A Novel Periplasmic Protein, VrpA, Contributes to Efficient Protein Secretion by the Type III Secretion System in *Xanthomonas* spp.

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Efficient secretion of type III effector proteins from the bacterial cytoplasm to host cell cytosol via a type III secretion system (T3SS) is crucial for virulence of plant-pathogenic bacterium. Our previous study revealed a conserved hypothetical protein, virulence-related periplasm protein A (VrpA), which was identified as a critical virulence factor for *Xanthomonas citri* subsp. *citri*. In this study, we demonstrate that mutation of *vrpA* compromises *X. citri* subsp. *citri* virulence and hypersensitive response induction. This deficiency is also observed in the *X. campestris* pv. *campestris* strain, suggesting a functional conservation of VrpA in *Xanthomonas* spp. Our study indicates that VrpA is required for efficient protein secretion via T3SS, which is supported by multiple lines of evidence. A CyaA reporter assay shows that VrpA is involved in type III effector secretion; quantitative reverse-transcription polymerase chain reaction analysis suggests that the *vrpA* mutant fails to activate citrus-canker-susceptible gene *CsLOB1*, which is transcriptionally activated by transcription activator-like effector PthA4; in vitro secretion study reveals that VrpA plays an important role in secretion of T3SS pilus, translocon, and effector proteins. Our data also indicate that VrpA in *X. citri* subsp. *citri* localizes to bacterial periplasmic space and the periplasmic localization is required for full function of VrpA and *X. citri* subsp. *citri* virulence. Protein–protein interaction studies show that VrpA physically interacts with periplasmic T3SS components HrcJ and HrcC. However, the mutation of VrpA does not affect T3SS gene expression. Additionally, VrpA is involved in *X. citri* subsp. *citri* tolerance of oxidative stress. Our data contribute to the mechanical understanding of an important periplasmic protein VrpA in *Xanthomonas* spp.

The type III secretion system (T3SS) and T3SS effectors (T3Es) are critical for pathogenicity of many important pathogenic bacteria, including animal pathogens (e.g., *Yersinia* and *Salmonella* spp., *Escherichia coli*, and *Shigella* spp.) and plant pathogens (e.g., *Xanthomonas*, *Ralstonia*, and *Pseudomonas* spp.) (Bliska et al. 2013; Diepold and Wagner 2014; Feng and Zhou 2012; Tampakaki et al. 2010). The T3SS apparatus of

animal pathogens typically forms needle structures (Fujii et al. 2012); however, the plant pathogen T3SS apparatus forms syringae structures (Lohou et al. 2013) to inject T3Es into host cells. T3SS in plant-pathogenic bacteria is encoded by hypersensitive reaction and pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes (Bogdanove et al. 1996). A typical T3SS consists of an extracellular nanopilus with a translocon, an inner membrane (IM), an outer membrane (OM) ring structure connected with a periplasmic rod, and a predicted cytoplasmic ring structure (Büttner 2012). Comparative sequence analyses indicate that at least 9 of more than 20 T3SS components are conserved among plant- and animal-pathogenic bacteria. The conserved T3SS components are likely the core components of the secretion apparatus in the IM and OM (Büttner 2012).

Due to the importance of T3SS and T3Es, dramatic progress has been made in understanding T3SS gene regulation, the T3SS apparatus assembly and architecture, and effector secretion. The assembly process is largely controlled by the T3SS gene cluster. It is assumed that T3SS assembles through a hierarchical process, wherein the extracellular components of the machinery are secreted prior to effectors, the ring structures in the IM and OM are assembled before the inner rod (Sukhan et al. 2001), and the export apparatus is assembled before the needle complex forms (Wagner et al. 2009).

Because the T3SS spans the membrane, periplasmic components have been shown to affect the T3SS through multiple mechanisms. For example, one study showed that peptidoglycan binds to HrpB1, which is a periplasmic component of T3SS and binds to T3S system components, including the inner membrane protein HrcD, the secretin HrcC, the pilus protein HrpE, and the putative inner rod protein HrpB2 (Hausner et al. 2013). Studies have shown that periplasmic lytic transglycosylases are required for efficient T3SS assembly because the natural pores of peptidoglycan are too narrow for T3SS apparatus formation (Garcia-Gomez et al. 2011; Zahrl et al. 2005). The lytic transglycosylases cleave the β -1,4-glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan during T3SS apparatus formation. CtpA is a soluble periplasmic carboxyl-terminal-processing protease protein. CtpA is essential for normal T3SS function in *Pseudomonas aeruginosa*, cytotoxicity toward the host cells, and virulence in a mouse model of acute pneumonia. It was suggested that CtpA activity might be required for proper T3SS assembly or function, even though experimental evidence is lacking (Seo and Darwin 2013). MreC, which is a periplasmic cytoskeletal element that controls cell shape in *Salmonella* spp., has also been reported to affect T3SS. The cytoskeleton is dispensable to T3SS structure assembly but is essential for *Salmonella*

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pathogenicity island-1 type (SPI-1) T3SS gene expression. The regulation is mediated by the two-component system sensor kinase RcsC, which detects changes at the cell surface caused by bacterial cytoskeleton disintegration (Bulmer et al. 2012).

In addition to periplasmic proteins, a potential link between outer-membrane protein (OMP) assembly and T3SS gene expression was suggested (Amy et al. 2004; Fardini et al. 2007). Mutation of *bamB*, which encodes a lipoprotein involved in OMP assembly, downregulated expression of SPI-1, SPI-2, and flagella genes as well as impaired the ability of *Salmonella* cells to invade epithelial cells and secrete T3SS-1 effectors and flagellar proteins (Amy et al. 2004; Fardini et al. 2007). Both the T3SS and flagella are membrane-spanning apparatuses and share high identity in many components (Kawamoto et al. 2013). Soscia and colleagues (2007) showed that T3SS and flagellar assembly exhibit negative cross-control. A *P. aeruginosa* strain without flagella showed increased T3SS gene expression, effector secretion, and cytotoxicity (Soscia et al. 2007). Interestingly, DsbA, which is a periplasmic disulfide-bonded oxidase, affects SPI-1 regulation and SPI-1 function through a feedback inhibition of SPI-1 transcription in *Salmonella* spp. (Lin et al. 2008). This inhibition depends on the flagellar protein FliZ and RcsCDB phosphorelay system (Majdalani and Gottesman 2005). Overall, previous studies suggest that the T3SS is affected by multiple factors at the cell wall.

