Interaction of Liberibacter Solanacearum with Host Psyllid Vitellogenin and Its Association with Autophagy

Poulami Sarkar,a Murad Ghanima

aDepartment of Entomology, Agricultural Research Organization, Volcani Institute, Rishon LeZion, Israel

ABSTRACT Candidatus Liberibacter solanacearum (CLso) haplotype D, transmitted by the carrot psyllid Bactericera trigonica, is a major constraint for carrot production in Israel. Unveiling the molecular interactions between the psyllid vector and CLso can facilitate the development of nonchemical approaches for controlling the disease caused by CLso. Bacterial surface proteins are often known to be involved in adhesion and virulence; however, interactions of CLso with carrot psyllid proteins that have a role in the transmission process has remained unexplored. In this study, we used CLso outer membrane protein (OmpA) and flagellin as baits to screen for psyllid interacting proteins in a yeast two-hybrid system assay. We identified psyllid vitellogenin (Vg) to interact with both OmpA and flagellin of CLso. As Vg and autophagy are often tightly linked, we also studied the expression of autophagy-related genes to further elucidate this interaction. We used the juvenile hormone (JH-III) to induce the expression of Vg, thapsigargin for suppressing autophagy, and rapamycin for inducing autophagy. The results revealed that Vg negatively regulates autophagy. Induced Vg expression significantly suppressed autophagy-related gene expression and the levels of CLso significantly increased, resulting in a significant mortality of the insect. Although the specific role of Vg remains obscure, the findings presented here identify Vg as an important component in the insect immune responses against CLso and may help in understanding the initial molecular response in the vector against Liberibacter.

IMPORTANCE Pathogen transmission by vectors involves multiple levels of interactions, and for the transmission of liberibacter species by psyllid vectors, much of these interactions are yet to be explored. Candidatus Liberibacter solanacearum (CLso) haplotype D inflicts severe economic losses to the carrot industry. Understanding the specific interactions at different stages of infection is hence fundamental and could lead to the development of better management strategies to disrupt the transmission of the bacteria to new host plants. Here, we show that two liberibacter membrane proteins interact with psyllid vitellogenin and also induce autophagy. Altering vitellogenin expression directly influences autophagy and CLso abundance in the psyllid vector. Although the exact mechanism underlying this interaction remains unclear, this study highlights the importance of immune responses in the transmission of this disease agent.

KEYWORDS vitellogenin, autophagy, outer membrane proteins, Liberibacter, psyllid
psyllid vectors (11–17). However, little attention has been given to the molecular interactions and the functional validation of Liberibacter and insect proteins that aid in the transmission process. Recently, several reports have unraveled at the transcriptional response of whole psyllids and organs to the acquisition and retention of Liberibacter species (3, 18–21). These studies have shown that the acquisition of different Liberibacter species, by their respective psyllid vectors, induced significant immune responses (22–25). Genome and transcriptome sequencing results have further shown that psyllids do not bear a complete immune response system as has been described in model insects such as Drosophila (14, 26, 27). Psyllids lack the adaptive immunity and the immune deficiency (Imd) pathway, which generally respond to invasion by Gram-negative bacteria, thus leading for example to the ability of Liberibacter species to invade tissues in psyllids where they are able to replicate (21, 23, 28). Such findings raise the hypothesis that psyllids use alternative immune responsive mechanisms for combating with the effects of invasion by the bacterium into host cells. On the other hand, psyllids bear an innate defense mechanism against pathogens, which involves both cellular and humoral immune responses (23, 28, 29). Cellular responses include phagocytosis, and humoral responses involve secretion of several antimicrobial peptides (23, 28, 30). The first line of defense involves recognition of conserved elicitors, molecules, or essential structures often known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by host pattern-recognition receptors (PRR). Bacterial outer membrane proteins (Omp), flagellins, and pili appendages are some of the known bacterial virulence factors involved in pathogenesis that elicit immune responses in the host (31–33). OmpA is a major unique integral transmembrane protein with amphipathic β-barrels which is often involved in cell adhesion and virulence (34–37). OmpA also has a direct role in virulence upon infection in the host cells for several human-pathogenic bacteria (36, 38) such as Escherichia coli (39, 40), Salmonella enterica (41), Leptospira interrogans (42, 43), and Neisseria gonorrhoeae (44). The fat body in insects is one of the major immune-responsive organs, where host PRRs against bacterial virulence factors are produced and then directly released into the hemolymph (45). Additionally, hemocytes act as macrophages that have phagocytic activity but also require the presence of PRRs for presenting the pathogen to these macrophages.

One of the major known PRRs is apolipoprotein or vitellogenin (Vg) which belongs to the large lipid transfer protein (LLTP) superfamily having opsonin activity (46–48). LLTPs consist of a large phosphoglycolipoprotein and a major egg yolk protein precursor (YPP) in insects. They are large molecules (200 kDa) synthesized in the fat bodies and midguts, transported through the hemolymph and sequestered by ovaries with the help of vitellogenin receptors (VgR) via receptor-mediated endocytosis, and are subsequently cleaved to generate the nutrient yolk protein vitellin required for the developing oocytes (49–51). Although Vg was initially considered a female-specific protein, males and sexually immature animals have also been shown to express Vg indicating several roles beyond the nourishment of developing oocytes (50, 52, 53). It provides host innate immunity with multifaceted functions during several extraneous factors, including chemical exposure, nutritional stress, and infection (47, 54). Insect Vg often acts as a pattern recognition molecule to recognize pathogens, enhances macrophage phagocytosis and autophagy, neutralizes viruses by creating cross-links between virions, and often kills bacteria by interacting with the lipopolysaccharides and lipoteichoic acid present in bacterial cell walls (49, 51, 53, 55, 56). For instance, silk-worm apolipoproteins inhibits Staphylococcus aureus by binding to cell surface lipoteichoic acids (57, 58). Mosquito Vg has been reported to have antiparasitic response against plasmidium (59). Bacterial membrane proteins, flagella, and pili often serve as PAMPs and immune elicitors that interact with Vg and act as PRRs which induce autophagy and transgenerational immune priming (36, 60–65).

In this study, we show that OmpA and flagellin of CLso interact with Vg of B. trigonica and induce autophagy in the psyllid cells. While both Vg and autophagy are
important in the immune response against CLso, each seems to negatively regulate the other, and both are important for regulating CLso titers, in addition to oocyte development, oviposition, and egg viability. The described immune responses in this study are crucial for CLso persistence in the insect and seem to be part of a larger mechanism regulated by both the insect and CLso for maintaining the balance between the vector and the pathogen.

RESULTS

**Liberibacter surface proteins interact with the von Willebrand factor type D (VWD) domain of host vitellogenin.** As OmpA contains a surface antigen domain, while Flg acts as a virulence factor (31), Liberibacter OmpA and Flg were used to screen for interacting proteins in the psyllid vector. A cDNA expression library was prepared using whole psyllids and was mated with the full-length CDS of OmpA/Flg expressed as a fusion protein with GAL4-DNA binding domain in Y2HGold that binds to promoters of four reporter genes (AUR1C, HIS3, ADE2, and MEL1). Around 54 isolated colonies were obtained in QDO plates for OmpA and 62 for Flg which were restreaked on QDO/Xgal and QDO/Xgal/Aba plates for confirmation of β-galactosidase activity. Four out of all the colonies for OmpA and three for Flg were identified as parts of VWD domain of Vg after DNA sequencing (Fig. S3). To verify the Y2H interaction, VWD domain of Vg was amplified separately from psyllid DNA, cloned into pGAD-T7 vector, and screened against OmpA/Flg once again which also showed strong β-galactosidase activity (Fig. 1A).

