Characterization of the Population Structure of a Grapefruit Isolate of *Citrus tristeza virus* (CTV) Selected for Pre-immunization Assays in Argentina

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ABSTRACT. The Argentinian *Citrus tristeza virus* (CTV) isolate C315-14 was collected from a vigorous symptomless Henninger Ruby Red grapefruit plant in an orchard at Calilegua (Jujuy) where the remaining plants were seriously affected by tristeza. This is one of the isolates that is being used in Argentina for pre-immunization in cross-protection trials of pigmented grapefruit. Graft-inoculation with C315-14 caused severe symptoms on Mexican lime, severe stem-pitting on Duncan grapefruit, and no symptoms on sweet orange. Population structure analysis of the p20 and p23 genes of C315-14 showed the presence of two genetically related variants, indicating the coexistence of two viral populations in the same host. The values of genetic diversity estimated for each genome region were similar between the variants and also similar to the global genetic diversity estimated for Argentinian grapefruit isolates so far studied. By using the PHYLPRO program, possible genetic recombination between haplotypes was identified. Estimated values of synonymous substitutions per synonymous site (dS), non-synonymous substitutions per non-synonymous site (dN), and dN/dS ratio were similar to those previously found for p20 in CTV isolates from other world citrus growing regions, and for p23 in other Argentinian isolates previously analyzed.

Gonsalves and Garnsey (9) proposed to use a mild strain of *Citrus tristeza virus* (CTV) for cross-protection against more severe strains of the same virus that can cause economic damage. Cross protection with mild strains is effective even against a severe challenge strain of the virus (7). The program using cross-protection to control losses by CTV isolates causing stem pitting in sweet orange and grapefruit in Brazil is the largest such program and the most successful use of cross-protection for control of a plant virus disease (19, 26). In Australia, cross protection trials conducted at two locations over more than 25 years showed the beneficial effects of the method as well as the effects of the climate (2). Cross protection has also been extensively used to control losses caused by stem pitting CTV isolates in South Africa (30). One of the problems that has limited the use of this control measure has been screening effectively for protective isolates (2, 3, 22, 27, 32). Biological and molecular properties of isolates can change after inoculation to a new host (16, 17,18) or after aphid transmission (10, 12). In recent years, it has been shown that sequence variants within CTV isolates (1, 13, 25) likely affect the biological properties of a given isolate. Several factors such as cultivar, environment, and multiple infections by repeated aphid inoculations affect the balance of sequence variants within a host (13, 23). Therefore, characterization of the population structure is crucial to understanding the biology and evolution of CTV isolates and may have important implications in the selection of pre-immunizing isolates.

CTV and its most efficient vector, *Toxoptera citricida* (Kirkaldy), were introduced into Argentina in the 1930s and subsequently caused the loss of more than 14 million citrus trees grafted on sour orange root-

stock. Since then, CTV has been endemic in all citrus-growing areas of the country. Measures to prevent losses caused by CTV in Argentina include the use of tolerant rootstocks and the application of a certification program for commercial propagation of citrus. In spite of these control measures, cultivars of pigmented grapefruits in the northwest of Argentina have been seriously affected by severe CTV stem pitting since 1990 and a great number of trees have died (5, 6). Significant effects on yield and fruit size of young pigmented grapefruit have resulted in the reduction of the economic viability of affected trees even if propagated on tolerant rootstocks (6). In 2000, stem-pitting symptoms were also detected in grapefruit trees in an orchard near Concordia in the northeast citrus region of Argentina.

With the goal being to reduce losses caused by severe CTV isolates on grapefruit cultivars, cross-protection trials were started with two isolates obtained from healthy trees in orchards where the remaining citrus plants were seriously affected by tristeza. Isolates selected were obtained from a Ruben Pink (C278-1) and from a Henninger Ruby Red (C315-14) grapefruit. The population structure and genetic diversity of the C278-1 isolate were previously studied by analysis of the p27, p25 and p23 genes (unpublished data). In the present work, we have studied the population structure and genetic diversity of the C315-14 isolate by sequence analysis of the p20 and p23 genes.

