Inefficient Transmission of Citrus Tristeza Virus from Grapefruit by Single Brown Citrus Aphids

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Abstract. Six severe and six mild Florida isolates of citrus tristeza virus (CTV) were used to evaluate the transmission efficiency of the virus from grapefruit seedlings by single brown citrus aphids (*Toxoptera citricida* Kirkaldy) (BrCA) from colonies initiated by aphids obtained from citrus groves in Fort Pierce, Fla. The transmission rate to 2120 receptor plants ['Mexican' lime (*Citrus aurantifolia*)] from grapefruit by single BrCA was 1.5%. Single BrCA transmitted four of the six severe isolates and three of the six mild isolates of CTV. The average transmission rate of severe isolates was 1.8%, higher than that (0.9%) of mild isolates. Severe isolate Y-7 had the highest transmission rate among six severe isolates, 3.6%. Mild isolate Y-23 had the highest transmission rate among the mild isolates, 3.0%. The transmission rates of CTV by alatae, apterae, or nymphs of BrCA were 1.5%, 1.5%, and 1.0%, respectively. The results suggested that BrCA is an inefficient vector of CTV when the source plant is grapefruit.

The brown citrus aphid (*Toxoptera citricida* Kirkaldy) (BrCA) (synonyms: *Aphis citricidus*, *A. aeglis*, *A. nigricans*, *A. tavarresi*, *Myzus citricidus*, and *Paratoxoptera argentinensis*) has been widely considered the most effective vector of citrus tristeza virus (CTV) since it was first demonstrated that BrCA had the ability to transmit CTV (Abate, 1988; Cambra et al., 1981; Essig, 1949). Studies carried out by Costa et al. (1951), Nickel et al. (1984), Yokomi and Damsteegt (1990), Kano and Koizumi (1991), and Broadbent et al. (1996) in different countries showed that the transmission rates of CTV by BrCA varied. The transmission rates of CTV by single and multiple (3–100) BrCA ranged from 0% to 66.6% and from 0% to 91.6%, respectively (Broadbent et al., 1996; Costa and Grant, 1951; Lastra et al., 1992; Nickel et al., 1984; Yokomi et al., 1994). The factors affecting the transmission efficiency of CTV by BrCA included the isolate of CTV, source plant of the virus and the morphological stage of the aphid (Abate, 1988; Broadbent et al., 1996; Costa and Grant, 1951;

Rocha-Pena et al., 1995; Sharma, 1989; Yokomi and Damsteegt, 1990; Yokomi et al., 1994; Yokomi and Garnsey, 1987). The effects of sweet orange [*Citrus sinensis* (L.)] and 'Mexican' lime [*C. aurantifolia* (Christm.)] on CTV transmission efficiency by BrCA were revealed from the studies carried out by Costa et al. (1951), Yokomi and Damsteegt (1990) and Broadbent et al. (1996). The transmission rates of CTV by single BrCA from sweet orange were 0% to 55% (Costa and Grant, 1951; Yokomi et al., 1994), and the transmission rates of CTV by single BrCA from 'Mexican' lime were 16.6% to 66.6% (Nickel et al., 1984). When compared to the results of CTV transmission experiments with other aphids, the transmission efficiency of CTV from sweet orange and 'Mexican' lime by BrCA was apparently much higher than that by other aphids. It was suggested that BrCA is the most effective vector of CTV from sweet orange and 'Mexican' lime plants (Abate, 1988; Broadbent et al., 1996; Michand, 1998; Rocha-Pena et al., 1995; Roistacher and Bar-Joseph, 1987; Yokomi et al., 1989; Yokomi and Garnsey, 1987; Yokomi et al., 1994). The aphid transmission rates of CTV by cotton aphid (*Aphis gossypii* Glover) varied with the virus source plants and with the severity of CTV isolate (Bar-Joseph and Loebenstein, 1973; Bar-Joseph et al., 1977). Even more, the results showed that the transmission rate of CTV from grapefruit (*C. paradisi*) or lemon

(*C. limon*) by cotton aphid was much lower than that from other source plants (Bar-Joseph et al., 1973, 1977). Otherwise, the transmission efficiency of CTV from grapefruit and other variety source plants by BrCA is still unknown.

