Superinfection of Orange Trees Containing Mild Isolates of Citrus Tristeza Virus with Severe Florida Isolates of Citrus Tristeza Virus

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ABSTRACT

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The ability of four mild isolates of citrus tristeza virus (CTV) to suppress the spread of Florida severe isolates of CTV into Valencia sweet orange trees propagated on sour orange rootstock was assessed by symptom development over an 8-yr period. Decline symptoms occurred in some of the trees containing each of the mild isolates, as well as the unprotected (no mild isolate) control trees, within 3 yr after planting. After 5 yr, the percentage of decline in trees infected with the four mild isolates was 28, 22, 27, and 25, respectively, compared with 39% for the unprotected control trees. After 8 yr, the percentage of trees with symptoms (stunting or decline) was 75, 76, 74, and 73, respectively, compared with 86% for the control. Eight years after planting, a monoclonal antibody to CTV (MCA13) reacted with 100, 99, 92, and 19% of the extracts from trees with decline and stunting, stunting, decline, or no symptoms, respectively. A second monoclonal antibody (3DF1) reacted with extracts from all of the trees.

Citrus tristeza virus (CTV) causes economically important diseases wherever citrus is grown (2,8). The virus can cause stunting, slow decline, quick decline, stem pitting, or no symptoms depending on the virus isolate, environmental conditions, and citrus cultivar (7,11). In Florida, only mild isolates, which cause no obvious symptoms, and severe isolates, which cause stunting and/or decline, are currently present. Some Florida decline isolates of CTV will cause a moderate seedling yellows reaction (6). The Florida severe isolates affect only citrus on sour orange or *Citrus macrophylla* P. J. Wester rootstocks; they have not been reported to induce severe stem-pitting symptoms or affect other rootstocks.

Currently, the effect of CTV can be controlled in Florida by propagating citrus on rootstocks other than sour orange. However, there are several reasons why it would be beneficial to protect sour orange rootstock from disease induced by CTV. First, there are many older productive groves in Florida that are propagated on sour orange rootstock. Second, many growers feel that the combination of local environmental conditions and sour orange rootstock are responsible for the high quality of their fruit. Third, all of the other rootstocks currently grown in Florida have at least one major disease or horticultural problem not prevalent with sour orange.

One possible mechanism to protect citrus on sour orange rootstock from severe isolates of CTV is mild strain cross-protection. This approach has been successful in controlling stem-pitting isolates of CTV in both Brazil (4) and South Africa (5). There are indications that cross-protection of sour orange against decline-inducing isolates of CTV may be effective (3,9,13,14,17), but the long-term usefulness of this protection in the field has not been demonstrated.

One of the difficulties in monitoring cross-protection by mild isolates of CTV is the inability to easily distinguish the mild isolates from severe isolates. A breakdown in cross-protection could only be evaluated based on symptoms, and superinfection with severe isolates could not be confirmed serologically. Recently, a monoclonal antibody to CTV that reacts with most decline-inducing isolates of CTV, but not with mild isolates, has been produced (12). This antibody may provide the means to document the superinfection of trees containing mild isolates of CTV with severe isolates (17).

The purpose of this study was twofold. First, the ability of four mild strains of CTV to protect sweet orange trees on sour orange rootstocks from Florida severe strains of CTV was determined. Second, the reliability of monoclonal antibodies to differentiate between mild and severe isolates of CTV and to confirm superinfection of symptomatic trees containing mild isolates of CTV with severe isolates was evaluated.

MATERIALS AND METHODS

Virus isolates and tree propagation. The four mild isolates of CTV included in this study are DD102bb, Guettler HS, DPI 136-53-4, and DPI 1-12-5-X-E. DD102bb originated from a Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) tree on sour orange rootstock (*C. aurantium* L.) in Winter Garden, FL, where CTV-induced disease was prevalent. The tree had remained symptomless for more than 20 yr. Guettler HS came from a symptomless Valencia sweet orange tree on sour orange rootstock from a grove in which 90% of the trees were in decline. DPI 136-53-4 and DPI 1-12-5-X-E were from symptomless old-line and nucellar Valencia sweet orange selections, respectively, on sour orange rootstock in Florida's budwood repository. Each isolate was obtained by graft inoculation of several greenhouse-grown, CTV-free Valencia sweet orange trees on sour orange rootstock with three scion bark chips from each source tree containing the mild isolates. The presence of CTV in the inoculated trees was

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confirmed by ELISA, and the mild nature of the isolates was confirmed by the symptomless nature of inoculated sweet orange trees on sour orange rootstock and by indexing on Mexican lime (*C. aurantifola* (L.) Swingle).

