Possible Resistance to *Citrus tristeza virus* in Red Shaddock

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ABSTRACT. Resistance to systemic infection by CTV occurs in trifoliate orange and in some hybrids of trifoliate orange and *Citrus* spp. A previous study indicated that pummelo and hybrids of grapefruit and trifoliate orange express resistance against some isolates of CTV, while other isolates can systemically infect these hosts. Since the isolates of CTV unable to replicate in these hosts were genetically similar to one another, seedlings of Red Shaddock pummelo were inoculated with single or mixed infections of isolates of CTV characterized as similar to the genetically distinct T30, T36, and VT isolates. Serological and molecular analysis showed that CTV with the T30 genotype systemically infected three of 17 inoculated seedlings, and CTV with the VT genotype systemically infected seven of 14 inoculated seedlings. In contrast, CTV with the T36 genotype systemically infected 17 of 17 inoculated seedlings. For both VT and T30 genotypes, systemic infection appeared delayed relative to inoculations to sweet orange control plants and to T36. The replication of the T36 genotype in all inoculated seedlings indicates a lack of resistance to the T36 genotype. The apparent lack of systemic infection of the T30 and VT genotypes in some inoculated seedlings indicated that resistance is expressed in some 'Red Shaddock' seedlings, but that there is variability in the expression of resistance among these seedlings.

Citrus tristeza virus (CTV) is a globally distributed pathogen of citrus. Infection with certain strains of the virus is associated with economically important diseases such as decline, an incompatibility occurring with various scions grafted on sour orange rootstock, and stem-pitting, a disease characterized by the development of pits in the wood of twigs, small and large lateral branches and also the main trunk, as well as reduction of the growth of the tree and a decline in fruit yield, fruit size and quality in severe cases (15).

Resistance to CTV infection has been demonstrated for *Citrus* relatives such as *Severinia buxifolia* and *Swinglea glutinosa* and trifoliate orange (8). Trifoliate orange expresses a general resistance to systemic infection by CTV *via* both graft inoculation with infected budwood and by inoculation with aphids. Recent work demonstrated that CTV replicates in trifoliate orange protoplasts, indicating that whole plant resistance might be due to restricted cell-to-cell or long distance virus movement, or possibly to an induced resistance mechanism (2). The trifoliate orange resistance gene has been introduced into species in the genus *Citrus* by sexual hybridization, creating hybrids such as citrumelos and citranges that are used extensively as rootstocks in many citrus growing areas. The trifoliate orange resistance gene also has been introduced into advanced scion breeding lines as a means of introducing this gene into economically important citrus lines including scion cultivars (3). Progress also has been made in the identification and isolation of the trifoliate orange resistance gene(s), providing a pathway of introduction of only the resistance gene directly into important citrus varieties by plant transformation and regeneration methodologies (4, 19).

Recent work showed that the intergeneric hybrid, Swingle citrumelo, and also varieties of pummelo express resistance effective against some isolates of CTV, but are susceptible to infection by others (5, 10). The studies undertaken by Fang and Roose (5) with "Chandler" pummelo used the California CTV strain T-514 as challenge inoculum, whereas the studies by Garnsey et

al. (10) tested seven isolates of CTV (B2, B3, B28 B31, B51, B52 and B280) from the USDA Exotic Citrus Pathogen Collection at Beltsville, MD (9) for their ability to systemically infect six pummelo varieties as well as Swingle citrumelo.

Recent analyses of the sequences of several isolates of CTV have shown that CTV isolates are genetically distinct (1, 7, 12, 17, 18) and can be distinguished by molecular genetic marker-based methods which assign a "genotype" to an unknown isolate by comparison of the marker profile to those of designated standard isolates (6, 7).

The aforementioned studies on resistance to CTV used isolates without knowledge of the isolate genotype. Marker analysis of isolates used in studies with pummelo and Swingle citrumelo (10) indicated these isolates had a T30 genotype (isolates B2, B51, B52), T36 genotype (B3), T3 genotype (B31) and non-standard genotypes (B280, B28). Studies on Chandler pummelo (5) used a T30 genotype, isolate T-514 (Hilf, unpublished data).

To assess the prevalence of selective resistance in pummelo and to determine if isolates with the same genotype are affected similarly by selective resistance, we selected nine independent Florida CTV isolates based on their assessed genotypes and inoculated these into small populations of seedlings of the pummelo cultivar Red Shaddock. We postulated that isolates which had the same genotype would behave similarly in the Red Shaddock seedlings. We report here the results of preliminary experiments to predict isolate behavior in pummelo hosts based upon viral genotype.

