Characterization of Citrus Tristeza Virus Isolates by SSCP of the Coat Protein Gene in Initially Healthy Sweet Orange Varieties After Three Years of Field Exposure

A. A. Souza, S. A. Carvalho, M. L. P. N. Targon, G. W. Müller, and M. A. Machado

ABSTRACT. Citrus tristeza virus (CTV) may be present as a complex mixture of strains, that affects symptom expression as a result of the strain composition and the susceptibility of the scion to the virus. The determination of the proportion and virulence of the strains in a complex may be made through techniques that allow a comparison of the strains of the virus. The identification of such complexes has mainly been carried out by the analysis of the viral genes. Natural CTV infection via *Toxoptera citricida* and the isolate diversity were studied on CTV-free plants of the following sweet orange varieties obtained through shoot-tip grafting: Baianinha, Pera Sta Alice, Pera GS 2000, Pera Rio, Pera IAC, Pera Olímpia, Pera Ipiguá, Westin, Rosa, Piralima and Serrana, all grafted on Rangpur lime and established in the field for 3 yr. Detection of CTV was made by DASI-ELISA, while isolate evaluation was made by single-strand conformation polymorphism (SSCP) of the coat protein gene. A mixture of CTV strains were detected in all the plants studied. SSCP analysis of the isolates collected in the second year showed very distinct patterns between varieties and clones. The patterns from isolates collected after 3 yr were less complex than that observed after 2 yr. Only two isolates maintained stable with similar SSCP patterns between the second and third years. Depending on its long-term stability and protective capacity, they may have potential as candidates for cross protection. Three years after planting, almost all isolates from Pera clones were similar. For almost all isolates, two fragments of higher intensity in SSCP were observed regardless of whether they were clones or varieties. The SSCP technique was satisfactory for monitoring the introgression of different isolates. An additional use for SSCP could be the evaluation of the stability of mild complexes for preimmunization.

Citrus tristeza virus (CTV) can be present as a mixture of strains that express various symptoms depending on the strain composition in the mixture and the susceptibility of the scion and rootstock combination. The determination of the number and severity of strains in a virus mixture can only be made through techniques that allow the effective comparison of these strains. The detection of those mixtures has mainly been carried out by the analysis of the coat protein gene (CPG) of the virus (6, 8, 9). Field isolates of CTV are presently being compared by single-strand conformation polymorphism (SSCP) analysis of RT-PCR products using primers for different genes. The procedure is also being applied to the study of cross protection between CTV strains (9).

The purpose of this work was: i) to characterize CTV isolates that became established in initially healthy sweet orange varieties during 3 yr of field exposure to the brown citrus aphid *Toxoptera citricida* (Kirkaldy); and ii) to determine the parameters that may contribute to the selection of mild CTV isolates for preimmunization programs.

MATERIALS AND METHODS

Plant material. The assay was carried out using tissue from the sweet orange varieties Baianinha, Pera Sta Alice, Pera GS 2000, Pera Rio, Pera IAC, Pera Olímpia, Pera Ipiguá, Westin, Rosa, Piralima and Serrana all grafted on Rangpur lime. The plants were from an experiment established in Sta Amélia farm, Araraquara County, São Paulo State, Brazil. All scions came from shoot-tip grafted plants grown under greenhouse conditions and free of CTV before being planted in the field. Plant tissue was

harvested in May after the second and third year after planting. From a group of three plants of each variety, one plant was chosen at random. Twigs were harvested from the north, south, west, and east quadrants of the selected tree and pooled to comprise a single sample of each variety. Samples of leaf and bark tissue were collected for dsRNA extraction using the CF-11 cellulose chromatography procedure of Valverde (14). In the third year, the plants were evaluated also for development, production of fruits and stem-pitting incidence.

DASI-ELISA analysis. The double antibody sandwich indirect (DASI) ELISA was used to determine CTV infection after the plants were in the field for 2 yr. The samples used for DASI-ELISA were collected as describe above. The DASI-ELISA was performed using the procedure of Garnsey and Cambra (4). The microtiter plates (Nunc) were coated with polyclonal antibody PCA 1006/BR (1:10,000). The monoclonal antibodies 3DF1 + 3CA3 (1:10,000) were used as secondary antibodies. Samples were considered positive when OD_{405} values were higher than three times the mean of the healthy controls.

