

## Molecular Characterization of *Citrus tristeza virus* Isolates from Panama

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**ABSTRACT.** Twelve isolates of *Citrus tristeza virus* (CTV) were collected from the main citrus growing regions in Panama and characterized at the molecular level. The CTV coat protein gene (CPG) was amplified by RT-PCR, and the amplified PCR products were cloned and sequenced. The sequences analysis showed the relatedness among the Panama isolates and with the CTV isolates available in Genebank. The CTV isolates were further examined using the multiple molecular markers (MMM) method to determine genotype. Four of the isolates were T30 genotype (CTVP-02, CTVP-14, CTVP-19, and CTVP-26). The rest of the isolates were identified as a mixture of two genotypes while isolate CTVP-18 appears to be a complex mixture of several genotypes and this was the only isolate tested which had a product amplified with the T36-Pol specific primers. Isolate CTVP-22 amplified only the universal marker, with the T36-CPG primer pair, and had no products amplified with any of the other MMM primer pairs. Based on the CPG sequence, three of the 12 isolates are reactive with the monoclonal antibody, MCA-13, used in Florida to identify CTV isolates which cause decline on sour orange rootstock. These results are being used to identify potential mild strains of CTV for further evaluations for mild strain cross protection.

*Index words.* Genotype, tristeza, RT-PCR, cross protection.

*Citrus tristeza virus* (CTV) is the etiological agent of tristeza disease. This disease is the most important citrus virus disease and has a global distribution (14). The presence of CTV in Panama was first reported in 1990 from a survey in the Capira locality, one year after the detection of the brown citrus aphid, *Toxoptera citricida*, in the country (13).

Citrus production in Panama is concentrated in four main areas: Capira, Coclé, Veraguas and Chiriquí (Fig. 1). Washington navel, red Valencia sweet orange, Persian lime, grapefruit, and mandarins are the predominant citrus varieties planted in the country. According to the 2000-2001 agricultural and forestry inventory, the total citrus production in Panama was estimated to be 2,052,494 hundred ton in 2001 compared to 3,030,920 hundred ton reported in 1971 (<http://FAOSTAT.FAO.ORG/collections?subset=agriculture>). This decline in production is mostly due to the effect of different diseases of citrus including CTV (1).



**Fig. 1.** Map of the Republic of Panama showing the major citrus-growing regions of Chiriquí, Veraguas, Coclé and Capira.

Most of the citrus in Panama is on sour orange rootstock. The symptoms associated with CTV infections range from mild to varying degrees of severity and cause decline on sour orange rootstock, seedling yellows, stem pitting and stunting (14).

Since both CTV and the efficient vector, *Toxoptera citricida*, are already present in all the citrus

growing regions of Panama, it is impossible to protect existing trees from becoming infected with CTV. Mild strain cross protection (MSCP) can be used, such as in South Africa, Brazil, and Australia, to control losses caused by stem pitting strains of CTV (2, 9, 15, 18). MSCP is a management strategy that uses mild strains to delay or prevent the symptoms expressed by severe strains (5). The mechanism of MSCP is not known. Empirical testing is used presently to select mild strains having cross protecting ability, although some progress has been made on using molecular methods to identify mild strains and to determine if the mild strain is mixed with potentially severe CTV strains (10). The use of MSCP would be desirable in the main citrus growing areas of Panama where CTV is a limiting factor of production. Until now, the characterization of CTV isolates in Panama has been done solely by ELISA using a polyclonal system for detection of all CTV isolates and also the monoclonal antibody, MCA-13, which discriminates between mild and decline strains of CTV in Florida (12). This study is a preliminary molecular characterization of the CTV isolates present in Panama. The sequence information of the coat protein gene (6) and the use of the multiple molecular markers (MMM) to quickly identify viral genotypes (4) should allow for identification of mild isolates which may be useful for MSCP. Biological assays of the isolates used in this study are in progress.

## MATERIALS AND METHODS

**CTV isolates.** All isolates used in this study were field collections from the two major citrus growing areas; Chiriquí and Veraguas (Fig. 1). Isolates CTVP-01, CTVP-02, CTVP-11, CTVP-14, CTVP-15, CTVP-17, and CTVP-18 were collected from Chiriquí. The isolates CTVP-19, CTVP-21, CTVP-22, CTVP-23 and

CTVP-26 were collected from Veraguas. New flushes with fully expanded leaves were collected from each tree, placed in a plastic bag, and transported on ice to the laboratory for further processing.