MATERIAL AND METHODS

Virus isolate and biological characterization. Isolate C315-14 was obtained from a symptomless Henninger Ruby Red grapefruit tree at Calilegua (Jujuy, northwest citrus growing region of Argentina). Biological indexing was carried out according to Garnsey et al. (8). This CTV isolate is part of the collection kept at the Estación Experimental Agropecuaria Concordia-INTA (Entre Ríos, Argentina).

cDNA synthesis and PCR amplification of p20 and p23 genes. Infected bark of young shoots was frozen in liquid nitrogen and pulverized in a mortar. Nucleic acids were extracted with phenol-detergent buffer. The dsRNA was purified by column chromatography on non-ionic cellulose (CF-11; Whatman) in the presence of 16.5% ethanol and precipitated as previously described (15). First-strand cDNA was synthesized using dsRNA as template. The dsRNA was heat denatured in the presence of 100 ng of primer p23B (5' ACGCGTCGACCAATCAGATGAAG TGGTG 3'). Methylmercury hydroxide (2.4 mM final concentration) was added to denature the dsRNA for cDNA synthesis. RNA was reverse-transcribed 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). For amplification by polymerase chain reaction (PCR) an aliquot (1 µl) of cDNA product was amplified in a 10 µl reaction 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 each of specific gene primer and 0.2 U of *Taq* DNA polymerase (Promega). The primers used for amplification of the p23 gene were p23A (5' CGAGCTC ATGGATGATACTAGCGGACA 3') and p23B, and for the p20 gene were p20 forward and p20 reverse (24). PCR conditions were: 94°C for 1 min; 35 cycles each at 94°C for 12 s, 50°C for 15 s, 72°C for 1 min; and one cycle of 72°C for 3 min.

RT-PCR products were cloned into pGem-T Easy vector (Promega), according to the manufacturer's instructions, followed by transfor-

mation into *Escherichia coli* DH5α (Promega). Clones containing recombinant plasmids were selected by PCR, using the conditions mentioned above.

Nucleotide sequence and statistical analysis. Multiple independent cDNA clones of each gene were sequenced in both directions. Complete sequences were established from partial sequences using PileUp, Assemble, Gap and BestFit programs of Genetics Computer Group (GCG) package (4). Multiple alignments of nucleotide sequences were obtained using Clustal-W (29) software.

Genetic diversity was calculated as the mean of nucleotide substitutions per site between pairs of sequence variants or between populations, using the Jukes-Cantor correction. Numbers of synonymous and nonsynonymous substitutions were estimated by the Pamilo-Bianchi-Li method (14, 21). Phylogenetic relationships were inferred by maximum parsimony using PAUP* version 4.0b6 (28). Phylogenetic trees were displayed with the TreeView 1.6.1 program (20). Recombination events were analyzed using the PHYLPRO program (31).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in GenBank under accession numbers AY962326 to AY962335 for clones of the p20 gene and AY962348 to AY962388 for clones of the p23 gene. The following CTV nucleotide sequences used in the analyses were obtained from GenBank: T30 (AF260651), SY568 (AF001623), T385 (Y18420), T36 (AY170468), Nuaga (AB046398) and VT (U56902).

RESULTS AND DISCUSSION

Genetic diversity and nucleotide substitutions calculated using p20 and p23 gene sequences. Although the original Henninger Ruby Red field tree was symptomless in the orchard, the C315-14 isolate caused severe stem-pitting symptoms when graft inoculated in Duncan grapefruit seedlings. The SSCP patterns of the RT-PCR products of the p20 and p23 genes were identical in the field host plant and in the Duncan grapefruit seedlings used in the biological indexing performed in the greenhouse (data not shown). This difference in the symptoms observed in the field and in the greenhouse could be due to environmental differences (temperature, humidity, etc.) or to a varietal difference between Duncan and Henninger Ruby Red grapefruits.

To study the population structure and the genetic diversity of isolate C315-14 several clones of the p23 and p20 genes were analyzed. Forty clones of the p23 gene and ten clones of the p20 gene were sequenced, and the genetic diversity and nucleotide substitution rates were estimated. The genetic diversity estimated for each gene (Table 1) was similar to total genetic diversity estimated for other Argentinian grapefruit isolates by analysis of p20 gene sequence variants (11). These data confirmed that Argentinian CTV isolates so far studied have a high genetic diversity. This high value of within-isolate genetic diversity was previously observed using single strand conformation polymorphism (SSCP) analysis of p20 gene. Although the SSCP analysis can overestimate the genetic diversity because it does not allow estima-

TABLE 1
GENETIC DIVERSITY AND NUCLEOTIDE SUBSTITUTION VALUES OF P20 AND P23 GENES OF CTV ISOLATE C315-14

	p20	p23
Dt*	0.051 (0.007)	0.069 (0.007)
*d*S	0.139 (0.025)	0.136 (0.021)
*d*N	0.019 (0.006)	0.044 (0.007)
*d*N/*d*S	0.14	0.32

