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# Cloning and Characterization of Exchangeable Effector Locus of *Pseudomonas syringae* pv. *averrhoi*, a New Pathogen on *Averrhoa carambola*

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## ABSTRACT

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Bacterial spot of carambola (*Averrhoa carambola*) caused by *Pseudomonas syringae* is not only a new record on this host, but also is a new epidemic disease in Taiwan. Based on extensive studies of its etiology and physiology, this pathogen is very distinct from reported pathovars of *P. syringae* and thus has been considered as a new pathovar, averrhoi. In this study, *P. syringae* pv. *averrhoi* was referred to this pathogen and abbreviated as Pav. To clone exchangeable effector locus (EEL) of Hrp pathogenicity island from Pav, a primer pair degenerated from the flanking sequence of EEL from *P. syringae* pv. *syringae* strain 61 (Psy 61) was applied to amplify ca 4.3 kb DNA fragment from Pav strain HL1. Sequencing data show that the amplified fragment contains a 3.5 kb of EEL and its flanking sequences including a complete sequence of t-RNA$^{Leu}$ and partial sequences of *hrpK* and *queA* gene. The EEL contains *shcA2* and a transposase sequence (encoded by ISPsy2) inserted *hopA2* gene, indicating that this EEL is dispensable in pathogenesis. Based on gene composition and sequence analysis, this EEL shows the highest similarity with that of *P. syringae* pv. *morsprunorum* PDDCC5795. Since no apparent *hrp* box sequence located upstream of *shcA2* gene was found, the genes in this locus might be a part of the same operon with *hrpK* gene. Moreover, *Escherichia coli* MC4100 carrying a modified Pav EEL locus, where ISPsy2 was deleted, had the HR eliciting activity on tobacco leaves. The intact HopA2 also slightly reduced the virulence of Pav on its host carambola, but did not apparently affect the pathogen multiplication in carambola leaves.

Key words: *Pseudomonas syringae* pv. *averrhoi*, exchangeable effector locus (EEL), avirulence

## INTRODUCTION

Strains of *Pseudomonas syringae* sharing similar host ranges are grouped as one of at least 50 pathovars in this species [31]. In host plants the bacteria typically grow to high population levels in intercellular spaces of leaves and then produce necrotic lesions. In nonhost plants or in host plants with race-specific resistance, the bacteria elicit the hypersensitive response (HR), a rapid, defense-associated

programmed death of plant cells in contact with the pathogen [2, 25]. The ability of *P. syringae* strains to trigger both types of interactions with plants is controlled by *hrp* (hypersensitive response and pathogenicity) pathogenicity island (Pai) with a tripartite mosaic structure [4]. Namely, the *hrp/hrc* (hypersensitive response and conserved) gene cluster at the center of this Hrp Pai mainly coding type III protein secretion system (T3SS) complex is conserved and is flanked by a unique exchangeable effector locus (EEL) and a conserved effector locus (CEL). The *hrc* genes represent a subset of *hrp* genes that have been renamed to reflect their conservation among T3SS of both plant and animal pathogens [7], such as animal pathogens in the genera *Yersinia*, *Salmonella*, *Shigella*, and *Escherichia*, and plant pathogens in the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* [16, 19, 21, 30, 44]. This T3SS is a device for pathogens to inject the effector proteins into their host cells and then to alter the host biochemical reactions [11]. Furthermore, some of the amino acid sequences of the T3SS components also share significant homology with some proteins of the bacterial flagellar export systems [16, 21]. According to the observation using an electronic microcopy, the appearance of this T3SS complex isolated from either *Salmonella typhimurium* or *Shigella flexneri* 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 flagellum [27, 37]. Taken together, it implies that the T3SS machinery may have evolved from the genes associated with flagellar export systems [16, 21].

T3SS substrates in *P. syringae* include Avrs (for the avirulence they can confer in test plants) and Hops (Hrp outer proteins) [11]. As for animal pathogens, Avr proteins of plant pathogens are also classified as effectors, which function primarily inside of the host cells [12, 21]. If Avr proteins are recognized by their corresponding R proteins of host plants, they will turn on the plant defense system, known as the HR [23]. However, some Avr proteins act as suppressors of plant defense system if host plants lack of corresponding R proteins [1, 15]. HrpA, one kind of Hops, is a pilus forming protein which provides a conduit for effectors into host cells [32]. Harpins, another kind of Hops, are glycine-rich, devoid of cysteine proteins and possess ability to elicit the HR when infiltrated into the intercellular spaces of plant leaves [17]. In *P. syrinage* pv. *phaeolicola*, harpin was evident to insert into lipid layer and forms an ionic channel [28]. Both harpin and HrpA that appear to interact with plant cell walls and membranes are helper proteins rather than true effectors [11].

The CEL carries several candidate effectors including AvrE, HrpW and so on, that are conserved between *P. syringae* pv. *syringae* (Psy) B728a and *P. syrinage* pv. *tomato* (Pto) DC3000. A large deletion in the Pto DC3000 CEL abolishes pathogenicity, suggesting that the CEL is one of several necessary contributors to pathogenesis [4]. The region residing between *hrpK* and $tRNA^{Leu}$ of the Hrp Pai is given the EEL designation because it contains completely different candidate effector genes in the different strains, which appear to be exchanged at this locus at high frequency [4]. Deletion of the EEL in Pto DC3000, which codes for ORFs with no similarity to any known effector proteins, partially reduces pathogen fitness *in planta*. It indicates that the ORFs might collectively contribute to parasitism. The Psy 61 EEL (2.5 kb in length) encodes one effector protein, HopPsyA (formerly known as HrmA and newly renamed as HopA1), which has avirulence activity when it is heterologously expressed in *P. syringae* pv. *tabaci* (Pta) cells [3, 18]. The 7.3-kb EEL of Psy B728a encodes three putative effectors which are homologous to AvrB/C of *P. syringae* pv. *glycinea*, AvrPphE of P. *syringae* pv. *phaseolicola* 1302A, and AvrRxv of X. *campestris* pv. *vesicatoria*, respectively. Deletion of this EEL causes reduced parasitic fitness and heterologous expression of these EEL effector genes in *P. syringae* pv. *tabaci* confers an avirulence phenotype as well [13]. Recently, Charity *et al.* [9] investigated EEL loci from 33 strains of *P. syringae* pv. *syringae* predominately representing Northern American area, and classified the EELs, the source of disparate effectors, into six families which display ten distinct types. They also proposed that EEL is evolved independently of core region in Hrp Pai, most likely through integron-like assembly of transposed gene cassettes.

