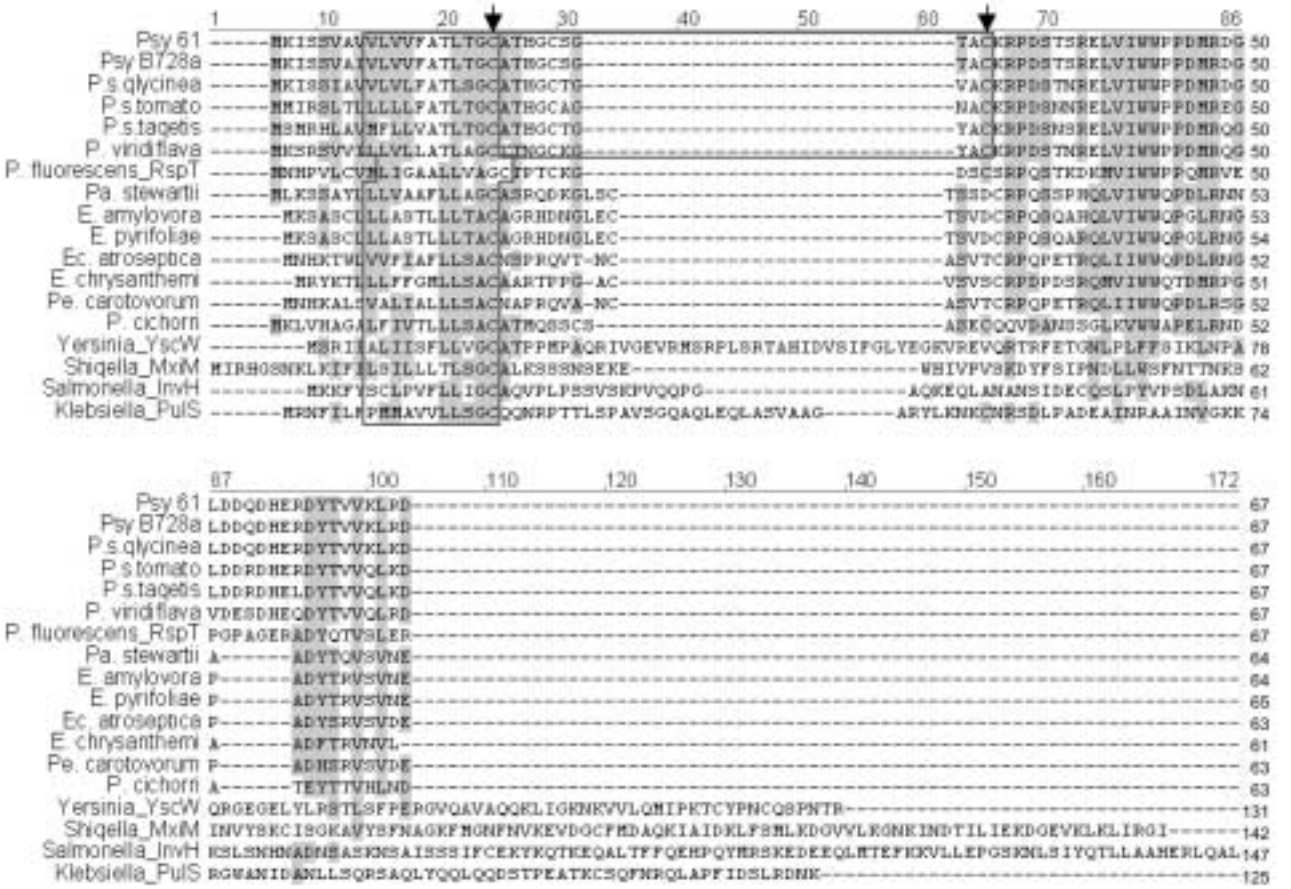

Fig. 5. Multiple alignment of the deduced amino sequence for HrpT from *P. s.* pv. *syringae* 61 (AAC35755) with its homologs. The consensus sequences for lipid attachment motif were shown in boxes. Residues for potential lipid attachment, C19 and C29, were indicated by the vertical arrow. This alignment was made by using CLUSTAL W program [52]. The HrpT sequences from *P. s.* pv. *syringae* B728a (YP_234293), pv. *glycinea* (AAC35806), pv. *tomato* (AAO54912), *pv. tagetis* (ABB91652), *P. viridiflava* (AAT96205), *P. cichorii* (ABA47302), *Erwinia amylovora* (AAB49180), *E. pyrifoliae* (AAS45459), *E. carotovora* subsp. *atroseptica* (CAG75003), *Pantoea stewartii* subsp. *stewartii* (AAG01464), *Pectobacterium carotovorum* subsp. *carotovorum* (AAQ73912), *E. chrysanthemi* (AAC31976), RspT sequence from *P. fluorescens* (AAK81928), YscW sequence from *Yersinia enterocolitica* (NP_052409), MxiM sequence from *Shigella flexneri* (CAC05818), InvH sequence from *Salmonella typhimurum* (AAL21780), and PulS sequence from *Klebsiella pneumoniae* (AAA61978) were used for comparision.

An interaction between HrpT and HrcC was detected in yeast two-hybrid assay

MATCHMAKER Two-Hybrid System 3 (Clontech) was used to determine the interaction between HrpT and HrcC *in vivo*. The interactions of murine p53 (p53) and SV40 large T-antigen (T) and SV40 large T-antigen and human lamin C (Lam) were used as a positive control and negative control, respectively. In this system, HrpT, HrcC, HrcQa and HrcQb were fused to GAL4 DNA binding domain (BD) in pGBKT7 and activation domain (AD) in pGADT7, and the recombinant constructs were cotransformed into yeast strain Y187 containing a *lacZ* reporter gene whose