All Five Host-Range Variants of *Xanthomonas citri* Carry One *pthA* Homolog With 17.5 Repeats That Determines Pathogenicity on Citrus, but None Determine Host-Range Variation

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Citrus canker disease is caused by five groups of *Xanthomonas citri* strains that are distinguished primarily by host range: three from Asia (A, A*, and A^w) and two that form a phylogenetically distinct clade and originated in South America (B and C). Every *X. citri* strain carries multiple DNA fragments that hybridize with *pthA*, which is essential for the pathogenicity of wide-host-range *X. citri* group A strain 3213. DNA fragments that hybridized with *pthA* were cloned from a representative strain from all five groups. Each strain carried one and only one *pthA* homolog that functionally complemented a knockout mutation of *pthA* in 3213. Every complementing homolog was of identical size to *pthA* and carried 17.5 nearly identical, direct tandem repeats, including three new genes from narrow-host-range groups C (*pthC*), A^w (*pthAW*), and A* (*pthA**). Every noncomplementing paralog was of a different size; one of these was sequenced from group A* (*pthA*-2*) and was found to have an intact promoter and full-length reading frame but with 15.5 repeats. None of the complementing homologs nor any of the noncomplementing paralogs conferred avirulence to 3213 on grapefruit or suppressed avirulence of a group A* strain on grapefruit. A knockout mutation of *pthC* in a group C strain resulted in loss of pathogenicity on lime, but the strain was unaffected in ability to elicit an HR on grapefruit. This *pthC*⁻ mutant was fully complemented by *pthA*, *pthB*, or *pthC*. Analysis of the predicted amino-acid sequences of all functional *pthA* homologs and nonfunctional paralogs indicated that the specific sequence of the 17th repeat may be essential for pathogenicity of *X. citri* on citrus.

Additional keywords: *avr*, gene-for-gene, resistance, specificity.

All strains of *Xanthomonas citri* cause hyperplastic cankers on citrus that are diagnostic of the disease, but host-range varies

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among strain groups (Brunings and Gabriel 2003; Gabriel 2001). The Asiatic (A) group (*X. citri* pv. *citri* A) has the widest host range and is widespread throughout the world. The B and C groups (*X. citri* pv. *aurantifolii* B and C) have only been found in South America and have a reduced host range compared with the A groups (Stall and Seymour 1983). New groups of *X. citri* pv. *citri* (A^w from Florida and A* from Southwest Asia) were more recently discovered that are restricted in host range and pathogenic primarily on Mexican lime (*Citrus aurantifolia*) (Sun et al. 2004; Vernier et al. 1998). Grapefruit (*C. paradisi*) can serve as a differential host that is resistant to the A*, A^w, and C strains; the A^w and C strains elicit a strong hypersensitive response (HR) in grapefruit, while some A* strains show reduced or no symptoms on grapefruit but without evidence of an HR (Brunings and Gabriel 2003; Table 1). The molecular basis for avirulence of the A*, A^w, and C strains on grapefruit or the wide-host range of the A strains is unknown.

Pathogenicity gene *pthA* is required for both growth and pathogenicity of group A strain 3213 on citrus (Swarup et al. 1991, 1992). *pthA* is capable of conferring ability to cause canker-like symptoms to strains that cannot otherwise cause canker, such as *X. campestris* pv. *citrumelo* (Swarup et al. 1991) or even *Escherichia coli* carrying a functional *hrp* system (Kanamori and Tsuyumu 1998). A nearly identical homolog, *pthB*, is similarly required for pathogenicity of group B strain B69 and is found on a self-mobilizing plasmid that can transfer among *X. citri* strains (El-Yacoubi et al. 2007). All strains of *X. citri* tested carry *pthA* homologs (Cubero and Graham 2002; Mavrodieva et al. 2004). When *pthA* is transiently expressed in citrus using either *Agrobacterium tumefaciens* or particle bombardment, small canker-like lesions are elicited (Duan et al. 1999). Therefore, *pthA* and its homologs encode the primary causal effectors of the citrus canker disease phenotype.

pthA is the first member of the *avrBs3/pthA* gene family demonstrated to function for pathogenicity (Swarup et al. 1991, 1992). The vast majority of cloned or described *Xanthomonas* avirulence genes belong to this family; many have demonstrated pathogenicity functions (Leach and White 1996). Members of this gene family typically show 90 to 97% identity at the protein sequence level and all members encode from 5.5 to 28.5 nearly identical, 34 amino-acid, tandemly arranged, direct repeats that determine both pathogenic host specificity and gene-for-gene avirulence specificity (Gurlebeck et al. 2006; Leach and White 1996). The N-terminal region upstream

from the repeats appears to be important for type III secretion into plant cells (Szurek et al. 2002), while the C-terminal region downstream from the repeats appears to be important for nuclear import (Szurek et al. 2001; Yang and Gabriel 1995a) and eukaryotic transcriptional activation (Szurek et al. 2002; Zhu et al. 1998). A portion of the C-terminal region of AvrXa7 outside of the repeats is also recognized by rice resistance gene *Xa7* for avirulence (Yang et al. 2005), but this may be exceptional; the C-terminal regions of most AvrBs3/*pthA* family members are not known to generally determine pathogenic or avirulence specificity. Swapping repeat regions between members of the gene family generally and *pthA* specifically results in chimeric genes that confer one or both of the pathogenicity and avirulence phenotypes expected from the source genes (Herbers et al. 1992; Yang et al. 1994).

To date, no particular repeat sequence has been associated with a particular pathogenic or avirulence specificity. The nearly identical tandem direct repeat structure of *pthA* readily allows intragenic recombination, resulting in paralogs with altered pathogenicity or avirulence phenotypes, or both, including those eliciting an HR on citrus (Yang et al. 1995b), indicating a potential role for some *pthA* homologs in restricting the host range of some strains on citrus.

