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# A single amino acid substitution in PthA of *Xanthomonas axonopodis* pv. *citri* altering canker formation on grapefruit leaves

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**Abstract** The typical citrus canker lesions produced by *Xanthomonas axonopodis* pv. *citri* are erumpent, callus-like, with water-soaked margins. Three novel atypical symptom-producing variants of *X. axonopodis* pv. *citri* were described recently in Taiwan. Only the variant designated as $A^f$ type produces typical erumpent canker lesions on Mexican lime (*Citrus aurantifolia*) but induces flat necrotic with water-soaked margin lesions on grapefruit leaves (*C. paradisi*). Two homologous *pthA* were cloned and characterized from strains XW19 (a typical canker lesion producing strain) and XW47 (a strain of $A^f$ type). The *pthA* homolog from XW19 was transformed into XW47. The transformant of XW47 induced typical erumpent canker lesions on grapefruit leaves. Sequence analyses of transformants XW19 and XW47 revealed over 99% homology in nucleotide and deduced amino acid sequences compared with *pthA* homologs deposited in GenBank. The amino acid residues located at positions 49, 286, 742 and 767 of PthA were different between XW47 and XW19. The PthA mutants with a single amino acid substitution at each of these four positions were constructed by site-directed mutagenesis. Modified PthA (S286P) from XW47 in transformant 47SP induced erumpent canker lesions on grapefruit leaves, whereas another modified PthA (P286S) from XW19 in transformant 47PS only induced flat necrotic lesions. These results suggested that a single amino acid substitution from either serine to proline or proline to serine at position 286 of PthA can alter canker formation by *X. axonopodis* pv. *citri* on grapefruit leaves.

**Keywords** Citrus · Citrus bacterial canker · *pthA* Site-directed mutagenesis · Proline

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## Introduction

Citrus canker, an important citrus disease worldwide, is caused by *Xanthomonas axonopodis* pv. *citri* with a

broad host range (Stall and Civerolo 1991). *X. axonopodis* pv. *citri* induces erumpent, callus-like lesions with a water-soaked margin. Severe symptoms include premature fruit drop and twig dieback. Recently, two 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 were phenotypically distinct from *X. axonopodis* pv. *citri* and their host was primarily limited to lime (Verniere et al. 1998; Sun et al. 2004; Bui Thi Ngoc et al. 2008). Three novel atypical symptom-producing variants of *X. axonopodis* pv. *citri* were discovered and identified in Taiwan, and designated as type *Xac*-$A^f$, *Xac*-$A^p$ and *Xac*-$A^r$ (Lin et al. 2005, 2008). *Xac*-$A^f$ strain XW47 caused typical canker lesions on Mexican lime (*C. aurantifolia*) but induced flat necrotic lesions on grapefruit (*C. paradisi*), sweet orange (*C. sinensis*) and lemon (*C. limon*). Although symptoms induced by *Xac*-$A^f$ strain on leaves of citrus species were similar to those induced by *Xac*-A* strains and *Xac*-$A^w$ strains, but *Xac*-$A^f$ could be differentiated from strains *Xac*-A* and *Xac*-$A^w$ by *lrp* sequence assay and PCR amplified DNA profiles (Lin et al. 2005). The question as to why these atypical symptom-inducing *X. axonopodis* pv. *citri* strains elicited different symptoms on citrus species remains unanswered.

The *avrBs3/pthA* (avirulence and pathogenicity) gene family, widely distributed in phytopathogenic *Xanthomonas* species, was involved in disease symptom expression and host defence response (Swarup et al. 1991, 1992; Fujikawa et al. 2006). Many members of this gene family are required for pathogenicity of *Xanthomonas* species (Leach and White 1996; Gabriel 1999). For example, the *X. axonopodis* pv. *citri pthA* gene is necessary for *X. axonopodis* pv. *citri* to cause citrus canker disease (Swarup et al. 1991). Transient expression of *pthA* could induce small raised cankers when they were introduced in citrus leaves either by particle bombardment or by *Agrobacterium tumefaciens*-mediated transformation (Duan et al. 1999). When *pthA* is transferred to strains of other *Xanthomonas* species, it enabled these strains to form canker on citrus and induce hypersensitive response on bean and cotton (Swarup et al. 1991; Swarup et al. 1992). Thus, *pthA* exhibits both pleiotropic pathogenicity and avirulence functions. This *pthA* gene contains a 102-bp tandem repeats in the central portion of the gene including 17.5 identical repeats. The repeat region is critical for host specific determination and avirulence specificity (Yang et al. 1994; AI-Saadi et al. 2007; Shiotani et al. 2007). A leucine zipper-like motif is contiguous with the 34 amino acid tandem repeats. Both types of motifs, the three nuclear localization signal sequences (Yang and Gabriel 1995b) and an acidic transcriptional activation domain in the C-terminus (Zhu et al. 1998), are required for activity. These structural features suggest PthA protein is secreted by the type III secretion system, which is a secretion apparatus present in Gram-negative bacteria, for delivering effector proteins into plant cells (Buttner and Bonas 2002).