**Nucleic Acid Extraction and cDNA synthesis.** For each sample, 4 g of bark tissue was frozen in liquid nitrogen then pulverized in a mortar and pestle. The pulverized tissue was added to a 50 ml tube containing 18 ml of 2× GPS (0.2 M glycine, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, and 0.6 M NaCl, pH 9.6), 2 ml 10% SDS (w/v), 200 µl 2-mercaptoethanol, 5 mg bentonite powder and 5 ml phenol-chloroform-isoamyl alcohol (24:24:1 v/v/v). After mixing well, the tubes were set on ice for 15-20 min, and centrifuged at 8,000 rpm for 15 min. The aqueous phase was transferred to a 50 ml centrifuge tube, and the volume was adjusted to 20 ml with 2× GPS buffer which was then divided into two 10 ml aliquots in separate tubes. To each tube, 1 ml of 3.5 M sodium acetate pH 5.2 (0.1 volume) and 25 ml of 95% ethanol (2.5 volumes) were added, mixed thoroughly and stored at -20°C. The total nucleic acid extracts were then shipped on dry ice to Riverside, CA for further processing.

A 2 ml aliquot of the ethanol-precipitated total nucleic acid extraction was transferred to a 2 ml microcentrifuge, centrifuged for 15 min at RT at 13,000 *x g*. The supernatant was then removed, and another 2 ml of the ethanol-precipitated total nucleic acid extraction was added, and the tube centrifuged as above. The supernatant was removed, and the air-dried pellet was resuspended in 350 µl of 1× STE (0.05 M Tris, 1 mM Na<sub>2</sub>EDTA, and 0.1 M NaCl, pH 6.9) and kept on ice for a minimum of 5 min with constant mixing. For dsRNA isolation, the tubes were centrifuged for 30 sec, 333 µl of the supernatant was transferred to a clean microcentrifuge tube, and 0.02 g of CF-11 cellulose powder and 67 µl of 95%

ethanol were added, mixed well, and then placed on a shaker at 4°C for 30 min. The suspension was centrifuged for 1 min, the supernatant was discarded and 400 µl of 16% wash buffer (1× STE with 16% ethanol) was added, vortexed to mix, and set on ice for 3 min. This wash step was repeated three times. The final suspension was centrifuged for 1 min, and the pellet was suspended in 400 µl of 1× STE (without ethanol), mixed well and set on ice for 3 min, then centrifuged for 5 min. The supernatant (containing the dsRNA) was transferred to a sterile microcentrifuge tube and precipitated with 0.1 volume of 3.5 M sodium acetate, pH 5.2 and 2.5 volumes of 95% ethanol overnight at -20°C. Samples then were centrifuged for 15 min and the supernatant was discarded. The pellet was washed with 100 µl of cold 70% ethanol, centrifuged for 15 min, the supernatant discarded, and the pellet dried by inverting the tube on a

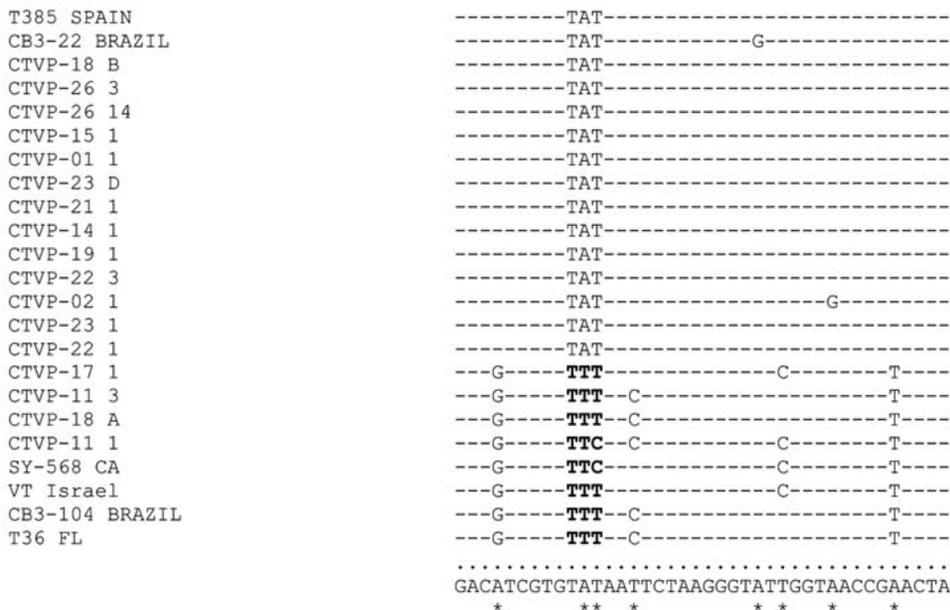
Kimwipe®, and then resuspended in 25 µl sterile water. Ten µl of the resuspended pellet (containing dsRNA) was used for cDNA synthesis using CTV specific primers and Superscript II reverse transcriptase in a 40 µl final reaction volume (10).

