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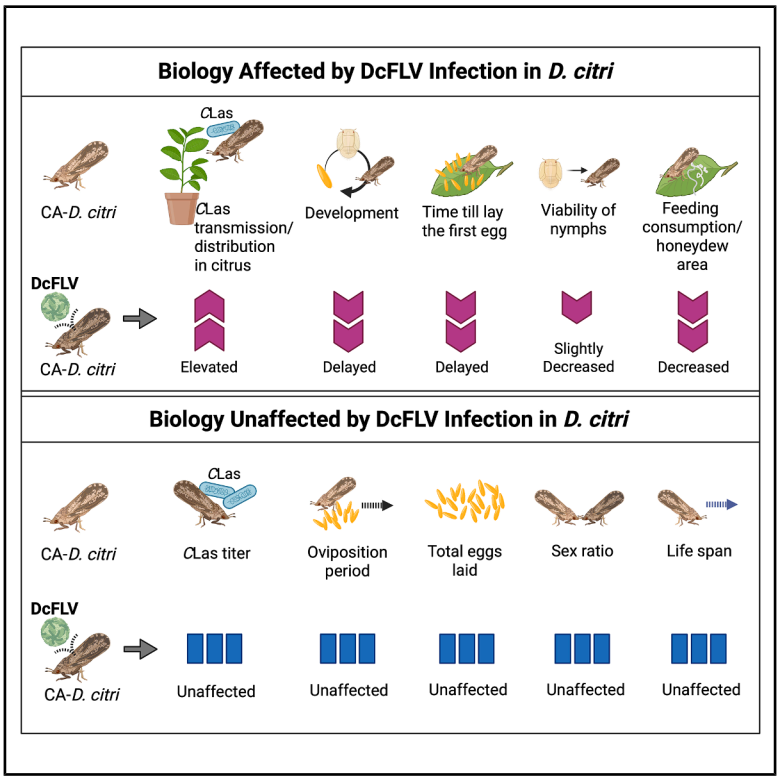
Graphical abstract



Highlights

- An insect-specific virus, DcFLV, delayed *Diaphorina citri* development
- DcFLV increased vector competence of *D. citri* for citrus pathogen *C*Las
- DcFLV delayed oviposition in female *D. citri*; total egg number unaffected
- DcFLV infection reduced feeding consumption in *D. citri*

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In brief

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Biological properties and vector competence of *Diaphorina citri* for *Candidatus* Liberibacter asiaticus modulated by an insect-specific virus

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SUMMARY

Insect-specific viruses (ISVs) infect only insects and have been studied primarily in mosquitoes. Here, we extend the concept of ISVs to include those found in plant-feeding insect vectors, such as *Diaphorina citri*, the vector of *Candidatus* Liberibacter asiaticus (*C*Las), the causal agent of citrus Huanglongbing. Using Diaphorina citri flavi-like virus (DcFLV) as a model, we examined its effects on host biology and *C*Las transmission. DcFLV infection delayed development and preoviposition and reduced feeding activity, whereas overall survival, oviposition, and sex ratio were unaffected. DcFLV-infected *D. citri* transmitted *C*Las with ~20% greater efficiency than uninfected controls, despite no significant difference in *C*Las titers. These findings suggest that ISVs can modulate the transmission dynamics of plant pathogens by insect vectors, adding complexity to vector-pathogen interactions.

INTRODUCTION

Citrus is one of the worlds' most important crops, but it is currently threatened by a devastating bacterial disease: Huanglongbing (HLB).[1] Of all the citrus-producing states in the United States affected by this disease, Florida experiences the most significant impact.[2] Since the disease was first discovered in Florida in 2005, the impact of HLB on citrus production has become increasingly severe, with yields decreasing by 74% in 2017–2018.[3,4] Yields have continued to decline steadily since then, with many groves experiencing substantial losses.[5] HLB is caused by a plant pathogenic bacterium, *Candidatus* Liberibacter asiaticus (*C*Las), which is transmitted by *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), the Asian citrus psyllid.[1] Previously, multiple viruses have been identified in various populations of *D. citri*.[6–8] Four of such viruses, Diaphorina citri picorna-like virus (DcPLV),[8] Diaphorina citri reovirus (DcRV),[9,10] Diaphorina citri densovirus (DcDV),[11] and Diaphorina citri flavi-like virus (DcFLV),[12] were shown to be vertically transmitted to the psyllid progeny. None of the viruses identified in *D. citri* have been identified in any plants or other insects and all studies to date indicate that the identified viruses specifically infect *D. citri*.[7,10–12]

DcFLV has previously been identified in Florida and China *D. citri* populations.[6,13] It shares genome organization and encoded protein similarity with other viruses in the family *Flaviviridae*. DcFLV has a positive-sense, single-stranded unsegmented RNA genome that encodes a polyprotein translated from a single open reading frame (ORF).[13] DcFLV is transmitted to the progeny through vertical transmission in *D. citri*.[12] Previous work also showed that DcFLV and *C*Las co-localized in midgut and salivary glands of *D. citri* and the co-occurrence of DcFLV and Diaphorina-citri-associated C virus (DcACV) with *C*Las in *D. citri* populations from Florida citrus groves.[12] However, there is no evidence showing if DcFLV affects the *D. citri* biology and/or its ability to transmit *C*Las to plant hosts. In this study, we report that DcFLV accumulates to different titers in different developmental stages and organs of *D. citri* and affected aspects of *D. citri* biology. More importantly, here we show that DcFLV enhances the vector competence of *D. citri* for transmitting *C*Las to plants. This is the first report showing that an insect-specific virus (ISV) modulates the biology of and plant pathogenic bacterial transmission efficiency by an insect vector.

ISVs were originally named for a group of mosquito insect viruses that only infect insects and are incapable of replicating in vertebrate cells.[14] The primary distinguishing feature of mosquito or phlebotomine sandfly ISVs is their inability to replicate in vertebrates, the hosts upon which the insects feed.[15] Most ISVs of mosquito belong to the RNA virus order *Bunyavirales* (negative (−) or ambisense single-stranded RNA [ssRNA]) or to the viral families *Flaviviridae* (positive (+) sense, ssRNA), *Reoviridae* (dsRNA), *Rhabdoviridae* (−ssRNA), and *Togaviridae* (+ssRNA). The main

mechanism of transmission and maintenance of the identified ISVs in mosquitos is thought to be vertical transmission. In this report, we extend the term ISVs to include viruses identified in insect vectors of plant diseases that can infect only the insect vectors and not plants in addition to those previously identified in mosquitoes. The biology of these viruses, specified as ISVs in prior studies, remains relatively underexplored but holds significant importance for understanding viral evolution and developing potential strategies to control insect vectors or plant diseases through the utilization or manipulation of ISVs. Recently, more ISVs have been identified in insect vectors other than mosquitoes, including the insect vectors transmitting plant diseases.[6,7] Although studies have shown that mosquito ISVs can affect mosquito vector competence for arboviruses such as dengue virus (DENV)[16] and West Nile virus (WNV),[17] only limited reports have explored whether ISVs in the insect vectors of plant diseases influence their biology or vector competence for plant pathogens. Here, we present additional evidence confirming that DcFLV is indeed an ISV and that it has significant effects on *D. citri* to transmit *C*Las to citrus plants.

RESULTS

DcFLV unable to infect citrus plants

To better understand DcFLV, we conducted assays to determine if DcFLV is indeed an ISV and thus unable to infect or replicate in citrus plants infested by *D. citri*. We first tested DcFLV transmission and infection in citrus plants using single psyllid with 2 days inoculation access period (IAP), leaving the psyllid on a citrus leaf for 2 days, for each repeat. Our results showed that the leaf tissues used for DcFLV-infected California *D. citri* (CA-*D. citri*) feeding assays were negative for DcFLV at all the tested time points (T0, D1, D3, D7, and D14) in our RT-qPCR assays (Figure S1). To further confirm that the negative results for DcFLV infection in citrus plants were not due to a low inoculum, we performed DcFLV transmission/infection experiments in citrus plants following the method used by Munster et al.,[18] using 10 DcFLV-infected psyllids with an 8-day IAP for each repeat. Our RT-qPCR assays consistently showed negative results for DcFLV in all feeding assays across all time points (Figure 1A). Negative DcFLV detection in leaf tissues at all time points indicated that DcFLV was not able to infect and replicate in the plant cells. This provides further confirming evidence that DcFLV is an ISV.

DcFLV infection delayed development, affected nymph viability but not sex ratio of *D. citri*

We conducted studies to investigate the influence of DcFLV infection on the biology of *D. citri*. Various assays were designed to assess different aspects of development and viability.