Multiple *pthA* homologs are always present in all strains of *X. axonopodis* pv. *citri* including $A^*$ and $A^w$ (Kanamori and Tsuyumu 1998; AI-Saadi et al. 2007). 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 (Kanamori and Tsuyumu 1998; AI-Saadi et al. 2007). A new functional chimeric *pthA* homolog cloned from a Japanese strain KC21 of *X. axonopodis* pv. *citri* was necessary for inducing defence response on the host but could only partially interrupt canker development (Shiotani et al. 2007). These results indicated the repeating units were important for the canker symptom development and might play a role for some *pthA* homologs in the host range restriction of some strains on citrus. Despite several distinct phenotypes of *X. axonopodis* pv. *citri* have been reported, the genetic basis to explain why symptoms vary on different citrus cultivars is still unclear.

In this study, the site-directed mutagenesis and a chimeric gene fusion method were used to investigate the mechanism of symptom variation on grapefruits caused by the typical phenotype *Xac*-A strain and an atypical symptom-producing *Xac*-$A^f$ strain originated from Taiwan. Nucleotide sequence of *pthA* gene was analyzed to determine the core of amino acid residues contiguous to N terminal region of leucine-rich repeats for canker formation on grapefruit by *X. axonopodis* pv. *citri*.

## Materials and methods

### Bacterial strains, plasmids, and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and its

**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 | Type A strain | (Wu et al. 1986) |
| XW47 | Type $A^f$ strain | (Wu et al. 1986) |
| 47MCS | XW47 containing pBBR1MCS-5; $Gm^r$ | This study |
| 4735 | XW47 containing pMCS1935; $Gm^r$ | This study |
| 4738 | XW47 containing pMCS1938; $Gm^r$ | This study |
| 47SP | XW47 containing pMCS47SP; $Gm^r$ | This study |
| 47PS | XW47 containing pMCS19PS; $Gm^r$ | This study |
| 47IM | XW47 containing pMCS47IM; $Gm^r$ | This study |
| 47MI | XW47 containing pMCS19MI; $Gm^r$ | This study |
| *X. axonopodis* pv. *citrumelo* | | |
| F2 | Pathotype E strain | (Wu et al. 1993) |
| F35 | F2 containing pMCS1935; $Gm^r$ | This study |
| F38 | F2 containing pMCS1938; $Gm^r$ | This study |
| F35W | F2 containing pMCS4735; $Gm^r$ | This study |
| F38W | F2 containing pMCS4738; $Gm^r$ | This study |
| F2SP | F2 containing pMCS47SP; $Gm^r$ | This study |
| F2PS | F2 containing pMCS19PS; $Gm^r$ | This study |
| Plasmids | | |
| pCR-XL-TOPO | PCR cloning vector; $Ap^r$, $Km^r$ | Invitrogen |
| pDrive | PCR cloning vector; $Ap^r$, $Km^r$ | Qiagen |
| pGEM-T easy | PCR cloning vector; $Ap^r$ | Promega |
| pBBR1MCS-5 | Broad host range cloning vector; $Gm^r$ | (Kovach et al. 1995) |
| pTOPO1938 | 3.8-kb *pthA* fragment from XW19 cloned into pCR-XL-TOPO; $Ap^r$, $Km^r$ | This study |
| pTOPO1935 | 3.5-kb *pthA* fragment from XW19 cloned into pCR-XL-TOPO; $Ap^r$, $Km^r$ | This study |
| pTOPO4738 | 3.8-kb *pthA* fragment from XW47 cloned into pCR-XL-TOPO; $Ap^r$, $Km^r$ | This study |
| pTOPO4735 | 3.5-kb *pthA* fragment from XW47 cloned into pCR-XL-TOPO; $Ap^r$, $Km^r$ | This study |
| pDrive085 | 0.85-kb *Xho*I-*Hind*III fragment-swapped chimeric gene from *pthA* fragment of XW19 cloned into pDrive; $Ap^r$, $Km^r$ | This study |
| pGEMT265 | 2.65-kb *Hind*III-*Xba*I fragment-swapped chimeric gene from *pthA* fragment of XW19 cloned into pGEM-T easy; $Ap^r$ | This study |
| pMCS1935 | 3.5-kb *pthA* fragment from pTOPO1935 subcloned into pBBR1MCS-5; $Gm^r$ | This study |
| pMCS4735 | 3.5-kb *pthA* fragment from pTOPO4735 subcloned into pBBR1MCS-5; $Gm^r$ | This study |
| pMCS1938 | 3.8-kb *pthA* fragment from pTOPO1938 subcloned into pBBR1MCS-5; $Gm^r$ | This study |
| pMCS4738 | 3.8-kb *pthA* fragment from pTOPO4738 subcloned into pBBR1MCS-5; $Gm^r$ | This study |
| pMCS47SP | pMCS4735 derivative, changing the amino acid $Ser_{286}$ to $Pro_{286}$ of 3.5-kb *pthA* fragment from XW47 | This study |
| pMCS19PS | pMCS1935 derivative, changing the amino acid $Pro_{286}$ to $Ser_{286}$ of 3.5-kb *pthA* fragment from XW19 | This study |
| pMCS47IM | pMCS4735 derivative, changing the amino acid $I_{49}$ to $M_{49}$ of 3.5-kb *pthA* fragment from XW47 | This study |
| pMCS19MI | pMCS1935 derivative, changing the amino acid $M_{49}$ to $I_{49}$ of 3.5-kb *pthA* fragment from XW19 | This study |

derivatives were grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) or LB agar plate at 37 C. *Xanthomonas* spp. was cultured on YPD medium (Vernière et al. 1991) at 30 C. Media were amended with 50 μg/ml of Kanamycin, 50 μg/ml of gentamicin, or 20 μg/ml of gentamicin as stated.