Budwood was collected from additional bark chip-inoculated trees, which had been maintained in the greenhouse, containing each of the mild isolates and from an uninfected Valencia sweet orange tree on sour orange rootstock. This budwood was used to propagate Valencia on sour orange trees that contained each of the four mild isolates of CTV or no CTV. The presence or absence of virus in the budded trees was confirmed with enzyme-linked immunosorbent assay (ELISA) by S. M. Garnsey and R. Yokomi (USDA, Orlando, FL).

Field plot design. Valencia sweet orange trees on sour orange rootstock, previously inoculated with one of four mild isolates of CTV or uninoculated, were randomly planted on raised beds in eight rows with 40 trees per row at the Agricultural Research and Education Center, Fort Pierce, FL, in the spring of 1981. Each of the five treatments was repeated eight times per row. The trees were planted approximately 30 m from a block of Temple orange trees on Cleopatra mandarin rootstock known to be infected with decline-inducing strains of CTV (based on ELISA with MCA13 antibody). The Valencia block was irrigated as needed with a microsprinkler system. Pathogens and pests were controlled as needed by application of pesticides according to University of Florida recommendations.

Each tree was evaluated annually in the spring for decline and/or stunting symptoms characteristic of infection by Florida severe isolates of CTV between 1983 and 1989. A tree was rated as "in decline" if its foliage was visually thinner than healthy trees. A tree was rated as stunted if it was visually smaller in height and canopy and had a significantly smaller trunk circumference (measured 10 cm above the graft union) than healthy trees.

Immunoassay. Indirect double-antibody sandwich (DAS)-ELISA was performed on each of the 320 trees in the plot using conventional protocol (1). Wells of Pro-Bind 96-well flat-bottom plates (Becton-Dickinson, Lincoln Park, NJ) were coated by incubation for 4 hr at 37 C with 1 μg/ml of rabbit polyclonal IgG to CTV (antibody number 1052) (12) in 0.05 M sodium carbonate buffer, pH 9.6. Four wells were incubated with each antigen overnight at 4 C. Antigens were prepared by triturating approximately 1 g of phloem-enriched (bark, petiole, and leaf midrib) young tissue in 7.5 ml of phosphate-buffered saline (PBS) (0.15 M sodium chloride and 0.015 M sodium phosphate, pH 7.4) containing 0.05% Tween 20 (TPBS) using a Tissumizer (Tekmar, Cincinnati, OH). The second antibody, 0.1 μg/ml of either MCA13, which reacts with most Florida severe but not mild isolates of CTV (12), or 3DF1 (15), which reacts with all Florida isolates of CTV, mouse monoclonal IgG in TPBS was incubated in each of two of the four wells for each antigen for 4 hr at 37 C. The conjugated (alkaline phosphatase) antibody, a 1–5,000 dilution of goat antimouse IgG in TPBS, was incubated in each well overnight at 4 C. The substrate was 1 mg/ml of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8. The absorbance in each well was measured spectrophotometrically at 405 nm with a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA) and recorded when the healthy extract control reached a value of 0.1 (usually after 1–3 hr). A sample was considered positive if the OD_{405} for both wells measured greater than 0.5 and negative if the OD_{405} for both wells measured less than 0.2. On rare occasions, when at least one well measured between 0.2 and 0.5, the tree was resampled and retested.

RESULTS

Table 1 shows the percentage of Valencia sweet orange trees on sour orange rootstock that displayed decline and/or stunting symptoms characteristic of infection by Florida severe isolates of CTV over an 8-yr period. Symptoms were first detected 2 yr after planting, and some trees of all of the treatments were in decline within 3 yr. The percentage of disease gradually increased for all the treatments; by year 8, three-fourths of the trees were affected. The percentages of diseased trees within any given year were not significantly different between treatments (Duncan's multiple range test, $P = 0.05$) indicating that none of the four mild isolates prevented or delayed CTV-induced decline.

Eight years after planting, the trees were evaluated visually by symptom type (Table 2) and by trunk circumference. Those trees rated with symptoms of stunting and decline, stunting, decline, or no symptoms had trunk circumferences (cm) of 16.1 ± 3.7, 16.0 ± 2.1, 34.6 ± 2.5, and 32.1 ± 1.6, respectively. Results showed that none of the four mild CTV isolates tested protected trees against development of decline symptoms. There was, however, significantly less stunting in trees initially infected with three of the mild isolates of CTV.

