# Molecular Differentiation of Mild and Severe *Citrus tristeza virus* Isolates in Mexico

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ABSTRACT. *Citrus tristeza virus* (CTV) is the most important viral disease of citrus worldwide. In the last 20 yr it has caused the death of more than 100 million trees in South America, California, Florida, Israel and Spain. CTV is transmitted by the aphid *Toxoptera citricida* and in infected propagating material. The presence of both the virus and the aphid in Mexico are of great concern, since most of the commercial plants are grafted on sour orange rootstocks that are susceptible to CTV. The molecular characterization of different CTV strains, including the complete sequence of the virus genome has opened the door to studies with the goal of diminishing the damage caused by this viral complex. The objectives of this study were to develop a method to differentiate CTV strains based on the molecular characterization of the p25 coat protein gene. The sequence of this gene was amplified, and RFLP polymorphisms were examined with restriction enzymes that allowed the differentiation of mild strains from severe ones. The results showed that digestion with the enzyme *Hae* III made it possible to accomplish this. In addition, the use of the enzyme *Kpn* I discriminated between the strains that cause stem pitting from those that induce tree decline. These results were also observed by *in silico* digestion, with the same enzymes, of several coat protein gene sequences deposited in Genbank. The derived dendrograms of the multiple alignments of the amino acids sequences of p25 gene showed the separation of the weak strains from the severe ones. The analysis of the nucleotide sequence of gene p25 at positions 49, 63 and 124 showed that the amino acids glycine, threonine and phenylalanine are conserved in severe strains.

*Citrus tristeza virus* (CTV) has worldwide distribution, and its strains vary in symptoms in different hosts, as well as in their transmissibility by aphids (16). CTV strains have been found that cause tree decline and death in orange, grapefruit and mandarin, grafted onto sour orange rootstock, while others cause stem pitting in various species. There are also isolates that do not cause any visible effects on the infected host (7).

This virus is disseminated in a semi-persistent way by several species of aphids; the most efficient vector being *Toxoptera citricida* (Kirkaldy). CTV is transmitted by the vector without a period of latency and it looses its efficacy of transmission 48 hr after acquisition (8, 15).

Most CTV isolates are complex populations containing mixtures of different viral genotypes (2). These mixtures include genetic variants, defective RNAs (dRNAs) and chimerical genomes that are possibly generated as consequence of recombination events. Recombination during transcription is considered one of the main factors that determine the evolution of positive sense RNA viruses.

It has been postulated that at least four species are the progenitors of current citrus varieties, and that the different strains of CTV evolved in the native citrus of Asia, and were dispersed around the world through infected buds (1). Strains considered mild, for example T-30, are genetically related to Asian strains, as well as other isolates from Colombia and California (isolates B272 and B354, respectively, of the USDA exotic CTV collection, Beltsville, MD, USA), and are related to isolate T385 from Spain, which is also considered mild. Phylogenetic analysis showed that T30 was introduced to Florida more than 200 yr ago and that its genetic changes are mostly related to the movement of the citrus throughout the world (16).

At present, the main detection method for CTV is an enzyme linked immunosorbent assay (ELISA). The monoclonal antibodies 3DF1 and 3CA5 are able to detect most of the known isolates of this virus (18). However, other antibodies such as MCA13 are specific for severe strains. The methods previously used to differentiate CTV strains include different monoclonal antibodies (13), the analysis of dsRNAs in infected plants, and molecular hybridization with probes of complementary DNA (cDNA) (11). The ability to differentiate strains based on single strand conformational polymorphism (SSCP) and restriction fragment length polymorphisms (RFLP) is due to a nucleotide change resulting in the DNA acquiring a different conformation. This allows the DNAs to migrate at different rates when analyzed in polyacrylamide gels.

Lozano (9) used RFLPs, digesting the amplified products of the reverse-transcription polymerase chain reaction (RT-PCR) with the restriction enzyme *Hinf* I and found that it was useful to characterize the adenovirus family. Studies carried out by Gillings (5) analyzing the p25 CTV coat protein gene digested with the same restriction enzyme *Hinf* I, generating a dendrogram of the similarity of different isolates which correlated with their biological characterization, allowing prediction as to whether these were mild or severe strains. This approach allowed differentiation of isolates of CTV without having to clone and sequence the p25 gene (4).

This study was conducted to develop a molecular method based on RT-PCR jointly with the use of RFLP using the coat protein gene and the virulence of CTV isolates to differentiate severe from mild strains.

