Molecular Cloning and Sequencing of Coat Protein Genes of Citrus Tristeza Virus Isolated From Meyer Lemon and Homely Tangor Trees in Florida

B. Cevik, S. S. Pappu, H. R. Pappu, D. Tight, D. Benscher, S. H. Futch, P. Rucks, R. F. Lee, and C. L. Niblett

ABSTRACT. Samples were taken from seven Meyer lemon and two Homely tangor trees naturally infected with citrus tristeza virus (CTV) in Florida. The capsid protein gene (CPG) of CTV was amplified by reverse transcription/polymerase chain reaction, cloned in the pUC118 plasmid vector and sequenced. Comparative analyses of nucleotide and deduced amino acid sequences of the CPGs of the nine isolates showed 91-99% similarity among themselves and with T36, a Florida decline-inducing strain of CTV. Computer-assisted phylogenetic analyses of the capsid protein (CP) amino acid sequences indicated that the Meyer lemon isolates clustered either with T36 or with known stem pitting strains of CTV. However, isolates from Homely tangor clustered with T30 and other known mild isolates of CTV. Some of the Meyer lemon isolates showed stem pitting in Duncan grapefruit in biological assays. Variability among nucleotide sequences of some clones from the same isolate suggests that some trees may be infected with more than one strain of CTV.

Meyer lemon, a dwarf lemon variety, was introduced into California from China in 1908 (17). It was later introduced into Texas and Florida around 1920. It became a desired variety of backyard ornamental, and some commercial plantings were made in Texas and Florida during the 1930s. Some small commercial plantings still exist in Florida (3) The presence of citrus tristeza virus (CTV) in nearly all true Meyer lemon trees tested in California and Texas in the 1950s suggested that some of the Meyer lemon brought from China were infected with CTV (17, 18). Since most Meyer lemon trees in Florida were vegetative descendants of the original introductions, they were indexed for CTV in 1964 and were found to be nearly all infected with CTV (3). Even though most of the Meyer lemon trees were infected with CTV, the transmission rate of CTV from Meyer lemon to other citrus plants was relatively low (5). Several attempts to transmit Meyer lemon CTV isolates to Mexican lime with *Aphis gossipii* Glover (10), *Toxoptera aurantii* (Boyer de Fonscolombe) and *Aphis spiraecola* Patch failed. However, *Toxoptera citricida* (Kirkaldy) was able to transmit the Meyer lemon CTV isolates (9). Recent, biological indexing of CTV isolates present in Meyer lemon indicated the presence of stem pitting strains of CTV which do not occur in commercial citrus in Florida (S. M. Garnsey, personal communication).

The capsid protein genes (CPGs) of several geographically and biologically different isolates of CTV have been cloned and sequenced (1, 7, 8 12). Comparative analyses of the nucleotide and deduced amino acid sequences of the CPGs showed a high degree of homology among different strains of CTV, and a correlation was found between the biological characteristics and their CPG sequences (12). Restriction analyses of CPGs of CTV have also been used for characterization and strain differentiation. Six restriction fragment length polymorphism (RFLP) groups were established based on the pattern produced by restriction digestion of CTV CPGs with two restriction enzymes, *Hinf*I and *Rsa*I (4).

In this study, CTV isolates collected from Meyer lemon and

Homely tangor were studied. Their CPGs were cloned and sequenced. Based on sequence analysis, the relationship was determined between these isolates and other known, well-characterized strains of CTV from different origins.

MATERIALS AND METHODS

CTV isolates. Isolates FL7, FL14, FL15, FS84, and T10 were from the collection of S. M. Garnsey. The isolates FL7, FL14 and FL15 had been collected from Florida field sources of Meyer lemon and caused moderate stem pitting on graft inoculated Duncan grapefruit seedlings. Isolates FS84, and T10 were originally obtained from Meyer lemon trees in the field in Florida in the 1960s. T10 was mechanically transmitted as a subisolate from FS98 and showed vein clearing in Mexican lime. All the isolates were maintained in Madam Vinous sweet orange.

