Suppression of Decline-inducing Isolates of Citrus Tristeza Virus by Nondecline-inducing Isolates

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Abstract. Bark chips from six container-grown citrus trees, infected with nondecline-inducing citrus tristeza virus (CTV) isolates and maintained in a vector-free greenhouse for 10 years, 15 commercial grapefruit (*Citrus paradisi* Macf.) trees, and 16 commercial sweet orange [*C. sinensis* (L.) Osbeck] trees were used to inoculate three indicator plants each of 'Madam Vinous' sweet orange [*C. sinensis* (L.) Osbeck], sour orange (*C. aurantium* L.), 'Duncan' grapefruit (*C. paradisi* Macf.), 'Mexican' lime [*C. aurantifolia* (Christm.)], Swingle citrumelo [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.], and sour orange grafted with 'Hamlin' sweet orange [*C. sinensis* (L.) Osbeck]. All plants providing bark chips had repeatedly tested positive by enzyme-linked immunosorbent assay (ELISA) for CTV [reacted with monoclonal antibody (MAb) 17G11], but tested negative for Florida decline-inducing isolates of CTV (did not react with MAb MCA13). After 6 months in vector-free greenhouses, all inoculated trees (except Swingle citrumelo, which is considered CTV resistant) were positive for CTV by 17G11 ELISA. In addition, some indicator plants inoculated from nine (two container, two commercial grapefruit, and five commercial orange trees) of the 37 bark chip source trees also were positive for decline-inducing CTV by MCA13 ELISA. Some of these positive indicators also showed vein-clearing symptoms characteristic of infection with a severe isolate of CTV. No control, noninoculated indicators in the same greenhouse, became infected with either decline-inducing or nondecline-inducing CTV. These results indicate that decline-inducing isolates of CTV can be present as a minor component of a mixture at levels undetectable by ELISA, and that these decline-inducing isolates can become detectable by ELISA and sometimes by symptoms when inoculated into indicator plants.

Citrus tristeza virus (CTV) is a serious pathogen of citrus worldwide. Some isolates cause stunting, slow decline, and/or quick decline of citrus grafted onto sour orange (*C. aurantium* L.) or *C. macrophylla* Wester rootstocks. Other isolates cause stem-pitting of the scion regardless of rootstock. Some isolates are mild and cause no apparent symptoms in commercial citrus. In Florida, only those isolates that affect citrus on sour orange rootstock or cause no symptoms are prevalent in commercial citrus. Recently, in many regions of Florida, there has been increased incidence of CTV-induced decline symptoms in grapefruit and sweet orange groves on sour orange rootstock (Brown and Davidson, 1997). This may be attributed to the establishment of the brown citrus aphid (*Toxoptera citricida* Kirkaldy) several years ago, followed by recent drought conditions.

Control of CTV-induced disease should begin with a vigorous budwood certification program to prevent distribution or introduction of harmful CTV isolates into commercial and noncommercial plantings. Another component of CTV disease control is cross-protection: the process of inoculating trees with nondisease-in-

ducing virus isolates with the hope that they will prevent infection or expression of symptoms caused by disease-inducing isolates.

Cross-protection has had both successes (Bar-Joseph et al., 1989; Muller and Costa, 1968; Muller and Costa, 1977; Powell et al., 1999; Yokomi et al., 1991) and failures (Powell et al., 1992). In the Florida budwood program, certification and cross-protection are often combined by propagating trees that are known to have nondisease-inducing isolates, but test negative for disease-inducing isolates of CTV.

Typically, a tree is certified as "no tristeza found" or "mild CTV" based on enzyme-linked immunosorbent assay (ELISA) tests performed at regular intervals. The certified tree is used as a budwood source for increase blocks or directly for commercial nursery stock. Such a program in Florida appears to have virtually eliminated detectable levels of decline-inducing isolates of CTV from several Florida nurseries (Powell, unpublished). However, detection of severe CTV strains depends mainly upon ELISA tests using the monoclonal antibody (MAb) MCA13 (Permar et al., 1990) which reacts with most decline-inducing, but not nondecline-inducing, isolates of CTV in Florida and the Caribbean basin. This raises concerns about "missing" exotic isolates that do not react with MAb MCA13, yet cause severe symptoms, or isolates that are present in low concentration.