Our results showed that development time was significantly different between DcFLV-uninfected and -infected *D. citri* in all development stages. The egg hatching time from pairs of male and female *D. citri* (22 pairs from DcFLV-uninfected *D. citri* and 17 pairs from DcFLV-infected *D. citri*) was shorter in the DcFLV-uninfected *D. citri* than the DcFLV-infected *D. citri*, ranging from 4.25 to 5.43 days and 4.33 to 5.80 days, respectively (Figure 1B). The difference of the egg hatching time between DcFLV-uninfected and -infected was significant (unpaired t test; $p = 0.0079$). The egg hatching time of CA *D. citri* was significantly delayed by DcFLV infection. Moreover, the development time of nymphs to adults for DcFLV-uninfected *D. citri* ranged from 9.36 to 12.5 days and from 9.6 to 13.87 days in DcFLV-infected *D. citri* (Figure 1C). The difference of the development time of nymph to adult between DcFLV-uninfected and -infected was significant (unpaired t test; $p = 0.0118$). Consequently, the total development cycle (eggs to adults) duration was significantly (unpaired t test; $p = 0.0001$) shorter in the DcFLV-uninfected *D. citri* (overall average 15.39 days) compared to DcFLV-infected *D. citri* (overall average 16.38 days) (Figure 1D).

While our results showed that DcFLV infection delayed the development of *D. citri* at different stages, the viability of *D. citri* at different stages might not be affected. Therefore, we collected and analyzed the data of the viability of eggs, nymphs, and adults of DcFLV-uninfected and -infected *D. citri*. Our results showed that although the average viability of eggs (number of eggs hatched into nymphs) and eggs to adults (viability total) of DcFLV-uninfected and -infected *D. citri* resulted in no significant differences (Figures 2A and 2C), the difference between the viability of the nymphs (number of nymphs emerged into adults) of DcFLV-infected (17 biological repeats) and DcFLV-uninfected *D. citri* (22 biological repeats) was significant ($p = 0.0394$; unpaired t test; Figure 2B), 87.99% and 78.40%, respectively. Moreover, the infection of DcFLV had no discernible influence on the sex ratio of *D. citri* (Figure 2D); both DcFLV-uninfected and -infected *D. citri* had more female progeny than male progeny.

DcFLV infection delayed preoviposition period but not oviposition time, fecundity, survival, or longevity

The preoviposition period was delayed significantly in DcFLV-infected *D. citri* females. The average time for DcFLV-infected *D. citri* females to lay the first egg on *Citrus macrophylla* plants was 10.94 days, whereas the corresponding duration for DcFLV-uninfected *D. citri* females was 5.38 days (Figure 2E). However, no significant differences were observed in oviposition time (days of females laying eggs) and fecundity (eggs per female) averages during the lifespan of *D. citri* females. DcFLV-uninfected females had an average oviposition time of 24.8 days and laid an average of 1,017 eggs. DcFLV-infected females had an average oviposition time of 18.33 days and laid an average of 879.2 eggs. These averages were calculated from 18 DcFLV-uninfected and 18 DcFLV-infected *D. citri* females (unpaired t test; Figures 2 F and 2G).

The lifespan of both DcFLV-uninfected and -infected *D. citri* females was significantly shorter compared to the males (Figure 3; A and C: simple survival analysis—Kaplan-Meier; B and D: unpaired t test; $p < 0.0001$ for both DcFLV-uninfected and -infected). Therefore, the infection of DcFLV did not significantly affect the lifespan/longevity of the CA-*D. citri*. The lifespan/longevity of female was shorter than that of male with or without DcFLV infection. For instance, DcFLV-uninfected *D. citri* females died by day 75, whereas 86.6% of DcFLV-uninfected *D. citri* males were still alive at that point (Figure 3A). The average longevity of DcFLV-uninfected *D. citri* females and males were 36.37 and 93.63 days, respectively (Figure 3B). DcFLV-infected

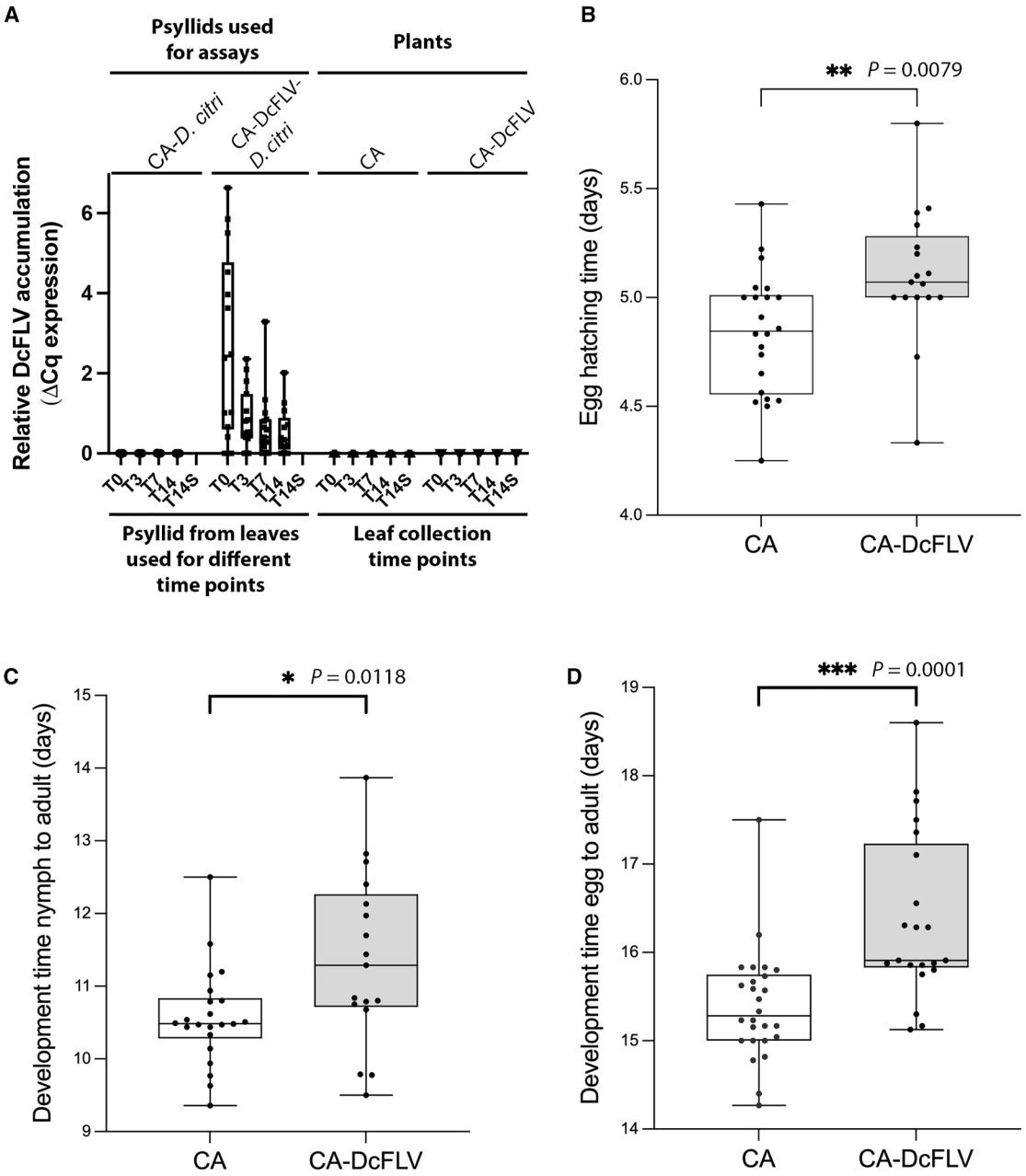


Figure 1. The infectivity of Diaphorina citri flavi-like virus (DcFLV) in citrus plants and the biological effects of the infection in *Diaphorina citri*

(A) DcFLV does not infect citrus plants but is only able to infect *Diaphorina citri* (Asian citrus psyllid) and gives delayed maturation development of *D. citri*. The *D. citri* used to feed on the *Citrus macrophylla* plants to test the DcFLV infectivity were all tested positive for DcFLV. None of the plants at any time points was tested positive for DcFLV. DcFLV-uninfected CA *D. citri* was used as negative controls. T0: 0 h after psyllid removal time; D3: 3 days after psyllid removal time; D7: 7 days after psyllid removal time; D14: 14 days after psyllid removal time; D14S: systemic leaf tissue collected at 14 days after psyllid removal time.

(B) Egg hatching time of CA *D. citri* was significantly delayed by DcFLV infection.

(C) The development time for nymph to adult stage of CA *D. citri* was delayed by DcFLV infection.

(D) The overall development time egg to adult stage of CA *D. citri* was significantly delayed by DcFLV infection. CA/CA-*D. citri*: *D. citri* originally collected from a California population. CA-DcFLV: California *D. citri* infected with DcFLV. *p = 0.0118; **p = 0.0079; ***p = 0.0001. The data were analyzed using two-tailed unpaired t-tests.

