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Molecular Characterization of Florida Citrus Tristeza Virus Isolates with Potential Use in Mild Strain Cross Protection

F. M. Ochoa, B. Cevik, V. J. Febres, C. L. Niblett, and R. F. Lee

ABSTRACT. Mild strain cross protection (MSCP) is an important strategy for maintaining citrus production in Florida. Recent surveys indicate that severe strains of citrus tristeza virus (CTV) are spreading following the arrival of the brown citrus aphid in 1995, thus, increasing the threat of CTV to the Florida industry. It is desirable to be able to detect and select mild strains for further evaluation as protective isolates with potential for MSCP. Recent progress on the characterization of the CTV genome has led to the development of several methods to differentiate strains, which previously could only be identified by time-consuming bioassays. In addition to the serological differentiation using MCA-13 monoclonal antibody for the selective detection of severe strains, recently developed methods are based on the major and minor coat protein gene sequences: strain specific probes and single stranded conformational polymorphism assays. These methods were applied to characterize isolates of CTV and aphid transmitted sub-isolates to identify the presence of mixed infections containing both mild and severe strains. We report here the screening of 20 CTV isolates collected from surviving trees in areas where CTV was causing decline on sour orange rootstock. The results indicate that 10 isolates were mild strains (T30-like), five were decline strains (T36-like), and five isolates were mixtures containing both mild and decline strains or mild and stem pitting strains of CTV.

Index words. Citrus tristeza virus, mild strain cross protection.

There are an estimated 15 million trees on sour orange rootstock in Florida (34) which are highly susceptible to decline induced by strains of citrus tristeza virus causing decline on sour orange (CTV-D). Previous work has demonstrated that some mild strains of CTV can minimize the symptoms induced by stem pitting strains (CTV-SP) when the mild strain is inoculated into the plant before the severe strain, a phenomenon often referred to as mild strain cross protection (MSCP) (6, 8, 11, 16, 23, 28). MSCP using selected mild strains has been shown to be useful to prolong the life expectancy of trees on sour orange rootstock in Florida (29, 35). The development of a better understanding of the basis of MSCP is an important long-term goal for continued protection of citrus from CTV in Florida. However, the selection of mild strains which are useful for MSCP has been an empirical and time-consuming process. While there are many mild CTV strains, only a few have any cross protection ability, about one out of 200 to 300

(14, 16, R. Lee and G. Müller, pers. comm.). In Florida, CTV mild strains for MSCP have been collected, mostly from surviving trees on sour orange rootstock from areas where CTV losses have been severe (14, 28). Five mild CTV strains have been extensively tested under field conditions in Florida (14, 28, 29, 31).

A better understanding of the CTV genome has resulted in progress being made on the development of rapid methods of CTV strain differentiation. This progress has been reviewed recently (15). A monoclonal antibody (MCA-13) has been selected that reacts selectively with most severe CTV strains, including CTV-D and CTV-SP strains (24, 27). MCA-13 has been useful in Florida and the Caribbean Basin to determine if a CTV isolate contains a severe strain (32). However, isolates have been found which contain CTV-SP, yet the isolate does not react against MCA-13 (2). Biological indexing (10, 14) thus provides the ultimate determination of the biological activity of a CTV isolate. Recently, methods for strain

differentiation of CTV have been developed based on nucleic acid sequence differences in the coat protein gene. The strain specific probe (SSP) method (4, 5) allows the differentiation of strains and enables detection of mixed infections containing severe CTV strains that may not be detected with serological testing or in the biological indexing. The SSP methodology is based upon the micro-heterogeneity of the coat protein gene and uses non-radioactive oligonucleotide probes (4, 5). Another method that has been useful to differentiate CTV strains has been single-strand conformation polymorphism (SSCP) of the PCR products of the coat protein (CP) or p27 gene (7, 33).

The purpose of this study was to evaluate the newly developed methods of SSP and SSCP for CTV strain differentiation to detect the presence of severe CTV strains in the isolates collected for evaluation for MSCP, and in single aphid transmitted sub-isolates from the original sources using the brown citrus aphid, *Toxoptera citricida*.

