Stability of the Mild Protective 'PIAC' Isolate of *Citrus tristeza virus*

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

ABSTRACT. An important aspect of tristeza control in Brazil is the isolation and testing of new mild strains of *Citrus tristeza virus* (CTV) for use in preimmunization, since the protection this affords can sometimes break down. To evaluate the stability of the mild isolate obtained from Pera IAC sweet orange (PIAC isolate), this was graft-inoculated to different species and varieties of citrus. After 4 yr, the plants were evaluated by single strand conformation polymorphism (SSCP) of the coat protein gene. The SSCP profile remained unchanged only in Pera IAC and Mexican lime. To test the protection given by the isolate, plants that had maintained the original SSCP profile were challenged by cross-inoculation using the severe isolate Barão B. After 45 days, 6 mo and 1 yr, samples were again analyzed by SSCP, and the plants still showed the original profile, and had only mild symptoms. When exposed to natural aphid inoculation in the field, the Pera IAC plants retained the original SSCP profile even after 1 yr, and after 2 yr these trees showed no symptoms. We conclude that the PIAC isolate was stable and that it had protected the trees from severe field strains of CTV.

Index words. SSCP, cross-protection.

Brazil is the main world producer of sweet orange, accounting for 36% of global production (13). *Citrus tristeza virus* (CTV) became a major threat to Brazilian citriculture in the 1940s, when it attacked all combinations using sour orange as rootstock. Since finding that certain combinations of rootstock and sweet orange scion, as well as preimmunized clones, are more tolerant to this virus, the Brazilian citrus industry has lived with the disease, although today all trees are infected with CTV to differing degrees. In Brazil preimmunization with mild isolates has been used for many years for controlling stem-pitting isolates of CTV on sweet orange. The mild isolate used must have desirable characteristics such as stability in the host and no influence on plant development (5). Recently stem-pitting has occurred on preimmunized plants, and the SSCP profile of the CTV coat protein gene obtained from these plants was different from that of the mild isolate used for preimmunization, suggesting breakdown of cross-protection (10, 11). These results underline the need to find new isolates suitable for use in preimmunization.

Most CTV isolates are actually a population of genetically related variants (4), and several approaches have been tested for the evaluation of such isolates. Single strand conformation polymorphism (SSCP) analysis provides rapid discrimination of DNA fragments of the same size but with variations in sequence, since small changes in sequence may alter the conformation of ssDNA and consequently its electrophoretic mobility. SSCP analysis using different genomic regions has been used to differentiate CTV isolates and monitor cross-protection between mild and severe isolates (1, 4, 6, 7, 8, 10, 11).

We report here experiments with a mild CTV isolate obtained from Pera IAC sweet orange. We used SSCP to assess the stability of the isolate in different citrus hosts, and examined its ability to cross-protect against challenge by severe strains of CTV.

MATERIALS AND METHODS

Plant material and CTV isolates. The mild CTV isolate 'PIAC' was selected from an outstanding Pera IAC sweet orange/Rangpur

lime combination in a variety trial at the Centro APTA Citros Sylvio Moreira, Cordeirópolis, SP, Brazil (12). The Barão B CTV isolate was collected from a Barão sweet orange/ Caipira sweet orange combination; this isolate has previously been used as a severe CTV control (8, 14).

Buds of shoot-tip grafted (virus free) sweet orange (cvs. Pera, Baia and Hamlin), Mexican lime, and Ponkan mandarin, were grafted on Rangpur lime rootstock. When the plants were 30 cm tall, two buds infected with PIAC were grafted into five virus-free plants of each species/variety. The plants were kept in a greenhouse at the Centro APTA Citros Sylvio Moreira. Four years later, samples of young bark were collected for CTV dsRNA purification. The Pera sweet orange and Mexican lime plants were challenge-inoculated by grafting with buds containing isolate Barão B. Samples were collected 45 days, 6 mo and 1 yr later. Symptoms (average of three different plants) were scored on a 0-5 scale (0 = none, 5 = very intense) after 1 yr. Eight Pera IAC plants containing isolate PIAC were taken to the field; four to the orchard at the Centro APTA Citros Sylvio Moreira and four to the Capão Bonito experimental station, southern region of São Paulo, with the highest incidence of the severe Capão Bonito CTV complex. Samples collected after 1 yr were re-analyzed by SSCP and re-evaluated for symptoms.