Conversely, the *pthA* homolog *aplI* from *X. citri* pv. *citri* group A has been suggested as a suppressor of the tobacco defense response (Ponciano et al. 2003), indicating a potential role for some *pthA* homologs in expanding an otherwise limited host range of some strains on citrus. Although *pthA* and its homolog *pthB* have been shown to be required for pathogenicity on citrus, no naturally occurring *pthA* homolog has been shown to play a role in determining the host range variation observed among the five known *X. citri* groups. *X. citri* group A strain 306 has been fully sequenced and four *pthA* homologs were identified (da Silva et al. 2002), but no functional information regarding these genes has been published to date.

The purpose of this study was to investigate the potential role of *pthA* homologs in determining the host-range variation observed among the five different *X. citri* groups on citrus. Representative strains from each of the five groups were analyzed to determine the likely number of *pthA* homologs carried, and DNA fragments of sufficient size to carry intact homologs of *pthA* were cloned and screened to identify all homologs from each strain. DNA fragments that hybridized with *pthA* from each of the three limited-host-range *X. citri* groups (C, A^W, and A*) were then screened to determine if they i) encoded functional homologs of *pthA* and ii) functioned for avirulence to limit host range on grapefruit. Grapefruit (*Citrus paradisi*) was chosen as a differential host, since it exhibits a readily scored HR against two of the narrow-host-range groups (Brunings and Gabriel 2003). DNA fragments that hybridized with *pthA* from wide-host-range *X. citri* group A strain 3213 were then screened to determine if they functioned to suppress avirulence on grapefruit in a narrow-host-range strain (A*). A secondary purpose was to compare the predicted amino acid sequences of the functional versus the apparently nonfunctional homologs from each of the five *X. citri* groups for sequence features that might be correlated with function.

RESULTS

Strains from all known *X. citri* groups carried a 3.4-kb *Bam*HI DNA fragment that hybridized with *pthA*.

All tested *X. citri* strains had at least two *Bam*HI DNA fragments of similar size that strongly hybridized to an internal 3.4-kb *Bam*HI fragment from *pthA* (Fig. 1A). Based on Southern hybridizations of all strains, using *Eco*R1 (Fig. 1B), *Pst*I, *Kpn*I, and *Eco*R1/*Kpn*I total DNA digests using the 3.4-kb

*Bam*HI probe (blots of the latter three digests not shown), all strains tested, other than 3213, appeared to carry only two DNA regions that hybridized with *pthA*. All strains tested, including 3213, appeared to have a common 3.4-kb *Bam*HI hybridizing fragment (of identical size to that of *pthA*), regardless of group, and at least one other band of slightly variable size, depending on the strain. Based on the hybridization intensities of both *Bam*HI- and *Eco*RI-digested DNA fragments from genomic (Fig. 1) and from plasmid DNA preps (not shown), all hybridizing DNA fragments appeared to be plasmid-borne regardless of strain, and the C and A^W strains appeared to carry their two hybridizing DNA fragments on one plasmid.

Each *X. citri* group carried one *pthA* homolog that was isofunctional to *pthA* and identical in size, with 17.5 repeats.

Using the internal *Bam*HI fragment of *pthA* as a probe, colony hybridization of *E. coli* carrying cloned group A^W strain X0053 plasmid DNA revealed eight colonies with hybridizing inserts. The plasmids from these colonies were designated as pAW5.1 to pAW5.8, and all carried 5-kb *Eco*RI-*Kpn*I hybridizing inserts of identical size (Table 2). Four of these were separately introduced into strain B21.2 (*pthA*::Tn5) and were screened for pathogenicity. All four clones complemented B21.2 to the same extent as pZit45 (*pthA*), pAB18.1 (*pthA* but trimmed of nearly all flanking DNA), and pQY96 (*pthB*), restoring ability to cause canker symptoms on both Duncan grapefruit and Mexican lime (Fig. 2; Table 1). The *pthA* homolog encoded on pAW5.2 was sequenced, was found to carry 17.5 tandem repeats, and was designated *pthAW* (GenBank accession number EF473085). The short stretch (460 bp) of upstream flanking DNA sequenced outside of the left terminal inverted repeat of *pthAW* was 99% identical to the sequence found flanking *pthA4* (corresponding to *pthA* of strain 3213) on plasmid pXAC64 of *X. citri* pv. *citri* 306 (GenBank accession number AE008925; da Silva et al. 2002), further confirming the taxonomic placement of X0053 in *X. citri* pv. *citri*.

Table 1. Phenotypic responses of all five *Xanthomonas citri* groups, mutant derivatives affecting *pthA* homologs and complementation of said mutant derivatives

Strains/plasmid	Mexican lime ^a		Duncan grapefruit ^b	
	Low	High	Low	High
3213 (A group)	+	+	+	+
B69 (B group)	+	+	+	+
C340 (C group)	+	+	HR	HR
Xc270 (A* group)	+	+	0	0
X0053 (A ^W group)	+	+	HR	HR
B21.2 (<i>pthA</i>)	0	0	0	0
B21.2/pZit45 (<i>pthA</i>)	+	+	+	+
B21.2/pAB18.1 (<i>pthA</i>)	+	+	+	+
B21.2/pAW20.2 (<i>pthA</i>)	+	+	+	+
B21.2/ pAW20.4 (<i>pthA1-3213</i>)	0	0	0	0
B21.2/ pAW20.7 (<i>pthA2-3213</i>)	0	0	0	0
B21.2/ pAW20.11 (<i>pthA3-3213</i>)	0	0	0	0
B21.2/pQY96(<i>pthB</i>)	+	+	+	+
B21.2/ pQY222 (<i>pthB₀</i>)	0	0	0	0
B21.2/pQYCI.1 (<i>pthC</i>)	+	+	+	+
B21.2/pQYCI.1 (<i>pthC₀</i>)	0	0	0	0
B21.2/pAW5.2 (<i>pthAW</i>)	+	+	+	+
B21.2/pAW12.1 (<i>pthA</i> *)	+	+	+	+
B21.2/pAW12.3 (<i>pthA</i> *2)	0	0	0	0
CIM1 (<i>pthC</i>)	0	0	HR	HR
CIM1/pZit45 (<i>pthA</i>)	+	+	HR	HR
CIM1/pQY96 (<i>pthB</i>)	+	+	HR	HR
CIM1/pQYCI.1 (<i>pthC</i>)	+	+	HR	HR

^a Low and high = inoculum density of 10⁴ to 10⁵ or 10⁸ to 10⁹CFU/ml, respectively. + = canker response phenotype; 0 = null response;

^b HR = hypersensitive response.

