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The XopE2 effector protein of *Xanthomonas campestris* pv. *vesicatoria* is involved in virulence and in the suppression of the hypersensitive response

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ABSTRACT. Pathogenicity of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) causing bacterial spot disease on tomato (*Lycopersicon* spp.) and pepper (*Capsicum* spp.) requires the type III secretion system (T3SS) and T3SS effectors. In this study, we employed the AFLP technique to investigate the diversity of *X. campestris* pv. *vesicatoria* isolated in Taiwan, and consequently a XopE2 homologue was identified in all fourteen Xcv strains that have been classified into two groups. Phylogenic analysis of XopE2 amino acid sequences indicated that XopE2 of Xcv Xvt122 (group A) has a closer genetic distance to XopE2 of Xcv 85-10 than to that of Xcv Xvt45 (group B). Interestingly, although it was suggested that Xvt45 contains duplicated *xopE*2 genes, one being located on the genome and the other located on a large plasmid, a single copy deletion of *xopE*2 within the genome caused a substantial reduction in virulence, but no effect of *xopE2* mutation on virulence of Xcv 85-10 and Xvt122 was observed. Furthermore, our results revealed that XopE2 of Xcv Xvt122 or Xcv Xvt45 was able to suppress HR in a T3SS-dependent manner and the heterologously-expressed XopE2 was sufficient to modulate the virulence on susceptible tomato plants. Their biological functions are not dependent on the consensus catalytic triad (159th cysteine) and thiol-protease His residue (47th histidine) of XopE2.

Keywords: Amplified restriction fragment length polymorphism; Bacterial spot; Hypersensitive response; Type III secretion system; XopE2 effector.

Abbreviations: AFLP, Amplified restriction fragment length polymorphism; **T3SS,** type III secretion system; **HR,** hypersensitive response; **avr,** avirulence; **PCD,** programmed cell-death.

INTRODUCTION

Plants are armed with an elaborate network of defense mechanisms to protect themselves from the invasion of microorganisms. On the other hand, bacterial pathogens have evolved sophisticated strategies to conquer their plant hosts, i.e., they suppress the basic defense mechanisms in order to successfully establish the initiation of invasion (Nurnberger et al., 2004; Schechter et al., 2006). Similar to most Gram-negative phytopathogenic bacteria, *Xanthomanas* sp. possesses a conserved type III secretion (T3S) system which contains a needle-like structure and is encoded by *hrp* (hypersensitive response and pathogenicity)/ *hrc* (hypersensitive response and conserved) gene clusters. The T3S machinery secretes proteins into the extracellular milieu (e.g. harpin or pilus proteins) and translocates effector proteins (e.g. Xop or Avr proteins) into the plant cell (Grant et al., 2006; Gűrlebeck et al., 2006; Kay and Bonas, 2009). Some effectors designated as avirulence (avr) proteins are specifically recognized in resistant plants containing corresponding resistance (R) genes, usually resulting in a hypersensitive response (HR) that restricts bacterial growth via programmed cell-death (PCD) reactions (Klement, 1982; Staskawicz et al., 2001; Grant et al., 2006; Mudgett, 2005). The T3S mutants that are impaired in growth *in planta* also fail to cause disease symptoms in susceptible plants and lose the capacity to induce the HR in non-hosts or resistant hosts, indicating that T3S effectors may play essential roles in the interaction of bacteria with plants (Kay and Bonus, 2009).

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Individual *Xanthomonas* strains secrete a repertoire of 15 or more T3 effectors, but only a few effectors were shown to be major virulence factors because their mutation led to a dramatic loss of virulence (Thieme et al., 2005; Gűrlebeck et al., 2006; Kay and Bonas, 2009). For instance, AvrBs2 of *X. campestris* pv. *vesicatoria* causing bacterial spots on pepper and tomato significantly contributes to the symptom development, epiphytic survival, and growth *in planta* (Gűrlebeck et al., 2006). The XopX (*Xanthomonas* outer proteins) of *X. campestris* pv. *vesicatoria* promotes lesion development and growth of nonhost pathogens in *Nicotiana benthamiana*, suggesting that XopX suppresses basal plant defense (Metz et al., 2005). The members of *Xanthomonas avrBs3/pthA* gene family, such as *apl1*, *avrXa7*, and *avrXa10*, can suppress nonhost HR and down-regulate the expression of basal defense-associated genes, such as *RbohB*, *PR1*, and *PAL* (Fujikawa et al., 2006). In the genome sequence of *X. campestris* pv. *vesicatoria* 85-10, two new type III effector proteins, XopE1 and XopE2 belonging to the HopX family of *Pseudomonas syringae* were recently identified (Thieme et al., 2007). The N-termini of XopE1 and XopE2 encompass a putative N-myristoylation motif that mediates host cell membrane targeting. Interestingly, this conserved N-myristoylation motif of XopE1 was found to be essential for the induction of cell-death reactions in *N. benthamiana*, whereas such a membrane targeting signal motif is dispensable for the induction of the avirulence activity in *Solanum pseudocapsicum* by XopE2 (Thieme et al., 2007).

The causal agent of bacterial spot symptoms on tomato (*Lycopersicon* spp.) and pepper (*Capsicum* spp.) was originally identified to be *X. campestris* pv. *vesicatoria* (Jones and Stall, 1998). However, in the past two decades, strains of *X. campestris* pv. *vesicatoria* were determined to be composed of two genetically and phenotypically distinct groups, group A and group B, based on their amylolytic activities (Beaulieu et al., 1991), protein, and DNA polymorphisms (Bouzar et al., 1994; Jones and Stall, 1998; Stall et al., 1994; Vauterin et al., 1995). Furthermore, Vauterin et al. (1995 and 2000) reclassified and defined the group A and group B into species *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*, respectively. According to more refined technology, recently at least four taxonomically distinct xanthomonads that cause bacterial spot symptoms on pepper and tomato plants have been identified, e.g *X. euvesicatoria* (=*X. axonopodis* pv. *vesicatoria*), *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al., 2000; Stall et al., 2009). Nowadays, *X. campestris* pv. *vesicatoria* (*X. euvesicatoria*) strain 85-10 is often used as a model isolate for the study of the involvement of T3SS and its effectors in pathogenesis (Thieme et al., 2007; Kay and Bonas, 2009). To date, 17 type III effector proteins have been verified experimentally in *X. campestris* pv. *vesicatoria* 85-10 and its closely related strains (Gűrlebeck et al., 2006; Thieme et al., 2007).

Amplified restriction fragment length polymorphism (AFLP) has been developed as an efficient technique for fingerprinting plant or microbial genomes (Vos et al., 1995; Folkertsma et al., 1996; Lin et al., 1996; O'Neill et al., 1997). For the study on genetic diversity of strains of *X. axonopodis* pv. *manihotis* (Xam) causing bacterial blight disease on cassava, the AFLP primer combinations EcoRI+T/MseI+A were shown to be the most efficient in discriminating between pathogenic and nonpathogenic Xam strains and the sequence analysis of polymorphic bands obtained showed significant homology with genes involved in pathogenic fitness and regulators of virulence (Gonzalez et al., 2002). In *X. campestris* pv. *vesicatoria* 85-10, 30 HrpG-induced (*hgi*) and five HrpG-repressed (*hgr*) cDNA fragments were identified using cDNA-AFLP technique (Noël et al., 2001). In Taiwan, bacterial spot diseases of tomato and pepper are very destructive, especially in rainy seasons, and are mainly caused by *X. campestris* pv. *vesicatoria* group A (=*X. euvesicatoria* = *X. axonopodis* pv. *vesicatoria*,) and *X. campestris* pv. *vesicatoria* group B (=*X. vesicatoria*). In this study, we applied AFLP analysis to investigate the diversity between *X. campestris* pv. *vesicatoria* (Xcv) group A and B strains isolated in Taiwan. The virulence-associated locus *xopE2* was cloned and Southern blot analysis revealed that *xopE2* was highly conserved in *Xanthomonas* spp. Comparisons of the DNA sequences and genomic annotation surrounding *xopE2* suggested that the gene organization of the *xopE2* locus in the genome could be considered as a unique feature to discriminate the members of *X campestris* pv. *vesicatoria* group A from that of group B. The XopE2 mutant of group B exhibited reduced growth and symptom formation, while such phenotypes were not seen in the group A-derived XopE2 mutant. Furthermore, our results suggested that XopE2 from both groups of Xcv can efficiently suppress the hypersensitive response (HR) induced by the avirulence effector HopPsyA of *P. syringae* pv. *syringae* in a T3SS-dependent manner.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulation

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium at 37°C and *Xanthomonas* spp. strains were grown at 28°C in LB broth or 523 agar plate (sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, K_2HPO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 0.3 g/l). The concentrations of antibiotics used were as follows: ampicillin (Amp), 100 μg/ml; Chloramphenicol (Cm), 12.5 μg/ml; gentamycin (Gm), 10 μg/ml; kanamycin (km), 50 μg/ml; and tetracycline (Tc), 20 μg/ml. Plasmids were introduced into bacteria by electroporation (GenePulser, Bio-Rad). DNA manipulations and PCR were carried out according to standard procedures (Sambrook et al., 1989). All the primers used in this study are listed in Table 2. Colony hybridization and Southern blotting analysis were performed with DIG Luminescent Detection Kit (BM) according to the manufacturer's instructions. DNA sequencing was done with ABI 3700 DNA sequencer (in Biotechnology center,

National Chung Hsing University). Database searches were performed using gapped BLASTN and BLASTP (http://www.ncbi.nlm.nih.gov/).

AFLP analysis

The AFLP analysis was carried out according to the manufacturer's instruction (AFLP Expression Analysis Kit, LI-COR Biosciences). Briefly, a total of 250 ng genomic DNA was digested with *Taq*I/*Mse*I enzymes, and then ligated with the respective adapters. The pre-amplification (pre-amp) was performed using 1:10 dilution of the above ligation mixture as the template and the pre-amp primers (primer *Mse*I-N/primer *Taq*I-N), while *Mse*I primer/IRDye^{TM} 700-labeled *Taq*I primers and 1:10 dilution of the pre-amp DNA as template were used for the selective amplification. The PCR program was one cycle of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min, followed by 12 cycles of 0.7°C/per cycle down-gradient annealing temperature; and then additional 23 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. The amplified products were denatured at 95°C, chilled on ice and resolved by a 6.5% polyacrylamide denaturing gel (KB^{PlusTM}). Electrophoresis was carried out for 4 h at 45 V/cm and 45°C using 0.8 x TBE buffer. The polymorphic bands were viewed with LI-COR Odyssey^{TM} scanner. The bands of interest were then excised from gels using a razor blade, resuspended in 20 µl TE buffer, and the suspension was subjected to a series of freeze-thaws and finally the DNA was leaky out from the gel and collected by centrifugation at 15,000 *g* for 20 min at 4°C. The DNA was re-amplified by PCR and cloned into pGEM-T easy vector (Promega). Sequence of the DNA inserts was determined and analyzed.