Interaction between Vg and OmpA/Flg was further confirmed using a pulldown assay using OmpA as a bait. A band of approximately 48 kDa was observed in SDS-PAGE as well as Western blots using a monoclonal Anti-polyHistidine antibody produced in mouse (Sigma-Aldrich, Israel) when OmpA and Vg were included in the assay (Fig. 1B) or when Flg and Vg were used (Fig. 1C). No band was detected when GST control was used as bait, indicating a specific interaction between OmpA/Flg and Vg-VWD.
To further confirm the interaction, spatial localization of Vg was confirmed in dissected midguts from Ca. L. solanacearum infected psyllids using immuno-localization with specific antibodies for Vg and Ca. L. solanacearum. The signal observed in the midgut indicated a partial overlap in the fluorescent signals of Vg and Ca. L. solanacearum, which indicated a physical proximity in midgut cells (Fig. 1D). Vg localization and expression profiles in Ca. L. solanacearum-infected and Ca. L. solanacearum-free males and females are shown in Fig. S4.

**In silico analyses.** Only one Vg homolog was identified from the psyllid transcriptome (3). The coding sequence was validated by cloning and sequencing. Structural analysis of Vg revealed three major domains, which include Lipoprotein LPD_N-terminal domain, DUF1943, and VWD domain. (B) Three-dimensional structure of psyllid Vg, modeled by iTasser with highest C-score of 0.32 shows the two major domains: LPD_N (red) and VWD (blue). (C) Phylogeny of amino acid sequences of all known psyllid Vg proteins showing clustering within Hemipteran clade with T. evansi used as an outgroup.

To further confirm the interaction, spatial localization of Vg was confirmed in dissected midguts from Ca. L. solanacearum infected psyllids using immuno-localization with specific antibodies for Vg and Ca. L. solanacearum. The signal observed in the midgut indicated a partial overlap in the fluorescent signals of Vg and Ca. L. solanacearum, which indicated a physical proximity in midgut cells (Fig. 1D). Vg localization and expression profile in Ca. L. solanacearum-infected and Ca. L. solanacearum-free males and females are shown in Fig. S4.

**In silico analyses.** Only one Vg homolog was identified from the psyllid transcriptome (3). The coding sequence was validated by cloning and sequencing. Structural analysis of Vg revealed three major domains, which include Lipoprotein LPD_N-terminal domain, DUF1943, and VWD domain. (B) Three-dimensional structure of psyllid Vg, modeled by iTasser with highest C-score of 0.32 shows the two major domains: LPD_N (red) and VWD (blue). (C) Phylogeny of amino acid sequences of all known psyllid Vg proteins showing clustering within Hemipteran clade with T. evansi used as an outgroup.

Amino acid sequences of all known Liberibacter OmpA and Flg were aligned for sequence identity. Despite an overall high level of identity, conservation of sequences were found to be scattered for both OmpA and Flg. Domain analysis for OmpA revealed four polypeptide transport-associated domain (POTRA) and a bacterial surface antigen. Homology modeling also revealed a three-dimensional structure for OmpA with all the major domains (Fig. S5). Similar analyses for flagellin revealed a signaling domain and a polymerization domain and show minor heterogeneity when aligned with conserved sequences (Fig. S6).

**Ca. L. solanacearum induces vitellogenin and autophagy-related genes in psyllids.** Differential expression profiles of Vg and autophagy related genes were studied in Ca. L. solanacearum-free and Ca. L. solanacearum-infected psyllids. Immunostaining
revealed higher expression of Vg in the Ca. L. solanacearum-infected midguts and its expression was upregulated in Ca. L. solanacearum-free psyllids by 5.9-fold changes in whole body samples (in both males and females) and by 1.75-fold changes in the midguts (Fig. 3A and B).

Cathepsin-B and Caspase-I, known immunity genes involved in lysosomal functions against several pathogen invasions, were found to be upregulated in Ca. L. solanacearum-infected psyllid whole body and midguts. Additionally, the expression of autophagy genes Atg16, Atg2, and Atg5 involved in autophagosome formation was also upregulated in both midguts and whole psyllids (Fig. 3A). Higher lysosomal activity in Ca. L. solanacearum-infected midguts (Fig. 3C) and in ovaries (Fig. 3D) compared with Ca. L. solanacearum-free psyllids was observed when guts and ovaries were stained with Lysotracker, which specifically binds to acidic organelles, indicating higher formation of autolysosomes. The intensity of the Lysotracker signal in Ca. L. solanacearum-infected midguts were 3.12 ± 1.2 times more than Ca. L. solanacearum-free as validated using integrated intensity in ImageJ software.

**Inducing vitellogenin impairs autophagy and vice versa.** Vg expression was measured following psyllid treatment with the JH-III hormone, which is the main regulator of Vg production during oogenesis. After 16 h of exposure to JH-III, significant elevation of Vg expression was observed in whole bodies and midguts of both male and female with significantly higher induction in females (Fig. 4A). Female psyllids in which Vg was induced also had increased number of fat bodies as seen during dissection (data not shown). Induction of Vg also induced Liberibacter titer in the midguts as well as in the hemolymph as measured by qPCR and immunostaining (Fig. 4B to D), where elevated autolysosomal
activities were also observed (Fig. 3). Vg and Ca. L. solanacearum were seen to mostly colocalize in midguts and ovaries as validated by Pearson’s correlation coefficient ($R$ = 0.75). Induction of Vg expression, however, caused a significant downregulation of the autophagy-related genes in whole females (Fig. 5A) midguts (Fig. 5B) and ovaries (Fig. 5C). The presence of autolysosomes was almost negligible in the JH-III treated psyllid midguts (Fig. 5D) and ovaries (Fig. 5E) compared with the control treatments. Interestingly, application of thapsigargin, that specifically inhibits autophagy, reduced the expression of Vg along with all other autophagy genes, while causing an increase in Ca. L. solanacearum levels, as seen in qRT-PCR (Fig. 6A) and immunostaining (Fig. 6C). On the other hand, using the specific autophagy inducer rapamycin significantly induced autophagy and autophagy related genes and reduced the expression of Vg and Liberibacter titers in the psyllid midguts (Fig. 6B and C).

**Induction of Vg impairs egg development, oviposition, and viability.** Because Vg has an important role in oogenesis and egg development, we investigated its induction following JH-III treatment on mortality, oviposition, and fertility, compared with induction as a result of the presence of Ca. L. solanacearum. No significant mortality was observed in the JH-III exposed female psyllids compared to the controls. However, a significant reduction in the number of eggs laid by Vg induced female psyllids was obtained (Fig. 7A). JH-III application further induced oocyte development in female psyllids post-48-h treatment with higher number of mature oocytes that was observed compared with that of control females (Fig. 7B). Moreover, only 3% of the eggs laid by JH-III exposed females hatched compared with 83% viability in Ca. L. solanacearum-infected control eggs (Fig. 7C), with observed malformations and developmental defects in the laid eggs following JH-III treatment (Fig. 7D).
Absence of transovarial transmission. Because JH-III significantly induced Ca. L. solanacearum titers and ovary development in the psyllids, we tested whether these effects may cause the bacterium to be transferred to the developing oocytes by transovarial transmission. One out of 25 hatched nymphs that developed from eggs laid by Ca. L. solanacearum-infected females that were reared on Ca. L. solanacearum-free leaves tested positive for Ca. L. solanacearum, indicating very low or negligible transovarial transmission. Newly hatched nymphs that developed from eggs laid by Ca. L. solanacearum females that were reared on Ca. L. solanacearum leaves, were completely viable and efficiently acquired Ca. L. solanacearum when fed on Ca. L. solanacearum-

FIG 5 Effect of JH-III on autophagy. Relative expression of lysosomal and autophagy genes in whole bodies (A), midguts (B), and ovaries (C) showing downregulation of all the known genes upon JH-III application (P < 0.05). (D, E) Representative images showing reduction of autophagy and lysosomes in the JH-III applied psyllids. Staining of the midguts (D) and ovaries (E) with DAPI (blue) and lysosomes (green) with b and d showing magnified images of the insets in a and c, respectively. *, P < 0.05; ***, P < 0.01.