Each tree was also evaluated immunologically using two monoclonal antibodies previously reported to distinguish mild from severe isolates of CTV (12) (Table 3). Extracts from all of the trees, including those initially free of CTV, reacted with the nondiscriminating 3DF1 antibody, indicating that after 8 yr, all of the previously uninfected trees were

Table 1. Percentage of Valencia sweet orange trees on sour orange rootstock showing severe decline or stunting symptoms after preinoculation with mild isolates of citrus tristeza virus

Mild isolate[y]	Years after planting[z]						
	2	3	4	5	6	7	8
DD102bb	2	6	14	28	38	53	75
Guettler HS	0	4	19	22	40	40	76
DPI 136-53-4	3	8	22	27	40	40	74
DPI 1-12-5-X-E	1	6	23	25	42	58	73
None	5	10	33	39	47	63	86

[y] Each of 64 trees was propagated on sour orange rootstock using buds from Valencia sweet orange trees that had previously been infected with a specific mild isolate of CTV or no virus using bark chips.

[z] The percentage of 64 trees showing disease symptoms typical of infection with severe Florida isolates of CTV. None of the comparisons within any given year are significantly different ($P = 0.05$).

Table 2. Symptoms in Valencia sweet orange trees on sour orange rootstock cross-protected by mild strains of citrus tristeza virus 8 yr after planting

Protecting strain[y]	Symptoms (%)[z]			
	Stunting and decline	Decline	Stunting	Healthy
DD102bb	10 ab	42 a	23 ab	25 a
Guettler HS	14 a	47 a	15 a	24 a
DPI 136-53-4	14 a	40 a	20 a	26 a
DPI 1-12-5-X-E	7 b	47 a	19 b	27 a
None	13 a	43 a	30 b	14 a

[y] Each of 64 trees was propagated on sour orange rootstock using buds from Valencia sweet orange trees that had previously been infected with a specific mild isolate of CTV or no virus using bark patches.

[z] The percentage of 64 trees that showed various symptoms 8 yr after planting. Numbers followed by the letter "a" are significantly different from numbers in the same column followed by the letter "b" ($P = 0.05$). Trees with stunting and decline, decline, stunting, and no symptoms (healthy) had mean trunk circumferences (cm) of 16.1 ± 3.7, 16.0 ± 2.1, 34.6 ± 2.5, and 32.1 ± 1.6, respectively.

infected with at least one mild isolate of CTV. One hundred percent of the trees with both stunting and decline tested positive for severe CTV by reaction with the discriminating MCA13 antibody. Sixty-seven of 68 trees with stunting reacted with the MCA13 antibody; one tree did not. This tree may have a stunting isolate of CTV that does not react with the MCA13 antibody or it may be stunted for another reason. Ninety-two percent of the trees with decline symptoms tested positive for severe CTV. The 11 trees with characteristic CTV-induced decline symptoms that did not react with the MCA13 antibody probably are infected with severe CTV isolates that lack the epitope, recognized by MCA13, which is shared by most severe CTV isolates. It cannot be ruled out that their decline had alternative causes. Fourteen trees with no symptoms of infection by severe isolates of CTV reacted with the MCA13 antibody. These trees were most likely recent infections that have not yet developed symptoms. The results (Table 3) confirm that severe Florida isolates of CTV will superinfect trees previously infected with Florida mild isolates of CTV.

Documentation of the breakdown of cross-protection in sweet orange trees on sour orange rootstock infected with Florida mild isolates of CTV is summarized in Table 4. The percentage of trees that became infected with severe isolates of CTV is not significantly different ($P = 0.5$) between trees infected with four mild isolates of CTV and trees that initially did not contain any mild isolate of CTV, as judged by either symptoms or reaction with severe strain discriminating antibody.

DISCUSSION

Four Florida mild isolates of CTV clearly failed to protect sweet on sour orange trees from Florida severe isolates of CTV. This was demonstrated both by development of decline and/or stunting symptoms, as well as detection of severe CTV isolates in protected trees with monoclonal antibody. The reason for this breakdown in cross-protection is unknown. It is known that the effectiveness of field cross-protection against papaya ringspot virus can be dependent on disease pressure (16). Hot weather can prevent mild CTV from moving into new flushes and exposing unprotected tissue to infection (10,14). However, it remains unclear why mild CTV isolates propagated from sweet orange trees on sour orange rootstock, which have remained healthy for up to 20 yr in areas where severe CTV-induced disease is prevalent, do not effectively protect other sweet on sour orange trees grown under similar conditions.

