Eur J Plant Pathol (2013) 137:677–688
DOI 10.1007/s10658-013-0278-4

A *pthA* homolog from a variant of *Xanthomonas axonopodis* pv. *citri* enhances virulence without inducing canker symptom

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Accepted: 7 August 2013 / Published online: 22 August 2013

Abstract Three novel atypical symptom-producing types of *Xanthomonas axonopodis* pv. *citri* were described recently. The variants designated as *Xac*-A^p type produce large flat necrotic with water-soaked margin lesions instead of canker lesions on leaves of Mexican lime (*Citrus aurantifolia*) and grapefruit (*C. paradisi*). Random mutagenesis using transposon Tn5 in *Xac*-A^p type strain XL38 was used to isolate a virulence-deficient mutant T38, which induced small flat necrotic with water-soaked margin lesions on leaves. *PthAp* being inactivated by the transposon was cloned and characterized. Our result demonstrated that *pthAp* is responsible for XL38 to cause large flat necrotic with water-soaked margin lesions and bacterial multiplication but fails to confer the ability of inducing canker lesions. Sequence analysis reveled *pthAp* is a new member belong to the *pthA* homologs. The sequence is almost identical to the other homologs except for the number of direct tandem repeats in the central region of the gene. The *pthAp* contained an intact promoter and a full-length reading frame but with 18.5 direct repeats. Moreover, the amino acid at position 3rd in 1st, 2nd, 3rd repeats and at 13th position in 11th, 12th, 13th, 14th repeats were varied. Mutant T38 is fully complemented by *pthA* carried 17.5 direct repeats for the pathogenicity of *X. axonopodis* pv. *citri* to elicit canker lesions. The *pthAp* affected in planta bacterial growth and lesions in size but no ability of inducing canker symptom. These results suggested that *pthAp* is a new distinct virulence effector responsible for the enhancement of virulence.

Keywords Citrus · Citrus canker · Virulence-deficient · Mutagenesis · Pathogenicity

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Introduction

Asiatic citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. citri* subsp. *citri*) with a broad host range among members of the *Rutaceae* is one of the most serious problems in citrus production worldwide (Stall and Civerolo 1991). *X. axonopodis* pv. *citri* induces erumpent, callus-like lesions with water-soaked margin in citrus plants.

New groups of *X. axonopodis* pv. *citri* strains A* and A^w isolated from Mexican or key lime trees in southwest Asia, central Asia and Florida exhibit a narrow host range but shown to be genetically related to *X. axonopodis* pv. *citri* (Verniere et al. 1998; Sun et al. 2004; Bui Thi Ngoc et al. 2008, 2007). These strains elicited typical canker lesions on Mexican lime but induced flat water-soaked lesions or hypersensitive reaction on grapefruit (Rybak et al. 2009). Two groups of *X. axonopodis* pv. *citri* strains distinct in aggressiveness

on pummelo cultivars have been found among Japanese strains of *X. axonopodis* pv. *citri* (Shiotani et al. 2000). There are three novel atypical symptom-producing variants of *X. axonopodis* pv. *citri* were discovered in Taiwan, and designated as *Xac*- A^f, -A^r and -A^p type strains (Lin et al. 2005, 2008). These three type strains induced atypical symptom on citrus leaves. *Xac*-A^f type strains induce flat necrotic lesion with water-soaked margin and light chlorotic halo on leaves of grapefruit and Liucheng (*C. sinensis*), while typical canker lesions on Mexican lime. *Xac*-A^r type strains induce restricted and raised corky lesions with no water-soaked margin and light chlorotic halo on these leaves of *Citrus* species. *Xac*-A^p type strains induce large flat necrotic lesion with water-soaked margin and light chlorotic halo on these leaves of *Citrus* species. The mechanisms for the *Xac*-A^w, -A^f, and -A^r type strains inducing symptomatic variation in *Citrus* species, and the *X. axonopodis* pv. *citri* strains from Japanese distinct in aggressiveness on pummelo were well studied (Rybak et al. 2009; Lin et al. 2011, 2010; Shiotani et al. 2007). However, the molecular basis for avirulence of the *Xac*- A* type strains on grapefruit or *Xac*-A^p type strains inducing large flat necrotic lesion with water-soaked margin lesions on citrus leaves is still unclear.

The *avrBs3/pthA* (avirulence and pathogenicity) gene family, widely distributes in phytopathogenic *Xanthomonas* species, was involved in disease symptom expression and host defence response (Fujikawa et al. 2006; Swarup et al. 1991, 1992; Leach and White 1996). Many members of this gene family are essential for pathogenicity of *Xanthomonas* species. For example, pathogenicity gene *pthA* is necessary for *X. axonopodis* pv. *citri* to cause citrus canker disease (Swarup et al. 1991). This *pthA* gene shares unique structural features, composed of a central region of multiple, nearly identical repeats of 34 amino acids, a leucine zipper, three nuclear localization signals (NLSs), and an acid transcriptional activation domain (AAD) in the C terminus (Ponciano et al. 2003). The direct tandem 34-aa repeats confer host selectivity and are critical to the determination of pathogenicity (Swarup et al. 1992; AI-Saadi et al. 2007). Multiple *pthA* homologs are always present in all strains of *X. axonopodis* pv. *citri* also including A^*, A^w and A^f (Kanamori and Tsuyumu 1998; AI-Saadi et al. 2007; Lin et al. 2011). Only one *pthA* homolog carrying 17.5 nearly identical direct tandem repeats has the hallmark virulence function of canker formation, while functions of the other homologs were negligible or not measurable (AI-Saadi et al. 2007).

In this study, we utilized transposon mutagenesis for a strain of *Xac*-A^p type that induces large flat necrotic with water-soaked margin lesions in leaves of grapefruit and Mexican lime in order to characterize the gene involved in the symptomatic variation. The result of this experiment identified a new member of the *Xanthomonas avrBs3/pthA* gene family from *Xac*-A^p type strain XL38 that can enhance virulence without inducing canker symptom.