BrCA was first detected in Florida in early Nov. 1995 in Metropolitan Dade and Broward counties. With the northward movement of BrCA, the grapefruit industry in Florida may be at increased risk [≈48% of the grapefruit industry in Florida is still on sour orange rootstock, the most sensitive rootstock of citrus to CTV (Ed Stover, unpublished data)]. Grapefruit is an important Florida fruit crop, especially in the Indian River region (St. Lucie County and Indian River County). The production of grapefruit in the Indian River region is ≈60% of the state's total production. Florida produces ≈80% of grapefruit (total production is ≈2.6 million tons per year) in the United States (Jackson, 1991). The objective of this research was to evaluate the ability of BrCA to transmit CTV from grapefruit trees.

Materials and Methods

Virus isolates and source plants. Six CTV severe isolates (T-36, T-3, Y-3, Y-6, Y-7, and MG-3) and six mild isolates (T-30, T-26, T-4, Y-5, Y-23, and Y-26), collected from Florida field trees by budding or aphid transmission, were used to evaluate the transmission efficiency of CTV from grapefruit seedlings by single BrCA. The severity of the isolates was classified based on their reactions with CTV monoclonal antibodies (MAb) 17G11 and MCA13 in enzyme-linked immunosorbent assay (ELISA). Isolates that reacted with 17G11 and MCA13 were considered severe, decline-producing isolates; the ones that reacted with only 17G11 were considered mild, non-decline producing isolates (Table 1). Isolates Y-3, Y-7, Y-23, and Y-26 were collected from cross-protected grapefruit trees that were first inoculated with a mild isolate and then with a severe field isolate in Fort Pierce, Fla. Isolates Y-5 and Y-6 were field isolates collected directly from grapefruit groves in Fort Pierce, Fla. T-36, T-3, T-30, T-26, and T-4 were originally obtained from Florida citrus groves by aphid transmission many years ago and have been maintained in the greenhouse. Isolate MG-3 was a man-made isolate obtained by budding isolates T-36, T-30, T-26, T-4, and T-3 into a single plant. Isolates T-36 and T-3 cause severe vein clearing, stunting and stem pitting on 'Mexican lime', mild seedling yellow symptoms on 'Eureka' lemon and sour orange seedlings, and quick decline of sweet orange trees on sour orange rootstock. Isolates T-30, T-26, and T-4 caused mild to moderate symptoms on 'Mexican' lime, Eureka lemon, and sour orange seedlings and no symptoms on sweet orange trees on sour orange rootstock. The biological characteristics of isolates Y-3, Y-7, Y-5, Y-6, Y-23, Y-26, and MG-3 were not determined. All isolates used in this experiment were grafted into 'Duncan' grapefruit seedlings. Infection of the 'Duncan' grapefruit seedlings with dif-

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ferent isolates of CTV was confirmed by ELISA with CTV MAb 17G11, and these virus source plants were maintained in an insect-protected greenhouse at the Indian River Research and Education Center, Fort Pierce, Fla.

Receptor indicator plants. 'Mexican' lime seedlings were used as the receptor indicator plants in this experiment. Seeds of 'Mexican' lime were sown in trays filled with custom soil mix (Conrad Fafard, Apopka, Fla.), and grown under greenhouse conditions for 6–12 mo. The seedlings were then transplanted into plastic pots containing the above soil mix, one to three plants per pot. Seedlings with new flush were used as receptor indicator plants for single BrCA transmission of CTV. After inoculation with CTV by single BrCA, the receptor indicator plants were grown in an insect-free greenhouse, and tested twice by ELISA with CTV MAb 17G11 in 4–6 months for the confirmation of CTV infections.

