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Mutational analysis of type III effector genes from *Xanthomonas citri* subsp. *citri*

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Abstract *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker, relies extensively on a type III secretion system for infection by delivering type III effectors into host cells. In the genus *Xanthomonas*, two major regulators, HrpG and HrpX, are involved in the expression of genes encoding the type III secretion system. Twenty-three candidate type III effectors were identified as targets for analysis. The involvement in pathogenicity of 20 candidate effector genes in *X. citri* strain 306 (Xcc-306) was investigated using site-directed mutagenesis. Pathogenicity assays in grapefruit of 19 genes using site-directed mutagenesis revealed that none of the mutants demonstrated to have reduced ability to cause canker disease. A mutation in the TAL effector *pthA4*$^-$ resulted in loss of hypertrophy although no changes were observed in bacterial growth in leaves. Mutations in *hrpG*, *hrpX*, or *hrpA* genes displayed a complete loss of pathogenicity. Moreover, all mutants maintained the ability to trigger a hypersensitive response (HR) in non-host tomato. In contrast to previous studies, *hrpG*$^-$, *hrpX*$^-$ and *hrpA*$^-$ mutants also retained the ability to elicit an HR in tomato, indicating the presence of an Hrp independent elicitor in Xcc-306.

Keywords Citrus canker · Hypersensitive response · Pathogenicity · Type III secretion system · Virulence

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Introduction

Most phytopathogenic bacteria rely on complex gene networks to overcome the defences of their host plants (Nurnberger and Lipka 2005). *Xanthomonas citri* subsp. *citri* (*X. citri*), the causal agent of citrus bacterial canker, gains access into the host through wounds or natural openings (Brunings and Gabriel 2003). Once inside the plant, the bacteria release an array of proteins into the plant cells, including type III effectors (T3-effectors). These effectors are delivered via a conserved type III secretion system (T3SS), which is

encoded by a chromosomal gene cluster designated hypersensitive reaction and pathogenicity (*hrp*) gene cluster (Alfano and Collmer 2004; Cornelis and Van Gijsegem 2000). The T3-effectors interact directly and indirectly with plant substrates triggering two distinct outcomes. The first outcome, and the apparent principal function of the T3SS, is conversion of the host cell environment to facilitate bacterial growth. Considerable evidence indicates that many T3-effectors function to suppress host defence responses that are triggered by host recognition of a variety of pathogen molecules, which are referred to as pathogen associated molecular patterns or PAMPs (Büttner and Bonas 2010). PAMP triggered immunity (PTI) is part of the basal defence responses of plants. Recent results indicate that PTI can be elicited by components of the T3SS itself (Oh et al. 2010). A second outcome, depending on the genotype of the plant, is the activation of plant defences (Alfano and Collmer 2004; Büttner and Bonas 2006; Mudgett 2005). Specific recognition of pathogen effectors is often referred to as effector-triggered immunity (ETI), which generally culminates in the induction of a form of rapid cell death known as the hypersensitive response or HR (Block et al. 2008; Greenberg and Yao 2004).

In *Xanthomonas* species, T3SS is initiated upon phosphorylation of product of the *hrpG*, which is promoted by a yet unidentified environmental stimulus (Büttner and Bonas 2006, 2010). Phosphorylated HrpG, which is a member of the response regulator of two-component regulatory systems, in turn, activates transcription of *hrpX* and *hrpA* (Wengelnik and Bonas 1996). Consequently, HrpX, which is a member of the AraC family of transcriptional factors, activates expression of the genes in *hrpB* through *hrpF* operons by binding to a conserved promoter element known in *Xanthomonas* as the plant inducible promoter (PIP)-box, which is represented by the consensus sequence motif TTCGC-N15-TTCGC (Koebnik et al. 2006; Wengelnik and Bonas 1996). Another motif known as the –10 box-like motif (YANNNT; Y: C/T; N: A/T/C/G) is localized 30–32 base pairs downstream of the PIP box and has been proposed to be involved in the HrpX-mediated regulation (Cunnac et al. 2004; Furutani et al. 2006). However, HrpX also activates some genes that do not contain a PIP-box binding site motif (Tsuge et al. 2005).