MATERIAL AND METHODS

CTV isolates. The isolates characterized in this study were collected from surviving trees on sour orange rootstock from areas where CTV caused severe decline in commercial groves in Florida. The isolates were maintained as *in planta* cultures in Madame Vinous sweet orange at the University of Florida's Citrus Research and Education Center (CREC), Lake Alfred. The selection and collection procedure has been described recently (13) and is summarized here in brief. The samples were collected from many surviving trees from areas where trees were being debilitated by CTV decline on sour orange rootstock. The field isolates were then established *in planta* on several plants, and some of these plants were graft

challenged with several very severe Florida CTV strains, with the challenge buds remaining in the plant. If the challenged plant continues to grow for 2 to 3 flushes, the respective *in planta* culture for that isolate which has not been challenged is selected to evaluation for MSCP. The selected *in planta* isolates were also used as the source for single aphid transmissions using *T. citricida* being subjected to additional rounds of single aphid transmission with a 24-h acquisition feeding and a 24-h inoculation feed. These aphid-transmitted sub-isolates were designated with a one hundred number followed by an extension number. The CTV strains T36 (CTV-D) and T30 (CTV-mild) were used as controls for reference.

Indirect ELISA. Fresh, tender bark tissue samples were pulverized in liquid nitrogen and stored at -80°C until needed. Double antibody sandwich (DAS)-indirect ELISA assays were performed as previously described (9). Rabbit polyclonal antibodies, CREC 1052 (21) and CREC 1053 (31) were obtained from the CREC antisera collection, and were used for coating. Goat polyclonal antibody G604 (R.F. Lee and K.L. Manjunath, unpublished) and the monoclonal antibody MCA-13 (27) were used as secondary antibodies for broad spectrum and selective detection of severe CTV strains, respectively. Anti-goat and anti-mouse antibody-alkaline phosphatase conjugates (Sigma, St. Louis, MO.) were used as appropriate. The OD_{405nm} values three times that of the healthy was considered to be positive for CTV. Some ELISA plates were stored at -20°C for later use with immunocapture reverse-transcriptase-PCR (IC-RT-PCR) (22).

Extraction of nucleic acids, IC-RT-PCR. RNA extracts from fresh tissue were obtained by phenol: chloroform extraction and spin-column chromatography in Sephadex G-50 as described by Pappu et al.

(25). The sample consisted of about 1 cm² of leaf tissue (petiole or bark) pulverized in liquid nitrogen before addition of 0.6 ml of extraction buffer (10% SDS, 1.0 M Tris pH8, 0.5 M EDTA) and an equal volume of phenol: chloroform (1:1 v/v). The samples were incubated at 70°C for 5 min, then centrifuged 5 min at 14,000 rpm at room temperature. The supernatant was collected and a 17.5 µl aliquot used in a 50 µl RT-PCR reaction (10mM Tris HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 10 mM dithiothreitol; 2.5 mM MgCl₂; 0.1 M dNTPs and 100 pM of each primer). Occasionally, 25 µl reactions were performed by halving all the reaction component volumes. An alternate method to amplify CP and p27 CTV genes in a single reaction was sometimes used. These two genes are contiguous in the CTV genome. In this procedure, the CTV virions were first trapped onto microtiter plates and subsequently used for RT-PCR according to Nolasco et al. (22), with modifications. After performing the DAS-indirect ELISA, the plates (Immulon 2 HB, Dynex Technologies, Chantilly, VA) were washed twice each with PBS-T, then sterile distilled water and stored at -20°C. For IC-RT-PCR, the RT reaction was performed in the plate wells by adding 45 µl of disruption buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 10 mM dithiothreitol; 2.5 mM MgCl₂, 0.1 M dNTPs and 100 pM of each primer). The plates were then incubated at 70°C for 5 min, then allowed to cool to room

temperature. The following enzyme mixture was added: 10 U Avian Myeloblastosis virus reverse transcriptase (Promega, Madison, WI), 2.5 U Taq polymerase (Promega), and 20 U RNasin (Promega) in a final volume of 5 µl, and the plates incubated at 42°C for 45 min. The mixture was transferred to PCR tubes and amplified for 40 cycles at 90°C, 50°C, 72°C for 1 min each, and 10 min at 72°C for the final extension. The specific primers used for the CP gene (CN119 and CN120) and the p27 gene (CN167 and CN168) are described in Table 1. When the CP and p27 genes were amplified together, primers CN119 and CN168 were used. The RT-PCR products were separated in agarose gels and photographed.