Isolates ML94-3-5 (201A), ML94-3-8 (202B), HG92-2E#17 (203C) and HG92-2E#20-1 (204D) were from the collection of R. F. Lee. The isolates 201A and 202B were collected from Meyer lemon trees in the field in Florida and caused very severe seedling yellows (SY), quick decline (QD) of sweet orange scions on sour orange rootstock and stem pitting (SP) on Duncan grapefruit indicator plants. The isolates 203C and 204D were taken from Homely tangor trees in the field in Florida and tested in variety of hosts. They caused very weak SY on sour orange seedlings and did not cause QD or SP on any host.

Oligonucleotide primers. The primers were designed as complementary to the 5' and 3' ends of the CTV CPG (12), and the recognition sites of restriction enzymes *Xba*I and *Eco*RI were added to the 5' end of primers CN150 and CN151, respectively, to facilitate cloning. The respective sequences of these primers were CN150 5'ATATATTTACT CTAGATCTACCATGGACGACGAA ACAAA3' and CN151 5'GAATCG GAACGCAATTCTCAACGTGTGTT AAATTTCC3'.

Preparation of tissue extracts and amplification of the CTV CPG. Three g of young bark and/or leaf tissue were frozen in liquid nitrogen and ground to a powder. The powder was ground further with 50 mM Tris-HCl pH 7.5 containing 5% sucrose at the ratio of 4 ml/g. The extract was filtered through cheesecloth, lyophilized in 1 ml aliquots, and stored at -20°C until used. One hundred mg of freeze-dried tissue extract was mixed with 1 ml of extraction buffer [0.1 M Tris-HCl pH 8.0, 0.2 mM ethylenediamine-tetraacetic acid (EDTA) plus 2% SDS], 250 µl of phenol, and 250 µl of chloroform. After vortexing, the mixture was heated at 70°C for 5 min., vortexed again and centrifuged at room temperature for 5 min. The supernatant was fractionated on a Sephadex G-50 chromatography column (15) prepared in 1 ml sterile syringe (Monoject Inc., St. Louis, MO), flash-frozen in liquid nitrogen and stored at -80°C. A coupled reverse transcription/ polymerase chain reaction (RT/PCR) protocol was used to amplify the CPG of CTV (13).

Cloning and sequencing of CTV CPGs. Amplified DNA products were purified using the Wizard DNA purification system (Promega) according to the manufacturer's instructions. The purified DNA (50 µl) was digested with *Eco*RI and *Xba*I restriction enzymes and ligated into the pUC118 plasmid vector. Competent cells of the DH5α strain of *Escherichia coli* were transformed with the recombinant pUC118 plasmid vectors. Recombinant colonies were selected and tested for the presence of the CTV CPG as described (12). The CPG of CTV in the recombinant pUC118 plasmid was sequenced in both direction using the Sequenase ver-

sion 2.0 sequencing kit (U.S. Biochemical), ^{35}S labeled ATP (DuPont), and the M13 forward or reverse universal primers by the dideoxynucleotide sequencing method for dsDNA (16).

Sequence analysis of the CTV CPG. The nucleotide sequences were read from exposed X-Ray film, imputed into a computer and analyzed using Seqaid II (14), Clustal V (6), and GCG (2) sequence analysis computer programs.

RESULTS

Cloning and sequencing of the CPGs of the CTV isolates. Fragments of the expected size, about 700 bp, for CPGs were amplified by RT/PCR from Sephadex-purified crude nucleic acid extracts of all nine CTV isolates. The CPGs of these isolates were cloned into the *Eco*RI and *Xba*I cloning sites of pUC118 plasmid vector as described. Three clones from isolate 201A (201A-1, 201A-2, and 201A-3), and two clones each from isolates 202B (202B-1 and 202B-2), 204D (204D-1 and 204D-2) and T10 (T10-1 and T10-2) were sequenced. Only one clone each was sequenced from isolates 203C, FL7, FL14, FL15 and FS84.

