Titer Variation of Citrus Tristeza Virus in Aphids at Different Acquisition Access Periods and Its Association with Transmission Efficiency

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Abstract

Tristeza, caused by citrus tristeza virus (CTV; *Closterovirus, Closteroviridae*), is of significant economic importance. Tristeza epidemics have caused severe declines in productivity, and even death, of millions of citrus trees on sour orange rootstock in many regions all over the world. In the field, CTV is most efficiently vectored by the brown citrus aphid (*Toxoptera citricida* (Kirkaldy)) in a semipersistent manner. The transmission efficiency of the vector is influenced by its acquisition access period (AAP) for CTV. A real-time RT-PCR assay using SYBR Green fluorescent dye was used to estimate the CTV titers in groups of 15 aphids under AAPs after 0.5 to 48 h for three CTV isolates (CT11A, CT16-2, and CTLJ). Similar trends for CTV titer in viruliferous aphids were displayed for the three isolates. The maximum CTV titer was at AAP 6 h for isolates CT11A and CT16-2,

and at 4 h for isolate CTLJ. During the AAPs from 0.5 to 6 h, the mean CTV titer of CT16-2 increased from 7.8×10^4 to 1.71×10^7 copies per 15 aphids, and was correlated with an increase in transmission rate from 20 to 90.9%. This suggests that the transmission efficiency is positively correlated with viral titer in the insect from 0.5 h until 6 h AAPs. While a downward trend in CTV titer was observed after a 6-h AAP, the transmission rate remained higher than 90% up to 48 h. These results indicate that factors other than the virus titer in the vector contribute to successful transmission under long acquisition conditions. This is the first detailed quantitative analysis of CTV in its main vector species following different AAPs and its association with transmission efficiency, and should enhance our understanding of *T. citricida*-CTV interactions.

Citrus tristeza virus (CTV) isolates are members of *Closterovirus* genus (*Closteroviridae*) (Bar-Joseph et al. 1989; Cook et al. 2016; Karasev et al. 1995) that cause detrimental viral disease of citrus worldwide. One important disease is the rapid decline of trees on sour orange rootstock (Moreno et al. 2008; Roy et al. 2013), and another important disease is stem-pitting in susceptible scions, which occurs regardless of rootstock (Atta et al. 2012; Dawson et al. 2013). The CTV genome is approximately 20 kb of single-stranded positive-sense RNA (gRNA) encoding 12 open reading frames (ORFs) (Karasev et al. 1995). A high level of sequence conservation exists among isolates toward the 3' end, while sequence nearer the 5' terminus of the genome can be up to 40% divergent (Hilf et al. 2005). The two 5' proximal ORFs (1a and 1b) are directly translated from the gRNA, while 10 ORFs downstream, encoding the p33, p6, p65, p61, p27, p25, p18, p13, p20, and p23 proteins, are expressed through subgenomic RNAs (sgRNAs) (Karasev et al. 1997). Moreover, p25 and p27 are two coat proteins that encapsidate about 97% and 3% of the gRNA, respectively (Febres et al. 2004; Satyanarayana et al. 2004).

The virus is graft-transmitted, and several aphid species vector CTV in a semipersistent manner (Cambra et al. 2000; Killiny et al. 2016). Therefore, assessing the interactions between the main vector and CTV has become an important avenue of research into the epidemiology of the disease. Factors affecting aphid transmission include the genotypes of the CTV isolates or strains, aphid species, plant donors, receptor varieties, environmental conditions, and the number of aphids (Roistacher and Bar-Joseph 1987; Sharma 1989). *Toxoptera citricida* (Kirkaldy) is the most efficient vector of CTV compared

with other aphids that feed on citrus and can acquire the virus (Yokomi and Damsteegt 1991; Yokomi et al. 1994). Moreover, Sharma (1989) associated higher transmission rates with the symptomatically more severe strains as compared with the more mild strains. The high incidence of aphids carrying CTV is consistent with the high CTV prevalence and proliferation in the field (Marroquín et al. 2004). Yokomi and Damsteegt (1991) suggested that a minimum of 15 viruliferous aphids/plant were required for 100% transmission of CTV after 24 h of acquisition and inoculation. Moreover, the acquisition or infection feeding periods by insect vectors can also affect transmission efficiency (Bar-Joseph et al. 1977; Roistacher and Bar-Joseph 1987). Costa and Grant (1951) showed that a 4-h acquisition feeding period was requisite for maximum transmission by *T. citricida*. However, *Aphis gossypii* transmitted CTV after 30 min of feeding, thereafter showing moderate transmission efficiency levels after 2 h, and then near-maximal transmission efficiency after 6 h (Racchah et al. 1976).

