Genetic Structure of *Citrus tristeza virus* (CTV) Populations from Field Argentinian Grapefruit Isolates

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ABSTRACT. Commercial pigmented grapefruit in the Northwest region of Argentina have been seriously affected by *Citrus tristeza virus* (CTV) and a great number of trees have died. The population structure of 12 grapefruit isolates was assessed by single-strand conformation polymorphism (SSCP) analysis of the p20 gene. SSCP patterns showed that most isolates contained only one predominant sequence variant. Global genetic diversity values were similar to those found for CTV isolates from other world citrus growing regions. However, the within-isolate genetic diversity, quantified by the parameter heterozygosity, showed values higher than those found for CTV isolates from other citrus production areas. The existence of several haplotypes in the same isolate may be due to continuous aphid inoculations with isolates having divergent haplotypes. In Argentina, the main aphid CTV vector, *Toxoptera citricida*, is endemic. The high heterozygosity of Argentinian isolates makes probable the presence of sequence variants that cause severe symptoms, which could predominate under certain conditions. Analysis of phylogenetic relationships, using p20 gene sequences from GenBank, showed an unrooted tree in which most Argentinian isolates grouped with isolates VT and SY568.

Citrus tristeza virus (CTV) isolates differing in symptom expression in various citrus species and aphid transmissibility (2, 7) have been described worldwide. As with other RNA viruses, CTV-infected tissues contain a population of sequence variants, often referred to as quasispecies (1, 4, 5, 6, 21). The quasispecies nature implies a high adaptative potential, allowing the rapid selection of biologically different sequence variants with the highest fitness in new environments (14). In addition, the presence of divergent sequence variants has also been detected in some CTV isolates, indicating the possibility of population shift to a distinct predominant sequence (20). Therefore, studies of genetic structure and diversity would be important to understand the evolutionary mechanisms which generate and/or maintain variation in virus populations, which affect their evolution (14) and which may be highly relevant to the development of strategies for control of virus-induced diseases (6).

Since 1990, cultivars of pigmented grapefruits in the northwest region of Argentina have been seriously affected by stem-pitting and decline symptoms and a great number of trees have died. In 2000, these symptoms were also detected in grapefruit trees from an orchard near Concordia (northeast region). Biological evaluation of nineteen isolates from pigmented grapefruits showed the development of stem-pitting symptoms in Duncan grapefruit seedlings under greenhouse conditions.

In this work, we have studied the structure and genetic diversity of the p20 gene region from natural CTV isolates from the two main citrus growing areas of Argentina. The p20 gene was selected based on its size, utility for SSCP analysis, and on the existence of previous data about the variability of this gene in other world citrus-growing areas

(10, 21). By SSCP analysis of the p20 gene, we have estimated the within-isolate genetic variation. Analysis of haplotype nucleotide sequences was used to estimate the genetic diversity of isolates and to infer the phylogenetic relationships among them.

MATERIAL AND METHODS

CTV isolates. Twelve CTV isolates were collected from naturally infected grapefruit plants and analyzed by biological and molecular characterization. Eleven isolates were from the northwest (Jujuy) citrus-growing region of Argentina and one was from the northeast region (Concordia, Entre Ríos) (Table 1). Biological characterization of isolates was done as previously described (7, 8). Samples were multiplied by graft-inoculation on Duncan grapefruit seedlings. CTV isolates analyzed in this work belong to the collection kept at the Estación Experimental Agropecuaria Concordia-INTA.

cDNA synthesis and PCR amplification of the p20 gene. Infected bark of young shoots from field grapefruit trees was frozen in liquid nitrogen and pulverized in a mortar. Nucleic acids enriched in dsRNAs were extracted with phenol-SDS buffer. The dsRNAs were then purified by column chromatography on non-ionic cellulose (CF-11, Whatman), in the presence of 16.5% ethanol as previously described (15). First-strand cDNA was synthesized using 100 ng of dsRNA as template. The dsRNA was heat denatured in the presence of 100 ng of p20-reverse primer (21). RNA was reverse-transcribed by incubation at 42°C for 120 min in a reaction mix (10 µl) containing 1× *Avian myeloblastosis virus* RT (AMV) buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, and 10 mM DTT), 200 µM each dATP, dCTP, dGTP, dTTP, 7.2 U of RNAsin (Promega) and 5 U of AMV reverse transcriptase (AMV-RT) (Promega). An aliquot (1 µl) of the cDNA product was amplified by polymerase chain reaction (PCR) in a 10 µl reaction mix containing 1× *Taq* buffer (10 mM Tris-HCl pH 9; 50 mM KCl and 0.1% Triton X-100), 200 µM each of the four dNTPs, 2 mM $MgCl_2$, 0.5 µM of p20-forward and p20-reverse primers (21) and 0.2 U of *Taq* DNA polymerase