## PCR amplification, cloning and sequence analysis of *pthA* gene

Total DNAs from *X. axonopodis* pv. *citri* strains XW19 and XW47 were isolated by standard methods (Sambrook et al. 1989). The *pthA* genes of XW19 and XW47 were amplified by PCR using a primer pair pthAP7/AR2 described previously (Lin et al. 2005). The *pthA* gene without promoter region was amplified by primer pair pthA*XhoI* and pthA*XbaI* (Table 2). PCR 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 (BioBasic Inc., Canada) with proof-reading function, and DMSO 5 μl. The amplification condition consisted of 94C for 1 min, 63C for 1 min, and 72C for 5 min for 35 cycles with an initial step of 94C 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.

The PCR products were purified by the Viogene Gel-M™ Gel Extraction system (Viogene Corporation, Taiwan) and cloned into a pCR-XL-TOPO cloning vector (Invitrogen Corporation, Netherlands). Clones were selected on LB medium supplemented with kanamycin (50 μg/ml) after transformation into *E.coli* DH5α cells. DNAs of the recombinant clones containing the individual *pthA* gene from XW19 or XW47 were sequenced using an automatic DNA sequencing system (ABI-377-19; Perkin-Elmer Applied Biosystems, Foster City, CA).

DNA sequence data were analyzed with Blast program, running at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) network service. 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 Diago Supercomputing Center). The variant amino acid residues were analyzed with Vector NTI™ version 8.0 (Invitrogen, Madison, Wisconsin).

## Transformation of *X. axonopodis* pv. *citri* and *X. axonopodis* pv. *citrumelo* by electroporation

To transform strains XW47 of *Xac* and F2 of *X. axonopodis* pv. *citrumelo*, the competent XW47 and F2 cells were prepared as described by Francois et al. (1997). The *pthA* genes from XW19 and XW47 were further subcloned individually into the broad host range vector pBBR1MCS-5 (Kovach et al. 1995)

**Table 2** Oligonucleotide primers used in point-mutation

| Name | Sequences (5′ to 3′)[a] | Restriction enzyme |
|---|---|---|
| IM-1 | CTCGGCGGACG**ATG**TCCCGGACCCG | |
| AIM-1 | CGGGTCCGGGA**CAT**CGTCCGCCGAG | |
| SP-1 | GACGGGTGCC**CCC**CTGAACCTGACC | |
| ASP-1 | GGTCAGGTTCAG**GGG**GGCACCCGTC | |
| MI-3 | CTCGGCGGACG**ATT**TCCCGGACCCG | |
| AMI-3 | CGGGTCCGGGA**AAT**CGTCCGCCGAG | |
| SG-1 | ATCGCCAGCAAT**GGC**GGTGGCAAGCAG | |
| ASG-1 | CTGCTTGCCACC**GCC**ATTGCTGGCGAT | |
| DE-2 | CCTGACCCCG**GAG**CAGGTGGTGG | |
| ADE-2 | CCACCACCTG**CTC**CGGGGTCAGG | |
| pthA*XhoI* | GAAAACTCGAGAAAGAGGTATGCCTGATGGA | *XhoI* |
| pPS1 | GTTCAA**GCT**TGCACCCGTCAGTG | *Hind* III |
| pPS2 | GCACTGACGGGTGCA**AGC**TTGAACCTG | *Hind* III |
| pthA*XbaI* | GAAAATCTAGAGCCTCACTGAGGCAATAGCT | *XbaI* |

[a] Bold bases represent mutated nucleotides and underlined bases indicate restriction sites

before being transformed into both strains of XW47 and F2 by electroporation (Keen et al. 1990). Selection of transformants was accomplished on YPD plates supplemented with gentamicin (20 μg/ml).

## Southern blotting analysis

Total DNAs from XW19 and XW47 were digested with *Eco*RI restriction enzyme, 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 being hybridized with a biotin-labeled *pthA* homolog which 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

Amino acid substitutions were performed in clones pMCS4735 and pMCS1935 by site-directed mutagenesis introduced by a QuikChange XL site-directed mutagenesis kit (Stratagene) and a chimeric gene fusion method. For site-directed mutagenesis, the primers used in the construction were listed in Table 2. Five primer pairs, IM-1 and AIM-1, SP-1 and ASP-1, MI-3 and AMI-3, SG-1 and ASG-1, and DE-2 and ADE-2, were used for inducing single point mutation to create mutated residues at critical positions of clone pMCS4735 ($Ile_{49}$→$Met_{49}$ and $Ser_{286}$→$Pro_{286}$) and clone pMCS1935 ($Met_{49}$→$Ile_{49}$, $Ser_{742}$→$Gly_{742}$ and $Asp_{767}$→$Glu_{767}$).