FIG 6 Effect of Thapsigargin and Rapamycin on Vg, Ca. L. solanacearum, and autophagy. (A) Relative gene expression of Vg, autophagy genes and Ca. L. solanacearum titer in the psyllid midguts upon Thapsigargin application (P < 0.05). (B) Relative gene expression of Vg, autophagy genes, and Ca. L. solanacearum titer in the psyllid midguts upon Rapamycin application (P < 0.05). (C) Staining of lysosomes (green) and nuclei (blue) showing disintegrated nuclei and absence of autophagy upon Thapsigargin application (b), and increase in lysosomal activity upon Rapamycin application (c) compared with the control midguts (a). Lower panel showing decrease in vitellogenin and increase in Ca. L. solanacearum titer upon Thapsigargin application (e) and lower Ca. L. solanacearum abundance upon Rapamycin application (f) compared with control midguts (a). *, P < 0.05; ***, P < 0.01.
infected leaf flush. Interestingly, ovaries dissected from *Ca. L. solanacearum*-infected females treated with JH-III all tested positive for *Ca. L. solanacearum* (Fig. S7); however, the eggs laid were 100% unviable (Fig. 7C and D), suggesting that JH-III treatment indeed accelerates *Ca. L. solanacearum* penetrance into ovaries; however, this treatment is fatal for the eggs as it impairs autophagy.

**DISCUSSION**

Bacterial membrane proteins have virulence properties and often act as pattern recognition molecules which induce host immune response (32, 61). In this study, we used Liberibacter outer membrane protein (OmpA) and flagellin (Flg) as baits to screen for specific insect host proteins that interact with OmpA and Flg. Because both of these bacterial proteins bear an important role in adhesion and virulence, we expected that both proteins will interact with psyllid proteins either for adhesion during the transmission process or for bypassing the host immunity. Yeast two-hybrid assays using OmpA/Flg as baits revealed their interaction with the host Vg-VWD domain when screened against psyllid cDNA library (Fig. 1A). Because OmpA has a transmembrane domain, we used OmpA as a prey and used Vg-VWD as a bait to revalidate the interaction. Pulldown assays and spatial coimmunolocalization using confocal microscopy also indicated specific interactions between both bacterial proteins and Vg (Fig. 1B and C). The reason behind this spatial localization of Vg is that Vg is a secretory protein which is produced in cells and is often translocated to the midgut lumen and hemolymph. However, presence of Vg in the midgut epithelial cells help in pathogen movement across the midgut cells and in some cases opsonization (66–68). Further studies are needed to confirm the interaction in the lumen or in the hemolymph which can help us with a better rationale behind CLso-Vg interaction. In the Asian citrus psyllid *Diaphorina citri*, the expression of Vg is reported to be induced in response to CLas infection (69), similar to its induction in the potato psyllid upon CLso infection (18). Vg has also been reported to act as a pattern recognition molecule against pathogens.
and it has been shown to mediate their degradation by hemocytes through phagocytosis (59, 60, 70, 71). Both bacterial proteins also carry immune elicitors and are able to induce immunity in subsequent generation through the process known as transgenerational immune priming (TGIP) (30, 62, 72, 73). In this study, only one Vg homolog was identified from the carrot psyllid transcriptome (3) and the full-length coding sequence was assembled and cloned. It has two major domains: apolipoprotein domain (LPD_N), which helps in lipid transport; and VWD domain, a multifunctional domain involved in maintaining homeostasis (74, 75). Based on phylogenetic analysis, Vg clustered with the other two psyllid Vgs that clustered within the hemipteran clade (Fig. 2). The surface antigen region of OmpA (Fig. S5) and hypervariable region of flagellin (Fig. S6) are reported to possess adhesion-like properties and they often act as microbe-associated molecular pattern (MAMP) inducing virulence (35–37, 39, 76).

The known interaction between Vg and bacterial membrane proteins was a trigger to investigate the role of Vg in Liberibacter pathogenesis and host immunity response. The function of vitellogenin is often known to accompany programmed autophagy during development and under stress conditions, and a tight link between the two has been reported in various studies (56, 77, 78). In this study, we investigated the gene expression profiles of autophagy-related genes (available from the transcriptome) and the presence of autolysosomes in Ca. L. solanacearum+ psyllid midguts and ovaries as a result of Vg induction. The results showed a significant upregulation of Vg along with the autophagy-related genes (Atg2, Atg5, and Atg16) in Ca. L. solanacearum+ whole body as well as in midguts of carrot psyllids (Fig. 3A and B). The midgut, being the first barrier for Ca. L. solanacearum, is the major organ important for transmission and the first cellular organ to meet the pathogen where it invades intracellularly and activates immune responses. Thus, it is the most suitable organ for studying the interaction between the bacteria and psyllid proteins. Although the immune system is activated in the whole body, the molecular interactions in the midgut are expected to be more intense and possibly prime other responses in other parts of the body. In the midgut, the pathogen activates machineries for adhesion, cell invasion, and crossing the basal lamina to reach the hemolymph while avoiding the psyllid defenses (3, 16). Cathepsin B and caspase 1 involved in lysosomal activity were also upregulated in Ca. L. solanacearum+ psyllids (Fig. 3B). The presence of lysosomal bodies and autolysosomes were evidently higher in Ca. L. solanacearum+ psyllid midguts and ovaries (Fig. 3C and D). A higher number of lysosomes indicates higher lysosomal activity, and higher autophagy as autophagosomes delivers cytoplasmic materials or cellular debri to the lysosomes for degradation. These results explain the joint and orchestrated function of both Vg and autophagy-related genes upon Liberibacter infection for maintaining homeostasis, and the crucial role of these functions for maintaining the cell viability. However, when Vg expression was induced with the application of JH-III hormone (Fig. 4), there was a drastic reduction in autophagy and lysosomal activity (Fig. 5), and the expression of autophagy-related genes and lysosomal proteases were significantly downregulated (Fig. 4 and 6). Moreover, Vg induction drastically reduced oviposition and egg viability (Fig. 7). JH-III is a well-known regulator of vitellogenesis (79, 80). It is known that overexpression of Vg induces ageing and impairs the induction of autophagy and lysosomal genes required to maintain longevity (78) and autophagy is induced during the synthesis phase of Vg in the fat body to maintain developmental switches, regulate immunity, and recycle cellular components during development (77, 81, 82). On the other hand, upon autophagy arrest by thapsigargin, Vg expression was reduced along with the autophagy-related gene expression in the psyllids (Fig. 6A and C). This was surprising to us although the result is in congruence with previous reports where it has been shown that cellular calcium plays a basal regulatory role in Vg production independent of mTOR pathway which is the major regulator for autophagy as well as for Vg expression. Thapsigargin acts as calcium mobilizing agent while blocking autophagy, while inducing both ER stress and apoptosis (83–86). This induction of apoptosis is also known to activate Perk-eIF2 pathway and Ire-1 dependent decay (RIDD)
of mRNA, which results in reduced synthesis and degradation of Vg mRNA, respectively (83, 87). Additionally, Liberibacter titers increased significantly, and its signal was seen to be diffused in the psyllid midguts treated with thapsigargin. This experiment helped us solely to understand the effects of reduced autophagy on CLso abundance. Further studies can be done to silence specific autophagy-related genes to understand how Vg and autophagy process are connected. Interestingly, inducing autophagy and blocking the mTOR pathway by applying rapamycin, reduced Vg expression as well as Liberibacter in the psyllid midguts. The reduction in Liberibacter titer is believed to be a result of increased autophagy. This result is in correspondence with new findings in ticks where vitellogenesis is delayed by the application of rapamycin and is regulated by autophagic mTOR pathway (88). Overall, we know that Vg induction is mostly regulated by mTOR than by a calcium-regulated pathway. Disrupting these two pathways independently have similar effects on Vg, although, the negative effects of autophagy on CLso remains constant. As programmed autophagy is crucial for proper cell development, it will be interesting to study how Vg and autophagy regulate each other during egg maturation in psyllids. This indicates that both vitellogenesis and autophagy are important for cell survival and are integral parts of developmental process, which help in maintaining cellular homeostasis. Any imbalance between the two may disrupt the homeostasis and may lead to cell death (77, 78, 89, 90).