TABLE 1
GEOGRAPHIC ORIGIN AND PATHOGENICITY CHARACTERISTICS OF FIELD GRAPEFRUIT CTV ISOLATES

			Symptoms induced in different citrus hosts				
Isolate	Scion	Origin	Mexican Lime (VC, SP)	Duncan Grapefruit (SP)	Sour Orange (SY)	Sweet Orange (SP)	Biogroup
C315-1	Flame	Jujuy	+	+	+	—	4
C315-4	Star Ruby	Jujuy	++	++	++	—	4
C315-5	Ruben Pink	Jujuy	++	+	++	—	4
C315-7	Henninger Ruby	Jujuy	++	++	++	—	4
C315-8	Oran Red	Jujuy	++	++	++	—	4
C315-11	Burgundy	Jujuy	+	+	+	—	4
C315-12	Ray Ruby	Jujuy	++	++	++	—	4
C315-13	Star Ruby	Jujuy	++	++	++	—	4
C315-14	Henninger Ruby	Jujuy	+	+	+	—	4
C315-15	Henderson	Jujuy	+	+	+	—	4
C315-16	Rio Red	Jujuy	++	+	++	—	4
C320-3	Star Ruby	Entre Ríos	+	+	+	—	4

VC: vein clearing; D: decline; SY: seedling yellow; SP: stem pitting.

(Promega). PCR conditions used were: 94°C for 1 min; 35 cycles each of 94°C for 12 s, 50°C for 15 s and 72°C for 1 min; and 72°C for 3 min.

Cloning of PCR products. RT-PCR products were cloned into the p*Gem*-T easy vector (Promega) using T4 DNA ligase (Promega), according to the manufacturer's instructions, followed by transformation into *Escherichia coli* DH5α (Promega). Thirty clones of each isolate were selected by PCR, using the same conditions outlined above.

SSCP analysis. One microliter of the PCR product was mixed with 9 µl of denaturing solution (95% v/v deionized formamide, 20 mM EDTA pH 8.0, 0.25% w/v bromophenol blue and 0.25% xylene-cyanol), heated at 100°C for 10 min and chilled on ice. Samples were electrophoresed on a non-denaturing 10% polyacrylamide minigel (10 × 8 cm), using 1× TBE (89 mM Tris-borate, 2 mM EDTA pH 8.0) buffer. Conditions used were 200 V for 3 h 30 min at 20°C. A constant temperature was maintained in the gels during electrophoresis by water circulation and gels were stained with silver nitrate (3).

Nucleotide sequence and statistical analysis. cDNA clones representative of the major haplotype of each isolate were sequenced in both directions. Multiple alignments of nucleotide sequences were obtained using Clustal-W software (23). Heretozygosity was estimated according to Nei's equation 8.5 (16), using the number and frequency of SSCP haplotypes. Genetic diversity and nonsynonymous and synonymous substitutions were determined with MEGA 2.1 (12) and the Pamilo-Bianchi-Li method (18, 13) for nucleotide substitutions. Phylogenetic relationships were inferred by maximum parsimony using PAUP* version 4.0 b6 (22). Visualization of trees was done with TreeView 1.6.1 program (17).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers AY962336 to AY962347. Nucleotide sequences of other CTV isolates obtained from GenBank were: AF260651 (T30), AF001623 (SY568), Y18420 (T385), AY170468 (T36) and U56902 (VT).

