The Tad Pilus Apparatus of ‘*Candidatus Liberibacter asiaticus*’ and Its Regulation by VisNR

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Citrus huanglongbing (HLB) is one of the most destructive diseases affecting citrus plants. ‘*Candidatus Liberibacter asiaticus*’, an uncultivated α -proteobacteria, is the most widely spread causal agent of HLB and is transmitted by the Asian citrus psyllid *Diaphorina citri*. ‘*Ca. L. asiaticus*’ attachment to the psyllid midgut is believed to be critical to further infect other organs, including the salivary gland. In this study, the type IVc tight adherence (Tad) pilus locus encoded by ‘*Ca. L. asiaticus*’ was characterized. The Tad loci are conserved among members of Rhizobiaceae, including ‘*Ca. L. asiaticus*’ and *Agrobacterium* spp. Ectopic expression of the ‘*Ca. L. asiaticus*’ *cpaF* gene, an ATPase essential for the biogenesis and secretion of the Tad pilus, restored the adherence phenotype in *cpaF* mutant of *A. tumefaciens*, indicating CpaF of ‘*Ca. L. asiaticus*’ was functional and critical for bacterial adherence mediated by Tad pilus. Quantitative reverse transcription PCR (qRT-PCR) analysis revealed that ‘*Ca. L. asiaticus*’ Tad pilus-encoding genes and ‘*Ca. L. asiaticus*’ pilin gene *flp3* were upregulated in psyllids compared with in planta. A bacterial one-hybrid assay showed that ‘*Ca. L. asiaticus*’ VisN and VisR, members of the LuxR transcriptional factor family, were bound to the *flp3* promoter. VisNR regulate *flp3*. Negative regulation of the *flp3* promoter by both VisN and VisR was demonstrated using a shuttle strategy, with analysis of the phenotypes and immunoblotting together with quantification of the expression of the *flp3* promoter fused to the β -galactosidase reporter gene. Comparative expression analysis confirmed that ‘*Ca. L. asiaticus*’ *visNR* was less expressed in the psyllid than in the plant host. Further, motility and biofilm phenotypes of the *visNR* mutant of *A. tumefaciens* were fully complemented by expressing ‘*Ca. L. asiaticus*’ *visNR* together. The physical interaction between VisN and VisR was confirmed by pull-down and stability assays. The interaction of the *flp3* promoter with VisR was verified by electrophoretic mobility shift assay. Taken together, the results revealed

the contribution of the Tad pilus apparatus in the colonization of the insect vector by ‘*Ca. L. asiaticus*’ and shed light on the involvement of VisNR in regulation of the Tad locus.

Keywords: attachment, bacterial pathogenesis, citrus, greening, *Liberibacter*, pilus, regulation

Citrus huanglongbing (HLB), or citrus greening disease, is the most economically important disease affecting citrus production worldwide (Bendix and Lewis 2018; Clark et al. 2018; Li et al. 2019; Wang et al. 2017b). The most common causal agent associated with HLB, phloem-residing and vectorborne ‘*Candidatus Liberibacter asiaticus*’, has not yet been cultivated (Bendix and Lewis 2018; Coletta-Filho et al. 2014), which has hindered the understanding of its biology and virulence mechanism. ‘*Candidatus Liberibacter*’ spp. belong to the Rhizobiaceae family and cause diseases on many plant species. In addition to ‘*Ca. L. asiaticus*’, ‘*Ca. L. africanus*’ and ‘*Ca. L. americanus*’ also cause citrus HLB (Duan et al. 2009; Kuykendall et al. 2012; Wulff et al. 2014). ‘*Ca. L. solanacearum*’ causes diseases on members of the Solanaceae family (e.g., “zebra chip” disease in potato) (Wang et al. 2017a and 2017b). ‘*Ca. L. asiaticus*’ is transmitted by the phloem-feeding insect vector Asian citrus psyllid, *Diaphorina citri*. *D. citri* acquires ‘*Ca. L. asiaticus*’ from HLB-diseased plants as nymphs or adults, but the propagation of the pathogen will occur only after the colonization of the psyllid midgut by ‘*Ca. L. asiaticus*’ (Ammar et al. 2016; Sétamou et al. 2016). It is assumed that ‘*Ca. L. asiaticus*’ attachment to the psyllid midgut is critical to further infect other organs, including the salivary gland. Thus, understanding the ‘*Ca. L. asiaticus*’ adherence mechanism to the psyllid midgut, as well as to other psyllid tissues, might provide useful information to prevent ‘*Ca. L. asiaticus*’ transmission.

Comparative analysis of the genome sequences of ‘*Candidatus Liberibacter*’ spp. allowed a conserved gene cluster encoding the Tad (tight adherence) pilus apparatus (also termed type IVc pili) in their chromosomes to be identified (Duan et al. 2009; Ellison et al. 2017; Roux et al. 2012; Tomich et al. 2007). This gene cluster also is found in the genome of many bacterial species of Rhizobiaceae, including *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* (Capela et al. 2001; Wang et al. 2014). Interestingly, two gene clusters encoding the Tad pilus apparatus are found in the *S. meliloti* genome, and these genes are required for efficient nodulation of the alfalfa host (Barnett et al. 2001; Capela et al. 2001; Zatakia et al. 2014). Type IV pili

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are widespread appendages whose assembly initiates in the bacterial inner membrane and expands out to the extracellular environment (Costa et al. 2016; Leighton et al. 2015). They participate in adhesion, cell–cell interactions, DNA transport, and motility (Mignolet et al. 2018). During the assembly process the type IV pilins are oligomerized through the secretion system apparatus with energy generated by the ATPases (Chung et al. 2014). Type IV pili are classified into three types—type IVa, IVb, and IVc—based on the phylogenetic analysis and features of their structural proteins and pilins (Ellison et al. 2017; Roux et al. 2012; Thompson et al. 2018). Pilins are processed before oligomerization by a prepilin peptidase, which is an inner membrane enzyme (de Bentzmann et al. 2006). The prepilin peptidase recognizes and cleaves the amino-terminal leader peptide, which is short (five to six residues) in type IVa pilins and longer (13 to 30 residues) in type IVb and IVc pilins (Ellison et al. 2017; O’Connell Motherway et al. 2011). The primary structure of the type IVc or Tad pilins shows distinct regions: a leader sequence in the amino terminal, a flp (fimbrial low-molecular weight protein) motif, hydrophobic region, and nonconserved carboxy terminal. Tad pilus apparatus is encoded by a limited number of genes clustered into one or more operons that are likely acquired by horizontal transfer in some bacterial species (Angelov et al. 2015; Hansen and Forest 2006; Roux et al. 2012).