Analysis of the CPG nucleotide and amino acid sequences. The nucleotide and deduced amino acid sequences of the clones were examined for the presence or absence of the MCA13 epitope. The clones of isolates 203C and 204D had adenine (A) at position 371 in their nucleotide sequences and tyrosine (Y) at position 124 in their amino acid sequences (Fig. 1). Thus, the MCA13 epitope is not found in the CP of these isolates, suggesting that they are possibly mild strains of CTV. However, all clones from all the other isolates had thymine (T) at position 371 in the nucleotide sequence and phenylalanine (F) at amino acid position 124 (Fig. 1).

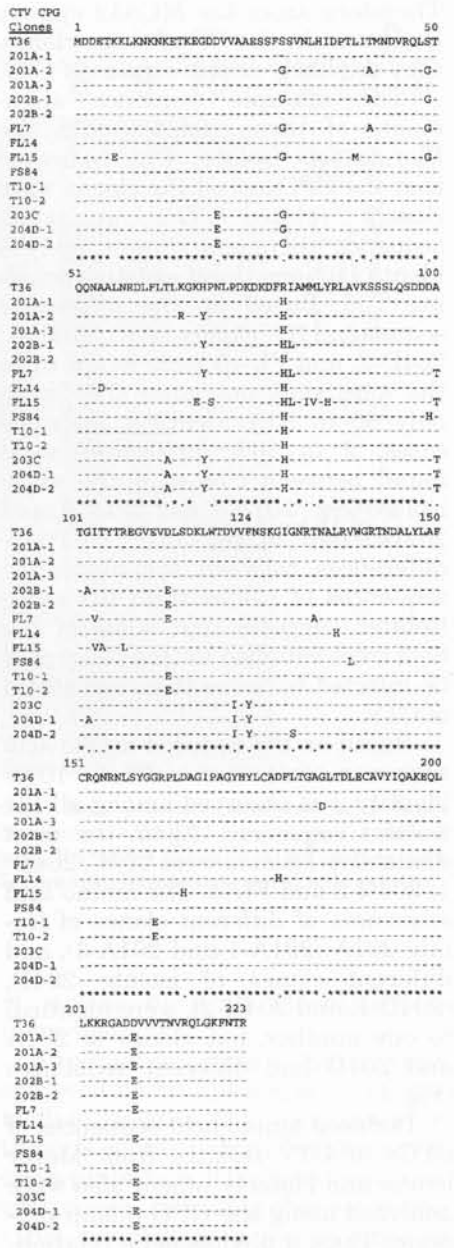

Fig. 1. Multiple alignment of deduced amino acid sequences of the CPGs of CTV isolates collected from Meyer lemon and Homely tangor trees with that of T36, a severe Florida strain. The alignment was produced using Clustal V. The asterisks and dashes indicate identical amino acids. Dots indicates substitution with different but similar amino acids.

Therefore, since the MCA13 epitope is present in the CP of these clones, they are likely severe strains of CTV.

The multiple sequence alignments of these isolates with the Florida QD isolate, T36, indicated that the CPGs of all the clones were closely related. The nucleotide sequence alignment showed 90-99% identity among these isolates including T36. Based on the nucleotide sequences, the clones 203C, 204D-1, 204D-2, and FL-15 were found to be the most different since they were only 90 to 93% identical. In some cases, 2 to 10% differences were found between clones of the same isolate (e.g., 201A-1 and 201A-2, and 202B-1 and 202B-2) (Fig. 1). These differences between the nucleotide sequences of clones from the same isolates suggests that some of the field trees sampled in this study may be infected by more than one strain of CTV.