Materials and methods

Bacterial strains, plasmids, and culture media

The information of bacterial strains and plasmids for this study was listed as Table 1. *Escherichia coli* DH5α and its derivatives were grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) or LB agar plate at 37 °C. The strains of *X. axonopodis* pv. *citri* were cultured on Yeast Extract Bactopeptone Dextrose (YPD) medium (Verniere et al. 1991) at 30 °C. Antibiotic selection was carried out using 50 μg ml^{-1} of kanamycin, 100 μg ml^{-1} of ampicillin, 20–50 μg ml^{-1} of gentamicin.

Transposon mutagenesis and pathogenicity screening

Plasmid pSUP2021 containing Tn5 was introduced into strain XL38 of *Xac*-A^p type (Table 1) by electroporation as described previously (Shiotani et al. 2007). Kanamycin-resistant mutants were picked from YPD agar plate with 26-gauge needle. Screening for pathogenicity and virulence was performed by inoculating mature attached leaves of grapefruit (*C. paradisi*) and Mexican lime (*C. aurantifolia*) with needle pricks. The inoculated plants were grown in a growth chamber with 65–90 % humidity and 12-h light at 30 °C and 12-h dark at 25 °C. The pathogenicity and virulence of each mutant were determined visually or with a dissecting microscope 30 days after inoculation. Putative clones that altered the symptom on citrus leaves were doubly confirmed by performing for the second test on the leaves of grapefruit and Mexican lime, and each mutant was evaluated by measuring the diameter of the lesions. To prepare inoculums, *X. axonopodis* strains were grown overnight in YPD broth, harvested by centrifugation 6,000×g for 5 min at

Table 1 List of bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	*Sup*E44 Δ*lacU*169 (Φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96thi-*1rel*A1	(Brown 1991)
X. axonopodis pv. *citri*		
XW19	Wild-type *Xac*-A strain inducing typical canker lesion	(Lin et al. 2005)
XL38	Wild-type *Xac*-A^{p} strain inducing large flat necrotic with water-soaked margin lesion	(Lin et al. 2008)
T38	Tn5 inserted mutant of XL38 inducing small flat necrotic with water-soaked margin lesion	This study
T38MCS	T38 containing pBBR1MCS-5; Gm^{r}	This study
T3836	T38 containing pMCS3836; Gm^{r}	This study
T3835	T38 containing pMCS1935; Gm^{r}	This study
T3835f	T38 containing pMCS4735; Gm^{r}	This study
T38SP	T38 containing pMCS38SP (PthAp S286P); Gm^{r}	This study
T3847SP	T38 containing pMCS47SP(PthA S286P);Gm^{r}	This study
X. axonopodis pv. *citrumelo*		
F2	Wild-type pathotype E inducing small flat necrotic with water-soaked margin lesion	(Wu et al. 1993)
F2MCS	F2 containing pBBR1MCS-5; Gm^{r}	This study
F3836	F2 containing pMCS3836; Gm^{r}	This study
Plasmids		
pSUP2021	Suicide vector containing Tn5; Ap^{r} Cm^{r} Km^{r}	(Simon et al. 1983)
pCR-XL-TOPO	PCR cloning vector; Ap^{r}, Km^{r}	Invitrogen
pBBR1MCS-5	Broad host range cloning vector; Gm^{r}	(Kovach et al. 1995)
pTOPO3836	3.6-kb *pthA* homolog fragment from XL38 cloned into pCR-XL-TOPO; Ap^{r}, Km^{r}	This study
pTOPO3829	2.9-kb *pthA* homolog fragment from XL38 cloned into pCR-XL-TOPO; Ap^{r}, Km^{r}	This study
pMCS3836	3.6-kb *pthA* homolog fragment from pTOPO3836 subcloned into pBBR1MCS-5; Gm^{r}	This study
pMCS3829	2.9-kb *pthA* homolog fragment from pTOPO3829 subcloned into pBBR1MCS-5; Gm^{r}	This study
pMCS1935	3.5-kb *pthA* fragment from XW19 subcloned into pBBR1MCS-5; Gm^{r}	(Lin et al. 2011)
pMCS4735	3.5-kb *pthA* fragment from XW47 subcloned into pBBR1MCS-5; Gm^{r}	(Lin et al. 2011)
pMCS38SP	pMCS3836 derivative, changing the amino acid Ser_{286} to Pro_{286} of 3.6-kb *pthA* homolog fragment from XL38	This study
pMCS47SP	pMCS4735 derivative, changing the amino acid Ser_{286} to Pro_{286} of 3.5-kb *pthA* fragment from XW47	(Lin et al. 2011)

4 °C (SCR20BA, Hitachi, Japan) and re-suspended in sterile distilled water to a concentration of approximately 10^{8} colony-forming units (CFU)/ml. For needle-prick inoculation, six wounds within a 1 cm^{2} area were made on citrus leaves with a standard 26-gauge needle. An aliquot (20 μl) of the bacterial suspension was dropped onto each wound, and the drops were wiped off with sterile cotton 2 min after inoculation.

Analysis of Tn5 insertion site by inverse-PCR

To identify the insertion site in mutant T38 (Table 1), 2 μg of genomic DNA of T38 was digested with restriction enzyme *Sal*I overnight at 37 °C. The digested DNA was separated using agarose gel and purified by Viogene Gel-M^{TM} Gel Extraction system (Viogene Corporation, Taiwan) and allowed to self-ligate in the presence of T4 DNA ligase in a 10 μl mixture for 4 h at 16 °C. The ligation mixture was electroporated into *E.coli* DH5α cells. The ligated DNA sequence was used as template for an inverse-PCR reaction using three outward-facing primers, Tn5IS (5′ GCAGTACGGCGAGGATCACC 3′), Tn5-L (5′ CTCATGCTGGAGTTCTTC 3′) and Tn5-R (5′ GCCGAAGTCGAGCACGTT 3′), which were designed based on the transposon sequence.