Collection and identification of aphids. Colonies of aphids collected from the citrus groves at Fort Pierce, Fla. were reared on virus-free citrus seedlings. Colonies were sampled, preserved in a mixture solution of 95% ethanol and 75% lactic acid (2:1), and sent to the Division of Plant Industry Entomology Section, the Florida Dept. of Agriculture and Consumer Services, for identification of species (Banziger, 1977; Denmark, 1978). Those verified as *Toxoptera citricida* (BrCA) were later used to establish virus-free aphid colonies for CTV transmission experiments.

Establishment of virus-free aphid colonies. Single adult aphids of BrCA were transferred to a piece of filter paper in a plastic petri plate and were allowed to produce nymphs. The newborn nymphs were immediately moved onto young tender shoots of healthy 'Duncan' grapefruit seedlings. A colony of virus-free BrCA developed from each nymph after a few weeks. Colonies of virus-free BrCA were maintained on the 'Duncan' grapefruit seedlings until used. The healthy seedlings were cut back every two or three weeks to stimulate new flushes for the virus-free BrCA. When the flushes of the rearing plants were getting old, the colonies of BrCA were moved to other uninfected plants with new flushes to maintain the reproduction of colonies.

Single aphid transmission. 'Duncan' grapefruit plants infected with the T-3, T-4, T-26, T-30, T-36, Y-3, Y-5, Y-6, Y-7, or MG-3 isolates of CTV were pruned several weeks before aphid acquisition feeding to stimulate flushes of new growth. When the new flushes had their leaves completely expanded, groups of 200–400 virus-free aphids were moved onto the new flushes for a 24-h acquisition feeding. Single aphids were then transferred from 'Duncan' grapefruit using a small paintbrush to a new flush on healthy 'Mexican' lime seedlings.

Different treatments were made with different morphological stages (nymph, alatae, apterae) of the aphids. The aphids were

allowed 24 h on the receptor seedlings for inoculation. After the inoculation feeding, pesticides (Marathon) or 1 soap : 1 oil : 30 water mixes were sprayed to kill the aphids. The inoculated 'Mexican' lime seedlings were kept under insect-free greenhouse conditions (23 to 30 °C) for 4–6 months. A total of 2120 healthy 'Mexican' lime seedlings were inoculated with 12 isolates of CTV by single BrCA in the experiment. The treatments were replicated over time. The transmission efficiency was defined as the ratio between the number of CTV-infected seedlings and the total number of aphid-inoculated seedlings.

Enzyme-linked immunosorbent assay (ELISA). Extractions for ELISA were prepared from 0.5 g of leaf tissues as described with 5 mL of 1× PBST buffer (Lin et al., 2000). Immulon 2 microtiter plates (Dynatech, Chantilly, Va.) were coated with rabbit polyclonal antibody 1212 at 1 µg·mL⁻¹ in 0.02 M sodium carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Then, the plates were washed with PBST-PVP (PBST with 2% polyvinylpyrrolidone, pH 7.0) three times, 3 min each time. One hundred micro-liters of the sample extracts were added into each well of the plates. The plates were incubated for 4 h at 37 °C, and washed with PBST-PVP three times as above. Again the plates were incubated with undiluted cell culture fluid of MAb 17G11 (a CTV specific MAb that reacts with most isolates of CTV) (100 µL each well) overnight at 4 °C, and washed with PBST-PVP three times. The plates were then incubated with alkaline phosphatase conjugated goat anti-mouse Ig (H+L)-AP (South Biotechnology Associates, Birmingham, Ala.) (100 µL each well) at a 1:2000 dilution

in PBST-PVP for 4 h at 37 °C and washed with PBST-PVP three times. Substrate reactions (1 mg/mL p-nitrophenyl phosphate (Sigma, St. Louis) in 0.1 M diethanolamine buffer, pH 9.8) were allowed to develop at room temperature, and absorbency values (415 nm) were determined using a Bio-Rad 3550 microtitre plate reader (Bio-Rad Laboratories, Richmond, Calif.).