Analysis of the genomic sequence of *X. citri* strain 306 (Xcc-306) revealed several putative T3-effectors (da Silva et al. 2002). However, the candidate effectors have not been assayed for involvement in Xcc-306 pathogenicity. To address their involvement, site-direct mutagenesis was employed to construct mutant strains for the predicted HrpX-regulon and other candidate pathogenicity genes of Xcc-306 for assessment of the effect of the mutations on infection of citrus leaves.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Supporting Information Table S1. *Escherichia coli* was cultivated in Luria-broth (LB) medium at 37° C (Sambrook et al. 1989). All strains of *Xanthomonas* were cultured at 28°C on nutrient agar (NA) medium. Conjugations were performed on NYG (nutrient yeast glycerol) agar plates as described previously (Daniels et al. 1984). Antibiotics were used at the following concentrations: ampicillin (Amp) 100 μg ml^{-1}; chloramphenicol (Cm) 100 μg ml^{-1}; kanamycin (Km) 50 μg ml^{-1}; spectinomycin (Spc) 100 μg ml^{-1}; rifampicin SV (Rif) 80 μg ml^{-1}; and tetracycline (Tet) 12.5 μg ml^{-1}.

DNA manipulation techniques, transformation and sequence analysis

Isolation and manipulation of DNA were performed using standard techniques (Sambrook et al. 1989). Individual constructs were transfected into *E. coli*-DH5α cells as described (Sambrook et al. 1989). Conjugations with *Xanthomonas* strains were performed following standard protocols (Daniels et al. 1984). DNA sequencing was performed at the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida—Gainesville, FL (UF-ICBR).

Generation of *X. citri* subsp. *citri* strain 306 mutants

For inactivation of the putative HrpX-regulon genes, partial fragments of each targeted gene were PCR amplified and cloned into the pCR2.1 vector using the TA cloning method according to the instruction of the supplier (Invitrogen Corp, San Diego, CA). The

inserts were sequenced in both strands for confirmation. The plasmids were introduced into Xcc-306 competent cells. The integration of plasmids containing the targeted gene lacking the 5′ and 3′ coding regions of the respective genes by single homologous recombination resulted in the disruption of the targeted gene (Sugio et al. 2005). The mutants were selected for resistance to kanamycin and confirmed by PCR using a primer flanking the upstream region of the targeted gene combined with pCR2.1 internal primers. The *pthA4* mutant (306Δ*pthA4*) was produced using the pOK1 suicide vector (Minsavage et al. 2004). Complementation of 306Δ*pthA4*, 306Ω*hrpG* and 306Ω*hrpX* mutant strains was performed by introducing pAW5.2 construct, which carries *pthA4* homologue from *X. citri* strain X0053 (Al-Saadi et al. 2007), into 306Δ*pthA4* mutant strain; and a pL22 construct, which contains *hrpX*, *hrpG* genes from *X. citri* strain A^w or Xcc-A^w (Rybak et al. 2009), into 306Ω*hrpG* and 306Ω*hrpX* mutant strains.

Plant materials and inoculation procedures

Grapefruit cv. Duncan (*Citrus paradisi*) and Key-lime (*Citrus aurantifolia*) plants were kept in the glasshouse at ambient temperatures ranging from 25–30°C. Tomato plants (*Solanum lycopersicum*) cultivar Bonny Best, approximately 6 weeks of age, were inoculated with bacterial suspensions and incubated in a growth room at constant temperature of 28°C.

For preparation of bacterial suspensions, *Xanthomonas* strains were grown on NA plates for 18 h at 28°C, and the cells were harvested and suspended in sterile tap water, and standardized to an optical density at 600 nm (OD_{600})=0.3 (5×10^8 cfu/ml). For pathogenicity assays, the suspensions of *X. citri* strains were infiltrated with a syringe equipped with a 27-gauge hypodermic needle into the abaxial surface of young grapefruit leaves, 14–21 days after new shoots had expanded following pruning. Disease development was scored daily for a period of 10 days after inoculation (DAI). Bacterial growth was monitored by removing three disks (diameter=8 mm) from individual infiltrated areas and homogenizing the leaf disks in 1.0 ml of sterile tap water. Bacterial populations (cfu per cm^2) were determined by plating dilutions of leaf homogenates onto NA plates.

For HR tests, bacterial suspensions of *X. citri* strains and mutants were adjusted to a concentration of 5×10^8 cfu/ml and infiltrated into the mesophyll of tomato cv. Bonny Best with a hypodermic syringe and needle. The inoculated plants were incubated in a growth room at 28°C and assessed for elicitation of an HR at 24 h after infiltration. All experiments were repeated at least three times.