expression is GAL4-dependent . The β-galactosidase activity in transformants was first monitored using colony-lift filter method, and the positive reaction was only observed with the transformants carrying both recombinant plasmids of interest. Subsequently, the β-galactosidase activity was quantified using ONPG (O-nitrophenyl β-D-galactopyranoside) as a substrate. Comparing with the positive control strain harboring the murine p53 and SV40 large T-antigen [30,37], the β-galactosidase activities in the transformants expressing AD-HrcQa/BD-HrcQb, AD- HrcC/BD-HrpT, and AD-HrcC/BD-HrcC pairs were about 16.9%, 7.7%, and 7.4% of the activity in the positive control strain, respectively (Table 2). Both HrpT and HrcC proteins harbor N-terminal transmembrane helices which might interfere with the interaction assay in yeast two-hybrid

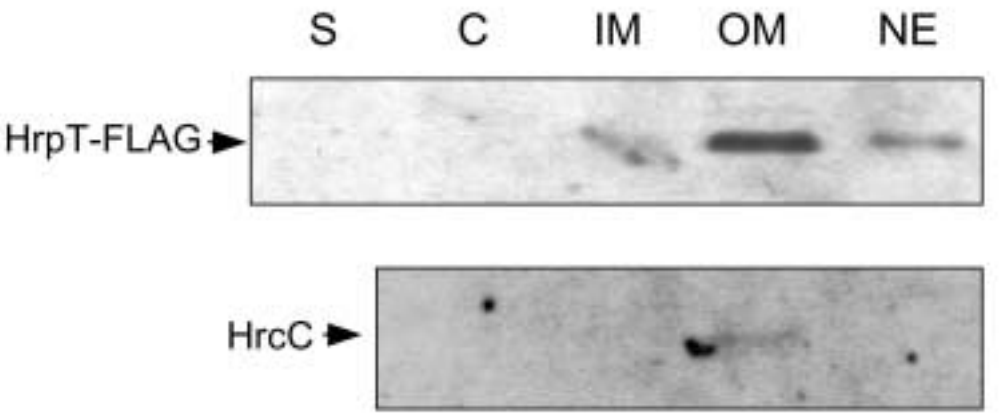

Fig. 6. Cellular localization of HrpT-FLAG protein in Psy61-N402 (pNCHU655) was detected by immunoblotting with anti-FLAG M2 antibody. Proteins prepared from culture supernatant (S) and the cell-bound fractions which were further separated into cytoplasmic (C), inner membrane (IM), outer membrane (OM) and non-extracted (NE) fractions were described in the experimental procedures and subjected to SDS-PAGE and immunoblot analyses. The distribution of HrcC protein shown in lower panel was detected with anti-HrcC antibody and used as an outer membrane marker.

system. However, an interaction of HrcC with itself could be detected, likely due to the formation of a HrcC multimeric complex in T3SS [17]. Similar β-galactosidase activities in the transformants expressing the recombinant AD-HrcC/BD-HrpT and AD-HrcC/BD-HrcC reveal that HrpT also interacts with HrcC *in vivo*.