Similarly, colony hybridization of cloned group A* strain Xc270 plasmid DNA revealed three hybridizing clones, designated as pAW12.1 to pAW12.3. The inserts carried on pAW12.1 and pAW12.2 were 22-kb *EcoRI/HindIII* fragments; pAW12.3 carried a 6-kb *EcoRI/HindIII* fragment. When transferred to B21.2, pAW12.1 restored ability to cause canker symptoms on both grapefruit and lime to the same extent as *pthA*, *pthB*, and *pthAW* (Table 1). The *pthA* homolog encoded on pAW12.1 was sequenced, was found to carry 17.5 tandem repeats, and was designated *pthA** (GenBank accession number EF473086). pAW12.3 did not complement B21.2 on either host (Table 1). The *pthA** paralog from pAW12.3 was also sequenced and designated *pthA**-2 (GenBank accession number EF473087). *pthA**-2 carried only 15.5 tandem repeats. To verify that the lack of evident activity of *pthA**-2 was not due to a cloning artifact, the promoter region and Shine-Dalgarno (SD) sequence were verified as present on pAW12.3, and no premature stop codons or frameshifts were found in *pthA**-2.

Similarly, colony hybridization of cloned group C strain C340 plasmid DNA revealed several hybridizing clones of two different sizes, and again, representative clones of both sizes were selected for complementation tests. pQYC1.1 (20-kb *SalI* insert) and pQYC2.1 (6-kb *SalI* insert) were mobilized into

B21.2; only pQYC1.1 was found to complement B21.2, restoring canker symptoms on both grapefruit and lime to the same extent as *pthA*, *pthB*, *pthAW*, and *pthA** (Table 1). The *pthA* homolog encoded on pQYC1.1 was designated as *pthC*, was recloned into high-copy vector pUC119, forming pQY103.5, was sequenced (GenBank accession number EF473088), and was found to carry 17.5 tandem repeats. The short stretch (550 bp) of flanking downstream DNA sequenced outside of the right terminal inverted repeat of *pthC* was 99% identical to the downstream flanking sequence of *pthB* on plasmid pXcB of *X. citri* pv. *aurantifolii* B69 (GenBank accession number AY228335; El Yacoubi et al. 2007), further confirming the taxonomic placement of C340 in *X. citri* pv. *aurantifolii*.

The presumed *pthC* paralog on pQYC2.1 was designated as *pthC*₀.

Functional *pthA* homologs from narrow-host-range strains C, A*, and A^w do not confer the avirulence phenotype of the source strain on grapefruit.

Even when inoculated at high concentrations, none of the new *pthA* homologs that complemented B21.2 from the narrow-host-range *X. citri* groups (*pthAW*, *pthA**, or *pthC*) elicited an

Fig. 1. Southern blot of genomic DNA from the indicated *Xanthomonas citri* strains hybridized with the ³²P-labeled, 3.4-kb *Bam*HI internal fragment of *pthA*. **A.** *Bam*HI restriction digest. The horizontal black line indicates the common 3.4-kb band. **B.** *Eco*RI restriction digest. M, λ marker DNA digested with *Hind*III; Group A: 3213; Group B: B69; Group C: C340; Group A*: Xc205, Xc270, Xc280, Xc290, Xc322, Xc406; and Group A^w: X0053.

HR on grapefruit in B21.2 (Table 1). The symptoms observed in B21.2 on grapefruit were not distinguished from those elicited by the wild-type, wide-host-range strain 3213, from which B21.2 was derived (not shown). Hence, the HR phenotypes elicited by the narrow-host-range strains C and A^w were not caused by members of the *pthA* gene family.

Inactivation of *pthC* in *X. citri* pv. *aurantifolii* resulted in loss of pathogenicity on lime, but not loss of avirulence on grapefruit.

In order to determine the role of *pthC* in the pathogenicity and host range of group C strain C340 in citrus, marker interruption mutagenesis was carried out. Southern blot analysis showed that *pthC* had been interrupted in CIM1 (not shown). CIM1 was unable to cause canker symptoms on lime but elicited an HR in grapefruit that was as strong as the HR elicited by the wild-type strain C340 (Table 1). CIM1 was fully complemented by pZit45 (*pthA*), pQY96 (*pthB*), and pQYC1.1 (*pthC*) to elicit a wild-type response in lime, and these genes had no effect on the HR phenotype elicited on grapefruit. (Table 1).

None of the *pthA* homologs from Group A strain 3213 increased the host range of group A* strain 270 to include grapefruit.

A genomic library of *X. citri* pv. *citri* A strain 3213 was constructed, and 18 randomly picked clones all gave different restriction patterns, indicating random representation of inserts; the average size of the inserts was 39 kb (data not shown). Based on the Clark and Carbon (1976) formula, 610 clones were required to cover the whole *X. citri* 3213 genome with 99% probability. A total of 750 clones were maintained in *E. coli* DH5α and were stored in 15% glycerol at -80°C.

All four *pthA* homologs from group A strain 3213 were isolated and cloned from a 3213 total DNA library by colony hybridization, using an internal fragment of *pthA* as probe; pAW20.2, pAW20.4, pAW20.7, and pAW20.11 carried *pthA*, *pthA1-3213*, *pthA2-3213*, and *pthA3-3213*, respectively. The latter three genes appeared identical in size to *pthA1*, *pthA2*, and *pthA3* of sequenced group A strain 306 (data not shown; *pthA* is identical in amino acid sequence to *pthA4* of strain 306). Of the four plasmids, only pAW20.2 (*pthA*) comple-