Bacterial spot of carambola (*Averrhoa carambola* L.) caused by *P. syringae* was first recorded in Island Hai-Nai, China [43] and primarily reported in 1997 in Taiwan [10]. The typical symptoms on leaves are purple spots surrounded by yellow haloes, and the infected leaves turn yellow and fall easily. This bacterium also causes the blight spots on the shoots and fruits, resulting in dysformation of the fruits as seriously infected [10, 43]. Back to 1995, Wen and Huang [43] purposed this causing agent as a new pathovar *averrhoi* to reflect the specificity of pathogenicity on carambola plants although the designation was not approved yet. In addition, this strain currently has been extensively characterized on the basis of etiology, ecology, and physiology in Taiwan, and the results support that this strain potentially can be classed into a distinct pathovar [10]. Herein, we followed a proposal of prefix designation for pathovars of *P. syringae* given by Vivian and Mansfield [41] and then used *P. syringae* pv. *averrhoi* (Pav) as the name for this pathogen. Also we followed proposed guidelines for designation of Hop effectors given by Lindeberg *et al.* [29] in this study. Since this pathogen is newly discovered

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

| Strain, plasmid, and primer | Relevant characteristic and sequence | Source and reference |
|---|---|---|
| ***Escherichia coli*** | | |
| DH10B | *endA1 hsdR17 recA1 relA* △*(argF-lacZYA) U169* Φ80d *lacZ* △*M15* | Life Sciences Technologies |
| HB101 | *F ' hsd 20 recA13 thr leu thi pro, Sm*$^r$ | (5) |
| BL21(DE3) | B strain F$^-$ *omp*T r$_b^-$ m$_b^-$ *hsdS gal* ( λ DE3 c*Its*857 *int*1 *sam*7 *nin*5 *lac*UV5-T7 gene1) | (35) |
| MC4100 | F$^-$ *araD139* △(*argF-lacZYA*) U169 *relA rpsL*150 *flb-5301 ptsF25 deoC1 thi*, Sm$^r$ | (8) |
| ***Pseudomonas syringae*** | | |
| pv. *syringae* (Psy) 61 | wild type isolated from wheat, a spontaneous Nx$^r$ mutant was used | (19) |
| *pv. averrhoi* (Pav) HL1, HL8, PA4, PA5, PA8, PA9, PA15 | wild type isolated from carambola, a spontaneous Nx$^r$ mutant was used | (10) |
| **Plasmids** | | |
| pBluescripII SK | Cloning vector, ColE1 mcs-lacZ, Ap$^r$ | Stratagene |
| pBBR1MCS-5 | Broad host range vector, Gm$^r$ | (26) |
| pGEM-T easy | T/A cloning vector for PCR product, Ap$^r$ | Promega Inc. |
| pRK2013 | Helper plasmid, IncP Tra RK2$^+$ △repRK2 *repE1*$^+$, Km$^r$ | (14) |
| pT7-5 | T7 RNA polymerase dependent expression vector, Ap$^r$ | (36) |
| pUC18, 19 | Cloning vector, Ap$^r$ | (46) |
| pHIR11 | Psy 61 *hrp/hrc/hrmA* cluster cloned in pLAFR3, Tc$^r$ | (19) |
| pHIR11BB8 | A 8.4 kb *Eco*RI-*Bam*HI fragment from pHIR11 subcloned into pLAFR3, Tc$^r$ | (19) |
| pCPP2071 | Tn*phoA* inserted in *hopPsyA* (*hrmA*; *hopA1*) gene of pHIR11 | (20) |
| pNCHU738 | A prEEL1/EEL2-generated 4.3 kb *XhoI-Eco*RI fragment containing the exchangeable effector locus from Pav HL1 cloned in pGEM-T easy | This work |
| pNCHU774 | A 1.7 kb *Eco*RI-*Bam*HI fragment containing *shcA2* and recombinant *hopA2* from Pav HL1cloned in pT7-5 | This work |
| pNCHU776 | A 1.7 kb *Eco*RI-*Bam*HI fragment containing *shcA2* and recombinant *hopA2* from Pav HL1 cloned in pBBR1MCS-5 | This work |
| pNCHU778 | A 3.7 kb *Eco*RI-*Bam*HI fragment containing *shcA* and *hopPsyA* from Psy 61 in pBBR1MCS-5 | This work |
| **Primers** | | |
| prEEL1 | 5 ' - GTGACTCGAGGCGTGGATTCAGGCAAAT-3' (*Xho*I) | (4) |
| prEEL2 | 5 ' - GCATGAATTCGGGATTGACAGGGCGCAT-3' (*Eco*RI) | (4) |
| prhopA-S1 | 5 ' - CAGGAGCTCCTGTACATCTACCTC-3' (*Sac*I) | This work |
| prhopA-S2 | 5 ' - CACAGGAGCTCAAAGCACAC-3 ' (*Sac*I) | This work |
| prhopA-B | 5 ' - CAGGGATCCATACTCATTCCA-3 ' (*Bam*HI) | This work |

for this host and nothing related to pathogenesis is known of its genetics, the EEL locus of Pav was initially cloned and characterized to reveal the diversity of EEL features found in *P. syringae* group.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, primers, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) medium [33] at 37℃. *Pseudomonas* strains were grown on King's B medium (KB) [24]. 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; rifampicin (Rf), 200; tetracycline (Tc), 20.

## Recombinant DNA technology

Restriction endonuclease digestion, agarose gel electrophoresis, plasmid extraction, DNA ligation, and bacterial transformation ($CaCl_2$ procedure) were performed as described by Sambrook *et al.*[33]. The DNA fragments were recovered from agarose gels using a gel filtration kit (QIAGEN, Chatsworth, CA).