Construction of the genomic Library

The genomic library was constructed using Copy-Control^{TM} BAC Cloning Kit (Epicentre) according to the manufacturer's instruction. Briefly, genomic DNA was partially digested with *Eco*RI and the resulting DNA fragments 10 to 25 Kb in length were collected and cloned into pCC1BAC. The transformants were selected on LB plates supplemented with chloramphenicol and incubated at 37°C overnight until colonies reached a diameter of 1 mm. Colony hybridization was applied to screen the clones of interest.

Generation of *xopE2* knocked out strains

To create *xopE2* deletion in Xcv Xvt122, an *nptII* gene lacking a Rho-independent transcription terminator (Beck et al., 1982; Alfano et al., 1996) was used to replace the coding sequence of *xopE2*. Briefly, 1 kb *Xba*I-*Hin*dIII fragment and 0.9 kb *Xbo*I-*Kpn*I fragment that encompassed the flanking region of *xopE2* were amplified using the primer pairs pXvt122-8K-7-*Xba*I-F and pXvt122-8K-7-*Hin*dIII-R, and pXvt122-8K-7-*Xho*I-F and pXvt122-8K-7-*Kpn*I-R, respectively. The amplified DNAs were then cloned into pCPP2988 and pBBR1MCS-5 to generate pNCHU1229 and pNCHU1300. Subsequently, a 2.5 Kb *Xba*I-*Xho*I fragment containing *npt*II gene was isolated from pNCHU1229 and subcloned into pNCHU1300 generating pNCHU1301 containing the up- and down-stream regions of *xopE2* in which the *xopE2* coding sequence was replaced with *npt*II gene (Figure 4A). To make *xopE2* mutant of Xct45, the plasmid pNCHU1362 (Figure 4B) was constructed using the similar procedure but with the primer pairs pXvt45857-*Xba*I-F/pXvt45857-*Hin*dIII-R and pXvt45852-*Xho*I-F/pXvt45852-*Kpn*I-R (Table 2). Finally, the resultant plasmid pNCHU1301 or pNCHU1362 was introduced into *Xanthomonas* strains by electroporation followed by subculturing 5 days in liquid broth to promote homologous recombination as reported (Huang et al., 1988). Putative mutants were screened for the Km-resistant and Gm-sensitive phenotypes. The genome types presented in Xvt122Δ*xopE2* and Xvt45Δ*xopE2* were verified by Southern hybridization.

Site-directed mutagenesis

The plasmids, pNCHU1913 and pNCHU1917 containing *xopE2* genes of Xvt122 and Xvt45 respectively, were used as the templates for the crossover PCR-based mutagenesis. Briefly, the primer pairs pXvt45-E3-F-XhoI (including start codon) / prH47A-1 (the 47th histidine was substituted by alaine) and prH47A-3 (the complementary sequence of prH47A-1)/ pXvt45-E3-R-XbaI (including stop codon) were used for amplification of the DNAs containing two partial xopE2 fragments. The DNAs were then used as templates for reamplification with the primer pair pXvt45-E3-F-XhoI / pXvt45-E3-R-XbaI, and the resulting *xopE2* mutant fragment were cloned into the pDrive vector (GIAGEN PCR Cloning Kit), resulting in recombinant plasmids pNCHU1914 (XopE2$_A$ H47A) and pNCHU1918 (XopE2$_B$ H47A) respectively. Similar procedure with different primer pairs was applied and the resulting plasmids containing different *xopE2* mutations were obtained and designated as pNCHU1915 (XopE2$_A$ C159A), pNCHU1919 (XopE2$_B$ C159A), pNCHU1916 (XopE2$_A$ H47A/C159A), and pNCHU1920 (XopE2$_B$ H47A/C159A). The DNA fragments containing various site-directed mutations of xopE2 in the above-mentioned plasmids were isolated by *Xho*I/*Xba*I digestion and subcloned into the broad host range vector pBBR1MCS-5 (Kovach et al., 1995). The resulting plasmids include pNCHU1921 (XopE2$_A$), pNCHU1922 (XopE2$_A$ H47A), pNCHU1923 (XopE2$_A$ C159A), and pNCHU1924 (XopE2$_A$ H47A/C159A), pNCHU1925 (XopE2$_B$), pNCHU1926(XopE2$_B$ H47A), pNCHU1927(XopE2$_B$ C159A), and pNCHU1928 (XopE2$_B$ H47A/C159A).

Plant bioassays

For bacterial multiplication assays in susceptible tomato (Bonny Best L305) leaves, the bacteria were suspended in distilled water at 10^4 ~10^5 cfu /ml for syringe infiltration. Tomato plants were incubated in a humid growth chamber (RH = 90%) with a light intensity of 150 µE/cm^2 and a

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

Designation	Relevant characteristics	Source or reference
Strains		
E. coli		
DH10B	*endA1 hsdR17 recA1 relA*Δ*(argF-lacZYA)U169*Φ80d *lacZ*Δ*M15*	Life sciences technologies (Gaithersburg, MD)
EPI300™	*E. coli* carrying inducible *trfA* gene for amplification of high copy number. Used for genomic DNA library.	CopyControl™ BAC cloning kit (Epicentre)
MC4100	F⁻*araD139*Δ*(argF-lacZYA)U169 relA rpsL150 flb-5301 ptsF25 deoC1 thi,* Sm^r	Casadaban (1976)
A. tumefaciens LBA4404	Wild type	Life technologies
X. c. pv. *vesicatora*		
Xvt12, Xvt28, Xvt48, Xvt122, Xvt185,	Wild types isolated from tomato, classified into group A, no amylolytic activity	Hsu (1998)
Xvp169, Xvp182, Xvp186, Xvp194, Xvp197	Wild types isolated from pepper, classified into group A, no amylolytic activity	Hsu (1998)
Xvt45, Xvt46, Xvt147, Xvt148	Wild types isolated from tomato, classified into group B, with amylolytic activity	Hsu (1998)
Plasmids		
pBluescript II SK$^+$	ColE1 mcs-*lacZ*, Ap^r	Stratagene
pBBR1MCS-5	A broad host range vector containing *lac* promoter, compatible to IncP, IncQ, or IncW group plasmids, Gm^r	Kovach et al. (1995)
pCC1BAC™	Used for construction of genomic DNA library, Cm^r	Epicentre
pGEM-T easy	T/A cloning vector, Ap^r	Promega Inc.
pDrive	T/A cloning vector carrying T7 & SP6 RNA polymerase, Km^r, Ap^r	Qiagen
pCPP2988	pBluescriptII SK⁻ carrying 1.5 kb *Hin*dIII-*Sal*I terminator-lacking *nptII* gene fragment from pRZ102	Aflano et al. (1996)
pHIR11	*P.s.* pv. sringae 61 *hrp/hrc/hrmA* cluster in pLAFR3, Tc^r	Huang et al. (1988)
pNCHU1068	1.3 kb pavrXacE3-F/pavrXacE3-R-generated fragment containing *xopE2* from *Xcv* Xvt122 cloned in pGEM-T easy	This study
pNCHU1070	1.1 kb pavrXacE3-F/pavrXacE3-R -generated fragment containing *xopE2* from *Xcv* Xvt45 cloned in pGEM-T easy	This study
pNCHU1200	1.1 kb pavrXacE3-F-*Xba*I/pavrXacE3-R-*Sma*I-generated fragment containing *xopE2* from *Xcv* Xvt45 cloned in pBI121	This study
pNCHU1201	1.3kb pavrXacE3-F-*Xba*I/pavrXacE3-R-*Sma*I-generated fragment containing *xopE2* from *Xcv* Xvt122 cloned in pBI121	This study
pNCHU1226	30 kb *Eco*RI fragment containing *xopE2* from *Xcv* Xvt45 cloned in pCC1BAC™	This study
pNCHU1227	7 kb *Eco*RI fragment containing *xopE2* from *Xcv* Xvt122 cloned in pCC1BAC™	This study
pNCHU1275	7.8 kb *Eco*RI-*Eco*RV fragment from pNCHU1226 subcloned in pBluescript II SK	This study
pNCHU1276	5.8 kb *Eco*RI-*Eco*RV fragment from pNCHU1226 subcloned in pBluescript II SK	This study
pNCHU1229	1 kb pXvt122-8K-7-*Xba*I-F/pXvt122-8K-7-*Hin*dIII-R-generated *Xba*I-*Hin*dIII fragment containing upstream region of *xopE2* from Xvt122 cloned in pCPP2988	This study
pNCHU1300	0.9 kb pXvt122-8K-7-*Xho*I-F/pXvt122-8K-7-*Kpn*I-R-generated *Xho*I-*Kpn*I fragment containing downstream region of *xopE2* from Xvt122 cloned in pBBR1MCS-5	This study

Table 1. (Continuing)

Designation	Relevant characteristics	Source or reference
pNCHU1301	2.5 kb *Xba*I-*Xho*I fragment from pNCHU1229 cloned in pNCHU1300, creating Xvt122 *xopE2* non-polar mutant.	This study
pNCHU1360	1 kb pXvt45852-*Xho*I-F/Xvt45857-*Kpn*I-R-generated *Xho*I-*Kpn*I fragment containing downstream region of *xopE2* from Xvt45 cloned in pBBR1MCS-5	This study
pNCHU1361	1 kb pXvt45857-*Xba*I-F/pXvt45857-*Hin*dIII-R-generated *Xba*I-*Hin*dIII fragment containing upstream region of *xopE2* from Xvt45 cloned in pCPP2988	This study
pNCHU1362	2.5 kb *Xba*I-*Xho*I fragment from pNCHU1361 cloned in pNCHU1360, creating Xvt45 *xopE2* non-polar mutant.	This study
pNCHU1921 (pNCHU1925)	1.1 kb pXvt45E3-F-*Xho*I/pXvt45E3-R-*Xha*I-generated fragment containing *xopE2* from pNCHU1068 (pNCHU1070) and cloned in pBBR1MCS-5	This study
pNCHU1922 (pNCHU1926)	1.1 kb pXvt45E3-F-*Xho*I/prH47A-1 and prH47A-3/pXvt45E3-R-*Xha*I-generated XopE2(H47A) fragment from pNCHU1068 (pNCHU107) and cloned in pBBR1MCS-5	This study
pNCHU1923 (pNCHU1927)	1.1 kb pXvt45E3-F-*Xho*I/prC159A-1 and prC159A-3/pXvt45E3-R-*Xha*I-generated XopE2(C159A) fragment from pNCHU1068 (pNCHU107) and cloned in pBBR1MCS-5	This study
pNCHU1924 (pNCHU1928)	1.1 kb pXvt45E3-F-XhoI/prC159A-1 and prC159A-3/pXvt45E3-R-*Xha*I-generated XopE2(H47AC159A) fragment from pNCHU1914 (pNCHU1918) and cloned in pBBR1MCS-5	This study

Table 2. Primers used in this study.