Based on the deduced amino acid sequence comparisons, 91 to 100% identity was observed among all the isolates sequenced. Again the most distinctive isolates were 203C, 204D-1, 204D-2 and FL15. The amino acid sequences of different clones of isolate 201A (201A-1 and 201A-3), and different clones of isolate 204D, (204D-1, and 204D-2), were identical to one another, but clones of 201A and 204D had different sequences (Fig. 1).

Deduced amino acid sequences of CPGs of CTV isolates from Meyer lemon and Homely tangor also were analyzed using the GCG pileup program. Thus, a phylogenetic relationship was determined among the amino acid sequences of the CPs of the CTV clones. The clones 201A-1, 201A-3, T10-1, T10-2, 202B-2, FS84, and FL14 clustered with T36, the QD strain from Florida. The clones 202B-1, 201A-2, and FL7 clustered with T3, a distinct QD strain from Florida which also causes strong SP on Mexican lime, and also with B227 and B53, known SP strains from India, and Japan, respectively. The clones 203C, 204D-1, and 204D-2 clustered with T30, a known mild strain from Florida. The isolate FL15 did not cluster with any known strain of CTV, and it was the most different isolates of CTV thus far sequenced (Fig. 2).

Restriction fragment length polymorphism (RFLP) characterization of the CPG of the CTV isolates. The restriction enzymes *Hinf*I and *Rsa*I were used by Gillings et al. (4) to propose groupings of CTV strains based on the RFLP analysis of their CPGs. In our study, computer assisted searches for the sites of these enzymes indicated that the isolates 201A-1, 201A-3, 202B-1, 202B-2, FS84, T10-1, and T10-2 have the same restriction patterns as RFLP group I which includes T36. The isolates 203C, 204D-1, 204D-2 have the restriction pattern of RFLP group IV, which includes only mild strains of CTV. The isolates FL7 and was similar to RFLP group VI. The isolates 201A-2, FL14 and FL15 did not fit into any of Gillings' RFLP groups.

DISCUSSION

Nucleotide and deduced amino acid sequences were determined for the CPGs of 14 clones from nine different CTV isolates, seven collected from Meyer lemon and two from Homely tangor trees. The nucleotide and deduced amino acid sequences of these clones showed 90-99% and 91-100% identity, respectively, among themselves and with T36, the QD strain from Florida. The degree of identity is similar to earlier reports on CPG sequences of various CTV isolates (1, 7, 8, 12).

Sequence analyses indicated that the isolates 203C and 204D from Homely tangor, are probably mild strains of CTV. However, the isolates 201A, 202B, F7, F14, F15, FS84 and T10 from Meyer lemon trees, are most likely severe strains