Table 2. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5α	F ⁻ , <i>endA1</i> , <i>hsdR17</i> (r _K ⁻ m _K ⁻), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i>	Gibco-BRL (Gaithersburg, MD, U.S.A.)
<i>Xanthomonas citri</i>		
3213	Group A, wild type. ATCC 49118.	Gabriel et al. 1989
3213Sp	Spontaneous Sp ^r derivative of 3213, Sp ^r	Gabriel et al. 1989
B21.2	<i>pthA</i> ::Tn5- <i>gusA</i> , marker-exchanged mutant of 3213Sp, Sp ^r Km ^r	Swarup et al. 1991
B69	Group B, wild type, ATCC51301	Gabriel et al. 1989
B69Sp	Spontaneous Sp ^r derivative of B69, Sp ^r	El-Yacoubi et al. 2007
C340	Group C, wild type. ATCC 51302	Gabriel et al. 1989
C340Sp	Spontaneous Sp ^r derivative of C340, Sp ^r	This study
Xc205	Group A*, wild type	Verniere et al. 1998
Xc205	Spontaneous Rif ^r derivative of Xc205, Rif ^r	This study
Xc270	Group A*, wild type	Verniere et al. 1998
Xc270Rif	Spontaneous Rif ^r derivative of Xc270, Rif ^r	This study
Xc280	Group A*, wild type	Verniere et al. 1998
Xc290	Group A*, wild type	Verniere et al. 1998
Xc322	Group A*, wild type	Verniere et al. 1998
Xc406	Group A*, wild type	Verniere et al. 1998
X0053	Group A ^w , wild type	Sun et al. 2004
X0053Rif	Spontaneous Rif ^r derivative of X0053, Rif ^r	This study
BIM2	<i>pthB</i> ::pUFR004, marker-integrated mutant of B69Sp	El-Yacoubi et al. 2007
CIM1	<i>pthC</i> ::pUFR004, marker-integrated mutant of C340	This study
Plasmids		
pRK2013	ColE1, Km ^r , Tra ⁺ , helper plasmid	Figurski and Helinski 1979
pRK2073	pRK2013 derivative, npt::Tn7, Km ^s Sp ^r , Tra ⁺ , helper plasmid	Leong et al. 1982
pUC119	ColE1, M13 lg, Ap ^r , <i>lacZα</i> ⁺	Vieira and Messing 1987
pUFR004	ColE1, Mob ⁺ , Cm ^r , <i>lacZ</i> ⁺	De Feyter et al. 1990
pUFR043	repW, Mob ⁺ , <i>lacZα</i> ⁺ , Gm ^r , Nm ^r , <i>cos</i> , shuttle vector	De Feyter and Gabriel 1991
pUFR047	repW, Mob ⁺ , <i>lacZα</i> ⁺ , Par ⁺ , Gm ^r Ap ^r	De Feyter et al. 1993
pUFR053	repW, ColE1, Gm ^r , Cm ^r , Mob ⁺ , mob(P), <i>lacZα</i> ⁺ , Par ⁺	El-Yacoubi et al. 2007
pUFR071	repW, ColE1, Mob ⁺ , Cm ^r , Gm ^r , <i>lacZ</i> ⁺ , Par ⁺	Castaneda et al. 2005
pZit45	4.5-kb fragment containing <i>pthA</i> from 3213 cloned in pUFR047, Ap ^r	Swarup et al. 1992
pYD9.3	<i>pthA</i> from pZit45 recombined in pUC118, with <i>EcoRI/XbaI</i> and <i>HindIII/BglII</i> sites immediately flanking the <i>pthA</i> coding region, Ap ^r	Duan et al. 1999
pAB18.1	<i>EcoRI/HindIII</i> fragment of pYD9.3, containing <i>pthA</i> , in pUFR47	This study
pQY96	14-kb <i>HindIII</i> fragment containing <i>pthB</i> from B69 cloned in pUFR053	El-Yacoubi et al. 2007
pQY222	4.4-kb <i>EcoRI</i> fragment containing presumed <i>pthB</i> ₀ (paralog) in pUFR053	El-Yacoubi et al. 2007
pQY103.5	5-kb <i>SalI</i> fragment containing <i>pthC</i> from C340 cloned in pUC119	This study
pQYC1.1	5-kb <i>SalI</i> fragment containing <i>pthC</i> cloned in pUFR047	This study
pQYC2.1	20-kb <i>SalI</i> fragment containing presumed <i>pthC</i> ₀ (paralog) cloned in pUFR047	This study
pAW5.1- 5.8	5-kb <i>EcoRI-KpnI</i> fragments containing <i>pthAW</i> from X0053 cloned in pUFR047	This study
pAW12.1-12.2	22-kb <i>EcoRI/HindIII</i> fragments containing <i>pthA</i> [*] from Xc270 cloned in pUFR071	This study
pAW12.3	6-kb <i>EcoRI/HindIII</i> fragment containing <i>pthA</i> [*] 2 (paralog) from Xc270 cloned in pUFR071	This study
pAW20.2	36-kb <i>MboI</i> fragment containing <i>pthA</i> from 3213 cloned in pUFR043	This study
pAW20.4	17-kb <i>MboI</i> fragment containing <i>pthA1-3213</i> (paralog) from 3213 in pUFR043	This study
pAW20.7	32-kb <i>MboI</i> fragment containing <i>pthA2-3213</i> (paralog) from 3213 in pUFR043	This study
pAW20.11	40-kb <i>MboI</i> fragment containing <i>pthA3-3213</i> (paralog) from 3213 in pUFR043	This study

^a Rif = rifampin, Sp = spectinomycin, Cm = chloramphenicol, Ap = ampicillin, Gm = gentamycin, Km = kanamycin.

mented B21.2. When each of these clones was conjugated into the A* strain Xc270, none extended the host range of the strain to include Duncan grapefruit, and none affected its pathogenicity on Mexican lime (Fig. 3).

All *pthA* homologs that were isofunctional with *pthA* carried 17.5 direct tandem repeats and the 17th repeat was highly conserved.

The DNA sequences of all 13 available *pthA* homologs from *X. citri* (nine previously published and four determined in this study) were analyzed, and the predicted amino-acid sequences were found to be >75% identical (Table 3). Perhaps surprisingly, the amino-acid sequence similarities among those PthA homologs experimentally shown to exhibit pathogenicity function on citrus (i.e., PthA, Apl1, PthB, PthC, PthAW, and PthA*) were less overall than the similarity among homologs of similar geographic origins, including those experimentally examined and evidently lacking in pathogenicity function on citrus (i.e., Apl2, Apl3, and PthA*2; Table 3; Fig. 4). With the notable exception of Apl3, all PthA homologs from *X. citri* strains originating in Asia were more closely related to each other (all >92% identical) than to the PthA homologs shown in this study or elsewhere to function for pathogenicity (all >87% identical; Table 3; Fig. 4).