The detection and quantitative determination of the number of virions carried by aphids provides a better understanding of the epidemiology of this disease (Wosula et al. 2013). The detection of viruses in aphid vectors has been achieved using PCR-based assays, either using RT-PCR or RT-nested PCR (Mehta et al. 1997; Olmos et al. 1999). Real-time RT-PCR (RT-qPCR) assays using TaqMan probes have been used for the detection of CTV in infected aphids (Bertolini et al. 2008; Saponari et al. 2008), as real-time quantitative PCR offers higher sensitivity than conventional PCR (Bustin 2000).

Various studies have attempted to estimate the number of viral particles carried in aphids which show nonpersistent transmission of the virus following specific acquisition periods (Betancourt et al. 2008; Moury et al. 2007). However, compared with viruses with nonpersistent transmission relationships, viruses transmitted in a semipersistent manner are just the aphid transmission characteristics. Although the main features of CTV transmission are well known, little is known regarding the number of virus particles carried in aphids after different acquisition access periods (AAPs). Moreover, the relationship between the CTV transmission rate and the number of virions carried by the aphids is not well understood.

In the present study, a real-time RT-PCR assay using SYBR Green fluorescent dye was used to quantify CTV in aphids after 10 access periods between 0.5 and 48 h. The relative transmission efficiencies

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among the different acquisition access times were studied. The objective was to determine the relationships between the number of viral targets acquired by the aphids, the transmission efficiencies, and the duration of the feeding period. The differences in CTV targets detected at the different access periods could contribute to our understanding of *T. citricida*–CTV interactions.

Materials and Methods

Virus isolates. The three CTV isolates used in this study were CT11A, CT16-2, and CTLJ, which were initially obtained with different single aphid transmissions. The three isolates are considered mild isolates that induce no apparent symptoms in several hosts, including ‘Symons’ sweet orange (*Citrus sinensis* L.), ‘Fenghuang’ pummelo (*C. grandis*), and ‘Duncan’ grapefruit (*C. paradisi*). According to the classification scheme of Gillings et al. (1993), CT11A and CT16-2 are classified in group III based on the results of a restriction fragment length polymorphism (RFLP) analysis of coat protein genes digested with the restriction enzyme *Hinf*I (CPG/*Hinf*I RFLP group III), while CTLJ belongs to CPG/*Hinf*I RFLP group IV. According to the classification scheme of Hilf et al. (2005), isolate CT11A and CT16-2 are not assigned a standard genotype, while CTLJ has a T30 genotype. An analysis of the completed genome sequence of CT11A (GenBank Accession No. JQ911664) indicated it is closely related to isolate T318A (GenBank Accession No. DQ151548, 98.9% identity), but is distantly related to the poorly transmissible isolate T36 (GenBank Accession No. U16034, 80.2% identity). Isolates on ‘Jincheng’ sweet orange (*C. sinensis* L.) were collected from the Citrus Research Institute, Southwest University / Chinese Academy of Agricultural Sciences. The viruses were graft-inoculated to Jincheng sweet orange (*C. sinensis* L.) seedlings and were kept in an insect-proof screen house for 5 years.

Aphid species. *T. citricida* colonies were established as reported by Broadbent et al. (1996) and reared on virus-free sweet orange plants in insectaries under controlled conditions (25°C and a photoperiod of 16/8 h, light/dark).

Aphid transmission. Jincheng seedlings about 1 1/2 years old were used as donor and receptor plants. These plants were pruned to stimulate regrowth, and the aphids were placed on new flush in insect-proof cages. All transmission experiments were repeated three times.