Statistical analyses. Transmission rate (%) data were subjected to an analysis of variance (ANOVA) by the SAS software program (SAS Inst., Cary, N.C.). Data were subjected to an arcsine square root transformation prior to conducting the ANOVA. Main effects were virus isolates and aphid morphology. A single degree of freedom contrast (Severe vs. mild isolates) was partitioned from the main effects of virus isolates. Since virus isolates × aphid morphology interaction was nonsignificant, only main effects were presented.

Results

Transmission efficiency of CTV from grapefruit by BrCA. The transmission rates of CTV isolates from grapefruit seedlings to 'Mexican' lime seedlings by single BrCA were low (Table 1). The total infection rate by single BrCA to 2120 receptor plants was 1.5%.

Differences in transmission rates of different isolates of CTV. Four (T-36, Y-3, Y-6, and Y-7) of six severe isolates and three (T-4, T-26 and Y-23) of six mild isolates of CTV used in the experiments were transmitted by single BrCA (Table 1). Isolates T-3, MG-3, T-30, Y-5, and Y-26 were not transmitted by single BrCA. The transmission

Table 1. Differences in transmission efficiency of CTV isolates from grapefruit to Mexican lime by single BrCA.

CTV isolate	Isolate source ^c	Severity ^y	No. positive/ no. inoculated ^a	Transmission rate(%) ^x
Y-7	BD	S	4/110	3.6
Y-6	BD	S	6/170	3.5
Y-23	BD	M	3/100	3.0
Y-3	BD	S	7/290	2.4
T-36	AT	S	7/360	2.0
T-4	AT	M	2/100	2.0
T-26	AT	M	2/170	1.2
T-30	AT	M	0/180	0.0
T-3	AT	S	0/100	0.0
MG-3	BD	S	0/310	0.0
Y-5	BD	M	0/180	0.0
Y-26	BD	M	0/50	0.0
Contrast ^w				
Severe isolates			24/1340	1.8
Mild isolates			7/780	0.9
Total			31/2120	1.5

^aAT = the isolates of CTV were obtained by aphid transmission from CTV-infected plants. BD = the isolates were obtained by budding (grafting) from CTV-infected plants.

^yS = isolates that reacted with CTV specific monoclonal antibodies 17G11 and MCA13 and had a severe phenotype in indicators. M = isolates that reacted with CTV specific monoclonal antibodies 17G11 but not with MCA13 and had a mild phenotype in indicators.

^xCTV infection was determined by ELISA tests with CTV specific monoclonal antibody 17G11. The transmission rate of each isolate was a total transmission rate by a combination of all stages of BrCA used in the test. Differences among treatment means were significant at $P \leq 0.01$.

^wThe transmission rates of severe or mild isolates were the total transmission rates by a combination of all stages of BrCA used in the test. The single degree of freedom contrast (severe vs. mild) was significant at $P \leq 0.05$.

efficiency of severe isolates of CTV was higher than that of mild isolates of CTV (Table 1). The average transmission rate of six severe isolates of CTV was 1.8% while the average transmission rate of six mild isolates of CTV was 0.9%. The highest transmission rate of a severe isolate was 3.6% from isolate Y-7 and the highest transmission rate of a mild isolate was 3.0% from isolate Y-23 (Table 1). The CTV isolate $\times$ aphid morphology interaction was nonsignificant.

Effect of aphid morphological stages on transmission rate of CTV. The morphological stage, nymph, alatae and apterae, of the aphid may have affected the ability of BrCA to transmit CTV from grapefruit plants (Table 2). The transmission rates of CTV by alate adults and apterous adults of BrCA from grapefruit to 'Mexican' lime seedlings were not different (both were 1.5%), but the transmission rate (1.0%) of CTV by nymphs of BrCA from grapefruit seedlings to 'Mexican' lime seedlings was lower (Table 2).