MATERIALS AND METHODS

Virus inoculum and plant inoculations. *In planta* cultures of CTV isolates FL-191, T-26, FL-188, T-30, FL-120, T-36, FL-207, FL-205 and FL-201 were maintained in seedlings of the sweet orange cultivar Madam Vinous under glasshouse conditions. Table 1 summarizes pertinent information on CTV isolates used in this study. Seeds of Red Shaddock pummelo were obtained from the Bureau of Citrus Budwood Registration, Dept. of Plant Industry, Florida Division of Consumer Services, Winter Haven, Florida, and planted in soil mix in a greenhouse for germination and growth under an appropriate temperature regime. Inoculation of individual seedlings with CTV was by budding with a mixture of three budeyes or blind-buds per plant. Grafts

TABLE 1
ORIGIN, MCA13 STATUS AND GENOTYPE OF CTV ISOLATES INOCULATED INTO RED SHADDOCK SEEDLINGS

Isolate	Origin	MCA13 Status	Genotype[4]
T26	Valencia	[-]	T30
T30	Mexican lime	[-]	T30
T36	Hamlin	[+]	T36
FL 120	Meyer lemon[1]	[+]	T36+VT
FL 188	Meyer lemon[2]	[+]	T30+T36
FL 191	Meyer lemon[2]	[+]	T30+T36
FL 201	Meyer lemon[1]	[+]	VT
FL 205	Meyer lemon[1]	[+]	VT
FL 207	Meyer lemon[3]	[+]	T36

[1]Commercial Meyer lemon propagations of virus-infected budwood.
[2]Commercial Meyer lemon propagations of virus-free budwood.
[3]Dooryard tree, original virus status unknown.
[4]Genotype determined by marker analysis using procedure of Hilf et al. (6).

were kept wrapped for 3 weeks and were observed for 1 week more. Plants with two or more live grafts after four weeks were included in the study. One set of plants was inoculated in April, 2003 and a second, smaller set of plants was inoculated in July, 2003.

Serological detection of CTV. Unless otherwise indicated, samples for serological testing for CTV were 0.5 g of petioles harvested from near to fully or fully expanded leaves of new growth. 'Red Shaddock' seedlings inoculated in April, 2003 were tested by ELISA at 62, 133 and 337 days post-inoculation (dpi). Seedlings inoculated in July, 2003 were tested at 54 and 256 dpi. ELISA was done within 24 to 48 h after collection. Samples for ELISA were stored at 4°C prior to analysis.

Samples for ELISA were pulverized in 5 ml of PBS-Tween, using a Kleco sample pulverizer (Kinetic Laboratory Equipment Co., Visalia, CA). The presence of CTV was determined by double antibody sandwich enzyme-linked-immunosorbent assay (DAS-ELISA) using a polyclonal coating antibody of purified IgG at 1 µg/ml. Detection was accomplished with a mixture of the monoclonal antibodies 3E10 and 11B1 (16) and the monoclonal antibody MCA13 was used for differentiation of isolates (14). Goat anti-mouse IgG conjugated to alkaline phosphatase was used to detect bound anti-CTV monoclonal antibodies (Sigma, St. Louis, MO). Lyophilized extracts from T36 and T30 infected Madam Vinous seedlings were used as positive controls for CTV detection with the mixture of 3E10 and 11B1 monoclonal antibodies and as positive and negative controls, respectively, for MCA 13. Lyophilized extracts from healthy (uninoculated) Madam Vinous seedlings were used as negative controls for ELISA.

CTV marker analysis of infected Red Shaddock seedlings. An immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) procedure used for genetic marker analysis of CTV-infected citrus tissue was performed as previously described (6, 7). Extracts for marker analysis were obtained from fresh or desiccated tissue prepared as for ELISA. Desiccated samples were re-hydrated in PBS-Tween for 10 min prior to extraction. Virions were immunocaptured from this extract using CTV polyclonal IgG attached to magnetic beads coated with goat anti-rabbit antibodies (Dynal, Lake Success, NY). Sequence specific primers (Table 2) for amplification of markers from the T36, T30 and VT genomes were derived from Genbank accessions AY170468, AF260651 and U56902 respectively. The markers T30-1, T30-2, VT-1, VT-3, T36-1 and T36-2 have not been reported previously. Primers to amplify these markers were derived from the aforementioned genomic CTV sequences and were used for marker characterization in place of the sequence selective 5' and k17 markers reported previously in conjunction with the pol markers (6, 7). Used in conjunction with the pol markers (Table 2), we found the newer markers to be as reliable for selective amplification for isolate characterization as the previously reported 5' and k17 markers (unpublished data).

For reverse transcription and amplification of sequence specific CTV molecular markers, 5 µl of CTV cDNA were used as template in a 25 µl reaction volume containing a 1× concentration of reaction buffer (supplied by Promega Corp., Madison, WI), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µm of each primer and 0.625 units of Taq polymerase (Promega Corp). Amplification profiles were 94°C for 30 s, 56°C for 60 s and 72°C for 60 s for 30 cycles. Reactions were incubated an additional 10 min at 72°C prior to maintenance at 4°C until analysis. Reaction products were analyzed on 1.5% agarose gels containing ethidium bromide at 200 ng per ml and run in TAE buffer.