Comparative analysis of the nearly identical, 34-aa direct repeat regions of all 13 genes revealed three primary regions of variation within each repeat, at positions 3 and 4 (region 1), positions 11 to 13 (region 2), and positions 30 to 32 (region 3) (Fig. 5). In region 1, no particular set of amino acids was uni-

versally conserved among any of the repeats of canker-active *pthA* homologs, although P(3) was always followed by an acidic amino acid [i.e., either P(3)D(4) or P(3)E(4)] in repeats 1, 3, 7, 9, 10, 11, 14, 16, and 17. In regions 2 and 3 and only in repeat number 17 in each gene, N(12)G(13) in region 2 and Q(31)A(32) in region 3 were always present in active *pthA* homologs.

DISCUSSION

Southern hybridization analyses of strains representative of all five described groups of *X. citri* strains revealed a common 3.4-kb *Bam*HI band that hybridized with *pthA* and at least one other hybridizing band of different size in each strain. With the exception of group A strain 3213, which has four *Bam*HI fragments of similar size that hybridize with *pthA* (Yang and Gabriel 1995b), all other strains from all other groups, including the A*, A^w, B, and C groups, had only two such *Bam*HI fragments. Due to the very high level of sequence conservation among members of the gene family generally (Leach and White 1996), the slight size differences of these bands were likely due to variable numbers of 102-bp direct tandem repeats within the *Bam*HI fragment. DNA sequence analyses of 13 *pthA* homologs and functional testing together revealed that i) the 3.4-kb *Bam*HI fragment represented *pthA* homologs carrying 17.5 tandem internal repeats and ii) only those homologs carrying 17.5 repeats appeared to encode a pathogenicity gene that was required for elicitation of citrus canker disease. This includes genes *pthA**, *pthAW*, and *pthC* cloned in this study

Fig. 2. Complementation of group A strain 3213 knockout B21.2 (*pthA*::Tn5) using different *pthA* homologs cloned from group A^w strain X0053 in citrus. pAW5.2, pAW5.4, pAW5.5, and pAW5.8 carry different fragments that hybridized with *pthA* inoculated on grapefruit (left) and Mexican (Key) lime (right).

from the narrow-host-range A*, A^w, and C strains, respectively, as well as *pthA* from a wide-host-range Asiatic group A strain (Swarup et al. 1992) and *pthB* from a wide-host-range but phylogenetically distinct South American group B strain (El-Yacoubi et al. 2007). All five of these genes were isofunctional and fully complemented a *pthA*::Tn5 knockout mutation in Asiatic strain B21.2 without distinction on both lime and grapefruit.

There was no evidence that *pthA**, *pthAW*, or *pthC* conferred an avirulence phenotype to B21.2, despite being members of an *avr* gene family and despite the avirulence of the respective source strains on grapefruit. Indeed, the *pthC* knockout mutation in CIM1 eliminated pathogenicity in lime but did not affect the HR in grapefruit, which remained as strong as that elicited by the wild type. The HR elicited by the wild-type C group strain C340 is therefore independent of *pthC*. These results also suggest that the C, A*, and A^w strains carry as-yet-to-be identified *avr* genes that limit host range on grapefruit and which most probably do not belong to the *pthA* family.

The additional C340 and group A* strain Xc270 DNA clones that each carried a *Bam*HI fragment of a size other than 3.4 kb that hybridized with *pthA* did not complement B21.2. The Xc270 fragment that failed to complement B21.2 was further analyzed, and the *pthA* paralog encoded on the fragment, *pthA**-2, was sequenced. This noncomplementing paralog carried 15.5 repeats and appeared to have an intact promoter, a

SD region, and a full-length open reading frame. This gene was 97% identical to PthA2 and Apl2 (Table 3; Fig. 4) and carried the same number of repeats. All three of these genes appeared to be intact, and yet, neither *apl2* (Kanamori and Tsuyumu 1998) nor *pthA**-2 in the present study exhibited any effect on pathogenicity in the functional tests used.

The other three *pthA* paralogs cloned from group A strain 3213 also did not complement B21.2, confirming and extending the functional work of Kanamori and Tsuyumu (1998) on group A strain L-9. Although no functional studies have been reported to date of the four *pthA* homologs identified in the strain 306 genome (da Silva et al. 2002), pathogenicity function of the four strain 306 *pthA* homologs may be inferred based on their close similarity with the homologs cloned from strain 3213 and functionally evaluated. Strain 3213 genes *pthA1*-3213, *pthA2*-3213, *pthA3*-3213, and *pthA* corresponded in size to *pthA1*, *pthA2*, *pthA3*, and *pthA4* of strain 306, respectively. Furthermore, based on sequence comparisons, *pthA4* of strain 306 appears to be the equivalent of *pthA* (Brunings and Gabriel 2003).

The fact that all group A strains, which have the widest host range of any of the *X. citri* strains, carry two additional *pthA* homologs that are not present in the more narrow-host-range B, C, A*, and A^w strains suggested a possible enabling role of these homologs in conditioning host range. Indeed, an enabling potential of these homologs is strongly suggested by the

Fig. 3. Lack of phenotypic change on grapefruit and lime of A* strain Xc270 by transfer of *pthA* (on pAW20.2 and pZit45) and its three paralogs (*pthA1*-3213 on pAW20.4, *pthA2*-3213 on pAW20.7, and *pthA3*-3213 on pAW20.11) from group A strain 3213.

report that *apl1*, a *pthA* homolog that is functionally equivalent to *pthA*, suppressed the tobacco defense response and HR (Ponciano et al. 2003). However, when *pthA* or any of its 3213 paralogs (*pthA1*-3213, *pthA2*-3213, or *pthA3*-3213) were transferred into Xc270, no increase in host range of A* strain Xc270 to include grapefruit was observed (Fig. 3). Although additional *pthA* homologs in a given *X. citri* strain may contribute marginally to pathogenicity (Kanamori and Tsuyumu 1998), the primary value of multiple copies of the gene family in a given strain may be to facilitate recombination and the potential for rapid adaptation to new hosts (Gabriel 1999; Yang and Gabriel 1995b).