Different isolates of CTV can occur as mixtures in the same tree. For example, sweet orange or grapefruit trees known to be infected with nondecline-inducing isolates of CTV can become superinfected with decline-inducing isolates of the virus (Powell et al., 1992). In addition, apparently healthy grapefruit trees on sour orange rootstock that were cross-protected with nondecline-inducing isolates of CTV that were MAb MCA13-negative in ELISA were shown to contain MAb MCA13 reactive isolates using an in situ immunoassay (ISIA) (Lin et al., 2002). The ISIA allows immunological detection of virus in thin tissue sections using a light microscope and is more sensitive than ELISA (Lin et al., 2002). These results raise concerns related to management of CTV through certification and quarantine. In mixed infections, can a nondecline-inducing isolate of CTV suppress a decline-inducing isolate to a concentration that is not detectable by ELISA? Is this a common occurrence? How might this affect current strategies to control the citrus tristeza disease through budwood certification and cross-protection? We report here the results of experiments to determine if container-grown and commercial citrus trees that assayed negative for CTV using MAb MCA13 could harbor undetectable concentrations of decline-inducing isolates of CTV.

Materials and Methods

Source plants. Two sources of plants were used for inoculum. The first source was six container-grown trees that contain nondecline-inducing isolates of CTV and have been maintained in an aphid-free greenhouse for at least 10 years. These container-grown source plants were two plants of *Citrus excelsa* Wester on rough lemon (*C. jambhiri* Lush) rootstock containing the T26 mild isolate of CTV, *C. excelsa* on rough lemon rootstock containing the T4 mild isolate of CTV, *C. macrophylla* Wester on rough lemon rootstock containing the T4 isolate of CTV, *Atalantia cylonica* (Arn.) Oliv. on Swingle [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.] rootstock containing the T30 isolate of CTV, and a *C. excelsa* on rough lemon rootstock containing the T30 isolate of CTV. None of the isolates (T4, T26, or T30) cause decline of sweet orange on sour orange rootstock or seedling yellows. T30 and T26 can cause mild vein-clearing, and T4 severe vein-clearing, in 'Mexican' lime [*C. aurantifolia* (Christm.) Swingle]. When tested with MCA13, each of these six source trees gave mean ELISA values no higher than healthy controls and similar to buffer controls in five different repetitions performed over a 1-year period.

The second type of inoculum source plant was trees from two commercial groves near Fort Pierce, Fla., where both decline and nondecline-inducing isolates of CTV are endemic and the brown citrus aphid is established. Fifteen of the source trees were 'Ruby Red' grapefruit on Swingle rootstock and sixteen were 'Valencia' sweet orange on Swingle rootstock. The trees were ≈ 15 years old. Each of the 31 trees indexed positive for

CTV (MAb 17G11), but negative for decline-inducing CTV (MAb MCA13) in five different ELISA tests performed 1 year, 6 months, and 3 months prior to collection of budwood, at the time of budwood collection, and 3 and 6 months after budwood collection.

ELISA. ELISA was performed using an indirect double antibody sandwich (DAS) protocol (Lin et al., 2000). The coating antibody was purified rabbit polyclonal 1212IgG used at a concentration of 1 µg/mL (Lin et al., 2000). The second antibody was either MAb 17G11, which detects most Florida isolates of CTV, or MAb MCA13, which specifically detects most Florida decline-inducing isolates (Permar et al., 1990). The third (detecting) antibody was alkaline phosphatase conjugated goat anti-mouse Ig (H&L) (Sigma Chemical Co., St. Louis). Both MAbs were undiluted cell culture fluid. Plants were considered positive if their mean OD in ELISA (based on four replications) was significantly higher (5% level) than healthy controls.