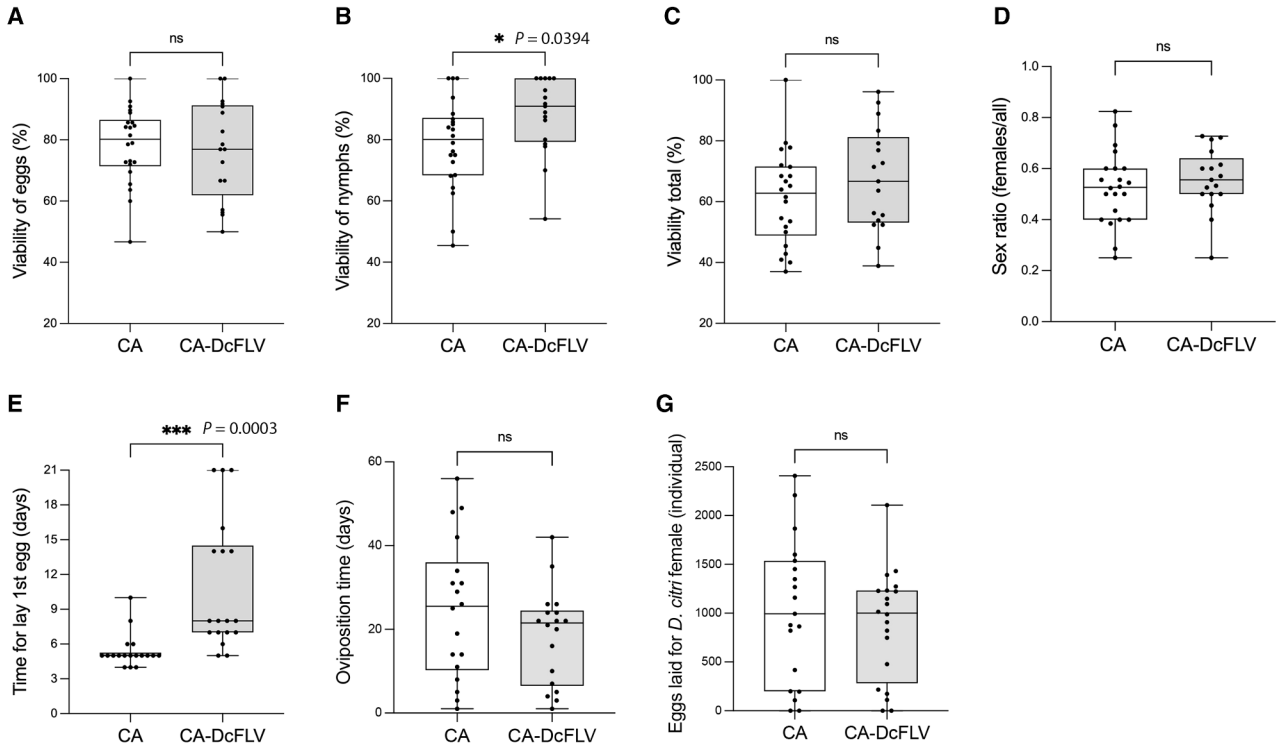


Figure 2. Effects of Diaphorina citri flavi-like virus (DcFLV) infection on the viability, sex ratio, preoviposition, oviposition time of, and total eggs laid by *Diaphorina citri*

(A–C) (A) The viability of *D. citri* eggs (the number of eggs hatch into nymphs) was not significantly affected, whereas (B) the viability of nymphs (the number of nymphs emerge into adults) was affected by DcFLV infection. However, (C) the overall viability (viability total, the number of eggs developed into adults) was not statistically significantly affected by DcFLV infection.

(D–G) (D) The sex ratio was not affected by DcFLV infection. Although the time for female *D. citri* to lay the first egg, the pre-oviposition time, (E) was significantly delayed, the oviposition time (F) and total eggs laid (G) by *D. citri* female were not affected. CA: *D. citri* originally collected from a California population. CA-DcFLV: California *D. citri* infected with DcFLV. *p = 0.0394; ***p = 0.0003. The data were analyzed using two-tailed unpaired t tests.

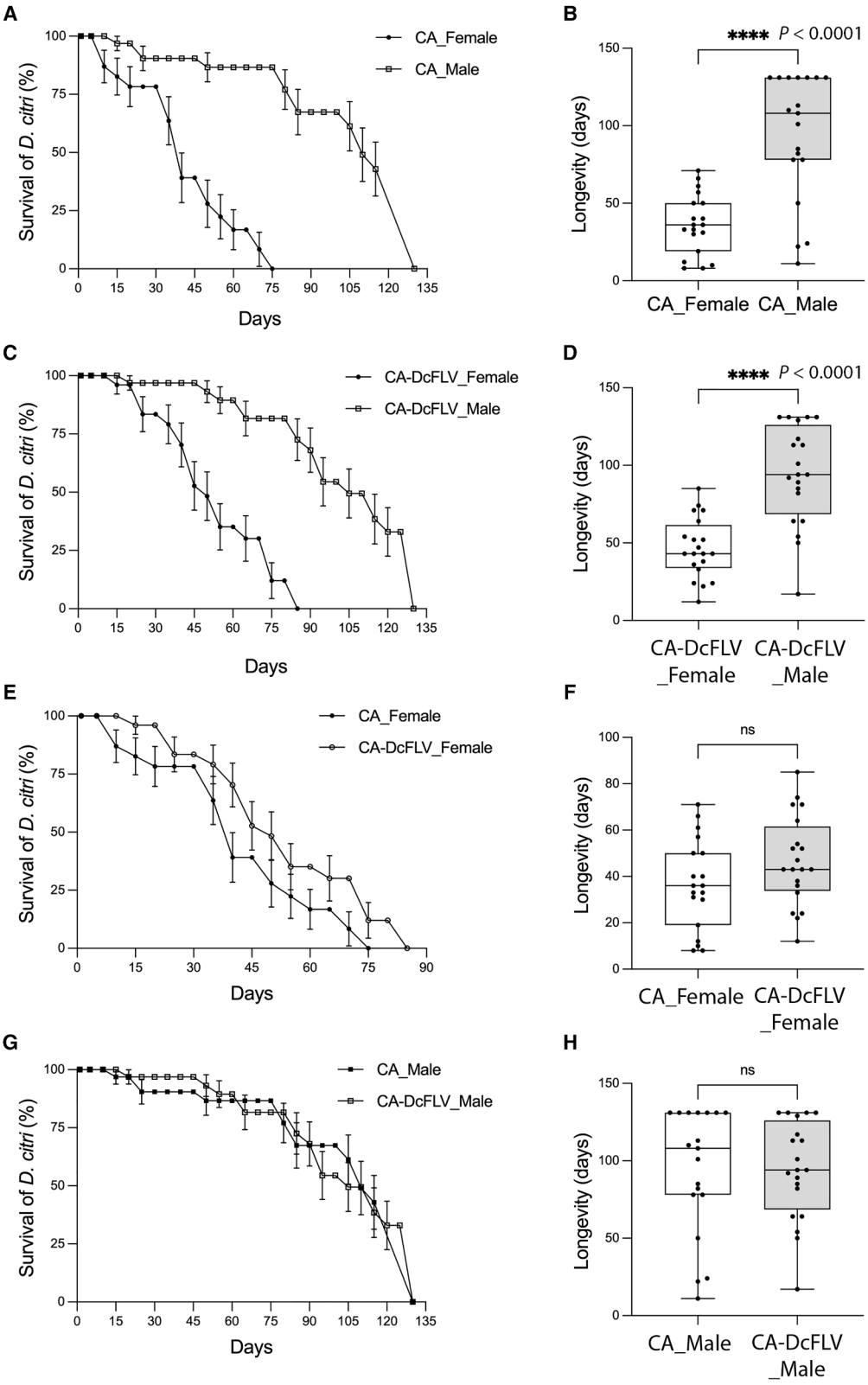
D. citri females died by day 85, whereas 72.57% of DcFLV-infected *D. citri* males remained alive (Figure 3C). Similarly, the average longevity of DcFLV-infected *D. citri* females was 46.55 days, whereas that of DcFLV-infected *D. citri* males was 94.10 days (Figure 3D). Nevertheless, no significant differences were exhibited between survival rates of DcFLV-uninfected and -infected *D. citri* females and males (simple survival analysis—Kaplan-Meier; Figures 3E and 3G). No statistically significant differences were observed in the longevity when DcFLV-uninfected and -infected *D. citri* females and males were compared (unpaired t test; Figures 3F and 3H). Overall, the longevity of DcFLV-uninfected and -infected *D. citri* females was significantly shorter than that of males, whereas there is no significant difference for the survival rate and longevity between the DcFLV-uninfected and -infected *D. citri*.

DcFLV infection affected feeding behavior of *D. citri*

To investigate the impact of DcFLV infection on the feeding behavior of California *D. citri* population, we quantified excreted honeydew produced while feeding on *C. macrophylla* plants for both DcFLV-infected and -uninfected *D. citri* (Figures S2A and S2B). The assessment was based on the measurement of the honeydew areas stained with ninhydrin on filter paper with 10 biological repeats (Figures S2C and S2D) and indicated that the DcFLV-uninfected *D. citri* had significant higher feeding consumption (unpaired t test; p = 0.0273; Figure 4A), ranging from 0.212 to 0.523 cm^2 of honeydew area, compared to the DcFLV-infected *D. citri*, ranging from 0.048 to 0.400 cm^2 of honeydew area (Figure 4A).