HrpT-FLAG interacts with HrcC in vitro.

To demonstrate that HrpT can directly bind to HrcC *in vitro*, a pull-down assay was applied. In the pull-down assay using an anti-FLAG affinity gel as a trap, soluble proteins extracted from the cell lysates of *E. coli* BL21 expressing HrcC (pNCHU316) and HrpT-FLAG (pNCHU639) were incubated with anti-FLAG affinity gel. The whole cell lysates and FLAG gel-bound proteins were resolved by SDS-PAGE and immuno-analyzed using antibodies that recognize either the FLAG epitope of HrpT-FLAG or HrcC. As presented in Fig. 7, HrcC was detected in the gel-bound fraction in the presence of HrpT-FLAG in *E. coli* BL21 carrying pNCHU639, revealing that HrcC binds to HrpT-FLAG *in vitro*, and these two proteins can be pulled down together by the FLAG affinity gel.

Conclusions

Secretins (e.g. PulD, InvG, MixD, YscC, and HrcC) belong to a large protein family whose members are widely dispersed across many species of Gram-negative bacteria. Not only are secretins present in T3SS, but they also participate in various macromolecular transport processes including the type II secretion, type IV pilus biogenesis and filamentous phage release [11,51]. However, secretins share neither sequence nor structural homology with any of the outer membrane components of the flagellar system. Individual monomers of secretin assemble into stable ring-like oligomers of 12-14 subunits with a central channel of 5-10 nm in diameter [14,33,39]. Biogenesis of the secretin ring begins with protein synthesis in the bacterial cytoplasm and export of the

Table 2. Interaction between HrpT and HrcC proteins detected by yeast two-hybrid tests

Sample combination		Colony-lift	Liquid culture assay
pGADT7-derived	pGBKT7-derived	filter assay [1]	(β-galactosidase units) [2]
pGADT7-T	pGBKT7-53	+++	6.64 ± 0.20
pGADT7-T	pGBKT7-Lam	-	0.12 ± 0.02
HrcQa	HrcQb	++	1.12 ± 0.19
HrcC	HrpT	+	0.51 ± 0.12
HrcC	HrcC	+	0.49 ± 0.11

[1] '+ ～ +++' signs indicate the relative intensity of the blue color developed after exposure of permeabilized cells to X-Gal (5-bromo-4-chloro-3-indoly-b-D-galacto-pyranoside) for 1 hr.

[2] Data represent the means of three replicates ± standard error.

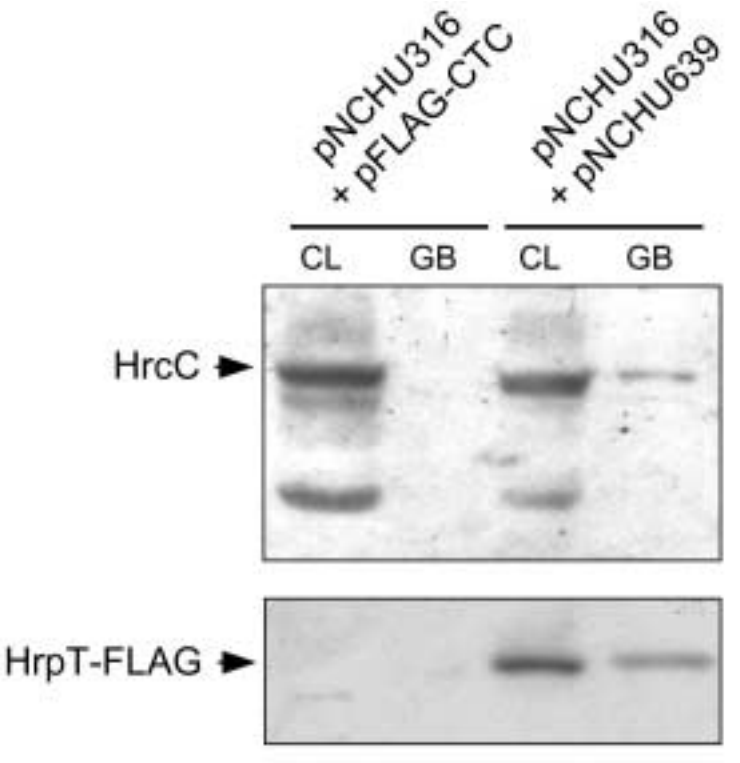

Fig. 7. Pull-down assay shows HrpT interacting with HrcC. The samples of soluble proteins collected from sonicated cultures of *E. coli* (pNCHU316) expressing HrcC and *E. coli* (pNCHU639) expressing Flag-tagged HrpT were mixed with anti-FLAG M2 affinity gel. The sonicated cell lysate (CL) and the gel-bound (GB) protein eluted from M2 affinity gel were immunoblotting with HrcC antiserum (upper panel) and anti-FLAG M2 antibody (lower panel).