TABLE 2
NUCLEOTIDE SEQUENCE AND GENOMIC LOCATION OF SEQUENCE SPECIFIC OLIGONUCLEOTIDES USED FOR GENETIC MARKER ANALYSIS OF CTV ISOLATES

Marker designation	Amplified region[1]	Oligonucleotide sequences (5'-3')	
T30-1	22-592	gtatctccggagctcgatc	[+]
		cagtagggtcaactagtttcgc	[-]
T30-2	792-1635	tacggcttggtgctctgaggcc	[+]
		acgcctgcgaaccgccgac	[-]
T30pol	10,772-11,467	gatgctagcgatggtcaaat	[+]
		ctcagctcgctttctcacat	[-]
VT-1	19-583	gtaccctccggaaatcacg	[+]
		ggtagggtctactcgtttcat	[-]
VT-3	2246-3070	caggtgagaattctccatcgt	[+]
		agaatcaggcaaacgccc	[-]
VTpol	10,745-11,440	gacgctagcgatggtcaagc	[+]
		ctcggctcgctttcttacgt	[-]
T36-1	68-662	agcctttaagctctaatatt	[+]
		accaagtcggctgtttcgtc	[-]
T36-2	855-1618	aaactgatttctccactcag	[+]
		acaatcgagccaggaacactg	[-]
T36pol	10,791-11,508	tgacgctaacgacgataacg	[+]
		accctcggcttgttttcttatg	[-]

[1]T36, T30 and VT primer sequences and marker positions correspond to Genbank accessions AY170468, AF260651 and U56902 respectively.

RESULTS AND DISCUSSION

Table 3 summarizes the results of serological and molecular analysis of inoculated Red Shaddock seedlings. Presence or absence of virus based upon ELISA with appropriate controls is indicated by "+" and "-", respectively. Genotype determination of CTV in Red Shaddock seedlings was done as outlined in Materials and Methods using markers amplified from the genomic regions indicated in Table 2.

Virus with the T30 genotype was inoculated into Red Shaddock seedlings individually (isolates T26, T30) or as part of a mixture with the T36 genotype (isolates FL 188 and FL 191). Seedlings 1030, 1117 and 1037 were three of seventeen Red Shaddock seedlings which became systemically infected by CTV with the T30 genotype (Table 3). The three seedlings 1030, 1031 and 1032 were inoculated at the same time, but only 1030 had become infected by 337 dpi. Seedling 1030 did not have CTV detectable by ELISA when tested at 62 and 133 dpi. Marker analysis indicated that CTV in seedling 1030 had the same marker profile as the parent source (Table 3). Three Madam Vinous seedlings budded similarly from the same source plant had virus detectable by ELISA by 56 dpi (data not presented).

CTV was detected in seedling 1117 by 256 dpi, but not at 62 dpi. It was not clear when the T30 genotype would have been detected by ELISA in seedling 1037, since the T30 and T36 genotypes cannot be serologically distinguished in a mixed infection and the marker analysis was not performed until near the last date for ELISA at 337 dpi. None of the fourteen other seedlings inoculated with the T30 genotype had virus detected by ELISA during the course of the experiment. Marker analysis was not performed on these

TABLE 3
SUMMARY OF REPLICATION OF DIFFERENT CTV GENOTYPES IN RED SHADDOCK SEEDLINGS

Isolate	Genotype	Seedling[1]	MMC/MCA13[2]	DPI[3]	Genotype[4]
T26	T30	1030	+/-	337	T30
		1031	-/-		n/d
		1032	-/-		n/d
T30	T30	1045	-/-		n/d
		1046	-/-		n/d
		1047	-/-		n/d
		1117	+/-	256	T30
		1118	-/-		n/d
		1119	-/-		n/d
T36	T36	1027	+/+	133	T36
		1028	+/+	62	T36
		1029	+/+	62	T36
FL 120	VT+T36	1024	+/+	62	T36
		1025	+/+	62	VT+T36
		1026	+/+	62	VT+T36
FL 188	T30+T36	1036	+/+	62	T36
		1037	+/+	62	T30+T36
		1038	+/+	62	T36
FL 191	T30+T36	1021	+/+	133	T36
		1022	+/+	62	T36
		1023	+/+	133	T36
		1120	+/+	256	T36
		1121	+/+	256	T36
FL 201	VT	1048	-/-		n/d
		1049	-/-		n/d
		1050	-/-		n/d
		1123	-/-		n/d
		1124	+/+	256	VT
		1125	+/+	256	n/d
FL 205	VT	1033	+/-	337	VT
		1034	+/+	133	VT
		1035	-/-		n/d
		1114	+/-	256	VT
		1115	+/+	256	VT
FL 207	T36	1039	+/+	62	T36
		1040	+/+	62	T36
		1041	+/+	62	T36

[1]Seedling numbers refer to a database record.
[2]Reaction to the mixture of monoclonal antibodies 3E10 and 11B1 and the single monoclonal MCA13.
[3]Days post-inoculation at which virus is first detected by ELISA.
[4]Genotype determined by sequence specific genetic markers. n/d indicates genotyping not done.

seedlings, so it is not clear if virus of the T30 genotype was present at a lower titer and could have been detected by PCR, a potentially more sensitive detection method.

