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Identification of *Xanthomonas citri* ssp. *citri* host specificity genes in a heterologous expression host

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SUMMARY

We provide the first conclusive evidence that *Xanthomonas axonopodis* pv. *citri* Asiatic strain (Xac-A) and, in particular, Xac-A^w, a unique citrus canker A strain isolated from Key lime in Wellington, Florida, induces a hypersensitive reaction (HR) in grapefruit leaves. Using the heterologous tomato pathogen *X. perforans*, as a recipient of the Xac-A^w genomic library, we identified a 1599-bp open reading frame responsible for HR in grapefruit, but not Key lime, and designated it *avrGf*1. Xac-A^{w}Δ*avrGf*1 produced typical, although visibly reduced, citrus canker symptoms (i.e. raised pustules) in grapefruit and typical canker symptoms in Key lime. We also determined that the *X. perforans* transconjugant carrying an Xac-A^w *hrpG* elicited HR in grapefruit and Key lime leaves, and that *xopA* in *X. perforans* was partly responsible for HR. Xac-A transconjugants carrying the *X. perforans xopA* were reduced in ability to grow in grapefruit leaves relative to wild-type Xac-A. The *X. perforans xopA* appears to be a host-limiting factor. An *avrBs3* homologue, which contained 18.5 repeats and induced HR in tomato, was designated *avrTaw*. This gene, when expressed in a pustule-minus Xac-A^w, did not complement pustule formation; however, *pthA^w*, a functional *pthA* homologue, complemented the mutant strain to produce typical pustules in Key lime, but markedly reduced pustules in grapefruit. Both *avrBs3* homologues, when expressed in a typical Xac-A strain, resulted in typical citrus canker pustules in grapefruit, indicating that neither homologue suppressed pustule size in grapefruit. Xac-A^w contains other unidentified factors that suppress development in grapefruit.

INTRODUCTION

Until recently, citrus canker was known to be initiated by two pathovars of *Xanthomonas axonopodis*. These pathovars, i.e. *X. axonopodis* pv. *citri* (Xac) and *X. axonopodis* pv. *aurantifolii* (Xaa), have been distinguished by genetic differences and phenotypic traits. Recently, a change in nomenclature of these two pathovars has been proposed to *X. citri* ssp. *citri* and *X. fuscans* ssp. *aurantifolii*, respectively (Schaad *et al.*, 2005). Groups of strains exist within each pathovar that have different pathogenicities. The most destructive strains causing Asiatic citrus canker, which are members of the pathovar *citri*, are termed the A form and are designated Xac-A. Xac-A strains have a broad host range within Rutaceae, including grapefruit and Key lime (Table 1). Other strains exist that are genetically very similar to Xac-A, but have a limited host range (Sun *et al.*, 2004; Verniere *et al.*, 1998). Verniere *et al.* (1998) designated strains restricted to Key/Mexican lime as Xac-A*, based on their physiological and genetic similarities and serological differences from Xac-A. Sun *et al.* (2004) designated strains that were pathogenic on Key lime, but that did not cause canker symptoms on grapefruit, as Xac-A^w (Table 1). More distantly related strains associated with citrus canker include Cancrosis B, or false canker (formerly known as B-strain canker), discovered on lemon (*Citrus limon*) in Argentina in 1923 (Civerolo, 1984), and found primarily on *C. limon* and *C. aurantiifolia*, but also affecting *C. aurantium*. Another strain, found to be associated with Key/Mexican lime cancrosis (formerly known as C-strain canker), was observed in Brazil on Key/Mexican lime (*C. aurantiifolia*) in 1963 and only infects this

Table 1 Reaction of citrus xanthomonads and *Xanthomonas perforans* on citrus.

Xanthomonad groups used in this study	Disease reaction in:		
	Grapefruit	Key lime	Tomato
X. axonopodis pv. *citri*			
Xac-A	+	+	(HR)
Xac-A^w	–	+	(HR)
Xac-A*	–	+	
Xaa-B	±	+	
Xaa-C	–	+	(HR)
X. perforans	– (NR)	– (NR)	+

+, typical disease reaction; ±, weak citrus bacterial canker disease; –, inability to cause typical disease reaction on host; HR, hypersensitive reaction; NR, null reaction.

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citrus host (Stall and Civerolo, 1991). The causal bacteria of the latter diseases that produce a very similar canker syndrome on their limited citrus hosts are genetically related to each other, but different from Xac-A, and therefore have been referred to as strains of *X. axonopodis* pv. *aurantifolii* (Xaa) (Gabriel *et al.*, 1989).

The symptoms of citrus canker include erumpent lesions on fruit, foliage and young stems of susceptible citrus cultivars (Gottwald *et al.*, 1997, 2002a,b). The occurrence of citrus canker lesions on fruit rind decreases the commercial quality, and infected fruit is not accepted by most important markets (Canteros, 2004). Warm weather, together with rains accompanied by the strong winds that occasionally occur, creates ideal conditions for the spread of Xac-A (Gottwald *et al.*, 1997, 2002a,b). Optimum temperatures for infection range between 20 and 30 °C (Koizumi, 1985).

The pathogen of Asiatic citrus canker was brought to North America in 1910 and became distributed throughout the Gulf States, according to the review by Loucks (1934). The eradication of the pathogen began in Florida in 1914 and was declared to be complete in North America in 1947 (Dopson, 1964). The Xac-A pathogen was rediscovered in 1986 in Manatee County, Florida and declared to be eradicated in 1994, but was found again in the area later (Cubero and Graham, 2002; Schubert *et al.*, 1996). A new focus of a different strain of Xac-A appeared in Miami, Florida, in 1995 (Gottwald *et al.*, 2002a; Schubert *et al.*, 2001), and an eradication programme was begun again. Successful eradication of Xac-A has occurred in the USA, South Africa, Australia and New Zealand. Xac-A is endemic to all countries in eastern and southern Asia, Argentina, Paraguay and Uruguay. An eradication programme currently exists in Brazil (Schubert *et al.*, 2001). As a result of the catastrophic weather conditions in 2004 and 2005, Xac-A spread extensively in Florida and eradication efforts were suspended in 2006.

Schubert *et al*. (1996) described at least two separate introductions of Xac in Florida after 1986. A group of strains that is pathogenic to Key lime and alemow plants, but not to grapefruit and orange, occurred in one of the introductions. The group was characterized (Cubero and Graham, 2002; Sun *et al.*, 2004) and found to be related genetically to Xac, rather than Xaa, and designated as Xac-A^w to represent the unique group of strains associated with Key lime but not grapefruit trees in Wellington, Florida. Sun *et al.* (2004) distinguished the Xac-A^w strains from the Xac-A* strains by physiological differences.

The aim of this work was to investigate the presence of host-limiting factors in the Xac-A^w strain. The Xac-A^w strain was thought to cause a hypersensitive reaction (HR) in grapefruit based on the observations of Sun *et al.* (2004), and was investigated. An HR caused by bacteria is usually the result of an avirulence gene in the bacterial genome interacting with a resistance gene (R) in the host (Bonas *et al.*, 1993; Crute, 1985; Minsavage *et al.*, 1990; Staskawicz *et al.*, 1984, 1987). If avirulence of the Xac-A^w strain in grapefruit is the result of the presence of a single gene in the bacterium, a change of virulence might occur by mutation (Dahlbeck and Stall, 1979; Gassmann *et al.*, 2000; Kearney and Staskawicz, 1990; Swords *et al.*, 1996). Analysis of the Xac-A^w strain was undertaken to determine whether a change in virulence to grapefruit of the Xac-A^w strain would occur. In addition, isolation of a postulated avirulence gene in the genome of Xac-A^w, which interacted with grapefruit leaves in a hypersensitive manner, was undertaken. Using a heterologous bacterial host (i.e. *X. perforans*, a tomato pathogen, which causes a null reaction when infiltrated into citrus leaves) as a recipient of the Xac-A^w library, factors associated with host specificity were identified and are presented.

RESULTS

HR in grapefruit leaves inoculated with Xac-A^w

Fleck lesions occurred in grapefruit leaves and typical canker lesions occurred in Key lime leaves when they were infiltrated with bacterial suspensions of Xac-A^w strain 12879 of approximately 10^3 colony-forming units (cfu)/mL (Fig. 1). Following isolation from approximately 30 representative fleck lesions, the bacterium was recovered from less than 25% of the lesions.