The Tad pili are usually not associated directly with motility. Instead, they are involved in adherence (e.g., the Cpa [Caulobacter pilus assembly] system in *Caulobacter crescentus* and Tad in *Aggregatibacter actinomycetemcomitans*) (Clock et al. 2008; Mignolet et al. 2018). For *Pseudomonas aeruginosa*, it has been demonstrated that the Tad pilus apparatus is required for bacterium-to-surface attachment, bacterium-to-bacterium contact and aggregation, and adhesion of bacterial cells to the host respiratory epithelial cells (Bernard et al. 2009). Interestingly, recent findings evidenced that the Tad pili apparatus in *C. crescentus* may sense the surface contact by resistance to pilus retraction, which is sufficient to stimulate holdfast synthesis that promotes the attachment to the surface (Ellison et al. 2017). In the early stage of Tad pili assembly, TadZ/CpaE functions as a hub for recruiting other pilus components (Tomich et al. 2007). In the current model of Tad pili biogenesis, the Flp pilins, TadE/CpaJ, and TadF/CpaK preproteins are targeted to the inner membrane, where they are processed by the TadV/CpaA prepilin peptidase. The TadE/CpaJ and TadF/CpaK pseudopilins may form an oligomeric structure in the periplasm, which guides pilus assembly and provides contact with the outer membrane complex. The TadD/CpaO and RcpB/CpaD proteins facilitate the assembly of the outer membrane RcpA/CpaC secretin complex. The TadA/CpaF ATPase provides energy for Flp pilin assembly into the filament, which is facilitated by the TadB/CpaG and TadC/CpaH inner membrane proteins. The TadG protein might serve as a membrane anchor for the Flp pilus. The Flp pilus is threaded through the RcpA/CpaC secretin complex in the outer membrane, which is gated by RcpB/CpaD protein (Mignolet et al. 2018; Roux et al. 2012; Tomich et al. 2007).

The signals that trigger the expression and assembly of the Tad pilus apparatus in each bacterium species may be different. For example, in the bacterial predator *Bdellovibrio bacteriovorus* the tad pilus genes are upregulated in the initial stage of the predation process, and studies have shown they are essential for predation through attachment to the prey (Avidan et al. 2017). Perhaps the activation of the tad pilus genes in *B. bacteriovorus* is related to the presence of the prey in the environment. For the phytopathogen *Pectobacterium*

atrosepticum, it was observed that the expression levels of the tad genes were increased under plant-mimicking conditions compared with rich growth medium (Nykyri et al. 2013).

In this study the Tad pilus of ‘*Ca. L. asiaticus*’ is characterized. The data indicate that the Tad pilus of ‘*Ca. L. asiaticus*’ is upregulated in psyllids and may contribute to the bacterial adherence and colonization of psyllid tissues and organs, such as the midgut. *A. tumefaciens* also was utilized as a model to reveal how the *flp3* pilin gene is regulated by VisN and VisR of ‘*Ca. L. asiaticus*’. This study advances our understanding of ‘*Ca. L. asiaticus*’ factors that are important for the colonization of psyllids and the spread of HLB disease and of how the ‘*Ca. L. asiaticus*’ attachment apparatus is regulated by VisNR.

RESULTS

Type IVc Tad pilus locus is conserved among ‘*Candidatus Liberibacter*’ spp.

Comparative genomic analyses showed that the Tad pilus-encoding gene cluster is conserved in the ‘*Candidatus Liberibacter*’ spp. whose genome sequences are available in GenBank. Its structure is similar to the Tad loci of other Rhizobiaceae species (e.g., *A. tumefaciens*) (Fig. 1). The Tad gene clusters in ‘*Candidatus Liberibacter*’ spp. encode for all proteins that are necessary for assembly and function of the Tad pilus apparatus. Recently, the gene annotated as *pilQ* in the Tad cluster, due to its homology with the T2SS-T3SS Pil-N domain of the secretin protein CpaC/RpaA/PilQ, was found to be important for Tad pilus assembly in *C. crescentus* and was reannotated as *cpaI* (Christen et al. 2016). Sequence alignment of CpaI homologs in liberibacters are shown in Supplementary Figure S1. The Tad pilin genes (Fig. 1) are multicopies from four to seven genes in the chromosomes of the ‘*Candidatus Liberibacter*’ spp., which indicates that duplication and divergence of *flp* genes may be important in the diversification of the colonization properties of these organisms (Avidan et al. 2017; Kachlany et al. 2001; Zatakia et al. 2014). The Flp pilins of ‘*Candidatus Liberibacter*’ spp. contain multiple conserved regions, a leader peptide sequence, a Flp motif, and a hydrophobic region. However, the carboxy-terminal region of the Flp pilins is not conserved, which may be important for the interaction of the “tip” of the Tad pilus with different surfaces (Roux et al. 2012). The comparative analysis of the primary structures of the seven pilins encoded by ‘*Ca. L. asiaticus*’ is shown in Supplementary Figure S2.

Ectopic expression of ‘*Ca. L. asiaticus*’ *cpaF* gene restored the adherence phenotype in the *cpaF* mutant of *A. tumefaciens*.

The *cpaF* gene encodes for the ATPase of the Tad pilus and is essential for the biogenesis and secretion of the pilins (Tomich et al. 2007). CpaF shares 70 to 100% identity among ‘*Candidatus Liberibacter*’ spp. and other members of Rhizobiaceae, such as *A. tumefaciens* (Duan et al. 2009; Wang et al. 2014). In this study *A. tumefaciens* was used as a model to study the function of CpaF of ‘*Ca. L. asiaticus*’ because it is not cultured in vitro. The *cpaF* gene of *A. tumefaciens* was inactivated by homologous recombination, and the *cpaF* deletion was confirmed by PCR (Fig. 2A). The Δ *cpaF* mutant strain showed a reduction of 50% in adherence to borosilicate glass tubes compared with the wild-type and complemented strains carrying ‘*Ca. L. asiaticus*’ *cpaF* or *A. tumefaciens* *cpaF* genes (Fig. 2B). The ectopic expression of CpaF in the complemented strains was confirmed by immunoblotting with antibodies against anti-FLAG Tag (Fig. 2C). The total protein extracts were verified using the loading control probed with anti-GroEL

(Fig. 2C). These data showed that CpaF of ‘*Ca. L. asiaticus*’ is functional and critical for bacterial adherence mediated by Tad pilus.

‘*Ca. L. asiaticus*’ Tad pilus genes are highly expressed during colonization of psyllids.

To investigate the function of Tad pilus genes of ‘*Ca. L. asiaticus*’, their gene expression in planta and in psyllids was compared. The presence of ‘*Ca. L. asiaticus*’ RNA in the leaf and psyllid RNA samples was verified by one-step reverse transcription PCR (RT-PCR) using the ‘*Ca. L. asiaticus*’ specific primers Las16SF and Las16SR (Fig. 3A). qRT-PCR analyses showed that the expression of Tad pilus-encoding genes was higher in the insect host than in the plant host, ranging from a two- to 12-fold increase (Fig. 3B). We also assessed the expression of the five genes encoding the Tad pilins that have a complete secretory signal (Fig. 3C and D). Interestingly, only the *flp3* gene was highly induced in psyllids, whereas the four other *flp* genes were downregulated in psyllids. Altogether, the comparative gene expression studies suggested that the Tad pilus apparatus and the Flp3 pilin may play important roles during the colonization of the insect host by ‘*Ca. L. asiaticus*’.

VisN and VisR, two LuxR transcriptional factors, regulate *flp3* expression in ‘*Ca. L. asiaticus*’.

After determining that Flp3 pilin is highly expressed in psyllids, an attempt was made to identify which transcriptional factor of ‘*Ca. L. asiaticus*’ regulates its expression. First, multiple regulators of ‘*Ca. L. asiaticus*’ were tested using a bacterial one-hybrid assay. For this assay, the *flp3* promoter region (–30 to –280) was fused with reporter gene *his3* using the plasmid pH3U3. Different ‘*Ca. L. asiaticus*’ transcriptional factors were cloned downstream of the *lacUV5* promoter and fused with the carboxi terminal of the *Escherichia coli* RNA polymerase omega subunit and FLAG Tag into the plasmid pB1H2ω2 (Noyes et al. 2008). All of the constructs were used

to transform the *E. coli* mutant strain ($\Delta\rho\sigma Z:zeo$, $\Delta hisB$), and selection assays in minimal media without histidine plus 3-aminotriazole and antibiotics were performed (Noyes et al. 2008).