The results of this study also show elevated titers of Liberibacter in the JH-III-treated midguts as well as in the hemolymph in the absence of autophagy (Fig. 4 and 6). Higher abundance of Liberibacter titer in the midguts and hemolymph suggests a role of Vg in presenting Liberibacter to the cells inducing autophagy, whose absence results in higher titers of the pathogen in the system. There might also be a role for Vg in transgenerational immune priming in Ca. L. solanacearum-infected psyllids and a possibility of transovarial transmission in the absence of autophagy. We could not detect Ca. L. solanacearum in viable ovaries and laid eggs, which indicates the absence of transovarial transmission. Nymphs developing from eggs laid by Ca. L. solanacearum-infected females that hatched and fed on Ca. L. solanacearum leaves were also negative for Ca. L. solanacearum. This implies that nymphs acquire Ca. L. solanacearum by feeding only on infected leaves with Ca. L. solanacearum, and not by transovarial transmission. Surprisingly, ovaries dissected from Ca. L. solanacearum+ females which were treated with JH-III tested positive for Liberibacter in the absence of autophagy. However, induction of Vg reduced egg viability although vitellogenic development in the oocytes and the number of ovarioles was greater compared with the control Ca. L. solanacearum+ psyllid ovaries (Fig. 7). This possibly happened due to the lack of autophagy, which disrupted proper cellular development. These results suggest that the ovaries tested positive for Ca. L. solanacearum because of Vg induction or reduction in autophagy. These results suggest a role for Vg in the defense mechanism and might be involved in TGIP, although an exact mechanism remains unknown. Although, the correlation between Vg and autophagy was tested in females with respect to oviposition and egg viability, it will be interesting to see if this interrelation and variability in expression is similar in the males. It will also be exciting to compare the immune response and liberibacter transmission competence between the male and the female psyllids following liberibacter acquisition.

In summary, the results presented in this study reveal that both vitellogenin and autophagy are essential in regulating Ca. L. solanacearum levels and possibly its persistence, transmission, and generated stress responses in the psyllid cells. Although the role of autophagy in CLso abundance seems to be inversely proportional, the exact role of Vg remains unclear. We believe one of the reasons behind CLso-Vg interaction is to regulate autophagy for easy pathogen persistence which also helps in maintaining a homeostasis between vitellogenesis and autophagy for host survival. It will be interesting to study how Vg interacts with autophagosomes or autophagy-related targets upon binding with CLso. This will further explain the relationship between vitellogenesis and autophagy process in psyllids upon CLso infection. Future studies are
imperative to investigate whether Liberibacter interacts with vitellogenin to manipulate the host immune response for its survival, or it is a host defense mechanism against Liberibacter to reduce cellular stress and maintain homeostasis.

MATERIALS AND METHODS

Maintenance of psyllid and Liberibacter. Ca. L. solanacearum-infected and Ca. L. solanacearum-free psyllids were maintained on 2 months old Parsley (Petroselinum crispum) in separate rooms, under 14-h photoperiodic light at 25 ± 2°C. The plants as well the psyllid population were tested for Ca. L. solanacearum routinely.

Plasmid vectors. For protein expression studies, we used pRSET-A and pFN2A Flexi vectors (Thermo Scientific) with competent BL21(DE3) and DH5α cells (NEB, USA). pGAD-T7-Rec (Clontech) was used for the cDNA library preparation, pGADT7-AD (Clontech) as prey vector for one to one assays, and were then transformed in Y2H Gold yeast cells. pGBK7 (Clontech) was used as a bait vector (DNA-BD) and was transformed into Y187 yeast cells.

Yeast two-hybrid bait constructs. Sequences of Liberibacter Outer membrane protein (OmpA) and Flagellin (Flg) were derived from the full genome sequence of Candidatus Liberibacter solanacearum (Haplotype D) with accession PKRU02000006.1 (91). Full-length coding sequence of OmpA and Flg were amplified from Liberibacter infected (C-loss) psyllids using Q5 DNA polymerase (NEB, USA), cloned into the bait vector-pGBK7 (EcoRI/BamHI) using In-Fusion HD cloning kit (TaKaRa) and screened for positive recombinants in DH5α cells. Recombinant OmpA-pGBK7 and Flg-pGBK7 were finally transformed into yeast two-hybrid Gold yeast strain separately using Yeastmaker yeast transformation system (TaKaRa, Clontech).

Psyllid library construction. Total RNA was extracted from around 100 C-loss + psyllids using TRIzol (Sigma) and purified using RNAeasy kit (Qiagen). First-strand cDNA was synthesized with 3.6 μg of total RNA using Make your own "mate & plate" library system (TaKaRa, Clontech) according to the manufacturer’s instruction. The first-stranded cDNA was next amplified to produce double-stranded cDNA in 20 amplification cycles by long-distance PCR using the Advantage 2 polymerase mix (TaKaRa, Clontech) following the manufacturer’s instructions. The double-stranded cDNA was purified using Chroma Spin + TE-400 to eliminate any products below 200 bp. The purified ds cDNA was finally cotransformed with pGAD-T7 Rec into competent Y187 using Yeastmaker yeast transformation system (TaKaRa, Clontech) and plated on SD-Leucine agar media. The plates were incubated at 30°C for 3 to 5 days. Around 2.6 million independent cDNA clones were obtained and the colonies were pooled using YPDA freezing media and stored in aliquots in −80°C.

Y2H assays. The two baits, OmpA-pGBK7 and Flg-pGBK7, were tested for self-activation and were further screened against the psyllid library individually, following Matchmaker Gold yeast two-hybrid user manual (TaKaRa, Clontech). A culture of the bait (Y2HGold) was allowed to mate with psyllid library and after mating, the cells were plated on QDO (SD-ATLH) media and incubated at 30°C for 8 to 10 days. Developed colonies were restreaked onto QDO/X-gal-plates to screen for the development of blue color for the β-galactosidase activity, and finally the blue colonies were further streaked onto QDO/Xgal/Aureobasidin (40 μg/mL). Plasmids were isolated from the colonies as previously described (92) and sequenced for identity.