In the single aphid transmission assays of isolates CT11A, CT16-2, and CTLJ, apterous adult aphids were transferred to virus source plants for a 24-h AAP, followed by a 24-h inoculation access period (IAP) on the healthy seedlings. Thirty receptor plants were used per isolate for each replicated experiment.

After feeding aphids on donor plants infected with the CT16-2 isolate for 24 h, either 1, 3, 5, 10, 15, or 25 aphids were released onto young flushes of each CTV-free sweet orange plant for a 24-h IAP to determine the effect of aphid number on transmission. Thirty receptor plants were used for each aphid group in each replicated experiment.

For testing the relationship between acquisition access period and CTV infection in the sweet orange plants, thousands of nonviruliferous aphids were placed on plants infected with the CT16-2 isolate for 0.5, 1, 2, 3, 4, 6, 9, 12, 24, and 48 h. At the completion of each feeding period, 375 aphids were directly transferred onto 25 healthy sweet orange seedlings with 15 aphids on each plant for each acquisition period and allowed to feed for a 24-h IAP.

After the 24-h IAP, the receptor plants were treated with insecticide and transferred to an insect-proof greenhouse maintained at 18 to 25°C. The incidence of CTV infection resulting from aphid transmission was determined 4 m post-IAP by a Direct Tissue Blot Immunoassay (DTBIA), using a commercially available CTV polyclonal antibody (Agdia, USA) (Lin et al. 2006).

Sample preparation and RNA extraction. To quantify the titers of the three isolates following AAPs of 0.5, 1, 2, 3, 4, 6, 9, 12, 24, and 48 h, the apterae of the adult aphids were placed on CTV-infected Jincheng seedlings (1 1/2 years old). Following each AAP, virus was quantified from three groups of 15 aphids. Individuals in each group of 15 aphids were randomly collected from several

CTV-infected sweet orange plants from each feeding period. This experiment was repeated three times.

For quantitation of CTV in individual aphids, 60 adult aphid apterae were allowed to feed on plants infected with the CT16-2 isolate for a 24-h AAP. Virus content was then determined by real-time RT-PCR immediately, or insects were preserved at –80°C until analysis.

Total RNA was extracted from the aphid samples using a TransZol up Kit (TransGen Biotech, Beijing, China) according to the manufacturer’s protocol. The purified RNA was resuspended in 20 µl of RNase-free water. The RNA extracted from *T. citricida* adults that were maintained on CTV-free plants was used as a virus-free control.

Primers designed for PCR. HD-F/HD-R primers were designed from the sequence within a highly conserved region of the *p25* ORF. A CTV-specific 136-bp nucleotide sequence was amplified by RT-PCR using the HD-F/HD-R primers. The CTV nucleotide sequences used for alignment were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), European Bioinformatics Institute (EMBL; <https://www.ebi.ac.uk/>), and DNA Data Bank of Japan (DDBJ; <https://www.ddbj.nig.ac.jp>) databases. Primer Express software (Beacon Designer 7, Premier Biosoft, USA) was used to obtain the optimal oligo and probe sequences. LD-T7-F/LD-R primers which flank the HD-F/HD-R primer sequences on the CTV genome were designed for standard curve. Primer LD-T7-F constitutes a modified version of the forward primer LD-F that included the T7-promoter sequence at its 5’ end (Table 1).

Standard curve. A CTV-specific nucleotide sequence of 399 bp, 373 nucleotides of the CTV target +26 nucleotides of the T7 promoter and protective bases, was amplified by RT-PCR using the LD-T7-F/LD-R primers. The purified products were used as targets in an in vitro transcription reaction using T7 RNA polymerase (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. The transcripts were digested at 37°C for 30 min with RNase-free DNase I (Promega, Madison, WI, USA). The RNA concentration of each preparation was determined using a UV-vis spectrophotometer (SmartSpec plus, Bio-Rad), and 10-fold serial dilutions containing 9.0×10^7 to 9.0×10^1 RNA copies were made. Aliquots of these dilutions were stored at –80°C until use. The number of copies of CTV-RNA was estimated as previously described (Ruiz-Ruiz et al. 2007).