RESULTS AND DISCUSSION

Detection of haplotypes and estimation of isolates population structure. The CTV p20 gene was reverse transcribed and amplified by PCR with specific primers using 100 ng of dsRNA per reaction as template to minimize the possibility of nucleotide misincorporation in the early PCR cycles (11). SSCP analysis was employed to assess the within-isolate haplotype population and profiles from thirty clones of each isolate showed that most isolates contained only one predominant sequence variant. Figure 1 shows the SSCP pattern corresponding to 15 clones of the C315-1 isolate. For several isolates the data

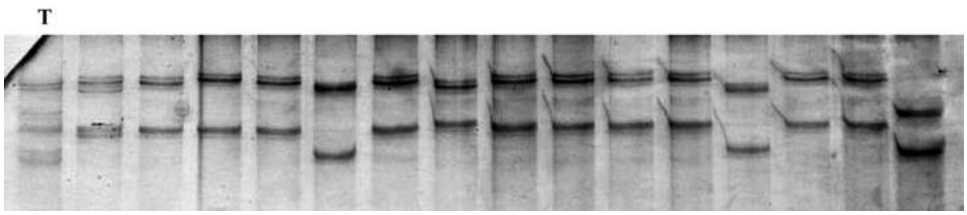

Fig. 1. Gel showing results of SSCP analysis of the p20 gene from isolate C315-1. Lane T shows the band pattern of the RT-PCR products from the parent source, whereas the other lanes show the band patterns of PCR products of individual cDNA clones for the same isolate.

indicate a typical quasispecies structure but this is not a general rule (4, 5, 6). The SSCP patterns of the C315-8 and C315-14 isolates showed two haplotypes in high frequency. Clones of the two different sequence variants present in each isolate were sequenced, and nucleotide sequence differences of 2.7% and 8% were found for the variants present in isolates C315-8 and C315-14, respectively.

Genetic diversity of each isolate was calculated from the haplotype frequencies by using the heterozygosity parameter (h) (Fig. 2) (16). Nine isolates (C315-1, C315-4, C315-5, C315-8, C315-12, C315-14, C315-15, C315-16 and C320-3) of the twelve (75%) showed h values higher than 0.5 and a mean heterozygosity value (H) of 0.74, indicating that these isolates are highly heterogeneous. The exceptions were isolates C315-7, C315-11 and C315-13, which showed h values lower than 0.5 and an H value of 0.4. These results showed that these isolates have great within-isolate sequence variability. CTV is endemic in citrus growing regions of Argentina due to the occurrence of the most efficient CTV vector, *Toxoptera citricida*. A large number of aphids frequently visit each citrus plant and probably caused re-infections with different sequence variants that could contribute to the great intra-population variability seen in the Argentinian isolates. The causes of within-isolate population changes after aphid transmission might be: i) uneven distribution of haplotypes within the infected source plant, so aphids can pick up and transmit different haplotypes depending on the plant tissue or area on which they feed; ii) differential selection of haplotypes during aphid acquisition and transmission (some haplotypes may be transmitted more effectively); iii) genetic shift due to the population bottleneck imposed by aphid transmission (19). The consequence of this variability in the population structure is an increased probability that severe variants of the virus can be present. Under conditions involving different environmental and host factors, these variants could predominate and produce the severe phenotype (20).

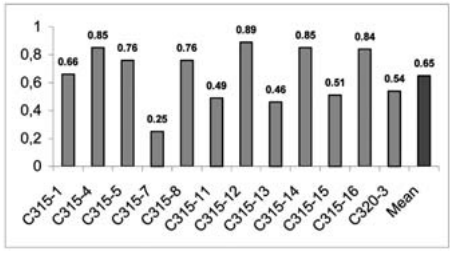

Fig. 2. Heterozygosity values of the p20 gene from twelve Argentinian CTV isolates.