RNA extraction, qRT-PCR analysis, and Liberibacter abundance. Single psyllids/guts/ovaries were used for RNA and DNA extraction, both from the same sample using CTAB (93) and as previously described (94). Males (1 week old) were only used for testing the expression of Vg. Females were used for all other experiments. The guts and ovaries were washed three times with PBS to remove any contaminants from the hemolymph before proceeding with RNA/DNA extraction. Final eluted nucleic acid was divided into two aliquots, one for RNA and one for DNA. DNA contaminations from the total RNA were removed with DNase I (Thermo) and used for cDNA synthesis using Verso cDNA synthesis kit (Thermo) following manufacturer’s instructions. RNA was removed from the DNA sample using RNaseI (Thermo) and was used to measure relative Liberibacter titer using qPCR. Real-time analyses were carried out using 2X Absolute Blue SYBR mix (Thermo) and 1 μL of diluted cDNA in a final volume of 20 μL. Threshold Ct values were calculated in StepOne real Time PCR system (Applied Biosystems) and normalized using the housekeeping genes (elongation factor). PCR efficiencies of all new primers were tested using a standard curve, and differential gene expression were analyzed using 2–ΔΔCT quantitation methods (95). The primers used in this study are listed in Table 1. Statistical analyses of all qRT-PCR data were conducted by one-way ANOVA with Tukey’s post hoc test (P < 0.05). The accumulation of Ca. L. solanacearum in the hemolymph was also quantified with the method previously described (94) using a Nanoliter 2010 injector (World Precision Instruments, Sarasota, FL, USA). The hemolymph was diluted in 10 μL of water and was used directly for qPCR analysis with OmpA-specific primers. Statistical analysis was done using Student’s t test (P < 0.05).

Recombinant protein expression, in vitro translation, and pulldown assay. (i) Prey (vitellogenin). Full-length coding sequence for VWD domain of Vg was cloned into pRSET-A, and finally transformed into competent E. coli BL21(DE3) cells to express 6-His-Vg-VWD. The transformed clones were grown overnight in liquid LB at 37°C with agitation (200 rpm). A fresh media of 5 mL was seeded with 200 μL of this culture and grown for 4 h or until it reaches 0.6 optical density. At this point, a final concentration of 1 mM IPTG was added and was incubated with agitation at 30°C for additional 5 h. The cells were finally harvested at 6,000 rpm followed by protein extraction. The cells were resuspended in 200 μL of B-PER along with 1 μg of Lysozyme (Sigma-Aldrich) and DNase (Thermo), incubated for 15 min with shaking at room temperature and centrifuged at a high speed for 10 min. The supernatant was collected as purified protein and was used for further protein assays.
### Table 1: Primers used for PCR detection, qRT-PCR

| Primer name          | Sequence 5' > 3'                                   | Target                                  | Product size (bp) | Reference                  |
|----------------------|-----------------------------------------------------|-----------------------------------------|-------------------|----------------------------|
| Ef1α_Fq              | CCACCCACCAACACATCTAC                                | B. trigonica elongation factor 1α       | 119               | 109                        |
| Ef1α_Rq              | ACTTCTTCTCTCCTCTCTCATCT                            |                                        |                   |                            |
| qVWD_Fq              | GAGAACCACTATCGCTCATAT                              | B. trigonica Vg                        | 197               | This study (GenBank MW316414) |
| qVWD_Rq              | TTGGGGGGAAGGATCCAC                                 |                                        |                   |                            |
| qOmp_Fq              | CCATATCCAATTTCAAGAAACC                             | Ga. L. solanacearum ompA                | 152               | 110                        |
| qOmp_Rq              | ATGCCACGTTAAGGATTTG                               |                                        |                   |                            |
| Actin_F              | AGATGACCCAGATCATGTGTGA                             | B. trigonica Actin                     | 140               | 110                        |
| Actin_R              | AGGGCCTAACCCTCCATAGT                              |                                        |                   |                            |
| CathB_Fq             | CAAAGTCATGTTGTTGTTGAAACAGCA                      | B. trigonica CathespinB                 | 125               | This study (GenBank OK188783) |
| CathB_Rq             | TGTTCCAGGATTTGGCACAT                               |                                        |                   |                            |
| Caspaseel_Fq          | GTCTGAGGAACACGCTACC                                | B. trigonica Caspaseel                  | 165               | This study (GenBank OK188782) |
| Caspaseel_Rq          | TACCAAGACCTACACGCC                                |                                        |                   |                            |
| Atg2_Fq              | TGTTGGCCACATGTTGTATCA                              | B. trigonica Atg2                      | 147               | This study (GenBank OK188779) |
| Atg2_Rq              | CTGGATCTGCTCTTGCCCTT                               |                                        |                   |                            |
| Atg5_Fq              | TGGCCATACCTATGTATTGCT                             | B. trigonica Atg5                      | 205               | This study (GenBank OK188780) |
| Atg5_Rq              | TCTGCATGTTGGAAACATCTGT                              |                                        |                   |                            |
| Atg16_Fq             | AGAAGGAGCAAGGACATG                                | B. trigonica Atg16                     | 157               | This study (GenBank OK188781) |
| Atg16_Rq             | CCGTCCACAGGACTCCACT                                |                                        |                   |                            |
| OA2-F (Lib16s_F)     | GCGCTTTATTTTATAGAGAGGACAAGCCAC                      | Ga. L. solanacearum 16s rRNA           | 1168              | 111                        |
| O2c-R (Lib16s_R)     | GCGCTTTAGAGAGGACAAGCCAC                            |                                        |                   |                            |
| ompA_full_F          | ATGGGCAAAGAAAAAAAGGACTC                           | Ga. L. solanacearum OmpA (full length) | 2331              | This study                 |
| ompA_full_R          | CTAATTTGCCCCGGATTCCCCGA                           |                                        |                   |                            |
| ompA_bait_F          | CGCGGATCATCGGCGGACTCCCGA                          | Ga. L. solanacearum OmpA as bait (pGBK7) | 2331              | This study                 |
| ompA_bait_R          | CGCGGATCATCGGCGGACTCCCGA                          |                                        |                   |                            |
| Omp-pFN2_sgf_F       | AGCTGGCGATCGCCATGAGGAGAAGAAAAAGGACT              | Ga. L. solanacearum OmpA (Infusion cloning into pFN2A) | 2331              | This study                 |
| Omp-pFN2_pme_R       | CGCGGCTTTAATCTATCGGCGGACTCCCGA                    |                                        |                   |                            |
| Flagellin_F          | ATGACTATGATTTCTAATCTACCAC                          | Ga. L. solanacearum Flagellin (full length) | 1362              | This study                 |
| Flagellin_R          | ATGACTATGATTTCTAATCTACCAC                          |                                        |                   |                            |
| Flagellin_bait_F     | CGCGGATCATCGGCGGACTCCCGA                          | Ga. L. solanacearum flagellin as bait (pGBK7) | 2331              | This study                 |
| Flagellin_bait_R     | CGCGGATCATCGGCGGACTCCCGA                          |                                        |                   |                            |
| Flg_pFN2_sgf_F       | GACCGCGATCGCCATGAGGAGAAGAAAAAGGACT               | Ga. L. solanacearum flagellin (Infusion cloning into pFN2A) | 2331              | This study                 |
| Flg_pFN2_pme_R       | GACCGCGATCGCCATGAGGAGAAGAAAAAGGACT               |                                        |                   |                            |
| Vg_F                 | ATGGTCTTCATCAATCTACC                              | B. trigonica Vitellogenin (full length) | 2331              | This study                 |
| Vg_R                 | TTAGCGGGCCAAAAACACATTTTG                          |                                        |                   |                            |
| VWD_full_F           | GAGAACCACTATCGCTCATAT                              | B. trigonica Vitellogenin-VWD domain (full length) | 2331              | This study                 |
| VWD_full_R           | TTAGGCGCCAAAAACACATTTTG                           |                                        |                   |                            |
| VWD_pSET_Xhol_F      | CCCCTCGAAGGAGAAGAAGCACATCCTCATAT                | Cloning VWD into pSETA                  |                   | This study                 |
| VWD_pSET_EcoRI_R     | CCCGAAATTTCTAGGCGGACACACATTTTG                    |                                        |                   | This study                 |
| VWD_pGAD_F (EcoRI)   | CCCGAAATTTCTAGGCGGACACACATTTTG                    | Cloning VWD into pGADT7                |                   | This study                 |
| VWD_pGAD_R (Xhol)    | CCCGAAATTTCTAGGCGGACACACATTTTG                    |                                        |                   | This study                 |
(ii) **In vitro translation of Bait protein.** Full-length CDS of OmpA/Flg was cloned into pFN2A (GST) Flexi vector separately for in vitro translation. Briefly, Liberibacter OmpA was amplified using Sgfl and Pmel restriction sites (Table 1) and was digested using Flexi Enzyme Blend (Promega). Similarly, pFN2A was also digested and finally ligated to OmpA/Flg using T4 ligase (Thermo). The ligation mixture was used to transform E. coli DH5α for screening a positive recombinant pFN2A-OmpA/pFN2A-Flg vector. Following this, the recombinant plasmid was isolated and sequenced for confirmation. For *in vitro* translation, we used TNT Quick coupled transcription/Translation System (Promega) following the manufacturer's instructions.