In this study, we reported one novel hypothetical periplasmic protein, XAC3049, which is designated as virulence-related periplasmic protein A (VrpA), and revealed that the secretion of T3SS proteins and T3Es are affected in *Xanthomonas citri*

subsp. *citri*. *X. citri* subsp. *citri* is a gram-negative aerobic bacterium that causes citrus canker, which is one of the most devastating diseases for most commercial citrus cultivars and is responsible for significant economic losses worldwide (Gottwald and Riley 2005). *Xanthomonas* spp. are an important group of plant-pathogenic bacteria that infect a wide range of hosts, including at least 68 plant families and more than 240 genera, as well as many economically important crops such as rice, citrus, banana, cabbage, tomato, pepper, and bean (Ryan et al. 2011). Previous studies from our laboratory showed that an XAC3049 EZ-Tn5 insertion mutant did not produce visible citrus canker symptoms on grapefruit (Yan and Wang 2012). In this study, we further studied the localization of VrpA, its effect on secretion of extracellular components of T3SS and T3Es, its interactions with T3SS components, and its homologs in other xanthomonads.

RESULTS

VrpA contributes to *X. citri* subsp. *citri* virulence in citrus plant and hypersensitive response induction in nonhost tobacco.

A previous study from our laboratory showed that an EZ-Tn5 insertion mutant, 416G9, produced no visible citrus canker symptoms on grapefruit (Yan and Wang 2012). The *vrpA* open reading frame (ORF) (XAC3049) was inserted by EZ-Tn5 in both 416G9 and 329E2 mutants (Yan and Wang 2012) (Fig. 1A). To confirm whether *vrpA* is involved in *X. citri* subsp. *citri* virulence, the *vrpA* deletion mutant was generated through

Fig. 1. VrpA is required for virulence in *Xanthomonas citri* subsp. *citri*. **A**, Schematic diagram of *vrpA* (XAC3049), the upstream XAC3048, and the downstream *btuB* gene loci. The single-head arrows represent the gene location and orientation. The two *Pst*I sites used for Southern blot analyses are located at 17 and 2,555 bp, respectively, from the left end. The EZ-Tn5 insertion sites in both the 416G9 and 329E2 mutants are indicated. The deletion and complementary regions are shown using dotted lines. **B**, Pathogenicity assay. Cells were used to inoculate grapefruit leaves (*Citrus paradise* Macf. 'Duncan') at 10^6 CFU ml⁻¹. Leaves were photographed 6 days postinoculation. Experiments were repeated more than three times with similar results, and only one representative leaf is presented. **C**, *X. citri* subsp. *citri* growth curve in planta. The bacterial cells were used to inoculate grapefruit leaves at 10^6 CFU ml⁻¹, and the leaves were recovered at different time-points. Values represent the means from three replicates. Means $\pm$ standard deviation are plotted. **D**, Wild type, *vrpA* mutant, and insertion complementary strain were inoculated into 4-week-old fully expanded leaves of *Nicotiana benthamiana* at the concentration of 10^8 CFU ml⁻¹. Hypersensitive response induction were observed and photographed around 10 days past-inoculation. All experiments were repeated three times with the similar results, and only one leaf was presented.

double crossover homologous recombination using the pOK1 suicide plasmid. Southern blot analyses indicated that the entire ORF of the *vrpA* gene was deleted from the chromosome (Supplementary Fig. S1). The *vrpA* mutant was subsequently used to inoculate Duncan grapefruit and Valencia sweet orange. Similar to the insertion mutants 416G9 and 329E2, the *vrpA* deletion mutant did not cause visible canker symptoms, which indicates a crucial role for *vrpA* in *X. citri* subsp. *citri* virulence (Fig. 1B). A similar phenotype was observed using a Valencia sweet orange plant (data not shown). The complementary strain induced canker symptoms typical for wild-type Xac306 on the grapefruit (Fig. 1B) and sweet orange plants (data not shown). In addition, the bacterial growth curve in planta indicated that the wild-type and complementary strain populations were similar, whereas the *vrpA* mutant population was significantly lower than the wild-type and complementary strains (Fig. 1C). No dramatic difference was observed between growth of the wild-type, complementary, and *vrpA* mutant strains in nutrient broth (NB), XVM2, M9, and plant-extraction media (Supplementary Fig. S2).

In addition, a tobacco inoculation assay was performed to test whether VrpA is involved in induction of the hypersensitive response (HR) in nonhost plants. The wild-type, *vrpA* mutant, and *vrpA* insertion complementary strains were inoculated on 4-week-old tobacco leaves at a cell density of 10^8 CFU ml⁻¹ (optical density at 600 nm [OD₆₀₀] = 0.3) by needleless infiltration. The results showed that wild-type 306 and the *vrpA* insertion complementary strain induced an HR on tobacco leaves at 10 days postinoculation (dpi), while the *vrpA* mutant failed to elicit HR or elicited a delayed and weakened HR compared with wild-type and complementary strains (Fig. 1D). Taken together, our results indicate that VrpA is an essential protein contributing to virulence and HR induction.

VrpA is a highly conserved protein in phytopathogenic xanthomonads.

Protein BLAST showed that VrpA in *X. citri* subsp. *citri* shares approximately 90% similarity with its homologs in other *Xanthomonas* spp. (Supplementary Fig. S3). In addition, *Stenotrophomonas* and *Xylella* spp. harbor VrpA homologs of approximately 76 and 65% identity, respectively. Notably, *Pseudoxanthomonas suwonensis* 11-1 and *Pseudomonas geniculata* N1 contain VrpA homologs with the similarities of 76 and 62%, respectively. An N-terminal 23-amino-acid signal peptide was predicted by the SignalP 4.1 program. Interestingly, the SignalP prediction revealed that all of the VrpA's homologs contain an N-terminal signal peptide, which suggests that the evolutionarily conserved VrpA proteins in *Xanthomonas* spp. and other species may have similar localization and biochemical functions. However, a bioinformatics analysis of the VrpA protein failed to provide additional insight into its biochemical function, and no known domain and motif was identified.