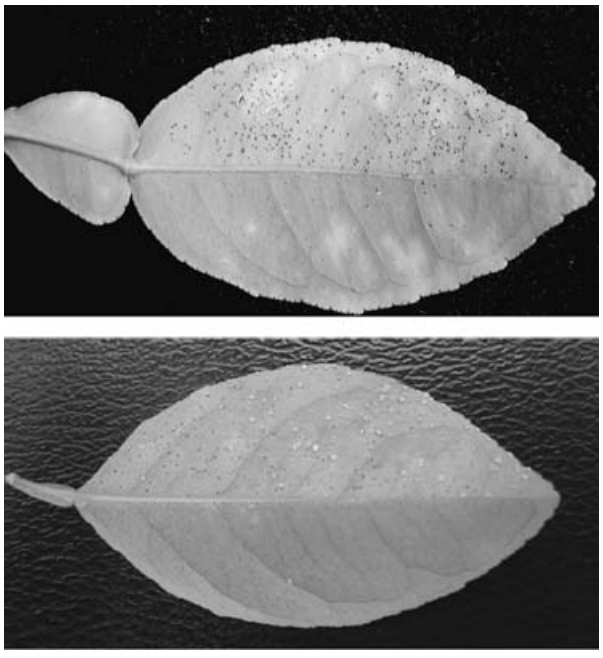

Fig. 1 Fleck lesions (above) and typical citrus canker lesions (below) caused by *Xanthomonas citri* ssp. *citri* strain Xac-A^w in grapefruit and Key lime leaves, respectively, 30 days after infiltration of the top half of each leaf with inoculum (10^3 cfu/mL).

Representative colonies from the remaining isolations, when inoculated into grapefruit and Key lime, produced typical canker symptoms in Key lime, but a necrotic reaction in grapefruit, indicative of an incompatible interaction. After infiltration of grapefruit leaves with inoculum adjusted to 5×10^8 cfu/mL, internal bacterial populations of Xac-A (strain A 40) and Xac-A^w (strain 12879) were similar through the second day, but populations of Xac-A were significantly greater than those of Xac-A^w after 6 days (similar to the data shown in Fig. 4, bottom). Electrolyte leakage from leaf tissue samples inoculated with Xac-A^w was similar to that of leaf tissue samples inoculated with Xac-A after 2 days. However, electrolyte leakage values for leaves infiltrated with the Xac-A^w bacterium were significantly greater than those of leaves infiltrated with the Xac-A bacterium at 4 and 6 days (similar to the data presented in Fig. 4, bottom). The data are typical for HR caused by the Xac-A^w strain, and similar to HR occurring in other plants and caused by other xanthomonads. However, HR in grapefruit leaves differs from that in other systems by being slow to occur (Astua-Monge *et al.*, 2000; Minsavage *et al.*, 1990, 2004).

Selection of host-limiting genes from the strain Xac-A^w library

Three clones were selected from a genomic library of the 12879 strain of Xac-A^w that caused rapid necrosis in grapefruit leaves, but not in tomato leaves when they were expressed in *X. perforans*. These three clones were designated pL22, pL689 and pL799. All three clones were successfully transferred from *Escherichia coli* by triparental matings into a strain (Xac-A 40) that causes Asiatic citrus canker in grapefruit and Key lime leaves. Only one of the three clones (pL799) caused HR in grapefruit leaves and a susceptible reaction in Key lime leaves when expressed in the Xac-A strain. The disease reaction of Xac-A containing the pL799 clone, when infiltrated into grapefruit leaves, was typical of the reaction of the Xac-A^w strain on these hosts. The presence of pL22 and pL689 clones in Xac-A 40 had no effect on disease reaction. Four clones were selected that caused HR in tomato, but not in grapefruit. All of these clones were investigated further.

DNA analysis of pL799

A 1599-bp open reading frame (ORF) was found within the nucleotide sequence of DNA from a 2.3-kb subclone from pL799 that caused HR in grapefruit leaves. The complete sequence of the ORF, designated as *avrGf*1, was submitted to GenBank and was assigned accession number DQ275469. The upstream region of *avrGf*1 does not contain an *hrp* box, but does contain an imperfect PIP box, TTCGT-N10-TTCGC (Huguet and Bonas, 1997), 80 bp upstream of the start codon. A GenBank search identified significant homology of *avrGf*1 with gene XCC3600, which is a hypothetical protein (AM428701) in the genome of *X. campestris* pv. *campestris* (Xcc33913), a hypothetical protein (ABM30884.1) in *Acidovorax avenae* ssp. *citrulli*, *HopG1* (AAZ34904.1) in *Pseudomonas syringae* pv. *phaseolicola*, HopG1 (NP_794468.1) in *P. syringae* pv. *tomato*, a putative type III effector protein (CAD17474.2) in *Ralstonia solanacearum*, and a hypothetical conserved protein (ABC92870.1) in *Rhizobium etli*. Alignment analysis of *avrGf*1 with AM428701 from *X. campestris* pv. *campestris* using Clustal (1.82) multiple sequence alignment indicated 84.99% identity at the amino acid level.

Presence of *avrGf*1 in xanthomonads pathogenic to citrus

Primers were selected for the amplification of a fragment containing *avrGf*1 by polymerase chain reaction (PCR). A fragment of the expected size was amplified from the genomic DNA of four Xac-A^w strains, but not from the genomic DNA of three Xac-A strains. In Southern hybridizations with the labelled DNA of clone pU799-3 as a probe, the probe hybridized strongly with the DNA of the Xac-A^w strain, but not with the DNA of Xac-A strains. Reactions did not occur with the DNA of a strain of each of the B and C groups of Xaa, with the DNA of four strains of the Xac-A* group or with the DNA of a strain of *X. axonopodis* pv. *citrumelo*, the pathogen causing bacterial spot of citrus (Fig. 2). The Xaa-B and *X. axonopodis* pv. *citrumelo* strains did not cause rapid necrosis in grapefruit leaves. The Xaa-C strain caused rapid necrosis in grapefruit leaves phenotypically different from that caused by Xac-A^w. In addition, some Xac-A* strains caused rapid necrosis in grapefruit leaves, and one of the strains, Xac-A* 1974, was included in probing. The probe hybridized to a band in the DNA of *X. campestris* pv. *campestris* (Xcc) strain 8004, but the hybridization pattern was different from that of the DNA of Xac-A^w (Fig. 2). Strain Xcc 8004 contained gene XCC3600 described above and caused rapid necrosis in grapefruit leaves. The *avrGf*1 probe did not hybridize to the plasmid DNA of Xac-A^w strain 12879 (Fig. 2).

Effect of knockout of *avrGf*1 in Xac-A^w on disease reaction in grapefruit

A strain with a mutated *avrGf*1 gene (Xac-A$^w\Delta$*avrGf*1) was compared with the wild-type Xac-A and Xac-A^w strains by inoculation into grapefruit leaves by a pin-prick method with inocula adjusted to 5×10^8 cfu/mL. Visually, the symptoms caused by the Xac-A$^w\Delta$*avrGf*1 strain were more similar to those produced by the wild-type Xac-A strain than to those produced by the wild-type Xac-A^w strain, in that raised pustules were observed and an expanding lesion developed (Fig. 3a). The symptoms of Xac-A$^w\Delta$*avrGf*1 were less severe than those produced

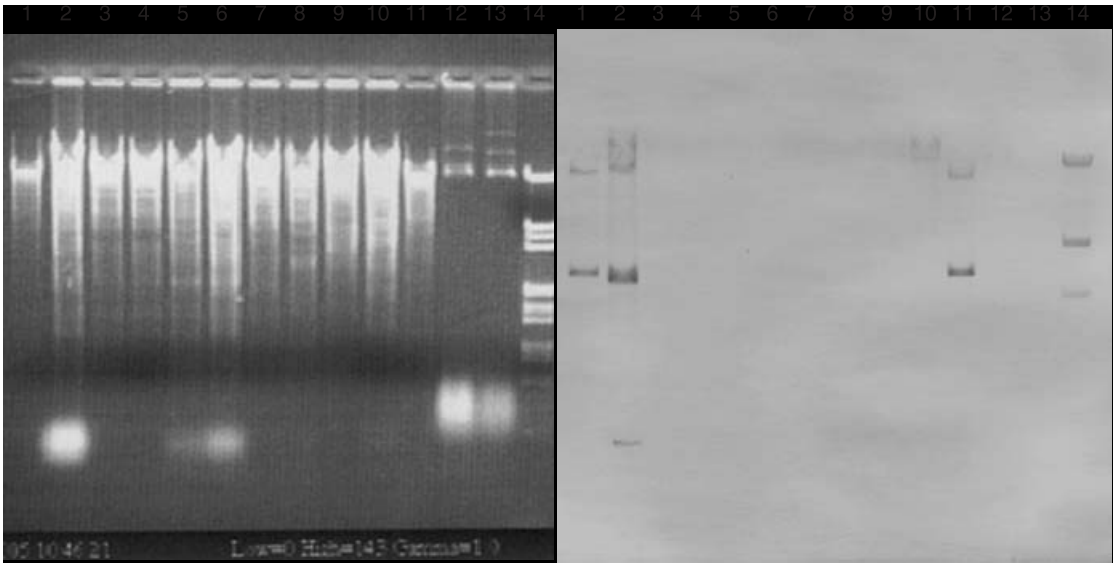

Fig. 2 Hybridization (right) of the subclone pU799-3 containing *avrGf*1 with total genomic DNA of *Xanthomonas* strains digested with *Hin*dIII and plasmid DNA (left). Lane 1, Xac-A^{w} 12879; lane 2, Xac-A^{w} Ω; lane 3, Xac-A 40; lane 4, Xac-A 306; lane 5, Xac-A* 1974; lane 6, Xac-A* 1975; lane 7, Xaa-B; lane 8, Xaa-C; lane 9, Xac-E 1887; lane 10, Xcc 8004; lane 11, Xac-A^{w} 12879; lane 12, plasmid Xac-A^{w}; lane 13, plasmid Xac-A 40; lane 14, λ marker digested with *Eco*RI and *Hin*dIII.

by the Xac-A strain. Nevertheless, mutation of the *avrGf*1 gene in Xac-A^{w} resulted in a disease reaction in grapefruit leaves.