X. citri has a highly clonal population structure involving two basic clades, the Asiatic groups A, A*, and A^w and the South American groups B and C (Gabriel et al. 1988). Surprisingly, sequence variation among the known functional *pthA* genes from both groups (PthA, PthAW, PthA*, PthB, and PthC) was greater than the variation among the *pthA* homologs, both functional and evidently nonfunctional, within the A group (Table 1). Even the apparently nonfunctional paralogs within the A group were closer in sequence identity to the functional homologs within the A group than to the functional

homologs from the B and C groups. Since the functional PthA homologs from *X. citri* strains originating in South America (i.e., PthB and PthC) were 98% identical in sequence, these results taken together indicated independent and convergent evolution of the known functional *pthA* genes in the Asian and South American clades. These results also indicated that a detailed analysis of the tandem repeat region, which is known to determine the host specificity of these *pthA* genes, might be productive in terms of identifying regions critical for eliciting citrus canker disease.

All *pthA* homologs that are required for citrus canker disease from all five known *X. citri* groups carried exactly 17.5 repeats. All other homologs, even those nearly identical to *pthA* (e.g., from *X. citri* pv. *citri* group A) were evidently not required for canker and had a different number of repeats. However, deletion mutants of various repeats and numbers of repeats in *pthA* can result in a gene that confers a weak canker phenotype in citrus to B21.2 (Yang and Gabriel 1995b). In that study, tandem repeat numbers 1 through 5 and 16 to 17 were not affected in deletion derivatives capable of conferring canker. This indicates that the number of repeats may be less important than the relative location of the specific repeats within the gene.

Table 3. Amino-acid sequence identity values (%) among PthA homologs from all known host range groups of *Xanthomonas citri* strains

	PthA 17.5 ^a	PthA4 17.5	Apl1 17.5	PthAW 17.5	PthA* 17.5	PthA*2 15.5	PthA1 16.5	PthA2 15.5	PthA3 15.5	Apl2 15.5	Apl3 23.5	PthB 17.5	PthC 17.5
PthA	100	100	100	99	98	92	95	93	92	94	84	87	87
PthA4	...	100	100	99	98	92	95	93	92	94	84	87	87
Apl1	...	...	100	99	98	92	95	93	92	94	84	87	87
PthAW	...	...	...	100	97	92	95	93	92	93	84	87	87
PthA*	...	...	...	...	100	92	95	93	92	93	84	87	87
PthA*2	...	...	...	...	...	100	95	96	97	97	79	82	83
PthA1	...	...	...	...	...	...	100	95	95	95	81	84	85
PthA2	...	...	...	...	...	...	...	100	98	99	79	82	82
PthA3	...	...	...	...	...	...	...	...	100	98	79	82	82
Apl2	...	...	...	...	...	...	...	...	...	100	80	82	82
Apl3	...	...	...	...	...	...	...	...	...	...	100	75	75
PthB	...	...	...	...	...	...	...	...	...	...	...	100	98
PthC	...	...	...	...	...	...	...	...	...	...	...	...	100

^a Number of repeats.

Fig. 4. Neighbor-joining dendrogram depicting phylogenetic relationships among the predicted, published PthA homologs based on pairwise comparisons of PthA family members from different species and pathovars of *Xanthomonas citri*. AvrBs3 from *X. campestris* pv. *vesicatoria* is used to root the tree. Numbers at the nodes represent bootstrap values >50 (based on 100 replicates). Shaded homologs indicate experimental evidence of pathogenicity function on citrus except in the case of PthA4 of strain 306, which is predicted to be the functional equivalent of PthA, based on comparisons of all four strain 306 homologs with all four homologs found in strain 3213.

Three somewhat variable regions were found in the repeats of each of the sequenced genes, specifically at amino acid positions 3 and 4, 11 through 13, and 30 through 32 in each repeat (Fig.5). The aligned repeat regions of all active genes revealed that only amino acids N(12)G(13) in the second and Q(31)A(32) in the third variable regions of the 17th repeat were absolutely conserved, and P(3) was always followed by an acidic amino acid [i.e., either P(3)D(4) or P(3)E(4)] in the first variable region of all canker-active genes in repeat number 17. No such conservation of identical amino acids was found in any other repeat (Fig. 5). Interestingly, in the third variable region, the South American group B and C strains show sequence identity to Asiatic strains only in the 17th repeat [Q(31)A(32)]; none of the other repeats in this region of group B and C strains share this identity. Indeed, Q(31)A(32) is not present in any other PthB repeat. In addition, the deletion mutants evaluated by Yang and Gabriel (1995b) never affected the 17th repeat. Finally, P(3)E(4), N(12)G(13), and Q(31)A(32) are found in the last complete repeat of Apl3, a PthA paralog with no known active function. This may mean that even if *pthA* or a functional equivalent were mutated or lost entirely in any canker strain, any intragenic recombination event within the *apl3* homolog that resulted in a deletion derivative with 17.5 repeats might become an active *pthA* allele. Taken together, these results suggest that the specific sequence of the 17th repeat may be critical for pathogenicity of *X. citri*. This hypothesis is currently being tested.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media.

Strains of *E. coli*, *X. citri*, and plasmids used in this study are listed in Table 2, along with their relevant characteristics and source or reference. *E. coli* strains were grown in Luria-Broth (LB) medium at 37°C (Sambrook et al. 1989). *X. citri* were grown in PYGM (peptone yeast extract-glycerol-MOPS) medium at 30°C as described (Gabriel et al. 1989). Unless otherwise specified, antibiotics were used at the following final concentrations (µg/ml): rifampin (Rif), 75; spectinomycin (Sp), 35; chloramphenicol (Cm), 35; ampicillin (Ap), 100; gentamycin (Gm), 5; and kanamycin (Km), 25.

Recombinant DNA techniques.

