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# Evaluation of Changes Which Occurred in a Mild Protective Citrus Tristeza Virus Isolate in Pera Sweet Orange Trees by Using RFLP and SSCP Analyses of the Coat Protein Gene

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**ABSTRACT.** The cross protection program to control citrus tristeza virus (CTV) in Pera sweet orange through the use of preimmunization with a mild isolate of CTV has been working satisfactorily for 30 yr with approximately 80 million preimmunized trees in the State of Sao Paulo (SP), Brazil. Recently, however, 3- to 4-yr-old plants (daughter plants) grown from budwood selected from 20-yr-old vigorous preimmunized Pera sweet orange trees are showing severe CTV symptoms. The protective CTV isolate present in the mother trees was compared with the isolates occurring in the daughter plants that were showing severe symptoms by restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analyses of the CTV coat protein gene (CPG). RFLP analyses detected no polymorphism between the CTV isolates of the mother trees and the original isolate maintained in stock mother plants in Cordeirópolis County, Sao Paulo, Brazil. However, the CTV in the daughter plants were quite different from the CTV isolates present in the mother plants. SSCP analyses revealed complex and different patterns in almost all the CTV isolates, including both groups of mother plants. The ramification of these results are discussed in relation to symptoms in the daughter plants and with cross protection.

The control of severe citrus tristeza virus (CTV) isolates in Pera sweet orange scions budded on rootstocks has been satisfactorily achieved by preimmunization with a mild isolate of CTV. Presently, 80 million preimmunized trees are growing in the State of Sao Paulo and other parts of Brazil as nursery plants, young or bearing orchards. However, more recently, 3- to 4-yr-old plants propagated from 20-yr-old vigorous preimmunized Pera sweet orange trees have been showing severe CTV symptoms. This could be attributed to a segregation of the protective isolate or the arising of severe isolates able to supplant the former after many years of exposure.

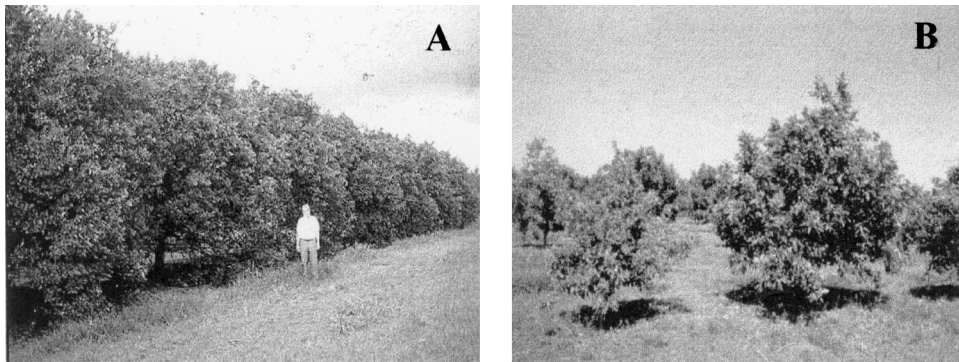
Techniques such as RFLP (restriction fragment length polymorphism) and SSCP (single-strand conformation polymorphism) of coat protein gene (CPG) have been used in order to characterize CTV isolates (2, 5). RFLP consists in the digestion of DNA from PCR amplifications using restriction enzymes. Fragments of different sizes are generated and separated by electro-

phoresis. SSCP analyses is based on the fact that denatured double-stranded DNA from PCR amplifications migrates as two single stranded DNA (ssDNA) bands in non-denaturing polyacrylamide gel electrophoresis. The migration of the two strands depends on the sequence of nucleotides and, therefore, on their conformation under the electrophoretic conditions used. It has been demonstrated that small changes in the sequence may alter conformation of the ssDNA and therefore their electrophoretic profile (4).

The purpose of this work was to compare the RFLP and SSCP patterns of the CPG of the CTV mild protective isolate from the 20-yr-old Pera IAC trees used as mother trees with those from the isolates found in daughter plants showing severe symptoms.