First strand cDNA synthesis and coat protein (CP) gene amplification. Double-stranded RNA was used as template for the first strand cDNA synthesis using random primers. The dsRNA was denatured at 75ºC for 10 min and chilled on ice. The cDNA reaction was carried out at 37ºC for 60 min, using M-MLV reverse transcriptase (GIBCO) (10). About 1/10 of the cDNA reaction was used for the polymerase chain reaction (PCR) to amplify the CP gene using CTV specific primers (RFL33 5'TCAACGTGTGTTGAATTT3' and RFL34 5'ATGGACGACGAAACAAAG3'). The DNA amplification was performed in 35 cycles of denaturation for 2 min at 94ºC, annealing for 2 min at 55ºC, and synthesis for 2 min at 72°C, followed by a single chain extension period of 10 min at 72°C. The success of the amplification was determined by analyzing an aliquot on 1% agarose gels. The gels were electrophoresed at 100 V for 30 min, stained with ethidium bromide and observed under UV light.

SSCP analysis. SSCP analysis was performed directly on the PCR products obtained from the CP gene. Usually, 1 to 3 ml of the PCR reaction were mixed with equal volumes of denaturing solution (95% formamide, 20 mM EDTA and 0.05% xylene-cyanole and 0.05% bromophenol blue), heated for 10 min at 95ºC, and chilled on ice. In preliminary experiments, several conditions (gel size, voltage, acrylamide concentration, run time, temperature, and presence or absence of glycerol) were assayed. The denatured DNA was electrophoresed using non-denaturing 8% polyacrylamide gels (16 × 20 cm × 0.75 mm) using TBE 0.5 × as electrophoresis buffer (10) and a constant voltage of 200 V for 7h at 25ºC. The gels were stained with silver nitrate (2).

RESULTS AND DISCUSSION

Agronomic characteristics of the plants evaluated are shown in Table 1. Among the Pera clones (susceptible to CTV) and the other varieties (CTV tolerant), the Pera GS 2000 and Baianinha varieties presented the best values of mean fruit yield. Furthermore, the Pera GS 2000 showed no stem pitting and surpassed development all other plants in mean plant growth (Fig. 1A). In general, all plants showed no or low stem pitting, except for the Pera Olímpia variety that had the highest stem pitting score and one of the lowest growth scores (Table 1). However, yield for this variety was as poor as other varieties having low or no stem pitting values. All trees evaluated by DASI-ELISA gave positive CTV reactions (Table

TABLE 1
AGRONOMIC CHARACTERISTICS OF THE SWEET ORANGE VARIETIES AFTER THREE YEARS OF FIELD EXPOSURE

Variety	Size[z]	Stem pitting[y]	Yield[x] Kg/tree	Yield[x] (bx/tree)
Baianinha	4.0	1.0	189	4.6
Pera Sta Alice	2.6	0.0	108	2.7
Westin	4.4	1.0	86	2.1
Rosa	3.0	0.0	81	1.9
Pera GS 2000	5.0	0.0	216	5.3
Pera Rio	3.3	1.0	84	2.0
Piralima	3.7	0.0	81	1.9
Serrana	3.0	0.0	84	2.0
Pera IAC	4.0	2.0	108	2.6
Pera Olímpia	3.3	3.0	89	2.1
Pera Ipiguá	4.0	1.0	81	1.9

[z]Average scores from three examinators: 1 = small size / bad development; 5 = big size/ good development (mean of three plants).
[y]Average scoring from three examinators: 0 = absence of stem pitting; 6 = pronounced stem pitting (mean of four twigs).
[x]97/98 harvest.

2) indicating that after 2 yr in the field, all plants were infected with CTV. After reverse transcription polymerase chain reaction (RT-PCR) using CP gene specific primers, all the isolates showed DNA fragments of the expected size (11).

The SSCP analysis of the RT-PCR products from the isolates collected on the second year (Fig. 2A) showed very distinct patterns between varieties and clones. Similar SSCP patterns were observed only in lanes 1 and 3. Through the SSCP analysis, different profiles and several bands were detected, indicating the occurrence of different isolates in the varieties and strain mixtures between them. However, the number of isolates can be greater than this procedure suggests, since during SSCP some virus strains may have very similar conformations, and it is not possible to separate those bands during electrophoresis using the conditions determined in this work.