## MATERIALS AND METHODS

**Virus isolates.** CTV isolates were obtained from sweet orange field trees in northeast Mexico. Isolates NL1, NL2, V1 and H33 showed typical CTV symptoms of either stem pitting or tree decline, and consequently were considered severe. But, isolates NL3-7, T2 and V2 did not showed any CTV symptoms and were thus considered mild isolates. Biological characteristics of Mexican CTV isolates were also evaluated in sweet orange, Mexican lime and grapefruit plants. Sequences of the coat protein gene from mild strains T30 and T385, and severe strains SY568 and T36 were also included as reference CTV strains.

**CTV reverse transcription, PCR and cloning.** Primers R731-7 (5'-cggaacgcaacagatcaacg-3') and R731-6 (5'-attatggacgacgaaacaaa-3') were designed for performing RT-PCR of the coat protein, producing an expected product of 688 base pairs (bp) (8). Viral dsRNA was purified from young bark tissue by a Trizol method and was used as templates for RT-PCR. Reverse transcription using dsRNAs was performed at 70°C for 5 min in a 15 µl reaction volume containing 2 µl (2 mg) of dsRNA, 0.5 mM of reverse primer, followed by incubation at 42°C for 50 min in a 25 ml reaction containing 1× M-MLV buffer, 0.5 mM dNTPs, 15.6 units of Rnasin, 200 units of M-MLV reverse transcriptase. PCR was performed in 25 µl reactions containing 2.5 ml of the RT solution, 1× Taq buffer, 1.5 mM $MgCl_2$, 0.5 mM each primer 0.2 mM dNTPs and 1.25 units of Taq polymerase. The samples were incubated first at 94°C for 5min, followed by PCR for 30 cycles of 94°C (30 sec), 50°C (30 sec), 72°C (1 min) followed by a final 7 min extension at 72°C. RT-PCR products were directly cloned using pCR® 2.1

(Invitrogen) as described by the manufacturer. RT-PCR and PCR products were detected by electrophoresis in a 2% agarose gel.

**RFLP analysis of the coat protein gene.** PCR products were digested with restriction endonucleases *Hae* III and *Kpn* I at 37°C for at least 2 h according to manufacturers' instructions (Gibco BRL). The digests were resolved by electrophoresis with 2% agarose gels in Tris-borate-EDTA buffer at 5V/cm for 2h. The reference severe strain was H33 from Texas (6). The RFLP migration pattern of p25 was observed and photographed under a UV transilluminator

**Nucleotide sequences and computational analysis.** The cloned cDNAs were sequenced using Sequi Therm Excel II DNA sequencing in a Li-Cor sequencer (LI-COR, Inc., Nebraska, USA). Sequences were analyzed by a BLAST program at Gen Bank (www.ncbi.nlm.nih.gov). The multiple sequence alignment program Clustal W (version 1.6) (17) was used to obtain an optimal nucleotide or amino acid sequence alignment file. Phylograms for the entire sequence were obtained by PHYLIP (14) based on aligned nucleotide sequences. The statistical significance of phylogenies constructed using DNADist/Neighbor was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets.

## RESULTS AND DISCUSSION

**Coat protein RT-PCR.** Fig. 1 shows the 688 bp fragments amplified by RT-PCR, corresponding to the coat protein gene of several CTV isolates from Nuevo Leon (NL), Tamaulipas (T) and Veracruz (V). The last lane corresponds to CTV H33, a severe virus strain from Texas used as a reference. One sample, NL1, yielded poor amplification, in spite of an increase in the total RNA concentration and modification of several PCR reaction conditions, such as the annealing temperature. It may have been due to low virus concentration affecting the quantity of the RNA template. Similar results were reported by Bar-Joseph, (3) where they showed that low concentrations of viral RNA cause defective amplification.

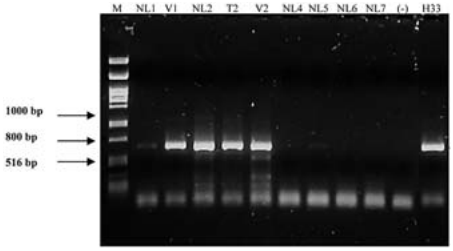


**Fig. 1. RT-PCR of p25 (CP) gene. Each lane corresponds to a CTV isolate from Nuevo León (NL), Tamaulipas (T) or Veracruz (V). The PCR products were analyzed in a 2% agarose gel. The amplified p25 gene corresponds to a fragment of 688 bp.**

**RFLP analysis of the coat protein gene.** The RFLPs of the amplified CP genes digested with the restriction enzyme *Alu* I showed that it was not possible to differentiate the isolates according to their RFLP patterns. (Data not shown) Furthermore, when the profiles were grouped, based on the pattern of bands, the dendrogram showed similar results with no evident relationship among CTV isolates. The severe strain H33 was grouped with the mild isolates V2 and NL1 (data not shown).