Primer	Sequence	Restriction enzyme
pavrXacE3-F	5’-GTGAGGCGAAGCGAAGCGGA-3’	
pavrXacE3-R	5’-TCACCAACTCAAGGGGGGGC-3’	
pavrXacE3-F-*Xba*I	5’-AGCCTCTAGAACCATGGGGCGGAGCGAA-3’	*Xba*I
pavrXacE3-R-*Sma*I	5’-ATTCACCCCGGGTTTCACCAACTCAAGGG-3’	*Sma*I
pXvt122-8K-7-*Xba*I-F	5’-ATCGCCTCTAGACATGCGATGGAGAACC-3’	*Xba*I
pXvt122-8K-7-*Hin*dIII-R	5’-GCGATGAAGCTTTCGAGTTCGCCAACGG-3’	*Hin*dIII
pXvt122-8K-7-*Xho*I-F	5’-TGACGCTCGAGCAAGCCGGATGAGCG-3’	*Xho*I
pXvt122-8K-7-*Kpn*I-R	5’-GGCCGGTACCGCCTGGACGAACTCG-3’	*Kpn*I
pXvt45857-*Xba*I-F	5’-GCGGTCTAGACCGTTTGCCCGAGCTG-3’	*Xba*I
pXvt45857-*Hin*dIII-R	5’-CCGAAAGCTTGGCTGGGATGGCGAAG-3’	*Hin*dIII
pXvt45852-*Xho*I-F	5’-GACGCTCGAGTAAACCGGATGAGCG-3’	*Xho*I
pXvt45857- *Kpn*I-R	5’-TGGCGGTACCGATCAACGCAACCTTG-3’	*Kpn*I
pXvt45-E3-F-*Xho*I	5’-CGCCACTCGAGCCTCTACAGTCACTG-3’	*Xbo*I
pXvt45-E3-R-*Xba*I	5’-GGTTTTCTAGAGCGTCACCAACTCAAG-3’	*Xha*I
prH47A-1	5’-CACCAAGCCAGCCAGGCTGGGTG-3’	
prH47A-3	5’-CACCCAGCCTGGCTGGCTTGGTG-3’	
prC159A-1	5’-TGTGGTCAGCGTTGCCTGCC-3’	
prC159A-3	5’-GGCAGGCAACGCTGACCACA-3’	

photoperiod of 16 h at 25-28°C. Three 0.6-cm-diameter leaf disks of each treatment were sampled 0, 3, 6, and 9 dpi (days post inoculation) and were collected and blended in 200 µl 10 mM $MgCl_2$. Population of bacteria grown in leaf disk was measured by serial dilution methods. For symptom formation analysis, tomato plants were grown to the five-leaf stage followed by dipping in bacteria suspensions (5×10^7 cfu/ml) containing 0.025% silwet L-77. Symptom formation was scored with 0-11 scales according to the percentage of diseased area appearing in the plants: The scales are: 0, 0%; 1, 1-3%; 2, 3-6%; 3, 6-12%; 4, 12-25%; 5, 25-50%; 6, 50-75%; 7, 75-88%; 8, 88-94%; 9, 94-97%; 10, 97-100%; 11, 100% infected area of plantlets (Horsfall and Barratt, 1945).

For the HR suppression assay with a transient expression of XopE2 on tobacco leave mediated by *Agrobacterium* infection, *A. tumefaciens* LBA4404 containing the pBI121 binary vector harboring wild type *xopE2* gene was infiltrated into tobacco leaves. In brief, overnight cultures of *A. tumefaciens* LBA4404 and its derivative strains were washed and resuspended in 5 mM MES (pH 5.6) to OD_{600} of 0.4. The bacterial suspensions were incubated with 200 µM acetosyringone 2 h prior to infiltration into leaves of *N. tabacum* L. cv. Van-Hicks or *N. benthamiana* plants (Kang et al., 2004). The leaves were then challenged with an incompatible bacterium *P. syringae* pv. *syringae* 61 (Psy61) after 24 h of the Agro-infiltration. Moreover, to confirm the suppression of HR by XopE2 via the T3SS, the plasmid pBBR1MCS-5 harboring full length *xopE2* or *xopE2* mutant gene was transformed into *E. coli* strain MC4100 (pHIR11 containing a functional T3SS and an effector *hopPsyA* gene) (Huang et al., 1988; Alfano et al., 1997) and 5×10^8 cfu/ml of the transformants were infiltrated into tobacco (*N. tabacum* L. cv. Van-Hicks) leaves to assess the XopE2-mediated suppression of HR. The appearance of HR on tobacco leaves was examined 24-48 hpi (hour post inoculation).

RESULTS

AFLP analysis of the *X. campestris* pv. *vesicatoria* strains

A total of fourteen strains of *X. campestris* pv. *vesicataoria* (Xcv) isolated from tomato (Xvt) or pepper cultivars (Xvp) have previously been grouped into A or B group based on amylolytic activity assay (Table 1) (Hsu, 1998). Herein, AFLP was employed to search for a genetic marker that will allow further classification of these strains. As shown in Figure 1, the DNA banding patterns resulted from the AFLP analysis appeared to be highly polymorphic among the strains in group A rather than in group B. Nevertheless, six polymorphic fragments designated Xvt12-1, Xvt48-3, Xvt122-4, Xvt148-6, Xvp186-8, and Xvp197-10, were isolated and cloned into pGEM-T easy vector prior to sequence determination. The fragment Xvt12-1 appeared to be unique in the strains from which the band was isolated, Xvt148-6 was absent in group A, Xvt122-4 and Xvp197-10 were absent in group B group, while 186-8 was present in all group B strains and some group A strains, and Xvt48-3 was present in some strains isolated from tomato but not from pepper. Except for the fragments (12-1, 148-6, and 186-8) with no similarity found, the results of the sequence analysis revealed that Xvt48-3 encodes a type I site-specific deoxyribonuclease with 98% sequence homology to that of Xcv 85-10 (accession no. YP_362244); Xvp197-10 encodes a putative transposase of Tn*5044*/Tn*3926* (accession no. YP_001972246); Xvt122-4 encodes a protein with 85% sequence homology to AvrXacE3 of *X. axonopodis* pv. *citri* 306 (accession no. AAM39257) and 92% homology to XopE2 of Xcv 85-10 (accession no. CAJ23957). The XopE2 of Xcv 85-10, a novel type III effector, has recently been shown to play a critical role in triggering cell death in solanaceous plants (Thieme et al., 2007). Whether Xvt122-4 (XopE2 homolog) functions differentially between the two groups was subsequently investigated.

The *xopE2* is conserved in all the *X. campestris* pv. *vesicatoria* strains

To investigate whether the *xopE2* contained in Xvt122

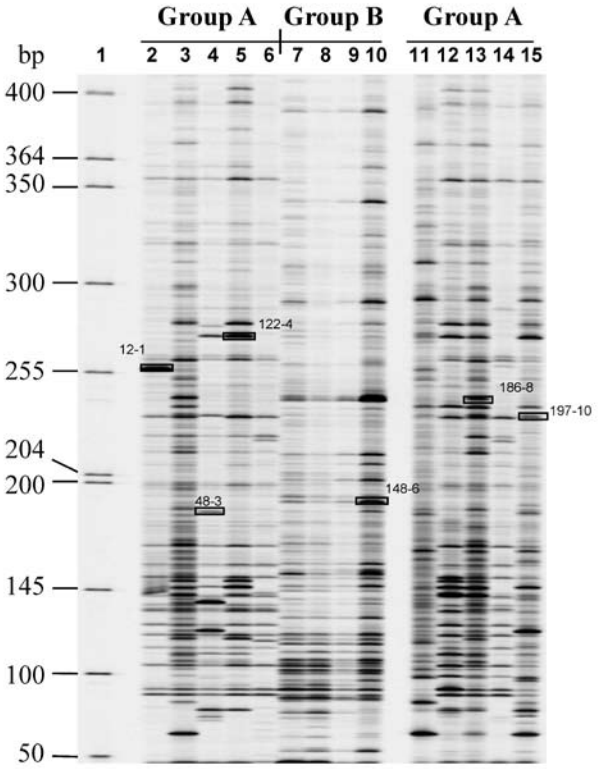

Figure 1. AFLP fingerprints of the *Xanthomonas campestris* pv. *vesicatoria* strains. The genomic DNA isolated from each of the bacteria was digested with *Eco*RI/*Mse*I and then subjected to PCR using primers IR700/MseI-GA and IR700/Mse-GT. Lanes 1: DNA marker; 2 to15: Xvt12, Xvt28, Xvt48, Xvt122, Xvt185, Xvt45, Xvt46, Xvt147, Xvt148, Xvp169, Xvp182, Xvp186, Xvp194, and Xvp197. The six polymorphic bands isolated for sequence determination are marked with rectangles.

is group A-specific as shown in the AFLP analysis (Figure 1), Southern blot analysis of the genomic DNA isolated from all 14 strains (Figure 2A) hybridized with the *xopE2* probe was carried out. Interestingly, the *xopE2* gene appeared to be present in all fourteen strains as well as in *X. axonopodis* pv. *citri* XW19 and *X. campestris* pv. *oryzae* 84 (Figure 2B). On the other hand, the XopE2 encoding gene was not found in *X. campestris* pv. *campestris* 70 and *X. campestris* pv. *diffenbachiae* 49. Some of the group A strains as well as *X. campestris* pv. *oryzae* 84 appeared to carry a single copy of *xopE2*, while other group A strains (Xvp169, Xvp182, Xvp197) and all the group B strains carried two copies of *xopE2* (Figure 2B).