To test whether the VrpA homologs have similar function, we tested the function of VrpA in *X. campestris* pv. *campestris*. The VrpA homolog (XCC2866, designed as VrpA_{Xcc} herein) in *X. campestris* pv. *campestris* ATCC 33913, which causes black rot on the most cultivated cruciferous vegetables worldwide, including the model plant *Arabidopsis thaliana* (Vicente and Holub 2013), was knocked out and the virulence of *vrpA*_{Xcc} mutant was tested. Wild-type Xcc33913 induced chlorotic and necrotic symptoms on *Arabidopsis* ecotype Col-0 at 5 dpi, while the *vrpA*_{Xcc} mutant-inoculated leaves remained symptomless (Fig. 2A). Introducing the complementation plasmid p53-VrpA_{Xcc} restored virulence in the *vrpA*_{Xcc} mutant. In addition, the VrpA homologs in *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*, which causes bacterial leaf spot on pepper and tomato, complemented the virulence of an

X. citri subsp. *citri* *vrpA* mutant. At 7 dpi, the *X. citri* subsp. *citri* *vrpA* mutants with p53-VrpA_{Xcc} and p53-VrpA_{Xcv} induced typical citrus canker symptoms, similar to wild-type 306 (Fig. 2B). The in planta bacterial population indicated that ectopic

Fig. 2. VrpA_{Xcc} and its homologs VrpA_{Xcc} and VrpA_{Xcv} are functionally conserved. **A**, Pathogenicity assay for *Xanthomonas campestris* pv. *campestris* on the *Arabidopsis thaliana* ecotype Col-0. Leaves were infiltrated with the wild type, a *vrpA*_{Xcc} mutant, and a complementary strain of *X. campestris* pv. *campestris* 33913 using a bacterial suspension adjusted to 5×10^5 CFU ml⁻¹. Photographs were collected 5 days postinoculation (dpi). **B**, Pathogenicity assay for *X. citri* subsp. *citri* in citrus. Bacterial cells were used to inoculate Duncan grapefruit leaves at 10^8 CFU ml⁻¹. Leaves were photographed 6 dpi. Pathogenicity assays using *Arabidopsis* and citrus were repeated more than three times with similar results, and only one representative leaf is presented. **C**, *X. citri* subsp. *citri* bacterial population in Duncan grapefruit. Bacterial cells were used to inoculate Duncan grapefruit leaves at 10^8 CFU ml⁻¹. Bacterial cells were recovered from the inoculated leaves 0, 4, and 8 dpi, and the values are the means from three repeats. Means $\pm$ standard deviation are plotted. Asterisks indicate a statistically significant difference (* indicates $P < 0.01$ versus the wild type).

expression of VrpA_{Xcv} and VrpA_{Xcc} restored the growth of the *X. citri* subsp. *citri* *vrpA* mutant to wild-type level (Fig. 2C). Although VrpA_{Xcv} and VrpA_{Xcc} contain 2 amino acid mutations (A18P and G130T) and 13 amino acid mutations (Y3H, M12 L, T19S, Q27A, L20I, S77T, G122A, G129N, G131A, V133A, A146S, S270N, and V279I), respectively, compared with VrpA in *X. citri* subsp. *citri*, our results suggest that VrpA and its tested homologs are functionally conserved.

VrpA is localized and functional in the periplasm.

VrpA contains a 23-amino-acid signal peptide at the N terminus and the cleavage site was predicted to be between amino acids 23 to 26. To examine whether VrpA is secreted to bacterial periplasm, we made an N-terminal 26-amino-acid deletion mutant, VrpA_{ΔN26}, which lacks a complete signal peptide. The full-length VrpA protein and truncated mutant were expressed in pTF53 plasmid in-frame with a 3xFLAG tag, and the corresponding constructs were ectopically expressed in the *vrpA* mutant under the control of the P_{trp} promoter. Cells expressing VrpA-FLAG and VrpA_{ΔN26}-FLAG were harvested at the stationary phase and fractionated into a cytoplasm and membrane (CM) fraction and a periplasmic (PP) fraction through osmotic shock, whereas the bacterial culture supernatant (SN) yielded the extracellular fraction (EC) (Matsui et al. 2009). The fractions and total extracts (TEs) were subjected to immunoblotting using an anti-FLAG antibody to detect VrpA. RNA polymerase was exclusively observed in the TE and the

CM fraction but not in the PP fraction (Fig. 3A), suggesting no bacterial lysis occurred and there was similar protein loading. As expected, VrpA-FLAG was only present in the PP fraction but not the CM fraction. VrpA_{ΔN26}-FLAG was only detected in the CM fraction but not the PP fraction. Neither VrpA-FLAG nor VrpA_{ΔN26}-FLAG were detected in the EC fraction, which suggests that VrpA is not secreted into the extracellular environment (data not shown).

To further confirm that VrpA is localized in the periplasm, an *E. coli*-based alkaline phosphatase (PhoA) assay was performed (Ammerman et al. 2008). PhoA is active only when translocated after export to the periplasm, and the phosphatase activity can be visually detected by adding a chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to the bacterial medium (Bronstein et al. 2005). To generate pJDT-VrpA and pJDT-VrpA_{ΔN26}, the full-length VrpA and truncation mutant were fused in-frame with the PhoA N terminus devoid of its native signal peptide. Two plasmids and an empty vector control were transformed into *E. coli* DH5α cells. The transformants were streaked on Luria-Bertani (LB) plates containing BCIP at 90 μg ml⁻¹ and ampicillin (Amp) at 100 μg ml⁻¹. The cell colonies with pJDT-VrpA turned blue, whereas the cell colonies with pJDT-VrpA_{ΔN26} and an empty vector did not (Fig. 3B). In summary, our localization studies suggest that VrpA is secreted into the bacterial periplasm.

The periplasmic localization prompted us to postulate that VrpA is functional in the periplasm, and the signal peptide is

Fig. 3. VrpA is localized and functional in the bacterial periplasm. **A**, VrpA detection in different cell fractions through immunoblotting. The *vrpA* mutant expressing full-length VrpA-Flag or VrpA_{ΔN26}-Flag was inoculated in XVM2 medium, and fractions enriched in the cytoplasm and membrane (CM) and periplasm (PP) were separated by osmotic shock. Total extract (TE), CM, and PP fractions were analyzed through immunoblotting using antibodies specific for the FLAG tag and RNA polymerase (control). **B**, Alkaline phosphatase gene fusion assay. *Escherichia coli* strains carrying pJDT1-VrpA, pJDT1-VrpA_{ΔN26}, and an empty vector were streaked onto Luria-Bertani plates supplemented with 5-bromo-4-chloro-3-indolyl phosphate at 90 μg ml⁻¹, 75 mM Na₂HPO₄, and ampicillin at 100 μg ml⁻¹. **C**, Pathogenicity assay for citrus. Bacterial cells from different *X. citri* subsp. *citri* strains were used to inoculate grapefruit leaves at 10⁶ CFU ml⁻¹. Photographs were collected 6 days postinoculation (dpi). **D**, *X. citri* subsp. *citri* strain bacterial populations in planta were determined 0, 4, and 8 dpi with the initial bacterial concentration 10⁸ CFU ml⁻¹. All experiments were repeated more than three times with similar results, and only one representative leaf is presented. Means ± standard deviation are plotted. Asterisks and pounds denote a statistically significant difference (* indicate *P* < 0.01 versus wild type and # indicates *P* < 0.01 versus wild type and *vrpA* mutant).