However, we found that restriction enzymes *Hae* III and *Kpn* I allowed the separation of severe strains from mild by analyzing the coat protein gene (data not shown). Over 25 sequences of the p25 gene from mild and severe strains were deposited in GeneBank and these were analyzed by DNA STRIDER software (10) with 20 different restriction enzymes. This analysis showed that the CTV strains designated as mild strains did not contain the *Hae* III and *Kpn* I restriction sites. On the other hand, the severe strains that cause tree decline had only the *Hae* III restriction site, but not the *Kpn* I site. An interesting point is that virus strains that induce stem pitting present both restriction sites *Hae* III and *Kpn* I. However it would be necessary to confirm this observation with larger numbers of biologically-characterized CTV isolates.

Isolates NL1, V1 and NL2 and reference strain H33 have the *Hae* III and *Kpn* I restriction sites, and isolates T2 and V2 do not (Fig. 2). Gillings (5) found that RFLPs generated by digesting the p25 gene with the restriction enzyme *Hinf* I proved useful separating strains of CTV. Seven groups were defined, some of which included isolates with similar biological characteristics. In our case, *Hae* III was useful for discriminating the mild from severe strains. We consider that the protocol developed in this work offers advantages to those previously reported, because a single digest could discriminate between mild and severe strains. A method to differentiate mild or severe strains based on monoclonal antibodies (11) has already been superseded by new molecular techniques. For such a reason, we consider that our method could help because is straightforward and less expensive.

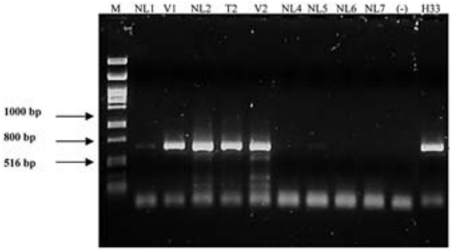
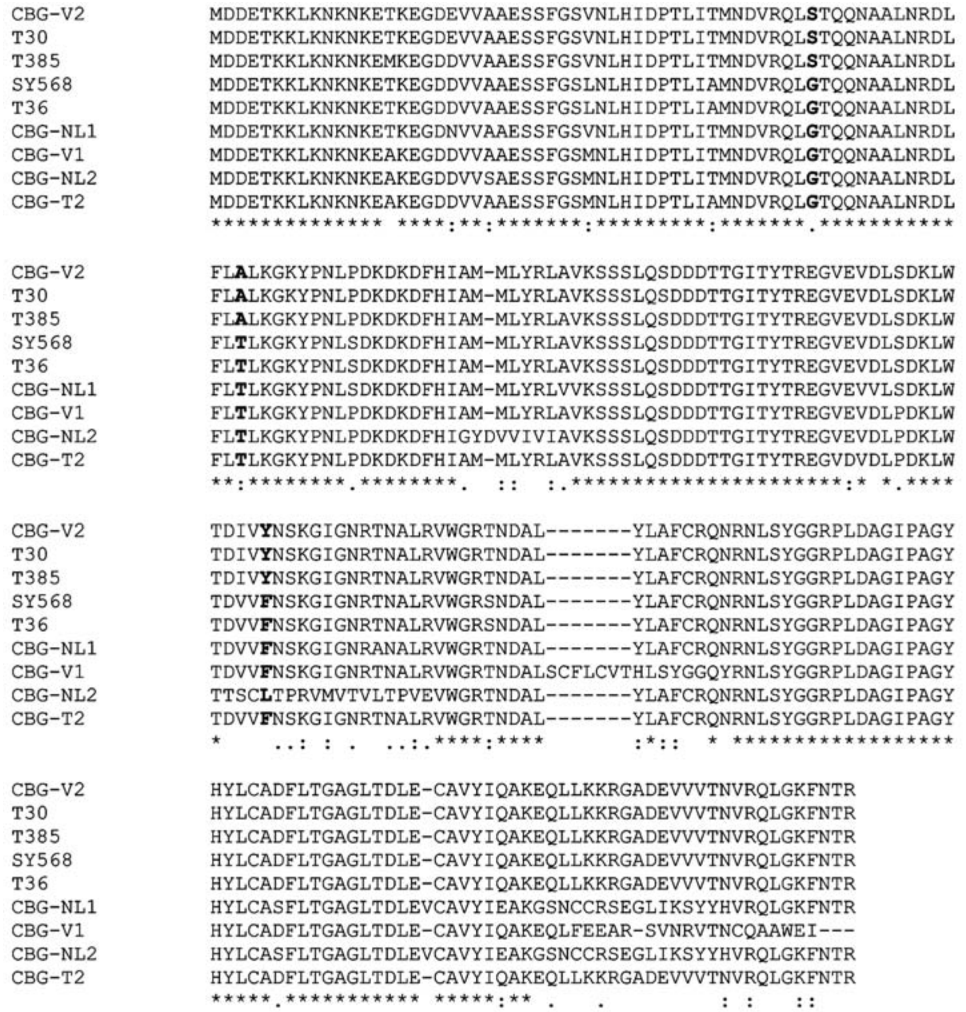