The *xopE2* (XACb0011, previously named *avrXacE3*) of *X. axonopodis* pv. *citri* 306 has been shown to be plasmid encoded (da Silva et al., 2002). To determine whether any copy of the *xopE2* genes are derived from a plasmid, plasmids from the fourteen strains and *X. axonopodis* pv. *citri* XW19 were isolated and subjected to Southern hybridization analysis. As shown in Figure 2C, a copy of the *xopE2* gene in group B strains and some group A strains (Xvp169, Xvp182, Xvp197) is located on the plasmid. Consistent with the finding for *X. axonopodis* pv. *citri* 306, the *xopE2* gene of *X. axonopodis* pv. *citri* XW19 appeared to be plasmid-encoded (Figure 2C).

XopE2 of the group A strains has closer genetic distance to XopE2 of *X. campestris* pv. *vesicatoria* 85-10 than to that of the group B strains

Since *xopE2* sequence was shown to be present in two groups of Xcv, we further investigated whether the sequence variation existed in the two groups to validate the result of AFLP analysis. The intact *xopE2* gene fragments from 14 Xcv strains were cloned by PCR using primer pairs pavrXacE3-F/pavrXacE3-R. The amino acid sequence analysis of XopE2 proteins revealed about 99%-100% identity among the strains in the same group, while 93% sequence identity was found between the group A (e.g. XopE2 of Xvt122) and B (e.g. XopE2 of Xvt45). In addition, all 14 XopE2 proteins contain a conserved N-myristoylation motif (G2) and a catalytic triad, which are also present in the HopX protein family (Figure 3A; Nimchuk et al., 2007). The comparison of the XopE2 between Xcv 85-10 and *X. axonopodis* pv. *citri* 306, Xcv Xvt122, Xcv Xvt45 resulted in amino-acid sequence identity of 98%, 97%, and 92%, respectively. Less sequence identities were observed between the HopPmaB (HopX2 subgroup) of *P. syringae* pv. *maculicola* (*Pma*) and the XopE2 of Xcv 85-10, Xcv Xvt122 and Xcv Xvt45 (79%, 78%, and 77% amino-acid identity, respectively) and the AvrXccE1 of *X. campestris* pv. *campestris* (71%). On the other hand, the XopE2 of Xcv Xvt122 and Xcv Xvt45 shared 62% and 61% amino-acid identity with the XopE1 of Xcv 85-10. Phylogenetic analysis of the XopE2 using vector NTI (Informax) and SDSC Biology Workbench (http://workbench.sdsc.edu) revealed a closer genetic distance between the A-group Xvt122 and Xcv 85-10 than between the B-group Xvt45 and Xcv 85-10 (Figure 3B).

The genomic sequences surrounding *xopE2* could be used to distinguish the members of group A from group B

Although *avrPphE*, a member of the HopX1 subfamily, and its homologues are present in all races of *P. syringae*

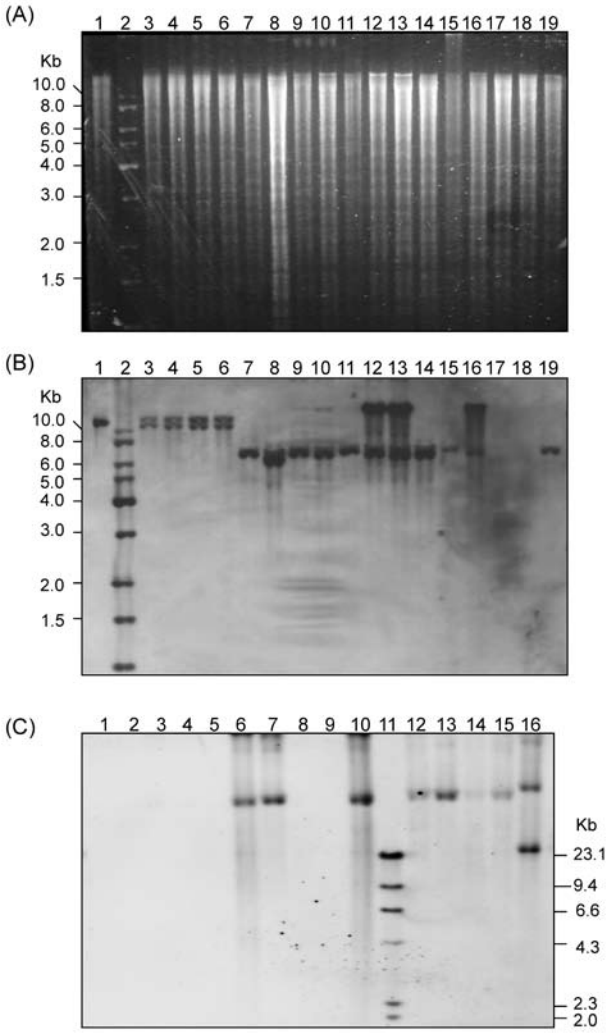

Figure 2. Southern blot analysis of the *Xanthomonas campestris* pv. *vesicatoria* strains. The genomic DNA was isolated, subjected to *Eco*RI digestion, separated on 1% agarose gel, and the gel stained with ethidium bromide (A) or subjected to Southern blot hybridization with the Dig-labeled *XopE2* (B). Lane 1, XacXW19 (*X. axonopodis* pv. *citri* XW19); Lane 2, 1-Kb ladder; Lanes 3-19, XcvXvt45, Xvt46, Xvt147, Xvt148, Xvt12, Xvt28, Xvt48, Xvt122, Xvt185, Xvp169, Xvp182, Xvp186, Xvp194, Xvp197, Xcc70 (*X. c.* pv. *campestris* 70), XcdA49 (*X. c.* pv. *diffenbachiae* 49), and Xco84 (*X. c.* pv. *oryzae* 84) respectively. (C) The plasmid DNA was isolated, separated on 0.7 % agarose gel, and hybridized with the Dig-labeled *XopE2*. Lanes 1-10, XcvXvt12, Xvt28, Xvt48, Xvt122, Xvt185, Xvp169, Xvp182, Xvp186, Xvp194, and Xvp197; 11, λ/*Hin*dIII DNA marker; Lanes 12-16, Xvt45, Xvt46, Xvt147, Xvt148, and XacXW19.

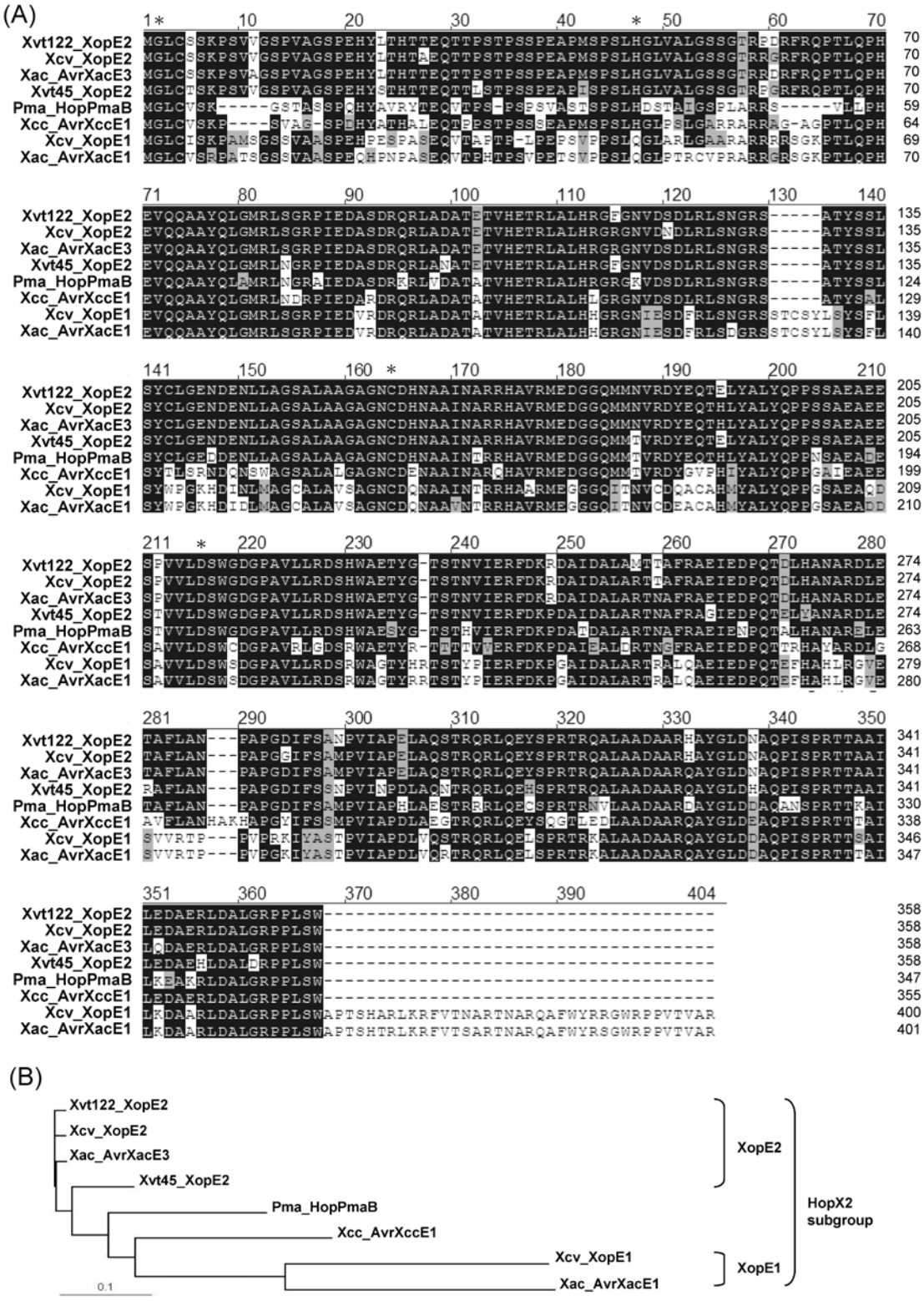

Figure 3. Sequence comparison between XopE2 and its homologues. (A) Amino acid sequence alignment of XopE2 and its homologues according to CLUSTAL W. The conserved residues are highlighted and the GenBank accession numbers for the following proteins are CAJ23957 (Xcv XopE2, *X. campestris* pv. *vesicatoria* 85-10), AAM39257 (Xac AvrXacE3, *X. axonopodis* pv. *citri* 306), AAL84240 (Pma HopPmaB, *P. syringae* pv. *maculicola*), AAM40923 (Xcc AvrXccE1, *X. c.* pv. *campestris*ATCC33913), CAJ21925 (Xcv XopE1, *X. c.* pv. *vesicatoria* 85-10), AAM35178 (Xac AvrXacE1, *X. axonopodis* pv. *citri* 306). The asterisk indicates the N-myristoylation motif (G2), the consensus catalytic triad, C159, D211 (Nimchuk et al., 2007) and the Thiol-Protease His residue (H47) based on PSORT II prediction. (B) Phylogenetic analysis using vector NTI (Informax). Each of the horizontal branched distance is proportional to the estimated numbers of amino acid substitutions.