Real-time RT-PCR. To optimize the real-time RT-PCR protocol, the annealing temperature from 53°C to 63°C and primer concentrations of 800 nM, 600 nM, 500 nM, 400 nM, 300 nM, and 200 nM were tested. Following optimization, real-time RT-PCR was performed in a 20-µl reaction volume that contained 1× SYBR Premix Ex Taq II (TAKARA, Japan), 0.6 µM primer of HD-F, 0.6 µM primer of HD-R (Table 1), and 2 µl of cDNA. The PCR was performed with a preincubation at 42°C for 30 min and denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 15 s, and an extension at 72°C for 20 s/cycle. A melting curve analysis was done immediately after the final PCR cycle to determine the specificity of the reaction by incubating it at 95°C for 15 s, annealing at 65°C for 30 s, and slowly increasing the temperature to 95°C. Data acquisition and analysis were performed with the iCyclerIQ Real-Time PCR Detection System (Bio-Rad, USA). For each amplification run, standards were loaded on the same plate to build the appropriate standard curve.

Table 1. Primers used in real-time RT-PCR to detect citrus tristeza virus

Primers	Primer sequence 5’-3’	Product size (bp)
HD-F	CGAGTCTGGGGTAGAAGTAACGA	136
HD-R	GGTCAAGAATCTGCACACAGG	
LD-T7-F ^z	GATCACTAATACGACTCACTATAGGG TCATTGCAAAGTGACGACGAC	
LD-R	TGCTGACATTGGTAACTACG	399

^z Primer LD-R and primer LD-T7-F containing 26 nucleotide bases of the T7 promoter were used to establish the standard curves.

Statistical analysis. Statistical analysis was performed using the statistics software SPSS12.0 (SPSS Inc., USA). The parameters measured on the quantitative CTV levels in *T. citricida* and transmission rate were calculated using Analysis of Variance (ANOVA) and Duncan's multiple comparisons at the $P < 0.05$ level of significance. Correlation analysis was performed using Microsoft Excel (Microsoft Corporation, Albuquerque, NM, USA).

Results

Effects of aphid number on the transmission of CTV. After 24 h of acquisition and inoculation with three replicates, the single aphid mean transmission rates of the different CTV isolates were 65.6, 15.6, and 4.4% for CT11A, CT16-2, and CTLJ, respectively. Significant differences were found in the single aphid transmission efficiency of the three CTV isolates tested ($P < 0.05$) (Fig. 1). Moreover, mean transmission rates of 15.6, 46.7, 68.9, 81.1, 92.2, and 96.7% were obtained with 1, 3, 5, 10, 15, and 25 aphids per test plant for the CT16-2 isolate, respectively. Significant differences were observed between the transmission rates of the different aphid number tested except between 15 and 25 aphids per test plant ($P < 0.05$) (Fig. 2).

Real-time RT-PCR protocol optimization. Different cycling conditions were tested to optimize the real-time RT-PCR. The primer set (HD-F/HD-R) yielded the most sensitive detection for all CTV isolates when the annealing temperature was 60°C by providing the lowest Ct values. Of the concentrations tested, the results indicated that 600 nM was the optimal concentration for these primers (data not shown), as it had the lowest Ct value and a high level of amplification efficiency (104.7%). The melting curve provided by the iCycler iQ software showed a single peak in all cases, and no melting curve was obtained from the nonviruliferous aphids (data not shown), which further confirmed the absence of significant amounts of nonspecific amplification products.

Detection of CTV in individual aphids. We used the real-time RT-PCR assay to detect the CTV RNA copy numbers in 60 individual aphids that had fed for 24 h on plants infected with isolate CT16-2. The results showed that after 24 h, 100% of the aphids had acquired CTV. The amounts ranged from 2.5×10^3 to 1.24×10^6 copies with 91.7% of the individual aphids carrying 10^4 – 10^5 copies. No CTV RNA was detected in the control group of the aphids feeding on non-infected citrus plants (data not shown).