DNA sequence alignment and phylogenetic analysis

Primers were designed from genome sequences of Xcc-306 based on the *hrpW* sequence available in GenBank (accession no.°AAM37767.1), and the region amplified corresponded to the entire gene. The purified PCR products for all strains tested here were sequenced in both directions and the nucleotide sequence alignments were done using the Clustal W Program (Thompson et al. 1994), available at http://www.ebi.ac.uk/clustalw. The dendrogram generated by PAUP* version 4.0b10 (Swofford 2003) showed phylogenetic relationship among *X. citri* strains. *X. campestris* pv. *campestris* strain 33913 (accession no. AAM40517.1) and *Ralstonia solanacearum* strain UW551 (accession no. ZP_00943832.1) served as the out-group. Maximum parsimony (MP) and the maximum likelihood (ML) trees were performed by tree bisection/reconnection (TBR) branch swapping and heuristic searches with random stepwise addition of 1000 replicates for MP and 1000 for ML. The ML trees were analyzed on the basis of hierarchal likelihood ratio tests performed by Modeltest 3.7 (Posada and Crandall 1998). The best-fit model (GTR + G) selected by Akaike's information criterion (AIC), and ML was re-run with these parameters. The MP and ML consensus trees were identical. Branch support for the MP tree was estimated using nonparametric bootstrapping by TBR swapping and 1,000 replicates (Felsenstein 1985).

Results

Candidate *HrpX*-regulated and T3-effector genes of *X. citri* strain 306

Genes that were targeted for mutagenesis were selected on the basis of the presence of type III regulatory motifs and relatedness to known type III effectors in other plant pathogenic bacteria. The mutational strategy was designed to integrate an incomplete region of the coding sequence of each

gene in a manner that would generate two truncated copies of each gene. The insertion of the fragment was assessed by PCR using primers upstream of the targeted gene and in the vector, and sequencing of the resulting fragment. Global *hrp* regulatory genes *hrpG* and *hrpX*, and the *hrp* structural gene *hrcC*, were mutated and used as controls for the effectiveness of the strategy. Complementation tests were performed on a select number of mutants to determine if the mutant phenotypes were attributable to the targeted gene. Twenty-three candidate genes were selected for study based on the presence of PIP box motif sequences or relatedness to known T3-effectors (da Silva et al. 2002). Six genes, XAC0076, XAC0393, XAC0416, XAC0061, XAC2370 and XAC3309 contain perfect PIP-box sequences, and eleven genes contain imperfect motifs (Table 1; da Silva et al. 2002). Four genes, XACb0065 (*pthA4*), XAC0415 (*hrcC*), XAC0543 (*xopX*) and XAC3224 (*avrXacE2*), did not have PIP box-like sequences. We also indentified the presence of another *cis* element, the –box-like sequence, which was found in eleven genes (Table 1).

Table 1 Candidate *hrpX* regulon and putative effector/avirulence genes of *X. citri*

ORF number	(Putative) ORF product	PIP box sequence[a]	Distance (bp)[b]	–10 box[c]
hrp structural candidates				
XAC0415	*hrcC*	None		
XAC0416	*hpa1*	TTCGC-N_{15}-TTCGC	31	**TA**CTG**T**
XAC1265	*hrpG*	None		
XAC1266	*hrpX*	None		
hrp regulon candidates				
XAC0277	Conserved hypothetical protein	TTCG**G**-N_{15}-TTCGC	30	**TA**GCA**T**
XAC0661	endopolygalacturonase	TTCGC-N_{15}-TTCGC	30	**TA**GAG**T**
XAC1706	Alkanal monooxygenase	TTCG**T**-N_{15}-TTCG**T**		
XAC1886	β-K-adipate enol-lactone hydrolase	TTCG**T**-N_{15}-TTCG**T**		
XAC2370	Endopeptidase	TTCGC-N_{15}-TTCGC	30	**TA**TAG**T**
XAC2374	Polygalacturonase	TTCG**G**-N_{15}-TTCG**T**	32	**AA**GCT**T**
XAC2534	Conserved hypothetical protein	TTCGC-N_{15}-TTCG**T**	30	**TA**TGG**T**
XAC2922	*hprW*	TTCGC-N_{15}-TTCG**G**	31	**GA**TGA**T**
XAC3230	Actin-ADP-ribosylating toxin	TTCG**T**-N_{15}-TTCG**G**	30	**CA**AAC**T**
XAC3309	aminopeptidase	TTCGC-N_{15}-TTCGC		
Putative effector/avirulence				
XAC0076	*avrBs2*	TTCGC-N_{15}-TTCGC		
XAC0286	*avrXacE1 (avrPphE1)*	TTCG**T**-N_{15}-TTCG**G**	30	**CA**AAC**T**
XAC0393	*hpaF*	TTCGC-N_{16}-TTCGC	30	**TA**GCC**T**
XAC3090	Leucine rich protein	TTC**CA**-N_{15}-TTCGC		
XAC3224	*avrXacE2 (avrPphE2)*	None		
XACb0011	*avrXacE3 (avrPphE3)*	TTCGC-N_{15}-TTCG**G**		
XACb0065	*pthA4*	None		
Xanthomonas outer protein				
XAC0543	*xopX*	None		
XAC4330	*xopQ*	TTCG**T**-N_{15}-TTC**AC**	31	**TA**ACG**T**