DcFLV genome accumulation level in different organs and developmental stages of *D. citri*

Different organs (Figure 4C), and developmental stages (Figure 4B) of DcFLV-infected *D. citri* were collected to determine the level of DcFLV genome. DcFLV accumulation level in midguts and heads, 3.40 and 3.34 log copy number.ng^{-1}, respectively, were significantly higher than those in ovaries (2.12 log copy number.ng^{-1}), testes (2.15 log copy number.ng^{-1}), and hemolymph (2.19 log copy number.ng^{-1}) (one-way ANOVA, multiple comparisons; Figure 4C). Furthermore, the DcFLV accumulation levels were significantly higher in 1 or 10 days post-emergence adult males and females (Figure 4B; my, fy, mm, fm), compared to other developmental stages except in eggs (Figure 4B). The average DcFLV accumulation level in eggs, first instar, second instar, third instar, fourth instar, fifth instar, and 10 days post-emergence males (mm) and



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females (fm) were 3.70, 3.39, 3.27, 2.83, 3.08, 3.25, 3.57, and 3.66 log copy number.ng^{-1}, respectively (Figure 4B). Groups sharing the same letter in Figures 4B and 4C are considered not statistically different from each other (one-way ANOVA, multiple comparisons; Table S1).

DcFLV infection did not affect *C*Las accumulation level but increased *C*Las transmission efficiency by *D. citri*

To investigate whether DcFLV modulated the vector competence of *D. citri* for *C*Las, we conducted transmission assays using DcFLV-infected and -uninfected *D. citri*. The day that the *D. citri* nymphs emerged into adults on the *C*Las-positive *C. macrophylla* plants, these newly emerged adults were transferred to healthy, *C*Las-free *C. macrophylla* plants for 7 or 14 days (Figure S5). Two age stages of *D. citri*, 7-day and 14-day post-emergence adult *D. citri* (7 and 14 days after the *D. citri* moved onto the healthy plants), were used for *C*Las transmission assays to individual healthy (*C*Las-free) *C. macrophylla* plants, one psyllid per plant. DcFLV-uninfected and -infected *D. citri*, from both ages, were moved to *C*Las-free *C. macrophylla* individually for 48-h inoculation access period (IAP). After the IAP, the *D. citri* were collected for analyses by qPCR for *C*Las (Figure S5). The *C*Las accumulation levels in DcFLV-uninfected and -infected *D. citri* in the same age stage, comparing both at 7-day or 14-day post-emergence, showed no significant differences (unpaired t test; Figure 4D). These data also indicated that the *C*Las acquisition percentage in DcFLV-uninfected and -infected *D. citri* from *C*Las-infected *C. macrophylla* plants was 100%. However, the *C*Las accumulation levels of DcFLV-infected and -uninfected *D. citri* at 14-day post-emergence were significantly higher, compared to those at 7 days post-emergence (one-way ANOVA—Kruskal-Wallis test; $p < 0.0001$; Figure 4D), indicating active infection and replication of *C*Las, suggesting that the accumulation level of *C*Las was not affected by DcFLV infection. We then tested the receptor *C. macrophylla* plants at 21 days, 2 months, and 6 months after the IAP (mo.a.i.). At 21 days post-IAP, the *C. macrophylla* leaves inoculated by *D. citri* during the IAP (top leaf, T) were tested for *C*Las (Table 1). Nine and twelve percent of *C. macrophylla* leaves inoculated by 7- and 14-day post-emergence DcFLV-uninfected *D. citri* were positive for *C*Las, whereas 0% and 12% of *C. macrophylla* leaves inoculated by 7- and 14-day post-emergence DcFLV-infected *D. citri* were positive for *C*Las (Table 1). Twenty-one days post-inoculation is very early for *C*Las infections to develop in plants; therefore, we next tested plants at 2 months after IAP to gain a more in-depth view of *C*Las transmission and subsequent infection of whole plants. We collected and tested *C. macrophylla* leaves that were distal from the inoculation site and thus not fed on by *D. citri*. These analyses showed that *C*Las detection for plants inoculated by the 7- and 14-day post-emergence DcFLV-uninfected *D. citri* was 18% and 25%, respectively. By comparison, the plants inoculated by the DcFLV-infected 7- and 14-day post-emergence *D. citri* were 14% and 44%, respectively (Table 1). We again tested these same plants at 6 months post-IAP to ensure that our analyses reflected transmission of *C*Las to plants, which resulted in systemic plant infection. The percentages of *C*Las-positive *C. macrophylla* inoculated by 7- and 14-day post-emergence DcFLV-uninfected *D. citri* were 41% and 68%, respectively, whereas those inoculated by 7- and 14-day post-emergence DcFLV-infected *D. citri* were 62% and 88%, respectively, at 6 mo.a.i (Table 1). Thus, these results showed that the *C*Las transmission efficiency was ~20% higher with DcFLV-infected *D. citri* than with DcFLV-uninfected *D. citri* for both 7- and 14-day post-emergence age stages of *D. citri*. The data were used for summary data graph, and the transmission results at 6 mo.a.i. showed statistically significant difference between DcFLV-infected and -uninfected *D. citri* vectored transmission using paired t test analysis (Figure 4E; $p = 0.0155$). Combining the *C*Las titer data of *D. citri*, our results showed that the difference in *C*Las transmission competency between DcFLV-uninfected and -infected individuals was not attributed to variations in *C*Las titer in the insect vector, *D. citri*. (Figure 4D; Table 1). The *C. macrophylla* plants used in the negative controls, inoculated by *C*Las-free *D. citri*, with and without DcFLV infection, remained *C*Las-negative throughout the assays (Table S2).

*C*Las distributed in more leaves when the plants inoculated by DcFLV-infected *Diaphorina citri*

We further analyzed the *C*Las distribution in the plants at the 2 mo. a.i. All the 18% *C*Las-positive plants inoculated by the 7-day post-emergence DcFLV-uninfected *D. citri* were with only one leaf tested positive with *C*Las (Figure 5E): 4.5% tested *C*Las positive in L1 leaf and ~13.5% tested positive in L3 (Figure 5A). Whereas, the 14% *C*Las-positive *C. macrophylla* plants inoculated by the DcFLV-infected 7-day post-emergence *D. citri* were with 1, 2, or 3 leaves tested positive with *C*Las per plant (Figure 5F): 14% out of the 14% were tested positive in L1, 5% out of the 14% were tested positive in L2, and 10% out of 14% were tested positive in L3 leaf (Figure 5B). The 25% *C*Las-positive *C. macrophylla* plants, inoculated by the DcFLV-uninfected 14-day post-emergence *D. citri*, were with 1 or 2 leaves tested positive with *C*Las

Figure 3. The survival rate and longevity of Diaphorina citri flavi-like virus (DcFLV)-uninfected and -infected *Diaphorina citri*

The survival rate and longevity of male *D. citri* were significantly higher than those of female psyllids for both DcFLV-uninfected and -infected *Diaphorina citri* (A–D). However, there were no significant differences of the survival rate and longevity between the DcFLV-uninfected and -infected California *D. citri* (E–H).

(A) Survival rate of DcFLV-uninfected California *D. citri* (female and male).

(B) Longevity of DcFLV-uninfected California *D. citri* (female and male).

(C) Survival rate of DcFLV-infected California *D. citri* (female and male).

(D) Longevity of DcFLV-infected California *D. citri* (female and male).

(E) Survival rate of DcFLV-uninfected and -infected California *D. citri* (female).

(F) Longevity of DcFLV-uninfected and -infected California *D. citri* (female).

(G) Survival rate of DcFLV-uninfected and -infected California *D. citri* (male).

(H) Longevity of DcFLV-uninfected and -infected California *D. citri* (male). CA: *D. citri* originally collected from a California population. CA-DcFLV: California *D. citri* infected with DcFLV. ****$p < 0.0001$; ns: not significant. The data were analyzed using two-tailed unpaired t tests.