Sequence analysis. The most frequent haplotype of each isolate was sequenced. Sequences were used to estimate the genetic diversity, the number of nonsynonymous and synonymous substitutions (Table 2) and the phylogenetic relationships. The estimated total genetic diversity was similar to the genetic diversity found by other authors for the same CTV genome region in isolates from other citrus-growing areas (10, 19). Values of synonymous substitutions were similar to previous ones found for other genome regions (p23, p25, and p27) from Argentinian isolates (unpublished data). The *d*N/*d*S ratio for the

TABLE 2
NUCLEOTIDE DIVERSITY OF ARGENTINIAN CTV ISOLATES FROM GRAPEFRUIT

Dt	0.051 (0.0058)
*d*S	0.1343 (0.0194)
*d*N	0.016 (0.0042)
*d*N/*d*S	0.12

Dt: total nucleotide diversity. Standard errors are indicated between parentheses.
*d*S: average number of synonymous substitution per synonymous site.
*d*N: average number of nonsynonymous substitution per nonsynonymous site.
*d*N/*d*S: average of the ratio between nonsynonymous and synonymous substitutions.

p20 gene indicate a high negative selective pressure for most of the amino acid changes. These values were similar to those estimated for Californian and Spanish isolates (21) and were similar to values for capsid protein genes of field Argentinian isolates (unpublished data).

An unrooted phylogenetic tree was obtained using the major haplotype nucleotide sequence of p20 for each isolate and p20 sequences derived from the complete genome region of isolates T30, T385, SY568, VT and T36 (Fig. 3). The Argentinian isolates grouped in two different clades, both with a bootstrap value higher than 97. Ten of twelve Argentinian isolates (C315-1, C315-4, C315-8, C315-11, C315-12, C315-13, C315-14, C315-15, C315-16 and C320-3) clustered with VT and SY568 isolates. This group included the C320-3 isolate, obtained from an orchard near Concordia. The phylogenetic analysis indicates that this isolate is genetically related to those from the northwest region. The remaining two Argentinian isolates (C315-5 and C315-7) clustered in a second group. These isolates showed an average nucleotide difference of 9% with respect to the other 10 Argentinian isolates. Despite the fact that all the Argentinian grapefruit isolates exhibited severe stem-pitting symptoms on Duncan grapefruit and seedling yellows on sour orange under greenhouse conditions, they clustered in different phylogenetic groups. There was no correlation between the biological characters of the isolates and specific features of nucleotide sequence of the p20 gene.

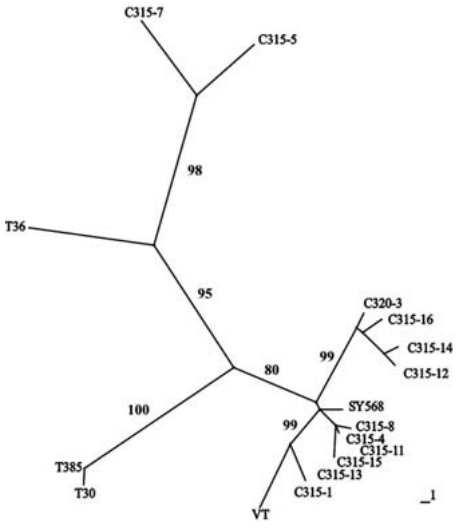

Fig. 3. Unrooted parsimony tree of p20 gene sequences from Argentinian grapefruit isolates of CTV. Sequences of T36, T385, T30, VT and SY568 isolates were included as reference standards. Bootstrap values for 1,000 replicates are indicated. Branch lengths are proportional to number of genetic changes.

High within-isolate heterogeneity of haplotypes was found for the Argentinian grapefruit isolates studied. This finding was confirmed by the analysis of ten clones of the p20 gene and forty clones of the p23 gene from isolate C315-14 (9). Intra-isolate genetic diversity values obtained were similar to total genetic diversity calculated for the twelve isolates analyzed in this work (9).

The data indicated that a high intra-isolate variability is a characteristic of Argentinian CTV isolates. This variability constitutes a serious potential problem in the control of CTV in grapefruit in Argentina and represents a real difficulty in the selection of isolates for cross-protection trials. Presently, pigmented grapefruit in Argentina are seriously affected by stem-pitting symptoms and many trees from orchards of the northwest region of the country must be eliminated.