Hybridization and detection by strain specific probes (SSP).

The CP-PCR products were blotted to a nylon membrane and fixed by UV cross linking (UV Stratalinker 1800, Stratagene, La Jolla, CA). After pre-hybridization, 50 ng of the desired oligonucleotide probe (with a biotin label) was added, and the hybridization conducted for 1 hr at 37°C with gentle agitation. After washing at the appropriate temperatures (50°C for probe I, 58°C for probe V, and 48°C for probe VI and VII), the membrane was then incubated in 0.1 ml/cm² of streptavidin-horseradish peroxidase (SA-HRP) conjugate with gentle agitation for 45min., rinsed and incubated with Super Signal CL-HRP, a chemiluminescent substrate (Pierce Inc., Rockford, IL). The reactions were then visualized on X-ray film.

TABLE 1
OLIGONUCLEOTIDE PRIMERS USED FOR CP AND P27 RT-PCR AMPLIFICATION OF
CITRUS TRISTEZA VIRUS

Primer	Sequence	Gene	Product size
CN119	5'AGATCTACCATGGACGACGAAACAAAG3' (sense)	CP	672 bp
CN120	5'GAATTCGCGCCGCTCAACGTGTGTTAAATTCC3'	CP	
CN167	5'CTATAAGTACTTACCCAAATC3'	p27	723 bp
CN168	5'AAGCTTCTAGAACCATGGCAGGTTATACAGTAC3' (sense)	p27	

After each development, the probes were removed from the membrane with 0.4 N NaOH at 42°C for 30 min. The same membrane was rehybridized successively with each of the oligonucleotide probes.

Single strand conformation polymorphism analysis (SSCP).

The SSCP analysis was similar to the protocol described by Febres et al. (7) and Rubio et al. (33). Depending on the DNA concentration after PCR, the p27 products were mixed with a denaturing solution (96% formamide, 20mM EDTA, 0.005% xylene cyanol and 0.005% bromophenol blue) to a final volume of 10 µl. The samples were denatured at 100°C for 10 min, then chilled on ice for 2 to 5 min. Five-µl samples were loaded onto a 8% polyacrylamide gel (30:1 acrylamide: bis-acrylamide) prepared with TBE buffer (53 mM Tris, 53 mM boric acid, 1.5 mM EDTA) and pre-cooled overnight at 4°C in a mini Protean II unit (Bio-Rad. Laboratories, Inc., Hercules, CA). The gel was then electrophoresed at 200 V for 4 h at room temperature using TBE as the running buffer. The unit was placed at 4°C if the unit started to get too hot, the gel was then silver stained.

RESULTS

Indirect ELISA, IC-RT-PCR and RT-PCR. Serological screening using G604 and MCA-13 antibodies, for broad spectrum and severe strain detection of CTV, respectively, allowed the differentiation of the CTV isolates into two preliminary groups of either mild or severe isolates (Table 1). All isolates reacted with G604 indicating that all plants were infected with CTV. Mild isolates (101-3, 102-3, 158-3, 170-17, 175-12, 181-3, 194-3) were negative with MCA-13 but positive with G604. The isolates 4-39, 7-26, 96-3a, 96-3b, 96-4b, and 161-30 were positive with both MCA-13 and G604 indicating severe strains were present in the

isolate. Seven isolates (93-17a, 96-3c, 96-3d, 96-9-6, 138-30, 163-30, 191-26) did not react with MCA-13 suggesting they were mild or that the titer of severe strains, if present, was too low for detection at the time of the sampling. For all isolates tested, CP and p27 products were obtained by either IC-RT-PCR or/and RT-PCR, which enabled further testing with SSP and SSCP.