PCR analysis was performed in a 50 μl mixtures containing 10 ng template DNA, 1× *Taq* buffer, each primer at a concentration of 5 μM, each deoxynucleoside triphosphate at a concentration of 2.5 mM, 1U of Taq Plus DNA polymerase (BioBasic Inc., Canada) with proof-reading function, $MgCl_2$ 25 mM and DMSO 3 μl. The amplification condition consisted of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 5 min for 35 cycles with an initial step of 94 °C for 10 min and a final step of 72 °C for 10 min. The PCR products were separated by electrophoresis in 1.5 % agarose gel, stained with ethidium bromide and visualized under a UV light. The PCR products were purified by Viogene Gel-M™ Gel Extraction system and cloned into a pCR-XL-TOPO cloning vector (Invitrogen Corporation, Netherlands). Clones were selected on LB medium supplemented with kanamycin (50 μg ml^{-1}) after transformation into *E.coli* DH5α cells. DNAs of the recombinant clones containing specific DNA fragements were sequenced by an automatic DNA sequencing system (ABI-377-19; Perkin-Elmer Applied Biosystems, Foster City, CA), and further mapped to the *X. axonopodis* pv. *citri* genome.

PCR amplification, cloning and sequence analysis of *pthA* homologs

Genomic DNA from *X. axonopodis* pv. *citri* strain XL38 was isolated by standard methods (Sambrook et al. 1989). Two *pthA* homologs of XL38 were amplified by PCR using primer pair pthAP7/pthAR2 or pthA*XhoI*/pthA*XbaI* described previously (Lin et al. 2005, 2011). PCR analysis with minor modification from previous report (Lin et al. 2011) was performed in a 50 μl mixtures containing 150 ng template DNA, 1× *Taq* buffer, each primer at a concentration of 1 μM, each deoxynucleoside triphosphate at a concentration of 300 μM, 1U of Taq Plus DNA polymerase (Bio Basic Inc., Canada) with proof-reading function, and DMSO 5 μl. The amplification condition consisted of 94 °C for 1 min, 63 °C or 58 °C for 1 min, and 72 °C for 5 min for 35 cycles with an initial step of 94 °C for 10 min and a final step of 72 °C for 10 min. The PCR products were separated by electrophoresis in 1 % agarose gel, stained with ethidium bromide and visualized under a UV light. Purfication of the PCR products and DNAs sequencing were performed as described previously.

DNA sequence data for *Xanthomonas* strains causing citrus bacterial canker were analyzed by Blast program at the National Center for Biotechnology Information (NCBI) network service (http://www.ncbi.nlm.nih.gov). The encoded amino acid sequences were analyzed with the Translate program of the SeqWeb sequence analysis system of the GCG software (Accelrys Inc., San Diego, CA). Alignments of deduced sequences of PthA proteins were performed with the Clustalw version 3.2 (Biology Workbench of San Diego Supercomputing Center). The varied amino acid residues were analyzed with Vector NTI™ version 8.0 (Invitrogen, Madison, WI).

Transformation into a mutant strain

Transformation into virulence-deficient mutant T38 was performed by electroporation as previously described (Keen 1990). The *pthA* homologs from XL38 were further subcloned individually into the broad host range vector pBBR1MCS-5 (Kovach et al. 1995) before being transformed into mutant T38. Selections of transformants were accomplished on YPD plates supplemented with antibiotics.

The plasmids from other clones listed in Table 1 were also transformed into mutant T38 and evaluated for pathogenicity individually.

Southern blotting analysis

Genomic DNA from XL38 and mutant T38 were digested with *BamH*I or *EcoR*I restriction enzymes, and electrophoresed on an agarose gel (1 %). The gel was then transferred onto a nylon membrane (Zeta-Probe® Blotting Membranes, Bio-Rad Laboratories, CA) followed by hybridizing with a biotin-labeled *pthA* homolog that was amplified from a total DNA of the XW19 strain by PCR using primer pair pthAP7/pthAR2 (Lin et al. 2005).

Construction of amino acid substitution in the *pthA* homolog

The amino acid substitution was performed in clone pMCS3836 containing a 3.6 kb *pthA* homolog fragment from strain XL38 by site-directed mutagenesis introduced by a QuikChange XL site-directed mutagenesis kit (Stratagene). The primers, SP-1 and ASP-1 (Lin et al. 2011) were used for inducing single point

mutation to create a mutated residue at a critical position of clone pMCS3836 (Ser_{286}→Pro_{286}). The identity of the insert in resulting plasmid described above was confirmed by DNA sequencing.

Bacterial population in grapefruit leaves

Based on previous study (Lin et al. 2011), for preparation of bacterial suspensions, bacterial cells grown overnight in YPD broth with or without antibiotics were harvested by centrifugation. Cultures were re-suspended in sterile distilled water to a concentration of approximately 10^5 CFU/ml. Young fully expanded citrus leaves with similar size and thickness were injection-infiltrated with the bacterial suspension into leaf tissues by pressing the opening of a syringe (without a needle) against the leaf surface. The conditions for inoculated plants were the same as previous described.

The leaves were inoculated with the injection-infiltration method. Leaf disks (9 mm in diameter) were sampled with a cork-borer randomly by punching within the inoculated area at various time intervals after inoculation. Twelve leaf disks were sampled for each time interval and three replicates for each strain. Leaf disks were soaked in 1 % sodium hypochlorite for 1 min, and then rinsed in sterile distilled water before being ground in phosphate buffered saline (PBS) (Verniere et al. 1998). The appropriate dilutions of the ground suspension were plated with a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, England) on YPDAC plates (Verniere et al. 1998) or YPDAC plates containing gentamicin (20 μg ml^{-1}) and the inoculated plates were incubated at 30 °C. The number of colonies was counted 3 days after incubation. The results of bacterial populations were presented as log CFU/cm^2.