## Cloning and sequencing of EEL locus from Pav

Chromosomal DNAs isolated from Pav strains HL1, HL8, PA4, PA8, PA9 and PA15 were prepared as previously described[33] and used as templates for amplification of EEL by polymerase chain reaction (PCR). Primers prEEL1 and prEEL2 (Table 1) were designed from the conserved sequences of *hrpK* and *queA* in Psy 61[4, 18]. *PCR* was performed with 1x *Taq* reaction buffer, 100 ng DNA, 0.5 μM of each primer, 0.4 mM 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℃ 5 min, thirty cycles for amplification, and 72℃ 10 min for extension. The PCR product gained from Pav HL1 as a template was cloned into pGEM-T easy vector (Promega), resulting in pNCHU738. In order to accomplish sequencing of the insert, this insert was digested with appropriate restriction enzymes and subcloned into pBluescript SK, pUC18 or pUC19. The resulting plasmids were subjected to DNA sequencing using an automated DNA sequencer, model 377 (ABI) at Biotechnology Center in National Chung Hsing University. Also, the obscure sequences were confirmed by using proper synthetic primers. The nucleotide and derived amino acid sequences were analyzed with programs of Vector NTI (Informax Inc. MD, USA) and SDSC Biology Workbench (http:// workbench. sdsc.edu/). Homology search was performed with BLAST program in NCBI web site (http://www.ncbi.nlm.nih.gov), and multiple alignments and phylogenetic analysis were performed with CLUSTAL W algorithm[38].

## Construction of recombinant plasmid pNCHU776 and pNCHU778

In Pav HL1 strain, *hopA2* gene is disrupted by a transposase ISPsy2, thus it is assumed to be nonfunctional. Therefore, in order to determine any biological function of HopA2 protein of Pav, an intact *hopA2* gene was reconstructed using PCR technology (Fig. 1). Primers were first designed based on the two sides of border sequences of transposase gene from Pav HL1 EEL. In brief, a 0.6 kb PCR product was amplified using a primer pair prhopA-S1/prEEL2 and pNCHU738 as a template, and then cloned into pT7-5 at *Sac*I/*Eco*RI sites. Another 1.1 kb PCR fragment amplified using a primer pair prhopA-B/prhopA-S2 was cloned into aforementioned pT7-5 derivative at *Bam*HI/*Sac*I sites, resulting in pNCHU774. Then the 1.7 kb *Eco*RI-*Bam*HI fragment isolated from pNCHU774 was cloned into pBBR1MCS-5, resulting in pNCHU776 which thus contains *shcA2* and intact hopA2. In order to compare with the activity of *HopA2* from Pav, a 3.7 kb *Eco*RI-*Bam*HI fragment containing the sequences of Psy 61 *shcA* and *hopPsyA* genes was isolated from pHIR11BB8[19] and subcloned into pBBR1MCS-5, creating pNCHU778.

## T7 RNA polymerase-dependent expression of *hopA2* gene in *E. coli*

The product of recombinant *hopA2* gene contained in plasmid pNCHU774, was analyzed by T7 polymerase-dependent expression system in *E. coli* BL21(DE3). Bacteria were grown in LB broth to an $OD_{600}$ of 0.5-0.6 at 37℃. After two-hour induction with 0.5 mM IPTG, rifampicin was added to the bacterial cultures at a final concentration of 200 μg/ml to inhibit the bacterial RNA polymerase-mediated transcription; then the culture was continuously incubated for an additional 2 hours. Bacteria were collected by centrifugation and then resuspended in 200 μl of 2× loading buffer (0.625 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 2% β-mercaptoethanol). The resuspended cells were sonicated (Heat system ultrasonics XL-2020 Sonicator) for one minute, heated to 100℃ for 5 min. Proteins resolved by SDS-12%polyacrylamide gel electrophoresis (PAGE) was visualized with Coomassie Brilliant Blue R250 staining.

## Triparental mating

The triparental mating was performed to transfer constructed plasmids into Pav strains[14]. In brief, the recipient *P. syringae* pv. *averrhoi*, donor *E. coli* DH10B (carrying the constructed plasmids), and helper strains *E. coli* HB101 (pRK2013) were mixed at a ratio of 10:1:1 on KB agar plated and incubated at 30℃ for 16 h. The mating mixtures were then spotted on KB agar supplemented with nalidixic acid, getamycin, and kanamycin at 30℃ for 2-3 days. A single colony of transconjugants was streaked out and purified for further assay.

## 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 prepared by suspending 24 h cultured cells grown on KB agar plates in distilled water at a density of $10^8$ cfu/ml. Inoculations