Internal bacterial populations and electrolyte leakage from grapefruit leaves infiltrated with strains Xac-A 40, Xac-A^{w} 12879, Xac-A 40 (pU799-3), Xac-A^{w}Δ*avrGf*1 and Xac-A^{w}Δ*avrGf*1 (pU799-3), at a concentration of 5×10^{8} cfu/mL, were compared (Fig. 4). Populations of all strains were about equal for the first 4 days after inoculation. However, at day six, the population of the Xac-A^{w} strain was significantly smaller than that of the Xac-A strain. At day ten, the population of the Xac-A strain was largest, and the population of the Xac-A^{w} strain was about 1.5 log units lower. The population of the Xac-A^{w}Δ*avrGf*1 strain was intermediate between those of the Xac-A and Xac-A^{w} strains. The strains containing the pU799-3 clone (*avrGf*1) showed the smallest populations. Complementation of the mutated Xac-A^{w} strain, Xac-A^{w}Δ*avrGf*1, with pU799-3 was successful. The strains Xac-A 40, Xac-A^{w} 12879, Xac-A^{w}Δ*avrGf*1 and Xac-A^{w}Δ*avrGf*1 (pU799-3) all multiplied equally for 12 days in Key lime leaves after infiltration of a low concentration (5×10^{5} cfu/mL) of inoculum into the leaves (data not included).

The electrolyte leakage from leaf tissue inoculated with the Xac-A^{w} strain and strains containing *avrGf*1 (pU799-3) started to increase at day four relative to the leakage from leaf tissue infiltrated with the Xac-A and Xac-A^{w}Δ*avrGf*1 strains (Fig. 4). At day eight, the strains with *avrGf*1 caused significantly greater electrolyte leakage than the strains that did not contain the gene. Electrolyte leakage caused by the Xac-A and Xac-A^{w}Δ*avrGf*1 strains was similar.

*avrGf*1 requires a functional type III secretion system (TTSS) for HR

Transconjugants of *X. perforans* strains 91-118*hrp*$^{-}$ and 91-118*hrp*$^{+}$ containing the clone pU799-3 (*avrGf*1) were grown overnight, and inocula were prepared and adjusted to 5×10^{8} cfu/mL and were infiltrated into grapefruit leaves. The *hrp*$^{-}$ strain did not cause HR, but the *hrp*$^{+}$ strain did. Thus, an active TTSS appears to be necessary for HR produced by *avrGf*1.

Identification of non-host effector by overexpression of *X. citri* ssp. *citri* HrpG in *X. perforans*

Clones pL22 and pL689 were determined to be overlapping on the basis of their similar restriction enzyme digestion fragment profiles using electrophoresis. Partial digestion of pL22 with *Sau*3A resulted in a subclone containing 5041 bp, which produced an HR in grapefruit leaves when expressed in *X. perforans*. The nucleotide sequence of the subclone contained ORFs that were homologous to *hrpX*, *hrpG* and *Hsp*90Xo (heat shock protein molecular chaperone) genes. Subsequently, a subclone containing only *hrpG* (p0346) was obtained, which produced an HR in grapefruit leaves when expressed in *X. perforans*, but not in cells of Xac-A. As *hrpG* is a key regulatory gene for the transcriptional activation of pathogenicity genes (Wengelnik *et al.*, 1996, 1999), a factor in addition to *hrpG* in *X. perforans* was speculated to be necessary to elicit HR in grapefruit leaves. Therefore, we attempted to determine the factor(s) involved in the elicitation of HR by *X. perforans* in

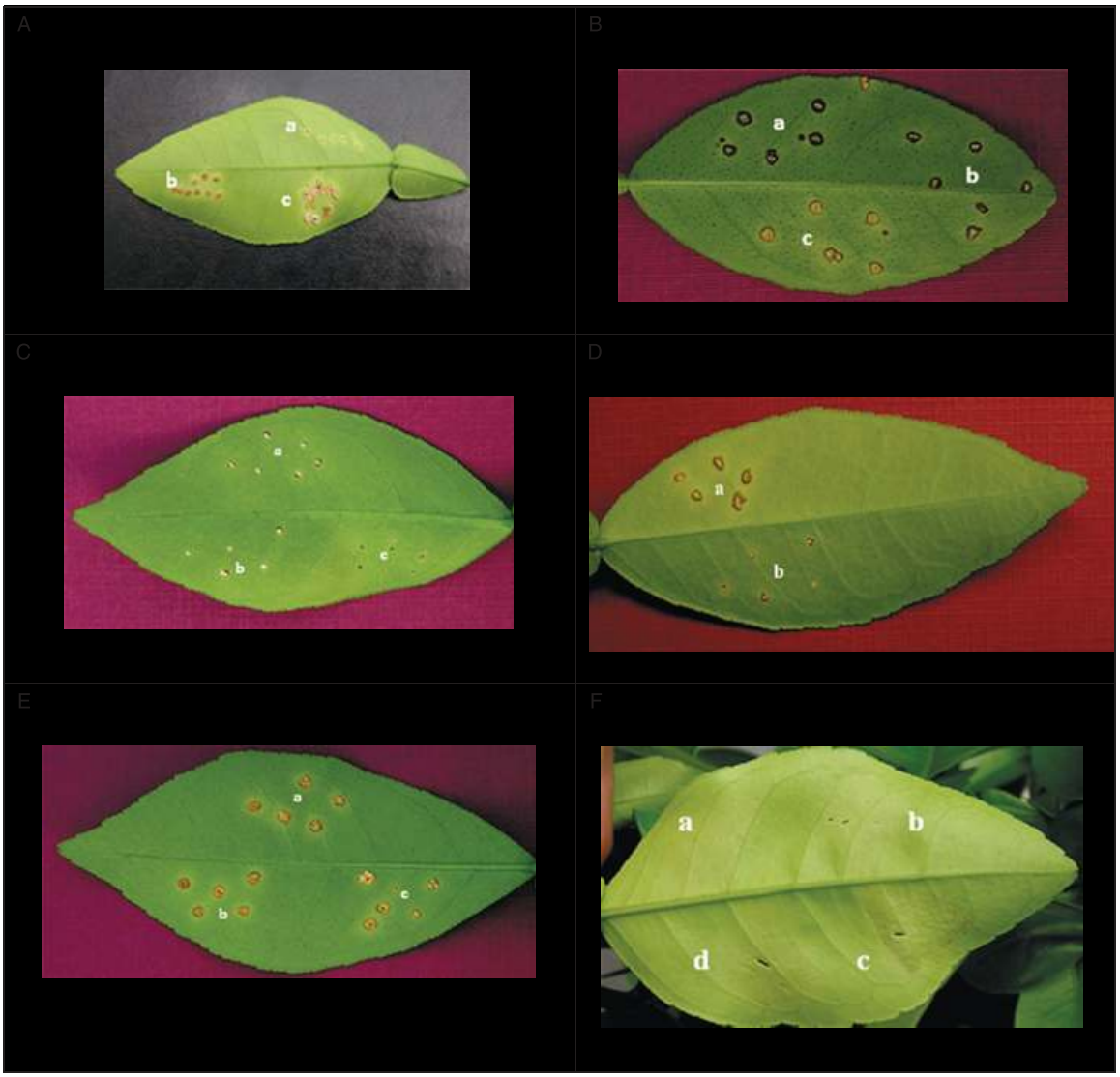

Fig. 3 Disease symptoms. (A) Grapefruit leaf after inoculation by needle punctures with Xac-A^{w} (a), Xac-A^{w} Δ*avrGf*1 (b) and Xac-A 40 (c). (B) Key lime leaf following inoculation with Xac-A^{w} Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pUFR034 (a), Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pAW5.2 (b) and Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pUFR80.1 (c). (C) Grapefruit leaf following inoculation with Xac-A^{w}Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pAW5.2 (a), Xac-A^{w}Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pUFR80.10 (b) and Xac-A^{w}Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pUFR034 (c). (D) Grapefruit leaf following inoculation with Xac-A^{w} Δ*avrGf*1 (a) and Xac-A^{w}Δ*avrGf*1Δ*avrTaw*D*pthA*^{w}::pAW5.2 (b). (E) Grapefruit leaf following inoculation with Xac-A40::pAW5.2 (a), Xac-A40::pUFR80.1 (b) and Xac-A40::pUFR034 (c). (F) Grapefruit leaf following inoculation with *Xanthomonas perforans* strains 91-118D*xopA* (a), 91-118 (b), 91-118+*hrpG* (c) and 91-118 D*xopA*+*hrpG* (d).