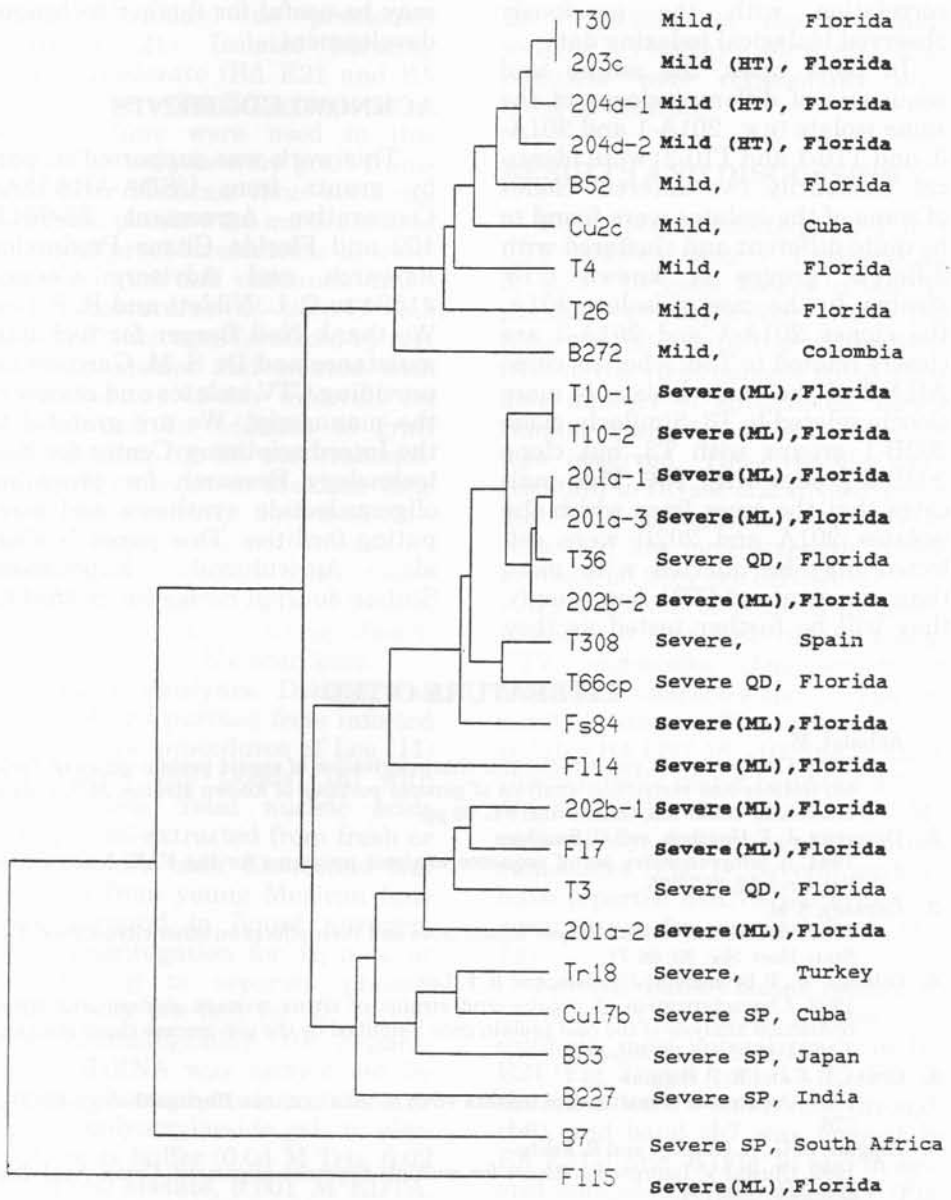

Fig. 2. A dendrogram showing the relationships of the deduced amino acid sequences of CPs of various strains of CTV. Biological activity such as mild (vein clearing on Mexican lime only), severe QD (quick decline on scions on sour orange rootstocks) and SP (stem pitting on grapefruit or sweet orange), and the country of origin of each strain are indicated where known. Sources of isolates sequenced in this study also are indicated (ML) = from Meyer lemon and (HT) = Homely tangor both from Florida.

of CTV. This was supported first by the absence or presence of the epitope for the monoclonal antibody MCA13 (11); second, based on the phylogenetic relationships among the CP sequences of various strains of CTV; and third, computer-assisted analysis of sites of the restriction enzymes *Rsa*I and *Hinf*I (4). The sequencing data showed a strong

correlation with the previously observed biological indexing data.

In most cases, the amino acid sequences of different clones of the same isolate (e.g., 201A-1 and 201A-3, and T10-1 and T10-2) were identical. Contrarily, two different clones of some of the isolates were found to be quite different and clustered with different groups of known CTV strains. In the case of isolate 201A, the clones 201A-1 and 201A-3 are closely related to T36; whereas clone 201A-2 of the same isolate is more closely related to T3. Similarly, clone 202B-1 groups with T3, but clone 202B-2 groups with T36. This indicates that the trees from which the isolates 201A and 202B were collected may be infected with more than one strain of CTV. Accordingly, they will be further tested as they may be useful for further technique development.