To test the feasibility of this assay, the first selection test was performed with the plasmids harboring, respectively, the promoter of the *ldtP* gene and its transcriptional regulator *ldtR* in ‘*Ca. L. asiaticus*’, which were previously characterized (Pagliai et al. 2014 and 2017). The transactivation of the promoter *ldtP* by LdtR was verified (Supplementary Fig. S3), indicating the feasibility of the bacterial one-hybrid assay.

The *flp3* promoter was activated by in-trans expression of the full-length *visR* or the carboxi terminal of both *visR* and *visN* that encode a DNA-binding domain helix-turn-helix motif (Fig. 4A). However, the expression of the full-length *visN* did not activate the *flp3* promoter under the tested conditions (Fig. 4B). VisN and VisR are two LuxR homologs encoded by several Rhizobiaceae species (Rotter et al. 2006; Sourjik et al. 2000; Xu et al. 2013). The expression of different ‘*Ca. L. asiaticus*’ transcriptional regulators fused to the omega subunit in this assay was confirmed by immunoblotting (Fig. 4C). The transactivation assays indicated that VisN and VisR can bind to the *flp3* promoter and regulate its expression.

Ectopic expression of ‘*Ca. L. asiaticus*’ *visN* and *visR* restores the phenotypes in the *A. tumefaciens visNR* mutant.

The *visN* and *visR* genes belong to one operon that is conserved among rhizobia bacteria. VisN and VisR act together to regulate the cellular motility, depending on flagellum, exopolysaccharide production, and biofilm formation (Hoang et al. 2008; Sourjik et al. 2000; Tambalo et al. 2010; Xu et al. 2013). The function of ‘*Ca. L. asiaticus*’ *visN* and *visR* was investigated, using *A. tumefaciens* as a model, by testing whether ‘*Ca. L. asiaticus*’ *visN* and *visR* could complement the

Fig. 1. Type IVc tight adherence (Tad) pilus-encoding gene cluster in ‘*Candidatus Liberibacter asiaticus*’. Comparative genomics analysis revealed the presence of gene clusters encoding the Tad pilus in ‘*Candidatus Liberibacter*’ spp. The Tad pilus-encoding gene homologs in *Agrobacterium tumefaciens* C58 also are indicated. The gene cluster comparison was based on genomic and protein sequence analyses utilizing BLASTn and BLASTp (National Center for Biotechnology Information). Genes encoding for pseudopilins (tadE/cpaJ and tadF/cpaK); pilotin (tadD/cpaO); tadG, involved in stabilization of the pilus filament; cpal; and Flp pilins are shown. Dotted, dashed, and shaded arrows represent essential genes for biogenesis of the Tad pilus apparatus that encodes the prepilin peptidase (cpaA), secretin (cpaC), periplasmic subunits (cpaB and cpaD), inner membrane core (tadB/cpaG and tadC/cpaH), and ATPase (cpaF). CLas: ‘*Ca. L. asiaticus*’ (CP001677); CLaf: ‘*Ca. L. africanus*’ (CP004021); CLam: ‘*Ca. L. americanus*’ (CP006604); CLso: ‘*Ca. L. solanacearum*’ (CP002371); Lcc: *Liberibacter crescens* BT-1 (CP003789); Atu: *A. tumefaciens* C58 (CP014259).

A. tumefaciens visNR mutant. Both *visN* and *visR* coding sequences in *A. tumefaciens* were deleted to produce the double-mutant $\Delta visNR$. The mutant strain was confirmed by PCR (Supplementary Fig. S4A). The swimming experiments exhibited loss of motility in the $\Delta visNR$ mutant strain or $\Delta visNR$ harboring constructs containing the *A. tumefaciens visN* or *visR* genes alone. The motility phenotype was restored by ectopic expression of *A. tumefaciens visNR* together, similar to previous studies (Xu et al. 2013). Likewise, the exopolysaccharide production, which was assessed using the polysaccharide-binding dye Congo red, and biofilm formation phenotypes were only fully restored to the wild-type traits by ectopic expression of both VisNR transcriptional regulators in the *A. tumefaciens* $\Delta visNR$ mutant strain. Taken together, these data indicate that in *A. tumefaciens visN* and *visR* work together to regulate motility, exopolysaccharide production, and biofilm formation. As previously shown, both VisN and VisR negatively regulated biofilm formation of *A. tumefaciens* on PVC 96-well microtiter plate surfaces (Fig. 5) (Xu et al. 2013). Surprisingly, both VisN and VisR positively regulated biofilm formation of *A. tumefaciens* in borosilicate glass tubes.

Next, the ability of '*Ca. L. asiaticus*' *visN* and *visR* to complement the *A. tumefaciens* $\Delta visNR$ double mutant was evaluated. Expression of '*Ca. L. asiaticus*' *visN* or *visR* alone partially restored swimming motility but did not recover exopolysaccharide production and biofilm formation on a PVC 96-well microtiter plate surface in the *A. tumefaciens* $\Delta visNR$

mutant (Fig. 5A, 5B, and 5C). The phenotypes were fully restored in the *A. tumefaciens* $\Delta visNR$ mutant harboring both '*Ca. L. asiaticus*' *visN* and *visR* (Fig. 5A, 5B, and 5C). These results suggest that VisN and VisR homologs from *A. tumefaciens* and '*Ca. L. asiaticus*' work together to recognize and bind to the same target promoters in *A. tumefaciens*. Next, the ability of '*Ca. L. asiaticus*' VisN and VisR to positively regulate the expression of the '*Ca. L. asiaticus*' *rem* gene was tested. Rem, the regulator of exponential growth motility, is the master regulator of the flagellar genes and a participant in VisNR cascade in Rhizobiaceae (Rotter et al. 2006; Tambalo et al. 2010). The nonpolar mutant Δrem in *A. tumefaciens* was generated, and its impaired swimming motility was complemented by ectopic expression of '*Ca. L. asiaticus*' *rem* (Supplementary Fig. S5A). Expression of '*Ca. L. asiaticus*' *rem* was activated, and its proteins levels were significantly increased by ectopic expression of *A. tumefaciens* or '*Ca. L. asiaticus*' VisN and VisR together in the *A. tumefaciens visNR* mutant strain, but not by expression of VisN or VisR alone. Altogether, these data suggest that VisNR and Rem in '*Ca. L. asiaticus*' have a similar function to their counterparts in *A. tumefaciens*.

Interaction between '*Ca. L. asiaticus*' VisN and VisR increases VisN stability.

Genetics data suggest that VisN and VisR act together to control the same targets in rhizobia bacteria (Sourjik et al.

Fig. 2. Ectopic expression of the '*Candidatus Liberibacter asiaticus*' *cpaF* gene restored adherence phenotype in the *Agrobacterium tumefaciens cpaF* mutant. **A**, Colony PCR confirmed the deletion of the *cpaF* gene in *A. tumefaciens*. $\Delta cpaF1$ and $\Delta cpaF2$ represent independent mutant strains obtained by two steps of homologous recombination. $\Delta cpaF1$ was used in the complementation assays. **B**, Quantitative biofilm assay in borosilicate glass tubes comparing the ability of the wild-type *A. tumefaciens*, $\Delta cpaF1$ mutant, and complementation strains ($\Delta cpaF1_Atu$ and $\Delta cpaF1_Las$) to form biofilm. The biofilm assay was repeated three times, and means of four replicates and standard deviation values are indicated. The asterisk indicates a statistically significant difference ($P < 0.05$ according to Student's *t* test). **C**, Expression of *Atu_CpaF* and *Las_CpaF* in the complementation strains was verified by western blot. Anti-GroEL level was assessed as a loading control. M: DNA molecular marker; WT: *A. tumefaciens* wild-type strain; $\Delta cpaF1$ and $\Delta cpaF2$: *A. tumefaciens* strains carrying an in-frame deletion within the *cpaF* gene; $\Delta cpaF1_Atu$ and $\Delta cpaF1_Las$: mutant $\Delta cpaF1$ complemented with the *A. tumefaciens cpaF* gene and with the '*Ca. L. asiaticus*' *cpaF* gene, respectively; L: DNA ladder.