grapefruit leaves when *hrpG* was present *in trans*. The possibility that *xopA* might be involved was suggested from a similar study with *X. axonopodis* pv. *glycines* (Kim *et al*., 2003). The gene *hpaG*, together with an additional copy of *hrpG*, was necessary for HR in tobacco caused by that bacterium. Therefore, a clone (pLXOPA) containing *xopA*, a homologue of *hpaG*, was identified in a pLAFR3 cosmid library of *X. perforans* 91-118 by PCR. The clones p0346 (*hrpG*) and pLXOPA (*xopA*) were conjugated into Xac-A 306 individually or in combination. A strong HR occurred in grapefruit leaves inoculated with Xac-A 306 containing both p3046 and pLXOPA, but not with Xac-A 306 cells alone, or containing p0346 or pLXOPA alone. The populations of the four strains in grapefruit leaves were also determined at different times after the infiltration of leaves with inocula adjusted to 5×10^5 cfu/mL. The populations of Xac-A 306 were 100-fold higher than those of Xac-A 306 containing both p0346 and pLXOPA, 15 days after inoculation of the leaves (Fig. 5). The populations of Xac-A 306 were 10-fold higher than those of

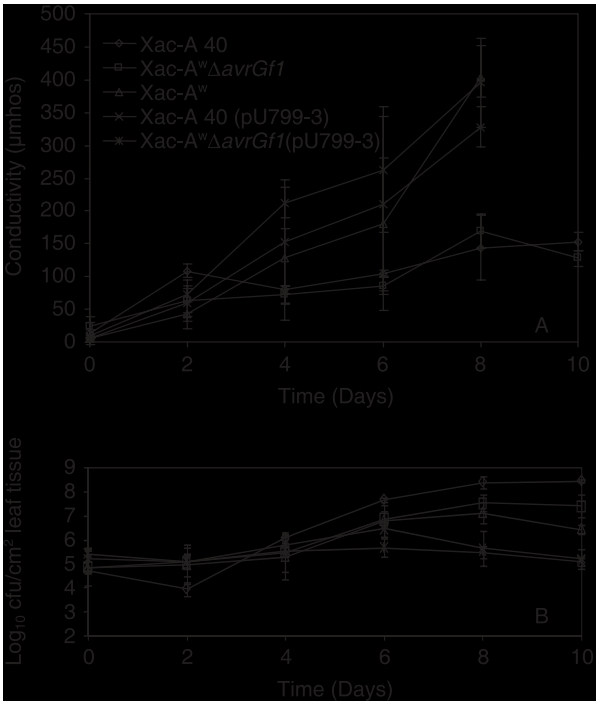

Fig. 4 Conductivity readings (top) and bacterial populations (bottom) in grapefruit leaves at different times after inoculation with 5×10^8 cfu/mL of Xac-A 40, Xac-A^{w}Δ*avrGf*1, Xac-A^w 12879, Xac-A 40 (pU799-3) or Xac-A^{w}Δ*avrGf*1 (pU799-3).

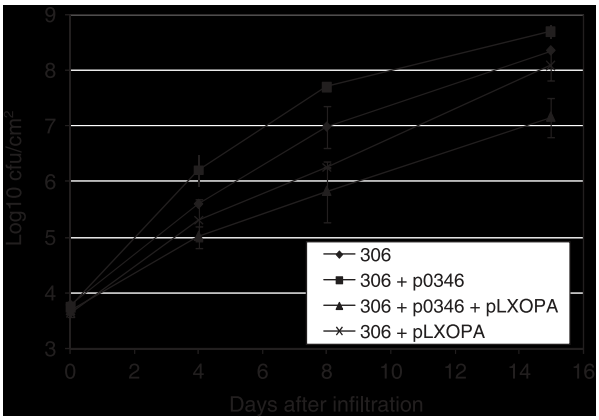

Fig. 5 Populations of Xac-A 306, Xac-A 306 containing p0346 (plasmid containing *hrpG* from Xac-A^w), p0346+pLXOPA (pLAFR3 cosmid that contains the *xopA* gene from *Xanthomonas perforans*) and pLXOPA alone in grapefruit leaves at different times after infiltration of 5×10^5 cfu/mL of each strain into the mesophyll.

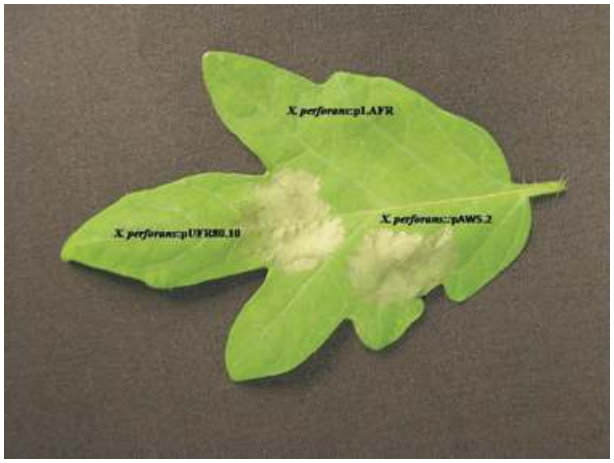

Fig. 6 Reaction of Bonny Best tomato leaflet following infiltration with *Xanthomonas perforans* transconjugants carrying pAW80.1 (subclone of pL80), pthA^w and pLAFR. Note that clones carrying pAW80.1 and pAW5.2 induced a hypersensitive reaction.

Xac-A 306 containing pLXOPA alone. The populations of Xac-A 306 containing p0346 were significantly larger than those of Xac-A 306.

In order to determine the role of the *xopA* gene in *X. perforans*, the gene was deleted by suicide-assisted mutagenesis, and the clone containing *hrpG* was conjugated into the mutated *X. perforans* strain 91-118::Δ*xopA*. Following the infiltration of grapefruit leaves with a cell suspension of the mutant strain adjusted to 5×10^8 cfu/mL, there was an observable reduction in necrosis compared with wild-type *X. perforans* containing an additional *hrpG* clone (Fig. 3f). The reaction could not be considered as a null reaction, but the deletion of *xopA* had a pronounced effect on the development of HR. However, there may be another gene in the pLXOPA clone that also contributes to HR in grapefruit leaves caused by wild-type *X. perforans* containing an additional *hrpG*.

Role of *avrBs3* homologues from Xac-A^w in tomato HR and in virulence in grapefruit and Key lime

Strains of both Xac-A and Xac-A^w caused HR in tomato. Four clones (pL80, pL104, pL115 and pL622) were selected from the Xac-A^w library that caused HR in tomato (Fig. 6), but not in grapefruit (data not shown), when expressed in *X. perforans*. These clones all contained an *avrBs*3 homologue, based on hybridization to a probe containing *avrBs*3-2 from *X. euvesicatoria* (Jones *et al.*, 2004) in Southern hybridizations. We determined that the *avrBs3* homologue, *pthA^w* (pAW5.2), from Xac-A^w, previously determined to be necessary for typical canker lesions including typical raised pustules (Al-Saadi *et al.*, 2007; Table 2), when expressed in *X. perforans*, resulted in HR in tomato (Fig. 6). None of the four clones, when expressed in a strain of *X. axonopodis* pv. *citrumelo*

Table 2 List of bacterial strains and plasmids used in this study.