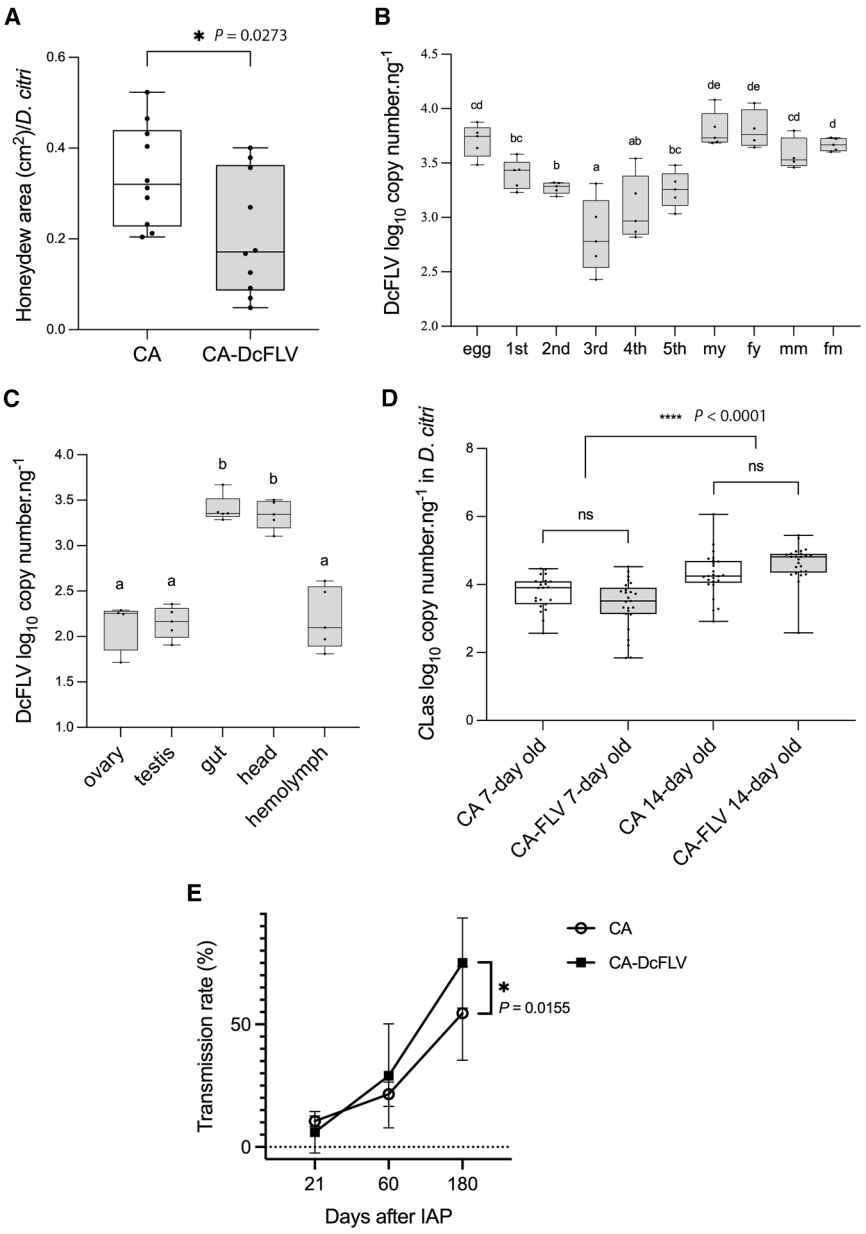

Figure 4. Higher titer of Diaphorina citri flavi-like virus (DcFLV) in adults and in guts and heads of *Diaphorina citri* while the *Candidatus* Liberibacter asiaticus (CLas) titers in both DcFLV-uninfected and -infected *D. citri* were not affected by DcFLV infection

(A) Feeding consumption was performed through the reactions of honeydew and ninhydrin on filter paper. The results showed that the feeding consumption of DcFLV-infected was significantly less than that of DcFLV-uninfected *D. citri*. CA: *D. citri* originally collected from a California population. CA-DcFLV: California *D. citri* infected with DcFLV. The data were analyzed using two-tailed unpaired t tests.

(B) DcFLV accumulation level Log per nanogram of total DNA extracted from single psyllid in different stages: egg, first instar (1st), second instar (2nd), third instar (3rd), fourth instar (4th), fifth instar (5th) of nymph, male young adults (my, one-day post emergence), female young adult (fy, one-day post emergence), male mature adult (mm, 10-day post emergence), and female mature adult (fm, 10-day post-emergence), in the *D. citri* life cycle.

(C) DcFLV accumulation level was found to be higher in California *D. citri* gut (including the Malpighian tubules) and head tissues, compared to the titers in ovary, testis, and hemolymph.

(D) *C*Las copy numbers in Log per nanogram of extracted total DNA in 7-day post-emergence adult California *D. citri* that were uninfected (CA 7-day-old) or infected with DcFLV (CA-FLV 7-day-old) and in 14-day post-emergence adult California *D. citri* that were uninfected (CA 14-day-old) or infected with DcFLV (CA-FLV 14-day-old). *C*Las titer in DcFLV-infected *D. citri* was not significantly different compared to that in the DcFLV-uninfected California *D. citri*. However, the *C*Las titer in 14-day post-emergence *D. citri* was significantly higher compared to that in 7-day post-emergence *D. citri*.

(E) The *Citrus macrophylla* plants used in the *C*Las transmission assays vectored by DcFLV-infected and -uninfected *D. citri* were detected at 21, 60 (2 months), and 180 (6 months) days after inoculation access period (IAP) (Table 1). The results at 180 d.a.i showed statistically significant difference ($p = 0.0155$; paired t test) between the transmission results of DcFLV-infected and -uninfected *D. citri* vectored transmission. Bars headed by different letters in (B and C) indicate statistically significant differences ($p < 0.05$) between different insect stages (B) and between different dissected organs (C). *$p = 0.0273$ (A); *$p = 0.0155$ (E); ****$p < 0.0001$.

per plant (Figure 5G): 8% out of the 25% were tested positive in L1, 12% out of the 25% were tested positive in L2, and 4% out of 25% were tested positive in L3 leaf (Figure 5C). Whereas, the 44% *C*Las-positive *C. macrophylla* plants, inoculated by the DcFLV-infected 14-day post-emergence *D. citri*, were with 1 (16%), 2 (8%), or 3 (20%) leaves tested positive with *C*Las per plant (Figure 5H): 36% out of the 44% were tested positive in L1, 24% out of the 44% were tested positive in L2, and 32% out of the 44% were tested positive in L3 leaf (Figure 5D). These results showed that only the plants inoculated by DcFLV-infected *D. citri*, 7-day post-emergence and 14-day post-emergence, were with *C*Las positive in three leaves (L1, L2, and L3; Figures 5F and 5H) in a single plant. Additionally, the number of the *C*Las-positive L1 and L3 leaves (Figures 5C and 5D; Table S2) was statically significantly higher (chi-squared test; L1: $p = 0.0169$, L3: $p = 0.0100$) when transmitted by 14-day post-emergence DcFLV-infected *D. citri* compared to 14-day post-emergence DcFLV-uninfected *D. citri*. When comparing the distribution of *C*Las inoculated by 14-day post-emergence DcFLV-uninfected and -infected *D. citri* (Figures 5G and 5H), the spread of *C*Las was also statistically significantly higher when transmitted by DcFLV-infected *D. citri* ($p = 0.0041$, chi-squared test). Although the distribution of *C*Las in the inoculated *C. macrophylla* plants was enhanced, with more leaves tested positive with *C*Las per plant,

Table 1. Proportion of *Candidatus* Liberibacter asiaticus (CLas)-positive *Citrus macrophylla* fed by 7- and 14-day post emergence DcFLV-uninfected (CA) and DcFLV-infected CLas-positive *D. citri* (CA + DcFLV)

	7-day post-emergence[a]			14-day post-emergence[a]		
D. citri	21 d.a.i.[b]	2 mo.a.i.[c]	6 mo.a.i.	21 d.a.i.	2 mo.a.i.	6 mo.a.i.
CA[d]	2/22 (9%)	4/22 (18%)	9/22 (41%)	3/25 (12%)	5/25 (25%)	17/25 (68%)
CA-DcFLV[e]	0/21 (0%)	3/21 (14%)	13/21 (62%)	3/25 (12%)	11/25 (44%)	21/24 (88%)

[a]Number of CLas-positive *C. macrophylla*/number tested.
[b]d.a.i.: days after inoculation access period (IAP).
[c]mo.a.i.: months after IAP.
[d]CA: *D. citri* originally collected from a California population.
[e]CA-DcFLV: California *D. citri* infected with DcFLV.

the overall CLas titer was not significantly higher in the plants inoculated by DcFLV-infected *D. citri* (Table S3).

Psyllids used in the studies harbored the same endosymbionts except for DcFLV

To ensure that DcFLV-uninfected and -infected California *D. citri* reared in the CRF facility harbored the same endosymbionts, except for DcFLV, we performed detections of other known *D. citri*-associated viruses and bacteria, including DcDV, DcRV, DcPLV, DcACV, *Wolbachia pipientis*, *Candidatus* Profftella armatura, and *Candidatus* Carsonella ruddii, using RT-qPCR or qPCR.[9,11,19,20] The TW *D. citri* harboring DcDV, DcRV, and DcPLV was used as positive controls for the detection of these three viruses, whereas DcACV-infected CA *D. citri* was used as the positive controls for DcACV detection. The primers and probes used for these detections are listed in