*Definitions of Dt, *d*S, *d*N, and *d*N/*d*S are as described in the text. Standard error values for each parameter are in parentheses.

tions of genetic distances between sequence variants, the data obtained in this work confirmed the results obtained using the SSCP method (11). The number of synonymous substitutions per synonymous site (dS) was the same for both genes, and was similar to values assessed for CTV genome regions of other Argentinian isolates (11). However, the number of non-synonymous substitutions per non-synonymous site (dN) was different between the two genome regions, with the value for p20 smaller than the value for p23. This suggests a negative selective pressure higher for the p20 genome region than for the p23 one. The dN/dS ratio allows quantification of this selective pressure. The dN/dS value obtained for the p23 gene of C315-14 was similar to values previously estimated for other Argentinian isolates studied, whereas the dN/dS value obtained for the p20 gene of C315-14 was similar to values found for known structural proteins (CP and CPm genes) of CTV.

Phylogenetic analysis. Unrooted phylogenetic trees were obtained for p20 and p23 sequences by applying the parsimony method (Fig. 1).

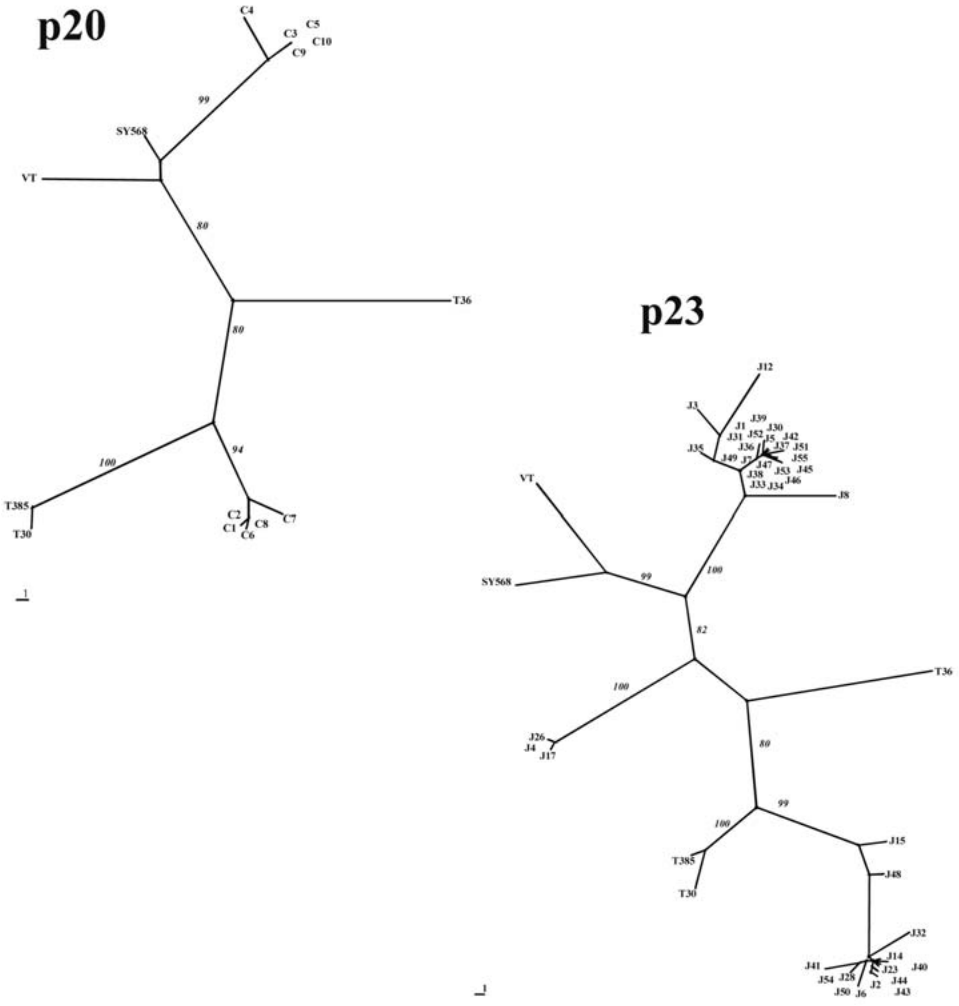

Fig. 1. Unrooted parsimony trees of p20 and p23 genes from C315-14 isolate. Bootstrap values for 1,000 replicates are indicated. Branch lengths are proportional to number of genetic changes.