First-strand cDNA synthesis and coat protein gene amplification. Double-stranded RNA was isolated according to the procedure of Valverde et al. (15). This RNA was used as template for first-strand cDNA synthesis using random primers. The dsRNA was denatured at 75°C for 10 min and chilled on ice. The cDNA reaction was carried out at 37°C for 60 min, using M-MLV reverse transcriptase (GIBCO) (9). About 1/10 of the cDNA product was used for the polymerase chain reaction (PCR) to amplify the entire CP gene (p25 region) using specific primers (forward 5'ATGGACGAC-GAAACAAAG 3' and reverse 5'TCAACGTGTGTTGAATTT3') (3). Amplification was performed in 35 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 55°C, and synthesis for 2 min at 72°C, followed by single chain extension for 10 min at 72°C. The products were analyzed on 1% agarose gels (electrophoresed at 100 volts for 30 min, stained with ethidium bromide and observed under UV light).

SSCP analysis. This was done on the CP gene PCR product obtained. Usually 1-3 µl of the PCR reaction were mixed with an equal volume of denaturing solution (95% formamide, 20 mM EDTA, 0.05% xylene-cyanole and 0.05% bromophenol blue), heated for 10 min at 95°C, and chilled on ice. The denatured DNA was separated by electrophoresis using non-denaturing 8% polyacrylamide gels (16 × 20 cm × 0.75 mm BIO-RAD Protean 11 A) and 0.5× TBE (10) as eletrophoresis buffer. A constant 200 volts was applied for 7 h, at 25°C. The gels were stained with silver nitrate (2).

RESULTS

All PCR tests revealed a 670 bp product matching the CP gene size. Negative control plants (grafted with virus-free buds) gave no amplification (results not shown).

The SSCP results after four years are illustrated in Fig. 1. Almost all profiles showed more than two bands, indicating that each CTV isolate contained different types. The profile generated four years after inoculation with PIAC was the same as the original only in Pera IAC sweet orange and Mexican lime (Fig. 1). In the other varieties/ species some of the original types were not found, indicating selection for certain variants in these plants.

To test the protection given by PIAC in Pera IAC sweet orange and Mexican lime, (the only ones that

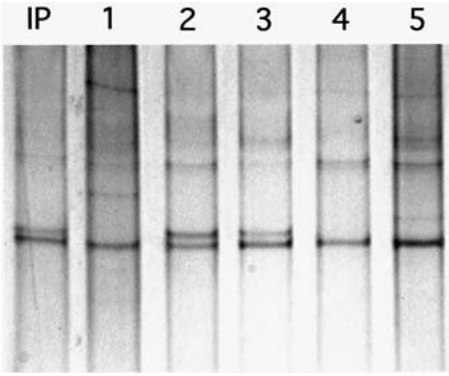

Fig. 1. SSCP profile of the CP gene from isolates of *Citrus tristeza virus* in different species and varieties of citrus evaluated 4 yr after preimmunization with a mild isolate from Pera IAC sweet orange (PIAC) IP, Original mild isolate; 1, Ponkan mandarin; 2, Mexican lime; 3, Pera IAC sweet orange; 4, Baia Tremembé sweet orange; 5, Hamlin sweet orange.