2000; Tambalo et al. 2010; Xu et al. 2013). To test whether ‘*Ca. L. asiaticus*’ VisN and VisR interact with each other, the pull-down assay was performed. It confirmed that ‘*Ca. L. asiaticus*’ VisN and VisR form a protein heterocomplex. The constructs 6xHis-VisR and VisN-FLAG were expressed in *E. coli*, and the total protein extracts were used in the assay. Protein interactions were evaluated in Ni-NTA agarose beads (QIAGEN). The input and eluted proteins were analyzed by immunoblotting with anti-His Tag and anti-FLAG Tag antibodies (Fig. 6A).

The recombinant protein ‘*Ca. L. asiaticus*’ VisN showed a lower amount of protein and was less soluble than ‘*Ca. L. asiaticus*’ VisR when expressed in *E. coli* alone. It was hypothesized that VisR may be important for interacting with and assist VisN to fold correctly in the cell. Therefore, VisN was expected to be more resistant to degradation by proteases in the presence of VisR. To test this hypothesis, the stability or degradation ratio of the ‘*Ca. L. asiaticus*’ VisN-FLAG expressed in the *A. tumefaciens visNR* double mutant, with and without ‘*Ca. L. asiaticus*’ VisR, was checked at different time points after inhibiting the translation process with spectinomycin (Fig. 6B). The degradation of VisN-FLAG was significantly reduced in presence of VisR in the bacterial cell. Anti-GroEL levels were analyzed as a loading control (Fig. 6B).

Ectopic expression of ‘*Ca. L. asiaticus*’ VisNR negatively regulates the expression of *flp3* pilin.

To understand how VisN and VisR regulate the *flp3* promoter in vivo, the promoters of the three ‘*Ca. L. asiaticus*’ genes *cpaA*, *cpaF*, and *flp3* were fused to the *lacZ* reporter gene cloned in the pRA301 plasmid (Akakura and Winans 2002; Wang et al. 2014). The constructs were used to transform *A. tumefaciens* wild-type and $\Delta visNR$ mutant strains and evaluate the β -galactosidase activity under the tested growing conditions. The β -galactosidase activity of the $\Delta visNR$ mutant strain carrying the *flp3* promoter was about three times that in the wild type (Supplementary Fig. S6). However, there was no significant difference for the promoters of the *cpaA* and *cpaF* genes between the wild-type and $\Delta visNR$ mutant strains. Accordingly, the results suggest that the LuxR-like proteins VisNR regulate the *flp3* promoter.

Next, *flp3* protein levels under the control of a constitutive promoter (*trp*) and its native promoter in *A. tumefaciens* wild-type, $\Delta visNR$, and complemented strains were analyzed. The expression of *flp3* under control of the constitutive promoter was similar between wild-type and $\Delta visNR$ mutant strains (Fig. 7A). In the *A. tumefaciens* strains carrying *flp3* regulated by its native promoter, Flp3 protein levels were higher in the mutant strains $\Delta visNR$ and $\Delta visNR$ harboring either *visN* or *visR* than in the wild-type and $\Delta visNR$ strains carrying both *visN* and

Fig. 3. Expression analysis of tight adherence (Tad) pilus-encoding genes in the psyllid and plant host infected with ‘*Candidatus L. asiaticus*’. **A**, Reverse transcription PCR (RT-PCR) analysis to confirm infectivity of ‘*Ca. L. asiaticus*’ in the insect vector (*Diaphorina citri*) and plant host (*Citrus sinensis*). RNA samples extracted from psyllids and sweet orange leaves (midrib and petiole) were subjected to one-step RT-PCR testing using Las16SF and Las16SR primers that specifically target the 16S rRNA of ‘*Ca. L. asiaticus*’. PCR products were loaded and visualized in a 1.2% agarose gel. All samples, including positive and negative controls, were performed in triplicate. Only one representative result for each sample is shown. CR: negative control using water as template; INC and PNC: ‘*Ca. L. asiaticus*’-free psyllid and sweet orange plant, respectively; I-Las and P-Las: ‘*Ca. L. asiaticus*’-positive psyllid and sweet orange plant, respectively. **B** and **C**, RNA samples from psyllids and plants infected with ‘*Ca. L. asiaticus*’ were used to analyze the expression of the genes encoding structural Tad pilus components and genes encoding pilins (*flp1* through *flp7*), respectively, using one-step qRT-PCR. Statistical analysis of all data were conducted using Student’s *t* test ($P < 0.05$). Results represent the mean and standard deviation of three independent experiments, with four replicates each. **D**, RT-PCR products of the pilin-encoding genes (*flp1* through *flp7*) amplified from the infected psyllid RNA samples were loaded and separated in 1.2% agarose gel. L: DNA ladder; Flp: PCR products of the ‘*Ca. L. asiaticus*’ genes coding fimbrial low-molecular weight protein (flp); C: ‘*Ca. L. asiaticus*’-free psyllid as a negative control.

visR (Fig. 7B). VisN and VisR of ‘*Ca. L. asiaticus*’ and *A. tumefaciens* were able to negatively control the expression of Flp3 together. These data suggest that during colonization of the insect vector ‘*Ca. L. asiaticus*’ VisNR are downregulated or inhibited by unknown signal molecules to promote upregulation of the Flp3 pilin that is secreted by the Tad pilus apparatus. To test this hypothesis, a comparative analysis of gene expression in the psyllid and plant host was conducted, and ‘*Ca. L. asiaticus*’ *visN* and *visR* were observed to be less expressed in psyllid (Supplementary Fig. S7), which corroborates findings from previous studies (Yan et al. 2013).

Transcriptomic studies of *A. tumefaciens* revealed several genes regulated by VisN and VisR, including *rem* and flagellar genes, but the promoter target sequences for VisN and VisR were not determined (Xu et al. 2013). Analysis of the ‘*Ca. L. asiaticus*’ *rem* and *flp3* promoter sequences via alignment identified a conserved motif, 5'-GTWAA-TAWMRTTWATA-3', in the positions -127 to -112 and -96 to -81, respectively. The results suggest ‘*Ca. L. asiaticus*’ VisN and VisR act together to regulate Rem and Flp3 protein levels.

Physical interaction between VisR and the *flp3* promoter.

The results suggest both ‘*Ca. L. asiaticus*’ VisN and VisR regulate the *flp3* promoter. The next goal was to provide

experimental evidence for the physical interactions between the *flp3* promoter and VisN or VisR. Despite multiple attempts, the experiment failed to purify the ‘*Ca. L. asiaticus*’ VisN recombinant protein or the complex VisNR protein in *E. coli*. Nonetheless, the recombinant ‘*Ca. L. asiaticus*’ VisR expressed in *E. coli* was purified. The 6HisVisR recombinant protein was purified by affinity chromatography and dialyzed against phosphate-buffered saline (PBS) buffer containing 2 mM dithiothreitol (DTT) at 4°C overnight. The purified 6HisVisR protein samples were resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) and stained with Coomassie blue (Fig. 8A).