Indexing. For the container-grown sample trees, 54 bark chips were collected from each of the six trees. Three of the bark chips, each from a different region of the sample tree, were inserted into bark flaps of an indicator plant, wrapped with budding tape, and allowed to heal for 2 weeks before the tape was removed. Each of the six sample trees was indexed on three of each indicator type. The indicator types were 'Madam Vinous', sour orange, 'Duncan' grapefruit, 'Mexican' lime, and Swingle citrumelo seedlings and 'Hamlin' sweet orange grafted onto sour orange rootstock. For the commercial grove trees, three newly produced shoots, each from a different region of the tree, were collected, sealed in a plastic bag, placed in a cooler, and transported back to the greenhouse. Three bark chips, each from a different shoot from the same sample tree, were used to inoculate each indicator plant. Each of the 31 field trees was indexed on three of each indicator type, as described above. The indicator types were 'Madam Vinous', 'Mexican' lime, and 'Duncan' grapefruit seedlings, and 'Hamlin' on sour orange. All bark chip inoculation was done in the fall (October–November). After 6 months, the indicators were assayed for CTV by indirect DAS–ELISA using monoclonal antibodies 17G11 and MCA13 and observed for foliar and/or xylem stem-pitting symptoms.

Results

Container-grown trees. After inoculation with bark chips from container-grown citrus infected with mild isolates of CTV, all of the 'Madam Vinous', sour orange, 'Duncan' grapefruit, and 'Hamlin' on sour orange indicator plants contained CTV detectable by ELISA using MAb 17G11 (Table 1). The mean OD value for all the indicators was 0.726 ± 0.115 with no significant difference among indicator types. The mean OD for healthy controls was 0.068 ± 0.034 , also, with no significant difference among indicator types. Only one of the Swingle indicator plants was positive for CTV by ELISA using this antibody. This plant was inoculated using a bark chip from

a T4-infected source plant. Only one of the three replicate Swingle indicators was positive (0.356 ± 0.022 compared to 0.033 ± 0.028 for healthy Swingle). This Swingle-infecting isolate has not yet been characterized further by bioassay or PCR.

Interestingly, two of the 'Madam Vinous' indicators ($OD 0.334 \pm 0.017$ and 0.336 ± 0.063 compared to 0.067 ± 0.023 for healthy controls) and one of the 'Duncan' grapefruit indicators (0.319 ± 0.006 compared to 0.066 ± 0.003 for healthy controls) were also positive for CTV using MAb MCA13, which is selective for Florida decline-inducing isolates of CTV. One of the 'Madam Vinous' MCA13 positives was inoculated from a T26-infected tree, and the other from a T4-infected tree. The 'Duncan' grapefruit which was MCA13 positive originated from the same T26-infected tree from which a decline-inducing CTV isolate (MCA13 positive) was detected when bark chip grafted into 'Madam Vinous'. In each of these three cases, only one of the three replicate indicators was positive. This shows that, in at least three cases, container-grown citrus trees that were negative for decline-inducing CTV by MCA13 ELISA, but positive for mild CTV strains, were carrying a decline-inducing isolate that became detectable when the virus was transferred into another host by grafting.