(iii) **Pulldown and Western blot assay.** The vector pFN2A was genetically modified to remove the Barnase gene and to express just the GST as control (Fig. S1). For pulldown assays, we used Magne-GST Pull-Down Systems (Promega, USA). The bait, pFN2A-OmpA/pFN2A-Flg, and control GST was immobilized onto Magne-GST particles following the manufacturer's instructions. Total soluble fractions from prey protein lysate (i.e., His-Vg-VWD) was incubated with the bait immobilized to Magne-GST particles for capture. After washing, the bound proteins were finally eluted by boiling in 1 × SDS buffer, separated on 10% SDS-PAGE for analysis and detected by Western blot using monoclonal anti-polyHis antibody produced in mouse (Sigma-Aldrich, Israel).

(iv) **Homology modeling and in silico studies.** The open reading frames of the sequences derived from the yeast two-hybrid assays were annotated using BLASTp and NCBI Conserved Domain Database (CDD) search (96) databases and checked for in-frame reading sequences. Structural analysis for domain identifications for was done by Pfam (97) and NCBI-CDD. Full-length VWD domain of Vg was amplified from carrot psyllids and was used for all further studies. For phylogenetic studies, *Ca. L. solanacearum* full-length Vg sequence was aligned with 20 other insect Vgs in Mega 7.0 software with arachnid Vg as an outgroup. The phylogenetic relationship was assessed in CIPRES gateway using MrBayes XSEDE tool with fixed LG+G substitution model and 1 million generation. The tree was finally edited in Figtree program v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree). Three-dimensional model structure for Vg was generated by I-TASSER (98) with the highest C-score. All known full-length sequences of OmpA/Flg from Liberibacter species were aligned in Mega7.0 (99) and similarity scores with consensus sequences were obtained in ESPript 3.0 (100). Three-dimensional model structure for OmpA/Flg was generated by Swiss-Model Tool (101) and the server Orientations of Proteins in Membranes (OPM) (https://omp.phar.umich.edu/) and for Flg by Swiss-Model.

**JH-III hormone treatment.** JH-III (Sigma, Israel), which is the principle regulator of Vg synthesis in Hemipterans (79, 102), was dissolved in ethanol at a concentration of 5 µg/µL. This concentration and application protocol was optimized after trying three different concentrations adapted from different reports. The one with low lethal activity and high effects on gene expression was chosen (103–107). To induce the expression, JH-III was applied to a flush of parsley in an incubation box with 20 adult female psyllids (up to 1 week old with unknown mating status) for 16 h. Ethanol was used in the control set of experiment. The psyllids were collected and were used for oviposition, DNA/RNA isolation, and immunostaining analyses. The experiments were done in triplicate with minimum of six samples each for qRT-PCR/q-PCR.

**Induction and repression of autophagy.** Autophagy was induced by treating the psyllids with Rapamycin (Sigma, Israel) (a potent mTORC1 inhibitor). The experiment was set up similar to the dsRNA experiments as mentioned previously (94). Fresh leaf flush was placed in a microcentrifuge tube, applied with 10 µM Rapamycin (dissolved in ethanol). This concentration was again optimized based on low lethal effects. Twenty adult female psyllids (up to 1 week old) were released into each jar containing the leaf flush and were allowed to feed for 24 h. Similarly, Thapsigargin (Enco, Israel) was used for autophagy inhibition at a concentration of 10 µM and the application was similar to that of JH-III. Ethanol was used as control. DNA/RNA was extracted from the psyllids, midguts, and ovaries for qPCR and qRT-PCR analyses. Midguts were also used for immunostaining. Each experiment was conducted three times with a minimum of six samples each time for a total of minimum 18 samples.

**Immunolocalization.** Immunostaining for Vitellogenin, Liberibacter, and auto-lysosomes were done according to the protocol described previously (94). Psyllid midguts/ovaries were dissected out in PBS, fixed in 4% paraformaldehyde, treated with TritonX-100 and incubated in 1.5% blocking buffer for 1 h. Following this, the guts/ovaries were incubated with rabbit-polyclonal Anti-Vg antibody (Abcam) or Anti-Omp8 antibody produced in rabbit (GenScript Corp., USA) (108) for 1.5 h followed by secondary antibody conjugated with Cy3/Cy5 counterstained with DAPI. The colocalization of Liberibacter and Vg was validated using the Colocalization Finder plugin of Imagej with Pearson’s correlation coefficient (R value) using five different images (https://imagej.nih.gov/ij/plugins/colocalization-finder.html). LysoTracker Green DND-26 (Invitrogen) was used to locate auto-lysosomes according to the manufacturer’s instructions. At least eight midguts were used for each immunolocalization experiments to confirm the consistency of the results obtained. The differences in the signals for auto-lysosomes in Ca. L. solanacearum-free and Ca. L. solanacearum-infected midguts were validated using ImageJ software with area integrated intensity and mean gray value. A minimum of 10 images were used for measurement for each ImageJ analysis.