[a] PIP box sequence and the distance in base pairs between the two conserved motifs. Nucleotides deviating from the consensus are underline

[b] Distance in base pairs between the end of PIP box and the –10 promoter motif

[c] –10 promoter motif sequence and –10 conserved base pairs are shown in bold. Nucleotides deviating from the consensus are underline

Most mutants of candidate effector genes produced typical symptoms in grapefruit leaves

In total, twenty-three mutants were screened in grapefruit leaves following infiltration of bacterial suspensions adjusted to 5×10^8 cfu/ml. These pathogenicity assays demonstrated that the regulatory mutants 306Ω*hrpG* and 306Ω*hrpX*, and the T3SS structural gene 306Ω*hrcC* were incapable of inducing visible citrus canker-like symptoms in grapefruit leaves (Fig. 1a). Complementation tests were performed on *hrpG*⁻ and *hrpX*⁻ mutants by introduction of plasmid pL22, carrying wild type *hrpG* and *hrpX*. Both mutants fully restored the wild-type phenotype (Fig. 1a). None of the additional nineteen mutants tested showed any observable phenotypic difference when compared with symptoms induced by wild type Xcc-306, with the exception of one strain that was derived from the mutagenesis of *hrpW* (Table 2).

Mutant 306Ω*hrpW* resulted in reduced virulence in grapefruit. However, several attempts to restore the wild-type phenotype with the full-length *hrpW* clone failed. The mutant was then screened for complementation using a library from *X. citri* strain A44. One clone (pL03) restored the virulence. Sequence analysis of pL03 indicated the clone contained a copy of *pthA4* gene homologue. A previously cloned version of *pthA4* from Xcc-X0053, pAW5.2 (Al-Saadi et al. 2007), was used for complementation and the wild-type phenotype was restored.

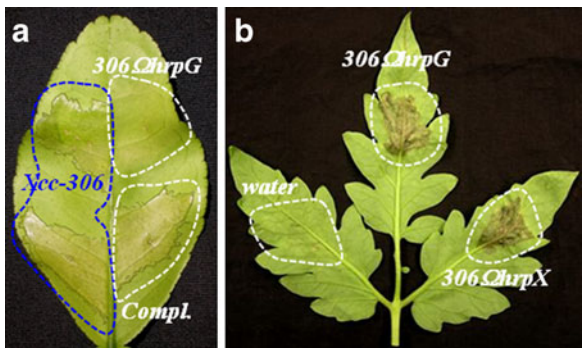

Fig. 1 Phenotypic analysis of *X. citri* subsp. *citri* strain 306 and Xcc-306 mutants into host grapefruit and nonhost tomato. **a** Xcc-306 wild type, 306Ω*hrpG* mutant and mutant complemented strains in grapefruit. Leaves were syringe infiltrated at concentrations of 5×10^8 cfu/mL and photographed six days after inoculation. **b** HR in tomato leaves by 306Ω*hrpG* and 306Ω*hrpX* mutant strains. Leaves were infiltrated as in A and photographed 24 h after inoculation

Type III defective mutants trigger hypersensitive response in non-host tomato plants

Because T3-effectors can induce a defence-related-response when injected into plant cells upon recognition by host receptor sites, we investigated if these mutations could interfere with the HR in non-host tomato plants. Under normal conditions Xcc-306 induces a strong HR in tomato plants 24 h after inoculation. All 20 mutants representing the putative T3-effectors genes induced strong HR 24 h after inoculation in tomato leaflets (Table 2). Interestingly, the regulatory 306Ω*hrpG* and 306Ω*hrpX*, and structural 306Ω*hrcC* mutant strains also triggered HR in tomato plants (Fig. 1b). This result indicates that an Hrp independent elicitor factor is involved in this HR induction.