**Fig. 2. RFLP profile of p25 *Hae*III digested. Lanes: M, *Pst*I λ phage; 2) NL1: undigested PCR fragment; 3) NL1, digested PCR fragment; 4) V1 undigested PCR fragment; 5) V1 digested PCR fragment; 6) NL2 undigested PCR fragment; 7) NL2 digested PCR fragment; 8) T2 undigested PCR fragment; 9) T2 digested PCR fragment; 10) V2 undigested PCR fragment; 11) V2 digested PCR fragment; 12) H33 PCR fragment undigested; 13) H33 PCR fragment digested.**

**Sequence analysis of the p25 gene.** The amino acid sequences of isolates NL1, V1, NL2, V2 and T2 showed an 85% homology to one another. Pappu (12) found similar results when they analyzed the relationship between the virulence of different CTV isolates and the p25 gene sequences. The isolates NL1, NL2 and V1 showed 96% similarity with the severe CTV strain H33. Isolates T2 and V2 showed 99% of identity with strain T30 (characterized as mild).

The multiple alignment of CP amino acid sequences of strains T30, T385, SY568 and T36 showed that severe strains have conserved residues at positions 49, 63 and 124 corresponding to glycine (G), threonine (T) and phenylalanine (F) respectively, while strains considered as mild had serine (S), alanine (A) and a tyrosine (Y), respectively at the same positions (Fig. 3). The first two amino acid changes have not been reported in the literature before. However, Pappu (12) stated that severe strains have conserved phenylalanine (F), while the mild strains have conserved tyrosine (Y) at the same position, with this being the only distinguishing characteristic among the previously reported severe and mild strains. We believe that these residues could play an important role in the CTV pathogenesis that would be worthwhile to study in the future.

Isolates V2 and T2 do not possess the restriction sites *Hae* III and *Kpn* I, reported from mild strains. However, severe strains such as T36 that cause tree decline, have only the restriction site *Hae* III, while severe strains such as SY568 that also induce stem pitting, have both the *Hae* III and *Kpn* I restriction sites. Isolates NL1, NL2 and V1 have both these sites. From the multiple alignments of