**Oviposition and egg hatching.** After JH-III treatment, the insects were released on fresh leaf flush for 72 h and the number of laid eggs (oviposition) by 10 female psyllids was counted on each leaf flush and were monitored for hatching (fertility). Eggs from Ca. L. solanacearum+ psyllids treated with ethanal were used as a control. To test the presence of transovarial transmission, the leaf flush (for control and JH-treatment) along with oviposited eggs were washed for 2 min in 0.05% bleach followed by 50% alcohol and finally washing in sterile distilled water thrice. The eggs were then carefully separated from the leaves and placed on a Petri dish (Fig. S2) and then incubated in the plant growth room. The newly hatched nymphs were allowed to feed on the uninfected leaves and were tested for Ca. L.
solanacearum in the third instar stage. For control experiments, the eggs were placed on sterile Ca. L. solanacearum-infected leaves. The differences were analyzed by Student’s t test (P < 0.05).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

We thank Eduard Belausov for technical help with the confocal microscope and members of the Ghanim laboratory for technical support and for providing comments on preliminary versions of the manuscript text.

This research was supported by grant 1163/18 from the Israel Science Foundation to M.G.

REFERENCES

1. Ghanim M, Achor D, Ghosh S, Kontsedalov S, Lebedev G, Levy A. 2017. ‘Candidatus Liberibacter asiaticus’ accumulates inside endoplasmic reticulum associated vacuoles in the gut cells of Diaphorina citri. Sci Rep 7: 16945. https://doi.org/10.1038/s41598-017-16905-w.

2. Mawassi M, Dror O, Bar-Joseph M, Piasezky A, Sjolund JM, Levitzky N, Shoshana N, Meselin L, Haviv S, Porat C, Katsir I, Kontsedalov S, Ghanim M, Zelinger-Reichert E, Arnsdorf YM, Gera A, Bahar O. 2018. ‘Candidatus Liberibacter solanacearum’ is tightly associated with carrot yellows symptoms in Israel and transmitted by the prevalent psyllid vector Bactericera trigonica. Phytopathology 108:1056–1066. https://doi.org/10.1094/PHYTO-10-17-0348-R.

3. Ghosh S, Jassar O, Kontsedalov S, Lebedev G, Wang C, Turner D, Levy A, Ghanim M. 2019. A transcriptomics approach reveals putative interaction of Candidatus Liberibacter solanacearum with the endoplasmic reticulum of its psyllid vector. Insects 10:279. https://doi.org/10.3390/insects10090279.

4. Capoor SP, Rao DG, Viswanath SM. 1967. Diaphorina citri Kuway., a vector of the greening disease of citrus in India. Indian J Agric Sci 37:572–576.

5. Ammar ED. 1994. Propagative transmission of plant and animal viruses by insects: factors affecting vector specificity and competence. Adv Dis Vector Res:289–331.

6. Ammar E, Shatters RG, Jr, Hall DG. 2011. Localization of Candidatus Liberibacter asiaticus, associated with citrus Huanglongbing disease, in its psyllid vector using fluorescence in situ hybridization. J Phytopathol 159: 726–734. https://doi.org/10.1111/j.1439-0442.2011.01836.x.

7. Cooper WR, Sengoda VG, Munyaneza J. 2014. Localization of ‘Candidatus Liberibacter solanacearum’ (Rhizobiales: Rhizobiaceae) in Bactericera cockerelli (Hemiptera: Triozidae). Ann Entomol Soc Am 107:204–210. https://doi.org/10.1093/anies/107.1.204.

8. Cicero JM, Fisher TW, Qureshi JA, Stansly PA, Brown JK. 2017. Colonization and intrusive invasion of potato psyllid by ‘Candidatus Liberibacter solanacearum.’ Phytopathology 107:36–49. https://doi.org/10.1094/PHYTO-03-16-0149-R.

9. Clark K, Franco JY, Switzer S, Pang Z, Hawara E, Liebrand TWH, Pagliaccia D, Zeng L, Gurung FB, Wang P, Shi J, Wang Y, Ancona V, van der Hoorn RAL, Wang N, Coaker G, Ma W. 2018. An effector from the Huanglongbing-associated pathogen targets citrus proteases. Nat Commun 9:1–11. https://doi.org/10.1038/s41467-018-0140-9.

10. Levy JG, Gross R, Mendoza-Herrera A, Yang J, Jiang L, Kuhl JC, Dibble MS, Xiao F, Tamborindeguy C. 2020. Lso-HPE1, an effector of Candidatus Liberibacter asiaticus on the phytopathology of the vector Diaphorina citri. J Appl Microbiol 121:1718–1726. https://doi.org/10.1111/jam.13302.

11. https://doi.org/10.1146/annurev-phyto-080516-035513.

12. Molki B, Ha PT, Mohamed A, Killiny N, Gang DR, Omsland A, Beyenal H. 2019. Physiochemical changes mediated by ‘Candidatus Liberibacter asiaticus’ in Asian citrus psyllids. Sci Rep 9:1–9. https://doi.org/10.1038/s41598-019-52692-7.

13. Sarkar P, Ghanim M. 2020. Unravelling the pathogenesis and molecular interactions of Liberibacter phytopathogens with their psyllid vectors. Agronomy 10:1132. https://doi.org/10.3390/agronomy10081132.

14. Wang N, Pierson EA, Setubal JC, Xu J, Levy JG, Zhang Y, Li J, Rangel LT, Martins J. 2017. The Candidatus Liberibacter–host interface: insights into pathogenesis mechanisms and disease control. Annu Rev Phytopathol 55:451–482. https://doi.org/10.1146/annurev-phyto-080516-035513.

15. Fisher TW, Vyas M, He R, Nelson W, Cicero JM, Willer M, Kim R, Kramer R, May GA, Crow JA, Soderlund CA, Gang DR, Brown JK. 2014. Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative liberibacter transmission. Pathogens 3:875–907. https://doi.org/10.3390/pathogens3040875.

16. Ibanez F, Levy J, Tamborindeguy C. 2014. Transcriptome analysis of Candidatus Liberibacter solanacearum’ in its psyllid vector, Bactericera cockerelli. PLoS One 9:e100955. https://doi.org/10.1371/journal.pone.0100955.

17. Vyas M, Fisher TW, He R, Nelson W, Yin G, Cicero JM, Willer M, Kim R, Kramer R, May GA, Crow JA, Soderlund CA, Gang DR, Brown JK. 2015. Asian citrus psyllid expression profiles suggest Candidatus Liberibacter asiaticus-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity. PLoS One 10:e0130328. https://doi.org/10.1371/journal.pone.0130328.

18. Ammar E-D, Ramos JE, Hall DG, Dawson WO, Shatters RG, Jr. 2016. Acquisition, replication and inoculation of Candidatus Liberibacter asiaticus following various acquisition periods on huanglongbing-infected nymphs by nymphs and adults of the Asian citrus psyllid. PLoS One 11:e0159594. https://doi.org/10.1371/journal.pone.0159594.

19. Arp AP, Hunter WB, Pelz-Stelinski KS. 2016. Annotation of the Asian citrus psyllid genome reveals a reduced innate immune system. Front Physiol 7:570. https://doi.org/10.3389/fphys.2016.00570.

20. Canale MC, Tomasetto AF, Haddad M, de L, Della Coletta-Filho H, Lopes JRS. 2017. Latency and persistence of ‘Candidatus Liberibacter asiaticus’ in its psyllid vector, Diaphorina citri (Hemiptera: Liviidae). Phytopathology 107:264–272. https://doi.org/10.1094/PHYTO-02-16-0088-R.

21. George J, Ammar E-D, Hall DG, Shatters RG, Lapointe SL. 2018. Prolonged phloem ingestion by Diaphorina citri nymphs compared to adults is correlated with increased acquisition of citrus greening pathogen. Sci Rep 8:1–11. https://doi.org/10.1038/s41598-018-28442-6.