required for virulence. To test this hypothesis, the wild type, a *vrpA* mutant, a chromosomal insertion complementary strain, and a *vrpA* mutant with constitutive VrpA-FLAG or VrpA_{ΔN26}-FLAG expression were used to inoculate Duncan grapefruit leaves at 10⁸ CFU ml⁻¹ (OD₆₀₀ = 0.3). The wild-type, chromosomal insertion complementary, and full-length VrpA-FLAG-expressing strains produced citrus canker symptoms (Fig. 3C). However, the *vrpA* mutant and VrpA_{ΔN26}-FLAG-expressing strains did not induce citrus canker symptoms (Fig. 3C). In addition, bacterial populations of the wild-type, insertion complementary, and VrpA-FLAG-expressing strains were significantly higher than the *vrpA* mutant and VrpA_{ΔN26}-FLAG-expressing strains (Fig. 3D). The reduced population of VrpA-FLAG expressing cells was observed and may be due to the accumulation of secretory proteins being toxic to the cell (Schlegel et al. 2013). Taken together, our results strongly support the notion that VrpA is a periplasmic protein secreted via a Sec-dependent pathway, and VrpA's periplasmic localization is required for its function in *X. citri* subsp. *citri* virulence.

VrpA contributes to secretion of T3SS proteins and effectors via T3SS.

Due to the significant effect of *vrpA* mutation on *X. citri* subsp. *citri* virulence and HR induction, we postulated that VrpA affects T3SS, which is crucial for pathogenicity and HR induction of many gram-negative phytopathogenic bacteria (e.g., *Xanthomonas*, *Pseudomonas*, *Erwinia*, and *Ralstonia* spp.) (Büttner 2012; Büttner and He 2009). To determine whether VrpA plays a role in protein secretion via T3SS, an adenylate cyclase (CyaA) fusion assay was performed (Sory et al. 1995). The CyaA is a calmodulin-dependent reporter protein that produces measurable cyclic AMP (cAMP) levels only when it is inside a plant cell but not in bacteria or the plant apoplast. The first 100 amino acids of an exogenous effector protein, AvrXv4, were used to fuse with CyaA. The AvrXv4₁₋₁₀₀::CyaA construct was introduced into the wild type, *vrpA* mutant, insertion complementary strain, and T3SS component *hrcV* and *hrcN* mutants. Similar expression levels and stability of the chimeric protein in all transformants were observed by immunoblot in XVM2 medium (Supplementary Fig. S4A). The corresponding strains were used to inoculate tobacco leaves at 10⁸ CFU ml⁻¹ (OD₆₀₀ = 0.3), and the cAMP levels were measured 24 h postinoculation. cAMP levels in the leaves inoculated with wild-type or insertion complementation strains were significantly higher than those inoculated the with *vrpA* mutant and T3SS-defective *hrcV* and

hrcN mutants (Fig. 4A). These experiments showed that VrpA affects T3E secretion via T3SS.

A recent study showed that *CsLOB1* is a citrus canker susceptibility gene that induces pustule formation, and the expression of *CsLOB1* can be induced by a transcription activator-like effector (TALE) PthA4 in *X. citri* subsp. *citri* (Hu et al. 2014). Thus, *CsLOB1* expression level can be used to evaluate PthA4 translocation in the *vrpA* mutant strain compared with the wild type. For this test, the wild type, *vrpA* mutant, inserted complementary strain, *pthA4* mutant, and *hrcV* and *hrcN* mutants were used to inoculate Valencia sweet orange at 10⁸ CFU ml⁻¹. *CsLOB1* expression was strongly upregulated upon inoculation of the wild-type and complementary strains. However, the *vrpA* mutant as well as *hrcV* and *hrcN* mutants failed to induce the expression of *CsLOB1* to the wild-type level (Fig. 4B), indicating that translocation of PthA4 by the T3SS was compromised in *vrpA* mutant.

Fig. 4. VrpA affects protein translocation through a type III secretion system (T3SS). **A**, Adenylate cyclase (CyaA) translocation assay. The AvrXv4₁₋₁₀₀-CyaA fusion construct was transferred into the wild type, a *vrpA* mutant, the inserted complementary strain, and the *hrcV* and *hrcN* mutants. The derivative strains were then used to inoculate *Nicotiana benthamiana* leaves. The cAMP level was determined 24 h postinoculation. Standard errors are indicated. Each experiment was repeated at least twice with similar results. **B**, Relative fold-change expression levels of *CsLOB1* were determined using a quantitative reverse-transcription polymerase chain reaction after inoculation with wild-type 306, *vrpA* mutant, inserted complementary strain, *pthA4* mutant, and two T3SS-deficient mutants, *ΔhrcV* and *ΔhrcN*. Bacterial strains were used to inoculate sweet orange at 10⁸ CFU ml⁻¹. The total RNA from inoculated zone was isolated and analyzed 0, 10, and 20 h postinoculation. Fold change values are shown as 2^{-ΔΔCT} and were normalized to *CsLOB1* expression at 0 h after wild-type infiltration and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*Cs5g06870*) as an internal control for equal total RNA input. Error bars are indicated. **C**, Protein in vitro secretion assay. Strains 85* (wild type) and 85* *ΔvrpA* harboring pGM-AvrGfI, pTF53-XopN, pTF53-HrpF, and pTF53-HrpE were incubated in a secretion-inducing medium. The total protein extraction (TE) and supernatant (SN) fractions were analyzed through immunoblotting.

The effect of VrpA on secretion of T3SS proteins and T3Es was further tested using an extracellular secretion assay with *X. campestris* pv. *vesicatoria* 85* and the 85* *vrpA* mutant instead of *X. citri* subsp. *citri* strains because *X. campestris* pv. *vesicatoria* 85* expresses a constitutively activated form of *hrpG* (*hrpG**); therefore, T3SS is constitutively activated in *X. campestris* pv. *vesicatoria* 85* (Rossier et al. 1999; Wengelnik et al. 1999). The constitutively activated T3SS in *X. campestris* pv. *vesicatoria* 85* renders it easy to measure the T3SS secretion in vitro. We investigated secretion of the *X. citri* subsp. *citri* specific effector AvrGF1, the XopN proteins that are present in both *X. citri* subsp. *citri* and *X. campestris* pv. *vesicatoria*, T3SS pilus protein HrpE, and translocon proteins HrpF. Protein samples from the TE and culture SN were isolated and used for immunoblotting. RNA polymerase was used as a control to ensure that no detectable bacteria lysis occurred. Secretion of all analyzed proteins was reduced in the *vrpA* mutant compared with the wild type (Fig. 4C), suggesting that VrpA plays a significant role in secretion of T3SS proteins and T3Es. Intriguingly, we also observed that $\Delta vrpA_{xcc}$ was unable to induce HR on nonhost tobacco; the concentration of cAMP in the $\Delta vrpA_{xcc}$ -inoculated leaves was approximately fourfold lower than the wild type (Supplementary Fig. S5). In summary, our data indicate that VrpA contributes to secretion of T3SS proteins and effectors via T3SS.