SSP analyses. The hybridization of the CTV-CP products with the different strain specific probes enabled the differentiation of the isolates in mild (reactivity with probes 0, VI and VII), decline (reactivity with probes 0 and I only), mixture of mild and decline strains (reactivity with probes 0, I, VI, and VII), and mixture of mild, decline and stem pitting (reactivity with probes 0, I, V, VI, and VII). All isolates reacted against the universal probe 0. Ten isolates (93-17a, 96-9-6, 101-3, 102-3, 138-3, 158-3, 163-30, 175-12, 181-3, 194-3) reacted against mild strain probes VI and VII, but not against any other probes for stem pitting strains (probes II, III, IV, and V). Five isolates (7-26, 96-3c, 96-4b, 161-30, 191-26) reacted with probe I indicating the isolates contain decline strains of CTV. Furthermore, a third group of isolates hybridizing to probes I (decline probe) and VI and VII (mild probes) (96-3a, 96-3b, 96-3d, 170-17) suggesting a mixture of decline and mild strains present in the isolates. The isolate 4-39 reacted weakly against probe I, and strongly with probes V (stem pitting probe), VI and VII suggesting a mixture of decline, stem pitting, and mild strains present in the isolate. None of the isolates tested reacted against probes II, III, IV (Fig. 1, Table 1).

SSCP patterns. The SSCP patterns of the p27 PCR products of some of the isolates were compared with the patterns obtained for the Florida strains T36 (decline), T30 (mild), and isolate B249, a Venezue-

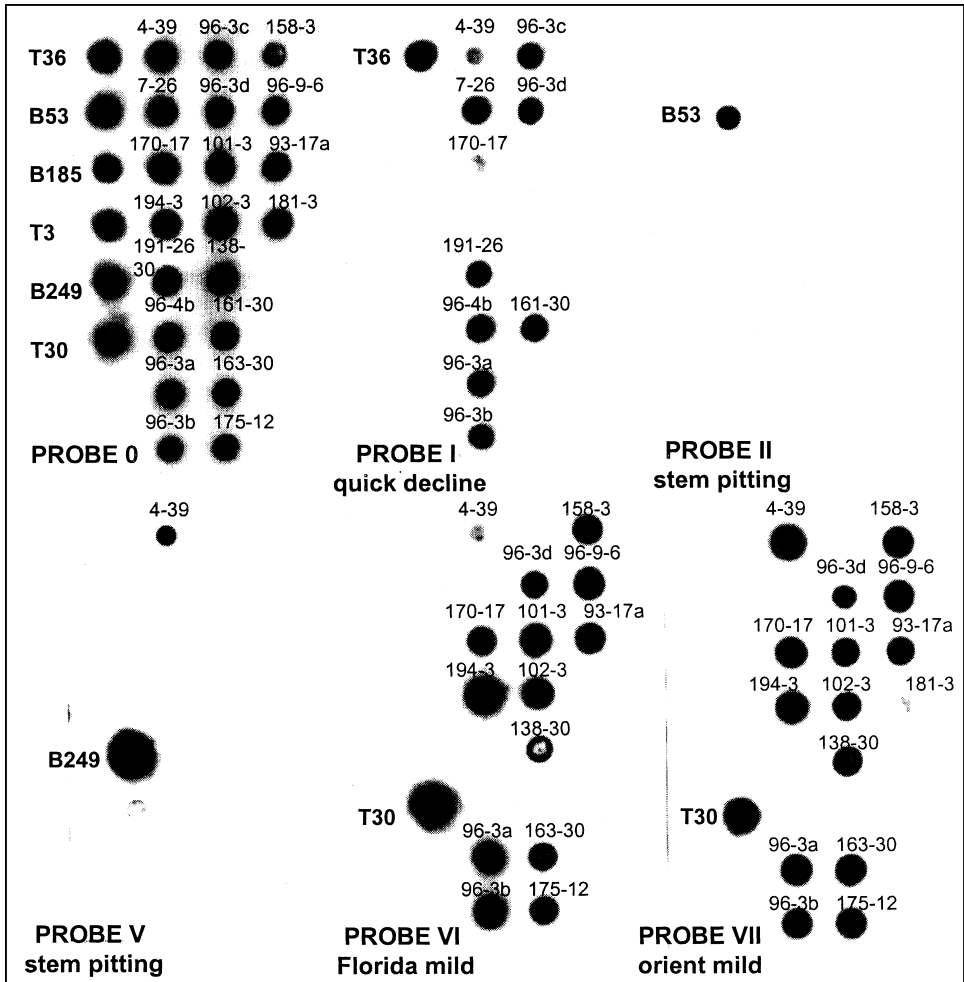


Fig. 1. Hybridization blots of Florida isolates with CP-CTV biotin-labeled probes. Probe 0, from a conserved CP region. Probe I, T36 and T66 (QD). Probe II, B1 and B53 (SP from Australia and Japan). Probe V, B128 and B249 (SP from Colombia and Venezuela). Probe VI, T26 and T30 (Florida mild). Probe VII, B188 and B215 (Orient mild).