22. Wulff NA, Zhang S, Setubal JC, Almeida NF, Martins EC, Harakava R, Kumar D, Rangel LT, Foissac X, Bové JM, Gabriel DW. 2014. The complete genome sequence of ‘Candidatus Liberibacter americanus’, associated
Liberibacter Interacts with Vitellogenin

Microbiology Spectrum

July/August 2022 Volume 10 Issue 4 10.1128/spectrum.01577-22

37. Bunpa S, Chaichana N, Teng JLL, Lee HH, Woo PCY, Sermwittayawong D, Vallet-Gely I, Lemaitre B, Boccard F. 2008. Bacterial strategies to over-

45. Feldhauser H, Gross R. 2008. Cellular and molecular mechanisms of insect immunity. Insect Physiol Ecol 9:179–212.

54. Tetreau G, Dhinaut J, Gourbal B, Moret Y. 2019. Trans-generational immune priming in invertebrates: current knowledge and future prospects. Front Immunol 10:770.

66. Coons LB, Lamoreaux WJ, Rosell-Davis R, Tarnowski BI. 1989. Onset of vitellogenin synthesis in telotrophic male and female meconium. J Biol Chem 264:9239–9245.

77. Li Z, Zhang S, Liu J, Liu Z. 2009. Regulation of vitellogenin production and vitellogenesis, and their relationship to changes in circulating testosterone. Mol Cell Endocrinol 302:95–102.

88. Roy-Zokan EM, Cunningham CB, Hebber LK, McKinney EC, Moore AJ. 2015. Temporal and spatial detection of Candidatus Liberibacter asiaticus in citrus huanglongbing. Mol Plant Microbe Interact 28:1330–1337.
87. Metcalf MG, Higuchi-Sanabria R, Garcia D, Tsui CK, Dillin A. 2020. Beyond the cell factory: homeostatic regulation of and by the UPRER. Sci Adv 6:eabb9614. https://doi.org/10.1126/sciadv.aabb9614
88. Behri M, Teshima H, Kutsuwada K, Nakatake S, Ogihara MH, Taylor D. 2022. Production of the yolk protein precursor vitellogenin is mediated by target of rapamycin (TOR) in the soft tick Ornithodoros moubata (Acari: Argasidae). Insect Sci.
89. Raikhel AS. 1986. Lysosomes in the cessation of vitellogenin secretion by the mosquito fat body: selective degradation of Golgi complexes and secretory granules. Tissue Cell 18:125–142. https://doi.org/10.1016/0040-8166(86)90012-1.
90. Raikhel AS. 1986. Role of lysosomes in regulating of vitellogenin secretion in the mosquito fat body. J Insect Physiol 32:597–604. https://doi.org/10.1016/0022-1910(86)90088-0.
91. Katsir L, Zhepu R, Santos Garcia D, Piasseyz A, Jiang J, Sela N, Freilich S, Bahar O. 2018. Genome analysis of HaploType D of Candidatus Liberibacter Solanacearum. Front Microbiol 9:2933.
92. Hoffman CS, Winston F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene 57:267–272. https://doi.org/10.1016/0378-1119(87)90131-4.
93. Doyle JJ, Doyle JL, Doyle JA, Doyle FJ. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15.
94. Sarkar P, Kontsedalov S, Lebedev G, Ghanim M. 2021. The actin cytoskeleton mediates transmission of “Candidatus Liberibacter solanacearum” by the carrot psyllid. Appl Environ Microbiol 87:e02393-20. https://doi.org/10.1128/AEM.02393-20.
95. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402–408. https://doi.org/10.1006/meth.2001.1262.
96. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DJ, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH. 2015. CDD: NCBI’s conserved domain database. Nucleic Acids Res 43: D222–D226. https://doi.org/10.1093/nar/gku1221.
97. El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, Qureshi M, Richardson LJ, Salazar GA, Smart A, Sonnhammer ELL, Hirsh L, Paladin L, Piovesan D, Tosato SC, Finn RD. 2019. The Pfam protein families database in 2019. Nucleic Acids Res 47:D427–D432. https://doi.org/10.1093/nar/gkz995.
98. Yang J, Zhang Y. 2015. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res 43:W174–W181. https://doi.org/10.1093/nar/gkv342.
99. Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1876–1879. https://doi.org/10.1093/molbev/msw516.
100. Robert X, Gout P. 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res 42:W320–W324. https://doi.org/10.1093/nar/gkw316.
101. Waterhouse A, Bento M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, Schwede T, Schwede T. 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46:W296–W303. https://doi.org/10.1093/nar/gky427.
102. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, Hughes KA, Robinson GE. 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc Natl Acad Sci U S A 104:7128–7133. https://doi.org/10.1073/pnas.0701909104.
103. Wilson TG, Fabian J. 1986. A Drosophila melanogaster mutant resistant to a chemical analog of juvenile hormone. Dev Biol 118:190–201. https://doi.org/10.1016/0012-1606(86)90087-4.
104. Shemeshedi L, Wilson TG. 1990. Resistance to juvenile hormone and an insect growth regulator in Drosophila is associated with an altered cytosolic juvenile hormone-binding protein. Proc Natl Acad Sci U S A 87: 2027–2026. https://doi.org/10.1073/pnas.87.6.2027.
105. Kotaki T. 1996. Evidence for a new juvenile hormone in a stink bug, Pliata stali. J Insect Physiol 42:279–286. https://doi.org/10.1016/0022-1910(95)00113-1.
106. Bearfald JC, Henry AG, Tittger C, Blomquist GJ, Ginzel MD. 2009. Two regulatory mechanisms of monoterpenoid pheromone production in lps spp. of bark beetles. J Chem Ecol 35:689–697. https://doi.org/10.1007/s10886-009-9652-z.
107. Arredondo J, Ruiz L, López G, Pérez-Staples D, Díaz-Fleischer F. 2020. Female presence enhances sexual performance of sterile Anastrepha ludens males of the Tapachula-7 GSS strain. Entomol Exp Appl 168: 626–634. https://doi.org/10.1111/eea.12947.

108. Lin H, Lou B, Glynn JM, Doddapaneni H, Civerolo EL, Chen C, Duan Y, Zhou L, Vahling CM. 2011. The complete genome sequence of Candidatus Liberibacter solanacearum, the bacterium associated with potato zebra chip disease. PLoS One 6:e19135. https://doi.org/10.1371/journal.pone.0019135.

109. Bassan MM, Angelotti-Mendonc A JS, Alves GR, Yamamoto PT, Moura O Filho F. d A. 2017. Selection of reference genes for expression studies in Diaphorina citri (Hemiptera: Liviidae). J Econ Entomol 110:2623–2629. https://doi.org/10.1093/jee/tox253.

110. Ghosh S, Sela N, Kontsedalov S, Lebedev G, Haines LR, Murad G. 2020. An intranuclear sodalis-like symbiont and spiroplasma coinfect the carrot psyllid, Bactericera trigonica (Hemiptera, Psylloidea). Microorganisms 8:692. https://doi.org/10.3390/microorganisms8050692.

111. Munyaneza JE, Fisher TW, Sengoda VG, Garczynski SF, Nissinen A, Lemmetty A. 2010. Association of “Candidatus Liberibacter solanacearum” with the psyllid, Trioza apicalis (Hemiptera: Triozidae) in Europe. J Econ Entomol 103:1060–1070. https://doi.org/10.1603/ec10027.