VrpA interacts with periplasmic domains of HrcJ and HrcC.

Because VrpA is localized to the bacterial periplasm and contributes to protein secretion through T3SS, we postulated that

VrpA may directly or indirectly associate with the T3SS apparatus (i.e., periplasmic components). To investigate whether VrpA physically interacts with the T3SS periplasmic components, we performed a yeast-two hybrid (Y2H) assay to analyze the interactions between VrpA and five T3SS apparatus proteins: HrcJ, HrcC, HrpB1, HrpB2, and HrpD5. In animal pathogens, crystal structure studies revealed that EscD/PrpH/YscD family and EscJ/PrpK/YscJ family proteins are major components of the IM ring and EscC/InvG family members form the OM ring (Marlovits et al. 2004). Periplasmic domains of the OM and IM rings interact and compose the T3SS periplasmic rod (Spreter et al. 2009). HrpD5, HrcJ, and HrcC are isoforms of the EscD, EscJ, and EscC families, respectively. Recent studies show that HrpB2 is a periplasmic protein that interacts with HrpB1 in *X. campestris* pv. *vesicatoria* (Hartmann et al. 2012; Hausner et al. 2013). Because HrpD5, HrcJ, and HrcC are partially localized in the periplasm and contain a transmembrane domain, which may yield false-negative results, we used the periplasmic region of each protein without the transmembrane domain fused to the GAL4 DNA binding domain (BD) to monitor the interaction of the native form of VrpA_{AN26} fused to the GAL4 activation domain (AD), as indicated (Fig. 5A). The SV40-p53 interaction was used as the positive control. The cells grown on double dropout medium (DDO) without tryptophan and leucine indicate that both the BD and AD vectors were successfully co-transformed into the Y2HGold strain (Fig. 5A, left panel). Two reporter genes, AUR-C and MEL1, were used to detect the positive protein interactions that enable yeast cells to grow on DDO plates in the presence of the highly toxic drug Aureobasidin A and to show a blue color upon adding the chromogenic substrate

Fig. 5. VrpA interacts with the periplasmic domains of HrcJ and HrcC. **A**, Yeast-two hybrid (Y2H) assay. Full-length HrpB1, HrcJ₂₇₋₂₀₇, HrcC₃₃₋₃₇₀, full-length HrpB2, and HrpD₅₃₉₋₃₁₂ fused to the GAL4 DNA binding domain (BD) were expressed in combination with VrpA_{AN26} fused to the GAL4 activation domain (AD) in the yeast strain Y2HGold. Strains were grown on double dropout medium (DDO) with -Trp and -Leu (left panel) as well as on DDO medium supplemented with X- α -Gal and Aureobasidin A (DDO/X/A, right panel). SV40 large T-antigen (fused with BD) and murine p53 (fused with AD) were used as positive controls; the empty BD vector was used as the negative control. **B**, Glutathione-S-transferase (GST) pull-down assay. GST-HrcJ₂₇₋₂₀₇, GST-HrcC₃₃₋₃₇₀, GST-HrpB2, and GST-HrpD₅₃₉₋₃₁₂ were expressed in *Escherichia coli*, immobilized on glutathione sepharose beads, and incubated with *E. coli* lysates containing His-VrpA_{AN26}. Total cell extract (Input) and eluted protein (Elute) were immunoblotted using the anti-GST and anti-His antibody. Asterisks indicate GST fusion proteins.

X- α -Gal. All proteins synthesized in yeast strains were subjected to immunoblotting analysis (Supplementary Fig. S6). The results showed that HrcJ₂₇₋₂₀₇ and HrcC₃₃₋₃₇₀ but not HrpB1, HrpB2, and HrpD₅₃₉₋₃₁₂ interact with VrpA_{AN26} (Fig. 5A). To confirm these interactions, a glutathione-S-transferase (GST) pull-down assay was performed (Fig. 5B). The *E. coli* cell lysates that expressed GST-HrcJ₂₇₋₂₀₇, GST-HrcC₃₃₋₃₇₀, GST-HrpB2, GST-HrpD₅₃₉₋₃₁₂, and GST (negative control) were bound to glutathione sepharose beads and mixed with His-VrpA_{AN26}-expressing cell lysate. After incubation for 2 h, the GST fusion proteins were washed and eluted. The eluted proteins were immunoblotted to detect GST-fused proteins and His-VrpA_{AN26}. Specific signals were detected only in the HrcJ₂₇₋₂₀₇ and HrcC₃₃₋₃₇₀ elutions, which indicates that VrpA can interact with HrcJ and HrcC in vitro (Fig. 5B). Taken together, our results suggest that VrpA physically interacts with the periplasm domain of HrcJ and HrcC proteins.

The expression of VrpA is induced in planta and in XVM2 but the mutation of VrpA does not affect *hrp* or *hrc* gene expression.

In light of the intimate connection between VrpA and T3SS, we investigated the gene expression of VrpA and T3SS proteins. We found that *vrpA* is induced in citrus plants and plant-mimic medium XVM2 (Supplementary Fig. S7). Total RNA was isolated from inoculated leaves at 4, 7, or 14 dpi. The quantitative reverse-transcription polymerase chain reaction (QRT-PCR) revealed that the expression of *vrpA* was induced in planta at 4 dpi, culminated at 7 dpi, and reduced again after 14 dpi. In XVM2, the total RNA was extracted from inoculated bacteria at early stationary phase. The *vrpA* gene expression level in XVM2 was approximately fivefold higher than that in NB medium. In addition, we investigated the expression of T3SS genes *hrcQ*, *hrcU*, and *hrpB1* and two T3SS master regulators, *hrpG* and *hrpX*, in the wild type and *vrpA* mutant. QRT-PCR results showed that all tested genes had a similar expression level in the wild type and mutant. HrpG can be activated by a sensor kinase HpaS through phosphorylation and upregulates the expression of HrpX, which binds to a consensus sequence motif (TTCGC-N15-TTCGC) located in a plant-inducible promoter (PIP), known as PIP box, to promote T3SS gene expression (Fenselau and Bonas 1995; Guo et al. 2011; Li et al. 2013). However, no PIP box was identified at the upstream sequence of *vrpA*, suggesting that the expression of *vrpA* is *hrpX* independent. In support of this, previous studies have shown that *vrpA* is not regulated by HrpX or HrpG (Guo et al. 2011; Kogenaru et al. 2012).