X. citri total DNA was prepared as described (Gabriel and De Feyter 1992). Plasmids were isolated by alkaline lysis from *E. coli* (Sambrook et al. 1989) and *X. citri* (De Feyter and Gabriel 1991) as described. Southern hybridization was performed by using nylon membranes as described (Lazo et al. 1987).

DNA library construction.

Genomic DNA from the wide-host-range *X. citri* pv. *citri* group A strain 3213 was partially digested with *Mbo*I and size-fractionated on a sucrose gradient. Cosmid vector pUFR043 was used to make a DNA library of 3213 DNA

	Repeat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	Gene																							
Variable region I	PthA	PE	PE	PE	PE	PA	PE	PD	PQ	PE	PE	PE	PE	LD	PE	PD	PE	PE						
	PthA4	PE	PE	PE	PE	PA	PE	PD	PE	PE	PE	PE	PE	LD	PE	PD	PE	PE						
	Apl1	PE	PE	PE	PE	PA	PE	PE	PE	PE	PE	PE	PE	LD	PE	PD	PE	PE						
	PthA*	PE	PQ	PE	PE	PA	PE	PD	PQ	PE	PE	PE	LD	PE	PD	PE	PE	PE						
	PthAW	PE	PE	PE	LD	PE	PE	PE	PE	PE	PE	PE	PE	LD	PE	PD	PE	PE						
	PthB	PD	PA	PD	PA	PD	PA	PD	PA	PD	PD	PD	PD	PD	PD	PD	PD	PD						
	PthC	PD	PD	PD	PD	PD	PD	PD	PA	PD	PD	PD	PD	PD	PD	PD	PA	PD						
	PthA*-2	PE	PG	PE	PA	PA	PA	PE	PE	PE	PE	PA	PE	PE	PE	PE	PA							
	PthA1	PE	PD	PA	PA	PA	PA	PE	PE	PE	PA	PE	PE	PE	PE	PA	PE	PE						
	PthA2	PE	PE	PE	PE	PE	PD	PD	PQ	PE	PE	PE	PE	PE	LD	PE	PE	PE						
	PthA3	PE	PE	PE	PE	PE	PE	PE	PD	PD	PD	PD	PD	PE	PE	PE	PE							
	Apl2	PE	PE	PE	PE	PE	PE	PD	PQ	PE	PE	PE	PE	PE	LD	PE	PE							
	Apl3	PE	PE	PE	PE	PE	PE	PD	PQ	PE	PE	PE	PE	PE	PE	PE	PE	PE	PD	PE	PE	PE	PE	PE
Variable region II	PthA	SNI	SNG	SNI	SNI	SNI	SNG	SHD	SNG	SHD	SNG	SNG	SNG	SNG	SNS	SHD	SHD	CNG						
	PthA4	SNI	SNG	SNI	SNI	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNG	SNG	SNS	SHD	SHD	CNG						
	Apl1	SNI	SNG	SNI	SNI	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNG	SNG	SNS	SHD	SHD	CNG						
	PthA*	SNI	SNG	SNI	SHD	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNG	SNS	SHD	SNS	CNG	SNG						
	PthAW	SNI	SNG	SNG	SNG	SNS	SHD	SHD	SNS	SHD	SNG	SNC	SNG	SNG	SNS	SHD	SHD	CNG						
	PthB	SHD	SNG	SHD	SNG	SNI	SNG	SHD	SNG	SHD	SNI	SNI	SHD	SHD	SHD	SHD	SNG	SNG						
	PthC	SHD	SNG	SHD	SHD	SNI	SNG	SNI	SNG	SNI	SNI	SHD	SNG	SHD	SHD	SHD	SNG	SNG						
	PthA*-2	SNI	SNI	SN-	SNI	SN-	SNI	SHD	SHD	SNG	SNI	SHD	SNI	SHD	SHD	SHD								
	PthA1	SNI	SNG	SNI	SN-	SNI	SHD	SHD	SN-	SNI	SHD	SNI	SHD	SN-	SNI	SHD	SHD							
	PthA2	SNI	SHD	SNI	SHD	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNG	SNG	SNI	SNI								
	PthA3	SNI	SHD	SNI	SHD	SNI	SNG	SHD	SNG	SNG	SNG	SNI	SNI	SNI	SNI	SHD								
	Apl2	SNI	SNG	SNI	SHD	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNG	SNG	SNI	SHD								
	Apl3	SNI	SHD	SNI	SHD	SNI	SHD	SHD	SNG	SHD	SHD	SNG	SNI	SHD	SHD	SNI	SHD	SHD	SNG	SHD	SNG	SNG	SNI	SHD
Variable region III	PthA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	PthA4	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	Apl1	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	PthA*	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	PthAW	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	PthB	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ						
	PthC	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CEQ	CQA	CEQ	CEQ	CEQ	CEQ	CEQ	RQA	CEQ	CEQ						
	PthA*-2	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA							
	PthA1	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA						
	PthA2	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA							
	PthA3	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA							
	Apl2	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA							
	Apl3	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA	CQA

Fig. 5. Sequence alignment of the predicted amino acids encoded in the main variable portions of the repeat region of all 13 PthA homologs. Boxed areas indicate the regions in the 17th repeat that are conserved among all *pthA* homologs with experimental evidence of active pathogenic function. Variable regions I, II, and III represent the 3 to 4, the 11 through 13, and the 30 through 32 amino acids of each repeat, respectively.

fragments. This cosmid vector was split into two pools and cut with either *EcoRI* or *SaII* restriction enzyme to produce the two arms and treated with shrimp alkaline phosphatase. To create common cloning ends, the arms were then cut with *BamHI* and were used for ligations to the 20- to 25-kb 3213 DNA fraction. Recombinant DNA was packaged using Giga-pack III gold packaging extract (Stratagene, La Jolla, CA, U.S.A.) and was introduced into *E. coli* DH5 α via transfection as described by the manufacturer's protocol. Positive white plaques were then picked and placed onto LB plates containing Km. Colonies were transferred into 96-well microtiter plates containing liquid LB (with 14% glycerol) and were stored at -80°C . DNA from 18 randomly selected library clones was extracted, was digested with *BamHI* and was run on agarose gels in order to estimate insert size and evaluate the quality of the library.