pv. *phaseolicola*, the DNA sequence variations of those homologues were responsible for the different level of virulence in certain cultivars of bean plants (Stevens et al., 1998). To address whether the sequence variation of *xopE2* (HopX2 subfamily) could also result in differential virulence and to determine the genomic organization of the *xopE2* in two groups, genomic libraries for Xvt122 (as a representative strain of group A) and Xcv Xvt45 (as a representative strain of group B) were constructed. By colony hybridization of the libraries using a Dig-labeled *xopE2* fragment as a probe, two clones, pNCHU1227 (from Xvt122) and pNCHU1226 (from Xvt45) were obtained. The 30-kb DNA insert from pNCHU1226 was digested with *Eco*RI/*Eco*RV and then the 7.5-Kb and 5.8-Kb fragments were subcloned into pBluescript II SK^+ (Stratagene) to generate plasmids pNCHU1275 and pNCHU1276, respectively. The sequences of inserts in pNCHU1227, pNCHU1275 and pNCHU1276 were then determined.

As shown in Figure 4A, the insert in pNCHU1227 (accession no. HM125707) encompasses 5 open reading frames (ORFs) flanking the *xopE2* gene of Xvt122. The gene organization appeared to be identical to that of Xcv 85-10 (Thieme et al., 2005) except that the XCV2281 is replaced with a hypothetical protein encoding gene XACb0012 (accession no. AAM39258). On the other hand, the flanking sequences of Xvt45 *xopE2* are different from that of the group A strain Xvt122. As shown in Figure 4B, in addition to the *xopE2*, the genomic DNA (accession no. HM125708) contained in pNCHU1275 and pNCHU1276 encodes nine ORFs and two incomplete ORFs which share significant similarities with the known bacterial genes. These include the genes encoding DNA-methyltransferase (*Vibrio alginolyticus* 12G01, EAS74231), phage-related integrase (*Stenotrophomonas* sp. SKA14, EED40494), conserved hypothetical protein (*Stenotrophomonas* sp. SKA14, EED37125), hypothetical

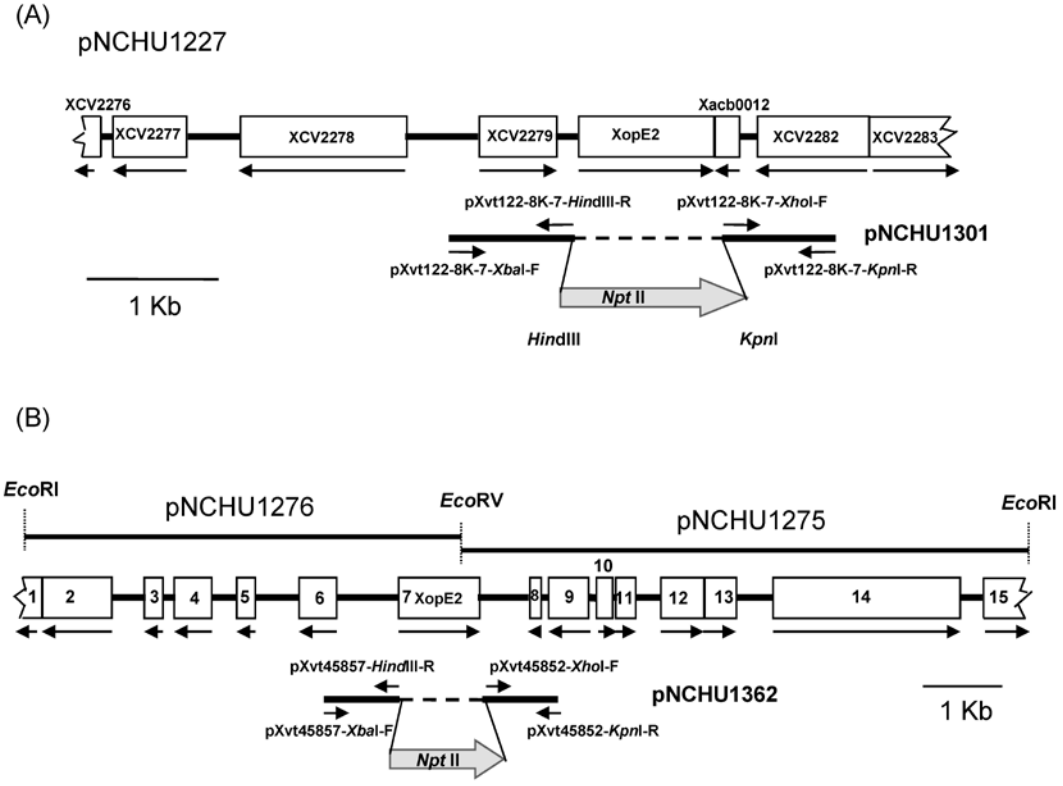

Figure 4. Organization of *xopE2* gene and its neighboring genes. (A) The insert from Xcv Xvt122 cloned into pNCHU1227 contains the *xopE2* and its flanking genes including an incomplete XCV2276 (hypothetical protein), XCV2277 (putative secreted protein), XCV2278 (pectate lyase precursor), XCV2279 (cointegrate resolution protein T), XCV2280 (XopE2), XACb0012 (hypothetical protein), XCV2282 (hypothetical protein), and incomplete XCV2283 (hypothetical protein). (B) The *xopE2* and the flanking genes of Xcv Xvt45 encoded on pNCHU1275 and pNCHU1276 are shown. The ORFs are: 1, DNA-methyltransferase (*Vibrio alginolyticus* 12G01); 2, phage-related integrase (*Stenotrophomonas* sp. SKA14); 3, conserved hypothetical protein (*Stenotrophomonas* sp. SKA14); 4, hypothetical protein Xvt45-1 (Xcv Xvt45); 5, hypothetical protein XALc0184 (*X. albillineans*); 6, hypothetical protein Xvt45-2 (Xcv Xvt45); 7, XopE2 (Xcv 85-10); 8, hypothetical protein SSKA14_4431(*Stenotrophomonas* sp. SKA14); 9, hypothetical protein XF2127 (*Xylella fastidiosa* 9a5c); 10, hypothetical protein XF2126 (*X. fastidiosa* 9a5c); 11, hypothetical protein Xvt45-3 (Xcv Xvt45); 12, hypothetical protein Xvt45-4 (Xcv Xvt45); 13, hypothetical protein XALc0195 (*X. albillineans*); 14, putative primase (*Stenotrophomonas* phage S1); 15, putative terminase small subunit (*Stenotrophomonas* phage S1). The arrows show the direction of transcription of the ORF. The construction maps for nonpolar mutations of the *xopE2* genes of Xvt122 and Xvt45 are also shown below the gene organization maps.

protein XALc0184 (*X. albillineans,* CBA14730), hypothetical protein SSKA14_4431 (*Stenotrophomonas* sp. SKA14, ACF52220), hypothetical protein XF2127 (*Xylella fastidiosa* 9a5c, AAF84926), hypothetical protein XF2126 (*X. fastidiosa* 9a5c, AAF84925), hypothetical protein XALc0195 (*X. albillineans,*CBA14740), putative primase (*Stenotrophomonas* phage S1, ACJ24725), and putative terminase small subunit (*Stenotrophomonas* phage S1, ACJ24727). In addition, a consensus nucleotide sequence 5'-TTCG-N16-TTCG-3', the plant-inducible promoter box or PIP box (Fenselau and Bonas, 1995), was identified upstream of the non-coding sequence of both xopE2 genes. The presence of the locus coding for cointegrate resolution protein T, XCV 2279 (Figure 4A) or phage-related integrase (Figure 4B) suggests that the *xopE2* allele may be a result of horizontal gene transfer from other bacteria.

The *xopE2* mutant in group B strain but not in group A strain reduced virulence in its tomato host

The XopE2 of *X. campestris* pv. *vesicatoria* 85-10 has recently been shown to be able to trigger avirulence activity in *Solanum pseudocapsicum*, suggesting a critical role in pathogenesis (Thieme et al., 2007). To study the biological functions of XopE2 protein in two groups of Xcv on their hosts, the x*opE2* nonpolar mutants of two groups were generated using marker-exchange mutagenesis by transforming Xvt122 (group A) and Xvt45 (group B) with pNCHU1301 (Figure 4A) and pNCHU1362 (Figure 4B), respectively. Southern blot analysis confirmed the *xopE2* deletion in Xvt122Δ*xopE2*, while one copy of the *xopE2* remained intact in *Xvt45*Δ*xopE2* (data not shown).

The *xopE2* deleting effect on Xvt122Δ*xopE*2 or *Xvt*45Δ*xopE*2 was then assessed on the basis of the bacterial growth and symptom development in a susceptible tomato cultivar (Bony Best L305). As shown in Figure 5A, the Xvt122Δ*xopE2* mutant retained its ability to develop disease symptoms similar to that of its wild type Xvt122 on tomato Bony Best L305. Both growth of Xcv Xvt122 and Xvt122Δ*xopE2* increased from 10^2 cfu/cm^2 to 10^5 cfu/cm^2 at 3 dpi and up to 10^6~10^7 at 9 dpi (Figure 5B). In contrast, the *xopE2* mutation in Xvt45 caused 10% to 20% reduction in symptom formation and the affected phenotypes were persistent for three weeks (Figure 5C). As shown in Figure 5D, the bacterial population of Xvt45Δ*xopE2* dropped about ten-fold compared to the wild type Xvt45 at 3 dpi. A hundred-fold reduction in growth at 9 dpi was observed for Xvt45Δ*xopE2* compared to that of Xvt45 (Figure 5D).