To construct chimeric gene fusions, two primer pairs pthA*XhoI*/pPS1 and pPS2/pthA*XbaI* (Table 2) were used for inducing single point mutation to create mutated residue at critical position of clone pMCS1935 ($Pro_{286}$→$Ser_{286}$). Two fragments, 0.85 kb *Xho*I-*Hind*III and 2.65 kb *Hind*III-*Xba*I, of chimeric gene were performed in pMCS1935 plasmid as a template amplified by PCR. Two fragments were digested with *Hind*III, *Xho*I or *Xba*I and ligated into the cloning vector pDrive (Qiagen) or pGEM-T easy (Promega) to obtain plasmids pDrive085 and pGEMT265, respectively. Furthermore, pDrive085 and pGEMT265 were digested with the same enzymes that were used to obtain 0.85 kb fragment and 2.65 kb fragment; they were ligated into the cloning vector pBBR1MCS-5 to produce pMCS19PS ($Pro_{286}$→$Ser_{286}$) and yielded a chimera. The identity of the insert in all resulting plasmids described above was confirmed by DNA sequencing.

## Plant inoculation

Citrus plants (*C. paradisi*, grapefruit; *C. aurantifolia*, Mexican lime) grown in 8-inch pots in a greenhouse were used. Plants inoculated with various strains were kept in a growth chamber with 65–90% humidity and 12-hr light at 30°C and 12-hr dark at 25°C.

To prepare inoculums, *X. axonopodis* pv. *citri* strains were grown overnight in YPD broth, harvested by centrifugation 6000×g for 5 min at 4°C (SCR20BA, Hitachi, Japan) and resuspended in sterile distilled water to a concentration of approximately $10^8$ colony-forming units (CFU)/ml. In needle-prick inoculation, six wounds in a 1 $cm^2$ area were made on young fully expanded 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. Symptoms were observed visually or examined with a binocular dissecting microscope. For injection-infiltration inoculation, bacterial cells grown overnight in YPD broth with or without gentamicin were harvested by centrifugation, and were resuspended 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.

## Light microscopy

For light microscopy observation, the leaf tissue containing a lesion was excised with a dissecting knife 24 days after inoculation with the needle-prick method, and was immediately fixed in a 20% gelatin solution. Sample was placed onto a frozen metal specimen holder, and then sectioned with a Freezing Microtome (model FX-801, Yamato Kohki Industrial Co. Ltd, Japan). Sections were examined under a light microscope (Optiphot, Nikon, Japan).

## Bacterial population in grapefruit leaves

For leaves that were inoculated with the injection-infiltration method, leaf disks (9 mm in diameter)

**Table 3** Symptoms observed on leaves of grapefruit and Mexican lime induced by *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas axonopodis* pv. *citrumelo* strains or transformed strains

| Strain | Genotype | Symptoms on grapefruit | Symptoms on Mexican lime |
|---|---|---|---|
| XW19 | Wild type | Canker | Canker |
| XW47 | Wild type | Flat necrosis | Canker |
| F2 | Wild type | Flat necrosis | Flat necrosis |
| 47MCS | XW47 derivative carrying pBBR1MCS-5 | Flat necrosis | Canker |
| 4735 | XW47 derivative carrying pMCS1935 | Canker | Canker |
| 4738 | XW47 derivative carrying pMCS1938 | Canker | Canker |
| 47SP | XW47 derivative carrying pMCS47SP(PthAS286P) | Canker | Canker |
| 47PS | XW47 derivative carrying pMCS19PS(PthAP286S) | Flat necrosis | Canker |
| 47IM | XW47 derivative carrying pMCS47IM(PthAI49M) | Flat necrosis | Canker |
| 47MI | XW47 derivative carrying pMCS19MI(PthAM49I) | Canker | Canker |
| F35 | F2 derivative carrying pMCS1935 | Weak canker | Weak canker |
| F38 | F2 derivative carrying pMCS1938 | Weak canker | Weak canker |
| F35W | F2 derivative carrying pMCS4735 | Flat necrosis | Weak canker |
| F38W | F2 derivative carrying pMCS4738 | Flat necrosis | Weak canker |
| F2SP | F2 derivative carrying pMCS47SP(PthAS286P) | Weak canker | Weak canker |
| F2PS | F2 derivative carrying pMCS19PS(PthAP286S) | Flat necrosis | Weak canker |

were removed with a cork-borer randomly by punching within the inoculated area at various time intervals after inoculation. Twelve leaf disks per time interval and three replicates for each strain were assayed. 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) (Vernière 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 (Vernière et al. 1998) or YPDAC plates containing gentamicin (20 μg/ml) and the inoculated plates were incubated at 30 C. The number of colonies was counted 3 days after incubation. Bacterial populations were expressed as log CFU/disk.

The stability of plasmids of the derivative strains 47SP and 47PS in leaves was determined by plating the above mentioned leaf extracts on YPDAC plates with or without the addition of gentamicin. Colonies developed from each time interval were screened for antibiotic resistance markers on the plasmid. The loss of plasmid over time was expressed as a percentage of *Xanthomonas* cells displaying the plasmid encoding antibiotic resistance.