individual monomers to the periplasm by the sec-dependent pathway. Subsequent folding and insertion into the ourter membrane are crucial steps to ensure proper oligomerization and channel formation. A specialized class of proteins known as pilotins (pilot proteins) has been shown to mediate this process [12,14,15,20,36,46,54]. Pilotins are a sequencedivergent family of small lipoproteins that are localized to the outer membrane and bind to the C termini of their cognate secretins. The recent crystal structure of *Shigella* MixM, a T3SS pilotin, has shed light on how this protein might assist its cognate secretin MixD in its insertion process [36]. A principal feature of the conical-shaped MixM protein is a "cracked-barrel" domain that generates a hydrophobic cavity which can bind acyl chains of bacterial lipids in the center of the protein. The lipidated N terminus of MixM, which is located in close proximity to this lipid-binding cavity, might bind here for stability before interacting with the outer membrane and MixD. Another possibility is that MixM could extract the lipid from the outer membrane by using this cavity to enable proper insertion of MixD [36,54].

In this study, we hypothesize that HrpT may be a pilotin of HrcC based on the following results obtained here: (i) The sequence analysis of HrpT indicates that it is a putative lipoprotein and possesses a cysteine residue at #19 as a putative lipid binding site which is also very conserved in the family of pilotins including PulS, YscW, InvH and MixM (Fig. 5). An unlipidated form of the YscW protein caused mislocalization of YscC, although it still interacted with the secretin, suggesting that the lipidation mediated by this cysteine residue is very important [12] (ii) Based on the western blot (Fig. 6), HrpT is an outer membrane protein. *hrpT* gene is located in the same operon with its cognate secretin gene *hrcC* and adjacent to it. Other pilotin genes including *mixM*, *invH*, and *yscW*, are also closed to their cognate secretin genes. (iii) HrpT is clearly interacted with HrcC protein according to the yeast two-hybrid and pull-down assays (Table 2; Fig. 7). It will be worthy of further investigation on how HrpT promotes the biogenesis of HrcC on forming a ring structure on the outer membrane.

ACKNOWLEDGEMENTS

This work was supported by NSC 93-2752-B-005-003-APE and NSC 93-2317-B-005-009.

LITERATURE CITED

1. Aizawa, S. I. 2001. Bacterial flagella and type III secretion systems. FEMS Microbiol. Lett. 202: 157-164.
2. Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpin, Avr proteins, and death. J. Bacteriol. 179: 5655-5662.
3. Alfano, J. R., Charkowski, A. O., Deng, W.-L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. Proc. Natl. Acad. Sci. USA 97: 4856-4861.
4. Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels, M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4: 593-601.
5. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman. J. G., Smith, J. A., and Struhl. K. E. 1995. Short protocols in molecular biology, 3rd ed. John Wiley & Sons, New York, N. Y.
6. Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn5 mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plama membrane K^+/H^+ exchange in tobacco. Phytopathology 77: 1268-1272.
7. Bauer D. W., Bogdanove A. J., Beer S. V., and Collmer A. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. 7: 573-581.
8. Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H.-C., Hutcheson, S. W., Panopoulos, N. J., and van Gijsegem, F. 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathologenic bacteria. Mol. Microbiol. 20: 681-683.
9. Bonas, U., Schulte, R., Fenselau, S., Minsavage G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4: 81-88.
10. Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. 169: 5626-5632.
11. Brissette, J. L., and Russel, M. 1990. Secretion and membrane integration of a filamentous phage-encoded morphogenetic protein. J. Mol. Biol. 211:565-580.
12. Burghout, P., Beckers, F., de Wit, E., van Boxtel, R., Cornelis, G. R., Tommassen, J., and Koster, M. 2004. Role of the pilot protein YscW in the biogenesis of the secretin YscC in *Yersinia enterocolitica*. J. Bacteriol.186: 5366-5375.
13. Collmer, A., Lindeberg, M., Petnicki-Ocwieja, T., Schneider, D. J., and Alfano, J. R. 2002. Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. Trends Microbiol. 10: 462-469.
14. Crago, A. M., and Koronakis, V. 1998. *Salmonella* InvG forms a ring-like multimer that requires the InvH lipoprotein for outer membrane localization. Mol. Microbiol. 30: 47-56.
15. Daefler, S., and Russel, M. 1998. The *Salmonella typhimurium* InvH protein is an outer membrane lipoprotein required for the proper localization of InvG. Mol. Microbiol. 28: 1367─1380.
16. Deng, W.-L., Preston, G., Collmer, A., Chang, C.-J., and Huang, H.-C. 1998. Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovars *syringae*, *tomato*, and *glycinea* and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hypersensitive response and disease in plants. J. Bacteriol. 180: 4523-4531.
17. Deng, W.-L., and Huang, H.-C. 1999. Cellular locations of *Pseudomonas syringae* pv. *syringae* HrcC and HrcJ proteins, required for Harpin secretion via the type III pathway. J. Bacteriol. 181: 2298-2301.
18. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium melioti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
19. Frederick, R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodriguez, A. S., Manulis, S., and Coplin, D.L. 2001. Genetic organization of the *Pantoea stewartii* subsp. *stewartii hrp* gene cluster and sequence analysis of the *hrpA*, *hrpC*, *hrpN*, and *wtsE* operon. Mol. Plant-Microbe Interact. 14: 1213-1222.
20. Hardie, K. R., Lory, S., and Pugsley, A. P. 1996. Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. EMBO J. 15: 978-988.
21. Hayashi, S., and Wu, H. C. 1990. Lipoprotein in bacteria. J. Bioenerg. Biomember. 22: 451-471.
22. He, S. Y., Huang, H.-C., and Collmer, A. 1993.