One of the problems of conducting a field experiment with a highly mobile pathogen is that the treatments become confounded. Within 8 yr, all of the control trees that were originally free of CTV became infected. Some of the control trees were infected with mild CTV isolates and some were infected with severe or severe and mild isolates. It is currently not possible to distinguish between the two latter conditions because a probe that specifically reacts with only mild isolates of CTV is not available. Thus, although our results clearly demonstrate the rapid superinfection of trees containing at least one of four mild isolates of CTV with severe CTV and the consequent rendering of all treatments unproductive, it cannot be ruled out that mild strain(s) infection delayed severe strain superinfection and symptom development. If trees could have been maintained free of mild CTV throughout the experiment, they may have declined faster than trees previously infected with mild isolates.

Most previous reports of successful field protection by mild isolates of CTV have been against stem-pitting CTV (4,5). The biology and physiology of disease induced by these isolates, which are exotic to Florida and affect all rootstocks, are quite different from the decline and stunting induced by isolates that are endemic to Florida and affect only trees on sour orange or *C. macrophylla* rootstocks. These Florida cross-protection results do not contradict results from other parts of the world.

The ELISA results demonstrate the use of the MCA13 antibody for distinguishing between dwarfing or decline-inducing isolates of CTV and mild isolates of CTV under field conditions. ELISA, using this antibody, had a success rate of at least 99% for dwarfing isolates and at least 92% for decline-inducing isolates. This antibody will be useful in future cross-protection studies by enabling early, rapid, preliminary mild strain evaluation.

The MCA13 antibody was also used to unequivocally show that superinfection occurred and that this superinfection correlated with severe symptoms. It is not known whether superinfection of trees containing mild CTV isolates by severe CTV isolates is limited to cells or flushes that are free of the mild isolate or whether both isolates can multiply in the same cell. The answer to this question has important implications both for genetically engineered protection and CTV evolution. Trees with mixed infections currently are being

Table 3. Reaction of Valencia sweet orange trees on sour orange rootstock displaying various symptoms with monoclonal antibodies to citrus tristeza virus 8 yr after planting

	Antibody reaction[w]		
Symptom[v]	MCA13+ 3DF1+[x]	MCA13− 3DF1+[y]	MCA13− 3DF1−[z]
Stunting and decline	37/37	0/37	0/37
Stunting	67/68	1/68	0/68
Decline	129/140	11/140	0/140
Healthy	14/75	61/75	0/75

[v] Trees with stunting and decline, stunting, decline, and no symptoms (healthy) had mean trunk circumferences (cm) of 16.1 ± 3.7, 16.0 ± 2.1, 34.6 ± 2.5, and 32.1 ± 1.6, respectively.
[w] The reaction of bark tissue of trees with various symptoms with monoclonal antibodies 8 yr after planting.
[x] Number of trees whose bark extracts reacted with both MCA13 and 3DF1 antibodies over the total number tested. Most Florida severe strains of CTV react with MCA13. All known Florida mild strains of CTV react with 3DF1 but not MCA13.
[y] Number of trees of which bark extracts reacted with 3DF1 antibody but not MCA13 antibody over the total number tested.
[z] Number of trees of which bark extracts reacted with neither MCA13 antibody nor 3DF1 antibody over the total number tested.

Table 4. Percentage of Valencia sweet orange trees on sour orange rootstock originally inoculated with various mild isolates of citrus tristeza virus (CTV) that have been superinfected by severe CTV isolates as judged by symptoms and antibody reaction

	Infection by severe CTV (%)	
Protecting strain[x]	Symptoms[y]	MCA13[z]
DD102bb	75	78
Guettler HS	76	72
DPI 136-53-4	74	72
DPI 1-12-5-X-E	73	75
None	86	87

[x] Each of 64 trees was propagated on sour orange rootstock using buds from Valencia sweet orange trees that had previously been infected with a specific mild isolate of CTV using bark patches.
[y] Percentage of trees showing thin foliage, dwarfing, or both. The numbers are not significantly different ($P = 0.05$).
[z] Percentage of trees with bark extracts which reacted with MCA13 antibody. The numbers are not significantly different ($P = 0.05$).

examined at the cellular level.

This experiment does not eliminate cross-protection as an approach to control of CTV-induced sweet on sour decline in Florida. Some naturally occurring or engineered mild strains may be effective as part of an integrated management strategy. Experiments are currently in progress to evaluate other mild isolates of CTV in the field by monitoring severe and mild strain spread over a period of several years.

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