Growth of *X. citri* strains is not affected by mutation in the *pthA4*

The virulence of 306Δ*pthA4* mutant was reduced as compared with the Xcc-306 wild-type strain. Therefore, growth of 306Δ*pthA4* mutant was investigated in grapefruit leaves. After 10 days, 306Δ*pthA4* mutant multiplied in the mesophyll of grapefruit leaves at levels similar to the wild-type strain, without triggering any visible raised pustule typical of citrus canker. The non-pathogenic 306Ω*hrpG* mutant, at the same time, showed lower growth rate than the wild-type strain (Fig. 2a). In order to support the results reported here, we performed a population analysis using the polymutant strain of Xcc-A^w (AΩ4), which is lacking the effectors *avrGf1, avrTaw* and *pthAw* (Rybak et al. 2009). In agreement with the results obtained with 306Δ*pthA4* mutant, the AΩ4 polymutant showed similar growth patterns in Key lime leaves when compared with Xcc-A^w wild type (Fig. 2b). Note that results were not significantly different from the results shown with Xcc-306 strains and mutants.

hrpW gene is highly conserved among *Xanthomonas citri* strains worldwide

We analyzed the full-length sequence of *hrpW* gene from a collection of *X. citri* strains. Based on PCR analysis, we detected *hrpW* in all 17 different *X. citri* strains tested, of which nine strains were from a

Table 2 Xcc-306 mutants pathogenicity phenotype in grapefruit and HR in tomato

Designtion	(Putative) ORF product	Grapefruit[a]	Tomato[b]
Xcc–306	Wild-type strain	+	+
306Ω*hrcC*	*hrpA*	−	+
306Ω*hpa1*	*hpa1*	+	+
306Ω*hrpG*	*hrpG*	−	+
306Ω*hrpX*	*hrpX*	−	+
306Ω2277	Conserved hypothetical protein	+	+
306Ω0661	endopolygalacturonase	+	+
306Ω1706	Alkanal monooxygenase	+	+
306Ω	β-K-adipate enol-lactone hydrolase	+	+
306Ω2370	Endopeptidase	+	+
306Ω2374	Polygalacturonase	+	+
306Ω2534	Conserved hypothetical protein	+	+
306Ω*hrpW*	*hrpW*	+	+
306Ω3230	Actin-ADP-ribosylating toxin domain	+	+
306Ω3309	aminopeptidase	+	+
306Ω*avrBs2*	*avrBs2*	+	+
306Ω*avrPphE1*	*avrPphE*	+	+
306Ω*hpaF*	*hpaF*	+	+
306Ω3090	Leucine rich protein	+	+
306Ω*avrPphE2*	*avrPphE2*	+	+
306Ω*avrPphE3*	*avrPphE3*	+	+
306Δ*pthA4*	*pthA4*	−	+
306Ω*xopX*	*xopX*	+	+
306Ω*xopQ*	*xopQ*	+	+

[a] Bacterial Pathogenicity: (+) typical canker symptom development including raised pustules (singular), (−) no symptoms

[b] HR induction: (+) positive for HR

worldwide collection and eight were collected in Florida, USA. Phylogenetic analysis at the nucleotide level demonstrated that all the strains were clustered in the same clade with Xcc-306. Moreover, CLUSTAL W analysis showed that the strains are 100% identical at the amino acid level. The phylogenetic analysis observed here indicates that *hrpW* is highly conserved among *X. citri* strains.