lan CTV isolate which causes stem pitting and which reacts with SSP probe V. Isolates 93-17a, 138-3, 163-3, 170-17, 175-12, 181-3 and 194-3 had SSCP patterns typical of T30 (mild strains). Isolate 102-3 and 181-3 had SSCP patterns similar but not exactly like isolate T30, suggesting these isolates could be a different mild strain (Fig. 2).

DISCUSSION

Until recently, the selection of mild strains for use in MSCP was

entirely an empirical process. In the past few years, we have been collecting apparently mild isolates of CTV for evaluation for MSCP with the desire to select additional mild strains for MSCP before *T. citricida*, present in Florida since November 1995, spreads CTV-D and CTV-SP strains to the point where the mild strains are no longer apparent in the field. Data from Australia (3) and our experience, as reported here, suggests that it may take four sequential single aphid transmissions to obtain a "pure" strain of CTV.

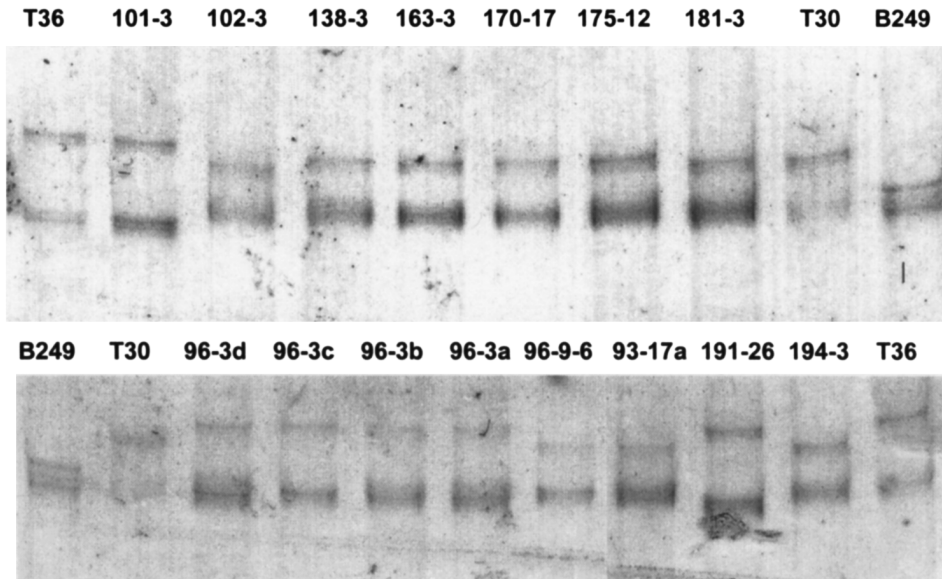


Fig. 2. SSCP patterns of the p27 gene of some Florida CTV strains. Selected controls: T36 (Florida QD), T30 (Florida Mild), B249 (Venezuela SP).

The recent advances in the knowledge of the CTV genome (4, 5, 12, 24) have resulted in the development of methods to differentiate CTV strains. In this study we have evaluated the recently developed methods of differentiation (serological differentiation of mild and decline CTV strains, PCR amplification of the coat protein gene followed by the SSP hybridization, and SSCP analysis of the p27 gene) to screen CTV isolates collected from surviving trees in areas severely affected by CTV decline on sour orange rootstock to determine if the isolates contained only mild, only severe, or mixtures of mild and severe strains. The evaluation for differentiation of CTV strains, as described in this paper, is applied for the detection of mild and severe strains from the original *in planta* culture as well as aphid-transmitted sub-isolates. We hope that application of these methods of differentiation of CTV strains will enable faster, and ultimately, better selection of mild strains, which may be useful for MSCP. It is interesting to