Quantification of viral targets in *T. citricida* after different acquisition times on different CTV isolates. The quantification of CTV targets acquired in *T. citricida* after different acquisition periods is shown in Table 2. Similar trends were observed for the CTV titer in 15 aphids of each of the three isolates after 0.5–48 h of acquisition. For the shortest period (0.5 h AAP), the mean number of CTV targets quantified was the lowest, estimated to be 2.08×10^5 , 7.80×10^4 , and

5.95×10^4 copies for CT11A, CT16-2, and CTLJ, respectively, in the aphids. Maximum viral targets were observed at 6 h for CT11A and CT16-2, and at 4 h for CTLJ. The highest mean values were 2.32×10^7 , 1.71×10^7 , and 9.38×10^6 copies for CT11A, CT16-2, and CTLJ, respectively. The copy numbers were lower for all three isolates at the 9, 12, and 24 h times. The copy number increased again at 48 h compared with 24 h for CT11A and CT16-2. Both CT11A and CT16-2 showed significant differences from the other AAPs at 6 h, whereas CTLJ was significantly different from the other AAPs at 4 h in the mean number of CTV targets (Table 2).

The CTV titer in aphids which acquired the highly transmissible isolate CT11A were all higher than those for the less transmissible isolates CT16-2 and CTLJ after AAPs of 0.5 h to 48 h, except at 12 h for CTLJ. Overall, the mean number of CTV targets in the 15 aphids which acquired CT11A for 1 h was nearly 10 times that for CT16-2 (Table 2).

Correlation analysis of CTV target titer and transmission rate after different acquisition periods. The mean transmission rate and mean number of CTV targets quantified increased with the acquisition access period from 0.5 h to 6 h for the CT16-2 isolate. However, there was no significant difference in mean CTV genome copy number for AAPs 0.5 to 4 h, even when significantly higher rates of transmission for AAPs of 2, 3, 4, and 6 h was found. From 0.5 until 6 h of acquisition time, the transmission rate ranged between 20 and 90.9%, and the CTV titer was between 7.80×10^4 and 1.71×10^7 copies per 15 aphids. Moreover, statistically significant correlation between the mean CTV titer and mean transmission rate during this period was found at $P < 0.01$ ($R = 0.9150$). The transmission rates were over 90% after the 6-h AAP, reaching 100% at 9- and 12-h AAPs for the CT16-2 isolate. However, a downward trend in CTV titer was observed after the 6-h AAP. Overall, the transmission rates from 0.5 to 48 h of acquisition showed a weak but not statistically significant correlation with the titer of the CTV targets of the CT16-2 isolate ($R = 0.4780$) (Table 3).

Discussion

Real-time RT-PCR has been widely used in the detection and quantification of specific viruses during the last few years (Moreno et al. 2009; Olmos et al. 2005). A real-time RT-PCR assay using SYBR Green was developed for the quantitative detection of CTV in infected plants by Ruiz-Ruiz et al. (2007). The primer set PM197F/PM198R, designed from conserved sequences in ORFs 1b and 2, was used to amplify the genomic RNA in these assays. In the present study, a real-time RT-PCR was used to quantify CTV copies in the aphid vector *T. citricida*. In this system, a set of primers HD-F/HD-R targeting a conserved sequence of *p25*, the gene of the coat protein, was designed and used. In the individual aphids,

Fig. 1. Variability in transmission of different citrus tristeza virus isolates by single *Toxoptera citricida*. Transmissibility is the percentage of plants infected. Error bars indicate standard deviations among three replicated experiments. Analysis of variance with Duncan's multiple comparisons was done at the significance level of $P < 0.05$. The same letter indicates no significant difference at $P < 0.05$.

Fig. 2. The effects of *Toxoptera citricida* numbers on the transmission of the CT16-2 isolate. Transmissibility is the percentage of plants infected. Error bars indicate standard deviations among three replicated experiments. Analysis of variance with Duncan's multiple comparisons was done at the significance level of $P < 0.05$. The same letter indicates no significant difference at $P < 0.05$.

the number of CTV copies ranged from 2.5×10^3 to 1.24×10^6 (with only one of 60 aphids carrying more than 10^6 copies, data not shown), which is similar to the result of Bertolini et al. (2008) using TaqMan probes. In their assay, between 4.73×10^3 and 1.23×10^5 CTV copies were carried by individual *A. gossypii*. Our results also confirm that the real-time technique can be used to detect CTV in plant tissues (data not shown).