Pseudomonas syringae pv. *syringae* Harpirn$_{Pss}$: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73: 1255-1266.

23. He, S. Y., and Jin, Q. 2003. The Hrp pilus: learning from flagella. Curr. Opin. Microbiol. 6: 15-19.
24. Hirano, S. S., and Upper, C. D. 1990. Population biology and epidemiology of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 28: 155-177.
25. Huang, H.-C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutchson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. J. Bacteriol. 170: 4748-4756.
26. Huang, H.-C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning *hrp* proteins. Mol. Plant-Microbe Interact. 4: 469-476.
27. Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: an envelope protein required for elicitation of the hypersensitive response in plants. J. Bacteriol. 174: 6878-6885.
28. Huang, H.-C., Lin, R.-W., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colineraly with *Yersinia ysc* homologues. Mol. Plant-Microbe Interact. 8: 733-746.
29. Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245: 1374-1377.
30. Iwabuchi, K., Li, B., Bartel, P., and Fields, S. 1993. Use of the two-hybrid system to identify the domain of p53 involved in oligomerization. Oncogene 8:1693-1696.
31. Keen, N. T., Tamaki, S., Kabayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.
32. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44: 301-307.
33. Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G. R., and Tommassen, J. 1997. The Outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. Mol. Microbiol. 26: 789-797.
34. Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E., and Aizawa, S. I. 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science 280: 602-605.
35. Labes, M., Puhler, A., and Simon, R. 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. Gene 89: 37-46.
36. Lario, P. I., Pfuetzner, R. A., Frey, E. A., Creagh, L., Haynes, C., Maurelli, A. T., and Strynadka, N. C. 2005. Structure and biochemical analysis of a secretin pilot protein. EMBO J. 24: 1111-1121.
37. Li, B., and Fields, S. 1993. Identification of mutations in p53 that affect its binding to SV40 T antigen by using the yeast two-hybrid system. FASEB J. 7: 957-963.
38. Lidell, M. C., and Hutcheson S. W. 1994. Characterization of the *hrpJ* and *hrpU* operons of *Pseudomonas syringae* pv. *syringae* Pss61: similarity with components of enteric bacteria involved in flagellar biogenesis and demonstration of their role in harpin$_{Pss}$ secretion. Mol. Plant-Microbe Interact. 7: 488-497.
39. Linderoth, N. A., Simon, M. N., and Russel. 1997. The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. Science 278: 1635-1638.
40. Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168: 512-522.
41. Lindgren, P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. 35: 129-152.
42. Richael, C., and Gilchrist, D. 1999. The hypersensitive response: a case of hold or fold? Physiol. Mol. Plant Pathol. 55: 5-12.
43. Sambrook, J., and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
44. Schnaitman, C. A. 1971a. Effect of ethylene diaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. 108: 553-563.
45. Schnaitman, C. A. 1971b. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. 108: 545-552.