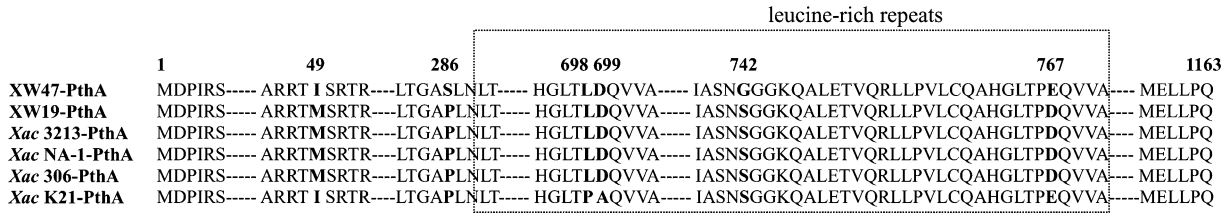



**Fig. 1** Sequence aligment of PthA proteins from *Xanthomonas axonopodis* pv. *citri*, including XW47-PthA (GenBank accession no. GU181332), XW19-PthA (GenBank accession no. GU181333), *Xac* 3213-PthA (GenBank accession no. U28802.1), *Xac* NA-1-PthA (GenBank accession no. AB021363.1), *Xac* 306-PthA (GenBank accession no. NC003922.1) and *Xac* K21-PthA (GenBank accession no. AB206388.1). Varied amino acid residues are shown in *boldface*

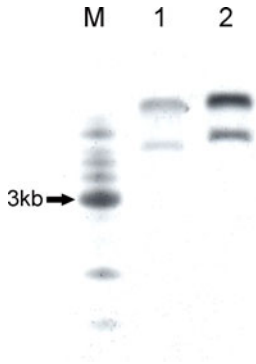



**Fig. 2** Detection of *pthA* homologs in two strains of *Xanthomonas axonopodis* pv. *citri*. The *Eco*RI-digested genomic DNAs from *X. axonopodis* pv. *citri* XW47 (lane 1) and *X. axonopodis* pv. *citri* XW19 (lane 2) were hybridized with a biotin-labeled 3.8 kb DNA fragement containing an entire length of *pthA* from strain XW19. M represents the molecular weight marker

## Results

### Cloning and expression of *pthA* gene

PCR-amplified *pthA* genes were cloned from both XW19 and XW47 strains and transformed into XW47 and F2 (Table 1). The nucleotide sequences of these *pthA* genes showed over 99% homology to *pthA* genes from *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.AB206388.1). The transformants 4735, 4738, F35 and F38 with the *pthA* from XW19 were able to induce canker lesions on leaves of grapefruit and Mexican lime, while the transformants F35W and F38W with the *pthA* from XW47 induced flat necrosis lesions on grapefruit leaves (Table 3). The 3.5-kb fragment of *pthA* homolog without promoter from strains XW19 and XW47, cloned into pBBR1MCS-5, with the *Xho*I site proximal to the vector *lac* promoter produced canker or flat necrosis lesions on citrus leaves. This result indicated that transcription of the *pthA* gene in pMCS1935 and pMCS4735 were dependent on the vector *lac* promoter.