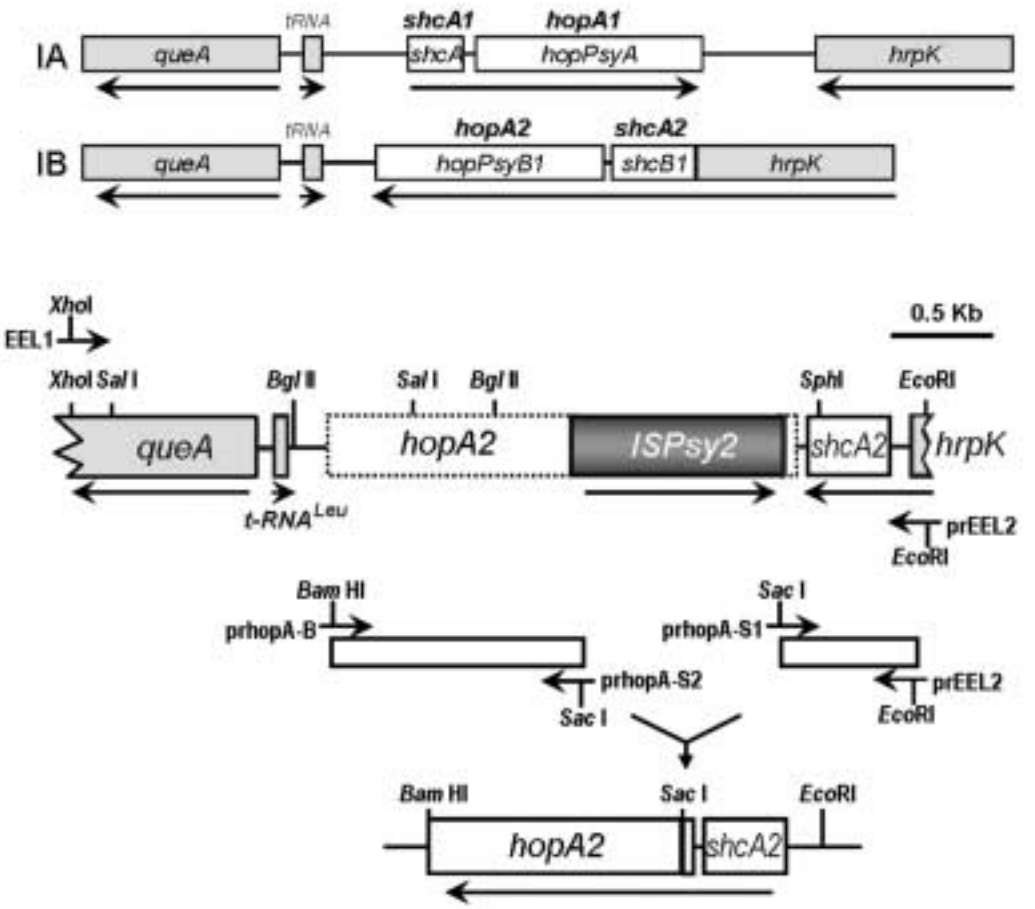


Fig. 1. Gene organization of exchangeable effector locus (EEL) and construction of the intact *hopA2* from *P. syringae* pv. *averrhoi* HL1. The EEL was amplified by using a primer pair prEEL1 and prEEL2 in PCR, while primer pairs prhopA-B/prhopA-S2 and prhopA-S1/prEEL2 were used to amplify two DNA fragments for constructing the intact *hopA2* and *shcA2* genes. The broken lines and arrows indicate nonfunctional genes and transcriptional direction, respectively. EELs of IA or IB family were already published elsewhere [9]. Genes in IA or IB shown on the top were newly named by Lindeberg *et al.* [29].

were performed by pricking leaves with a dissecting needle and then pressing the blunt end of the tuberculin syringe against the leaf surface while supporting the leaf with a finger [6]. The development of HR was observed within 24 hours at room temperature.

### Pathogenicity assay on carambola plant

The overnight cultures of Pav and its derivatives grown on KB plate containing appropriate antibiotics were suspended in water and were then adjusted to concentration of $10^8$ cfu per ml as an inoculum before spreading onto leaves of carambola plants which were grown and regularly maintained in the green house. The inoculation was performed using spraying bottle and evenly spread on leaflets which then were covered with plastic bags for 24 h. The symptoms were recorded six days after inoculation. For measurement of bacterial multiplication, the bacterial suspensions of Pav HL1 and its derivatives at concentration of $10^7$ cfu/ml were infiltrated into leaves with a needless syringe [6] and leaf disks were sampled from infiltrated area 0, 1, 2, 3, 5, 7, 9, 11, and 13 days post inoculation. To determine the bacterial population, leaf disks were collected in a microcentrifuge tube containing 0.5 ml distilled water and homogenized with a glass pestle, and the homogenates were subjected to a series dilution and spread on KB agar plates supplemented with appropriate antibiotics.

## RESULTS

### Sequence analysis of EEL locus from Pav HL1

The size of PCR products amplified from six strains of Pav containing EEL loci was equivalent, so that a 4.3 kb PCR

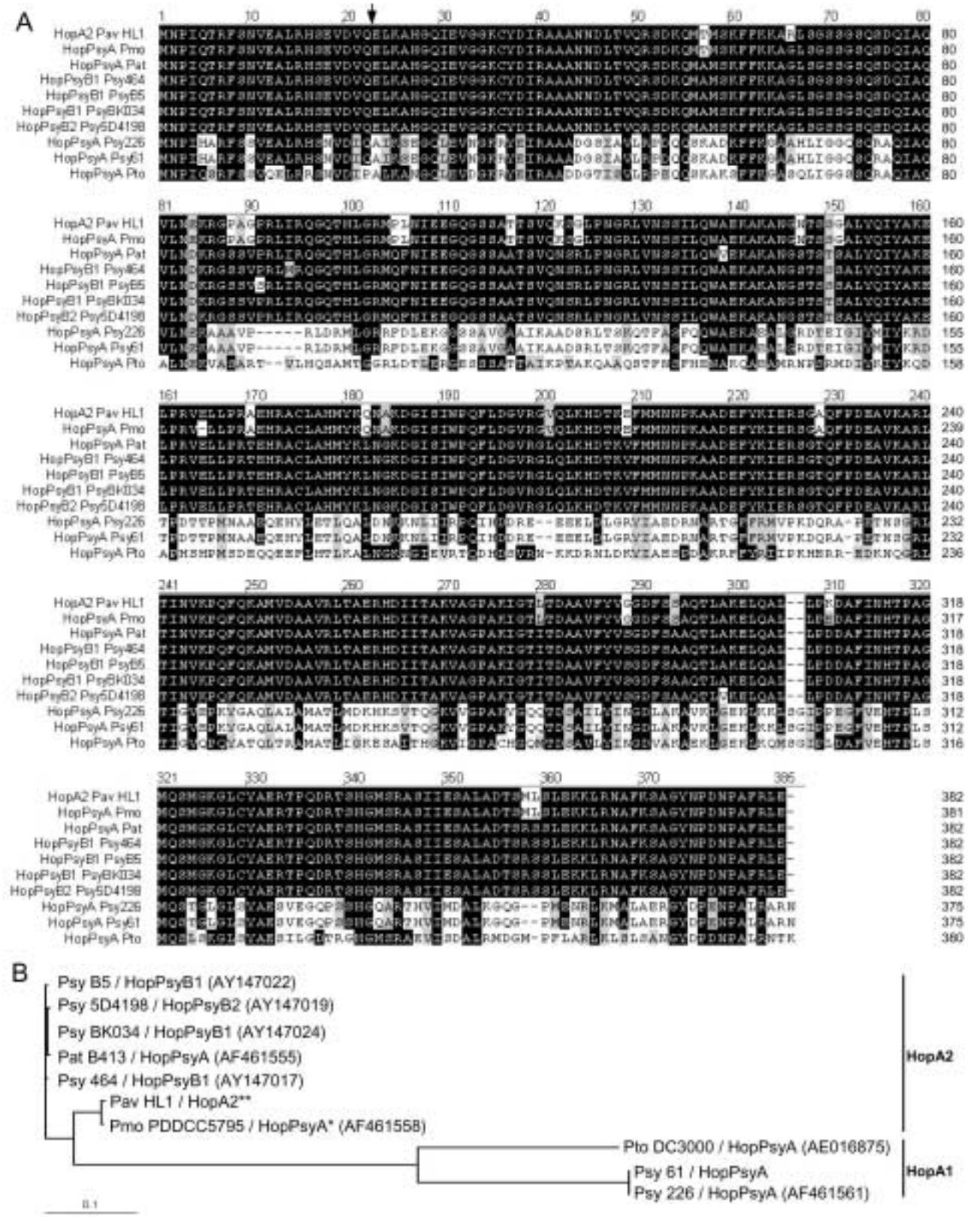


Fig. 2. Multiple alignment of HopA2 with its homologs (A) and a phylogenetic tree showing a evolutionary relationship (B). The phylogenetic analysis was performed as described in the text. Amino acids in the shadow are identical. HopPsyA* (in B) refers to the presence of a nonsense mutation between the amino acid sequence#164 and #165 (in A). HopA2** (in B) indicates that the sequence has been reconstructed by the deletion of ISPsy2 sequence between #22 and #23 shown in the vertical arrow (in A). The accession numbers for *hop* genes are indicated in parentheses.