We quantified the number of CTV copies in *T. citricida* at 10 acquisition periods ranging from 0.5 to 48 h postinfection. Bertolini et al. (2008) quantified the number of acquired CTV targets from adult *A. gossypii* that had been released on detached CTV-infected leaves after only three acquisition access periods (1, 24, and 48 h). Nevertheless, there is no information regarding CTV in *A. gossypii* for acquisition times between 1 h and 24 h. In the aforementioned study, CTV could be acquired after a short access period (1 h) on an infected plant, but high rates of acquisition were only achieved when the aphids fed for a long time (from 24 to 48 h) (Bertolini et al. 2008). Our results showed that the quantity of CTV in a group of 15 aphids that fed on plants infected with three different CTV isolates showed the highest copies at AAP 6 h for CT11A and CT16-2 or AAP 4 h for CTLJ. The quantity of CTV acquired gradually increased with increasing acquisition times up to 6 h, and then decreased after 6 h AAP for CT11A and CT16-2 or 4 h AAP for CTLJ. The quantity of CTV acquired by aphids depends on vector activity, particularly aphid feeding behavior. The duration of phloem feeding was found to be most strongly correlated with viral acquisition (Jiang et al. 2000). Using an electrical penetration graph technique, Carmo-Sousa et al. (2014) reported a sharp change in aphid probing behavior over time when exposed to plants infected with cucumber mosaic virus (CMV), which is transmitted in a nonpersistent manner. They found that at the second hour of recording (a later stage), aphids spent much less time in phloem salivation and ingestion (E1 and E2) on CMV-infected plants than on mock-inoculated plants, indicating that the aphids reduced their feeding on CMV-infected plants. It is possible that virus vector stylet activity can promote changes in the plant volatile emission profile of infected plants, thereby mediating the feeding behaviors of the insect vector (Mauck et al. 2012). If such is the case for CTV, *T. citricida* might decrease its feeding time, thereby causing a decline in CTV accumulation after 6 h. In addition, CTV might induce an immune response from the aphids which results in a decline in CTV accumulation (Cassone et al. 2014).

The percentage of viruliferous migrant aphids is a major factor influencing virus epidemiology (Betancourt et al. 2008; Moreno et al. 2009). In this study, differences in CTV titers in aphids were observed at different access periods. However, little is known about the influence of the number of CTV particles acquired by aphids on transmission efficiency. In this study, we found that the quantity of CTV isolate CT16-2 acquired by 15 aphids was higher than 7.80×10^4 genomes, with a transmission efficiency of only about 20%, which is similar to a transmission efficiency of 15.6% caused by 91.7% of individual aphids carrying 10^4 – 10^5 CTV copies for

CT16-2. However, a transmission efficiency of 20% was reported for a single aphid with an average of 6,529 RNA targets of the non-persistently transmitted plum pox virus (PPV) (Moreno et al. 2009). Moreover, the quantities of virus targets detected in a single aphid were found to range from 40 to more than 2×10^3 targets for PPV (Olmos et al. 2005), while for CTV this ranged from 2.5×10^3 to 1.24×10^6 targets.