Discussion

The results showed that BrCA is an inefficient vector of CTV when the source plant is grapefruit. Transmission rates from grapefruit by single BrCA were markedly lower than reported transmission rates from sweet orange and 'Mexican' lime. The transmission rates by single BrCA were 1% to 25% from sweet orange by Yokomi and Damsteegt (1990), 0% to 55% from sweet orange by Broadbent et al. (1996) and 16.6% to 66.6% from 'Mexican' lime by Nickel et al. (1984). In our hands, single BrCA transmitted T36 and T30 from sweet orange to 'Mexican' lime with effectiveness of 8% and 5%, respectively, based on ≈ 100 attempts (Powell and Lin, unpublished). In experiments on CTV transmission with other aphids, Bar-Joseph et al. (1977) also pointed out that the transmission rate of CTV by aphids was influenced by the variety of citrus from which the virus was acquired. The transmission rate of CTV from grapefruit (*C. paradisi*) or lemon (*C. limon*) by the cotton aphid (*Aphis gossypii*) was much lower than that from other source plants. Taken together, these results suggested that the virus source plant markedly influenced the transmission efficiency of CTV and that grapefruit is not an efficient source plant of CTV for aphid transmission. The low efficiency may be associated with the distribution of CTV in grapefruit plants. Lee et al. (1988) reported that CTV was unevenly distributed in a grapefruit tree, especially in the

late summers. The relationship between the distribution of the virus in host plants and the transmission efficiency of the virus by vector aphids needs to be further studied. Since single BrCA transmits CTV inefficiently from grapefruit, large numbers of this aphid, *A. gossypii* and *A. spiraecola* are required for rapid spread of the virus in grapefruit groves. *A. spiraecola* with high populations were usually observed in citrus groves in Florida and California (Pelosi et al., 1996; Roistacher et al., 1980).

The results also indicate that there are differences in transmission efficiency of different isolates of CTV by single BrCA. The severe isolates were more easily transmitted than the mild isolates of CTV used in the experiment (1.8% and 0.9%, respectively). This is in agreement with the previous results reported by Sharma (1989), Yokomi and Damsteegt (1990), and Broadbent et al. (1996). Their results disclosed that there were differences in transmission rates of different isolates of CTV from sweet orange or 'Mexican' lime plants by BrCA. They pointed out that BrCA may transmit severe isolates and severe latent-isolates of CTV efficiently. Bar-Joseph et al. (1977) also reported that the aphid transmission rates of CTV by cotton aphid also varied with the severity of CTV isolate.

The results showed that the morphological stage of BrCA might have an effect on its ability to transmit CTV. The ability of single adult of BrCA to transmit CTV from grapefruit to 'Mexican' lime was higher than that of BrCA nymphs. However, the ability of alatae and apterae of BrCA to transmit CTV from grapefruit to 'Mexican' lime were not different. These differences are not nearly as pronounced as those reported by Nickel et al. (1984). The transmission rates of CTV from 'Mexican' lime plants by alatae, apterae, and nymphs of BrCA reported by Nickel et al. (1984) were 66.6%, 25%, and 16%, respectively. The results reported by Costa et al. (1951) were different from that by Nickel et al. (1984) and our experiments. Their transmission rates of CTV from sweet orange by the single adults and nymphs of BrCA were 17% and 16%, respectively, and the transmission rates of CTV from sweet orange by multiple adults and nymphs (25 aphid/plant) were 21% and 75%, respectively. However, all results mentioned above indicate that the morphological stage of BrCA has apparent effects on its ability to transmit CTV. The apterae would be the most important for CTV movement under the natural conditions in the field.

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Table 2. Transmission efficiency of CTV from grapefruit to 'Mexican' lime within main effects of Morphological stages of BrCA.

Morphological stage	No. positive/no. inoculated	Transmission rate (%)
Apterae	13/881	1.5 a ²
Alatae	16/1037	1.5 a
Nymphs	2/202	1.0 b

²Mean separation with a column by Duncan's multiple range test, 5% level.

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