Molecular Characterization of *Citrus tristeza virus* Isolates from Veracruz and Tamaulipas States, Mexico

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ABSTRACT. Double-stranded RNA from 56 samples of *Citrus tristeza virus* (CTV) obtained from Veracruz and Tamaulipas states of México were analyzed by single-strand conformation polymorphism (SSCP) of the p25 (CP) and p23 genes to determine the population structure. Selective PCR amplification using primers PM33, PM34 and PM35 was performed to discriminate between mild and severe isolates. SSCP analysis indicated that samples from Veracruz were more similar as a group than were those from Tamaulipas. It was possible to characterize the isolates preliminarily by the selective PCR amplification. All samples from Veracruz appeared to be mild, and only two from Tamaulipas appeared to be moderately severe isolates such as the T-36 type. Very severe isolates such as the T-388 type were not detected. The p25, p27 and p23 genes and the 3'-end sequence of some of the isolates of CTV from Veracruz were cloned, sequenced and compared with published sequences. The CP and p27 sequences were identical to the mild isolate T30 and the 3'-end was similar to the mild isolate T385. These results were consistent with those obtained with selective PCR amplification.

Citrus tristeza virus (CTV) strains differ in their biology, serology and in their symptoms expression (11). To design effective management strategies for a viral disease it is important to establish the isolate identity and to study its epidemiological behavior (11, 13). Molecular approaches have been used recently with success for characterization of CTV isolates (1, 2, 7, 9, 11).

Comparison of genomic sequences of several CTV strains revealed a 97% or higher nucleotide sequence identity in the 3' end, whereas comparisons found an identity of 40% or less in the 5' end of the genomic RNA. This variation has led to speculation that, in most CTV infected citrus trees, particularly those exposed for long periods to recurrent aphid feeding, the isolates are populations of genomic variants (haplotypes), with one variant being dominant (1, 4, 8).

Single Stranded Conformational Polymorphism (SSCP) analysis has been used to characterize populations of CTV and to identify haplotypes in these populations. SSCP can give a preliminary result regarding the potential symptom severity or population complexity (6, 8, 16). Polymerase chain reaction analysis (PCR) with specific primers derived from the sequence of isolates previously characterized for their pathogenicity also allows screening many isolates in a short time (1).

In Mexico, isolates have been found which react to the monoclonal antibody MCA13, which commonly identifies isolate which can cause severe symptoms (such as for Florida isolate T36). However, these isolates do not show the characteristic severe symptoms reported for Florida (12, 18).

In this work isolates from Tamaulipas and Veracruz were characterized with SSCP analysis, selective and specific amplifications with PCR and cloning and sequencing of some CTV ORFs. Knowledge

derived from these analyses could be useful to design management strategies for this disease in specific zones and to understand potential epidemic outbreaks with the introduction of the effective aphid vector, *Toxoptera citricida*.

Alemow and/or sour orange plants were graft-inoculated with buds from field trees from Tamaulipas and Veracruz which were previously shown to be positive for CTV by DAS-ELISA using a mixture of the monoclonal antibodies 3DF1 and 3CA5 (AGDIA, Inc.™). The extraction of dsRNA was done by a modified phenol-detergent method (5).

Two step reverse-transcription-polymerase chain reaction (RT-PCR) and SSCP were performed with primers specific for the p25 gene (Table 1) as outlined previously (8, 16), and gels were silver stained (3). The T-36 and T-385 isolates were included as standards. The p23 gene was similarly analyzed in isolates which showed variation in SSCP patterns for p25.

To discriminate isolates further samples were analyzed by differential PCR with primers PM34, PM35 and PM33 to detect mild isolates similar to T-385, or severe isolates similar to T-36 and T-388, respectively (Table 1). Results obtained with SSCP and differential PCR analyses were confirmed by cloning and sequencing using standard procedures (17). For cloning, cDNA was obtained by RT-PCR using oligonucleotide CN304, which is specific for the 3'-end of CTV. The CP, p27-CP and last 1202 bp of

TABLE 1
PRIMERS USED FOR RT-PCR AND SEQUENCING OF FRAGMENTS OF CTV

Primers[1]	Primer sequence (5' to 3')	Binding site	Reference
CPF	AAC GCC CTT CGA GTC TGG GGT AGG A	16554	8
CPR	TCA ACG TGT GTT GAA TTT CCC AAG C	16763 (T36)	
p23F	ACT AAC TTT AAT TCG AAC A	18347	16
p23R	AAC TTA TTC CGT CCA CTT C	19026 (T36)	
PM33 +	CCC GTA CCC TCC GGA AAT CAC G	16-37 (VT)	1
PM34 +	TGG TGT AAA TCC CAA CCA GAC GGT TG	57-82 (T385)	1
PM35 +	ATC GCG CAT CTG GCG CAA AC	170-189 (T36)	1
RF-137	CCG TAG AGG GAC TAT CGG C	266-284	1
CN304[2]	atg c*GG ATC CAT GGG CCC* TGG ACC TAT GTT GGC CCC CCA T	19296 (T36)	Febres, 2001. Unpublished
VF16[3]	TTA TTA T*GC GGC CGC* ATG GAC tAa tAA ACA AAG AAA TTG AAG	16155 (T36)	Febres, 2001. Unpublished
VF12[3]	AAA CTC C*CT CGA G*CC GAT AGA AAC CGG GAA TCG G	16871 (T36)	
VF36[4]	TAT ATA *CTC GAG* ATG AGG TAC ATG AGT TCT TAG TCA CAC C	18094 (T36)	Febres, 2001. Unpublished
VF37[4]	ATA TAT *GGG CCC* TGG ACC TAT GTT GGC CCC CCA aTA GG	19296 (T36)	
VF53	GTC ATA TGA GCA GAG ACG TGG C	15291	Febres, 2001.
VF54	TGA AAC TCC ACC ATC CCG ATA	16873	Unpublished

