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Detection of Divergent Sequence Variants within *Citrus tristeza virus* (CTV) Isolates

L. Rubio, J. Guerri, and P. Moreno

ABSTRACT. *Citrus tristeza virus* (CTV) isolates consist of a population of sequence variants whose composition likely affects their biological properties. Here, the population structure of six Spanish isolates was examined by single-strand conformation polymorphism (SSCP) analysis of the *p20* gene, and their genetic variation was estimated by comparing the nucleotide sequence of the different sequence variants detected in the SSCP analysis. In three of the isolates, the SSCP pattern of all cDNA clones analyzed was identical, suggesting the presence of a very predominant sequence variant, whereas the population of the other three isolates was more heterogeneous and the cDNA clones were grouped in two or more SSCP patterns. In four cases, genetic diversity (average genetic distance between two sequence variants selected randomly) between isolates was higher than within-isolate diversity, whereas the other two isolates showed very high within-isolate diversity. The within-isolate diversity of these two isolates was sometimes higher than their between-isolate diversity. Phylogenetic analysis showed that the divergent sequence variants were significantly more similar to variants from other isolates than to variants from the same isolate, suggesting that they likely originated from a double infection rather than by mutations accumulated during replication in the same plant. The presence of divergent sequence variants within CTV isolates opens the possibility of population shift to a distinct predominant sequence, following aphid transmission or host change, with potential implications for the biological characteristics of the isolate.

Citrus tristeza virus (CTV), genus *Closterovirus*, family *Closteroviridae* (24), is the most economically important virus affecting citrus. It is phloem-limited and semipersistently transmitted by several aphid species. The virions are filamentous flexuous particles about 2,000 × 11 nm in size, with two coat proteins (CP and CPm) covering 95 and 5% of the particle length, respectively (12). The genome is a single-stranded, positive-sense, RNA molecule of ~20 kb organized in 12 open reading frames encoding at least 19 proteins (20).

CTV isolates differing by the type and intensity of symptoms induced in various citrus hosts have been reported worldwide (6, 35). Control measures are highly dependent on the types of CTV present in each citrus area; biological and molecular characterization of CTV isolates are therefore needed for a sound control program. To date, serological reactivity with monoclonal antibodies (7, 34), peptide mapping (1, 15), double-stranded RNA (dsRNA) analysis (10, 30), molecular hybridization (31, 37), restriction-fragment length polymorphism (RFLP) analysis (14) and single-strand conformation polymorphism (SSCP) analysis (39) have been used for characterization and differentiation of CTV isolates (27).

The complete nucleotide sequence of the genomic RNA (gRNA) of CTV isolates T36 and T30 from Florida (3, 20, 32), VT from Israel (26), T385 from Spain (45), and SY568 from California (46) have been obtained. These and partial gRNA sequences of several origins have also been used to compare CTV isolates of different pathogenic characteristics (4, 18, 22, 25, 33).

CTV isolates are not static entities, but for years it has been observed that their biological and molecular properties could change after processes such as thermal treatment (8), inoculation to a new host (11, 28, 29) or aphid transmission (16, 19). Those changes were explained assuming that some isolates contained several CTV strains, some of which would be filtered out in the indicated processes. Recently, it has

been shown that, as with other RNA viruses, CTV isolates have a population of sequence variants (5, 21, 40) whose composition likely affects their biological properties. Therefore, characterization of this population structure is crucial to understand the biology and evolution of CTV isolates, which may have important implications in their epidemiology and management. In this work we have assessed the population structure of six Spanish CTV isolates by SSCP analysis and their genetic variation by nucleotide sequence analysis.

MATERIALS AND METHODS

CTV isolates. The CTV isolates used in this study are from the collection of the Instituto Valenciano the Investigaciones Agrarias (IVIA), Moncada, Spain, and are maintained in navel orange plants grafted on Carrizo citrange in an insect-proof screenhouse. Biological characteristics available of these isolates are summarized in Table 1 (6, and unpublished data).

Synthesis and cloning of complementary DNA. Total nucleic acids were extracted from sweet orange bark infected with the different CTV isolates and enriched in dsRNA by nonionic cellulose column chromatography in the presence of 16.5% ethanol, as previously described (30).