46. Schuch, R., and A. T. Maurelli. 2001. MxiM and MxiJ, base elements of the Mxi-Spa type III secretion system of *Shigella*, interact with and stabilize the MxiD secretin in the cell envelope. J. Bacteriol. 183: 6991-6998.
47. Sekiya, K., Ohishi, M., Ogino, T., Tamano, K., Sasakawa, C., and Abe, A. 2001. Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc. Natl. Acad. Sci. USA 98: 11638-11643.
48. Studier, W. F., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185: 60-89.
49. Tabor, S., and Richarson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82: 1074-1078.
50. Tamano, K., Aizawa, S. I., Katayama, E., Nonaka, T., Imajoh-Ohmi, A., Kuwae, A., Nagai, S., and Sasakawa, C. 2000. Supramolecular structure of the *Shigella* type III machinery: the needle part is changeable in length and essential for delivery of effectors. EMBO J. 19: 3876-3887.
51. Thanassi, D. G., and Hultgren, S. J. 2000. Multiple pathways allow protein secretion across the bacterial outer membrane. Curr. Opin. Cell. Biol. 12: 420-430.
52. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.
53. van Dijk, K., Tam, V. C., Records, A. R., Petnicki-Ocwieja, T., and Alfano, J. R. 2002. The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of *Pseudomonas syringae*. Mol. Microbiol. 44: 1469-1481.
54. Yip, C. K., and Strynadka, N. C. J. 2006. New structural insights into the bacterial type III secretion system. Trends Biochem. Sci. 31: 223-230.

摘 要

林元春[1,2,3]、徐世典[1]、黃秀珍[2,4]. 2006. 菜豆細菌性斑點病菌第三型分泌系統 HrpT 與 HrcC蛋白間之交互作用. 植病會刊 15: 171-185. ([1]台中市國立中興大學植物病理學系；[2] 生物科技學研究所; [3] 台中市私立中臺科技大學通識教育中心; [4] 聯絡作者，電子郵件: hchuang@dragon.nchu.edu.tw ；傳眞: +886-4-22853527)

選殖自菜豆細菌性斑點病菌 Psy61 的 *hrp*/*hrc* 致病基因組至少可轉譯 28 個蛋白，其中多數的 Hrc/Hrp 蛋白在細菌內外膜上組成第三型分泌系統。前人研究顯示，將 *hrp/hrc* 基因組之 *hrpT* 基因突變後，Psy61 菌株在煙草上引起過敏性反應的能力及 harpin 蛋白的累積分泌均受到抑制。爲了探討 HrpT 蛋白在整個分泌過程中所可能扮演的角色，研究中構築一 *hrpT*-FLAG 基因並利用 M2 單株抗體偵測其所表現的融合蛋白。將 *hrpT*-FLAG 進一步次選殖於廣寄主載體並送入 *hrpT* 基因突變菌株後，可恢復突變菌株所喪失的引起過敏性反應以及累積並分泌 harpin 蛋白的能力，此結果亦顯示 HrpT 蛋白之 C 端融合”FLAG”胜肽，並不影響其原有功能。利用細胞成份分離法及免疫法進行分析，得知 HrpT-FLAG 與 HrcC 蛋白二者均分布於外膜部位；此外以酵母菌雙雜合系統 (yeast two-hybrid system) 及 pull-down 方法，可偵測到 HrpT 與 HrcC 蛋白於生體內外均有相互結合的現象。依據過去研究顯示，HrcC 蛋白就如其同源蛋白 YscC、InvG、MxiD 或 PulD 蛋白，以自體聚合方式組成一個碟狀結構，構成第三型分泌胞器跨越外膜部位的基座，但這些外膜蛋白尚須依靠導引蛋白幫助才能穩定存在於外膜。不同的導引蛋白彼此間序列相似程度不高，但都是位於外膜的小分子脂蛋白。HrpT蛋白大小爲 7.5 kDa，其N端序列含 18 個疏水性氨基酸以及脂質結合部位，本研究證實 HrpT蛋白位於菌體外膜且與 HrcC 蛋白間有交互作用，因此推測 HrpT 極可能擔任 HrcC 的導引蛋白，進一步參與第三型分泌胞器的組裝。

關鍵詞：菜豆細菌性斑點病菌、第三型分泌系統、導引蛋白