Interestingly, p20 and p23 trees showed the same general topology. The phylogenetic tree for the p20 gene showed two groups of sequence variants, each one with a bootstrap value higher than 94. The presence of two haplotypes in the C315-14 isolate had been previously detected by SSCP analysis of the p20 gene (11). The mean of the nucleotide differences between both groups present in the isolate was 8%, with a within-group variation of 0.6% for each group. The phylogenetic relationships of p23 sequence variants also showed two main groups of sequences. The mean nucleotide difference between both groups was 10%. A third cluster in the p23 gene tree included sequences representing 7.5% of the analyzed clones. In both phylogenetic trees, one group of sequence variants clustered with the isolates VT and SY568 (severe isolates) and the other group clustered with the isolates T30 and T385 (mild isolates) (Fig. 1).

Regardless of origin of haplotypes, the data indicate that there are two populations of related sequences in relatively equal proportion present in the C315-14 infected host plant. Differences in the fitness of each population or selection during aphid transmission could cause the displacement of one of them, resulting in possible changes in the symptoms expressed in infected host plants.

Estimation of possible genetic recombination between haplotypes. Possible recombination events in the p23 sequence were estimated using phylogenetic correlation. Recombination evidence was obtained in four clones of p23 gene (J8, J12, J15 and J48) (Fig. 2). These sequences were located distantly from the other sequences in their respective group. Recombination events between the two main populations found in the C315-14 isolate were not detected. These populations could be located in different plant tissues or areas, or simply recombination was not produced or not detected between them. No evidence for recombination was obtained for the p20 sequences.

The presence of divergent sequences within the isolate and the possibility of recombination among different sequence variants suggest the potential for emergence of new haplotypes in the host plant, increasing the genetic diversity of the isolate.

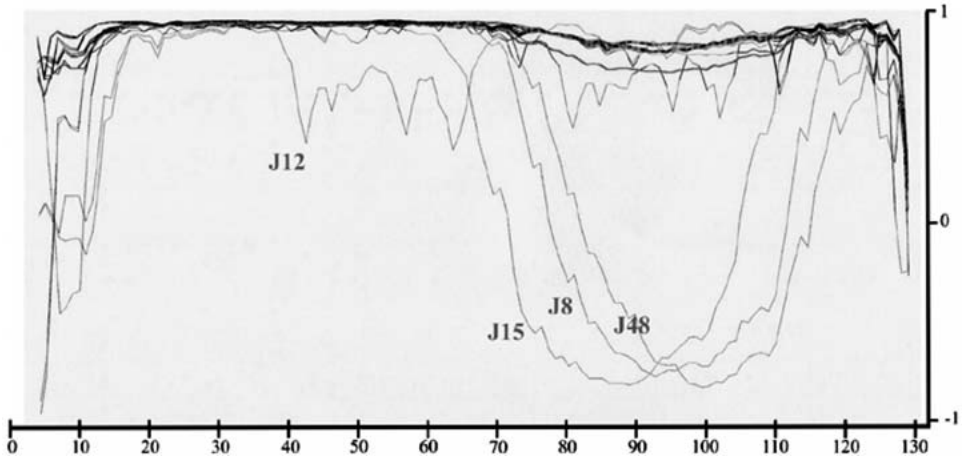

Fig. 2. Phylogenetic correlation profiles of p23 sequences cloned from CTV isolate C315-14. Variable sites are represented in the graph. A window of 40 nucleotides was used. Numbers under low phylogenetic correlation areas (possible recombination signals) indicate the recombinant clone.

Amino acid sequence alignments. Alignment of deduced amino acid sequences of p20 and p23 clones is shown in Fig. 3. Figure 3a shows the alignment corresponding p20 haplotypes of the isolate. The genetically related sequence variants showed a very high amino acid identity. The amino acid differences are indicated in the figure, and correspond to 5% of the sites. Interestingly, six of the nine amino acid changes were found in a region of 33 amino acids located at the carboxyl terminal half of the protein, suggesting that different parts of the coding region were under different evolutionary constraints.