note that of the nine isolates tested were collected directly from horticulturally superior appearing trees, one isolate (4-39) apparently was a mixture of mild, decline, and stem pitting strains; three isolates (96-3a, 96-3b, and 96-3d) apparently were mixtures of mild and decline strains; three isolates (7-26, 96-3c, 96-4b) apparently contained only decline strains, not mild; and only two isolates (93-17a and 96-9-6) appeared to be only mild strains. Previous study (30) suggests that isolates which give a high value in MCA-13 ELISA, and moderate to high values in broad spectrum ELISA, such as isolates 7-26 and 161-30 (Table 1), probably do not contain isolates useful for MSCP. Isolates which give low to negative values in MCA-13 ELISA, such as 4-39, 96-3a, 96-3b, 96-3d, may contain mild strains useful for MSCP. This may be due to the presence of mild strains which suppress the titer of the severe strains which may be present. These results, also confirm previous reports on the use of this methodology as a diagnostic strat-

TABLE 2
SEROLOGICAL AND MOLECULAR DIFFERENCES AMONG SOME FLORIDA ISOLATES OF
CITRUS TRISTEZA VIRUS

Isolate	ELISA		Biotin-labeled probes								SSCP	
	MCA-13	G604	0	I	II	III	IV	V	VI	VII	p27	Comment
4-39	0.028	0.585	+	+/-	—	—	—	+	+/-	+	n.a.	Mixture
7-26	1.388	0.187	+	+	—	—	—	—	—	—	n.a.	QD
93-17a	0.000	0.092	+	—	—	—	—	—	+	+	T30-like	Mild
96-3a	0.031	0.253	+	+	—	—	—	—	+	+	T36-like	Mixture
96-3b	0.045	0.517	+	+	—	—	—	—	+	+	T36-like	Mixture
96-3c	0.000	0.485	+	+	—	—	—	—	—	—	T36-like	QD
96-3d	0.000	0.364	+	+	—	—	—	—	+	+	T36-like	Mixture
96-4b	0.028	0.067	+	+	—	—	—	—	—	—	n.a.	QD
96-9-6	0.000	0.019	+	—	—	—	—	—	+	+	T30-like	Mild
101-3	0.000	0.396	+	—	—	—	—	—	+	+	T30-like	Mild
102-3	0.006	0.637	+	—	—	—	—	—	+	+	T30-like	Mild
138-30	0.000	0.260	+	—	—	—	—	—	+	+	T30-like	Mild
158-3	0.004	0.541	+	—	—	—	—	—	+	+	n.a.	Mild
161-30	1.654	0.133	+	+	—	—	—	—	—	—	n.a.	QD
163-30	0.000	0.124	+	—	—	—	—	—	+	+	T30-like	Mild
170-17	0.001	0.340	+	+/-	—	—	—	—	+	+	T30-like	Mixture
175-12	0.007	0.015	+	—	—	—	—	—	+	+	T30-like	Mild
181-3	0.005	0.518	+	—	—	—	—	—	+	+/-	T30-like	Mild
191-26	0.000	0.054	+	+	—	—	—	—	—	—	T36-like	QD
194-3	0.006	0.038	+	—	—	—	—	—	+	+	T30-like	Mild
T30	0.007	0.211	+	—	—	—	—	—	+	+	T30-like	Mild
T36	0.654	0.214	+	+	—	—	—	—	—	—	T36-like	QD
Healthy	0.003	0.003	—	—	—	—	—	—	—	—		
Blank	0.001	0.001										

+ = positive reaction.

— = negative reaction.

+/- = weak reaction.

n.a. = data not available.

QD = Quick decline.

egy to predict biological activity of CTV isolates from samples collected in Florida, Morocco, Mexico, Portugal, Spain, Colombia, and Venezuela (1, 18, 19, 20, 24, 26).

Studies of CTV sub-isolates obtained as a result of single aphid transmission using *T. citricida* (3, 17) indicates that it is possible to segregate CTV strains from a source isolate. Eleven single aphid transmitted sub-isolates were included in this study (isolate numbers greater than 100). Of these 11 isolates, only one (170-17) apparently is a mixture of mild and decline strains. Isolates 96-3c, and 191-26 did not react with

MCA-13 in ELISA, but reacted with SSP probe 1 and the SSCP pattern both indicated the presence of a decline strain of CTV.

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