Physical interaction between the purified ‘*Ca. L. asiaticus*’ 6HisVisR and the *flp3* promoter region (-198 to +1) was confirmed by electrophoretic motility shift assay (EMSA) (Fig. 8B), and no binding was observed when *cpaA* promoter was incubated with 6HisVisR (data not shown). Disruption of the binding of labeled *flp3* probe with VisR by 10× and 100× unlabeled *flp3* probe indicates the specificity of the interaction.

DISCUSSION

Liberibacter bacteria, including ‘*Ca. L. asiaticus*’, form biofilm (Cicero et al. 2016 and 2017; Ghanim et al. 2016; Kruse et al. 2017; Wang and Trivedi 2013) inside the midgut lumen of

Fig. 4. The transcriptional regulators VisN and VisR bind to the promoter of *flp3* that encodes the most abundant ‘*Candidatus L. asiaticus*’ pilin in infected psyllids. **A**, To evaluate the activation of the *flp3* promoter fused to the reporter gene *his3* by ‘*Ca. L. asiaticus*’ transcriptional factors, *Escherichia coli* cells carrying the constructs were plated in M9 minimal medium with 5 mM 3-AT. The minimal medium with histidine served as a control in each assay. Cells harboring the *flp3* promoter fused to *his3* were transformed with different ‘*Ca. L. asiaticus*’ genes encoding transcriptional factors and plated in triplicate in the selective media. The mean and standard deviation colony counts are shown. Error bars represent the standard deviations ($n = 3$). The asterisk indicates a statistically significant difference ($P < 0.05$, according to Student’s *t* test). **B**, A representative selection test is displayed with a control plate containing histidine and another plate without histidine plus 5 mM 3-AT. **C**, Expression of the ‘*Ca. L. asiaticus*’ transcription factors under control of the P_{tac} promoter in transformed *E. coli* cells were confirmed by immunoblotting using anti-FLAG antibodies. EV: empty vector. VisR, VisN, LdtR, CtrA, LysR, and Rem are transcriptional factors coded by the ‘*Ca. L. asiaticus*’ genome. CT-VisR and CT-VisN represent the carboxi-terminal region that encodes the DNA-binding domains of VisR and VisN, respectively.

psyllids before moving through the midgut epithelial wall to enter the hemocoel and eventually infecting the salivary gland (Ghanim et al. 2016; Wang and Trivedi, 2013). Attachment is the initial step in biofilm formation. The Tad pilus apparatus was shown to be important for adherence of bacteria to host tissues during the colonization process (Kawahara et al. 2016; Li et al. 2012; Nykyri et al. 2013; O'Connell Motherway et al. 2011; Tomich et al. 2006; Wairuri et al. 2012). The involvement of Tad pilus in adherence of *A. tumefaciens* is supported by the data related to mutation of *cpaF*, an ATPase essential for the biogenesis and secretion of the type IVc pilus. Because of the high identity between the Tad loci of *Agrobacterium* spp. and '*Ca. L. asiaticus*' and complementation of the *cpaF* mutant of *A. tumefaciens* with '*Ca. L. asiaticus*' *cpaF*, we reasoned that the type IVc pilus might be involved in attachment to the epithelial cells of the psyllid midgut and to other tissues and organs inside the psyllid body. This is consistent with the higher expression level of Tad genes of '*Ca. L. asiaticus*' in psyllids compared with in planta. Some Tad genes (e.g., *flp3*) are found among '*Ca. L. asiaticus*' transcripts, with the highest abundance found in transcriptome analysis of the total RNA extracted from isolated bacteria colonizing the psyllid midgut (*unpublished data*). Additionally, it was shown that '*Ca. L. solanacearum*' produces pili- and flagella-like surface appendages and can form biofilm inside the potato psyllid (*Bactericera cockerelli*) (Cicero et al. 2016 and 2017). Tad genes are highly expressed in '*Ca. L. solanacearum*' haplotypes isolated

from psyllids (Mendoza-Herrera et al. 2018). Liberibacter genomes encode for all of the genes needed to build a complete and functional Tad pili apparatus (Fig. 1), which might indicate that the Tad pili perform important functions in the adaptation of liberibacters to specific environments. Indeed, conservation of the Tad pilus-encoding loci among liberibacters supports the notion of a ubiquitous pili-mediated insect vector colonization mechanism for insectborne liberibacters.

The Tad genes were initially described for the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (Tomich et al. 2007). Transcriptome analysis of the *Bifidobacterium breve* in a murine colonization model revealed differential expression of Tad pilus-encoding genes. Mutational analysis demonstrated that the *tad* gene cluster is essential for efficient in vivo murine gut colonization (O'Connell Motherway et al. 2011). Further, mutational analysis in *Pectobacterium* spp. demonstrated that the Tad pilus apparatus had a significant impact on the maceration ability of the pathogen in potato tubers, indicating that the Tad pilus-encoding gene cluster represents a novel virulence determinant in *Pectobacterium* spp. (Nykyri et al. 2013). Likewise, Tad pili were shown to be essential for the pathogenicity of *Ralstonia solanacearum*, as the *tadA2* mutant was impaired in its ability to cause wilting of potato plants. In Rhizobiaceae, the Tad pilus apparatus was shown to increase the initial adherence of the bacterial cell to surfaces and to the host tissue (Thompson et al. 2018; Wang et al. 2014; Zatakia et al. 2014). Because '*Ca. L. asiaticus*' has

Fig. 5. Ectopic expression of '*Candidatus Liberibacter asiaticus*' *visNR* homologs restores motility, exopolysaccharide production, and biofilm formation in the *visNR* double mutant of *Agrobacterium tumefaciens*. **A**, Effect of ectopic expression of '*Ca. L. asiaticus*' *visN* or *visR* alone and *visNR* together on the swimming motility of the *A. tumefaciens visNR* double mutant. **B**, Exopolysaccharide production in *A. tumefaciens* strains was evaluated by growing the cells in modified ATGN Congo Red media. **C**, Biofilm formation of the *A. tumefaciens* wild-type, *visNR* mutant and complemented strains was assessed in PVC 96-well microtiter plates at 28°C for 48 h. Mean values plus/minus standard deviations ($n = 4$) are plotted. Mean values were compared with the wild type; asterisks indicate statistically significant differences ($P < 0.05$, according to Student's *t* test). WT: *A. tumefaciens* wild-type strain carrying the empty vector; M: double-mutant *visNR* of *A. tumefaciens* carrying the empty vector; M+VisR, VisN, and VisNR: double-mutant *visNR* of *A. tumefaciens* carrying the '*Ca. L. asiaticus*' *visR*, *visN*, and *visNR* gene, respectively.

not been cultured in vitro, we are unable to provide direct genetic evidence regarding the function of Tad genes. Using *A. tumefaciens* as a model, we are able to show that some of the Tad genes are functional. For example, *cpaF* is functional in restoring the adherence phenotype of the *cpaF* mutant of *A. tumefaciens*.