The mean number of CTV targets quantified was the lowest (7.80×10^4) for the shortest acquisition period (0.5 h) of CT16-2, but this indicated that *T. citricida* could acquire a significant amount of virus within 30 min, a result similar to what was observed in similar research by Raccach et al. (1976) and Bar-Joseph et al. (1989). Likewise, Limburg et al. (1997) suggested that beet yellow virus (BYV), another *Closterovirus*, is acquired by aphids during the phloem ingestion phase within a minimum time period (20 min) (Limburg et al. 1997). However, high CTV transmission rates were achieved only when the aphids fed for at least 4 or 6 h (Costa and Grant 1951; Raccach et al. 1976). Furthermore, BYV was found to possess maximum transmission efficiency at 6 h after acquisition, and no differences were observed at 6, 12, and 24 h of acquisition (Limburg et al. 1997). In our study, at an acquisition period of 6 h, the mean number of CTV targets quantified (1.71×10^7) was the highest for CT16-2. The transmission rate increased to more than 90% as the acquisition time was increased to 6 h. After 6 h of acquisition, a downward trend was observed in the CTV titer, but the transmission rate remained higher than 90% as the feeding time increased to 48 h. Overall, there was no significant correlation between acquired CTV targets and transmission efficiency from 0.5 h until 48 h of acquisition time. These results indicate that additional factors other than the virus titer in the vector contribute to successful transmission under long acquisition conditions. Chen et al. (2011) showed that the transmission of lettuce infectious yellows virus (LIYV), which also is transmitted in a semipersistent manner, is determined by a virion retention mechanism mediated by the minor coat protein in the anterior foregut or cibarium of whitefly vectors. Using fluorescently labeled virions of the poorly transmissible isolate T36, Killiny et al. (2016) found that CTV bound specifically to the lining of the cibarium of the aphid after membrane feeding. It was reported that three CTV proteins, p27, p61, and p65 may be involved in virus binding to the foregut of its aphid vector (Killiny et al. 2016). If virus retention is correlated with transmissibility of CTV, the binding of CTV to this retention site on the cibarium of the aphid might be sufficient to maintain a higher level of transmission, although the CTV targets decreased in aphids after 6 h acquisition. Also, it seems to indicate that after a saturation level is attained, further AAP time is still needed to maintain CTV retention on the cibarium to attain maximum transmission rates. In addition, despite the fact that CT16-2 was detected in 100% of single aphids tested, only 15.6% of the inoculations resulted in systemic infections. Moreno et al. (2009) found that the frequency of PPV detection in leaf discs collected immediately after the inoculation process was higher than the effective transmission rate, and they also demonstrated that approximately half of

Table 2. Quantification by real-time RT-PCR of citrus tristeza virus (CTV) targets acquired by *Toxoptera citricida* after different acquisition periods

Acquisition period	Number of CTV targets ^a		
	CT11A	CT16-2	CTLJ
0.5 h	$2.08 \times 10^5 \pm 0.43 \times 10^5$ c	$7.80 \times 10^4 \pm 1.54 \times 10^4$ b	$5.95 \times 10^4 \pm 2.56 \times 10^4$ c
1 h	$1.93 \times 10^6 \pm 0.71 \times 10^6$ c	$2.00 \times 10^5 \pm 0.53 \times 10^5$ b	$9.78 \times 10^5 \pm 2.14 \times 10^5$ bc
2 h	$5.95 \times 10^6 \pm 2.01 \times 10^6$ bc	$1.66 \times 10^6 \pm 0.86 \times 10^6$ b	$2.41 \times 10^6 \pm 0.10 \times 10^6$ bc
3 h	$5.00 \times 10^6 \pm 1.36 \times 10^6$ bc	$4.79 \times 10^6 \pm 3.95 \times 10^6$ b	$4.41 \times 10^6 \pm 2.60 \times 10^6$ bc
4 h	$1.39 \times 10^7 \pm 0.80 \times 10^7$ ab	$5.14 \times 10^6 \pm 1.59 \times 10^6$ b	$9.38 \times 10^6 \pm 2.97 \times 10^6$ a
6 h	$2.32 \times 10^7 \pm 0.55 \times 10^7$ a	$1.71 \times 10^7 \pm 0.37 \times 10^7$ a	$6.09 \times 10^6 \pm 1.83 \times 10^6$ ab
9 h	$9.24 \times 10^6 \pm 4.81 \times 10^6$ bc	$5.24 \times 10^6 \pm 1.49 \times 10^6$ b	$3.23 \times 10^6 \pm 0.87 \times 10^6$ bc
12 h	$3.27 \times 10^6 \pm 0.78 \times 10^6$ bc	$2.56 \times 10^6 \pm 0.75 \times 10^6$ b	$3.86 \times 10^6 \pm 1.80 \times 10^6$ bc
24 h	$2.64 \times 10^6 \pm 0.54 \times 10^6$ bc	$1.61 \times 10^6 \pm 0.47 \times 10^6$ b	$1.96 \times 10^6 \pm 1.33 \times 10^6$ bc
48 h	$9.63 \times 10^6 \pm 1.65 \times 10^6$ bc	$5.40 \times 10^6 \pm 1.10 \times 10^6$ b	$1.94 \times 10^6 \pm 0.63 \times 10^6$ bc

^a Mean number of estimated RNA target copies $\pm$ SE. Values in columns followed by the same letter are not significantly different at $P < 0.05$.