Complementary DNA (cDNA) was synthesized by reverse transcription (RT) and PCR amplification using dsRNA-rich preparations as templates and the primers p20-forward (5’-ACAATATGCGAGCT-TACTTTA-3’) and p20-reverse (5’-AACTACACGCAAGATGGA-3’), corresponding to both ends of the CTV *p20* gene. Approximately 200 ng of dsRNA were denatured at 95ºC for 10 min, chilled on ice and immediately used for single-step RT-PCR in a 20-µl reaction volume containing 2 µl 10 × PCR buffer, 1.5 mM $MgCl_2$, 1 mM of each of the four dNTPs, 100 ng of each primer, 0.1 units of *Avian myeloblastosis virus* (AMV) reverse transcriptase and 0.5 units of *Taq* DNA polymerase (Promega Corp). RT was performed at 42ºC for 45 min, and after 4 min at 94ºC, PCR included 30 cycles of 30 s at 94ºC, 30 s at 36ºC and 40 s at 72ºC, followed by a final extension of 2 min at 72ºC. PCR products were analyzed by electrophoresis in a 2% agarose gel and those showing the expected size were directly cloned using the pGEM-T Vector system (Promega), according to the manufacturer instructions. Cloned cDNA was later PCR amplified in the same conditions using 2 µl of the recombinant bacterial culture as template.

SSCP and nucleotide sequence analysis. For SSCP analysis, 1 µl of cDNA (from the RT-PCR product or

TABLE 1
SYMPTOMS CAUSED BY THE DIFFERENT CTV ISOLATES

Isolate	Mexican lime vein clearing[a]	Mexican lime stem pitting[a]	Sweet orange stem pitting[a]	Seedling yellows in grapefruit [a]
T300	2	1	0	–
T308	3	3	0	+
T373	2	1	0	–
T397	2	2	0	–
T398	2	2	2	–
T399	2	3	N	–

[a]Symptoms induced by CTV isolates in different hosts. Symptom intensity was scored from 0 (no symptoms) to 3 (very intense symptoms). Seedling yellows reaction in grapefruit is indicated as + or –, indicating presence or absence of this reaction, respectively.
N: Characterization not done.

amplified from a recombinant bacterial culture) was added to 9 µl of denaturing solution (95% formamide, 20 mM EDTA (PH 8.0), 0.1% bromophenol blue), boiled for 5 min and then chilled on ice. DNA strands were separated by electrophoresis in a non-denaturing polyacrylamide minigel (8% acrylamide) at 200 volts and 4ºC for 3 h, and visualized by silver staining (39, 40).

Clones showing different SSCP patterns were sequenced in both directions with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple alignments of the nucleotide sequences were done with the CLUSTAL W program (44). Nucleotide distances (number of nucleotide substitutions per nucleotide position) between sequence variants were estimated with the DNADIST program of PHYLIP package version 3.573 (13), using the Jukes and Cantor method for correction of superimposed substitutions. Phylogenetic relationships were inferred using the PHYLIP program NEIGHBOR, which implements the neighbor-joining method from a nucleotide distance matrix. SEQBOOT (1000 repetitions) and CONSENSE were used for bootstrap analysis.

Genetic diversity (average genetic distance between two sequence variants selected randomly) was as described by Lynch and Crease (23). Genetic diversity within isolates was estimated using the formula $D = 2/(n(n-1)) \Sigma n_i n_j d_{ij}$, where n is the number of clones analyzed per isolate, n_i and n_j are the number of clones of sequence variant i and j respectively, and dij is the nucleotide distance between sequence variants i and j. Genetic diversity between two isolates (k and l) was estimated using the formula $D_{kl} = \Sigma x_i x_j d_{ij} - 1/2\ (D_k + D_l))$, where dij is the nucleotide distance between the ith sequence variant from isolate k and the jth sequence variant from isolate l, x_i and x_j the frequency of sequence variants i and j in isolates k and l, respectively, and D_k and D_l are the genetic diversities within isolates k and l, respectively.

RESULTS

Differentiation of sequence variants within isolates by SSCP analysis. The population structure of the Spanish isolates T300, T308, T373, T397, T398 and T399 was preliminarily assessed by comparing the SSCP pattern of five clones of the p20 gene per isolate with the pattern of the corresponding RT-PCR product. Clones differing by their SSCP pattern were considered distinct variants and were sequenced.

Isolates T300, T373 and T399 had a rather homogenous population, as all clones showed the same SSCP pattern (Fig. 1). This was identical to the pattern predominant in the original RT-PCR product from which the clones had been obtained. Contrarily, two or three SSCP patterns were observed among clones from isolates T308, T397 and T398, suggesting that these have a more heterogeneous population, albeit the most frequent pattern also corresponded to the pattern predominant in the original RT-PCR product (Fig. 1).