Field trees. CTV was detected (by ELISA using MAb 17G11) in the indicator plants inoculated from all 15 commercial grapefruit (0.514 ± 0.056 compared to 0.083 ± 0.015 for healthy controls) and 16 commercial sweet orange trees (0.885 ± 0.178 compared to 0.042 ± 0.027 for healthy controls) (Table 2). In all cases, at least two of the three replicate indicators, and usually three, were positive. Decline-inducing CTV was detected (by ELISA using MAb MCA13) in indicator trees inoculated from two of the field grapefruit (mean OD for positives was 0.319 ± 0.061 compared to 0.058 ± 0.028 for negatives and 0.081 ± 0.040 for healthy controls) and five of the sweet orange trees (mean OD for positives was 0.410 ± 0.050 compared to 0.076 ± 0.041 for negatives and 0.047 ± 0.023 for healthy controls). This was unexpected since each of the 31 trees had previously tested negative (0.056 ± 0.016) for decline-inducing CTV by ELISA using MCA13 five times over a one-year period, and once again (0.041 ± 0.030) at the conclusion of the indexing on indicator plants. In most cases, only one of the three replicate indicators was positive for decline-inducing CTV, suggesting that either the decline-inducing isolate was unevenly distributed in the donor field tree or it frequently remained at an undetectable concentration in the indicator tree. The severe isolate from grapefruit detected in the 'Madam Vinous' and 'Duncan' grapefruit indicators was from the same source plant as one of the 'Hamlin' on sour orange severe positives. The severe CTV isolate detected in five sweet orange trees using 'Madam Vinous' indicators was also detected in four of the same trees using 'Mexican' lime indicators. The MCA13 positive 'Hamlin' on sour indicators were stunted, and the MCA13 positive 'Mexican' lime in-

Table 1. Transfer of CTV from container-grown citrus into indicators.^z

Source plant ^x	Indicator ^w	Isolate type detected ^y	
		Severe	Mild
<i>C. excelsa</i> with T26	None	0/5	5/5
	MV	1/3	3/3
	ML	0/3	3/3
	SO	0/3	3/3
	DG	1/3	3/3
	Sw	0/3	0/3
<i>C. excelsa</i> with T26	H/S	0/3	3/3
	None	0/5	5/5
	MV	0/3	3/3
	ML	0/3	3/3
	SO	0/3	3/3
	DG	0/3	3/3
<i>C. excelsa</i> with T4	Sw	0/3	0/3
	H/S	0/3	3/3
	None	0/5	5/5
	MV	1/3	3/3
	ML	0/3	3/3
	SO	0/3	3/3
<i>C. macrophylla</i> with T4	DG	0/3	3/3
	Sw	0/3	0/3
	H/S	0/3	3/3
	None	0/5	5/5
	MV	0/3	3/3
	ML	0/3	3/3
<i>A. cylonica</i> with T30	SO	0/3	3/3
	DG	0/3	3/3
	Sw	0/3	0/3
	H/S	0/3	3/3
	None	0/5	5/5
	MV	0/3	3/3
<i>C. excelsa</i> with T30	ML	0/3	3/3
	SO	0/3	3/3
	DG	0/3	3/3
	Sw	0/3	0/3
	H/S	0/3	3/3
	None	0/5	5/5
Healthy	MV	0/3	0/3
	ML	0/3	0/3
	SO	0/3	0/3
	DG	0/3	0/3
	Sw	0/3	0/3
	H/S	0/3	0/3

^zThree of each indicator variety were inoculated using bark chips from six container-grown citrus trees that were negative for decline-inducing CTV by ELISA.

^yNumber of indicators positive by ELISA vs number tested. Severe-positive ELISA reaction using MAb MCA13, mild-negative ELISA reaction using MAb MCA13, but positive reaction using MAb 17G11.

^xContainer-grown trees containing a mild isolate of CTV. See materials and methods for details.

^wNone = ELISA tests were conducted on tissue directly from the container-grown tree. Four tests were conducted prior to collecting bark chips for indexing, and one was conducted after indexing tests were completed, MV = 'Madam Vinous', ML = 'Mexican' lime, SO = sour orange, DG = 'Duncan' grapefruit, Sw = Swingle, and H/S = 'Hamlin' sweet orange scion grafted onto sour orange seedlings.

Table 2. Transfer of CTV from commercial citrus into indicators.²

Indicator	Grapefruit ^a		Sweet orange ^b	
	Severe	Mild	Severe	Mild
Madam Vinous	1/15	15/15	5/16	16/16
Mexican lime	0/15	15/15	4/16	16/16
Duncan grapefruit	1/15	15/15	0/16	16/16
Hamlin/sour ^c	2/15	15/15	0/16	16/16

^aThree of each indicator variety were inoculated using bark chips from 15 commercial grapefruit and 16 commercial sweet orange trees that tested negative for decline-inducing isolates of CTV by ELISA.