## MATERIALS AND METHODS

The evaluations were carried out using 10 20-yr-old Pera IAC mother trees (preimmunized) without symp-



**Fig. 1. A). Twenty-yr-old Pera TAC (preimmunized) sweet orange plant used as mother trees. B) Three- to 4-yr-old trees raised from tree shown in (A); tree on the left is showing severe CTV symptoms and the tree on the right is a good plant.**

toms (Fig. 1A), and a tree between 3- to 4-yr-old showing severe decline with severe stem pitting and which was representative of several other trees (Fig. 1B) propagated from budwood from the mother trees. The trees were growing in the Matão County, SP. The control mild protective CTV isolate was that in a Pera IAC tree maintained in the greenhouse at the Centro de Citricultura Sylvio Moreira (CCSM), Cordeirópolis County, São Paulo, Brazil. Young leaf and young bark samples collected from the plants were lyophilized and stored at  $-20^{\circ}\text{C}$  until used for CTV dsRNA extraction.

Ds RNA was isolated according to modifications of the procedure described by Valverde et al. (8). The dsRNA was used as a template for the first strand cDNA using random primers. The dsRNA was denatured at  $75^{\circ}\text{C}$  for 10 min and chilled on ice. The cDNA reaction was carried out at  $37^{\circ}\text{C}$  for 60 min, using M-MLV reverse transcriptase (GIBCO) (6). About  $1/10$  of the cDNA reaction was used for the PCR to amplify the CPG 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  $94^{\circ}\text{C}$ , annealing for 2 min at  $55^{\circ}\text{C}$ , and synthesis for 2 min at  $72^{\circ}\text{C}$ , followed by a single

chain extension period of 10 min at  $72^{\circ}\text{C}$ . The visualization of the amplified product was carried out by electrophoresis in 1% agarose gels containing ethidium bromide and observed under UV light.

The amplified CPG was digested with Hinf I, previously described for discriminating CTV isolates (2), according to the manufacturer's instructions (Biolabs). Digests were analyzed by electrophoresis in 8% polyacrylamide gels ( $16 \times 20\text{cm} \times 1\text{mm}$  BIO-RAD Protean II) using TBE 0.5 X (6) as electrophoresis buffer, and constant voltage of 200 V for 2 h at  $25^{\circ}\text{C}$ . The gels were silver stained using the procedure of Beidler et al. (1). The RFLP patterns were evaluated in function of size and number of fragments generated after digestion.

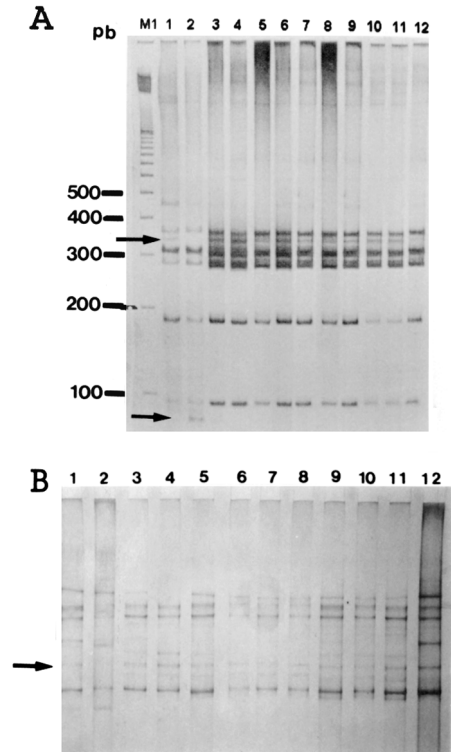
SSCP analysis was performed directly on the PCR products of the CPG of CTV isolates. Usually, 1 to  $3 \mu\text{l}$  of the PCR product was mixed with equal volume of the denaturing solution (95% formamide, 20 mM EDTA, 0.05% xylene-cyanole and 0.05% bromophenol blue), heated for 10 min. at  $95^{\circ}\text{C}$ , and chilled on ice. In preliminary experiments, several conditions (gel size, voltage, polyacrylamide concentration, run time, temperature, and presence or absence of glycerol) were determined. The denatured DNA was elec-

rophoresed using the conditions determined: non-denaturing 8% polyacrylamide gels (16 × 20 cm × 1mm BIO-RAD Protean II xi) using TBE 0.5 X (6) as electrophoresis buffer, and constant voltage of 200 V for 7h, at 25°C. The gels were silver stained using the procedure of Beidler et al. (1). The SSCP patterns were evaluated in function of position and number of electrophoretic bands.