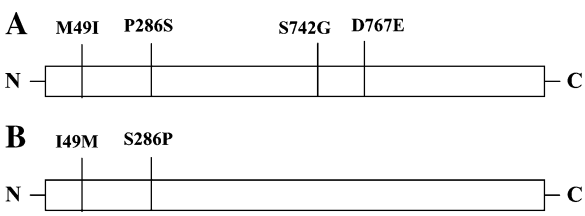


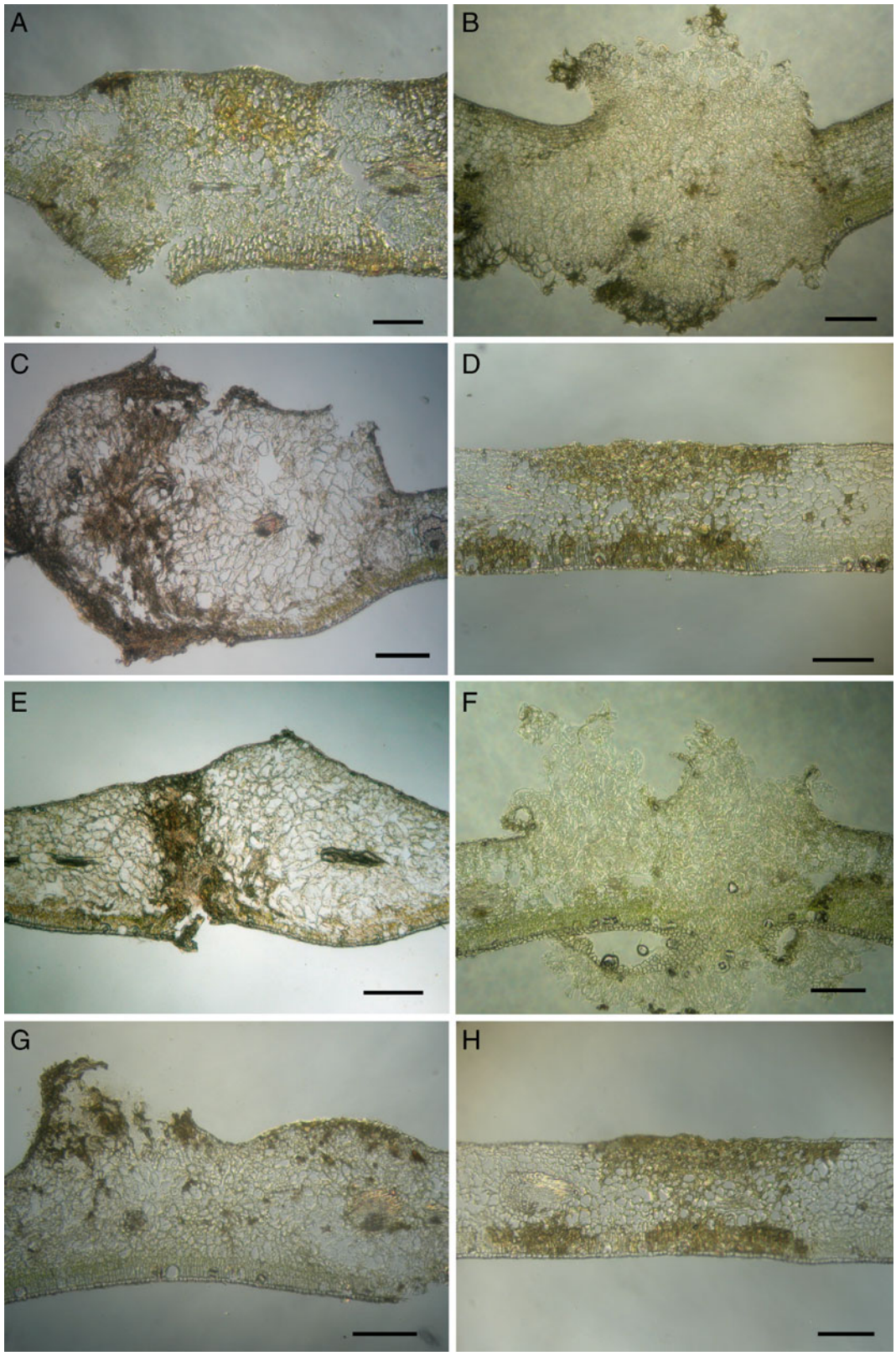

**Fig. 3** Position of point mutations in PthA protein of *Xanthomonas axonopodis* pv. *citri*. A: Amino acid residues Met49, Pro286, Ser742, and Asp767 in PthA of XW19 were substituted with Ile49, Ser286, Gly742, and Glu767, respectively; B: Amino acid residues Ile49 and Ser286 in PthA of XW47 were substituted with Met49 and Pro286, respectively

### Characterization of *pthA* gene

The nucleotide sequence analysis revealed that a 3.8 kb DNA fragment containing promoter region and the *pthA* gene encoding an open reading frame (ORF) of 3757 nucleotides was cloned from strains XW19 ( GenBank accession no. GU181333) and XW47 (GenBank accession no. GU 181332). Sequence of the ORF contained 17.5 of 102 bp tandem repeats with each encoding 34 amino acids in the central portion, a leucine zipper, three nuclear localization signals and an acid transcriptional activation domain (AAD) in the C-terminus. Multiple sequence alignment revealed the identity of amino acid sequences among PthA proteins from these *X. axonopodis* pv. *citri* strains was over 99%. Upon this region in XW47, four various amino acid residues were found by comparing with the amino acid sequences of PthA of *X. axonopodis* pv. *citri* 3213 *pthA*, *X. axonopodis* pv. *citri* NA-1 *apl1, X. axonopodis* pv. *citri* 306 *pthA4*, *X. axonopodis* pv. *citri* K21 *pthA* and *pthA* homolog from XW19. The positions of these four various residues in ORF of PthA from XW47 strain were at 49, 286, 742 and 767 (Fig. 1). These various amino acid residues of PthA between XW19 and XW47 were analyzed with a Vector NTI™ software package (version 8.0). The results indicated that the residue $Pro_{286}$ located immediately at N terminal domain of leucine-rich repeats of PthA may be required for maintaining the structural integrity of N terminal local domain of leucine-rich repeats, suggesting that this structural alteration might influence the interaction between PthA and host cells.

### Southern blotting analysis of *pthA* gene in *X. axonopodis* pv. *citri*

To determine the copy number of *pthA* homolog in strains XW19 and XW47, a 3.8-kb fragment of *pthA* gene from pTOPO1938 plasmid was used as the


A
B
C
D
E
F
G
H

◀ **Fig. 4** Histopathology of lesions induced by derivatives of *Xanthomonas axonopodis* pv. *citri* with an amino-acid residue substitution in the PthA on leaves of Mexican lime (**a**, **c**, **e**, **g**) and grapefruit (**b**, **d**, **f**, **h**) 24 days after needle-prick inoculation. **a** and **b**: The sections of typical erumpent canker lesions induced by strain 47SP (PthA S286P); **c**: A section of typical erumpent canker lesion induced by strain 47PS (PthA P286S); **d**: A section of flat necrotic lesion induced by strain 47PS; **e** and **f**: The sections of typical erumpent canker lesions induced by strain 47MI (PthA M49I); **g**: A section of typical erumpent canker lesion induced by strain 47IM (PthA I49M); **h**: A section of flat necrotic lesion induced by strain 47IM. (Bars=125 μm)

probe to hybridize *EcoR*I-digested genomic DNAs isolated from both XW19 and XW47 strains. The results showed at least two copies of *pthA* gene existing in both strains (Fig. 2).