The data revealed that in '*Ca. L. asiaticus*' the expression of the *flp3* gene is under the control of the two LuxR-type transcriptional regulators, VisN and VisR. *visN* and *visR* genes are localized in the same operon and conserved among members of the Rhizobiaceae. In *S. meliloti* in-frame deletions of *visN* and *visR* resulted in mutant strains that were nonmotile and non-flagellated due to impaired flagellar synthesis (Sourjik et al. 2000). Complementation by expression of the homologous wild-type alleles completely restored swimming and swarming proficiency in both mutant strains. The data clearly suggest that VisN and VisR act in trans and are both required for flagellar synthesis (Sourjik et al. 2000). Furthermore, *A. tumefaciens* produces a unipolar polysaccharide (UPP) adhesin at cell poles that contact surfaces. Transposon mutagenesis screening identified *visN* and *visR* as UPP-negative regulators with hyper-adherent phenotype. Likewise, as shown in *S. meliloti*, *visN* and *visR* are activators of flagellar motility in *A. tumefaciens*, in addition to inhibiting UPP and cellulose production (Xu et al. 2013). In this study, we advanced our understanding of the

regulatory roles of VisN and VisR by showing that they negatively regulate the *flp3* gene, the pilin gene with the highest expression in psyllids among the seven '*Ca. L. asiaticus*' pilin genes. Inversely, '*Ca. L. asiaticus*' *visN* and *visR* expression was shown to be downregulated in psyllids. Regulation of *flp3* by VisNR might allow '*Ca. L. asiaticus*' to switch between forming biofilm and circulative status inside the psyllid hemocoel to facilitate its infection and transmission, considering the role of pilin in adherence. Indeed, a similar connection between Tad pilus function and differential expression of *flp* pilins was reported in previous studies for other bacteria species (Avidan et al. 2017; Kachlany et al. 2001; Zatakia et al. 2014). VisN and VisR share similarities within their C-terminal regions with the DNA-binding domains of LuxR and FixJ homologs, as well as to each other. However, the N termini of VisN and VisR do not resemble either the acyl-homoserine lactone-binding motif of LuxR homologs nor the phosphate-receiver domain of FixJ. The N termini of VisN and VisR also do not resemble each other. Indeed, the DNA-binding domain encoded by the carboxi-terminal region of VisN and VisR in *A. tumefaciens* and '*Ca. L. asiaticus*' showed high identity of more than 80%. In contrast, the amino terminal of the LuxR homolog proteins VisN and VisR are less conserved, with about 40% of identity. Thus, the N termini of *A. tumefaciens* and '*Ca. L. asiaticus*' VisN and VisR regulators might bind to different

Fig. 6. Pull-down and stability assays show a physical interaction between '*Candidatus L. asiaticus*' VisN and VisR. **A**, The protein 6xHisVisR was used as bait for the pull-down assay. Protein 6xHisVisR was expressed in *Escherichia coli*, immobilized and washed on nickel-IMAC beads (Thermo Fisher Scientific), and incubated with *E. coli* lysates containing 4xFLAG-VisN. Total cell extracts (inputs) and eluted protein (elutes) were immunoblotted using anti-FLAG and anti-6xHis antibodies. **B**, Stability of '*Ca. L. asiaticus*' VisN was increased when coexpressed with VisR. The *Agrobacterium tumefaciens visNR* double-mutant strain harboring '*Ca. L. asiaticus*' *visN* was cotransformed with empty plasmid pBKT or pBKT-*visR*. Cells of both strains were cultured, and protein synthesis was blocked by addition of spectinomycin at 1 mg/ml into bacterial culture. Samples collected at the indicated time were analyzed by immunoblotting. Anti-GroEL antibody was used to normalize the amount of protein loaded. EV: *A. tumefaciens* double-mutant *visNR* carrying '*Ca. L. asiaticus*' *visN* and pBKT empty plasmid; VisR: mutant *visNR* carrying both *visN* and *visR* genes of '*Ca. L. asiaticus*'.

signal molecules. We cannot rule out the possibility that the upregulation of *flp3* in psyllids is not only due to down-regulation of '*Ca. L. asiaticus*' *visN* and *visR* gene expression but also to other mechanisms, such as the binding of specific molecules to the amino terminal of both proteins, which may regulate their function.

In conclusion, we have shown that '*Ca. L. asiaticus*' contains a functional Tad pili that may be involved in attachment to and colonizing of psyllid tissues and organs such as the midgut. VisN and VisR act together to negatively regulate the *flp3* gene by binding to its promoter region. Inhibition of '*Ca. L. asiaticus*' attachment to the epithelial cells in the psyllid midgut and to other psyllid tissues via Tad pilus may present a new path for reducing the colonization and transmission of '*Ca. L. asiaticus*' by the psyllid vector.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Tables S1 and S2. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium (1% tryptone [wt/vol], 0.5% yeast extract [wt/vol], 1% sodium chloride [wt/vol], pH 7.5). *A. tumefaciens* wild-type strain C58 and the mutant strains were grown in 2xTY medium (1.6% tryptone [wt/vol], 1.0% yeast extract [wt/vol], 0.5% sodium chloride [wt/vol], pH 7.5) or ATGN medium (79 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 5 mM MgSO₄·7H₂O, 1 mM CaCl₂, 0.0071 mM MnSO₄·7H₂O, 0.125 mM FeSO₄·7H₂O, 0.5% glucose [wt/vol], pH 7) with or without 1.5% (wt/vol) agar at 28°C (Morton and Fuqua 2012; Wise et al. 2006). For *sacB* counter-selection, 5% sucrose was added to LB medium. Plasmids were introduced into *E. coli* by heat-shock at 42°C

and into *A. tumefaciens* by electroporation (Weigel and Glazebrook 2006). For plasmid selection, the antibiotics were used at the following concentrations: kanamycin at 50 mg/ml; ampicillin at 100 mg/ml; spectinomycin at 50 mg/ml; and gentamicin at 10 mg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used when necessary. For Congo red plates, the dye was dissolved in methanol at 20 mg ml⁻¹. Filtered Congo red dye was added to ATGN medium (final concentration approximately 80 µg ml⁻¹).

Fig. 8. VisR binds to the promoter region of '*Candidatus L. asiaticus*' *flp3*. **A**, Purified 6HisVisR protein samples were resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and stained with Coomassie blue. **B**, Electrophoretic mobility shift assay of 6HisVisR incubated with the biotin-labeled *flp3* promoter fragment. From left to right: lane 1, free *flp3* probe; lane 2, probe with purified 6HisVisR; lane 3, probe with 6HisVisR and 10x nonbiotinylated *flp3* promoter; lane 4, probe with 6HisVisR and competitor 100x nonbiotinylated *flp3* promoter. Shift: the complex formed by 6HisVisR and biotinylated *flp3* promoter.

Fig. 7. VisN and VisR regulate the expression of '*Candidatus L. asiaticus*' *flp3*. **A**, Ectopic expression of *flp3* under control of the constitutive promoter P_{trc}. Cells of *Agrobacterium tumefaciens* wild-type and *visNR* mutant strains harboring the *flp3* coding region fused to P_{trc} were grown to an optical density (OD) at 600 nm = 1 on modified ATGN medium and collected. Total cell extracts were subjected to immunoblotting. **B**, '*Ca. L. asiaticus*' VisN and VisR regulate the expression of *flp3*. *A. tumefaciens* wild-type and *ΔvisNR* mutant strains carrying the plasmidborne '*Ca. L. asiaticus*' *flp3* gene were cotransformed with '*Ca. L. asiaticus*' *visN*, *visR*, and *visNR* or *A. tumefaciens visNR* genes as indicated. Bacterial cells grown in modified ATGN medium were collected at OD₆₀₀ = 1. Protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were probed with anti-FLAG antibodies. Anti-GroEL antibody was used to normalize the amount of protein loaded. Protein A conjugated with horseradish peroxidase was used to detect the antibody.