VrpA is involved in *X. citri* subsp. *citri* tolerance against oxidative stress.

Because VrpA is a periplasmic protein, we hypothesized that VrpA may be involved in tolerance against environmental stresses. The *vrpA* mutant showed reduced tolerance against H₂O₂ compared with the wild-type strain because the *vrpA* mutant exhibited the lower (Student's *t* test, *P* < 0.05) OD₆₀₀ at 100, 150, 200, and 250 μ M H₂O₂ compared with the wild type (Supplementary Fig. S8). We also examined other virulence traits (e.g., biofilm formation, lipopolysaccharide [LPS], and exopolysaccharide [EPS]) of the *vrpA* mutant and the wild-type strains. No difference was observed for biofilm formation, LPS, and EPS between the *vrpA* mutant and the wild-type strains (Supplementary Fig. S9).

DISCUSSION

Our study indicates that VrpA is required for efficient protein secretion via T3SS, which is supported by multiple lines of evidence. The CyaA gene fusion assay (Fig. 4A) showed that

the T3E secretion in *vrpA* mutant was reduced. This reduced secretion was also supported by reduced expression of citrus *CsLOB1* gene (Fig. 4B). *CsLOB1* was identified as the susceptibility gene for citrus canker. PthA4, a TALE in *X. citri* subsp. *citri*, induces hypertrophy and hyperplasia by binding to the effector binding element in the promoter of the *CsLOB1* gene to activate transcription of the *CsLOB1* (Hu et al. 2014). No hypertrophy and hyperplasia symptoms were observed on Duncan grapefruit with inoculation of *vrpA*, *pthA4*, *hrcV*, and *hrcN* mutants, which further supported the notion that *CsLOB1* induction was compromised or abolished in the mutants compared with the wild type under the experimental conditions. Moreover, the in vitro secretion studies showed a reduced secretion of T3SS pilus, translocon, and two effector proteins, indicating that full function of T3SS was systematically affected (Fig. 4). Therefore, it is conceivable that *X. citri* subsp. *citri vrpA* mutant has a much reduced virulence compared with the wild-type strain. The similar expression level of *hrp* or *hrc* genes in the wild type and *vrpA* mutant suggests that VrpA regulates T3SS through a novel mechanism rather than regulating T3SS gene expression. We did observe similar expression patterns of *vrpA* and *hrp* genes in planta and in XVM2 (Astua-Monge et al. 2005; Jalan et al. 2013). However, *vrpA* is not regulated by HrpX or HrpG, the master regulators for *hrp* genes (Guo et al. 2011; Kogenaru et al. 2012). No PIP box was identified in the promoter region of the *vrpA* gene. Overall, our data suggest that the *hrp* genes and *vrpA* are not coexpressed despite the effect of VrpA on T3SS.

The effect of VrpA on tolerance against oxidative stress likely contributes to the virulence deficiency of the *vrpA* mutant. Plants produce an oxidative burst, an important component of the plant defense responses, that characterizes an excessive amount of reactive oxygen species (ROS), which are toxic derivatives of oxygen, including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH•) (Baker and Orlandi 1995; Hammond-Kosack and Jones 1996). The oxidative burst by plants in response to microbial pathogen attack has been reported in both compatible and incompatible interactions (Bolwell et al. 2002; Wojtaszek 1997). Peak concentrations of H₂O₂ were observed at 24 h and between 8 and 10 days after inoculation in sweet orange Hamlin infected by *X. citri* subsp. *citri* (Kumar et al. 2011). ROS are capable of oxidizing DNA, lipid membranes, and proteins to result in cell damage (Lee et al. 2004; Levine et al. 1994). Thus, the ROS encountered by the *vrpA* mutant in planta may contribute to its reduced growth (Fig. 1). How VrpA contributes to tolerance against oxidative stress remains to be determined.

Our data indicate that VrpA in *X. citri* subsp. *citri* localizes to the bacterial periplasmic space, and periplasmic localization is required for full VrpA function and *X. citri* subsp. *citri* virulence (Fig. 3). In gram-negative bacteria, the periplasmic space is responsible for establishing a connection between the extracellular environment and bacterial intracellular biochemical reaction; thus, proteins localized to the periplasm often play a role in protein quality control and signal transduction during bacterial pathogenesis (Merdanovic et al. 2011). Due to the periplasmic localization of VrpA, we investigated the protein interaction of VrpA with the T3SS periplasmic component proteins. VrpA interacted with the periplasmic domain of HrcJ and HrcC, which was demonstrated using the Y2H and GST pull-down assays (Fig. 5). This physical interaction is specific because no interaction was observed for VrpA with HrpB1, HrpB2, and HrpD5. HrcJ and HrpD5 are major components of the IM ring, whereas HrcC is an OM ring component (Marlovits et al. 2004) with periplasmic domains that compose the T3SS periplasmic rod (Spreter et al. 2009). HrpB2 is required for the formation of the inner rod of T3SS (Hartmann et al. 2012;

Hausner et al. 2013), which might explain why VrpA does not physically interact with HrpB2. Our results suggest that VrpA may affect protein secretion of T3SS by physically interacting with T3SS periplasmic components, which most likely affects activation of secretion and assembly or stability of the T3SS apparatus.

VrpA is conserved in *Xanthomonas* spp. VrpA and its homologs most likely play a similar role in virulence in *Xanthomonas* spp. Our results show that mutation of both *vrpA*_{Xac} and *vrpA*_{Xcc} significantly reduce *X. citri* subsp. *citri* and *X. campestris* pv. *campestris* virulence (Figs. 1 and 2). *vrpA*_{Xcv} and *vrpA*_{Xcc} complemented the *vrpA* *X. citri* subsp. *citri* mutant (Fig. 2B and C). However, *vrpA* homologs were also identified in other genera of the Xanthomonadaceae family; for example, *Xylella* (Simpson et al. 2000), *Rhodanobacter* (Weon et al. 2007), *Stenotrophomonas* (Ramos et al. 2011), and *Pseudoxanthomonas* (Yoo et al. 2007). but not in other families, except for Xanthomonadaceae. *Xylella*, *Rhodanobacter*, *Stenotrophomonas*, and *Pseudoxanthomonas* do not contain the T3SS, and the latter three genera are not plant pathogens. Thus, we expect that *vrpA* may have other functions in addition to its interaction with the T3SS, which is beyond the scope of this study.