Bacterium or plasmid	Relevant characteristics	Source or reference
Xanthomonas. perforans		
91-118	Wild-type, pathogenic to tomato, Rif^r	Jones *et al.* (2004)
91-118hrp$^-$	Same as 91-118, hrp cluster mutated with NTG, complemented with hrp clone, Rif^r	G. V. Minsavage
91-118*ΔxopA X. axonopodis* pv. *citri*	XopA-RifR	Hert (2007)
Xac-A 40	Wild-type, Asiatic strain, isolated in Argentina, Rif^r	DPI
Xac-A 306	Wild-type, Asiatic strain, isolated in Brazil, Rif^r	DPI
Xac-A^w 12879	Wild-type, Wellington strain, pathogenic to Key lime	DPI
Xac-A^{w}ΔavrGf1	Wellington strain, Ω cassette inserted into *avrGf*1	This work
Xac-A^{w}Ω4	Wellington strain, Ω cassette inserted into *avrGf*1, *pthA^w* and *avrTaw*	This work
Xac-A* 1974	A* strain (Xc290)	(Sun *et al.*, 2004; Vernier *et al.*, 1998), DPI
Xac-A* 1975	A* strain (Xc280)	(Sun *et al.*, 2004; Vernier *et al.*, 1998), DPI
X. axonopodis pv.*aurantifolii*		
Xaa-B 1622	B-69, isolated in Argentina	DPI
Xaa-C 5979	Xc 70, isolated in Brazil	DPI
X. axonopodis pv. *citrumelo*		
Xac-E 1887	Wild-type	DPI
X. campestris pv. *campestris*		
Xcc 8004	Wild-type, Rif^r	Metz *et al.* (2005)
Escherichia coli		
DH5α	F$^-$recA hsdR17(rk-mk+) Φ80dLacZ	Bethesda Research Laboratories, Bethesda, MD, USA
HB101	F$^-$*recA*	Maniatis *et al.* (1982)
Plasmids		
pLAFR3	Tra$^-$Mob$^+$, RK2 replicon, tet^r	Staskawicz *et al.* (1987)
pLAFR6	pLAFR1 with *trp* terminators	Bonas *et al.* (1989)
pUFR034	Inc W, Km^r, Mob$^+$, lacZα, Par$^+$, cosmid	De Feyter *et al.* (1990)
pUFR051	pLAFR3 derivative	De Feyter *et al.* (1990)
pBluescript II SK(+)	Phagemid, pUC derivative, Amp^r	Stratagene (La Jolla, CA, USA)
pHOKmGus	Km^r Ap^r Tn*3-uid*A fusion	Bonas *et al.* (1989)
pUC8Ω	Vector with Ω cassette	B. Staskawicz
PGEM®T Easy Vector	Multiple site cloning vector	Promega
pOK1	Suicide vector, pKNG101 derivative, Sm^r/Suc^s	Huguet *et al.* (1998)
pRK2073	ColE1 replicon, TraRK$^+$, Mob$^+$, Spec^r	Figurski and Helinski (1979)
pAW5.2	5-kb *Eco*RI-*Kpn*I fragment containing *pthA^w* from X0053 cloned in pUFR047 that complements pustule formation in a pustule minus strain	Al-Saadi *et al.* (2007)
pL799	pLAFR3 with DNA fragment from Xac-A^w that contains *avrGf*1	This study
pL799-1	pL799 with Tn*3*-Gus insert in *avrGf*1	This study
pL799-2	3.0-kb fragment from pL799 in pLAFR6	This study
pU799-3	2.3-kb fragment containing *avrGf*1 in pUFR034	This study
pBs3.0	pBluescript II KS with 3.0-kb insert from pL799-1 containing Tn*3*-KmGus	This study
pL22	XacA^w pLAFR3 cosmid that contains *hrpX*, *hrpG* and *hsp*90Xo homologues	This study
pL22E	Xac-A^w pLAFR3 clone containing *hrpG*	This study
p0346	Xac-A^w pUFR034 clone containing *hrpG*	This study
pL689	Similar to pL22	This study
pLXOPA	91-118 pLAFR3 cosmid that contains the *xopA* gene	This study
pL80	XacA^w pLAFR3 clone that contains *avrBs3* homologue, designated *avrTaw*	This study
pL104	Similar to pL80	This study
pL115	Similar to pL80	This study
pL622	Similar to pL80	This study
pUFR80.1	Subclone of pL80 containing only *avrTaw* homologue that does not complement pustule formation	This study
pLAT211	pLAFR3 containing *avrBs3-2*	Bonas *et al.* (1991)

DPI, Division of Plant Industry of the Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA; NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; B. Staskawicz, University of California, Berkeley, CA, USA.

following conjugation by triparental matings, caused symptoms similar to Xac-A when infiltrated into grapefruit leaves (data not shown). The insert from pL80 was subcloned and transferred to pUFRO34 to contain a single ORF which included the *avrBs3* homologue, and was designated pUFR80.1. The ORF in pUFR80.10 was sequenced and was determined to contain an *avrBs3* homologue with 18.5 tandem repeats consisting of a combination of 33 and 34 amino acids; it was designated *avrTaw* for HR in tomato by an *avrBs3* homologue from the Wellington strain of *X. citri* ssp. *citri* group A. The sequence for the tandem repeats was assigned accession number FJ360749. This clone, pUFR80.1, when expressed in Xac-A^{w}Δ*avrGf*1Δ*avrTaw*Δ*PthaA*^{w}, mutated in *avrGf*1, and both copies of the *avrBs3* homologues (Duan *et al.*, 2004), deficient in pustule formation, did not complement pustule formation in grapefruit or Key lime (Fig. 3b,c), whereas pAW5.2 containing *pthA*^{w} complemented the triple mutant strain for pustule formation in Key lime, but not in grapefruit (Fig. 3c). Pustules were produced in grapefruit with Xac-A^{w}Δ*avrGf*1 (Fig. 3a), in which both Xac-A^w*avrBs3* homologues, *avrTaw* and *pthaA*^{w}, were present. In a separate experiment in which we inoculated grapefruit leaves by pin-prick with Xac-A^{w}Δ*avrGf*1 or Xac-A^{w}Δ*avrGf*1Δ*avrTaw*Δ*pthaA*^{w}:pAW5.2, typical pustules were only formed when Xac-A^{w}Δ*avrGf*1 was used as inoculum (Fig. 3d). It is clear that, at least one other host-limiting factor is present in Xac-A^w, given the pronounced reduction in pustule formation associated with Xac-A^{w}Δ*avrGf*1 in grapefruit (Fig. 3a) relative to Key lime (Fig. 3c).

Given that pustule formation in grapefruit by Xac-A^{w}Δ*avrGf*1 was significantly reduced relative to that in Key lime, we attempted to determine what other gene(s), in addition to *avrGf*1, was responsible for the reduced host range. Shiotani *et al.* (2007) demonstrated that an *avrBs3* homologue with 15.5 repeats was associated with reduced pustule formation in *C. grandis*. It was logical to determine whether the *avrBs3* homologues present in pUFR80.1 were involved in any way with reduced disease development in grapefruit. We transferred both *avrBs3* homologues present in Xac-A^w, *avrTaw* and *pthaA*^{w} expressed in pUFR80.1 and pAW5.2, respectively, to the Xac-A strain by conjugation, and determined that pustule formation was not reduced in grapefruit when inoculated with the Xac-A transconjugants carrying either *avrBs3* homologue (Fig. 3e).

DISCUSSION

The multiplication patterns of the two strains of *X. axonopodis* pv. *citri* (Xac-A^w and Xac-A) in Duncan grapefruit and Key lime leaves that occurred in this study were very similar to those reported by Sun *et al.* (2004). Maximum populations of the Xac-A^w strain were 10–100-fold less than those of the Xac-A strain in grapefruit leaves. The multiplication of each strain was no different after infiltration into Key lime leaves.

The relationship between confluent necrosis and the cell population, after high concentrations (5×10^8 cfu/mL) of Xac-A and Xac-A^w bacteria were infiltrated separately into leaves of grapefruit and Key lime, has not been reported previously (Sun *et al.*, 2004), and is indicative of an incompatible interaction in grapefruit leaves (Minsavage *et al.*, 1990). Necrosis, as determined by electrolyte leakage, occurred earlier and with smaller populations after inoculation of grapefruit leaves with cells of the Xac-A^w strain relative to those of the Xac-A strain. The time to necrosis and the cell populations were similar for each strain (data not presented) after inoculation of Key lime leaves. These data are consistent with the concept that the Xac-A^w strain caused HR in grapefruit leaves. The more rapid necrosis in grapefruit caused by Xac-A^w relative to Xac-A could easily be discerned visually. It should be noted that necrosis associated with both HR and the susceptible reaction occurs relatively slowly in grapefruit leaves inoculated with the citrus canker bacterium relative to that observed in some other host–pathogen systems (Minsavage *et al.*, 1990). This is the first report of the association of an avirulence gene with this type of interaction in citrus, although Khalaf *et al.* (2008) identified a general non-host interaction between *X. citri* ssp. *citri* and *Fortunella margarita*.