The stability of plasmids of the derivative strains in leaves was determined by plating the previously mentioned leaf extracts on YPDAC plates with or without adding of gentamicin. Colonies developed from each time interval were screened for antibiotic resistance markers on the plasmid. The loss of plasmid over time was presented as a percentage of *Xanthomonas* cells displaying the plasmid encoding antibiotic resistance.

Nucleotide sequence accession numbers

The nucleotide and amino acid sequence data for the *pthAp*, *pthA*, *pthA4*, *apl1*, *pthA-KC21*, *pthA**, *pthA^w*, *pthAf*, *pthA-XW19*, *pthB*, *pthC*, *pthA1*, *pthA2*, *pthA3*, *pthA*2*, *apl2*, *apl3*, and *hssB3.0* regions are available at the GenBank database under accession no. JX310119.1, U28802.1, NC_003922.1, AB021363.1, AB206338.1, EF473086.1, EF473085.1, GU181332.1, GU181333.1, AY228335.1, EF473088.1, NC_003921.1, NC_003921.1, NC_003922.1, EF473087.1, AB021364.1, AB021365.1, and AB175482.1, respectively.

Results

Mutant T38 alters disease symptom on leaves of Mexican lime and grapefruit

To determine whether transposon insertion affected the ability of *X. axonopodis* pv. *citri* strain XL38 to cause disease, 680 kanamycin-resistant transformants were individually inoculated in leaves of Mexican lime and grapefruit. The confirmed mutants with altered pathogenicity or virulence in the first round were inoculated and re-inoculated, resulting in a final eight mutants showing symptomatic variations. The single mutant *X. axonopodis* pv. *citri* T38 was obtained from mutants derived from strain XL38, which had shown smaller flat necrotic with water-soaked margin lesions on citrus leaves (Fig. 1c and d). Hybridization of *EcoR*I-digested DNA from T38 with the Tn5 probe revealed a single insertion site (data not shown). A clone containing Tn5 inserted region was isolated from genome of T38, and was sequenced from the end of Tn5. Approximately 460 bp of T38 sequence at the 5′ end of the transposon demonstrated high homology with the nuclear localization signals (NLSs) region and leucine zipper of *pthA* (Fig. 2).

Southern blot analysis of XL38 showed that two *BamH*I fragments (3.5 kb and 2.8 kb) hybridized with the probe corresponding to the *pthA* from XW19, which indicated that XL38 harbours two *pthA* homologs (Fig. 3a). The 3.5 kb *BamH*I fragment was absent in T38; however, 6.2 kb fragment containing the Tn5 fragment was detected (Fig. 3a). This result suggested that this 3.5 kb fragment is involved in the large flat necrotic with water-soaked margin lesion inducing on leaves of Mexican lime and grapefruit.

Cloning and DNA sequencing of the *pthA* homolog

PCR-amplified a 3.6 kb fragment containing a *pthA* homolog (designated as *pthAp* hereafter) and a 2.9 kb