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CBG-V2          MDDETKKLKNKNKETKEGDEVVAAESSFGSVNLHIDPTLITMNDVRQLSTQQNAALNRDL
T30             MDDETKKLKNKNKETKEGDEVVAAESSFGSVNLHIDPTLITMNDVRQLSTQQNAALNRDL
T385            MDDETKKLKNKNKEMKEGDDVVAAESSFGSVNLHIDPTLITMNDVRQLSTQQNAALNRDL
SY568           MDDETKKLKNKNKETKEGDDVVAAESSFGSLNLHIDPTLIAMNDVRQLGTQQNAALNRDL
T36             MDDETKKLKNKNKETKEGDDVVAAESSFGSLNLHIDPTLIAMNDVRQLGTQQNAALNRDL
CBG-NL1         MDDETKKLKNKNKETKEGDNVVAAESSFGSVNLHIDPTLITMNDVRQLGTQQNAALNRDL
CBG-V1          MDDETKKLKNKNKEAKEGDDVVAAESSFGSMNLHIDPTLITMNDVRQLGTQQNAALNRDL
CBG-NL2         MDDETKKLKNKNKEAKEGDDVVSAESSFGSMNLHIDPTLITMNDVRQLGTQQNAALNRDL
CBG-T2          MDDETKKLKNKNKEAKEGDDVVAAESSFGSMNLHIDPTLIAMNDVRQLGTQQNAALNRDL
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CBG-V2          FLALKGKYPNLPDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
T30             FLALKGKYPNLPDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
T385            FLALKGKYPNLPDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
SY568           FLTLKGKYPNLSDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
T36             FLTLKGKYPNLSDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLSDKLW
CBG-NL1         FLTLKGKYPNLSDKDKDFHIAM-MLYRLVVKSSSLQSDDDTTGITYTREGVEVVLSDKLW
CBG-V1          FLTLKGKYPNLPDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVEVDLPDKLW
CBG-NL2         FLTLKGKYPNLPDKDKDFHIGYDVVIVIAVKSSSLQSDDDTTGITYTREGVEVDLPDKLW
CBG-T2          FLTLKGKYPNLPDKDKDFHIAM-MLYRLAVKSSSLQSDDDTTGITYTREGVDVDLPDKLW
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CBG-V2          TDIVYNSKGIGNRTNALRVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
T30             TDIVYNSKGIGNRTNALRVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
T385            TDIVYNSKGIGNRTNALRVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
SY568           TDVVFNSKGIGNRTNALRVWGRSNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
T36             TDVVFNSKGIGNRTNALRVWGRSNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
CBG-NL1         TDVVFNSKGIGNRANALRVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
CBG-V1          TDVVFNSKGIGNRTNALRVWGRTNDALSCFLCVTHLSYGGQYRNLSYGGRPLDAGIPAGY
CBG-NL2         TTSCLTPRVMVTVLTPVEVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
CBG-T2          TDVVFNSKGIGNRTNALRVWGRTNDAL-------YLAFCRQNRNLSYGGRPLDAGIPAGY
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CBG-V2          HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
T30             HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
T385            HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
SY568           HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
T36             HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
CBG-NL1         HYLCASFLTGAGLTDLEVCAVYIEAKGSNCCRSEGLIKSYYHVRQLGKFNTR
CBG-V1          HYLCADFLTGAGLTDLE-CAVYIQAKEQLFEEAR-SVNRVTNCQAAWEI---
CBG-NL2         HYLCASFLTGAGLTDLEVCAVYIEAKGSNCCRSEGLIKSYYHVRQLGKFNTR
CBG-T2          HYLCADFLTGAGLTDLE-CAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
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**Fig. 3. Multiple alignment of amino acids sequences of p25gene. Asterisks (*) indicate identical amino acids, (:) indicates groups of strongly conserved amino acids, (.) indicates groups of weakly conserved amino acids. The distinctive residues 49, 63 and 124, Glycine (G), Threonine (T) and Phenylalanine (F), are conserved in CTV strains considered severe.**

our sequences, and with those already reported and biologically characterized, T36 (decline), SY568 (stem pitting), T30 and T385 (mild) (12), a dendrogram of similarity was generated with Protdist and Phylip software. Isolates NL1, NL2, V1 and T2 formed one group closely related to the severe isolate T36/SY568 cluster (all possessing the *Hae*III and *Kpn*I sites), while isolate V2, formed a cluster with the mild isolate T30. Isolates V2 and T30 were related with a genetic distance of 0, as was the cluster formed by the severe strains SY568 and T36. Isolate CBG-T2 showed a greater phylogenetic distance compared to other isolates where distances between 1.4 and 1.6 were observed (Fig. 4).

The RFLP analysis of the coat protein gene after digestion with *Hae* III and *Kpn* I restriction enzymes has the potential to differentiate mild strains from severe, but more characterized isolates need to be evaluated to determine if this pattern holds.

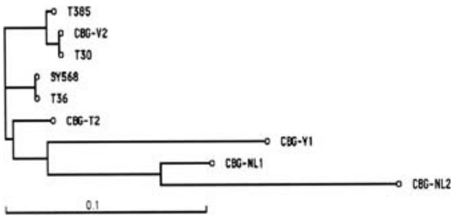

**Fig. 4. Similarity dendrogram of different CTV isolates. The reference CTV strains are T36 (severe, tree decline); SY568 (severe, stem pitting); T30 (mild) and T385 (mild). The bar indicates calculated changes per site.**