[1]CPF, CPR, p23F and p23R primers were used for SSCP analysis; PM33, 34, 35 and RF-137 primers were used for isolate characterization according to their severity. CN304, VF12, VF 16, VF 36, VF 37, VF 53 and VF54 primers were used for CP, 3'END and p27-CP cloning.
[2]This primer is specific for 3'END of CTV and contains extra *BamHI*, *NcoI*, *ApaI* restriction sites (italics) and sequence (lower case).
[3]These primers are specific for CP of CTV and they contain extra *NotI* and *XhoI* restriction sites (italics). VF16 primer has three point mutations at positions +7, +9 and +10 (lowercase) that add two stop codons and make the CP untranslatable.
[4]These primers are specific for the last 1202 bases of 3'end and they contain extra *Xho*I and *Apa*I restriction sites (italics). VF37 primer changes a G for an A (in lowercase) to eliminate a start codon in the antisense orientation.

the 3'-end were amplified using the specific internal primer pairs VF12/VF16, VF53/VF54 and VF36/VF37, respectively (Table 1).

The p25 gene from 56 CTV-infected samples from Tamaulipas and Veracruz was amplified and analyzed by SSCP. SSCP patterns of samples from Tamaulipas for the p25 gene of CTV were less similar to one another than were those from Veracruz. Specifically, samples Ee1a, Le8a and Le8b from Tamaulipas had different band patterns, and only one (Pd2a) had a SSCP pattern similar to those from CTV isolates from Veracruz. None of the 56 samples showed patterns similar to T-36 and T385 isolates (Fig. 1).

The SSCP patterns of Veracruz samples obtained by grafting were similar to the patterns from the original field samples, with all showing a characteristic three band pattern. Only samples Cz1e and Cz1c cloned from Cz1 tip-grafted on alemow showed two additional bands (Fig. 1).

SSCP analysis of the p23 gene also showed that Le8a and Ee1a from Tamaulipas were different from the rest of the isolates from this region, while isolate Pd2a again was similar to isolates from Veracruz.

None of the samples yielded product when analyzed with the 5'-end differential primer PM33 (which identifies VT type CTV). All samples, except Ee1a, yielded product with the PM34 primer (type T-385), and only Ee1a and Le8a from Tamaulipas yielded a product with the PM35 primer (type T-36 type CTV). Positive control samples for T-385 and T-36 analyzed with PM34 and PM35 yielded the correctly sized products.

Although there appeared to be no conserved patterns characteristic for a specific symptom, analyzed samples showed a more complex SSCP pattern for apparently severe isolates than for mild ones as previously reported (6, 8, 15). Most samples in this work showed three bands. For samples Ee1a (Tamaulipas), Cz1e and Cz1c (Veracruz), the presence of several bands could be indicative of a mix of variants in the same host (14, 16). Based on SSCP patterns, all samples from Veracruz appeared to be mild and only two from Tamaulipas appeared to be moderately severe isolates (similar to the T-36 type). Very severe isolates such as T-388 were not detected.

No typical CTV symptoms on the original hosts from Veracruz and Tamaulipas were observed, perhaps due to interference among isolates or cross protection (10). These results may have epidemiological implications, particularly if the isolates are differentially transmitted by aphids (2, 4). The Ee1a, Cz1e and Cz1c samples have been grafted-transmitted several times to segregate them as previously done (10) and to conduct further molecular and biological characterization of the new and original isolates.

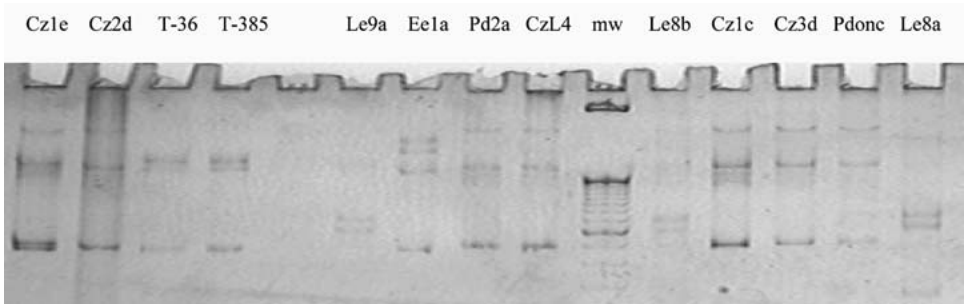

Fig. 1. SSCP patterns for CTV isolates from Tamaulipas (Le9a, Ee1a, Pd2a, Le8a, Le8b) and from Veracruz (Cz1e, L4, Cz1c, Cz3d and Pdonc).

SSCP analysis was useful for screening many samples very quickly to establish the degree of homogeneity in the virus population. Because SSCP analysis of the p23 and p25 genes gave similar results, either of these two regions can be used to differentiate isolates. The SSCP exploratory analysis also allowed us to determine which isolates to further characterize with differential PCR, which corroborated the SSCP results.

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