The SSCP patterns of the isolates collected from the plants 3 yr after planting (Fig. 2B) were less complex as compared to those observed in the plants 2 yr after planting. The low complexity of the isolates is sometimes related to the time of harvest (3); however, in this

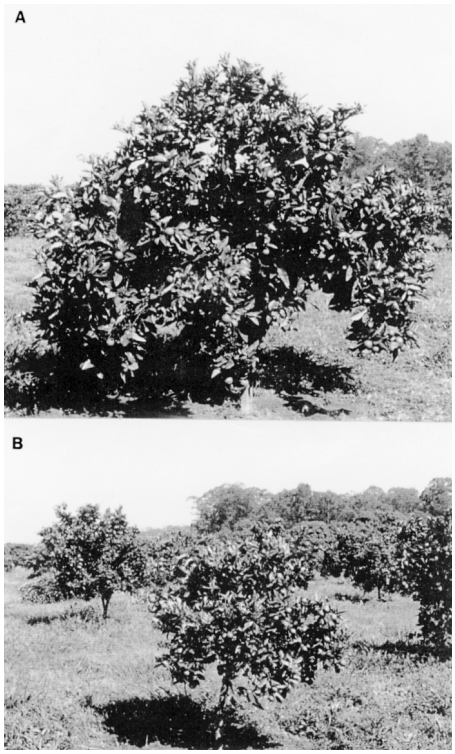

Fig. 1. A) Three-yr-old Pera GS 2000 with good growth. B) Same age Pera Olímpia showing little growth.

TABLE 2
ABSORBANCE VALUES (OD_{405}) FROM CITRUS TRISTEZA VIRUS IN SWEET ORANGE CULTIVARS ASSAYED BY DASI-ELISA

Variety	Mean OD_{405}
Baianinha	1.052
Pera Sta Alice	0.944
Westin	1.106
Rosa	1.065
Pera GS 2000	1.051
Pera Rio	1.049
Piralima	1.051
Serrana	0.911
Pera IAC	1.041
Pera Olímpia	0.922
Pera Ipiguá	0.902
Positive control	1.068
Negative control	0.022
Blank control	0.002

work sample tissue was collected at the same time each year. The lower complexity of isolates found in the plants 3 yr after planting, suggests the existence of competition between certain strains. Only the isolates contained in lanes 3 and 7 maintained themselves between years two and three, with patterns similar to those detected when the plants were 2-yr-old. The differences in the complexity in the CTV isolates found in the plants between the second and third year suggests that a selection may be occurring depending on the citrus variety, inoculation of different strains by *T. citricida*, and the interactions between the strains, since all plants were submitted to a same environment and collected at the same time. If the virus in Baianinha and Westin remain stable and protective over time, they may be potential candidates for cross protection.

Almost all CTV isolates from Pera clones (Fig. 2B, lanes 5, 6, 9, 10 and 11) were similar among themselves, however it can not be said that a selection of the strains of the complex occurred in the host. In spite of the CPG being one of the most studied regions of the virus (6, 8, 9, 11), it represents only 3.5% of the CTV genome, indicating that differences between the isolates may occur or that the conditions used for SSCP do not provide

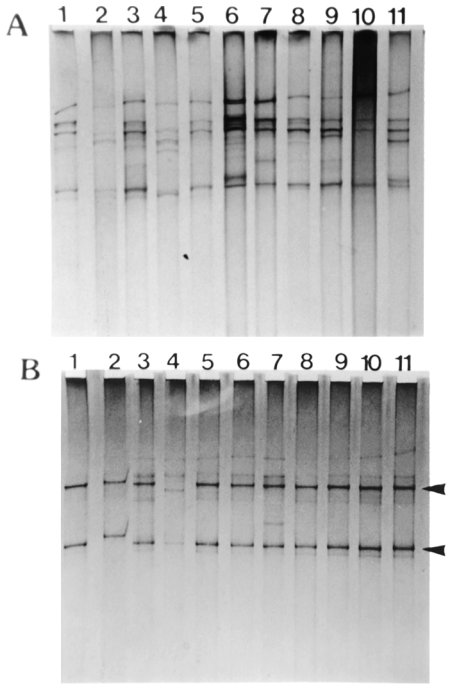

Fig. 2. Single-strand conformation polymorphism patterns of the CPG of CTV isolates collected from sweet orange varieties. 1. Baianinha; 2. Pera Sta Alice; 3. Westin; 4. Rosa; 5. Pera GS 2000; 6. Pera Rio; 7. Piralima; 8. Serrana; 9. Pera IAC; 10. Pera Olímpia; 11. Pera Ipiguá. A) 2 yr after planting B) 3 yr after planting.