In conclusion, we have provided a mechanistic understanding of the hypothetical periplasmic protein VrpA. We confirmed that VrpA is required for virulence of the canker pathogen *X. citri* subsp. *citri* and black rot pathogen *X. campestris* pv. *campestris*. VrpA is localized and functional in the bacterial periplasmic space and affects protein secretion via T3SS. VrpA physically interacts with the T3SS periplasmic components HrcJ and HrcC. Currently, the three-dimensional structure of the T3SS apparatus is unknown because it is not amenable to X-ray crystallography and solution nuclear magnetic resonance due to its inherent noncrystallinity and insolubility (Loquet et al. 2012). Physical interaction between VrpA and HrcJ as well as HrcC may aid in understanding the regulation of T3SS secretion. Additionally, VrpA may be an ideal drug target for citrus canker control because it is highly conserved in phytopathogenic xanthomonads and is absent in most environmental microbes and animal pathogens.

MATERIALS AND METHODS

Bacterial strains, growth conditions, primers, and plasmids.

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. The primers used in this study are listed in Supplementary Table S2. The *X. citri* subsp. *citri* wild-type strain and mutants were cultured at 28°C in NB (Difco Laboratories, Detroit), on nutrient agar (NA) plates, in a XVM2 medium (Wengelnik et al. 1996), on M9 minimal medium plates (Chong 2001), or in a peptone sucrose medium. The *E. coli* strains were grown in LB medium at 37°C. When required, antibiotics were used at the following concentrations: rifamycin at 50 µg/ml, kanamycin (Kan) at 50 µg/ml, Amp at 100 µg/ml, gentamicin (Gm) at 5 µg/ml, and spectinomycin (Spec) at 50 µg/ml.

Generation of the *vrpA* mutant and complementation strains.

The *vrpA* gene was deleted by integrating the suicide vector pOK1 into chromosomal DNA via double-crossover homologous recombination. Briefly, the 810-bp left border fragment and the 896-bp right border fragment were amplified using two pairs of primers, 3049 LB-F and 3049 LB-R, respectively, as well as 3049RB-F and 3049RB-R, with wild-type 306 genomic DNA as a template; they were then linked through double-joint PCR. The flanking fragment was digested by *Apa*I and *Xba*I and ligated with a similarly digested pOK1 vector to form pOK1-

VrpA. The resulting construct was transformed into Xac306 through electroporation. The transformed cells were selected on NA plates supplemented with Spec. The positive colonies were then replicated on NA plates supplemented with 5% (wt/vol) sucrose for negative selection. The sucrose-sensitive colonies were selected, cultured overnight, and dilution-plated on NA containing 5% (wt/vol) sucrose to select colonies with a second crossover event. The resulting *vrpA* mutant was confirmed through PCR and Southern blotting. The *vrpA* gene mutations in *X. campestris* pv. *campestris* ATCC 33913 and *X. campestris* pv. *vesicatoria* strains 85* were generated using a similar method with the suicide vector pNPTS138.

To complement through insertion, the entire ORF of the *vrpA* gene with the 480-bp upstream sequence was amplified and cloned into the plasmid pPM7 g to generate pPM7 g-VrpA. The resulting construct was then integrated into the *X. citri* subsp. *citri* *vrpA* mutant chromosome through single-crossover recombination with the α -amylase gene via electroporation. The transconjugated cells were selected on NA plates with Kan. The presence of *vrpA* was verified using PCR. For complementation of ectopic overexpression, the entire ORF of the *vrpA* gene was amplified and cloned into the pTF53 plasmid to generate pTF53-VrpA. *vrpA* gene expression is driven by the P_{trp} promoter. The resulting plasmid was transferred into the *vrpA* mutant through electroporation. The transconjugated cells were selected on NA plates with Gm.

Southern blot analysis.

The genomic DNA samples were isolated from both wild-type 306 and a *vrpA* mutant using a Wizard genomic DNA purification kit (Promega Corp., Madison, WI, U.S.A.); they were subsequently purified using phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol) in accordance with a standard molecular biology protocol (Chong 2001). After washing with 70% (vol/vol) ethanol and drying, the DNA samples were resuspended in RNase- and DNase-free water and then subjected to digestion with *Pst*I. The digested products were loaded on a 0.9% agarose gel for electrophoresis, and transferred to a positively charged nylon membrane (Roche, Indianapolis, IN, U.S.A.) using standard procedures (Guo et al. 2011). The *vrpA* gene left-border sequence was used as the hybridization probe (Fig. 1A). Probe labeling, hybridization, and chemiluminescence detection were performed using a digoxigenin-High Prime II DNA labeling and detection starter kit as recommended by the manufacturer (Roche).

Pathogenicity assay in plants and bacterial growth curve assay in vitro and in vivo.

As described in our previous study (Guo et al. 2011; Li and Wang 2011; Yan and Wang 2012), the pathogenicity assays using grapefruit (*Citrus paradise* Macf. Duncan) or sweet orange (*C. sinensis* (L.) Osbeck) were performed in a quarantined greenhouse at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. The pathogenicity assays using *Arabidopsis* Col-0 were performed as described (Meyer et al. 2005). Bacteria were grown in planta as described previously (Guo et al. 2011; Li and Wang 2011; Yan and Wang 2012). The in planta growth assay was repeated independently three times with at least three replicates for each time. The data from one representative experiment are presented. For the in vitro growth curves, the bacterial strains were grown overnight in 5 ml of NB supplemented with the appropriate antibiotics. Next, 3 ml of the cell suspensions ($OD_{600} = 0.3$) was transferred into 100-ml flasks containing 30 ml of NB medium or XVM2 medium with the appropriate antibiotics. The cultures were grown at 28°C with agitation at 200 rpm, and the bacterial population

(OD₆₀₀) was measured over 48 h using a spectrophotometer 289 (WPA CO8000 Biowave Cell Density Meter; Biochrom Ltd., Cambridge, U.K.). At least three replicates were included at each time point. The experiments were repeated twice.

Subcellular fractionation assay.