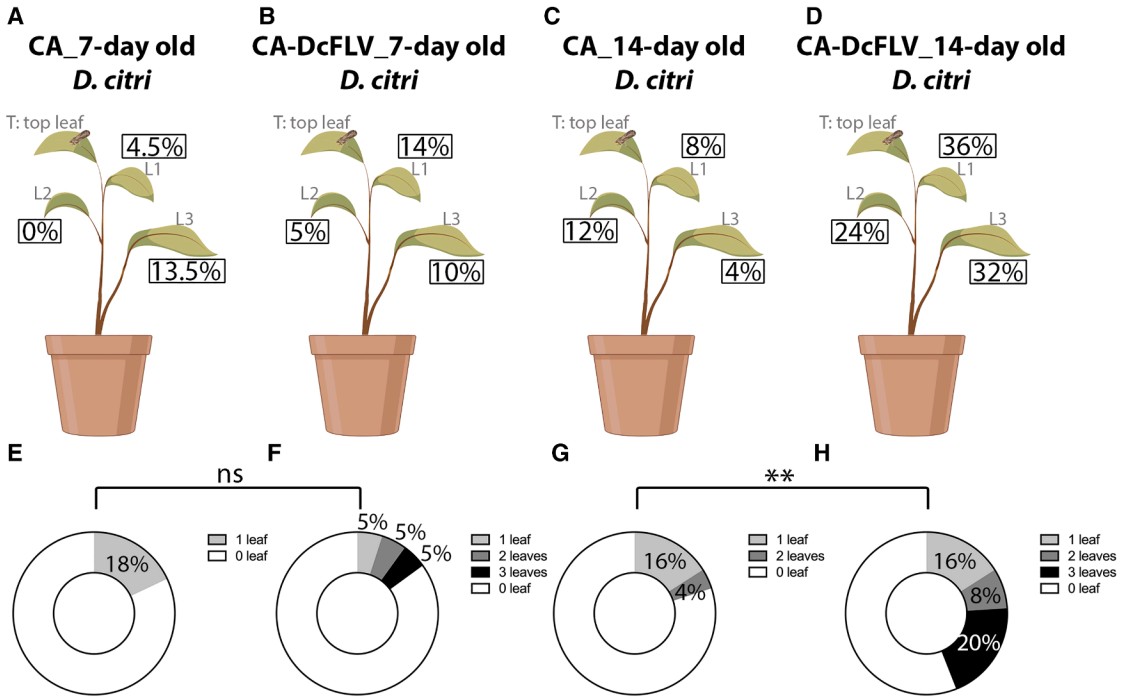

Figure 5. *Candidatus* Liberibacter asiaticus (CLas) was detected in more leaves in a single plant when plants were inoculated by Diaphorina citri flavi-like virus (DcFLV)-infected *Diaphorina citri* at 2-month post-inoculation access period (IAP) in transmission assays

(A–D) Percentage of leaves at different position infected by CLas. Individual DcFLV-infected and -uninfected *D. citri* that were infected by CLas was placed on the top leaf (T) of each plant, total of 21–25 plants for each assay, for 48 h (IAP). The first leaf down from leaf T (L1), the second leaf down from leaf T (L2), and the third leaf down from leaf T (L3) were tested for CLas infection.

(E–H) The percentages of plants infected with 0, 1, 2, or 3 leaves were analyzed. Only the plants inoculated by 7-day post-emergence and 14-day post-emergence DcFLV-infected *D. citri* showed plants with all three leaves (L1, L2, and L3) infected by CLas at 2-month post-IAP but not the plants inoculated by DcFLV-uninfected *D. citri*. (A and E): plants inoculated by California 7-day post-emergence DcFLV-uninfected *D. citri*; (B and F) plants inoculated by California 7-day post-emergence DcFLV-infected *D. citri*; (C and G) plants inoculated by California 14-day post-emergence DcFLV-uninfected *D. citri*; (D and H) plants inoculated by California 14-day post-emergence DcFLV-infected *D. citri*. Statistical comparison showed significantly greater CLas spread in DcFLV-infected *D. citri* ($p = 0.0041$, chi-squared test); ns, not significant. The cartoon images of plants in figures (A–D) were adopted from BioRender stock images and created in Adobe Photoshop.

Table 2. Primers and probes used in quantitative polymerase chain reaction (PCR) and/or reverse transcription PCR

Primer name	Forward (5′-3′)	Reverse (5′-3′)	Probe (5′-3′)
RT-qPCR			
DcFLV	GGAGATGCTGGGATTGCTATAA	CCTGCACTTATCCGGTCAATA	ACTGCTGTTCCAACTACACCGGAC
DcRV	CAGCATATCGTGAAGCAGTATC	TGCTTTATGCATCTTTTCAA TATAAGAATG	TCATGTTCGGTGTGGCATAGGGTT
DcPLV	CATGTATAATTGGGATGCAT GTAGCGGG	GAGTACGACATCGACAA TACGCCCTTT	TCATCGGGTATTCAGA GCCTATATTCCAGG
DcACV	AGCAGATCACTCCACGTTTG	CGAGATACCTGTCGTGGTAGTA	TAACATCTCGGGTGTGACGTGAGC
Actin	GACTCCGGAGATGGTGTCT	GCCAGATCCAGACGGAGGAT	CCATCTATGAAGGTTACGCCCTTCCC
qPCR			
CLas RNR β-subunit nrdB	CATGCTCCATGAAGCTACCC	GGAGCATTTAACCCCACGAA	CCTCGAAATCGCCTATGCAC
Carsonella	ATGGGAACGCCATATGCTAATA	CCAATGGGTTGTTCATCCTCTA	ACAAGACTATGATCCGTAGTTGGTCT
Profftella	CCTCATGGCCTTTATGGGTAG	CCGGACTACGATGCACTTT	TCGCCAATCTGCAAAGAGGAGCTA
Wolbachia	CTTTGCTGGAGCTCGTTACT	CAGCYTCTGCACCAACA	TGATAAGGCAGCTAAGGATGATACTGGT
DcDV	TGTGTAACCGACAAGCGAAC	CGACAACGGAGAACCTACAAC	ACGCCGATAAAGTTGCGCAACAGCA
Actin	TCGTGACATCAAGGAG AAGCTGTGC	TTGACCGTCGGGAAGTTC GTAGGAT	TCGCCCTGGACTTTGAACAGGAAA
COX	CGTCGCATTCCAGATTATCCA	CAACTAATATATAAGRRCCRRAAC	AGGGCATTCCATCCAGCGTAAGCA

Table 2. Our results showed that all DcFLV-uninfected and -infected *D. citri* were positive for *Wolbachia*, Profftella, and Carsonella with comparable level, but negative for DcDV, DcRV, DcPLV, and DcACV (Figure S4). Both groups of *D. citri* (DcFLV-infected and -uninfected) consistently carried the symbiotic bacteria *Wolbachia*, Profftella, and Carsonella. The abundance or load of these bacteria was similar between the two groups, indicating no detectable differences in the levels of these symbionts regardless of DcFLV infection status. This confirms that the DcFLV-uninfected and -infected *D. citri* used in our studies harbored the same endosymbiont background.

DISCUSSION

Recently several reports showed that certain ISVs could modulate vector transmission competence of arboviruses to their vertebrate hosts.[16,17,21–23] With the advancement of next-generation sequencing, ISVs have also been recently identified in plant-feeding insects[6,24]; nevertheless, very little is known about these ISVs in the insect vectors transmitting plant pathogens. Here, we report that DcFLV, one of the identified ISVs in *D. citri*, affected specific biological features of *D. citri*, but also increased vector competence in DcFLV-infected *D. citri* compared to uninfected *D. citri*.

DcFLV-infected *D. citri* required more time to complete their development, and this could indicate an energy cost related to DcFLV infection resulted in negative development fitness. Furthermore, this delay in development may have occurred as a result of the lower food consumption observed in DcFLV-infected *D. citri*. Generally, the difference in the food consumption of phloem-sucking insects is mainly due to differences in the quality and quantity of nutrients available from different host plant species or their varieties, affecting, for example, the insect development time.[25] A recent study showed morphological abnormalities in the nuclear structure in the DcFLV-infected *D. citri* gut cells,[26] which could potentially be one of the factors responsible for lower food consumption. Surprisingly, DcFLV-infected *D. citri* showed slightly higher viability during the nymphal stage, indicating that, despite reduced food consumption and longer development time, DcFLV did not negatively affect insects viability. Although DcFLV-infected *D. citri* females exhibited a longer preoviposition period compared to DcFLV-uninfected *D. citri* females, the fecundity was similar in both populations, suggesting that the presence of DcFLV does not significantly impact the oogenesis of *D. citri*. Some reports have shown the effect of ISVs in other insect vectors of plant pathogens. For instance, higher levels of ABV-1 (Aphid bunyavirus) reduced the development time of nymphs and increased the reproduction of the aphid *Acyrthosiphon pisum*.[27] However, Acyrthosiphon pisum virus (APV) significantly reduced the growth of the aphid and increased the time required to reach maturity.[28] Moreover, Aphis citricidus picornavirus (AcPV) altered the stylet penetration activity of brown citrus aphid, resulting in reduced phloem ingestion in citrus seedlings.[29]

Our studies also compared the DcFLV titer in different organs and hemolymph and all developmental stages of *D. citri*. The levels of DcFLV detected in the head and midgut were higher than in other organs, such as ovary, testis, and hemolymph, in *D. citri* (Figure 4C). Similarly, higher DcPLV titers were identified in the salivary glands and gut of *D. citri* from a China population.[8] Our data showed that the DcFLV accumulation levels were higher in the one-day post-emergence of *C*Las-free *D. citri* (male and female; 10 pooled *D. citri* per sample, 5 samples) adults compared to those in *C*Las-free nymphs (pooled 50 first instar, 40 second instar, 30 third instar, 20 fourth instar, and 10 fifth instar per sample, 5 samples), which differs from a previous report, showing no statistically significant difference of DcFLV titer between *C*Las-free nymphs and adults.[12] Further investigation is required for the correlations between the DcFLV titer pre- and post-*C*Las infection in *D. citri* and *C*Las transmission competency.

Our results showed that DcFLV-infected *D. citri* showed a greater transmission efficiency, compared to uninfected *D. citri*, of *C*Las, the bacterial pathogen of the citrus disease, HLB. This is the first report showing that an ISV can modulate vector competence of a plant-feeding insect vector of a plant pathogen.