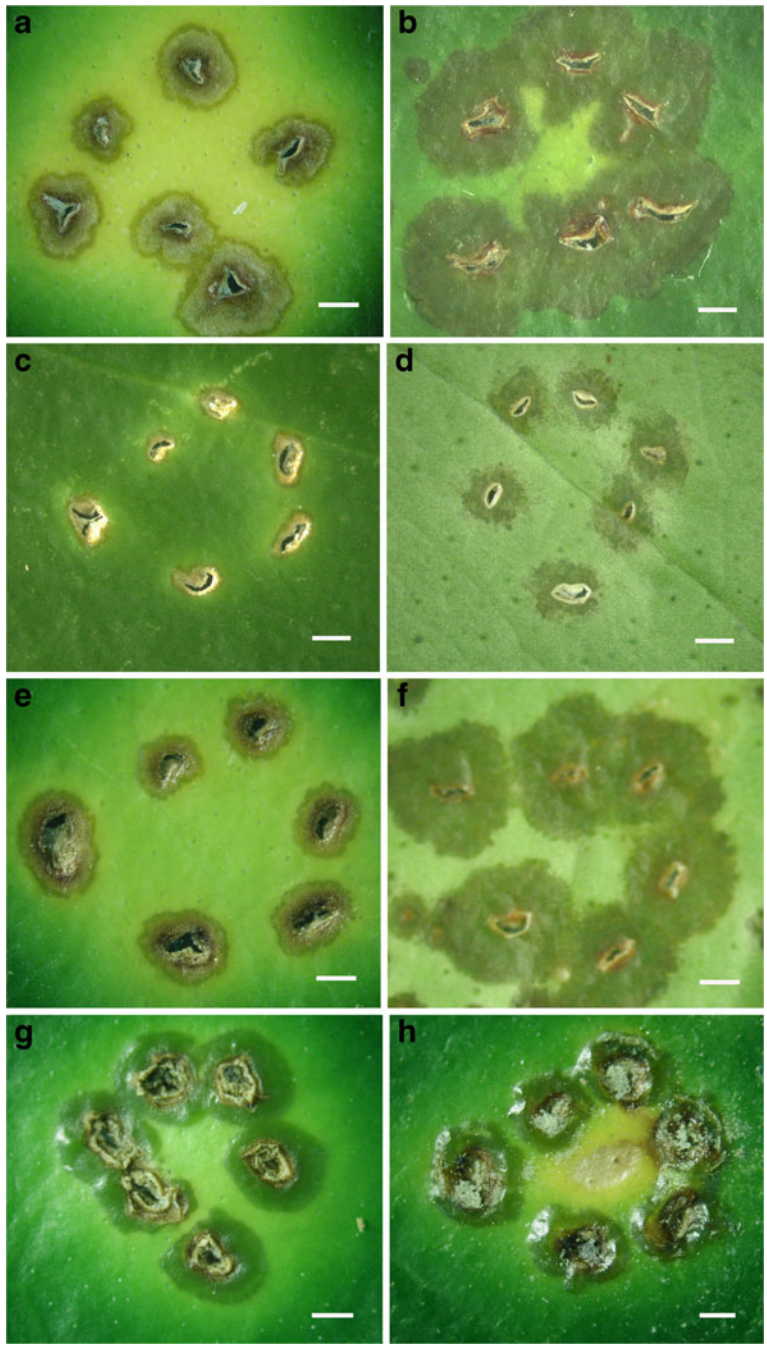

Fig. 1 Symptoms on leaves of grapefruit (**a**, **c**, **e**, **g**) and Mexican lime (**b**, **d**, **f**, **h**) induced by strains of *Xanthomonas axonopodis* pv. *citri* 30 days after inoculation. a and b: Large flat necrotic with water-soaked margin lesions induced by type *Xac*-A^P strain XL38; c and d: Small flat necrotic with water-soaked margin lesions induced by *pthAp*-deficient mutant strain T38; e and f: Large flat necrotic with water-soaked margin lesions induced by derivative strainT3836; g and h: Typical erumpent canker lesions with water-soaked margin induced by derivative strain T3835. (Bars=1 mm)

fragment were cloned from XL38 strain (Fig. 3b). The nucleotide sequences of the *pthAp* gene showed over 93 % homology to *pthA* genes from *X. axonopodis* pv. *citri* XW19 (GenBank accession no. GU 181333.1), *X. axonopodis* pv. *citri* XW47 (GenBank accession no. 181332.1), *X. axonopodis* pv. *citri* 3213 *pthA* gene (GenBank accession no.U28802.1), *X. axonopodis* pv. *citri* NA-1 *apl1* (GenBank accession no.AB021363.1), *X. axonopodis* pv. *citri* 306 *pthA4* (GenBank accession no.NC003922.1) and *X. axonopodis* pv. *citri* K21 *pthA* (GenBank accession no.AB206338.1). The nucleotide sequence analysis revealed that *pthAp* has an intact promoter, a SD (Shine-Dalgano) region, and a full-length open reading frame (ORF) (GenBank accession

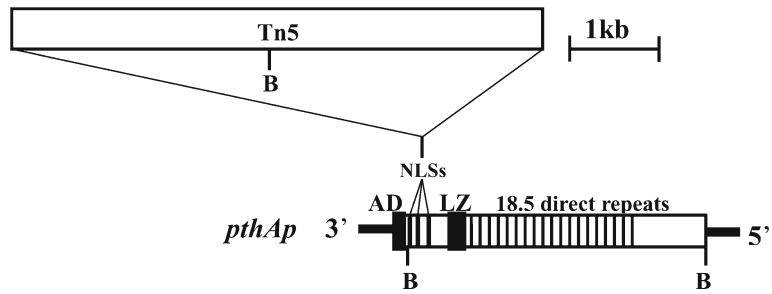

Fig. 2 Schematic presentation of Tn5 insertion into *pthAp* from mutant T38. Solid boxes represent the leucine zipper-like region (LZ), nuclear location signals (NLSs), and acidic transcriptional activation domain (AD). Restriction enzyme cleavage sites are show with a B for *BamH*I

no.JX310119.1). Sequence of this ORF contained 18.5 of 102 bp tandem repeats with each encoding 34 amino acids in the central region, a leucine zipper, three nuclear localization signals and an acid transcriptional activation domain (AAD) in the C-terminus (Fig. 2). Multiple sequence alignment revealed the identity of amino acid sequences among PthA proteins from these *X. axonopodis* pv. *citri* strains was over 93 %. Upon this region in XL38, an amino acid residue, serine, located at position 286 immediately at N terminal domain of leucine-rich repeat of PthA was the same as that of PthAf protein from XW47, but different from others PthA proteins previously published on GenBank. The result of aligned direct repeat region of this PthAp protein against other PthA proteins indicated that among 18.5 repeats in repeat region of PthAp protein, the third position of amino acid is serine in the first, second, and third repeats which is always proline in other previous published PthA proteins. Moreover, the 13th position of amino acid is alanine for the 11th, 12th, and 13th, and theronine for 14th repeats, while other amino acids were present as glycine, serine, aspartic acid, isoleucine for the rest of previous published ones (Table 2).

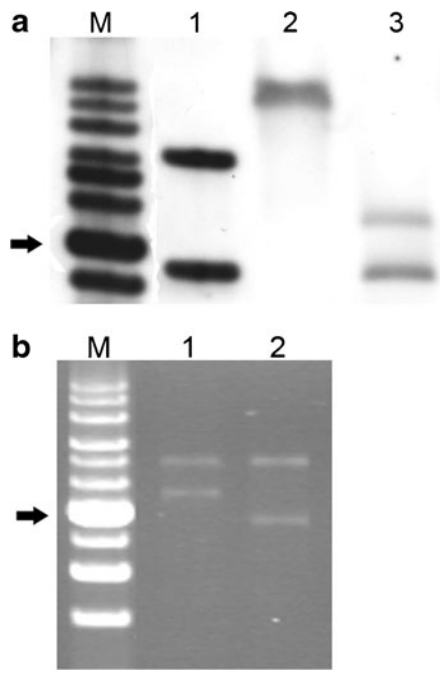

Fig. 3 **a** Southern hybridization showing Tn5 mutagenesis of *pthA* in *Xanthomonas axonopodis* pv. *citri*. The blot was probed with a biotin-labelled 3.5 kb DNA fragement containing an entire length of *pthA* from *X. axonopodis* pv. *citri* strain XW19. Lane 1: *BamH*I-digested total DNA from *X. axonopodis* pv. *citri* strain T38; Lane 2: *Eco*RI-digested total DNA from *X. axonopodis* pv. *citri* strain XL38; Lane 3: *BamH*I-digested total DNA from *X. axonopodis* pv. *citri* strain XL38. M represents the molecular weight marker. **b** Analysis of recombinant pMCS3836 and pMCS3829 by electrophoresis in agarose gel after digesting with restriction enzymes *Xho*I and *Xba*I. Lane1: pMCS3836, the 4.8 kb pBBR1MCS-5 vector with a 3.6 kb *pthA* homolog insertion fragment; Lane2: pMCS3829, the 4.8 kb pBBR1MCS-5 vector with a 2.9 kb insertion fragment. M represents the molecular weight marker. The 3 kb DNA fragment is marked with an arrow in the margin