**Cloning and sequencing of CTV coat protein gene (CPG).**

One microliter of cDNA from the reverse transcriptase reaction was used for CPG amplification using standard PCR protocols using the primers CN119 and CN120 (10). The PCR product was purified from agarose gels using the MinElute Gel Extraction Kit (QIAGEN) and cloned using the TOPO TA Cloning vector (Invitrogen). The cloned CPG was sequenced in the UC Riverside Core Instrumentation Facility using the ABI international protocol.

**Analysis of CPG sequence.**

Sequences were aligned using McClade (Sinauer Associates). The phylogenetic relationships of the Panama CTV isolates, plus the CPG



**Fig. 2. Multiple alignment of nucleotide sequences of the coat protein gene corresponding to the positions ranging from 361 to 400 of the Panama isolates. The codon for Phe (MCA-13 cryptic epitope) is in bold at position 371 in the isolates CTVP-11, CTVP-17, CTVP-18. The other isolates from Panama did not have this epitope which suggests they may be mild strains.**

sequence of six CTV isolates, VT (U56902), T-36 (NC001661), T-385 (Y18420), SY-568 (AF001623), CB3-10 (17) and CB3-22 (17), were deduced using PAUP version 4.10b, and Neighbor-Joining as the distance estimation method with Tajima & Nei's Algorithm (16). The bootstrap analysis was performed using 1,000 replicates.

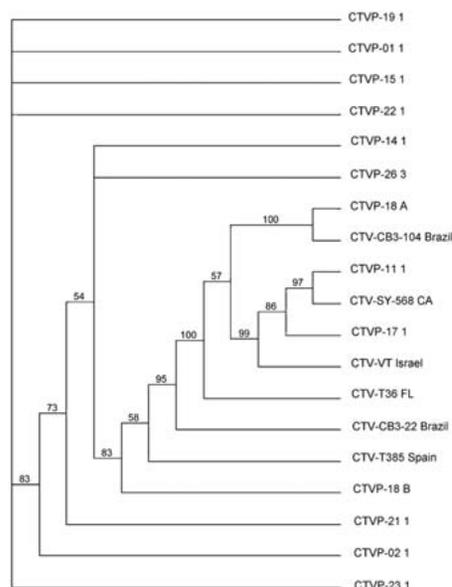
**Genotyping using Multiple Molecular Markers (MMM).** PCR amplifications were carried out using 1  $\mu$ l of cDNA. The eleven primer pairs and PCR conditions were employed as described by Hilf et al. (4). The PCR amplicons were analyzed by electrophoresis on 0.8% agarose gels containing 200 ng/ml of ethidium bromide. The genotypes of the CTV isolates from Panama were determined according to the amplification profile for each of the genotype-related primer sets.

## RESULTS AND DISCUSSION

The CTV CPGs amplified by RT-PCR from the different isolates of CTV from Panama had the expected size of 672 bp. The amplicons were cloned, and three clones from each isolate were sequenced. Analyses of the nucleotide sequences showed that only isolates CTVP-11, CTVP-17 and CTVP-18 exhibited the cryptic epitope (Phe coded by TTT or TTC at aa position 124 of the CPG, Fig. 2) that is needed for recognition by monoclonal antibody MCA-13, which is often characteristic of severe strains (11). Phylogenetic analysis grouped these isolates with SY568, VT and CB3-104, all which have severe biological activity (Fig. 3) although none of the trees in the field where these isolates were collected showed stem pitting. None of the Panama isolates grouped with the Florida decline isolate T-36. However, Kano et al. (3); and Mooney et al. (8), in Japan and New Zealand, respectively, reported the presence of the MCA-13 cryptic epitope in CTV isolates having mild symptoms based

on their biological indexing results. The rest of the Panama isolates of CTV did not have the MCA-13 cryptic epitope, suggesting that they might be mild strains. These isolates also grouped close to the mild strain T385 from Spain with very low genetic distances (Fig. 3).