### Phenotypic changes on citrus leaves by XW47 transformed with *pthA* gene containing a mutated residue

Six various substitutions of amino acid residues in PthA were obtained. The amino acid residue substitutions were the $Ile_{49}$ and $Ser_{286}$ in PthA of XW47 substituted with $Met_{49}$ and $Pro_{286}$ (PthA I49M and PthA S286P), and the $Met_{49}$ and $Pro_{286}$ in PthA of XW19 substituted with $Ile_{49}$ and $Ser_{286}$ (PthA M49I and P286S) (Fig. 3). Amplification products of the site-directed mutagenesis and the chimeric fusion genes were sequenced and further transformed into competent cells of XW47. Four derivative strains, individually contained a plasmid of mutant PthA with an amino acid residue substitution, were designated as 47SP (with PthA S286P), 47PS (with PthA P286S), 47IM (with PthA I49M), and 47MI (with PthA M49I) (Table 1). All of the derivative strains were inoculated onto leaves of grapefruit and Mexican lime. The inoculation revealed the hyperplasia and hypertrophy of mesophyll cells, the eruption of the abaxial epidermis and protrusion of the adaxial epidermis on sections of lesions induced by strain 47SP on Mexican limes and grapefruits 24 days post inoculation by a light microscopic examination (Figs. 4a and b). The similar histopathological symptoms to those induced by strain 47SP were found in Mexican lime leaves inoculated with strain 47PS (Fig. 4c). Strain 47PS, however, caused brownish necrotic and hyperplastic mesophyll tissues without hypertrophic cells and eruption of the epidermis in grapefruit leaves (Fig. 4d). A typical erumpent canker lesion induced by strain 47MI developed on leaves of Mexican limes and grapefruits 24 days post inoculation (Fig. 4e and f). The similar histopathological symptoms to those induced by strain 47MI were found on Mexican lime leaves inoculated with strain 47IM (Fig. 4g). However, a flat necrotic lesion induced by strain 47IM on grapefruit leaves showed brownish necrotic and hyperplastic mesophyll tissues without hypertrophic cells and eruption of the epidermis (Fig. 4h). This reversion analysis provided further evidence that the substitution of a proline for a serine at position 286 of ORF of PthA had produced a defective form of PthA. Amino acid substitutions of S742G and D767E in PthA of XW19 were not possible to complete. This might be due to technical difficulty that Ser742 and Asp767 were located in the repeat region.

### Bacterial growth in grapefruit leaves

All four strains XW19, XW47, 47SP and 47PS grew in grapefruit leaves. No significant difference in the growth rates was observed between XW19 and XW47 in grapefruit leaves (Fig. 5). The bacterial populations of both XW19 and XW47 strains increased gradually from $10^3$ CFU/disk to $10^8$ CFU/disk 20 days post inoculation. Similar increases were observed for strains 47SP and 47PS for the first 4 days after inoculation. Thereafter, the rates of growth differed. It was approximately 10-fold lower than that of XW19 or XW47 20 days post inoculation (Fig. 5). More than 90% of cells extracted from grapefruit leaves retained pMCS47SP and pMCS19PS within 20 days of inoculation based on the loss of the plasmid carrying the antibiotic marker.

## 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). Swarup et al. (1991) transformed a clone pSS10.35 carrying the *pthA* locus to *X. axonopodis* pv. *citrumelo* rendering the transformant to induce canker lesions on grapefruit leaves. In this study, the *pthA* homologs from XW19 and XW47 were subcloned into a vector pBBR1MCS-5 and then mobilized into *X. axonopodis* pv. *citrumelo* F2. The transformants harbouring a

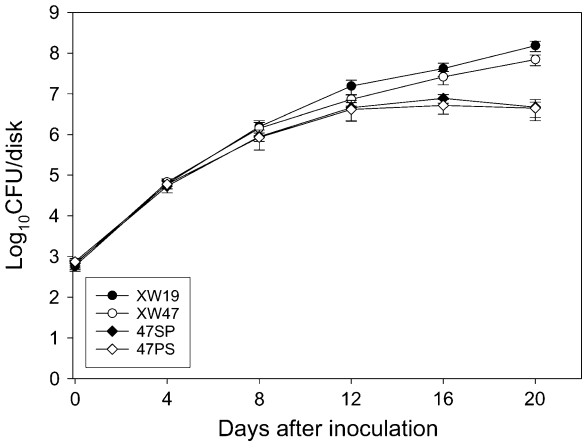



**Fig. 5** Populations of *Xanthomonas axonopodis* pv. *citri* strains XW19, XW47 and XW47 derivatives 47SP (PthAS286P) and 47PS (PthAP286S) in leaves of grapefruit. Each bacterial strain was inoculated into leaves by injection-infiltration at a concentration of $1\times10^5$ CFU/ml. Each value is the mean of three replicates and vertical bar represents the standard error