The overexpressed XopE2 is sufficient to modulate the virulence of *X. campestris* pv. *vesicatoria* on tomato

To investigate whether the overexpression of XopE2 allele in two groups of Xcv could alter the virulence in their hosts, two recombinant plasmids pNCHU1921 (carrying *xopE2* of Xvt122; designated XopE2$_A$) and pNCHU1925 (carrying *xopE2* of Xvt45; designated XopE2$_B$) were generated. After introducing pNCHU1921 or pNCHU1925 into Xcv Xvt122 and Xcv Xvt45, four recombinant bacterial strains were generated and named Xcv Xvt122/XopE2$_A$, Xcv Xvt122/XopE2$_B$, Xcv Xvt45/XopE2$_A$, and Xcv Xvt45/XopE2$_B$. Moreover, XopE2 contains a consensus catalytic triad (159th cysteine and 211th aspartic acid) of the HopX family type III effectors (Nimchuk et al., 2007) and a thiol-protease His residue (47th histidine) predicted by PSORT II (http://psort.ims.u-tokyo.ac.jp). To determine the role of the triad residues and the thiol-protease His residue in virulence, site-directed mutagenesis was employed to replace the C_{159} and H_{47} on XopE2-expressing pNCHU1921 (XopE2$_A$) or pNCHU1925 (XopE2$_B$) with alanine. The resulting XopE2 site-specific mutant plasmids were designated XopE2$_A$-H_{47}A (pNCHU1922), XopE2$_A$-C_{159}A (pNCHU1923), XopE2$_A$-H_{47}A/C_{159}A (pNCHU1924), XopE2$_B$-H_{47}A (pNCHU1926), XopE2$_B$-C_{159}A (pNCHU1927), and XopE2$_B$-H_{47}A/C_{159}A (pNCHU1928). In virulence assays on tomato inoculated with Xcv and its derivatives, a substantial reduction in bacterial numbers, by 1 order of magnitude compared to the population of Xcv Xvt122, was observed for Xcv Xvt122/XopE2$_A$, Xvt122/XopE2$_A$-H_{47}A, Xvt122/XopE2$_A$-C_{159}A and Xvt122/XopE2$_A$-H_{47}A/C_{159}A on tomato Bony Best L305 at 3 and 6 dpi as shown in Figure 6A. An identical pattern of growth was also observed for Xcv Xvt122/XopE2$_B$, Xvt122/ XopE2$_B$-H_{47}A, Xvt122/ XopE2$_B$-C_{159}A and Xvt122/XopE2$_B$-H_{47}A/C_{159}A (Figure 6B). The growth reduction was seen only at 6 dpi for Xvt45/XopE2$_A$, Xvt45/XopE2$_A$-H_{47}A, Xvt45/XopE2$_A$-C_{159}A and Xvt45/XopE2$_A$-H_{47}A/C_{159}A (Figure 6C). However, the reduction in growth on the tomato Bony Best L305 leaves at 3 dpi was also observed for Xvt45/XopE2$_B$, Xvt45/XopE2$_B$-H_{47}A, Xvt45/XopE2$_B$-C_{159}A and Xvt45/XopE2$_B$-H_{47}A/C_{159}A (Figure 6D). As shown in Figure 6E, the yellowing but not wilting symptom was observed for the leaves inoculated with Xcv Xvt122/XopE2$_A$ or Xcv Xvt122/XopE2$_B$ compared to that inoculated with the wild type. Taken together, results indicate that the overexpression of xopE2 allele appeared to ultimately reduce the growth of bacteria *in planta* and disease severity, and the consensus catalytic triad and a thiol-protease His residue in XopE2 is not required for this growth effect.

The XopE2-mediated HR suppression requires a type III secretion system

To verify whether the XopE2 confers the ability to suppress the HR on nonhost tobacco plants induced by *P. syringae* pv. *syringae* 61 (Psy61), the *Agrobacterium*-mediated transient expression of XopE2 system was employed. The recombinant *A. tumefaciens* LBA4404 was transformed with the control plasmid pBI121 and the XopE2 expressing plasmid, pNCHU1201 (XopE2$_A$) or pNCHU1200 (XopE2$_B$), and the resulting bacteria were designated as At-pBI121, At-XopE2$_A$ and At-XopE2$_B$, respectively. Aliquot of the diluted Psy61 was infiltrated into tobacco leaves as a challenge inoculum 24 h after inocula-

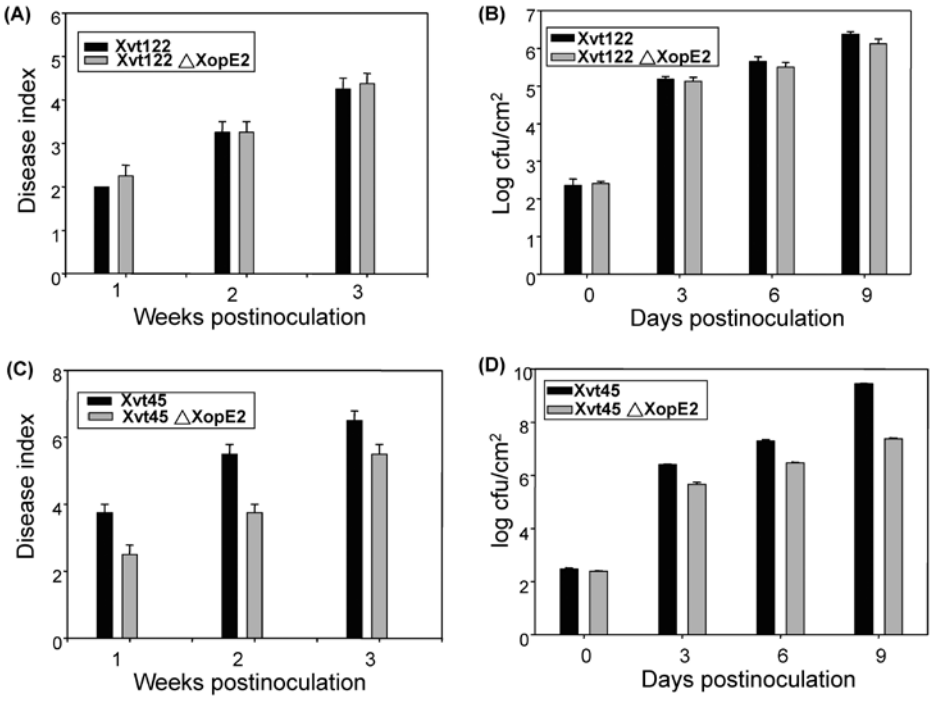

Figure 5. Symptom development and the bacterial growth on tomato leaves. (A) and (C) Leaves of tomato cultivar Bony Best L305 were dipped into 10^8 cfu/ml of bacteria culture and the disease symptom formation was scored following the rule described in the Materials and Methods. The disease index was the mean of the analysis of four independent plants. (B) and (D) The tomato leaves were infiltrated with 10^4 cfu/ml of bacteria suspension, and the growth was quantified at 0, 3, 6, 9 days after inoculation. The bacterial growth was determined as the mean of the bacterial number obtained from three independent plants. The experiments were repeated three times with similar results.

tion of *A. tumefaciens* derivatives. The HR symptom was investigated in the area of leaves where the infiltrations overlapped. As shown in Figure 7A (in *N, tabacun*) and B (in *N. benthamiana*), the Agro-mediated expression of XopE2 led to 12 to 24 h delay in HR induced by low titer of Psy61 inoculum (5×10^6 cfu/ml).

The cosmid pHIR11, carrying the *hrp/hrc* cluster of Psy61 encoding a functional T3SS and the effector HopPsyA, enables nonpathogenic bacteria, such as *P. fluorescens* 55 and *E. coli* MC4100, to elicit an HR in tobacco and several other plants (Huang et al., 1988; Alfano et al., 1997). To study if XopE2 could suppress the HR induced by the T3SS effector HopPsyA, *E. coli* MC4100 [pHIR11] was transformed with pBBR1MCS-5, pNCHU1921 (XopE2$_A$), or pNCHU1925 (XopE2$_B$). As shown in Figure 7C, an inoculation of tobacco (*N, tabacum*) leaves with 5 x10^8 cfu/ml of the *E. coli* MC4100[pHIR11/pBBR1MCS-5] elicited a typical HR at 2 dpi. The HR was not observed for the leaves inoculated with either *E. coli* MC4100[pHIR11/ XopE2$_A$] or MC4100[pHIR11/XopE2$_B$] (Figure 7C). Besides, the mutation effect of the catalytic triad residues and a thiol-protease His residue on HR suppression was also evaluated. As shown in Figure 7D, all the XopE2 mutants MC4100[pHIR11/ XopE2$_A$-H$_{47}$A], MC4100[pHIR11/ XopE2$_A$-C$_{159}$A], and MC4100[pHIR11/ XopE2$_A$-H$_{47}$A/C$_{159}$A] could inhibit the pHIR11-dependent HR. Taken together, the results indicate that XopE2 proteins derived from the two groups of Xcv have the ability to suppress the HR via the T3SS and the ability is not dependent on the consensus catalytic triad and a thiol-protease His residue.

DISCUSSION

In this study, AFLP analysis using two selective primer combinations *Taq*I-GA IR700 and *Mse*I-NN (NN means AC, AG, CA, CT, GA, GT, TC, TG) was applied to evaluate the diversity among 14 strains of *X. campestris* pv. *vesicataoria* (Xcv), which had been classified into A (=*X. axonopodis* pv. *vesicatoria*) and B (=*X. vesicatoria*) groups based on their amylolytic activities (Table 1). The polymorphic patterns shown in the 8 AFLP maps (one of them shown in Figure 1) appeared to be highly polymorphic among the strains in group A, but relatively similar in group B (unpublished, Lin). Nevertheless, one of polymorphic fragments was cloned and identified to be *xopE2* gene, encoding a T3S effector of *X. campestris* pv. *vesicatoria* 85-10 (Thieme et al., 2007). The features of XopE2 protein from both groups shown in this study revealed (*i*) the amino acid sequence variation of *xopE2* and its flanking genomic organization are dramatically distinct between both groups; (*ii*) *xopE2* mutation affects on the virulence of Xcv group B on its tomato host; (*iii*) overexpression of XopE2 in both groups reduces the bacterial multiplication and symptom severity; (*iv*) XopE2 from both groups

suppresses the HR elicited by *P. syirngae* pv. *syringae* in tobacco leaves.