**Genotype classification of isolates.** According to the MMM profiles, isolates CTVP-02, CTVP-14, CTVP-19, and CTVP-26 were classified as T30 genotype because two of the T30 specific primer pairs (T30Pol and T30-K17) gave amplicons with these isolates. All the remaining isolates, except for CTVP-



**Fig. 3.** Dendrogram showing the isolate groupings according to their CTV coat protein gene sequences. The phylogenetic tree was constructed using the Tajima & Nei algorithm (16), Neighbor-Joining estimation method and mid-point rooting. The first group is formed by presumptive mild isolates CTVP-19, CTVP-01, CTVP-15, CTVP-22, CTVP-23 which are essentially identical and closely related to CTVP-14, CTVP-26, CTVP-02, CTVP-21, and CTVP-18 and the mild isolate T385 from Spain. The second group is constituted by the reference isolates VT and SY568 and CTVP-17, CTVP-11 clone 1 (11 1). The third group consists of the reference isolate CTV CB3-104 from Brazil and isolate CTVP-18 A.

TABLE 1  
 MULTIPLE MOLECULAR MARKER (MMM) PROFILES OF SELECTED ISOLATES  
 OF CITRUS TRISTEZA VIRUS FROM PANAMA

Isolate No.	T36CP	T36POL	T36-5'	T36-K17	T30POL	T30-5'	T30-K17	VTPOL	VT-K17	VT-5'	T3-K17
CTVP-01	+a	-	+	-	-	+	-	-	+	+	-
CTVP-02	+	-	-	-	+	-	+	-	-	-	-
CTVP-11	+	-	-	-	-	-	-	+	-	+	-
CTVP-14	+	-	-	-	+	-	+	-	-	-	-
CTVP-15	+	-	-	-	+	+	+	-	-	+	+
CTVP-17	+	-	-	-	-	-	+	-	-	+	+
CTVP-18	+	+	-	+	+	+	+	+	+	+	+
CTVP-19	+	-	-	-	+	-	+	-	-	-	-
CTVP-21	+	-	-	-	+	+	+	+	-	+	-
CTVP-22	+	-	-	-	-	-	-	-	-	-	-
CTVP-23	+	-	+	-	+	-	+	-	-	-	-
CTVP-26	+	-	-	-	+	-	+	-	-	-	-

<sup>a</sup>Positive amplifications are indicated by a plus sign (+) while negatives by a minus sign (-).

22, seem to have mixed genotypes in the isolate (Table 1). For instance isolate CTVP-11 is a mixture of T30 (K17) and VT (Pol, 5') genotypes; isolate CTVP-21 is a mixture of T30 (Pol, 5', and K17) and VT (Pol, 5') genotypes; isolate CTVP-15 is a mixture of T30 (Pol, 5' and K17), T3 (K17) and VT (5') genotypes; and isolate CTVP-17 is a mixture of T30 (K17), VT (5'), and T3 (K17) genotypes. Isolate CTVP-01 also had a unique triple genotype profile: T36 (5'), T30 (5') and VT (5' and K17). Isolate CTVP-23 had genotypes similar to T30 (Pol and K17) and T36 (5'). Interestingly, the isolate CTVP-18 amplified with all markers except T36 5'. The 5' region primer pairs seem more reluctant to yield amplicons, probably due to the higher

variability of the genome sequence in this region of the CTV genome (7).

Isolates CTVP-02, CTVP-14, CTVP-19, and CTVP-26 appear to be composed of mild isolates with no indication of a potential severe strain of CTV being present based on the analyses of the CPG and genotyping using the MMM method. These isolates will be evaluated for their usefulness for MSCP following the completion of their biological activity on indicator plants.

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