Conclusive evidence for hypersensitivity in citrus leaves to strains of the citrus canker bacterium has not been reported previously. HR was suggested to be caused by a strain of Xaa-C when a suspension of 10^8 cfu/mL was infiltrated into grapefruit leaves (Stall *et al.*, 1982). The resulting rapid necrosis was compared with the relatively slow necrosis caused by cells of either Xac-A or Xaa-B strains in the same test. However, data on the relationship of populations of bacteria to necrosis were not reported. This relationship still needs to be determined for these strains.

A genomic library of a Xac-A^w strain was successfully produced and incorporated into *E. coli* DH5α. For successful screening of the library for an avirulence gene, one would normally transfer each clone into a strain that was pathogenic to the plant in question, in this case, into a strain of Xac-A. However, in previous experiments, the transfer of clones in the pLAFR3 cosmid library to strains of Xac-A from *E. coli* DH5α by triparental matings did not occur at high frequency. Clones of the library were transferred from *E. coli* to strain 91-118 of *X. perforans,* a highly promiscuous recipient, by triparental conjugations to circumvent this problem. The strain of *X. perforans* causes a null reaction in leaves of grapefruit, even though it contains the *hrp* genes necessary for the transfer of effector proteins into host cells (Bonas *et al.*, 1991). It was thought that an avirulence gene in a clone of genomic DNA of Xac-A^w would result in HR in grapefruit leaves when present in the strain of *X. perforans.* In fact, an avirulence gene in the Xac-A^w library was found. As this procedure was successful, it could possibly be used to locate other avirulence genes when the transfer of clones to a pathogen occurs very infrequently during triparental matings with *E. coli.*

The clone that was expressed in Xac-A and caused HR in grapefruit leaves did not cause HR in leaves of Key lime. This reflected the host range of the Xac-A^{w} strain. The importance of the avirulence gene in the determination of the host specificity of the Xac-A^{w} strain was further investigated by determining the presence of the gene in other bacterial strains pathogenic to citrus. Using PCR and Southern hybridization techniques, we could only detect the avirulence gene in Xac-A^{w} strains, although we did observe weak hybridization with the *X. campestris* pv. *campestris* strain which contained gene 3600. When the gene was mutated in a Xac-A^{w} strain, the symptoms caused by the mutated strain were similar to those caused by the Xac-A strains. However, the symptoms appeared to be less pronounced.

The growth of the Xac-A^{w} strain with *avrGf*1^{-} (Xac-A$^{w}\Delta$*avrGf*1) in grapefruit leaves was significantly greater than that of the wild-type Xac-A^{w} strain, but lower than that of the Xac-A strain, when both high and low levels of inoculum were used. The differences in the cell populations also reflected differences in the number of cells present at the time of necrosis in grapefruit leaves. Complementation of the mutant strain with the *avrGf*1 clone restored avirulence in grapefruit leaves for both the multiplication of cells and electrolyte leakage. The growth of Xac-A$^{w}\Delta$avrGf1 in Key lime leaves was equal to that of wild-type Xac-A and Xac-A^{w} strains (data not presented). Therefore, *avrGf*1 function did not appear to affect the virulence of the Xac-A^{w} strain in Key lime. The gene *avrGf*1 seems to be important in the delimitation of the host specificity of the Xac-A^{w} strain, but not in the virulence in Key lime.

Mutation of a hypothetical avirulence gene in the Xac-A^{w} strain would nullify HR and result in a susceptible reaction in grapefruit leaves, as has been observed in other systems (Dahlbeck and Stall, 1979; Gassmann *et al*., 2000; Swarup *et al*., 1992). Bacterial cells of Xac-A^{w} were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (Rybak, 2005). This is a powerful mutagenic agent that increases the frequency of mutation. NTG treatment increased the frequency of streptomycin mutants and white colonies in the population used to inoculate grapefruit leaves, attesting to the effectiveness of treatment. Rybak (2005) noted that no mutants of the Xac-A^{w} strain were found in these tests that were pathogenic on grapefruit leaves. This may indicate that an additional factor exists in Xac-A^{w}.

An assumption could be made that other resistance genes exist in grapefruit leaves that prevent the symptoms of the mutated *avrGf*1 strain from being equal to those of the wild-type Xac-A strain, and prevent the populations of the two strains from being equal. Other host-limiting genes in the genomic library of Xac-A^{w} that interact with resistance genes in grapefruit may be present in the library, because only 300 clones were screened. Clearly, more than one gene is present in Xac-A^{w} that affects its ability to cause typical citrus canker on grapefruit; however, it does not appear to be associated with HR and thus would not have been identified using the screening procedure employed to identify the other clones. We examined the possibility that *avrBs3* homologues could be involved in the suppression of growth in grapefruit, given that Shiotani *et al*. (2007) determined that a member of the avrBs3/*pthA* family, designated *hssB3.0*, with 14.5 tandem repeats, suppressed virulence, as manifested by a decrease in lesion size. However, neither of the *avrBs3* homologues (pUFR80.1 and pAW5.2) in Xac-A^{w} suppressed pustule formation in grapefruit when expressed in an Xac-A strain (Fig. 3d). Further work is necessary to identify other factors associated with the limitation of Xac-A^{w} in grapefruit.

We also provided indirect evidence that the *avrBs3* homologue, present in pUFR80.1, which contains 18.5 tandem repeats, is necessary for pustule formation in grapefruit. When Xac-Xac-A$^{w}\Delta$*avrGf*1Δ*avrTaw*Δ*PthaA*^{w} (deletion in *avrGf*1 and both *avrBs3* homologues), complemented with pAW5.2, was inoculated into grapefruit, pustules were not produced; they were only formed at sites inoculated with Xac-A$^{w}\Delta$*avrGf*1, which contained both copies of the *avrBs3* homologue. This second *avrBs3* homologue, although not essential for pustule formation in Key lime, appears to be important for pustule formation in grapefruit.

The protein expressed from *avrGf*1 may be an effector, which is delivered via TTSS into plant cells and has a role in virulence (Kjemtrup *et al*., 2000). Proteins expressed from other avirulence genes in *Xanthomonas* have been determined to be transferred to host cells by TTSS (Metz *et al*., 2005; Mudgett *et al*., 2000; Szurek *et al*., 2002), and thus were determined to be effectors that incited HR. Strains with mutation in the *hrp* gene cluster were used to prevent the transfer of proteins to host cells by TTSS in the experiments. HR did not occur in grapefruit leaves after the *avrGf*1 clone was conjugated into a strain of *X. perforans* that was mutated in the *hrp* locus. More work is needed to confirm that *avrGf*1 is transferred into grapefruit cells by the Xac-A^{w} strain, and thus is an effector.

The pL22 clone was unique because HR occurred in grapefruit leaves when it was expressed in *X. perforans*, but not when it was expressed in the Xac-A strain. Interestingly, the presence of a *hrpG* sequence in pL22 caused cells of *X. perforans* to incite HR rather than the null reaction in grapefruit leaves. As clone pL22, when expressed in *X. perforans*, but not in Xac-A, incited HR in grapefruit leaves, and did not contain an avirulence gene, it should be emphasized that any suspected clone must be transferred to a pathogen of the host to confirm that an avirulence gene exists.

Kim *et al*. (2003) reported that *X. axonopodis* pv. *glycines* did not produce HR in tobacco until a clone from *X. oryzae* pv. *oryzae*, which contained both *hrpX* and *hrpG*, was conjugated into *X. axonopodis* pv. *glycines*. The importance of either *hrpX*, *hrpG* or both in the production of HR in tobacco was not determined in this work. In our work, *hrpX* clearly was not necessary for HR in citrus caused by *X. perforans*. Kim *et al*. (2003) also gave evidence that the *hrpX* and *hrpG* clones increased the function

of *hpaG*, a homologue of *hrpN* of *Erwinia amylovora* and a known harpin gene, in *X. axonopodis* pv. *glycines* and, subsequently, HR in tobacco. The *hpa*G nucleotide sequence is a homologue of *xopA* in *X. campestris* pv. *vesicatoria* strain 85-10, but the XopA protein produced in *X. axonopodis* pv. *glycines* did not cause HR in tobacco (Kim *et al.*, 2003). Although both *X. campestris* pv. *vesicatoria* and *X. perforans* cause bacterial spot of tomato, they are genetically different (Jones *et al.*, 2004). However, the DNA sequences of *xopA* from both bacteria are almost identical (data not presented). The *xopA* gene in *X. perforans*, in conjunction with an additional *hrpG* copy, is at least partly involved in inducing HR in grapefruit leaves, but has no apparent effect on tomato. HrpG is reported to be the response regulator of a putative two-component system in which the unidentified sensor presumably detects environmental variables which induce the *hrc* and *hrp* genes (Wengelnik *et al.*, 1996). HrpG is thought to be at the top of the *hrc/hrp* regulatory cascade, as the mutant HrpG* constitutively activates all other *hrc* and *hrp* genes (Wengelnik *et al.*, 1999). Multiple copies of *hrpG in trans* may alter *hrp* gene expression and may perhaps lead to constitutive expression; this may have caused the increased delivery of Hrp-related proteins, such as XopA, which may have resulted in HR in grapefruit. Furthermore, expression of *xopA* and associated genes in Xac-A, with or without additional copies of HrpG, resulted in reduced growth in grapefruit. Therefore, *xopA* and/or other genes in the pLXOPA clone may be host-limiting factor(s) in citrus. A similar response was observed with *X. axonopodis* pv. *glycines*, in which the wild-type elicited HR in pepper, whereas the *hpaH* mutant did not (Kim *et al.*, 2003). It remains to be determined whether XopA has harpin-like activity in grapefruit leaves.