Various factors, such as the capacity of insects to be infected, carry, maintain, and transmit pathogenic viruses or bacteria, can contribute to vector competence,[30,31] and recently the role(s) of ISVs in vector competence have been increasingly recognized. It was reported that the ISV, Palm Creek virus, could regulate the infection of West Nile virus (WNV) in the mosquito, *Culex annulirostris*, and hence affected the transmission of WNV to vertebrate hosts.[21] Two other mosquito-specific ISVs, Phasi Charoen-like virus (PCLV) and Humaita Tubiacanga virus (HTV) were reported to increase arbovirus replication in mosquitoes, shortening the extrinsic incubation period (EIP) of ZIKV.[16] Our results revealed that the overall titer of *C*Las showed no significant differences between the DcFLV-uninfected and -infected *D. citri* (Figure 4D). This indicates that the observed increase in the *C*Las transmission efficiency among DcFLV-infected *D. citri* was not attributed to higher titers of *C*Las, but rather to other, as-yet-unknown effects of DcFLV infection. Additionally, our analysis of feeding behavior indicated reduced consumption by DcFLV-infected *D. citri*, which could potentially contribute to the delayed preoviposition period observed in female DcFLV-infected *D. citri*.

It has been conjectured that ISVs could induce antiviral immune responses and potentially serve as a strategy for controlling disease pathogen transmission. However, little is known about the immune responses triggered by ISVs in the insect vectors transmitting plant pathogens. Our results showed that DcFLV titer levels in midguts and heads were higher than in other organs, such as ovary, testis, and hemolymph in *D. citri* (Figure 4C). In addition to influencing feeding behavior and pathogen spread, insect immune responses could also affect vector competence. Evidence showed that AcPV could induce RNAi-based immunity in brown citrus aphids.[29] However, genes associated with antiviral pathways, Toll, IMD, Jak-STAT, autophagy, and RNA interference, were not found to be affected by the two ISVs, Humaita Tubiacanga virus (HTV) and PCLV, that infected *Aedes Aegypti*, which were reported to increase the ability of mosquitoes to transmit the arboviruses, DENV and ZIKV. Nevertheless, HTV and PCLV were found to specifically affect histone H4 expression, which was identified as an important pro-viral host factor *in vivo*.[16] Therefore, distinct ISVs within various insect hosts may elicit specific responses in their respective hosts. The involvement of various factors, including those associated in *C*Las spread and immune response in *D. citri*[32–34] could contribute to modulating *C*Las transmission.

Although several previous reports showed some effects and interactions between *C*Las and *D. citri*,[35–38] in this study, we present novel findings in the interactions/effects between *C*Las, *D. citri*, and DcFLV, demonstrating that the ISV, DcFLV, has the ability to modulate both the biology of the Asian citrus psyllid, *D. citri*, and the transmission of a pathogenic bacterium in citrus. This represents the first documented instance of such modulation in an insect associated with agriculture.

Limitations of the study

There are two main limitations in our study. First, the effects of DcFLV infection in *D. citri* were assessed only in the California population, due to the unavailability of virus-free *D. citri* populations from Taiwan and Uruguay in our facility. Second, we reared *D. citri* on a single host plant species, *C. macrophylla*, propagated from branch cuttings. It remains unclear whether similar conclusions would be reached for *D. citri* reared on other host plants. Future studies involving more diverse *D. citri* populations and host plants will be necessary to further refine and expand upon these findings.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Yen-Wen Kuo (ywkuo@ucdavis.edu).

Materials availability

Insect populations are available to be distributed to other institutions, subject to applicable USDA-APHIS shipping regulations and approval from the recipient institution's biosafety committee.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

D.M.G. designed and performed research, analyzed data, and wrote the manuscript. T.R. performed parts of research, analyzed data, and wrote a section of material and methods. W.I. performed parts of research. C.R.C. assisted in the design and early experiments of this work. G.R.A. assisted in the design and performed parts of this work. Y.-W.K. designed and performed parts of research, analyzed data, and wrote the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Bacterial strain and *D. citri* rearing

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Candidatus Liberibacter asiaticus	Hacienda Heights (HHCA) strain	N/A
Candidatus Carsonella ruddii	From California *D. citri* population	N/A
Candidatus Profftella armatura	From California *D. citri* population	N/A
Wolbachia pipientis	From California *D. citri* population	N/A
Diaphorina citri flavi-like virus	Florida isolate	GenBank: KX267823.1
Diaphorina citri picorna-like virus	Taiwan isolate	GenBank: KT698837.1
Diaphorina citri densovirus	Taiwan isolate	GenBank: KX165268.1
Diaphorina citri reovirus	Hawaii isolate	NCBI Taxonomy ID: 557218
Diaphorina citri-associated C virus	California isolate	NCBI Taxonomy ID: 1776154
Critical commercial assays		
Trizol Reagent	Thermo Fisher Scientific Inc.	Cat# 15596018
Trizol LS Reagent	Thermo Fisher Scientific Inc.	Cat# 10296028
RNeasy Plant Mini Kit	Qiagen	Cat# 74904
iQ™ Multiplex Powermix	Bio-Rad Laboratories™	Cat #1725849
Experimental models: Organisms/strains		
Diaphorina citri Kuwayama (Hemiptera: Liviidae)	California	N/A
Citrus macrophylla	Contained Research Facility, University of California-Davis	N/A
Oligonucleotides		
DcFLV (Table 2)	This paper, Matsumura et al.[13]	GenBank: KX267823.1
DcRV (Table 2)	This paper, Chen et al.[9]	NCBI Taxonomy ID: 557218
DcPLV (Table 2)	This paper, Nouri et al.[6]	GenBank: KT698837.1
DcACV (Table 2)	This paper	NCBI Taxonomy ID: 1776154
DcDV (Table 2)	This paper	GenBank: KX165268.1
Carsonella (Table 2)	This paper, Roldan et al.[20]	GenBank: AF211136.1
Profftella (Table 2)	This paper, Roldan et al.[20]	GenBank: MN928703.1
Wolbachia (Table 2)	This paper, Roldan et al.[20]	GenBank: KC539848.1 GQ385974.1 OP902291.1 KX198666.1 AF217721.1 KY472731.1
CLas RNR β-subunit nrdB (qPCR, Table 2)	This paper, Zheng et al.[39]	GenBank: JMIL00000000.2
Actin (qPCR, Table 2)	This paper	NCBI Ref. Seq.: XM_026821238.1
Actin (RT-qPCR, Table 2)	This paper	NCBI Ref. Seq.: XM_026821238.1
COX (Table 2)	Hughes et al.,[40] Kuo et al.[41]	N/A
Software and algorithms		
QUANT 1.0.1.	Alves et al.[42]	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strain and *D. citri* rearing

The Hacienda Heights (HHCA) strain of CLas[43] was used in this study. *D. citri* insects used in this study were reared in the Contained Research Facility (CRF) of University of California, Davis (UC Davis).[44,45] The insects were reared on *Citrus macrophylla* plants inside

mesh cages kept in a greenhouse at 27 ± 2°C under 14:10 (L:D) h photoperiod and 40 ± 10% relative humidity (RH). Two different *D. citri* populations were used in this study: DcFLV-infected and DcFLV-uninfected (DcFLV-free) California *D. citri*. DcFLV virions were originally partially purified, concentrated and filtered from naturally infected Florida *D. citri*. The virion preparation was micro-injected into California virus-free *D. citri*. The population originating from those injected CA-*D. citri* is 100% infected with DcFLV.

METHOD DETAILS

DcFLV infectivity assays in citrus plants

Two colonies of the California population of *Diaphorina citri* were used in the assays: DcFLV-uninfected CA-*D. citri* as the control, and DcFLV-infected CA-*D. citri*. Both colonies were synchronized for the age and stage of *D. citri*. The *D. citri* were left to mate for 5 days on a *Citrus macrophylla* plant inside mesh cages. After 5-day mating period, the *D. citri* were transferred to a fresh *C. macrophylla* plant inside mesh cages and left to lay eggs for 1 day and then all the adults were removed. The eggs were left to hatch and collected after the emergence of adults. The adults at 4 days post-emergence were used for the assay. Adult *D. citri* were picked up and checked under a dissecting microscope to determine their sex. One male and one female *D. citri* was put on individual leaves of one *Citrus macrophylla* plant with each leaf enclosed by a small mesh bag for the single psyllid assays. Five male and 5 female *D. citri* were used for the ten-psyllid assays.[18] After 48 h (single-psyllid assays) or 8 days (ten-psyllid assays) for inoculation access period (IAP), all the *D. citri* were collected, and this time point was marked as T0. Five repeats (5 pairs (single-psyllid assays) or 25 pairs (ten-psyllid assays) of *D. citri*, 5 plants) for each time point: time 0 h after psyllids removal (T0), 3 days after removal (D3), 7 days after removal (D7), and 14 days after removal (D14). The leaves the *D. citri* fed on were collected at their respective time points. The nearest leaf subsequent to the feeding leaf of the male or female psyllids was also collected on day 14 (D14S) to monitor viral movement in the citrus plants. The same experimental steps were done for both DcFLV-uninfected and -infected CA *D. citri*.