Table 3. Genome copy number and transmission rates of citrus tristeza virus (CTV) isolate CT16-2 after different acquisition access periods (AAPs)

AAPs	No. of CTV genomes ^w	Transmission rate (%) ^x	Correlation coefficient (R)
0.5 h	$7.80 \times 10^4 \pm 1.54 \times 10^4$ b	20.0 ± 2.3 f	0.9150 ^y
1 h	$2.00 \times 10^5 \pm 0.53 \times 10^5$ b	21.0 ± 3.5 f	
2 h	$1.66 \times 10^6 \pm 0.86 \times 10^6$ b	36.4 ± 2.3 e	
3 h	$4.79 \times 10^6 \pm 3.95 \times 10^6$ b	44.0 ± 4.6 d	
4 h	$5.14 \times 10^6 \pm 1.59 \times 10^6$ b	70.5 ± 2.7 c	
6 h	$1.71 \times 10^7 \pm 0.37 \times 10^7$ a	90.9 ± 2.7 b	0.4780 ^z
9 h	$5.24 \times 10^6 \pm 1.49 \times 10^6$ b	100.0 ± 0.0 a	
12 h	$2.56 \times 10^6 \pm 0.75 \times 10^6$ b	100.0 ± 0.0 a	
24 h	$1.61 \times 10^6 \pm 0.47 \times 10^6$ b	92.2 ± 2.3 b	
48 h	$5.40 \times 10^6 \pm 1.10 \times 10^6$ b	90.2 ± 1.3 b	

^w Mean number of CTV genomes $\pm$ SE. Values in a column followed by the same letter are not significantly different at $P < 0.05$.

^x Transmission rate = number of plants infected/number of test plants used $\times$ 100. Mean transmission rate $\pm$ SE. Values in columns followed by the same letter are not significantly different at $P < 0.05$.

^y Correlation coefficient between the mean transmission rate and the mean titer of CTV genomes following AAPs of 0.5 to 6 h. Significant correlation was found at $P < 0.01$.

^z Correlation coefficient between the mean transmission rate and the mean titer of CTV genomes following AAPs from 0.5 to 48 h.

the acquired PPV copies in aphids are responsible for a systemic infection. Soosaar et al. (2005) suggested that several factors involved in the viral invasion process can restrict virus movement in the plant and prevent systemic infection. Though virus systemic infection is complex, the quantitative detection of the number of CTV RNA targets acquired by aphids provides more information on the interaction between CTV isolates and their primary vector, *T. citricida*.

In the present study, our results demonstrated that the single aphid transmission rates of three mild CTV isolates were 65.6, 15.6, and 4.4% for CT11A, CT16-2, and CTLJ, respectively. The single aphid transmission rate of CT11A was higher than that of CT16-2 and CTLJ. The single aphid transmission rate of 65.6% for CT11A, which is a mild isolate, was surprising. In a prior study, CTV isolates were classified into three groups based on transmissibility, low (less than 5%), intermediate (5 to 20%), and high (over 21%) transmission rates (Racah et al. 1980). The virus titer of the different CTV isolates in the donor sweet orange host was estimated by real-time RT-PCR. However, no obvious relationship between CTV accumulation in the donor plant and aphid transmissibility was found (data not shown), which corroborates the findings of Yokomi and Garnsey (1987) and Zhou et al. (2011). Moreover, the CTV quantities acquired between 0.5 and 48 h of acquisition were higher in aphids fed on plants infected with CT11A than with isolates CT16-2 and CTLJ. These results indicated that *T. citricida* acquired more virions of the highly transmissible isolate. This is similar to the results of Zhou et al. (2011), who used CTV isolates with a different transmission rate at 24 h acquisition time. In addition, the full genome sequence of CT11A is closely related to isolate T318A, but is distantly related to the poorly transmissible isolate T36. This suggested that specific gene sequences of the CTV isolates are factors affecting aphid transmission, although characteristics of acquisition are important (Hilf et al. 2005; Killiny et al. 2016).

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