## RESULTS

After RT-PCR, the samples from CTV-infected plants produced a DNA fragment of approximately 670 bp, the expected size of the CP gene (7). The digestion of the CP gene with the enzyme *Hinf* I, revealed several restriction sites (Fig. 2A). The CTV protective isolate of the Pera IAC maintained in the greenhouse presented seven different restriction fragments (Fig. 2A, lane 1). The CPG amplified from the mother trees (Fig. 2A, lanes 3-12) presented the same restriction patterns as that of the amplified CPG from the protected Pera IAC. Polymorphism was detected between the isolate in the plant showing stem pitting symptoms and the other isolates, the first having a fragment under 100 bp (represented by the arrow) which was not detected in the other isolates and the absence of one fragment between 300 and 400 bp fragment (represented by the arrow) present in all isolates (Fig. 2A, lanes 1 and 2).

In the SSCP analysis, polymorphism was observed with nearly all the isolates (Fig. 2B). The isolate of the Pera IAC kept in the greenhouse (Fig. 2B, lane 1) differed from the isolate in the plant with stem-pitting (Fig. 2B, lane 2) by the presence of one fragment (represented by the arrow). None of the isolates from the mother trees presented patterns similar to the one plant with stem pitting. The SSCP patterns of the



**Fig. 2. (A) Restriction patterns of *Hinf* I digested citrus tristeza virus (CTV) coat protein gene (CPG). (B) Single-strand conformation polymorphism (SSCP) patterns of the CPG of CTV isolates. Lane M1 contains 100 bp DNA ladder (BRL); lane 1 C Pera IAC tree with the protective isolate kept in the greenhouse; lane 2 C Tree showing severe CTV symptoms; lanes 3 to 12 C Pera IAC (preimmunized) sweet orange plants used as mother trees.**

CTV isolates in preimmunized Pera IAC used as mother plants (Fig. 2B lanes 3, 5, 9, 10 and 11) were apparently identical, and similar to other source trees of Pera IAC represented in lanes 6, 7 and 8. The isolate in the tree represented in lane 4 differed from the isolates represented in lanes 3, 5, 9, 10 and 11 by the absence of the upper band, while the isolate represented in lane 12 presented a SSCP pattern very similar to that of the protected Pera IAC with the protective isolate (Fig. 2B, lane 1).

## DISCUSSION

RFLP analysis detected polymorphism in the CPG between the severe isolate and the isolates present in the mother trees. The disadvantage of this method is that mutations are recognized only when they occur in the sequence recognized by the restriction endonuclease, and that PCR products may need to be tested with many different restriction enzymes (3, 5). Because of this limitation, the SSCP technique is more appropriate for detecting point mutations and is more useful than RFLP to detect polymorphism (5).

The SSCP analysis distinguished mainly three groups. The SSCP patterns of different isolates presented bands with the same electrophoretic mobility, suggesting that probably these isolates have similar variants or that the variants present similar CPG conformations that can not be separated using the conditions described.

The protective CTV isolate present in the Pera IAC maintained under greenhouse conditions had a SSCP pattern similar to the isolate represented in Fig. 2 B, lane 12, but

differed slightly from the patterns of the 20-yr-old field mother trees. This suggests that other more competitive CTV isolate variants could establish themselves in the mother plants over time, promoting a partial alteration of the original protective isolate. However, the plant showing stem-pitting produced a CTV CP SSCP pattern that was not similar to any of the mother trees. If RFLP and SSCP patterns of CPG may be associated with stem-pitting severity in the field as proposed by Gillings et al. (2), different patterns between mother and daughter trees, excluding nursery contamination, may signify that the protective isolate may be changed by other isolates and some of them may become severe over the course of time.

In the present study, SSCP analysis of the CPG of CTV allowed the quick identification of groups of the preimmunized plants that best represented the field isolates studied. We found that SSCP analysis, more than RFLP, was satisfactorily for monitoring the protective isolate in preimmunized plants, as well as for the detection of severe isolates that, by chance, may become established.

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