The taxonomy and evolutionary relationships among the strains of the genus *Xanthomonas* causing bacterial spot diseases on tomato and pepper are considerably controversial (Jones et al., 2000). The strains classified in *X. campestris* pv. *vesicatoria* (Xcv) were reclassified into two genetically distinct groups by Stall et al. (1994) and Vauterin et al. (1990), in subsequent revision, these two groups were renamed as *X. axonopodis* pv. *vesicatoria* (Xav) or *X. vesicatoria* respectively (Jones et al., 2000). Since the strains of Xav (composed of A and C group) were found to be very heterogeneous, the strains belonging to C-group were later considered as a subspecies of Xav (Jones et

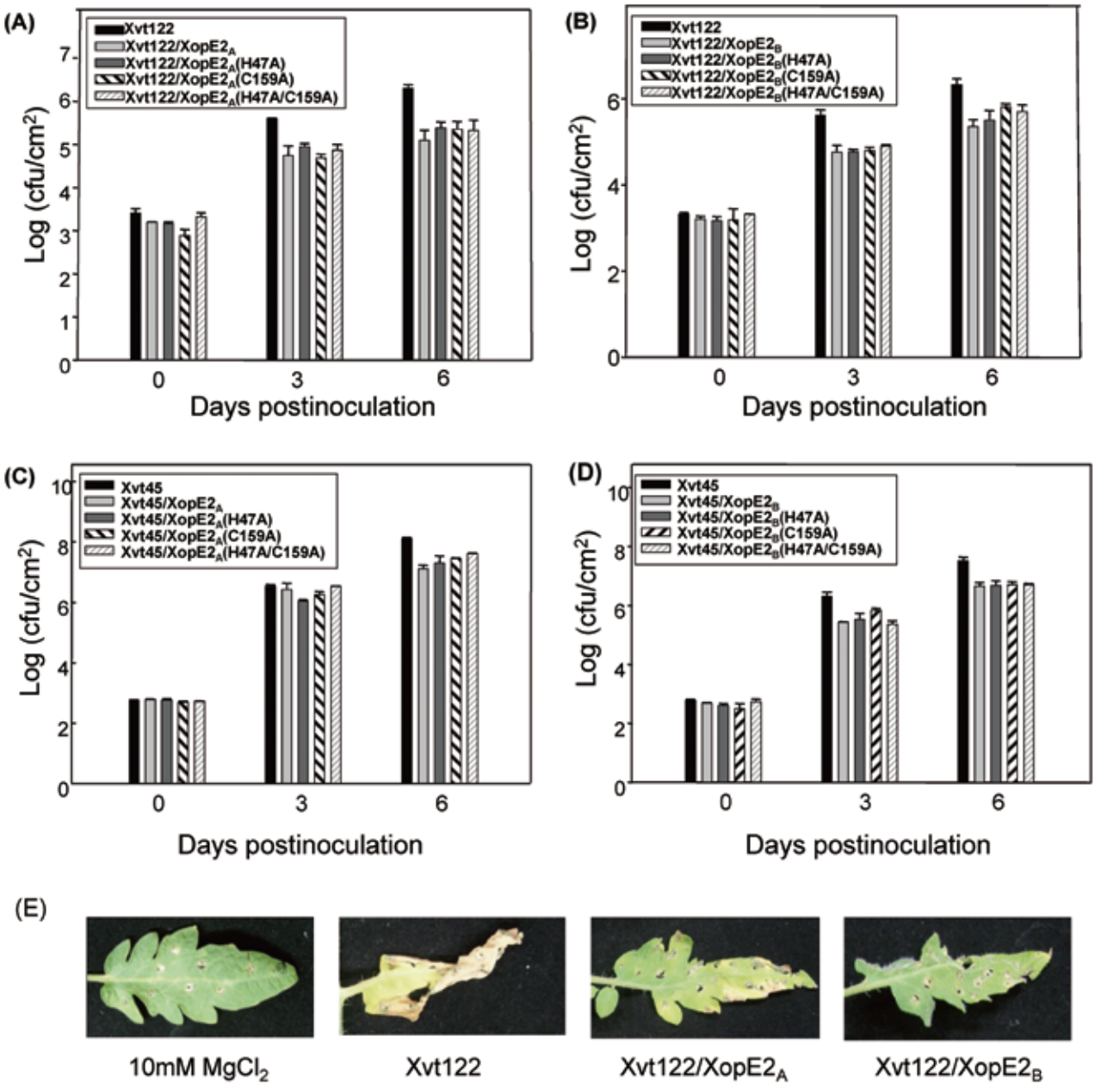

Figure 6. The overexpression of XopE2 suppressed the formation of symptom and bacterial growth on tomato leaves. The expression plasmids carrying $XopE2_A$ (pNCHU1921) or the $XopE2_A$ with site-directed mutation $XopE2_A$-$H_{47}A$ (pNCHU1922), $XopE2_A$-$C_{159}A$ (pNCHU1923), or $XopE2_A$-$H_{47}A/C_{159}A$ (pNCHU1924) were used to transform Xcv Xvt122 (group A) or Xcv Xvt45 (group B). The $XopE2_B$ expressing plasmids including pNCHU1925, pNCHU1926 ($XopE2_B$-$H_{47}A$), pNCHU1927 ($XopE2_B$-$C_{159}A$), and pNCHU1928 ($XopE2_B$-$H_{47}A/C_{159}A$) were also transformed into Xcv Xvt122 (group A) and Xcv Xvt45 (group B) individually. Bacterial growth was determined at 0, 3, 6 days after tomato leaves were inoculated with 10^5 cfu/ml Xvt122 or the derivative strains (A) and (B), or with 5×10^4 cfu/ml of Xvt45 and the derivative strains (C) and (D). The leaves were photographed 3 weeks after inoculation (E). The experiments were carried out twice and similar results were obtained.

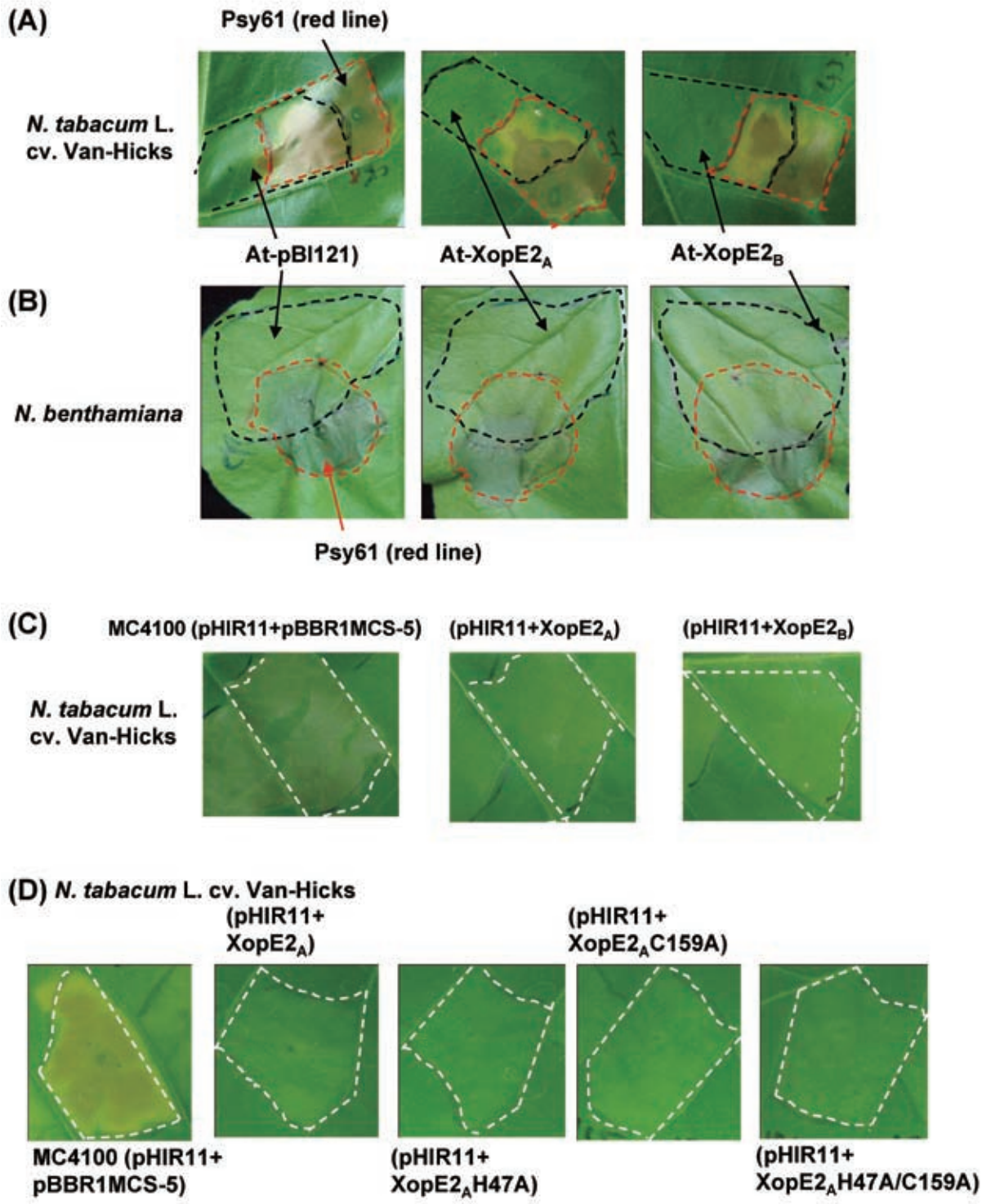

Figure 7. Suppression of the HR by the XopE2 of Xcv Xvt122 or Xcv Xvt45. The *Agrobacterium tumefaciens* LBA4404 carrying pBI121 or *xopE2* expressing plasmids was injected into the leaf of *Nicotiana tabacum* L. cv. Van-Hicks (A) or *N. benthamiana* (B) and the infiltrated areas were encircled with black dashed line. The leaves was challenged with 5×10^6 cfu/ml of *P. syringae* pv. syringae 61 (Psy61) 24 h after the infiltration. The second infiltrated sites were encircled with red dashed lines and photographs were taken 6 days after the challenge inoculation. (C) The tobacco leaves were infiltrated with 5×10^8 cfu/ml of *E. coli* MC4100 (pHIR11/ pBBR1MCS-5), MC4100 (pHIR11/ $XopE2_A$), and MC4100 (pHIR11/ $XopE2_B$) respectively and photographs were taken 48 h after the inoculation. (D) The tobacco leaves were infiltrated with 5×10^8 cfu/ml of *E. coli* MC4100 (pHIR11/pBBR1MCS-5), MC4100 (pHIR11/ $XopE2_A$); MC4100 (pHIR11/ $XopE2_A$-$H_{47}A$), MC4100 (pHIR11/ $XopE2_A$-$C_{159}A$), and MC4100 (pHIR11/$XopE2_A$-$H_{47}A$/ $C_{159}A$) individually, and photographs were taken 4 days after the inoculation. The experiments were repeated three times with similar results.

al., 2000). The strains of Xcv group A (=Xav) used in this study are also more genetically diverse based on AFLP analysis, so it is worthy to evaluate whether some of Xav strains collected in Taiwan can be reclassified into C-group.