Complementation of mutant T38

Clone pMCS 3836 containing *pthAp* was obtained from XL38 and being used to complement isolate T38 in the inoculation of leaves of grapefruit and Mexican lime. The large flat necrotic symptoms with water-soaked margin lesions were restored by transformant T3836 after 30 days of inoculation (Fig. 1e and f). Moreover, clone pMCS 3836 was transformed into competent cells of weakly aggressive *X. axonopodis* pv. *citrumelo* strain F2. The transformant F3836 induced large flat necrotic lesions instead of small flat necrotic lesions on citrus leaves (Table 3). This result indicated that *pthAp* gene confers an activity of enhancing virulence with *X. axonopodis* pathovars to induce large flat necrotic with water-soaked margin lesions.

Clones pMCS1935, pMCS4735, pMCS38SP and pMCS47SP (Table 1) were also employed to complement T38 in the inoculation experiments of leaves. The

Table 2 Aligment of the amino acid sequences encoded in the variable positions of the repeat region of 18 PthA homologs

Repeat		1	2	3	11	12	13	14
		Amino acid position 3			Amino acid position 13			
Gene								
PthAp	18.5[a]	S	S	S	A	A	A	T
PthA	17.5	P	P	P	G	G	G	S
PthA4	17.5	P	P	P	G	G	G	S
Apl1	17.5	P	P	P	G	G	G	S
PthA-KC21	17.5	P	P	P	G	G	G	S
PthA*	17.5	P	P	P	G	G	S	D
PthAw	17.5	P	P	P	G	G	G	S
PthAf	17.5	P	P	P	G	G	G	S
PthA-XW19	17.5	P	P	P	G	G	G	S
PthB	17.5	P	P	P	I	D	D	D
PthC	17.5	P	P	P	D	G	D	D
PthA1	16.5	P	P	P	I	D	_	I
PthA2	15.5	P	P	P	G	G	G	I
PthA3	15.5	P	P	P	I	I	I	I
PthA*2	15.5	P	P	P	D	I	D	D
Apl2	15.5	P	P	P	G	G	G	I
Apl3	23.5	P	P	P	G	I	D	D
HssB3.0	14.5	P	P	P	D	D	I	D

[a] Number of repeats

Table 3 Symptoms and lesion expansion observed on leaves of grapefruit and Mexican lime induced by *Xanthomonas axonopodis* wild-type strains and transformants

Strain	Genotype	Symptoms and Lesion diameter (mm) [a]	
		Grapefruit	Mexican lime
XW19	Wild-type	Canker (2.57±0.09a)	Canker (2.60±0.13b)
XL38	Wild-type	LFW (2.34±0.28a)	LFW (3.02±0.17a)
F2	Wild-type	SFW (1.22±0.10b)	SFW (1.54±0.12c)
T38	Tn5 inserted mutant of XL38	SFW (1.02±0.06b)	SFW (1.12±0.07c)
T38MCS	T38 carrying pBBR1MCS-5	SFW (1.06±0.07b)	SFW (1.13±0.05c)
T3836	T38 carrying pMCS3836	LFW (2.24±0.32a)	LFW (2.72±0.20b)
T3835	T38 carrying pMCS1935	Canker (2.58±0.18a)	Canker (2.63±0.17b)
F2MCS	F2 carrying pBBR1MCS-5	SFW (1.20±0.05b)	SFW (1.47±0.11c)
F3836	F2 carrying pMCS3836; Gm^r	LFW (2.31±0.23a)	LFW (2.62±0.29b)
T3835f	T38 carrying pMCS4735	LFW	Canker
T38SP	T38 carrying pMCS38SP (PthAp S286P)	LFW	LFW
T3847SP	T38 carrying pMCS47SP (PthAf S286P)	Canker	Canker

[a] Each value represents the mean diameter (mm) and standard error of 18 lesions for each strain 30 days after inoculation by pricking the attached citrus leaves. Data analyzed by unlike letters differ significant at α=0.01 according to the Tukey-Kramer honestly significant difference test

LFW large flat necrotic with water-soaked margin lesion; SFW small flat necrotic with water-soaked margin lesion

inoculation results revealed the canker lesions induced by the transformants T3835, T3835f, T3847SP on Mexican lime leaves except for T38SP while induced canker lesions on grapefruit leaves by T3835 and T3847SP except for T3835f and T38SP (Table 2).

Lesion expansion and bacterial growth

Based on the symptoms induced on leaves of grapefruit and Mexican lime, there was significant difference observed in lesion expansion on citrus leaves among the tested strains XW19, XL38, T38, T38MCS, T3836, T3835, F2, F2MCS and F3836 (Table 2). The lesions produced by T3836 after 30 days of inoculation were significantly larger than those caused by T38 or T38MCS. Moreover, the lesions produced by F3836 were significant larger than those caused by F2 or F2MCS.