Plant inoculations.

Duncan grapefruit and Mexican lime plants were grown, maintained, and inoculated under natural light in the quarantine greenhouse facility at the Division of Plant Industry, Florida Department of Agriculture (Gainesville, FL, U.S.A.). Temperatures in this greenhouse ranged from 25 to 35°C with 50 to 100% relative humidity.

Liquid cultures of *X. citri* strains were grown in PYGM at 30°C for 18 to 24 h. Cultures were centrifuged at 1,000 $\times$ g for 3 min at room temperature, were resuspended and adjusted to an optical density at 600 nm = 0.3 (approximately 10^8 CFU/ml) in sterile tap water saturated with CaCO_3 . Cultures were pressure-infiltrated at appropriate concentrations (10^5 CFU/ml for low and 10^8 for high) into the abaxial citrus leaf surface, using the blunt end of a tuberculin syringe as described (Gabriel et al. 1989). Observations were recorded 5 to 12 days after inoculation.

Southern hybridization analysis.

Genomic DNA from all canker-causing strains was isolated as described above, was digested with either *EcoRI* or *BamHI*, and was analyzed by electrophoresis on 0.6% agarose gels. DNA was then transferred onto GeneScreen Plus nylon membranes (DuPont, Wilmington, DE, U.S.A.) as described by the manufacturer. Membranes were hybridized with a ^{32}P -labeled *BamHI* internal fragment of *pthA*.

Colony hybridization.

Plasmid DNA from group A^w strain X0053 was digested with *EcoRI* and *KpnI* and was ligated into shuttle vector pUFR047. Recombinant DNA was transformed into DH5 α -competent cells, and transformed clones were selected on Ap and X-Gal/IPTG (isopropyl- β -D-thiogalactoside) in LB agar. White colonies were transferred onto a registry plate and pZit45 was included at specific positions as a control. Colonies from registry plates were replicated onto nylon membranes. Membranes were then placed colony side up on plain LB plates and were incubated for 2 to 4 h at 37°C. DNA was fixed on membranes as described by the manufacturer and were hybridized with a ^{32}P -labeled internal *BamHI* fragment of *pthA*. Similarly, plasmid DNA from group A* strain Xc270 was digested with *EcoRI* and *HindIII* and ligated into shuttle vector pUFR071; resulting plasmids were transformed into DH5 α , were selected on Cm and X-Gal/IPTG in LB agar, were replicated onto nylon membranes, and were hybridized as above. Similarly, plasmid DNA from Group C strain C340 was digested with *SaII*, was ligated into shuttle vector pUFR047 and transformed into DH5 α , was selected on Ap and X-Gal/IPTG in LB agar and replicated onto nylon membranes, and was hybridized as above.

Triparental matings.

Clones that hybridized to *pthA* were conjugated into *Xanthomonas* sp. strain B21.2 (*pthA*::Tn5) by triparental mating as described (De Feyter et al. 1990). Briefly, pRK2073 was used as a helper strain, B21.2 was concentrated 50- to 100-fold as recipient, and 10 μl each of recipient, donor, and helper grown in liquid overnight cultures without antibiotics were sequentially dropped, one on top of the other in the order given, on a PYGM plate without antibiotics and were allowed to grow overnight at 28°C. The mating mixture was then streaked onto PYGM plates containing Sp and Gm and was incubated at 28°C. Two to three days later, colonies that appeared were transferred onto fresh selection plates. Following single-colony purification, transconjugants were grown in liquid PYGM as described above and were infiltrated into Duncan grapefruit and Mexican lime for pathogenicity testing.

Marker integration mutagenesis.

Mutant CIM1 (*pthC*::pUFR004) was created by marker interruption mutagenesis exactly as described (El Yacoubi et al. 2007). Briefly, pYY40.10 (carrying a 2.0-kb internal *StuI*-*HincII* fragment of *pthA* cloned in pUFR004) was transferred by conjugation into C340Sp and was selected on Sp + Cm PYGM plates as described (El Yacoubi et al. 2007). The resulting colonies were screened by Southern blot for integration at *pthC*.

Sequence analysis of *pth* genes.

pthA homologs from the A*, A^w, and C strains were sequenced using primers based on the sequence of *pthA* (Swarup et al. 1992) and designed to cover the entire gene. Eight primers were used for sequencing reactions: DG8, gaggtgctgttgctaacgc; DG35, agttatctgccttgatc; DP35, caggtcactgaagctgccgc; DP36, gcgggcagcttcagtgcactg; DP37, ccgaaggttcgttcgaca; DP38, ctgtcgaacgaaccttcg; DP45, gcattggcgaatgcactgac; and YP03, tagtccatcaaccatgc. Sequencing was done at the University of Florida ICBR DNA Sequencing Core. When necessary, fragments were cloned into high-copy vectors such as pUC119 or pUC19 to obtain larger amounts of DNA.

Sequence assembly was by Vector NTI software (Invitrogen, Carlsbad, CA, U.S.A.). Nucleotide and predicted amino-acid sequence alignments were carried out with the program CLUSTAL W. The % amino acid identity was calculated using the needle program in EMBOSS, which uses the Needleman-Wunsch algorithm to do global alignment of sequences. The DNA sequences of *pthA* (Swarup et al. 1992) and *pthB* (El Yacoubi et al. 2007) were taken from GenBank accession numbers U28802 and AY228335, respectively. The DNA sequences of *pthA1*, *pthA2*, *pthA3*, and *pthA4* (da Silva et al. 2002) were taken from GenBank accession numbers NC_003921, NC_003921, NC_003922, and NC_003922, respectively. The DNA sequences of *apl1*, *apl2*, and *apl3* (Kanamori and Tsuyumu 1998) were taken from GenBank accession numbers AB021363, AB021364, and AB021365, respectively. The dendrogram showing phylogenetic relationships of these genes was generated with TREECON (version 1.3b) (Van de Peer and De Wachter 1994), using the neighbor-joining algorithm with Poisson correction. AvrBs3 from *X. campestris* pv. *vesicatoria* was used as an outgroup to root the phylogenetic tree. The percentage of 100 bootstrap resamples supporting the topology is indicated when the percentage is above 50.

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