The gene organization surrounding *xopE2* in Xvt122 (belonging to A-group) or in Xvt45 (belonging to B-group) are different, this can be very useful in discriminating both groups by using PCR technology. For example, we can design feasible primers according to the sequence of *xopE2* gene and its flanking sequences. Moreover, the sequences surrounding *xopE2* in both strains (Xvt122 and Xvt45) containing loci coding for cointegrate resolution protein T, XCV 2279 (Figure 4A) or phage-related integrase (Figure 4B), respectively, indicate that the *xopE2* gene was transferred from other bacteria via a horizontal transfer event. This event was believed to play some roles in the evolution of pathogenicity (Hacker and Kaper, 2000). For example, phytobacterial *avr* genes are acquired through horizontal gene transfer and are integrated into the bacterial genome because they confer some selective advantages in full virulence, symptom development or maximum growth rates *in planta* (Alfano and Collmer, 2004; Rohmer et al., 2004). Effect of *xopE2* mutation in B-group Xvt45 on virulence (Figure 5) echoes this significance of gene transfer event.

The HopX family including AvrPphE (recently renamed HopX), XopE1, XopE2, and homologs from subspecies of the plant pathogen genera *Pseudomonas*, *Ralstonia*, and *Xanthomonas*, is composed of two subfamilies HopX1 and HopX2 (Rohmer et al., 2004; Lindeberg et al., 2005; Nimchuk et al., 2007; Thieme et al., 2007). XopE1 and XopE2 from Xcv 85-10 (=Xcv groupA= *X. axonopodis* pv. *vesicatoria*) share 69% amino acid sequence identity and belong to the HopX2 subfamily (Thieme et al., 2007). As shown in this study, *xopE2* is present not only in the chromosomes of all strains of Xcv B-group (=*X. vesicatoria*), but also in the plasmid (Figure 2). In contrast, except for strains Xvp169, Xvp182, and Xvp197, most strains of Xcv groupA tested here only have a chromosomal copy of *xopE2*. Interestingly, *xopE1* seems to be absent in B-group strains tested in this study as inferred from the result of Southern blot assay probed with *xopE1* gene cloned from A-group Xvt122 (unpublished data, Lin). Altogether, presence or absence of *xopE1* and presence of a copy of plasmid-borne *xopE2* appear to be determined by different evolutionary paths in these two groups.

The *xopE2* mutation in A-group Xvt122 does not affect bacterial growth and symptom formation in susceptible tomato (cultivar Bony Best L305) plants. The same holds true for the Xcv 85-10 *xopE2* mutant on susceptible pepper plants (ECW) and a mutation in the *X. campestris* pv. *campestris* HopX2 homolog *avrXccE1* (Castañeda et al., 2005; Thieme et al., 2007). No contribution of the $xopE2_{A}$ and its homolog to bacterial virulence may be due to functional redundancy with repertories of T3S effectors (Alfano and Collmer, 2004, Kay and Bonas, 2009). Surprisingly, an *xopE2* mutation of B-group Xvt45 strain reduces symptom development and bacterial multiplication in susceptible tomato (cultivar Bony Best L305), although Xvt45 contains another copy of plasmid-borne *xopE2*. It implies that B-group strains of Xcv possess different T3S effectors (e.g. no *xopE1* gene) and the *xopE2* gene product encoded in the plasmid was not able to complement the function of chromosome-derived *xopE2* gene product. Moreover, overexpression of XopE2 proteins in both groups reduces the bacterial growth by one order of magnitude and symptom formation on their susceptible host (Figure 6), suggesting that XopE2 behaves like its homolog AvrPphE which sequence variations in alleles of several *P. syringae* pv. *phaseolicola* races were responsible for the different level of virulence in certain cultivars of bean plants (Stevens et al., 1998) and may act as a 'recognition rheostat' as proposed by Jones and Dangl (1996) and Mansfield et al. (1997), and functions in Xcv-tomato/pepper interactions through an unknown mechanism.

Effectors functioning in virulence by suppressing the effector-triggered immunity (ETI) in susceptible or nonhost plants had been reported in many cases (Abramovitch et al., 2003; Jamir et al., 2004; Kang et al., 2004; Fujikawa et al., 2006; Cunnac et al., 2009). The ETI is typically distinguished from PTI [PAMP (pathogen-associated molecular patterns)-triggered immunity] by elicitation of HR-associated localized programmed cell death (PCD) (Cunnac et al., 2009). Suppression of PCD is one of the mechanisms for plant-pathogenic bacteria to escape inhibitions imposed by the HR such as HopPsyA, and to ensure its survival in the host plant (Tsiamis et al., 2000; Abramovitch et al., 2003; Jamir et al., 2004; Kang et al., 2004; Fujikawa et al., 2006).To date, the biological functions of HopX family members were reported to be capable of eliciting cell death in *Nicotiana* spp. or suppressing ETI (Cunnac et al., 2009). For example, HopX1 of *P. syringae* pv. *tomato* DC3000 is capable of suppressing HR induced by HopPsyA in tobacco (Guo et al., 2009). In this study, XopE2 proteins from both groups were also shown to be capable of suppressing the HR of *Nicotiana* spp. induced by HopPsyA of *P. syringae* pv. *syringae* 61 and the reaction occurred within the plant cells after delivery of the XopE2 proteins by TTSS (Figure 7). In Xcv85-10, XopE1 is capable of eliciting cell death in *N. benthamiana*, whereas XopE2 can trigger cell death in *Solanum pseudocapsicum* (Thieme et al., 2007). Taken together, biological functions of XopE2 protein are more complex than expected since it plays at least two functions in different nonhost backgrounds.

The members of HopX family are modular proteins composed of a conserved potential cysteine-based catalytic triad of the TGase (transglutaminase) superfamily (Makarova et al., 1999; Nimchuk et al., 2007). Mutation in the residues of this putative catalytic triad of $HopX_{Pph\ race4}$ including C_{179}, H_{215}, and D_{233}, abolished avirulence activity on R2-expressing bean cultivars and also prevented initiation of cell death in Arabidopsis following transient conditional expression assays. Like $HopX_{Pph\ race4}$, both $HopX_{Pto\ DC3000}$ and $HopX_{Psy\ B728a}$ also triggered full R2-mediated

HR that was dependent on the proposed catalytic cysteine residue in each allele (Nimchuk et al., 2007). However, the mutation in the putative conserved catalytic amino acids (C_{159}) and (or) predicted Thiol protease His region (H_{47}) of XopE2 still display wild types phenotypes in terms of reduction in virulence on the susceptible tomato plants and suppression of HR (shown in Figures 6 and 7), implying that the target recognized by XopE2 inside the plants may be completely different from that for HopX or other functional domain(s) in XopE2 is (are) responsible for biological activities. XopE2 from the two Xcv groups also contains a conserved N-myristoylation motif which was previously shown to drive effectors to the host plasma membrane (Nimchuk et al., 2000; Shan et al., 2000; Robert-Seilaniantz et al., 2006; Thieme et al., 2007). This motif in Xcv85-10 XopJ is required for triggering cell-death reaction in *N. benthamiana*, in contrast, the mutant derivative XopE1(G2A) of Xcv85-10 triggers more faster and stronger cell-death reaction (Thieme et al., 2007). Whether the biological functions of XopE2 shown in this study require this motif awaits further evaluation.

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茄科細菌性斑點病菌 XopE2 有效蛋白具有毒性與抑制過敏性反應的功能

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由 *Xanthomonas campestris* pv. *vesicatoria* (Xcv) 引起的茄科細菌性斑點病，需透過第三型分泌系統的調控機制和其分泌的有效蛋白 (effectors) 引發其病原性。在此研究中，利用增幅限制片段核酸多型性技術 (AFLP) 調查台灣茄科植物細菌性斑點病菌的變異性，並選殖出一 *xopE2* 的同源基因。本實驗中所採用的 14 株菌株依據澱粉分解酶有無，可將菌株區分為 A、B 兩菌群。根據胺基酸序列所作樹狀親源分析，顯示 A 群菌株 Xvt122 其與 *X. campestris* pv. *vesicatoria* 85-10 的親源關係較之 B 群菌株 Xvt45 為近。雖然 Xcv Xvt45 存在有 2 套 xopE2 基因，一套座落在基因組上，另一套則位於質體上，剔除基因組上的 xopE2 會降低病原菌的感染力。然而，Xcv X85-10 和 Xvt122 的 *xopE2* 突變株並不會影響其病原性。此外，根據實驗結果顯示，無論是選殖自 Xcv Xvt122 或 Xcv Xvt45 的 *XopE2* 皆能透過第三型分泌系統抑制由 HopPsyA 所引發的過敏性反應，而異質表現 XopE2 則會降低其在感病番茄品系的毒性，而這些生物性功能和 XopE2 所具有的保留性三元催化胺基酸 (consensus catalytic triad)(159th cysteine) 與 His 硫醇蛋白酶胺基酸 (thiol-protease His residue)(47th histidine) 無關。

關鍵詞：增幅限制片段核酸多型性分析；茄科細菌性斑點病；第三型分泌系統；XopE2 蛋白；過敏性反應。