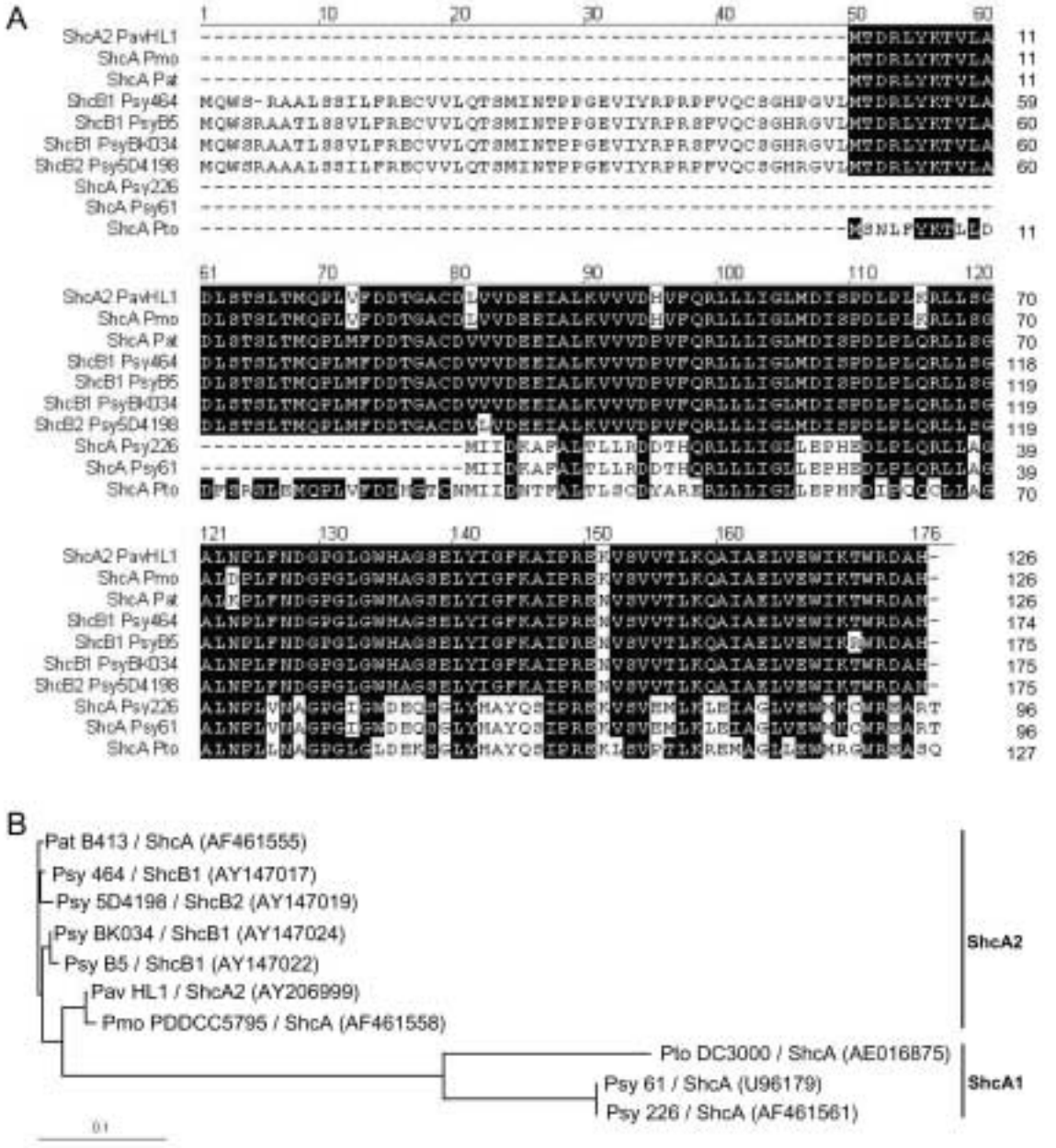


Fig. 3. Multiple alignment of ShcA2 with its homolgs (A) and a phylogenetic tree showing a evolutionary relationship (B). The phylogenetic analysis was performed as described in the text. Amino acids in the shadow are identical. The accession numbers for *shc* genes are indicated in parentheses.

fragment from Pav HL1 was cloned into pGEM-T easy vector, creating pNCHU738 for further characterization. The insert in pNCHU738 was subjected to sequence analysis. The nucleotide sequence reported here has been deposited in GenBank under accession number AY206999. Based on sequence analysis, the 4,291 bp fragment contains a partial *queA* (897 bp), $tRNA^{Leu}$ gene (84 bp), a ISPsy2 gene (972 bp) -inserted *hopA2* gene, *shcA2* (381 bp), and a partial *hrpK* gene (Fig. 1). There are two directed repeat sequences (ACAGG) located in the right/left borders (#634-#638, #1831-#1835) of ISPsy2 gene sequence, indicating a foot print of transposition. The amino acid sequence of ISPsy2 gene encoding transposase showed 98% in identity to those of *P. syringae* pv. *phaseolicola* (AAZ36389, AAZ33940)[22]. The G+C contents of *hopA2* and *shcA2* are 52% and 53% respectively, that is lower than 59-61% in average of *P. syringae hrp*/*hrc* core region, that displays the same character with the EEL loci in Hrp Pai of several pathovars of *P. syringae*[4, 9]. In general, the gene composition of EEL from PavHL1 is very similar to that EEL family IB such as *P. syringae* pv. *morsprunorum* (Pmo) strains PDDCC5795

[13] and 567, and some strains of *P. syringae* pv. *syringae* [9], except that a transposase ISPsy2 gene was inserted in *hopA2* gene in Pav HL1 strain, as shown in Fig. 1. Moreover, no obvious *hrp* box sequence [45] was found at the upstream region of *shcA2* gene, indicating that it is very likely transcribed along with *hrpK* as one transcript. Therefore, Pav EEL can be grouped to Family IB in term of the divergent gene compositions in EEL assigned by Hutcheson's group [9].