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## LITERATURE CITED

1. Albiach-Martí, M. R., M. Mawassi, S. Gowda, T. Satyanarayana, M. E. Hilf, S. Shanker, E. C. Almira, M. C. Vives, C. López, J. Guerri, R. Flores, P. Moreno, S. M. Garnsey, and W. O. Dawson
2000. Sequences of citrus tristeza virus separated in time and space are essentially identical. J. Virol. 74: 6856-6865.
2. Ayllón, M. A., L. Rubio, A. Moya, J. Guerri, and P. Moreno
1999. The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. Virology 25: 32-39.
3. Bar-Joseph, M., S. M. Garnsey, D. Gonsalves, and D. E. Purcifull
1980. Detection of citrus tristeza virus. 1. Enzyme-linked immunosorbent assay (ELISA) and SDS-immunodiffusion methods. In: *Proc. 8th Conf. IOCV,* 1-8. IOCV, Riverside, CA.
4. Cepeda-Nieto, A. C., and H. A. Barrera-Saldaña
1997. Cloning and sequencing of the coat protein gene of a new isolate of citrus tristeza virus from Mexico. Plant Dis. 81: 693.
5. Gillings, M., P. Broadbent, J. Indsto, and R. F. Lee
1993. Characterization of isolates of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. J. Virol. Methods 44: 305-317.
6. Herron, C. M., T. E. Mirkov, N. Solis-Gracia, C. J. Kahlke, M. Skaria, and J. V. da Graça
2005. Severity of *Citrus tristeza virus* isolates from Texas. Plant Dis. 89: 575-580.
7. Lee, R. F., and M. A. Rocha-Peña
1992. Citrus tristeza virus. In: *Plant Diseases of International Importance III.* A. N. Mukhapadhyay, H. S. Chaube, J. Kumar, and U. S. Singh (eds.), 226-249. Prentice Hall, NJ.
8. Lee, R. F., H. R. Pappu, M. A. Rocha-Peña, V. J. Febres, K. L. Manjunath, O. V. Nikolaeva, A. V. Karasev, B. Cevik, M. Akbulut, D. Benscher, E. J. Anderson, M. Price, F. Ochoa, and C. L. Niblett
1996. Progress on strain differentiation of citrus tristeza virus. Rev. Mex. Fitopatol. 14: 79-87.
9. Lozano, M. E., D. M. Posik, C. G. Albariño, G. Schujman, P. D. Ghiringhelli, G. Calderón, M. Sabattini, and V. Romanowski
1997. Characterization of arenaviruses using a family-specific primer set for RT-PCR amplification and RFLP analysis its potential use for detection of uncharacterized arenaviruses. Virus Res. 49: 79-89.
10. Marck, Ch.
1989. A program for DNA and protein analysis. Service de Biochimie, Département de biologie, Institute de Recherche Fundamentale CEA. France.
11. Moreno, P., J. Guerri, J. P. Aguilar, M. R. Albiach-Martí, L. Rubio, and M. A. Ayllón
1996. Caracterización de razas del virus de la tristeza de los cítricos mediante hibridación molecular y su aplicación al control de la enfermedad. Estudio preliminar del genoma del virus. Instituto Valenciano de Investigaciones Agrarias (IVIA). pp. 207-216.
12. Pappu, H. R., S. Pappu, C. L. Niblett, R. F. Lee, and E. L. Civerolo
1993. Comparative sequence analysis of the coat proteins of biologically distinct citrus tristeza closterovirus isolates. Virus Genes 7: 255-264.
13. Permar, T. A., S. M. Garnsey, D. J. Gumpf, and R. F. Lee
1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. Phytopathology 80: 224-228.
14. Retief, J. D.
2000. Phylogenetic analysis using PHYLIP. Methods Mol. Biol. 132: 243-58.

15. Rocha-Peña, M. A., R. F. Lee, R. Lastra, C. L. Niblett, F. M. Ochoa-Corona, S. M. Garnsey, and R. K. Yokomi
1995. Citrus tristeza virus and its aphid vector, *Toxoptera citricida*. Plant Dis. 79: 437-445.
16. Roistacher, C. N., and P Moreno
1991. The worldwide threat from destructive isolates of citrus tristeza virus—A review. In: *Proc. 11th Conf. IOCV*, 7-19. IOCV, Riverside, CA.
17. Thompson, J. D., D. G. Higgins, and T. J. Gibson
1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.
18. Vela C., M. Cambra, E. Cortés, P. Moreno, J. G. Miguel, C. Pérez de San Román, and A. Sanz
1986. Production and characterization of monoclonal antibodies specific for citrus tristeza virus and their use in diagnosis. J. Gen. Virol. 67: 91-96.