ACKNOWLEDGMENTS

This work was supported in part by grants from USDA-ARS/IFAS Cooperative Agreement 58-6617-102 and Florida Citrus Production Research and Advisory Council #1594 to C. L. Niblett and R. F. Lee. We thank Neil Berger for technical assistance and Dr. S. M. Garnsey for providing CTV isolates and review of the manuscript. We are grateful to the Interdisciplinary Center for Biotechnology Research for providing oligonucleotide synthesis and computing facilities. This paper is Florida Agricultural Experiment Station Journal Series No. R-05214.

LITERATURE CITED

1. Akbulut, M.
   1995. Citrus tristeza virus: Molecular characterization of capsid protein genes of Turkish isolates and restriction analysis of genome portions of known strains. M.S. Thesis. University of Florida, Gainesville, FL. 98 pp.
2. Devereux, J., P. Haerbeli, and O. Smithies
   1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Research 12: 387-395.
3. Garnsey, S. M.
   1970. Viruses in Florida's 'Meyer' lemon trees and their effects on other citrus. Proc. Fla. State Hort. Soc. 83: 66-71.
4. Gillings, M., P. Broadbent, J. Indsto, and R. F. Lee
   1993. Characterization of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. J. Virol. Methods 44: 305-317.
5. Grant, T. J. and R. P. Higgins
   1957. Occurrence of mixtures of tristeza virus strains in citrus. Phytopathology. 47: 272-276.
6. Higgins, D. G., A. Gleasby, and R. Fuchs
   1992. Clustal V: Improved software for multiple sequence alignment. Comp. Appl. Bioscience 8: 189-191.
7. Manjunath, K. L., H. R. Pappu, R. F. Lee, C. L. Niblett, and E. L. Civerolo
   1993. Studies on the coat protein gene of four isolates of citrus tristeza closterovirus from India: Cloning, sequencing and expression, p. 20-27 *In*: Proc. 12th Conf. IOCV., IOCV, Riverside, CA.
8. Mawassi, M., R. Gafny, and M. Bar-Joseph
   1993. Nucleotide sequence of the coat protein gene of citrus tristeza virus: Comparison of biologically diverse isolates collected in Israel. Virus Genes 7: 265-275.
9. Norman, P. A. and T. J. Grant
   1956. Transmission of citrus tristeza virus in Florida. Proc. Fla. State Hort. Soc. 66: 89-92.
10. Norman, P. A. and T. J. Grant
   1959. Transmission of T3 severe strain of citrus tristeza virus by the melon aphid. J. Econ. Entomol. 52: 632-634.
11. Pappu, H. R., K. L. Manjunath, R. F. Lee, and C. L. Niblett
   1993. Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. Proc. Natl. Acad. Sci. USA. 90: 3641-3644.

12. Pappu, H. R., S. S. Pappu, C. L. Niblett, R. F. Lee, and E. Civerolo
1993. Comparative analysis of the coat proteins of biologically distinct citrus tristeza closterovirus isolates. Virus Genes 7: 255-264.
13. Pappu, S. S., R. Brand, H. R. Pappu, E. P. Rybicki, K. H. Gough, M. J. Frenkel, and C. L. Niblett
1993. A polymerase chain reaction method adapted for selective amplification and cloning of 3' sequences of the potyviral genome: Application to dasheen mosaic virus. J. Virol. Methods 41: 9-20.
14. Rhoads, D. D. and D. S. Roufa
1985. Emetine resistance of Chinese hamster cells: Structure of wild type and mutant ribosomal protein S14 messenger RNA species. Mol. Cell Biol. 5: 1655-1659.
15. Sambrook, H., J. A. Fritsch, and T. Maniatis
1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Sanger, F., S. Niklen, and A. R. Coulson
1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA.74: 5463-5467.
17. Wallace, J. M. and R. J. Drake
1955. Tristeza virus in Meyer lemon. Citrus Leaves, Jan. 8 and 23.
18. Wallace, J. M., P. C. J. Oberholzer, and J. D. J. Hofmeyer
1956. Distribution of tristeza disease and the other diseases of citrus in propagative material. Plant Dis. Reptr. 40: 3-9.