Furthermore, based on homology analysis among EEL loci from various *P. syringae* strains, Pav HopA2 and ShcA2 showed the highest similarity to those from Pmo PDDCC5795 (98% and 97 % in identity, respectively) and ca 93 % in dentity to those from strains of *P. syringae* grouped in Family IB; and showed much lower in dentity with those from strains of *P. syringae* grouped in Family IA such as Psy 61 (37% and 54% identity respectively) (Fig. 2A and 3A) [9, 29]. Interestingly, Pmo PDDCC5795 *hopPsyA* also is a pseudogene due to a nonsense mutation existence in its coding sequence [13]. Psy 61 *hopPsyA* gene was sequenced again in this study and three errors (adding " cg " after #1130, and deleting " t " at #1202 and " c " at #1262) in the originally reported coding sequence (accession number L14926) [18] were detected. The new predicted amino acid sequence of HopPsyA is identical to that from Psy 226 (Fig. 2A). ShcA is a specific chaperone of HopPsyA effector, which is translocated into plant cell via T3SS and possesses an avirulent activity [40]. Moreover, phylogenetic analysis of *hopA2* and *shcA2* compared to their homologs was performed and phylogenetic trees were displayed by using neighbor-joining method. As shown in Fig. 2B and 3B, HopA2 and ShcA2 of Pav are in the same clade with those from Pmo, and the phylogenetic relationship among HopPsyA or ShcA homologs appear to be classified into two subfamilies A1 and A2, that results agreed with those gained by Lindeberg *et al.* [29].

## The modified HopA2 from Pav HL1 possesses the HR eliciting activity on tobacco leaves

In Pav HL1 strain, *hopA2* gene is disrupted by a transposase gene, thus it is assumed to be nonfunctional. In this study, we reconstructed *hopA2* gene by deletion of transposase sequence and the expressed 42 kDa HopA2 protein was detected by using T7 RNA polymerase dependent systems (Fig. 4). Next, to evaluate biological functions of HopA2 protein, a recombinant pNCHU776 containing an intact *hopA2* gene and *shcA2* was transformed to *E. coli* MC4100 (pCPP2071), a strain carrying a pHIR11 derivative in which Tn*phoA* was inserted in *hopPsyA* (=*hopA1*, *hrmA*) gene [3, 20, 39]. pHIR11 which contains a 25 kb *hrp*/*hrc*/*hrmA* gene cluster of Psy

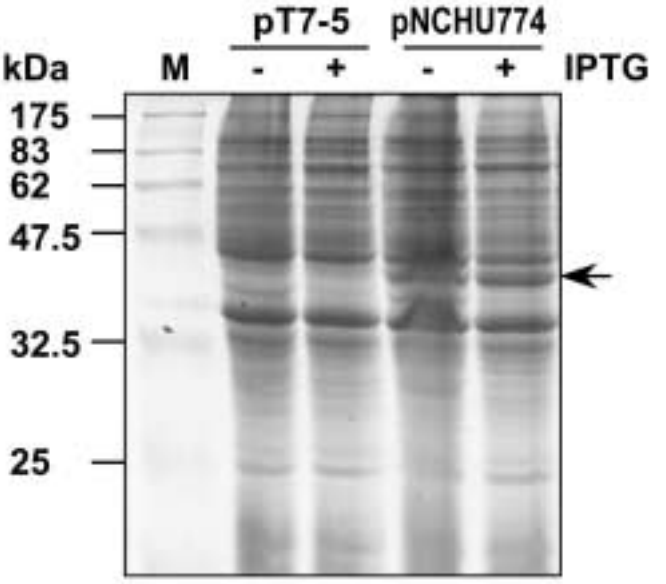


Fig. 4. Overexpression of *hopA2* in *E. coli* BL21(DE3) containing pNCHU774 by using T7 RNA polymerase dependent system. Proteins resolved by 12% SDS- PAGE were visualized with Coomassie Brilliant Blue R250 staining. "M" refers to the prestained molecular size marker. "-" and "+" denote the absence or addition of IPTG at a final concentration of 0.5 mM. The arrow indicates the expressed HopA2 with the estimated molecular weight of 42 kDa.

61 coding a complete T3SS and HopPsyA (an effector protein) enables nonpathogens such as *E. coli* MC4100 or *P. fluorescens* to elicit the HR in tobacco leaves, while pCPP2071 is defective in this activity (Fig. 5) [3, 20, 40]. So, *E. coli* MC4100 (pCPP2071) can be used as a strain to test for *hrp* T3SS-dependent effector activity. As shown in Fig. 5, pNCHU776 allows *E. coli* MC4100 (pCPP2071) to elicit the HR as the same as activity with pNCHU778 which contains *hopPsyA* from Psy 61. *E. coli* MC4100 carrying pNCHU776 or pNCHU778 individually could not induce the HR on tobacco leaves. Results here reveal that HopA2 can be secreted via Psy 61 T3SS and functions as an effector like HopPsyA.

## The modified HopA2 protein reduces the virulence of Pav strains in carambola

Both pNCHU776 and pNCHU778 were conjugated into wild type strains HL1 and PA5 using triparental mating [14], and then were inoculated into carambola leaves using a spreading method in pathogenicity test. The results showed that the number of spot lesions was significantly lower in Pav PA5 carrying pNCHU776 or pNCHU778 than in Pav PA5, while HopPsyA (pNCHU778) from Psy