Construction of the *A. tumefaciens* deletion mutants and complemented strains.

DNA manipulations and PCR were performed according to standard procedures (Sambrook and Russell 2006). Restriction digestion and DNA ligation were performed in accordance with the manufacturer instructions (New England Biolabs). To construct the *cpaF* (Atu0218), *rem* (Atu0573), and *visNR* (Atu0524-Atu0525) deletion mutants, approximately 1 kb of the upstream and downstream regions of the target genes was amplified by PCR from *A. tumefaciens* genomic DNA, and the two fragments were ligated to produce an in-frame deletion (Andrade et al. 2014). The generated fragments were then cloned into the *EcoRI* or *HindIII* restriction site of the pNPTS138 suicide vector, thus generating the plasmids pNPTS-*cpaF*, pNPTS-*rem*, and pNPTS-*VisNR*. These vectors were introduced into *A. tumefaciens* by electroporation, and the wild-type copy was replaced by the deleted version of each construct after two recombination events, as described previously (Andrade et al. 2014). To complement the generated knockout mutants, fragments including the deleted genes and approximately 500 bp of upstream sequence were amplified by PCR from *A. tumefaciens* genomic DNA and inserted into the pBBR1-MCS-2 vector at the *KpnI* and *XhoI* restriction sites, or the deleted coding region was fused to *lac* promoter in pBBR1-MCS-2. To verify whether the homologs encoded by '*Ca. L. asiaticus*' could complement the *A. tumefaciens* deleted mutant strains, the coding region of the genes CLIBASIA_03055 (*cpaF*), CLIBASIA_02065 (*rem*), and CLIBASIA_02905 and CLIBASIA_02900 (*visN* and *visR*, respectively) were amplified by PCR from '*Ca. L. asiaticus*' genomic DNA fused to *lac* or *trp* promoters and inserted into pBBR1-MCS-2 or pTF53. The genomic DNA for '*Ca. L. asiaticus*' was extracted from '*Ca. L. asiaticus*'-infected citrus grown in the psyllid room at the Citrus Research and Education Center (CREC, University of Florida, Lake Alfred, U.S.A.). The '*Ca. L. asiaticus*' genomic DNA was extracted using the Wizard DNA Genomic Purification Kit (Promega) following the manufacturer suggestions. The constructed plasmids were then transferred to the respective *A. tumefaciens* mutant strains by electroporation and selection for gentamicin or kanamycin resistance. All constructs were sequenced for confirmation.

Biofilm, exopolysaccharide, and motility assays.

Biofilm formation on glass surfaces and PVC 96-well microtiter plates was examined as described previously (Hibbing and Fuqua 2011; Li and Wang 2011), with modifications. The wild-type, mutant, and complemented cells were grown in 2×TY medium overnight at 28°C with shaking (180 rpm). Bacterial cells were pelleted and resuspended in ATGN medium to an optical density at 600 nm (OD₆₀₀) adjusted to 0.05 (UV/Vis Spectrometer Lambda Bio, PerkinElmer). A 200-μL portion (1.5-ml volume) of adjusted bacteria was placed in the glass tubes and plate wells, respectively, and incubated at 28°C without agitation. After 2 days, bacterial growth was checked by measuring the OD₆₀₀, and the culture was removed using a pipette. After repetitive water washes, the adhered cells were stained with 0.1% crystal violet (CV) (wt/vol) for 30 min at room temperature. After staining by CV, the unbound stain was removed by water washing, and the CV-stained cells were solubilized in 30% acetic acid and quantified by spectrophotometry at 600 nm. Ratio A₆₀₀/OD₆₀₀ represents the crystal violet quantification normalized by culture OD. The means of four replicates and standard deviation values are indicated. The motility assays were performed as previously described (Xu et al. 2013), with modifications. Bacteria were grown overnight in LB agar medium and bacterial cells were spotted on a plate containing ATGN medium plus 0.3% agar (wt/vol) (Difco).

Plates were incubated at 28°C for 48 h. The experiments were repeated three times, with three replicates each time. The exopolysaccharide production assay was conducted as described by Xu et al. (2013), with modifications. Bacterial cells were grown in LB medium overnight at 28°C and 180 rpm. The OD₆₀₀ was adjusted to 0.1 (UV/Vis Spectrometer Lambda Bio), and 3 μL of each strain was spotted on ATGN medium 1.5% agar (wt/vol) plus 2% glucose and Congo red dye (final concentration approximately 80 μg ml⁻¹). Plates were incubated at 28°C for 3 days.

RNA extraction and expression analysis of *Tad* pilus-encoding genes in infected citrus and psyllids by qRT-PCR.

Psyllid colonies were maintained on '*Ca. L. asiaticus*'-infected sweet orange ('Valencia') in a secured greenhouse with a temperature of 25 ± 3°C and relative humidity of 50% ± 5%. The '*Ca. L. asiaticus*' status of the insects and citrus plants was verified by qPCR (Yan et al. 2013). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN), according to the manufacturer instructions. For the extraction of RNA from '*Ca. L. asiaticus*'-infected plants, the midrib and petiole of symptomatic leaves were collected and ground in liquid nitrogen to a fine powder before RNA isolation. For RNA isolation from putative '*Ca. L. asiaticus*'-infected psyllids, 16 psyllids were pooled and ground in liquid nitrogen before RNA isolation. Purified RNA was treated with DNase I-Free (QIAGEN), and successful removal of genomic DNA was confirmed by PCR using specific primers that annealed only to the CLIBASIA_03015 promoter region. RNA quantity was determined on a ND-8000 Nanodrop spectrophotometer (NanoDrop Technologies). Isolated plant and psyllid RNA samples were subjected to one-step qRT-PCR assay to test for infection by '*Ca. L. asiaticus*' using primers Las16SF-Las16SR, which specifically target the 16S rRNA of '*Ca. L. asiaticus*' (Yan et al. 2013). Positive psyllid RNA samples for '*Ca. L. asiaticus*' were identified for further analysis of gene expression of the *Tad* pilus-encoding genes. All qRT-PCR analyses were performed using an Applied Biosystems 7500 Fast Real-time PCR system with a QuantiTect SYBR Green one-step RT-PCR kit (QIAGEN), as described by Yan et al. (2013). Relative expression levels of the *Tad* pilus-encoding genes, which were normalized using the reference gene *gyrB*, were calculated with the ΔΔCt method (Livak and Schmittgen 2001). Statistical analyses of all data were conducted by Student's *t* test (*P* < 0.05). Mean values derived from three independent experiments with four replicates each are shown. Primers were designed using Primer Express Software (Applied Biosystems).

Immunoblotting.

To analyze the specific protein levels in *E. coli* and *A. tumefaciens* strains, cells were grown in 2×TY or AT minimal media to an OD₆₀₀ of 0.5. Bacterial cells were harvested, and pellets were resuspended in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Tween 20, and 1 mg of lysozyme per milliliter. Cells were ruptured by sonication. Cell-free extract was prepared by centrifugation at 15,000 × *g* at 4°C for 45 min. Protein extracts were quantified by the Pierce BCA protein assay (Thermo Fisher Scientific). Equal amounts of proteins were loaded, separated by 15% SDS-PAGE, and transferred to a nitrocellulose membrane (GE Healthcare), followed by immunoblotting analysis. Rabbit polyclonal antibodies raised against anti-FLAG (Sigma-Aldrich) or anti-6xHis (Sigma-Aldrich) were diluted to 1:5,000. Rabbit antibodies were detected with staphylococcal protein A conjugated to horseradish peroxidase (Sigma-Aldrich). As a loading control for these experiments, immunoblotting was carried out for the same

protein extracts resolved on 15% acrylamide and probed with rabbit polyclonal antibodies raised against anti-GroEL (Sigma-Aldrich) diluted to 1:20,000. Anti-GroEL was detected with protein A conjugated to horseradish peroxidase (Sigma-Aldrich). Immunoblotting was developed with Pierce SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) and exposed to Pierce CL-XPosure film (Thermo Fisher Scientific).