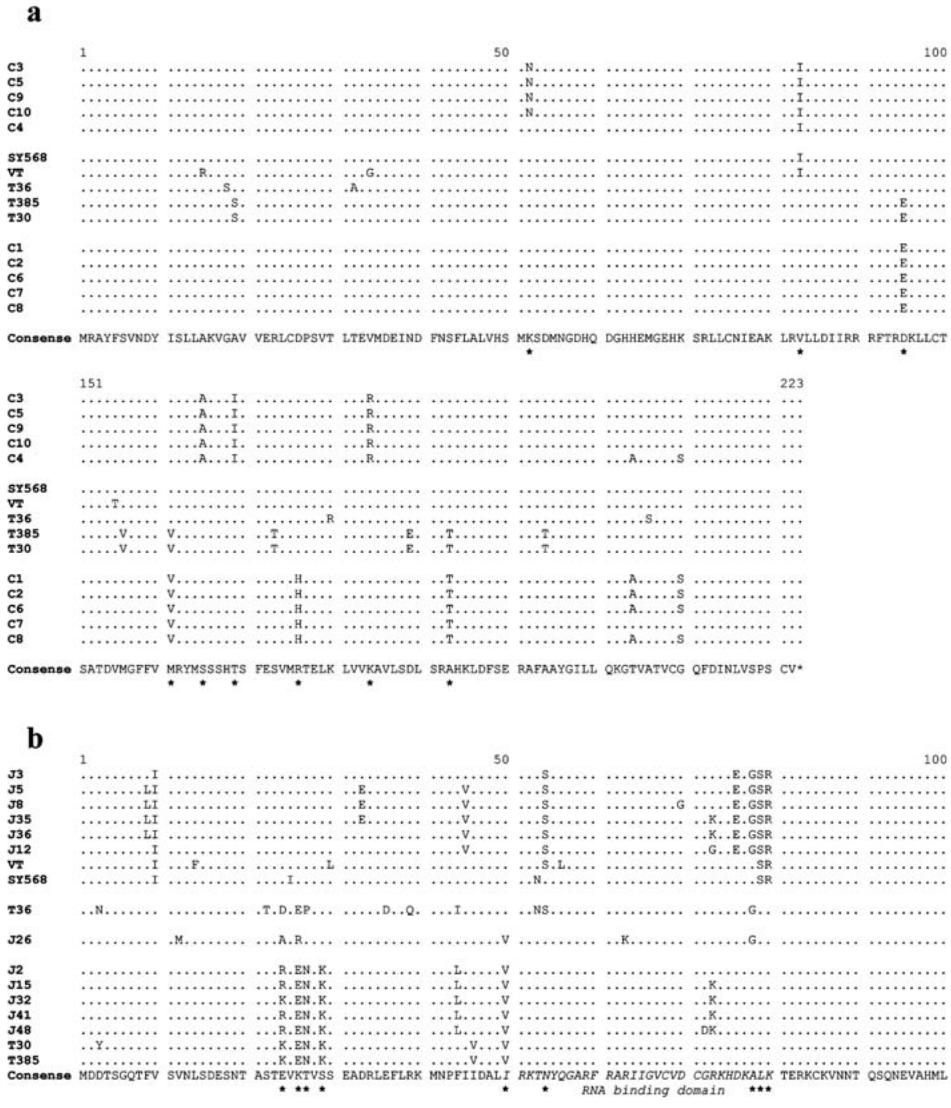

Fig. 3. Alignment of deduced amino acid sequences from clones of p20 and p23 genes from CTV isolate C315-14. Sequences from GenBank were included as references. a) Predicted amino acid sequence of the majority p20 haplotype of each isolate. Asterisks indicate variable sites between the two populations. b) First 100 amino acids of each selected haplotype sequence of p23 gene. The asterisks indicate amino acid differences similar to those described by Sambade et al. (25).

The alignment of representative amino acid sequences of selected haplotypes of the p23 gene are shown in Fig. 3b. As for p20, the genetically related sequence variants had a high amino acid identity. In three regions, the populations showed the group-specific amino acids at positions 78-80 described by Sambade et al. (25), Leu^{79} and Lys^{80} for the mild isolates, Ser^{79} and Arg^{80} for the severe group, and Gly^{78} for the atypical group. One of the populations showed the same amino acids associated with mild isolates (clones J2, J15, J32, J41, J48, etc.). The other showed amino acids similar to those found in severe CTV isolates (clones J3, J5, J8, J35, J36, J12, etc.). Haplotypes associated with an atypical phenotype were present in a minor proportion (clones J4, J17 and J26). These results suggest that the C315-14 isolate is composed of haplotypes which are related to known isolates with mild and severe phenotypes, and of haplotypes associated with an atypical phenotype.

The existence of two divergent sequences, one of them related to severe phenotypes of the virus, and the presence of the main aphid vector of CTV in Argentina, add uncertainty to the successful use of the C315-14 isolate in the initiated pre-immunization trials. Under certain conditions involving environmental and host factors, this isolate could produce a severe phenotype in the inoculated plants.

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