Seven tested strains were inoculated and grew in grapefruit leaves. The bacterial populations of XL38 strain increased gradually from 10^3 CFU/cm^2 to 10^7-10^8 CFU/cm^2 20 days after inoculation. Similar increases were observed for strain T3836 for the first 4 days after inoculation. Thereafter, the rates of growth were different. It was approximately 10–100 folds lower than that of XL38 20 days after inoculation. The bacterial populations of strains T38 and T38MCS increased gradually from 10^3 CFU/cm^2 to 10^4-10^5 CFU/cm^2 for the first 8 days after inoculation. Thereafter, the rates of growth declined. The bacterial populations of strains T38 and T38MCS were approximately 10^2 folds lower than those of T3836 20 days after inoculation (Fig. 4a). The bacterial population of strain F3836 increased gradually from 10^3 CFU/cm^2 to 10^6 CFU/cm^2 16 days after inoculation. Thereafter, the rate of growth declined. The bacterial populations of strains F2 and F2MCS increased gradually from 10^3 CFU/cm^2 to 10^4 -10^5 CFU/cm^2 for the first 16 days after inoculation and declined after that. The bacterial populations of strain F2 and F2MCS were approximately 10-folds lower than those of F3836 20 days after inoculation (Fig. 4b). These results indicated that *pthAp* gene confers the ability in enhancing bacterial multiplication.

Discussion

The *pthA* gene is the pathogenicity determinant for *X. axonopodis* pv. *citri* in the symptom development of citrus canker including hypertrophy, hyperplasia and cell death of host cells (Duan et al. 1999). AI-Saadi et al. (2007) reported that the pathogenicity of *X. axonopodis* pv. *citri* on citrus is determined by the specific sequence of the 17.5 direct repeats in the central region of the protein. In this project, the isolation and characterization of a new member of the *Xanthomonas avrBs3*/*pthA* gene family, *pthAp*, from a *Xac*-A^p type strain confers the abilities of enhancing lesion expansion and bacterial multiplication were performed. Moreover, we confirmed that the *pthAp* exhibited different ability for inducing flat necrotic symptom. These results suggested that the *pthAp* is an important virulence factor to perform a variety of tasks in enhancing bacterial virulence and proliferation.

In this study, the *pthAp* gene was cloned and a *pthAp* mutant of strain XL 38 was constructed. This mutant T38 was able to induce small flat necrotic lesion on citrus leaves. Furthermore, *pthAp* from XL38 was subcloned into vector pBBR1MCS-5 and then mobilized into weakly aggressive strain *X. axonopodis* pv. *citrumelo* F2 that induced small flat necrotic lesion on citrus leaves. The new transformant F3836 harbouring *pthAp* gene was able to elicit large flat necrotic lesion on citrus leaves, and bacterial population of F3836 was 10-folds higher than those of F2 20 days after inoculation. These results indicated that *pthAp* fail to induce the normal canker, however, the ability of bacterial virulence was enhanced.

The amino acid sequence analysis showed that the 3.5-kb *BamH*I fragment of *pthAp* containing 18.5 direct repeats that has not been reported in other pathogenic *X. axonopodis* pv. *citri* strains. The structure of *pthAp* belongs to typical *Xanthomonas avrBs3*/*pthA* gene family members. It has a central domain containing a series of 102-bp direct repeats (Gabriel 1999), three nuclear localization signals (Yang and Gabriel 1995b), and an eukaryotic transcriptional activation domain (Zhu et al. 1998). After aligned repeat regions of all PthA proteins, the results revealed that the individual repeat units are highly conserved apart from position 4, position 12 and 13 (hypervariable residues), and positions 31 and 32. This result is similar with previous study (AI-Saadi et al. 2007). However, PthAp protein is special at the third position of amino acid residue in the first, second, and third repeats. Moreover, the 13th position of amino acid residue in the 11th, 12th, 13th and 14th repeats of direct repeats are different from those of other AvrBs3/PthA family members. Based on the fact that *Xanthomonas*