One-hybrid system assay.

For the transactivation assay, the *flp3* promoter region (–22 to –439) was inserted into *Eco*RI and *Sal*I restriction sites of the plasmid pH3U3, which harbors a canonical Shine-Dalgarno sequence for *E. coli*. In addition, different transcriptional factors encoded by ‘*Ca. L. asiaticus*’ were cloned downstream of the *lacUV5* promoter and fused to the carboxi terminal of the *E. coli* RNA polymerase omega subunit and FLAG Tag into the *Kpn*I and *Xba*I restriction sites of the plasmid pB1H2 ω 2 (Noyes et al. 2008). All the constructs were used to cotransform the *E. coli* mutant strain (Δ *rpoZ*:*zeo*, Δ *hisB*, Δ *pyrF*) and proceed with selection assays in minimal media without histidine plus 3-aminotriazole and required antibiotics, as previously described (Noyes et al. 2008). To test the feasibility of this assay for ‘*Ca. L. asiaticus*’, the first selection test was performed with the plasmid pH3U3 harboring the *ldtP* promoter region (–15 to –380) with its transcriptional regulator *ldtR* cloned into pB1H2 ω 2, because the binding motif of *ldtR* to the *ldtP* promoter was previously well characterized in ‘*Ca. L. asiaticus*’ (Pagliai et al. 2014 and 2017).

β -Galactosidase assay.

Fragments containing the promoter elements upstream of *cpaA* (CLIBASIA_03080), *cpaF* (CLIBASIA_03055), and *flp3* (CLIBASIA_03105) were amplified from ‘*Ca. L. asiaticus*’ genomic DNA isolated from the plant host using Phusion DNA polymerase and primers. PCR fragments were agarose gel-purified, ligated into pGEM-T Easy, and verified by sequencing. Fragments were excised with the appropriate restriction enzymes and ligated into the compatibly cleaved vector pRA301. Fragments were designed to insert the promoter region and ribosome-binding site with the start codon of the gene of interest in frame with the promoterless *lacZ* gene. Fragments were confirmed by PCR, and the constructs were electroporated into *A. tumefaciens* wild-type and Δ *visNR* mutant strains. Bacterial cells were prepared for β -galactosidase assay by culturing in ATGN into the exponential phase measured for OD₆₀₀ and frozen at –80°C. For β -galactosidase activity, cells were permeabilized in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, pH 7.0) by the addition of three drops of 0.05% SDS and four drops of chloroform (Hibbing and Fuqua 2011). β -Galactosidase reactions were initiated by addition of 100 μ l of a colorimetric substrate *o*-nitrophenyl β -D-galactopyranoside (ONPG) solution concentration of 4 mg/ml and terminated with addition of 600 μ l of 6 M NaCO₃. Intact cells and debris were removed by centrifugation (10 min, 10,000 $\times$ g), free ONP was measured as A₄₂₀, and specific activity was reported in Miller units. At least three biological replicates were performed.

Pull-down assay.

E. coli cells expressing ‘*Ca. L. asiaticus*’ 6xHisVisR protein were washed in PBS and suspended in Lysis buffer (PBS, 20 mM imidazole, 100 μ g of lysozyme per milliliter, 5 mM MgSO₄, 40 μ g of DNaseI per milliliter, pH 7.4) and sonicated to generate the cell lysates. After centrifugation, the cell lysates were incubated with nickel-IMAC beads (Thermo Fisher Scientific). The beads were washed four times with PBS plus 20 mM imidazole at pH 7.4 to remove the unbound proteins and

incubated with *E. coli* cell lysates containing ‘*Ca. L. asiaticus*’ VisN-FLAG for 2 h at 4°C. After washing four times, the beads were resuspended in SDS-PAGE loading buffer, followed by boiling for 5 min. The samples are subjected to SDS-PAGE and immunoblotting using anti-His and anti-FLAG (Sigma-Aldrich) antibodies as described above.

Protein in vivo stability assay.

To test the in vivo stability of ‘*Ca. L. asiaticus*’ VisN-Flag in the presence of ‘*Ca. L. asiaticus*’ VisR, 10 ml of overnight *A. tumefaciens* cultures coexpressing VisN-Flag with VisR or an empty vector, as a control, were pelleted and resuspended in 10 ml of fresh medium for an additional 1 h of incubation. After incubation, spectinomycin solution was directly added to each culture at a final concentration of 1 mg/ml to block protein synthesis. A 1-ml aliquot was harvested at each time point and lysed in protein loading buffer followed by boiling for 5 min. The samples were subjected to SDS-PAGE and immunoblotting. The SDS-PAGE gel was transferred to a nitrocellulose membrane (Millipore) using a Trans-Blot Turbo system (Bio-Rad Laboratories). The membrane was blocked using 5% dry milk in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6). The membrane was incubated with anti-FLAG and detected by protein A conjugated to horseradish peroxidase. Immunoactive proteins were detected using a ECL detection kit and X-ray film. Anti-GroEL antibodies were used to normalize the amount of protein loaded.

Expression and purification of 6HisVisR in *E. coli*.

For expression of the recombinant protein 6HisVisR in *E. coli*, a fragment containing the *visR* coding region sequence was amplified by PCR using ‘*Ca. L. asiaticus*’ genomic DNA as template and the primers F-pETVisR and R-pETVisR. The amplified fragment was purified and digested with *Nde*I/*Hind*III and inserted into pET-28a (Novagen), resulting in pET-6HisVisR. Recombinant protein 6HisVisR was overexpressed in *E. coli* BL21 Star (DE3) pLysS (Novagen) by addition of 1 mM IPTG to cells grown to the midexponential phase at 37°C and 180 rpm. After addition of IPTG, the temperature was changed to 22°C, and the 6HisVisR induction occurred overnight. Next, bacterial cells were harvested by centrifugation, and cell pellets were resuspended in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mg of lysozyme per milliliter. Cells were ruptured by sonication. Cell-free extract was prepared by centrifugation at 14,000 $\times$ g at 4°C for 45 min. The recombinant protein 6HisVisR was purified by Pierce Ni-NTA affinity chromatography (Thermo Fisher Scientific) according to the manufacturer recommendations. The protein-containing fractions were concentrated, and the buffer was exchanged with PBS buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4) plus 2 mM MgCl₂, 2 mM DTT, and 5% glycerol. The protein concentration was estimated using a Pierce BCA method (Thermo Fisher Scientific) with bovine serum albumin as the standard. The purified protein was stored at –80°C.

EMSA.

For EMSA, the recombinant protein 6HisVisR was induced and purified as described above. Oligonucleotides Flp3p_Las-F and Flp3p_Las-R were used to amplify part of the *flp3* promoter region (–198 to +1) using PCR. The purified DNA fragment coding the *flp3* promoter was labeled using a Pierce Biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific). EMSA was conducted using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific). The binding reactions with the labeled probe incubated with 100 nM 6HisVisR were resolved in a

5% nondenaturing polyacrylamide TBE gel. The EMSA was developed in accordance with the manufacturer instructions.

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