Fractionation of the *X. citri* subsp. *citri* *vrpA* mutants harboring pTF53-VrpA or pTF53-VrpA_{ΔN26} was performed as described (Morita et al. 2006), with certain modifications. Briefly, the strains were grown in XVM2 overnight. An 8-ml aliquot of culture was centrifuged for 10 min at 8,000 × *g*. The SN was decanted and filtered through a 0.22-μm, sterilized, low-protein-binding filter. The protein in the SN was precipitated by adding 1 ml of cold 40% (wt/vol) trichloroacetic acid in acetone (−20°C) to 1 ml of SN. After incubating at −20°C for 4 h, the mixture was centrifuged for 30 min at 20,000 × *g* at 4°C. The pellet was washed twice with 100 μl of ice-cold 80% (vol/vol) acetone in water and dissolved in Laemmli sample buffer, which composed the EC. For the whole-cell fraction, the cells harvested from 10 min of centrifugation at 8,000 × *g* were suspended in 100 mM Tris-HCl (pH 8.0) with 20% (wt/vol) sucrose and adjusted to an OD₆₀₀ of 10. After adding the same volume of Tris-HCl with 5 mM EDTA and 20 μg of egg lysozyme, the samples were incubated for 30 min at room temperature. The cells were centrifuged at 10,000 × *g* for 20 min, and the SN was the PP. The precipitated pellet (spheroblast) was washed twice with cold phosphate-buffered saline (PBS) and resuspended in Laemmli sample buffer to generate the CM fraction.

PhoA reporter assays.

The *phoA* gene fusion assays were performed as described (Ammerman et al. 2008). Briefly, full-length VrpA and a VrpA_{ΔN26} truncation mutant were amplified through PCR with two pairs of primers, VrpAphoAinF and N26phoAinR as well as N26phoAinF and N26phoAinR, respectively. The amplified fragments were then inserted into an *Mfe*I- and *Sac*I-digested pJDT1 plasmid using an In-Fusion cloning kit (Clontech, Palo Alto, CA, U.S.A.) to produce an in-frame gene fusion between the PCR product and *phoA* gene. The resulting constructs and an empty vector were introduced into *E. coli* JM109 competent cells. The strains were cultured on LB plates supplemented with BCIP (the chromogenic PhoA substrate) at 90 μg/ml, 75 mM Na₂HPO₄ (to repress endogenous phosphatase activity), and Amp at 100 μg/ml. Pictures were taken 24 h postinoculation.

Cya protein translocation assay.

The Cya fusion protein activity was determined in vivo as described, with modifications (Roden et al. 2004). Briefly, the *avrXv4::cya* cassette was cut with *Xho*I and *Eco*RI from the pMS107 plasmid (a gift from the lab of J. B. Jones) and ligated into pBBRMCS-5, which produced pGM-XC1. The plasmid was then introduced into *X. citri* subsp. *citri* 306 (wild type), a *vrpA* mutant, a *vrpA* insertion complementary strain, and the *hrcV* and *hrcN* mutants. The cultured strains were suspended in 10 mM MgCl₂ at an OD₆₀₀ of 0.3 and then hand infiltrated into *Nicotiana benthamiana* leaves. A direct cAMP correlation enzyme immunoassay kit (Abcam) was used to process the leaf samples and measure the cAMP concentrations following the manufacturer's instructions. The protein content of each sample was determined using the Bio-Rad protein assay (Bio-Rad).

Secretion assay and protein analysis.

The type III effector AvrGf1, which induces HR in grapefruit (Rybak et al. 2009), was amplified using the primers GF1Xho-F and GF1Eco-R and ligated into a similarly digested pBBRMCS-5 plasmid, which generated pGM-AvrGF1. For

HrpE, HrpF, and XopN proteins, the entire ORFs were amplified using Q5 High-Fidelity DNA Polymerase (NEB). The fragments were inserted into pTF53 plasmid by In-Fusion cloning technology (Clontech). All constructs were introduced into the *X. campestris* pv. *vesicatoria* strains 85* (wild type) and 85* *ΔvrpA*. Growth of the derivative strains in secretion medium (minimal medium A supplemented with sucrose, casamino acids, bovine serum albumen, and thiamine) and the protein secretion assays were conducted as described (Büttner et al. 2002, 2006). To ensure that no bacterial lysis occurred, an antibody specific for the intracellular protein RNA polymerase was routinely added to the blots.

Y2H assay.

For the Y2H assay, the coding region of VrpA without the first 26 amino acids was amplified and inserted into the pGADT7 vector to generate an AD fusion. For the GAL4 DNA-binding (BD) fusion proteins, the following regions were amplified and inserted into the pGBKT7 vector: full-length HrpB1; HrcJ, 27-207; HrcC, 33-370; full-length HrpB2; and HrpD5, 39-312. Both the bait and prey were co-transformed into the yeast strain Y2HGold using the LiAc method. The auto-activation test was performed with pGADT7-VrpA alone in Y2HGold strain. The protein interactions were assayed as described (Matchmaker Gold Yeast Two-Hybrid System User Manual; Clontech).

GST pull-down assay.

E. coli cells expressing GST or GST fusion proteins were washed in PBS and suspended into CellLytic B Cell Lysis Reagent (Sigma-Aldrich) to generate the cell lysates. After centrifugation, the cell lysates were incubated with glutathione agarose beads in accordance with the GST Protein Interaction Pull-Down Kit instructions (Thermo Scientific). The beads were washed to remove the unbound proteins and incubated with *E. coli* cell lysates expressing VrpA_{ΔN26} for 1 to 2 h at 4°C. After washing four times, the beads were eluted with 10 mM glutathione, and the eluates were collected and immunoblotted using anti-His and anti-GST antibodies in accordance with the instructions.

RNA extraction and QRT-PCR.

The *X. citri* subsp. *citri* strains were cultured in an NB or XVM2 medium at 28°C with shaking at 200 rpm, and 10-ml samples of the bacterial cells were collected at 7, 14, and 19 h after inoculation. The RNA was immediately stabilized by mixing the culture with two volumes of RNeasy Protect Bacteria Reagent (Qiagen, Valencia, CA, U.S.A.) and extracted using the RNeasy Mini Kit (Qiagen). Contaminated genomic DNA was removed through treatment with a TURBO DNA-free kit (Ambion, Austin, TX, U.S.A.). The RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and adjusted to 75 ng/ml. The RNA obtained was subjected to a one-step QRT-PCR assay using a QuantiTect SYBR green RT-PCR kit (Qiagen) on a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). The *gyrA* gene was used as an endogenous control. The relative fold change in target gene expression was calculated using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). RNA extraction from plants was performed using the RNeasy plant mini kit (Qiagen), and the *CsLOB1* expression level was determined as described above.

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