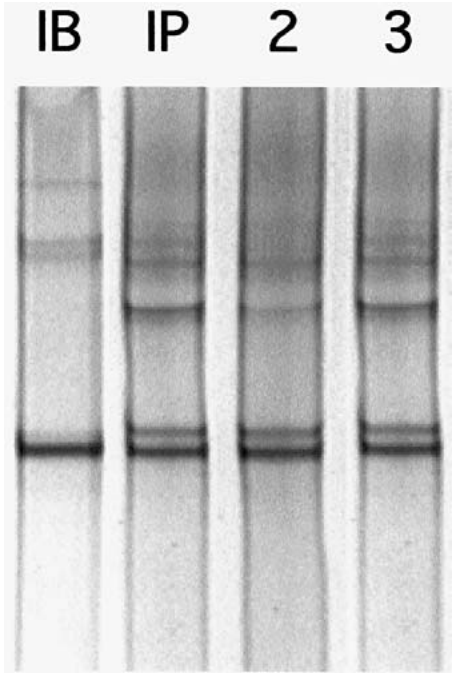

Fig. 2. SSCP profile of the CP gene from *Citrus tristeza virus* observed 1 yr after inoculation of the severe isolate Barão B in plants pre-immunized with the mild PIAC isolate. IB, pattern of Barão B; IP, profile of original PIAC; 2, Mexican lime; 3, Pera IAC sweet orange.

maintained the original SSCP profile), these were evaluated by challenging with the severe isolate Barão B. After 45 days, 6 mo and 1 yr samples were analyzed by SSCP and were seen to have maintained the original profile (Fig. 2).

These results were in good agreement with the symptoms observed in the greenhouse. Pera IAC sweet orange and Mexican lime inoculated only with Barão B were rated 4 on the symptom scale. On the other hand, the preimmunized plants challenged with Barão B showed much milder symptoms, rated 1, for Pera IAC sweet orange, and rated 2 for Mexican lime. This indicates that protection was given by the mild PIAC isolate.

Even after one year of exposure to aphids in the field, the Pera IAC plants retained a profile identical to that of the original protective isolate (Fig. 3). Two years from planting out, no CTV symptoms have been observed, even at Capão Bonito where the isolates are extremely severe and infect all varieties of sweet orange as well as the Rangpur lime rootstock.

DISCUSSION

The PIAC isolate was stable in Mexican lime and Pera sweet orange, where it also had a higher titer compared to other varieties suggesting increased replication in these hosts (14). Together with the mild symptoms observed in plants carrying the isolate and challenged with a severe isolate, and no symptom development in the field under severe pressure, these characteristics make it promising for the Brazilian preimmunization program.

Sambade et al. (8) analyzed two mild CTV isolates to monitor cross-protection in the greenhouse using SSCP analysis of four genes. These mild isolates did not protect sweet orange against challenge with severe isolates, since severe symptoms developed and a change in the SSCP profile was observed. The

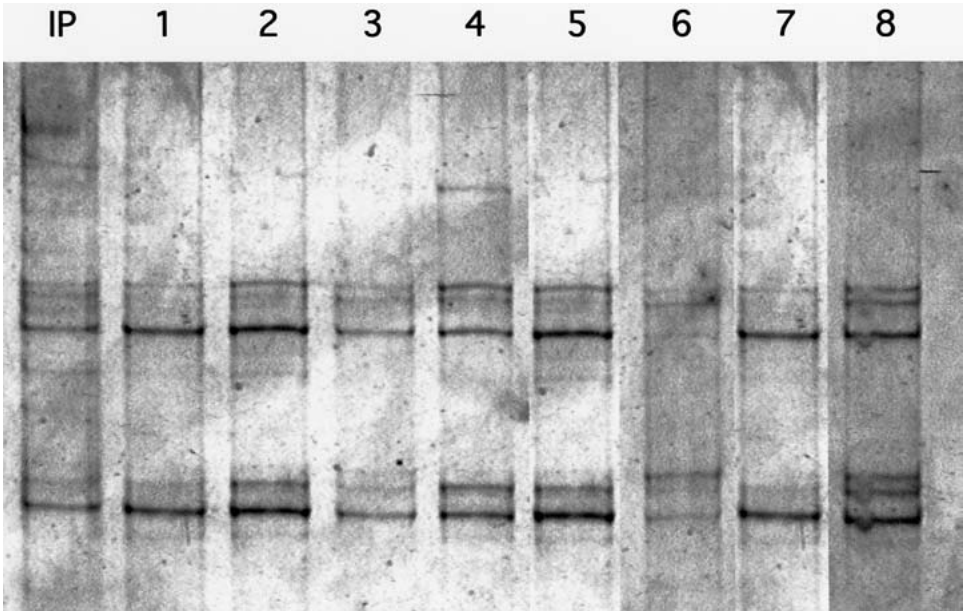

Fig. 3. SSCP profile of the CP gene from isolates of *Citrus tristeza virus* obtained from four plants pre-immunized with PIAC and challenged in the field. IP, profile of the original PIAC isolate; 1-4, plants challenged in the experimental field at the Centro APTA Citros Sylvio Moreira; 5-8, plants challenged in the experimental field at Capão Bonito.