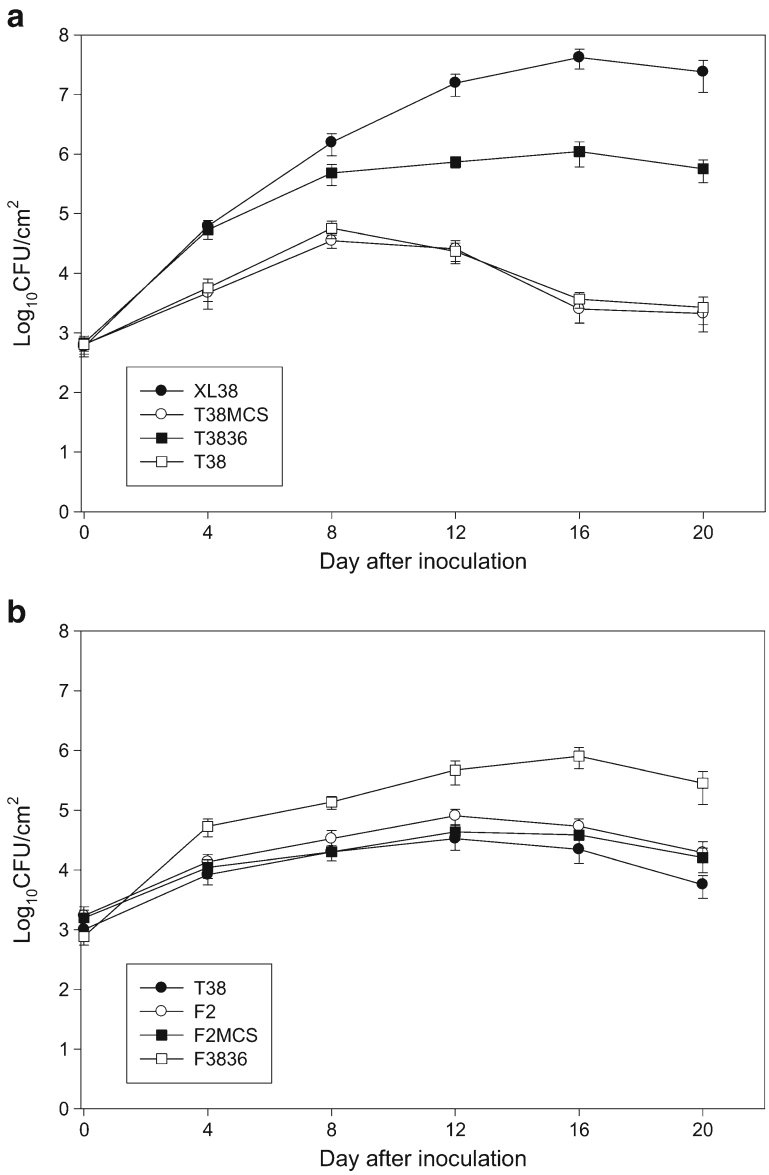

Fig. 4 Bacterial populations in grapefruit leaves. **a** The bacterial populations of strains XL38, T38MCS, T3836 and T38; **b** The bacterial populations of strains T38, F2, F2MCS and F3836. Each bacterial strain was inoculated into leaves by injection-infiltration at a concentration of 1×10^5 CFU/ml. Each value represents the mean of three replicates with standard error

translocates *avrBs3*/*pthA* gene family members into plant cells as transcriptional activators. They can be localized to the plant cell nucleus and modulate expression of plant gene (Kay and Bonas 2009). Therefore, the alteration of the amino acid residues in these specific sites in 17.5 direct repeats would affect the pathogenicity of PthAp protein. The specificity of *avrBs3*/*pthA* gene family members is encoded in the direct repeat region and each repeat corresponds to on DNA base pair. Two hypervariable amino acids [(known as repeat variable diresidues (RVDs)] at repeat position 12 and 13 determine the DNA base-pair recognition specificity of each repeat (Boch and Bonas 2010). Since most members of the AvrBs3/PthA protein family share high-sequence identity, it is postulated that, besides the variation in the number of direct repeats, the variable amino acid residues at the third position (P→S) in the 1st, 2nd, and 3rd repeats, and at the 13th position (G, I, D, S→A, T) in the 11th, 12th, 13th, and 14th repeats would alter a critical recognition in conferring the specificity required for the interaction with particular protein or DNA targets. Based on these observations that PthAp protein failed to induce erumpent canker symptom on citrus leaves. This finding suggested that a new number of direct repeats and alternation of amino acid residues at special positions

in these repeats can result in a new gene sequence with altered function.

Complementation of mutant T38 with clones pMCS1935, pMCS4735 and pMCS47SP with 17.5 direct repeat sequences (Table 1) from strains XW19 and XW47 of *pthA* gene respectively (Lin et al. 2011) successfully restored the ability of the T38 to elicit hyperplastic canker symptom on the leaves of Mexican lime. In the case of grapefruit, pMCS1935 and pMCS47SP also restored typical hyperplastic canker symptom expect clone pMCS4735 only elicited large flat necrotic lesion on leaves. These results suggested that the 17.5 direct repeat in *pthA* gene is required and critical for full pathogenicity of the bacterium. The amino acid residue, serine, located at critical position 286 in N terminus motif of leucin-rich repeat of PthAp was the same as that of PthAf protein (containing 17.5 direct repeats) from XW47 (Lin et al. 2011) can alter canker formation on grapefruit leaves. To investigate the effect of amino acid residue at position 286 of PthAp on symptom development induced by *Xac*-A^p type strain, mutated PthAp with a single amino acid substitution was constructed. The amino acid substitution S286P of PthAp (containing 18.5 direct repeats) from XL 38 resulted in no alternation of symptom for transformant T38SP to induce an erumpent canker symptom, whereas the amino acid substitution S286P of PthAf (containing 17.5 direct repeats) from XW47 could complement the canker-inducing ability of T38 on citrus leaves (Table 2). This result confirmed that the critical role of the specific sequence of 17.5 direct repeats that is responsible for inducing canker symptom by *X. axonopodis* pv. *citri*.

Many *X. axonopodis* pv. *citri* strains contain multiple variants of AvrBs/PthA proteins. The existence of multiple copies of *avrBs3*/*pthA* genes in a single strain is thought to facilitate recombination and rapid adaptation to new hosts (Yang and Gabriel 1995a), and variants of *pthA* from a single bacterial strain localize to nucleus of plant cells and form homo- and heterodimers to modulate host transcription (Domingues et al. 2010). In our study, Southern hybridization analysis revealed strain XL38 possessed two *BamH*I fragments (3.5-kb and 2.8-kb) hybridized with *pthA*, this result was the same as the strains of A*, A^w, B, C groups, had only two *BamH*I fragments except for the size differences (AI-Saadi et al. 2007). Based on high level of sequence conservation among members of *pthA* homologs, the size differences of these bands may due to variable numbers of 102-bp direct repeats within the *BamH*I fragment. The number of repeats to induce target gene expression, a minimum number of 6.5 repeats is necessary (Boch et al. 2009). We also cloned a 2.8-kb *BamH*I fragment with PCR-amplified method from genomic DNA of XL38 strain generated a 2.9-kb fragment (Fig. 3b) and sequenced. This 2.9-kb fragment is a *pthA* homolog with 11.5 direct repeats and appears to have an intact promoter, a SD region, and a full-length open reading frame (data not shown). Knockout mutation of this *pthA* homolog and its complementation are being investigated to provide further evidence for the relationship in symptom development. Due to *pthA* homologs from *Xac*-A^p type strain XL38 didn't possess normal 17.5 direct repeats that is essential for inducing canker symptom of *X. axonopodis* pv. *citri*. Taking altogether, our results demonstrated that features of these repeats contribute to the atypical symptom caused by strain XL38 that failed to induce canker lesions on leaves of grapefruit and Mexican lime.

Acknowledgments Our special thanks go to Professor K.-C. Tzeng (Department of plant pathology, National Chung-Hsing University, Taiwan) for many valuable suggestions. This research was supported in part of grant from National Science Council (NSC100-2313-B-235-004), Taiwan.

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