adequate resolution of the two strands of the CPG. Two fragments of higher intensity (represented by arrows) were observed in almost all isolates independently of whether they were clones or varieties.

The SSCP patterns of samples electrophoresed at 200V at 25°C for 7 h in polyacrylamide gels, allowed good separation of the two DNA strands and were reproducible. Rubio et al (9) detected differences in the CPG among CTV isolates using minigels and run times of 3 to 5 h. In this study, these conditions were not satisfactorily to distinguish the isolates, perhaps due to the complexity of the field isolates in Brazil. However, to detect a higher variability in the CPG among the isolates, it is necessary to use more than a single set of conditions.

The SSCP technique is applied to the study of the characterization, detection, population diversity and genetic variability of virus and viroids (1, 5, 7, 9, 12, 13). In this study, the SSCP technique was shown to be satisfactory for monitoring the introgression of different CTV isolates. A possible additional use for this technique could be the evaluation of the stability of mild CTV complexes for preimmunization.

LITERATURE CITED

1. Ayllon, 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. 255: 32-39.
2. Beidler, L. L., P. R. Hilliard, and R. L. Rill
1982. Ultrasensitive staining of nucleic acids with silver. Anal. Biochem. 126: 374-380.
3. Dodds, J. A., T. Jarupat, J. G. Lee, and C. N. Roistacher
1987. Effects of strain, host, time of harvest, and virus concentration on double-stranded RNA analysis of citrus tristeza virus. Phytopathology 76: 820-824.
4. Garnsey, S. M. and M. Cambra
1991. Enzyme-linked immunosorbent assays for citrus pathogens. In: *Graft-Transmissible Diseases of Citrus. Handbook for Detection and Diagnosis*. C. N. Roistacher (ed.), 48-50. FAO, Rome.
5. Koenig, R., P. Luddecke, and A. M. Haeberle
1995. Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphism of immunocapture RT-PCR products. J. Gen. Virol. 76: 2051-2055.
6. Gillings, M., P. Broadbent, J. Inosto, and R. F. Lee
1993. Characterization of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by polymerase chain reaction. J. Virol. Methods 44: 305-317.
7. Palacios, A. and N. Duran-Vila
1999. Single-strand conformation polymorphism (SSCP) analysis as a tool for viroid characterization. J. Virol. Meth. 77: 27-36.
8. Pappu, H. R., S. S. Pappu, C. L. Niblett, R. F. Lee, and E. L. Civerolo
1993. Comparative sequence analysis of the coat protein of biologically distinct citrus tristeza closterovirus isolates. Virus Genes 7: 255-264.
9. Rubio, L., M. A. Ayllón, J. Guerri, H. Pappu, C. Niblett, and P. Moreno
1996. Differentiation of citrus tristeza virus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. Ann. Appl. Biol. 129: 479-489.
10. Sambrook, J., E. F. Fritsch, and T. Maniatis
1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory. New York.
11. Sekiya, M. E., S. D. Lawrence, M. McMcaffery, and K. Cline
1991. Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. J. Gen. Virol. 72: 1013-1020.
12. Shi, N., J. Chen, T. M. A. Wilson, S. A. Macfarlane, J. F. Antoniw, and M. J. Adams
1996. Single-strand conformation polymorphism analysis of RT-PCR products of UK isolates of barley yellow mosaic virus. Virus Res. 44: 1-9.
13. Szychowski, J. A., R. Credi, K. Reanwarakorn, and J. S. Semancik
1998. Population diversity in grape vine yellow speckle viroid-1 and the relationship to disease expression. Virology. 248: 432-444.
14. Valverde, R. A., S. T. Nameth, and R. L. Jordan
1990. Analysis of double strand RNA for plant virus diagnosis. Plant Dis. 71: 255-258.