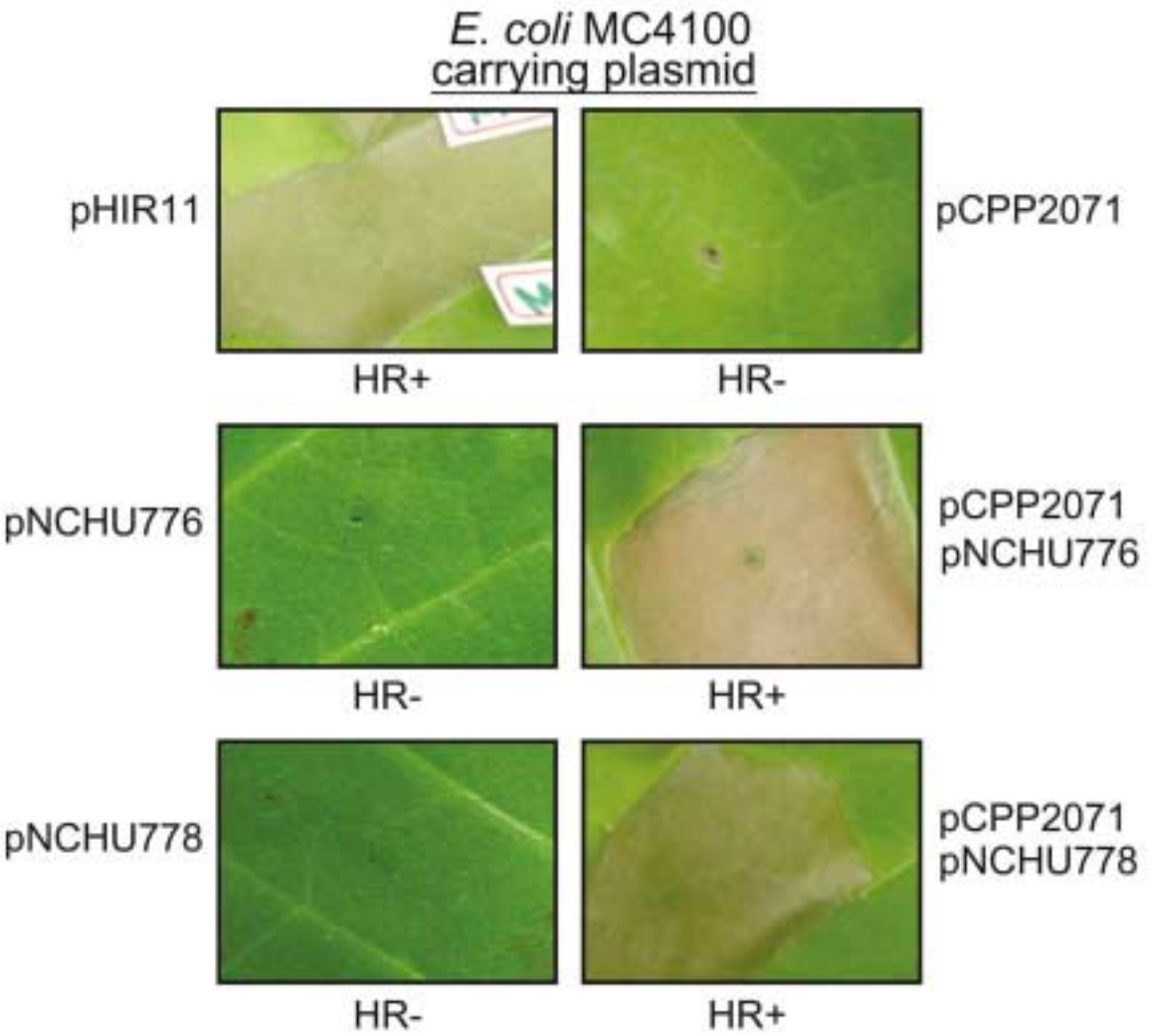


Fig. 5. The hypersensitive response on tobacco leaves elicited by *E. coli* MC4100 carrying plasmid vectors that encode T3SS (pHIR11 or pCPP2071) and HopPsyA (pHIR11 or pNCHU778) or HopA2 (pNCHU776) proteins. Tobacco (*Nicotiana tabacum* L. cv. van Hicks) leaf was infiltrated with bacteria at $10^8$ cfu per ml and photographed 3 days post inoculation.

Table 2. Effect of EEL loci from Pav HL1 or Psy 61 on virulence of *Pseudomonas syringae* pv. *averrhoi* HL1 and PA5 strains inoculated into carambola leaves at a concentration of $10^8$ cfu/ml

| Strain carrying plasmid | Lesion number / leaflet[1] | |
|---|---|---|
| | HL1 | PA5 |
| —[2] | 3.24 ±0.83 a[3] | 3.43 ±1.39 a |
| pNCHU776 | 0.73 ±0.44 b | 1.48 ±0.38 b |
| pNCHU778 | 0.24 ±0.16 b | 1.07 ±0.61 b |

[1] The number of lesions was scored after 6 days post inoculation.
[2] Wild-type strain carried neither pNCHU776 (recombinant EEL locus from PavHL1) nor pNCHU778 (EEL locus from Psy61).
[3] Data represent the means of three replicates ± standard error. Within each column, different letters indicate statistically significant difference between treatments (Duncan New Multiple Range, $\alpha = 0.05$).

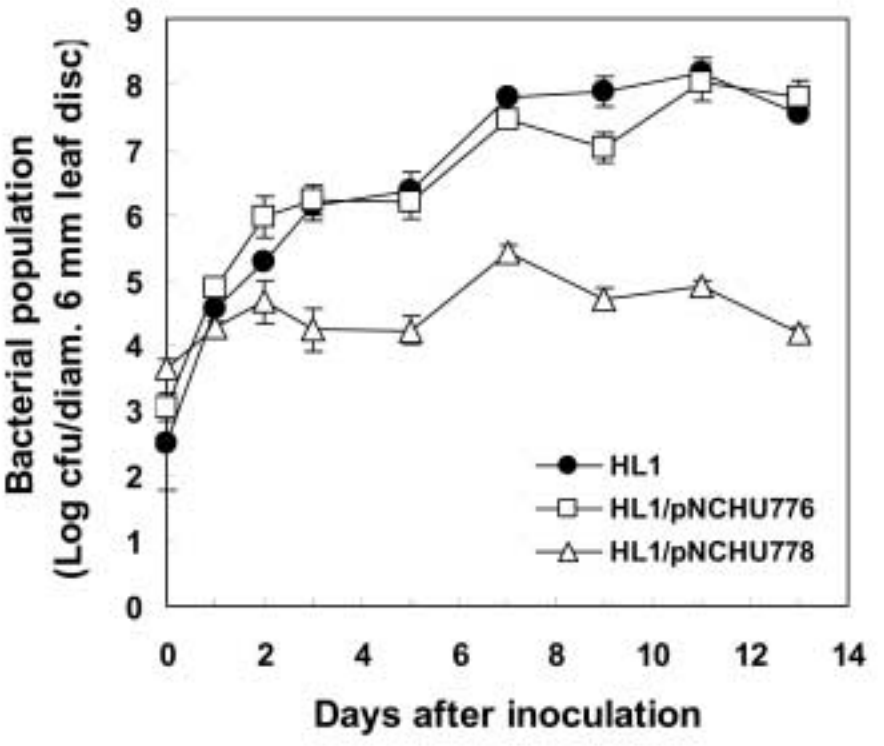


Fig. 6. Population changes of *P. syringae* pv. *averrhoi* HL1 and its derivatives carrying pNCHU776 or pNCHU778 grown on carambola leaves. Growth of the indicated strains was monitored at 0, 1, 2, 3, 5, 7, 9, 11 and 13 days post inoculation. Data represent the means of three replicates ± standard deviation.