Discussion

To characterize pathogenicity genes in *X. citri* strain 306, we analyzed 23 Xcc-306 mutant strains classified as putative HrpX-regulon genes, effector/avirulence genes and *hrp* genes. Based on the plant-inducible promoter (PIP) box and the -10 like box motifs, we determined that *pthA4*, *hrcC*, *avrXacE2* and *xopX*, carry neither of the motifs (Cunnac et al. 2004; Fenselau and Bonas 1995). The lack of a PIP-box in *avrXacE2* and *xopX* has been reported also in previous studies (da Silva et al. 2002; Koebnik et al. 2006). However, da Silva et al. (2002) did not report the presence of a PIP box-like sequence in XAC3090 (*leucine rich protein*), which was observed here.

In pathogenicity and HR tests, mutation of 19 putative effectors assessed in this study did not affect disease symptom development in susceptible grapefruit leaves or HR elicitation in non-host tomato plants. However, some of the genes analyzed here, such as *hpa1* and *hpaF,* have been reported to be involved in pathogenicity in other phytopathogens and when mutated in *X. oryzae* (Δ*hpa1,* Δ*hpaF*) and *X. glycines* (Δ*hpaF*), had significantly reduced virulence (Cho et al. 2008; Kim et al. 2003; Zhu et al. 2000).

XopX from *X. c.* pv. *vesicatoria* contributes to the virulence on host pepper and tomato plants, possibly targeting basic innate immunity in plants (Metz et al. 2005). XopX is also essential for pathogenicity in rice leaves but is not required for HR elicitation in tobacco by *X. o.* pv. *oryzae* (Yang et al. 2007). Several studies demonstrated that *xopQ* is highly conserved among