Total RNA extraction for virus detection

Individual or 3 pooled (for the ten-psyllid DcFLV citrus transmission/infection assays) psyllids were collected and placed into 2 mL screwcap tubes with 1.0 mm zirconia/silica beads (BioSpec Products Inc, Bartlesville, OK, USA). Psyllids were ground using a mini-beatbeater (Biospec) and extracted with TRIzol Reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. For the citrus plant RNA extraction, the individual leaves were collected and their midrib was cut into smaller pieces using a razor blade and placed into 2mL screwcap tubes with 2.3mm chrome steel beads (BioSpec Products Inc, Bartlesville, OK, USA). Guanidine Thiocyanate buffer (4M) was added to the tubes and ground using a mini-beadbeater. The total RNA of the *Citrus macrophylla* leaves was extracted using RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's protocol.

Biology of DcFLV-infected and control CA-*D. citri*

In the first assay, the development time, viability, and sex ratio of *D. citri* were evaluated. For this, a pair of 10-day-old adult *D. citri* was released on *C. macrophylla* plants with young flush shoots in a mesh sleeve cage until the female laid eggs. The female psyllid was allowed to lay eggs for one day (from 10 to 30 eggs) and then the pair of *D. citri* was removed, and the eggs were counted with a stereoscopic microscope. The hatched nymphs and dead nymphs were counted daily until the emergence of adults. In the second assay, the preoviposition period, the time of oviposition and total eggs laid on *C. macrophylla* plants by *D. citri* female, and the longevity of *D. citri* males and females were evaluated. For this assay, C. *macrophylla* branches with young flush shoots were cut and placed in a Falcon tube (50 mL) containing water and a lid with a hole for keeping the plant cutting in place. Those cuttings were confined in mesh cages. A pair of *D. citri* were observed daily, and the preoviposition period was determined from the first egg laid by the female. To determine the time of oviposition, the number of eggs laid by females was counted every other day and the females were transferred to new C. *macrophylla* cuttings with young flush shoots placed in Falcon tubes until the females stopped laying eggs. To evaluate the longevity and survival of tested *D. citri*, the pairs of *D. citri* were kept on the cuttings, observed daily until they died. Both assays were maintained in a climate-controlled greenhouse at a temperature of 27 ± 2°C, 40 ± 10% RH and a photoperiod of 14:10 (L:D) h. Statistical significance was analyzed using unpaired t-test.

Feeding behavior/consumption assays

We assessed *D. citri* feeding behavior following the technique for measuring honeydew excretion based on using ninhydrin assays on *D. citri* honeydew excreta.[46] The assays were done using mesh-ventilated transparent polypropylene plastic cups with lids that covered with filter papers (9 cm in diameter). The *C. macrophylla* cuttings were placed through the lid with the leaves and 6 *D. citri* (10 days-post-emergence) enclosed in an upside-down plastic cup. The filter paper was designed to catch honeydew produced from *D. citri* (Figures S1A and S1B). The *D. citri* were left on the plant for three days and the paper filters (discs) were removed to determine the amount of honeydew produced from the *D. citri* (Figure S1C). The filter paper discs are immersed in pure acetone + ninhydrin solution (198 mL + 2 g) for 3 min. After 24 h drying in room temperature (Figure S1D). The stained area was quantified in the software

QUANT 1.0.1.[42] Ten *C. macrophylla* cuttings were used for each *D. citri* population. The assay was performed in a climate-controlled greenhouse at a temperature of 27 ± 2°C, 40 ± 10% RH and a photoperiod of 14:10 (L:D) h.

Analyses of DcFLV titer in *D. citri*

Ten-day-post-emergence adult *D. citri* was collected from DcFLV-infected and DcFLV-uninfected *D. citri* populations/colonies that has been maintained on *C. macrophylla* plants in a greenhouse in the CRF. The collected *D. citri* adults were dissected in phosphate buffer (1× PBS) solution under a stereomicroscope (at 10×) to isolate ovary (Figure S2A), testis (Figure S2B), midgut (including the Malpighian tubules) (Figure S2C), and head (Figure S2D). RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, USA), following the manufacturer's protocol, from 10 pooled dissected midguts (including the Malpighian tubules), 10 ovaries, 10 testes and 10 heads. TRIzol LS (Thermo Fisher Scientific Inc., Waltham, USA) was used to extract RNA from hemolymph from 10 pooled *D. citri* following the manufacturer's protocol. RNA was also extracted from 100 pooled eggs, nymphs (50 first instar, 40 s instar, 30 third instar, 20 fourth instar, and 10 fifth instar), and adult males (10 pooled from each stage) and females (10 pooled from each stage) that were one day post-emergence and 10 days post-emergence. The RNA was resuspended in RNase-and DNase-free water and used for cDNA synthesis. Five biological replicates were analyzed from all treatments. Statistical significance was analyzed by one-way ANOVA, multiple comparisons.

cDNA synthesis and quantitative RT-qPCR

The cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, USA) following the manufacturer's instructions. The cDNA was used in quantitative reverse transcription-PCR (RT-qPCR) for quantifying DcFLV using iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) following manufacturer's instructions. The primer sequences (Table 2) were design using RealTime qPCR Assay platform (https://www.idtdna.com/primerquest/Home/Index) and *D. citri actin* was used as an internal control. The standard curve and quantification of DcFLV were generated by serial diluting of a recombinant plasmid containing DcFLV fragment (pCR-XL-2-TOPO-DcFLV-frag4).

CLas transmission assays

The CLas-negative DcFLV-infected and DcFLV-uninfected CA-*D.citri* populations (both previously mated) were placed into two different cages containing CLas-infected *C. macrophylla* plants. After 48hs, all *D. citri* were removed, and the eggs were maintained on the plants. On the day of emergence, *D. citri* adults were transferred to healthy (CLas-free) *C. macrophylla*. Two transmission assays with 2 different age stages were done for both DcFLV-infected and DcFLV-uninfected *D. citri* (Figure S5; total of 4 assays: DcFLV-infected-1, DcFLV-infected-2, DcFLV-uninfected-1, and DcFLV-uninfected-2). Assay 1 was done using the adult psyllids that were left on the CLas-free *C. macrophylla* for 7 and 14 days for assay 2. *D. citri* of each age group were individually transferred to CLas-free *C. macrophylla* plantlets, containing four leaves-T: top leaf, L1: the first leaf down from the top leaf; L2: the second leaf down from the top leaf; L3: the third leaf down from the top leaf. A single psyllid was confined on the plant top leaf (leaf T) in a mesh sleeve on each *C. macrophylla* plant for 48h for inoculation access period (IAP). Both transmission assays were done with 25 psyllid-plant replicates. Total DNA of the psyllids used in the transmission assays was extracted individually following the Dellaporta extraction method.[47] The extracts were then used for the CLas titer/copy number analyses by qPCR. Twenty-one days post inoculation (dpi), the midrib of the leaf T, where psyllids fed for 48 h, from each *C. macrophylla* were collected, and homogenized in Dellaporta buffer, using Mini-Beadbeater-96 (BioSpec), with steel beads (2.5mm) in the sample tubes. The second collection was done at two months post inoculation (mpi), during which leaves L1, L2 and L3 were individually collected and tested. Three leaves from the new growth were pooled for the collection 3 at 6 mpi. All the assays were performed in a climate-controlled greenhouse at a temperature of 27 ± 2°C, 40 ± 10% RH and a photoperiod of 14:10 (L:D) hr.

DNA extraction and qPCR

Total DNA of *D. citri* and midribs *C. macrophylla* leaves samples was extracted following Dellaporta extraction method.[47] The total DNA of leaf midribs was resuspended in 200 μL of nuclease-free water, while the total DNA of individual *D. citri* was resuspended in 20 μL of nuclease-free water.

CLas detection and quantification of both plant and *D. citri* samples was done using qPCR analyses using iQ™ Miltiplex Poermix (Bio Rad). Primers and fluorogenic probe of CLas *RNR β-subunit nrdB*[39] and *D. citri actin* were used for *D. citri* samples. Primers and fluorogenic probe of CLas *RNR β-subunit nrdB* and *mitochondrial cytochrome oxidase* (*COX*)[40,41] were used for plant samples. (Table 2). DcDV detection and quantification of *D. citri* samples were performed using iQ Miltiplex Powermix (Bio Rad) with DcDV and *D. citri actin* primers and probes (Table 2). *D. citri actin* and *C. macrophylla COX* were used as internal controls for *D. citri*, DcDV, and *C. macrophylla* leaves samples. The amplifications reactions were performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad™). The standard curve and quantification of CLas were generated by diluting a recombinant plasmid containing a CLas *RNR β-subunit nrdB* fragment in pGEM®-T vector (Promega).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data shown in Figures 1, 2, 3, and 4A were analyzed using two-tailed unpaired t-tests. Data in Figures 4B and 4C were analyzed using ordinary one-way ANOVA. Data in Figure 4D were analyzed using the Kruskal–Wallis test. Results in Figure 4E were analyzed using a paired t-test. Data in Figures 5E–5H were analyzed using the Chi-square test. All analyses were performed using GraphPad Prism (version 10.3.0 (461)), with statistical significance defined as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. Bars headed by different letters in Figure 4 section b and c indicate statistically significant differences ($p < 0.05$).