Four clones that caused HR in tomato were obtained from the 300 clones of the Xac-A^{w} library. All four of the clones contained an *avrBs3* homologue. Two homologues of this class are plasmid borne in Xac-A^{w} (Duan *et al.*, 2004), and therefore may have been over-represented in our library. All four of our clones were conjugated into a strain of *X. axonopodis* pv. *citrumelo*, a pathogen of citrus, and none of our clones caused the erumpent-type lesions in grapefruit or Key lime leaves when expressed in a strain of *X. axonopodis* pv. *citrumelo*, as do the *avrBs3* homologues designated as *pth*A (Swarup *et al.*, 1991, 1992). Two different *avrBs3* homologues have been reported to exist in the Xac-A^{w} strains (Duan *et al.*, 2004), and one was cloned and found to be related to *pth*A (Al-Saadi *et al.*, 2007). The second *avrBs3* homologue, *avrTaw1*, which was cloned by us and caused HR in tomato, did not produce the *pth*A effect in citrus leaves. This finding agrees with that of Al-Saadi *et al.* (2007), who determined that the pustule-forming genes, *pthA* homologues, contained 17.5 repeats, unlike *avrTaw1* which contained 18.5 repeats. It was not determined whether the *pth*A^{w} gene cloned by Duan *et al.* (2004) from another strain of Xac-A^{w} caused HR in tomato. It is known that the different *avrBs3* homologues have different specificities in host reaction causing HR and pathogenicity in plants (Kay *et al.*, 2005). Recently, Shiotani *et al.* (2007) identified a *pthA* homologue that induces a defence response in *C. grandis*. Perhaps one or more of these homologues, isolated from the Xac-A^{w} strain, is associated with the induction of a defence response in grapefruit. This could help to explain why the mutation experiments did not result in the identification of one or more mutants that were fully virulent on grapefruit. Further work is needed to address the issue of whether or not these *pthA* homologues are host-limiting factors.

EXPERIMENTAL PROCEDURES

Plants

Plants of grapefruit (*C. paradisi*), cultivar Duncan, and Key lime (*C. aurantifolia*) were kept in 12-in pots in a quarantine glasshouse at the Florida Department of Agriculture and Consumer Services, Division Plant Industry, Gainesville, FL, USA, which was kept between 20 and 35 °C. The plants were kept in vigorous growth by the application of 'Osmocote' fertilizer (19 : 6 : 12 ratio of nitrogen : phosphorus : potassium) as needed. Before inoculations, the plants were pruned to stimulate new growth. Leaves on the newly developed shoots were inoculated 14–21 days after the shoots began growth. The leaves chosen for inoculations were fully expanded or nearly so, but soft to the touch and not as fully green as mature leaves. By using this procedure, all the inoculated leaves were in a similar developmental stage. Tomato plants (cultivar Bonny Best) inoculated with clones in *X. perforans* were approximately 6 weeks of age. After inoculation with the clones, the plants were incubated in a growth room at 28 ± 2 °C.

Bacterial strains

The strains used in this research are listed in Table 1. All strains were maintained at −80 °C, and subcultured, when needed, on nutrient agar (NA). Antibiotic-resistant strains were obtained by plating 500 μL of 10^{9} cfu/mL onto NA supplemented with appropriate antibiotic. *Escherichia coli* strains were cultured on Luria–Bertani (LB) medium (Maniatis *et al.*, 1982). Conjugations were performed on nutrient-yeast-glycerol agar (NYGA) (Daniels *et al.*, 1984). The antibiotics and concentrations used in the media were as follows: rifamycin, 75 μg/mL; tetracycline, 12.5 μg/mL; kanamycin, 25 μg/mL.

Preparation of inocula

Suspensions of strains were each transferred to NA to determine the purity of the cultures. Several colonies from pure cultures were transferred to nutrient broth. After shaking overnight at 28 °C,

the bacterial suspensions were centrifuged and the cells were resuspended in sterile tap water, and standardized (0.3*A* at 600 nm wavelength) in a Spectronic 20 (UNICAM, Rochester, NY, USA) spectrophotometer. This optical density corresponded to a bacterial concentration of 5×10^8 cfu/mL. Other concentrations of inocula were obtained by dilution.

Electrolyte leakage

Leaves of grapefruit were inoculated with 5×10^8 cfu/mL by infiltrating areas of *c.* 5 cm^2 with a syringe and 27-gauge needle (Klement, 1963). The inoculated area was outlined by pin-pricking the infiltrated area with a needle. Each strain was placed in each leaf. Replicates involved different leaves. After 2 h and 2, 4, 6, 8 and 10 days, electrolyte leakage was measured from each of three leaves. Electrolyte leakage was determined as an increase in electrical conductivity over a 2-h period of a bath containing six 0.5-cm^2 leaf discs from an inoculated area that were added to 3 mL of de-ionized water (Klement *et al.*, 1990). The baths in 16×100-mm tubes were shaken at 200 r.p.m. A zero-time determination was subtracted from the 2-h determination to give the final value. The mean of the three values was used as the conductivity at each time period, but each value was used to determine the experimental error.

Bacterial populations

The populations of strains in grapefruit leaves were determined in the same leaves and at the same times as the electrolyte leakage determinations. From each leaf, 0.5 cm^2 of inoculated area was taken and triturated in 1 mL of sterile tap water; after appropriate 10-fold dilutions with sterile tap water, 50 μL were plated onto NA medium. The colonies were counted 3 days later. Three replicates were included at each time period. The experiments on population and electrolyte leakage were repeated three times, but only one experiment was included in the figures.

Recombinant DNA techniques

The techniques used for enzyme digestion, ligation, Southern transfer, plasmid alkaline lysis and agarose gel electrophoresis have been described by Maniatis *et al.* (1982). A genomic library of Xac-A^w (strain 12879) was constructed in the vector pLAFR3, as described by Metz *et al.* (2005). Individual clones were transfected into *E. coli* DH5α and maintained on LB medium. Each clone contained approximately a 25-kb fragment of Xac-A^w DNA. The helper plasmid pRK2073 in *E. coli* HB101 was used in conjugations involving triparental matings. Individual clones in *E. coli* DH5α were conjugated into strain 91-118 of *X. perforans* (Jones *et al.*, 2004). Each transconjugant was infiltrated into a 1-cm^2 area of leaf tissue with a syringe and 27-gauge needle. Grapefruit leaves were inoculated with *c.* 300 transconjugants individually. Five transconjugants were infiltrated into each leaf, which also contained a control suspension of strain 91-118. Tomato leaves were inoculated similarly as controls, because the Xac-A^w strain also elicits HR in tomato leaves. A clone that caused HR in grapefruit or tomato leaves was detected via necrosis in the infiltrated area of the leaf, which occurred more rapidly than with the control suspension of strain 91-118.

Subcloning of pL799

A clone (pL799) with HR activity in grapefruit, but not tomato, was subcloned to contain only the DNA necessary for HR activity. To identify the location of the avirulence gene in pL799, the gene responsible for HR activity was inactivated by the transposon pHoKmGus by the procedure described by Huguet and Bonas (1997). About 160 clones with kanamycin resistance were transferred to *X. perforans* and screened for the lack of HR in grapefruit. Three clones were selected for a lack of HR and one clone (pL799-1) was selected from the three for further work. The clone pL799-1 was restricted with each of several enzymes to find a fragment that contained the Tn*3*-Km*Gus* insert. A *Hin*dIII restriction fragment contained the Tn*3*-Km*Gus* insert and about 3.0 kb of DNA. This fragment was ligated into pBluescript II KS and labelled pBs3.0. A portion of the 3.0-kb insert in pBs3.0 was then sequenced (see below) using forward and reverse primers from the cloning vector. Custom oligonucleotide primers were designed for completion of the sequencing of the *Hin*dIII fragment.