SSCP profile of the CTV from challenged plants indicated a mixture of both isolates, with the bands characteristic of mild isolates often being less intense than those characteristic of the severe isolates. These changes occurred in 3-6 mo after challenge, with no variation after a further 6 mo.

The results of Sambade et al. (8) highlight the different behavior of the PIAC isolate, and although 1 yr is too short a time to be sure of commercially useful results, the stability observed for this isolate is encouraging, especially as Pera IAC sweet orange accounts for 41% of the Brazilian output (13).

LITERATURE CITED

1. 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 255: 32-39.
2. Beidler, L. L., P. R. Hilliard, and R. L. Rill
   1982. Ultrasensitive staining of nucleic acids with silver. Analyt. Biochem. 126: 374-380.
3. 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 polymerase chain reaction. J. Virol. Methods 44: 305-317.
4. Kong, P., L. Rubio, M. Polek, and B. W. Falk
   2000. Population structure and genetic diversity within California of *Citrus tristeza virus* (CTV) isolates. Virus Genes 21: 139-145.
5. Rezende, J. A. M. and G. W. Müller
   1995. Mecanismos de proteção entre os vírus e controle de viroses de vegetais por premunização. Rev. Anu. Patol. Plantas 3: 185-226.
6. 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.

7. Rubio, L., M. A. Ayllón, P. Kong, A. Fernández, M. Polek, J. Guerri, P. Moreno, and B. W. Falk
2001. Genetic variation of *Citrus tristeza virus* isolates from California and Spain: Evidence for mixed infection and recombination. J. Virol. 75: 8054-8062.
8. Sambade, A., L. Rubio, S. M. Garnsey, N. Costa, G. W. Müller, M. Peyrou, J. Guerri, and P. Moreno
2002. Comparison of viral RNA populations of pathogenically distinct isolates of *Citrus tristeza virus*: Application to monitoring cross-protection. Plant Pathol. 51: 257-265.
9. Sambrook, J., E. F. Fritsch, and T. Maniatis
1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory. New York.
10. Souza, A. A., G. W. Müller, M. L. P. N. Targon, and M. A. Machado
2000. Evaluation of changes which occurred in a mild protective citrus tristeza virus isolate in Pera sweet orange trees using RFLP and SSCP analyses of the coat protein gene. In: *Proc. 14th Conf. IOCV*, 131-135. IOCV, Riverside, CA.
11. Souza, A. A., G. W. Müller, M. L. P. N. Targon, H. D. Coletta-Filho, and M. A. Machado
2002. Avaliação de haplótipos do gene do capsídeo do *Citrus tristeza virus* em plantas pré-imunizadas de laranja 'Pêra'. Summa Phytopatol. 28: 154-159.
12. Teófilo Sobrinho, J., J. Pompeu Júnior, J. O. Figueiredo, C. G. B. Demétrio, and D. Barbin
1992. Competição entre quinze clones de laranjeira 'Pera' na região de Limeira. Rev. Bras. Fruticult. 14: 41-48.
13. Teófilo Sobrinho, J., G. W. Müller, J. O. Figueiredo, F. F. Laranjeira, and A. A. Salibe
2001. Laranja 'Pêra IAC 2000'. Laranja 22: 495-501.
14. Targon, M. L. P. N., M. A. Machado, S. A. Carvalho, A. A. Souza, and G. W. Müller
2000. Differential replication of mild and severe citrus tristeza virus isolates in species and varieties of citrus. In: *Proc. 14th Conf. IOCV*, 127-130. IOCV, Riverside, CA.
15. Valverde, R. A., S. T. Nameth, and R. L. Jordan
1990. Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. 74: 255-258.