These results are similar to previous findings that showed that CTV with the T30 genotype replicated in propagations of only two of six pummelo varieties tested (10) and systemically infected only a proportion of the progeny of crosses between 'Chandler' pummelo and trifoliate orange (5).

In contrast, CTV with the T36 genotype systemically infected all seventeen 'Red Shaddock' seedlings inoculated either singly (isolates T36 and FL 207), or as part of a mixture with either the T30 (isolates FL 188, FL 191) or VT (FL 120) genotype. In twelve of these seedlings virus was detected by ELISA as early as 62 dpi (Table 3). Seedlings 1027, 1021, 1023, 1120 and 1121 did not have detectable CTV as early as other Red Shaddock seedlings inoculated at the same time, but the seedlings most likely were systemically infected prior to the times indicated in Table 3. The apparent delay in systemic spread in these seedlings could be a characteristic of the seedling or could be due to variability in spread of the virus from the grafted inoculum budwood. Madam Vinous sweet orange seedlings inoculated from the same source plants had virus detected by ELISA at 56 dpi (data not shown).

An earlier study found that the T36 genotype systemically infected five of six pummelo propagations tested, as well as the intergeneric grapefruit hybrid Swingle citrumelo (10). Swingle is an artificial hybrid derived from a cross between grapefruit and trifoliate orange. Grapefruit is postulated to be derived from a natural hybridization of sweet orange and pummelo (13), so it is possible that seedlings of Swingle might show the selective resistance expressed by some pummelo varieties.

CTV with the VT genotype systemically infected seven of fourteen 'Red Shaddock' seedlings, whether inoculated singly (isolates FL 201 and FL 205) or as part of a mixture with the T36 genotype (isolate FL 120) (Table 3). In seedlings inoculated with only the VT genotype, systemic infection seemed delayed relative to the T36 genotype, as 133 dpi was the earliest the VT genotype was detected by ELISA in any of the seedlings. However, ELISA could not distinguish between the T36 and VT genotypes in seedlings 1025 and 1026, so it is not clear when the VT genotype systemically infected these seedlings. Madam Vinous seedlings inoculated from the same source plants had detectable levels of virus at 56 dpi (data not presented).

Marker analysis indicated that seedlings 1033 and 1114 were infected with CTV with the VT genotype, even though virus samples from these seedlings were not reactive with MCA13, as expected. Positive and negative ELISA controls for MCA13 reacted as expected and virus samples from Madam Vinous seedlings inoculated from the same source plants also reacted with MCA13 as expected (data not presented). Errors in sample preparation or handling could account for the lack of expected serological reactivity of these two samples.

All Red Shaddock seedlings inoculated with the T36 genotype were systemically infected by the end of the experiment. The majority were systemically infected by approximately 60 dpi, a period equivalent to that for systemic infection of Madam Vinous sweet orange seedlings. In contrast to the T36 genotype, both the T30 and VT genotypes showed either delayed systemic infection or systemic infection was not detected in Red Shaddock seedlings, while systemic infection of Madam Vinous seedlings with the same genotypes occurred within a 2-mo period. Studies indicated that the VT and T30 genotypes are more closely related to one another than either is to the T36 genotype (7). A common phylogeny for the T30 and VT genotypes might explain why they behaved similarly in Red Shaddock seedlings.

The lack of uniformity in the response of Red Shaddock seedlings to infection by VT and T30 genotypes suggests segregation for this resistance among seedlings. Since pummelos are open-pollinated and exhibit monoembryony (11), genetic variability of specific traits in seedlings is a reasonable finding. Resis-

tance has already been demonstrated in Chandler pummelo (5), and it would be interesting to determine if resistance is expressed in other pummelo varieties. Also, a revised experimental approach that uses budwood from healthy pummelo seedlings grafted to a CTV-infected rootstock, as was done in a previous study (10), would be a preferred method to the inoculation of individual seedlings, since this identifies susceptible and resistant seedlings and allows the maintenance of a virus-free source of budwood. In conclusion, the evidence corroborates earlier reports of CTV resistance in pummelo varieties and demonstrates that the CTV genotype is an important factor to consider when assessing CTV behavior in pummelo and possibly other citrus.

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