^bNumber of trees that tested positive by ELISA in at least one of the three indicators vs number of trees tested. Severe- positive ELISA reaction using MAb MCA13, mild- negative ELISA reaction using MAb MCA13, but positive reaction using MAb 17GII.

^cHamlin sweet orange scion grafted onto sour orange seedlings.

indicators showed severe vein-clearing. These data indicate that commercial grapefruit and sweet orange trees infected with mild isolates of CTV may harbor decline-inducing isolates that are at a concentration or distribution that was not detected by ELISA.

Discussion

The results confirm that decline-inducing and nondecline inducing isolates of CTV can co-infect the same citrus tree (Cevik et al., 1997; Moreno et al., 1991; Rosner et al., 1986). They also demonstrate that the nondecline-inducing isolate can predominate in this mixture with the decline-inducing isolate occurring at a concentration that is undetectable by ELISA. When bark chips are used to transfer the virus mixture to another citrus host, the concentration of the decline-inducing isolate can increase to a level where it is easily detected by ELISA, and can produce expected severe symptoms in the susceptible indicator plant. The results cannot be explained by transmission from contaminating aphids since the receptor plants were kept in an insect-free greenhouse, no aphid contamination was ever observed, and noninoculated "guard" citrus seedlings never became infected.

The mechanism by which one isolate of CTV predominates over another isolate in mixed infections is not understood. It may be through inhibition of virus replication, inhibition of virus movement, and an induction host plant defense mechanisms. It has been recently learned that aphids can acquire an undetectable severe CTV isolate in a mixed infection with mild isolates and transmit the severe isolate to susceptible citrus hosts (Bransky and Powell, unpublished). Perhaps the severe isolate is at low concentration when a whole plant or leaf is sampled, but predominates or is exclusively present in small numbers of cells.

It is not known whether the change in host or the grafting procedure itself (giving the viruses access to uninfected tissue) was responsible for the increase in concentration of the decline-inducing isolate. Experiments in which bark chips from the receptor trees containing a detectable decline-inducing isolate are used to inoculate the original host type in an attempt to reverse the phenomenon have not yet been completed.

A detailed characterization of the decline-inducing isolates has also not been completed. Preliminary work using PCR and isolate-specific primers suggests that the decline-inducing isolates recovered from the various field and container-grown citrus trees are not significantly different from each other or T36 (the decline-inducing isolate type commonly found in commercial citrus in Florida). However, subtle sequence differences would not have been detected.

The phenomenon of one isolate predominating in a mixed infection may be fairly common. It was observed in two of 15 (13%) grapefruit trees and five of 16 sweet orange trees (31%) tested. Admittedly, the sample size is not large enough to verify this hypothesis.

These results suggest that in a mixed infection with nondecline- and decline-inducing isolates of CTV, the decline-inducing isolate may occur at a concentration too low to detect by ELISA. These results have ramifications for both cross-protection and certification. A protecting isolate of CTV that is deliberately introduced into citrus trees may harbor an undetectable concentration of a decline-inducing or possibly even a stem-pitting isolate of CTV. This severe isolate could become apparent if it is moved into a different host or a different set of environmental conditions. Likewise, trees or budwood sources that contain nondecline-inducing isolates of CTV, but are negative for decline-inducing isolates by ELISA, could still contain a severe isolate that would be distributed in budwood. This could result in

the unknowing distribution of harmful isolates of CTV throughout Florida and other regions of the world. Cross-protection and budwood certification have been, and will continue to be, extremely important strategies for controlling the citrus tristeza disease. However, caution should be exercised when declaring a plant to be free of a virus based on any one procedure, especially when the dynamics of mixed infection with different biotypes is not understood.

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