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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. Ballester-Olmos, J. F., J. A. Pina, E. Carbonell, P. Moreno, A. Hermoso de Mendoza, M. Cambra, and L. Navarro
1993. Biological diversity of *Citrus tristeza virus* (CTV) isolates in Spain. Plant Pathol. 42: 219-229.
3. Caetano-Anollés, G. and P. M. Greeshoff
1994. Staining nucleic acids with silver an alternative to radioisotopic and fluorescent methods. Promega Notes 45: 13-18.
4. Domingo, E. and J. J. Holland
1994. Mutations rates and rapid evolution of RNA viruses. In: *The Evolutionary Biology of Viruses,* 161-184. S. S. Mores (ed.), Raven Press, New York, NY.
5. Eigen, M.
1996. On the nature of virus quasispecies. Trends Microbiol. 4: 216-218.
6. García-Arenal, F., A. Fraile, and J. M. Malpica
2001. Variability and genetic structure of plant virus populations. Annu. Rev. Phytopathol. 39: 157-186.
7. Garnsey, S. M., D. J. Gumpf, C. N. Roistacher, E. L. Civerolo, R. F. Lee, R. K. Yokomi, and M. Bar-Joseph
1987. Toward a standardized evaluation of the biological properties of citrus tristeza virus. Phytophylactica 19: 151-157.
8. Garnsey, S. M., E. L. Civerolo, D. J. Gumpf, R. K. Yokomi, and R. F. Lee
1991. Development of a worldwide collection of citrus tristeza virus isolates. In: *Proc. 11th Conf. IOCV*, 113-120. IOCV, Riverside, CA.
9. Iglesias, N. G., J. Marengo, K. Riquelme, N. Costa. M. I. Plata, and L. Semorile
2005. Characterization of the population structure in a grapefruit isolate of *Citrus tristeza virus* (CTV) selected for pre-immunization assays in Argentina. In: *Proc. 16th Conf. IOCV,* 150-158. IOCV, Riverside, CA.
10. Kong, P., L. Rubio, M. Polek, and B. W. Falk
2000. Population structure and genetic diversity within California *Citrus tristeza virus* (CTV) isolates. Virus Genes 21: 139-145.
11. Krawezak, M., J. Reiss, J. Schmiddtke, and U. Rösler
1989. Polymerase chain reaction: replication errors and reliability of gene diagnosis. Nucleic Acids Res. 17: 2197-2201.
12. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei
2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244-1245.
13. Li, W. H.
1993. Unbiased estimation of the rates of synonymous and non-synonymous substitution. J. Mol. Evol. 36: 96-99.
14. Lin, H. X., L. Rubio, A. B. Smythe, M. Jiménez, and B. W. Falk
2003. Genetic diversity and biological variation among California isolates of *Cucumber mosaic virus*. J. Gen. Virol. 84: 249-258.
15. Moreno, P., J. Guerri, and N. Muñoz
1990. Identification of Spanish strains of citrus tristeza virus (CTV) by analysis of double-stranded RNAs (dsRNAs). Phytopathology 80: 477-482.
16. Nei, M.
1987. *Molecular Evolutionary Genetics.* New York: Columbia University Press, pp. 179.
17. Page, R. D.
1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comp. Appl. Biosc. 12: 357-358.
18. Pamilo, P. and N. O. Bianchi
1993. Evolution of the Zfx and Zfy genes: rates and interdependence between the genes. Mol. Biol. Evol. 10: 271-281.
19. Rubio, L., J. Guerri, and P. Moreno
2000. Characterization of *Citrus tristeza virus* (CTV) isolates by single-strand conformation polymorphism analysis of DNA complementary to their RNA population. In: *Proc. 14th Conf. IOCV*, 12-17. IOCV, Riverside, CA.

20. Rubio, L., J. Guerri, and P. Moreno
2002. Detection of divergent sequence variants within *Citrus tristeza virus* (CTV) isolates. In: *Proc. 15th Conf. IOCV*, 60-68. IOCV, Riverside, CA.
21. Rubio, L., M. A. Ayllón, P. Kong, A. Fernández, M. I. Polek, J. Guerri, P. Moreno, and B. Falk
2001. Genetic variation of *Citrus tristeza virus* (CTV) isolates from California and Spain: evidence for mixed infections and recombination. J. Virol. 75: 8054-8062.
22. Swofford, D. L.
1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.
23. Thompson, J. D., D. G. Higgins, and T. J. Gibson
1994. CLUSTAL-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.