An ORF was identified in the sequence of the *Hin*dIII fragment. However, the ORF was at the end and was not complete. When the *Hin*dIII fragment was ligated into pLAFR6 (pL799-2) and conjugated into *X. perforans*, no HR occurred in grapefruit leaves. Primers were selected for sequencing beyond the end of the *Hin*dIII fragment in the original pL799 clone to obtain the complete ORF. Primers were then selected to amplify, by PCR, a 2.3-kb fragment that contained the complete ORF, which was then ligated into pGEM®T Easy Vector (Promega, Madison, WI, USA). The 2.3-kb insert was removed from pGEM®T Easy Vector with *Eco*RI enzyme and then ligated into the vector pUFR034, and designated pU799-3. The pUFR034 cosmid was used as a vector because DNA inserts in this vector could be conjugated into strains of Xac-A^w, whereas pLAFR3 derivatives could not be transferred to strains of Xac-A^w. The pU799-3 clone in *X. perforans* and Xac-A 40 caused HR in grapefruit leaves.

DNA sequence analysis

Sequencing of the DNA fragments was completed at the sequencing facility [Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville, FL, USA] with an Applied Biosystems model 373 system (Foster City, CA, USA).

Custom primers were synthesized at the ICBR facility with an Applied Biosystem model 394 DNA synthesizer to complete the sequencing of both strands of DNA. The computer program SEQAID II version 3.81 was used to analyse nucleotide sequence data and predicted protein products of the 2.3-kb region that contained *avrGf*1. A search for nucleotide and amino acid sequence homology in the gene bank was conducted using the BLASTN and BLASTP 2.2.11 programs (Altschul *et al.*, 1997).

Presence of *avrGf*1 in other xanthomonads

Primers were designed and custom-made to amplify a 199-bp fragment from within *avrGf*1 for its detection by PCR in the DNA of xanthomonads that cause disease in citrus plants. The primers used were as follows: forward, 5′-CGCCGGTTTCTGTCACTTG-3′; reverse, 5′-GCCGCCTTTGCCATCGACCAG-3′. PCRs were performed in a thermocycler (M.J. Research, Watertown, MA, USA).

Southern hybridization experiments were performed on Nytran (Schleicher and Scheull, Keene, NH, USA) membranes using the GENIUS non-radioactive DNA labelling and detection kit, according to the manufacturer's instructions (Boeheringer Mannheim Biochemicals, Indianapolis, IN, USA). Genomic DNA extractions were made using the Genomic Prep TM Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The isolated DNA preparations were restricted with *Hin*dIII endonuclease. Plasmid extraction from Xac-A and Xac-A^w strains was performed as described previously (Stall *et al.*, 1986). The restricted DNA and plasmids were electrophoresed in 0.75% agarose gel.

Mutation of *avrGf*1 in Xac-A^w

The *avrGf*1 gene in Xac-A^w was mutated to investigate the role of the gene in the host specificity of the bacterium. Mutation in *avrGf*1 in pGEM®T Easy Vector occurred by first cloning an Omega cassette (from pUC8Ω) into a *Bam*HI site in the gene. Then, the inactive gene was exchanged in the Xac-A^w strain using the suicide vector pOK1, following the procedure described by Huguet *et al.* (1998). Eventually, Xac-A^w (strain 12879) with the mutated avirulence gene was obtained and labelled Xac-A^{w}Δ*avrGf*1.

*avrGf*1 activity in a *hrp*$^-$ strain

A strain of *X. perforans* 91-118 that had been mutated in the *hrp* locus was on hand. This strain was obtained by NTG mutation and selected for not being pathogenic or hypersensitive on tomato. The strain was complemented to pathogenicity and hypersensitivity after the addition of plasmid pXV9, which contained the *hrp* cluster (Bonas *et al.*, 1991). The clone pU799-3 (*avrGf*1) was conjugated into *X. perforans* strains 91-118*hrp*$^-$ and 91-118*hrp*$^+$ and tested for reaction in grapefruit leaves as described above.

Hypersensitivity of pL22 in grapefruit

Clones pL22 and pL689, which caused HR in grapefruit when in cells of *X. perforans*, were restricted with several enzymes. Based on the restriction patterns, the clones were similar. Clone pL22 was chosen for further analysis and contained about 27 kb of inserted DNA. A partial digest of this cosmid was obtained by restricting it with *Sau*3A1 to obtain fragments of about 5 kb. The fragments were then ligated into pUFR051, transformed into competent cells of *E. coli* DH5α and conjugated into cells of *X. perforans.* Of the 180 conjugants, two gave HR in grapefruit leaves. These two clones did not produce HR in grapefruit when conjugated into strain 40 of Xac-A. The insert of one of the clones contained about 5 kb, which contained two *Bam*HI fragments. Each *Bam*HI fragment was cloned into pBluescript and sequenced. Three ORFs were found. With appropriate enzyme digestion, a fragment containing each ORF was transferred to pLAFR3, transformed into cells of *E. coli* DH5α, conjugated into *X. perforans* and inoculated into grapefruit leaves at a concentration of 5×10^8 cfu/mL.

Role of *X. perforans* XopA region in host specificity

A clone in pLAFR3 containing a x*opA* gene from *X. perforans* was selected from a previously developed library of strain 91-118 of *X. perforans* in pLAFR3 (Astua-Monge *et al.*, 2000). The clone was identified by amplifying by PCR the gene from pools of clones from the 91-118 library. The primers used in the amplification were as follows: forward, 5′-CATCTCGGAAAAGCARCTGGACC-3′; reverse, 5′-CATTCTKCGCCTGKARAATSTCTCC-3′. The clone containing *xopA* was labelled pLXOPA.

The *xopA* gene was deleted in strain 91-118. In the procedure, the *xopA* gene was amplified using the following primers: forward, 5′-GGGAAGCTTTGCTGCAAGAGGAAAAGCG-3′; reverse, 5′-GGGGAATTCAATCCGCGCGTGCGA-3′. The amplified product (1800 bp) was cloned into pGEM®T Easy Vector. The enzyme *Ava*I was used to delete 706 bp, which included the *xopA* gene (353 bp), 103 bp downstream and 250 bp upstream. The upstream deletion included a PIP promoter for *hpa*H running in the opposite direction. The deleted fragment was excised from the pGEM®T Easy Vector clone with enzymes *Apa*I and *Spe*I, and ligated into pOKI suicide vector that was cut with *Apa*I and *Xba*I. The suicide vector with the deletion fragment was then conjugated into strain 91-118 for deletion of *xopA* in that strain. This procedure was described by Huguet *et al.* (1998). Deletion of *xopA* in strain 91-118 was confirmed by PCR.

Clones with avirulence to tomato from Xac-A^w

The DNAs of four clones from the Xac-A^w library, which caused HRs in tomato leaves when conjugated in *X. perforan*s, were

digested with *Bam*HI and *Kpn*I restriction enzymes and subjected to electrophoresis. All four clones were similar based on the restriction fragment profiles. The DNAs of the clones were then hybridized with a probe (pLAT 211) (Bonas *et al*., 1993) containing *avrBs3-2* by the procedure described above. Genomic DNA from strain 82-8 of *X. euvesicatoria* and from a strain of Xac-A^w was included in the hybridization. These strains were known to contain an *avrBs3* homologue (Bonas *et al*., 1989; Duan *et al*., 2004).

Role of Bs3 homologues in HR and virulence

pUFR80.1 identified in this study and pAW5.2 containing the *avrBs3* homologue from Xac-A^w (Al-Saadi *et al*., 2007) were transferred to *X. perforans* by triparental mating, and suspensions from the resulting transconjugants were infiltrated into Bonny Best tomato at 5×10^8 cfu/mL. The reactions in tomato were compared with the reactions caused by inoculations with *X. perforans* at the same concentration.

Both pUFR80.1 and pAW5.2 were also transferred to strain Xac-A^{w}Δ*avrGf*1Δ*avrTaw*Δ*pthA*^{w}, which was also inactivated in *avrGf*1 and in both copies of the *avrBs3* homologues present in XacA^w, and which was deficient for pustule formation. The resulting transconjugants and Xac-A^{w}Δ*avrGf*1 were inoculated into grapefruit and Key lime by infiltration and the pin-prick method. The pin-prick method involves placing a drop of bacterial suspension on the adaxial surface of Key lime and grapefruit leaves and piercing a needle through the drop and through the leaf. The inoculated plants were incubated in the glasshouse and observed for disease development.

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