61 reduces the virulence of Pav HL1 much more than HopA2 (pNCHU776) from Pav (Table 2). Furthermore, using a leaf infiltration method, HopPsyA from Psy 61 inhibited Pav HL1 growth in carambola leaves with 100 time less, whereas HopA2 from Pav HL1 did not apparently affect on the growth of Pav HL1 (Fig. 6).

## DISCUSSION

According to cloning and sequence analysis of EEL and biological function assay, the results reveal that EEL locus of *P. syringae* pv. *averrhoi* is very similar to that in Pmo PDDCC5795. That is, the EEL locus contains *shcA2* and *hopA2*, but *hopA2* gene is inserted by a transposase gene, ISPsy2. This feature of transposase-containing EEL locus is not surprising since sequence analysis of the EELs isolated from quite a few pathovars of *P. syringae* reveals that those loci are rich in mobile genetic elements, nonsense mutation in coding sequences, and plasmid-related sequences [9, 13]. For examples, two isolated EELs from *P. syringae* pvs. *mori* and *morsprunorum* respectively have insertions and nonsense mutations in the sequences encoding putative effector proteins [13]. However, it is worth noting that *hopA2* genes from Pav and Pmo are pseudogenes and their sequences are nearly identical. Also, the hosts of Pav and Pmo are woody plants. So, it deserves further investigation to determine whether other *P. syringae* infecting woody host plants contain nonfunctional *hopA2* genes or not.

Despite of the effectors encoded by EEL not solely responsible for host range, the EEL appears to be highly variable among pathovars of *P. syringae* [9, 13] and may account for differentiation of pathogen-host interactions. The gene organization of EEL in Pav is obviously distinct from those published EELs of pathovars and *hopA2* in EEL seems to be evolved to be a pseudogene, suggesting that the strains infecting carambola can be classified as a new pathovar.

Up to date, homologs of *hopPsyA* and its chaperone *shcA* genes isolated from *P. syringae* obviously are divided into two subfamilies (Fig. 2 and 3) [29], A1 and A2. The HR eliciting activity of HopA1 in tobacco plant has been studied. Pta 11528 containing HopPsyA (=HopA1) protein of Psy 61 elicits defense associated HR rather than disease in its host *N. tabacum* [3], also the expression of *hopPsyA* gene in transgenic tobacco plant can convert the compatible plant-pathogen into incompatible interaction when inoculated with Pta 11528 [34]. Taken together, it

indicates that HopPsyA is an Avr-like protein. Moreover, heterologous expression of the Psy 61 EEL in Pav HL1 and PA5 strains reduces the growth and virulence of Pav on its host plant, carambola. It suggests that HopPsyA may be also recognized by a cognate resistant factor (R) in carambola besides an R factor from tobacco plant [34]. Currently, the N-terminus of Mcf2 protein, an insecticidal toxin produced by *Photorhabdus luminescens*, shows significant similarity to Psy 61 HopPsyA protein. Also the expression of c-Myc tagged the N-terminus of Mcf2 results in nuclear localization of the fusion protein and subsequent destruction of transfected mammalian cells [42]. So, the subfamily HopA1 protein can induce the host cell death and is contributed to a virulent factor in both animal and plant cells. Although the modified HopA2 protein from Pav shows 37 % identity to Psy 61 HopA1, it stills has the HR-eliciting ability on tobacco leave and less activity on its host carambola leaves. It may be due to the activity of this HopA2 evolved to loss in Pav. However, the molecular mechanism by which HopA proteins elicit the HR remains unknown. Also, it will be worthy to test more subfamily HopA2 proteins in elicitation of the HR, such as that from *P. syringae* pv. *atrofaciens* [13].

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# 摘 要

林元春[1,2,3]、胡育銘[2]、徐世典[1]、曾國欽[1]、黃秀珍[2,4]. 2006. 楊桃細菌性斑點病菌交換性有效因子區之選殖及特性分析. 植病會刊15:139-152 . ([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)

由 *Pseudomonas syringae* 所引起的楊桃細菌性斑點病爲台灣之新興病害，經生理生化特性及寄主範圍等測試，此病原菌與其他已知之病原型均有所差異，極可能歸屬於新的病原型，因此暫定名爲 *P. syringae pv. averrhoi* (Pav)。本研究以 PCR 技術選殖楊桃細菌性斑點病菌的交換性有效因子區基因序列，參考菜豆細菌性斑點病菌 Psy 61 具有保留性基因 *queA* 和 *hrpK* 的序列，合成一組引子對 prEEL1/prEEL2，以不同來源的楊桃細菌性斑點病菌之染色體 DNA 爲模板，皆可增幅出一段約 4.3 kb 的片段。選殖楊桃細菌性斑點病菌 HL1 菌株的交換性有效因子區增幅片段，經定序分析後得到全長爲 4291 bp 的核酸序列，此序列兩端有一完整的 $tRNA^{Leu}$ 以及部分 *queA* 與 *hrpK* 基因序列之外，主要含有 *shcA2* 基因以及被轉移酶序列 (*ISPsy2*) 插入的 *hopA2* 基因，顯示此交換性有效因子區域並非楊桃細菌性斑點病菌所必需。而根據此區域的基因組成及序列分析，楊桃細菌性斑點病菌與核果樹細菌性潰瘍病菌 *P. syringae* pv. *morsprunorum* PDDCC5795 的親緣關係最爲接近，且 PDDCC5795 菌株其 *hopPsyA* 基因也是不活化。研究中亦構築完整的 *hopA2* 基因以進一步分析其生物活性，結果顯示楊桃細菌性斑點病菌的 HopA2 蛋白具有非毒力蛋白的活性，並在菸草中可誘發過敏性反應；而攜帶完整 *hopA2* 基因的楊桃細菌性斑點病菌在楊桃植株中的生長不會受到影響，但對楊桃植株的致病力則有降低的趨勢。

關鍵詞：楊桃細菌性斑點病菌、交換性有效因子區、非毒力蛋白