*pthA* gene from XW19 were able to elicit canker lesions on grapefruit leaves. However, the transformants which contained a *pthA* homolog from XW47 were only able to induce flat necrosis lesions on grapefruit leaves. These results indicated the *pthA* homolog from XW19 strain has a normal canker-inducing function, whereas the *pthA* homolog from XW47 was deficient of the function for normal canker formation. The amino acid sequence analysis showed that four amino acid residues $I_{49}$, $S_{286}$, $G_{742}$ and $E_{767}$ in PthA in XW47 were different from those in PthA in XW19 or from other PthA deposited in GenBank. Predicted secondary structure of PthA analyzed by a Vector NTI™ software package (version 8.0) revealed that substitution of $P_{286}$ with $S_{286}$ in PthA would affect the structural conformation of PthA at N terminal region near leucine-rich repeats.

To investigate the effect of amino acid residue at position 49, 286, 742 and 767 of PthA on symptom development induced by *X. axonopodis* pv. *citri*, mutated PthA with a single amino acid substitution was constructed. The amino acid substitution P286S of PthA from XW19 resulted in the loss of the ability for transformant 47PS to induce an erumpent canker symptom on grapefruit leaves; instead, it induced flat necrotic lesion, whereas the amino acid substitution S286P of PthA from XW47 could complement the canker-inducing ability of XW47 on grapefruit leaves. Transferring the amino acid substitutions M49I and I49M of PthA from XW19 and XW47 respectively, into XW47 did not alter the symptom expression of XW47 on grapefruit leaves suggesting that $P_{286}$ in PthA appeared to play an important role in N terminus motif near leucine repeats. PthA could tolerate nonconservative amino acid changes in $M_{49}$ residue. The substitution of amino acid at position 286 of PthA could affect canker symptom expression induced by *X. axonopodis* pv. *citri* on grapefruit leaves but not on Mexican lime. Thus, P286S was predicted to cause break in the α-helix, and this structural alteration may influence local conformation in N terminal region of leucine-rich repeats of PthA. Therefore, an amino acid substitution at position 286 of PthA may modulate the phenotypes of plant interaction by activation of host plant gene expression between grapefruit and Mexican lime.

The *pthA* gene is one of the *avrBs3/pthA* family member in which the central region of the genes was composed of a number of 102 bp direct repeats. The number and the organization of the repeats are key factors determining the recognition specificity with plant (Yang et al. 1994; Yang and Gabriel 1995a). A novel region, *Hinc*II-*Sph*I region, in the 3′end of *pthA* gene from *X. axonopodis* pv. *citri* is essential for disease expression on citrus (Ishihara et al. 2003). Changes in the *Hinc*II-*Sph*I region can alter phenotypes dramatically. A natural variant of *X. oryzae* pv. *oryzae* that contained an allele of *avr Xa7* with only two amino acid changes in the *Hinc*II-*Sph*I region lost its avirulence function and exhibited reduced virulence function on rice (Cruz et al. 2000) indicating that the *Hinc*II-*Sph*I region in the 3′ end of the *avrBs3*/*pthA* gene family is important for avirulence and virulence functions. Results of this study provided evidence of the role a novel motif in N terminal region upstream the leucine-rich repeats of PthA plays in the alteration of the host phenotypes between grapefruit and Mexican lime.

The effect of amino acid substitutions of avirulence proteins on the virulence of the pathogen has been reported (Joosten et al. 1994; Shan et al. 2000a; Chang et al. 2001). Mutation of three AvrPto residues S94, I96, and G99 abolishes interaction with Pto and avirulence activity, but not virulence activity in tomato which enabled the identification of a core of amino acids that were required for function of AvrPto (Shan et al. 2000a, 2000b). AvrPto could tolerate substitutions at many positions and remained func-

tional (Chang et al. 2001). Joosten et al. (1994, 1997) reported that substitution of cysteine residues with tyrosine residues could affect the structure of AVR4 protein that resulted in a failure to bind the corresponding receptor. The mechanism of the recognition events involving PthA and receptor proteins occurred within the citrus cells was still unclear. In this study, mutation of PthA by changing residue Pro286 to Ser286 resulted in the abolishment of normal canker formation activity on grapefruit leaves by *X. axonopodis* pv. *citri*. Results from amino acid substitution experiments indicated that amino acid Pro286 appeared to play an important role in N terminus motif near leucine repeats. It might cause the PthA to interact with certain unknown receptors in host cells which in turn result in different symptom induction such as canker lesions or flat necrotic lesions. The amino acid residue Pro286 was highly conserved in many members of *avrBs3/pthA* gene family, and mutation in this position will affect the protein structure for symptom expression. This study reported for the first time that the alteration of canker lesion is caused by a single amino acid substitution in PthA of *X. axonopodis* pv. *citri*. Knockout mutation and complementation experiments of the *pthA* gene are being investigated to provide better evidence for their relationship.

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