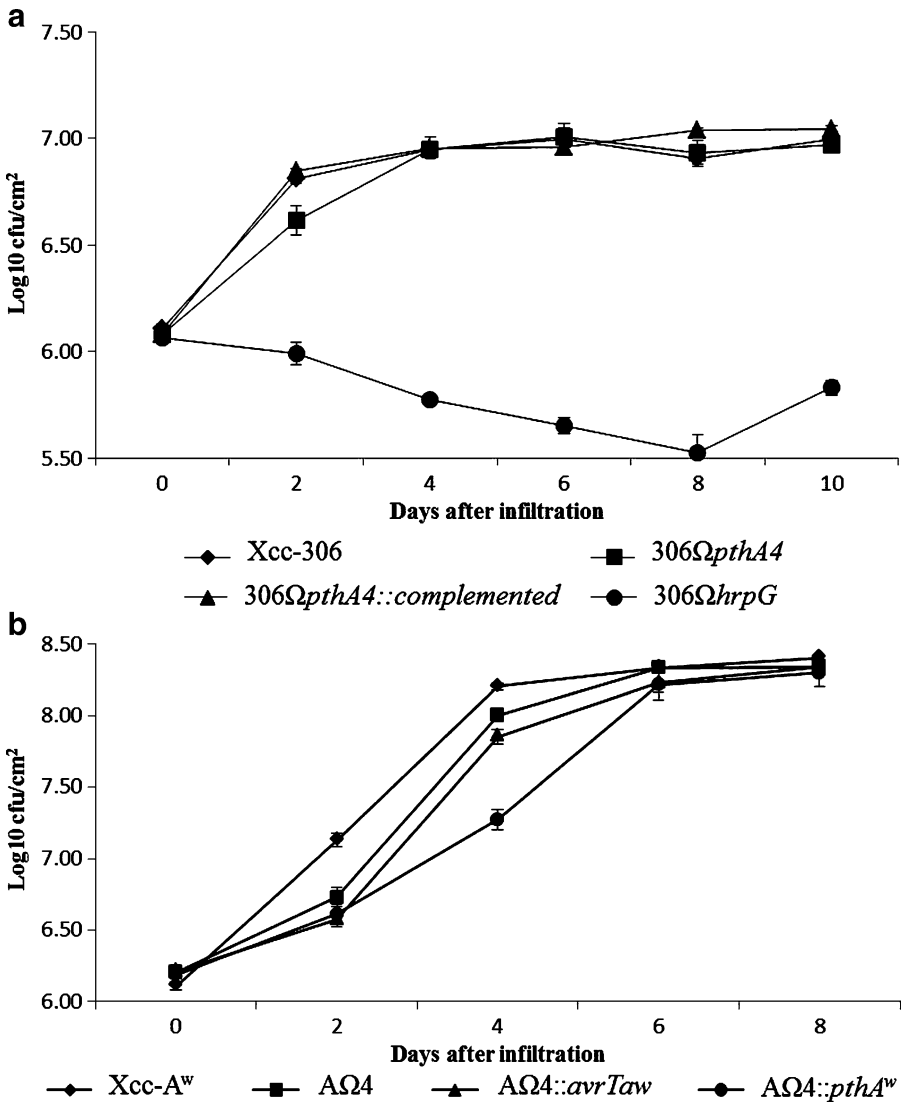

Fig 2 *Xanthomonas citri* subsp. *citri* strains carrying a defective *pthA* is not impaired with its ability to grow in host grapefruit and key-lime. **a** Bacterial growth in grapefruit leaves infiltrated with Xcc-306—wild-type, 306Ω*pthA4* mutant, 306Ω*pthA4* mutant complemented with pAW5.2 (306Ω*pthA4::complemented*), and 306Ω*hrpG* mutant; **b** Total bacterial cells recovered from Key-lime leaves infiltrated with Xcc-A^{w}—wild type, Xcc-A^{w}—triple knockout (AΩ4), AΩ4 mutant containing *avrTaw* (AΩ4*::avrTaw*), and AΩ4 complemented mutant strain (AΩ4::*pthA*^{w})

some *Xanthomonas* spp., such as *X. o.* pv. *oryzae*, *X. c.* pv. *vesicatoria* and *X. fuscans* subps. *aurantifolii* (Furutani et al. 2009; Koebnik et al. 2006; Moreira et al. 2010), as well as in Xcc-306, although *xopQ* from Xcc-306 is not required for virulence in grapefruit or HR elicitation in tomato. Although mutation in the *avrBs2* gene from *X. c.* pv. *vesicatoria* has been shown to affect virulence (Swords et al. 1996), Xcc-306 carrying a defective *avrBs2* did not present any visible alteration in disease development. In conclusion, we report that the putative effector genes tested here are not required for disease development and HR induction under greenhouse conditions. Our results indicate that these putative *hrpX*-regulons and effector/avirulence genes individually do not have any direct role in canker development. However, we cannot conclude that they are unnecessary in any stage of the bacterial survival *in planta*.

Disruption of either *hrpG* or *hrpX* eliminated induction of any symptoms in grapefruit leaves but both mutants retained the ability to trigger HR in non-host tomato plants. Similar results were reported for a *hrp*-regulatory Δ*hrcC* mutant of *P. syringae* pv. *tabaci* that was also able to trigger HR in non-host tomato plants in a *hrp*-independent manner and the elicitor, ultimately, was shown to be flagellin (Marutani et al. 2005). Based on these results, we speculate that an unknown T3SS-independent gene in Xcc-306 is the HR-elicitor. Future work is being conducted to clone and characterize the genes responsible for this reaction.

X. citri strain 3213 carrying a mutation in the *pthA* gene was previously shown to have lost the ability to induce pathogenic symptoms on grapefruit and incite an HR on the non-host California Light Red (*Phaseolus vulgaris*) plants (Swarup et al. 1991). Additionally, the growth of the bacterium in the plant was reduced when compared with the wild type strain. Here, we also demonstrated reduced symptoms in grapefruit leaves when infiltrated with 306Δ*pthA4* mutant. However, this mutant had a growth pattern similar to the wild-type strain, contrasting with the previously reported results (Swarup et al. 1991). We had similar results in a bacterial growth assay conducted with another strain Xcc-A^w, which carries two *pthA*-related genes, and a Xcc-A^w mutant (AΩ4), which is defective in both *pthA* homologues.

Comparison of phylogenies from sequences of conserved chromosomal genes is an effective method to evaluate the evolution among different pathovars in the same genus. Using the full *hrpW* sequence amplified from different *X. citri* strains, we demonstrated that *hrpW* gene is highly conserved among *X. citri* strains collected worldwide and could be used as a diagnostic marker for *X. citri* in citrus orchards. The *hrpW* homolog has been shown to be conserved in other plant pathogenic bacteria such as *R. solanacearum* – PopW protein (Li et al. 2010). Moreover, previous studies demonstrated that *hrpW* could be used as diagnostic marker for citrus canker (Park et al. 2006). Although the pathogenicity assay did not reveal that *hrpW* is essential for disease development, the presence in all *X. citri* strains and conserved nature of the sequence indicates that *hrpW* might be required by the bacterium in some unidentified stage.

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