Genetic diversity within and between isolates. SSCP analysis can very sensitively discriminate between sequence variants differing by one or a few nucleotides (39); however, it does not allow estimating genetic distances between sequence variants. To assess the genetic variation of isolates the nucleotide sequence of clones showing different SSCP pattern was determined and compared. The genetic or nucleotide distance between sequence variants, calculated as the number of nucleotide differences per nucleotide position, and their frequency (number of clones showing the same SSCP pattern), were used to estimate genetic diversity within and between isolates. The genetic diversity within isolates represents the average genetic distance between

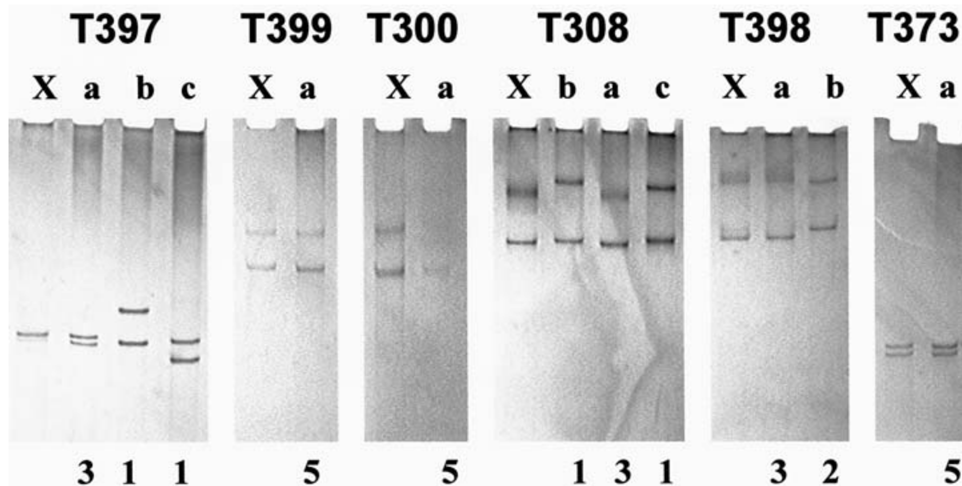

Fig. 1. Single-strand conformation polymorphism (SSCP) analysis of the *p20* gene of six Spanish *Citrus tristeza virus* (CTV) isolates (T300, T308, T373, T397, T398 and T399). SSCP analysis was performed by electrophoresis under non-denaturing conditions in 8% acrylamide gels, at 4°C, and 200 V for 3 h. Lanes X indicate the *p20* cDNA obtained from each isolate by RT-PCR, whereas the other letters (a, b, c) indicate individual clones from the same isolate. The number of clones showing each SSCP pattern is indicated below each lane.

pairs of sequence variants belonging to the same isolate, whereas diversity between isolates represents the average genetic distance between pairs of sequence variants belonging to different isolates.

Results summarized in Table 2 indicate that while the genetic diversity within isolates T300, T308, T373 and T399 was very low, isolates T397 and T398 were very diverse. Diversity between isolates was generally higher than diversity within isolates. Thus, the average of the within-isolate diversities was 0.016 ± 0.004, whereas the average of the between-isolate diversities was 0.065 ± 0.007. However, isolates T397 and T398 showed a within-isolate diversity higher than their diversity with respect to other isolates. For example, within-isolate diversity of T398 was 0.048 ± 0.01 but its diversity with respect to isolate T373 was 0.012 ± 0.016 (Table 2).

Phylogenetic relationships of sequence variants. The phylogenetic relationships of sequence variants of the p20 gene were estimated using the neighbor-joining method and are represented in an unrooted tree (Fig. 2). The predominant *p20* sequences of isolates T36 from Florida (20, 32), VT from Israel (26), T385 from Spain (45) and SY568 from California (46), were also included in this analysis.

Four groups of sequences could be distinguished. In one, the major components of T300 and T399, and one of the T398 variants, clustered with T385 in a very homogeneous group. In previous work, we observed that most Spanish and California isolates also had a high nucleotide identity with T385 (40). A second more diverse group was composed of two T397 variants, a second T398 variant, and the major component of T373. The third group, also diverse, included the major component of isolates VT and SY568. Previously, we observed that a few Californian isolates also belonged to this group, but none of the Spanish isolates studied did (40). The fourth group included the T397c variant, all T308 variants and T36. The three T308 variants and T36 clustered very closely, whereas the T397c variant was

TABLE 2
GENETIC DIVERSITY[a] OF *P20* GENE WITHIN AND BETWEEN *CITRUS TRISTEZA VIRUS* ISOLATES

	T300	T308	T373	T397	T398	T399
T300	**0.000** **(0.000)**					
T308	0.126 (0.013)	**0.003** **(0.002)**				
T373	0.080 (0.013)	0.104 (0.011)	**0.000** **(0.000)**			
T397	0.063 (0.012)	0.080 (0.018)	0.023 (0.009)	**0.044** **(0.018)**		
T398	0.023 (0.023)	0.091 (0.012)	0.012 (0.016)	0.015 (0.013)	**0.048** **(0.016)**	
T399	0.000 (0.000)	0.126 (0.013)	0.080 (0.013)	0.063 (0.012)	0.023 (0.023)	**0.000** **(0.000)**

[a]Genetic diversity (average nucleotide distance between two sequence variants selected randomly) estimated using the frequency of each sequence variant and the nucleotide distances calculated as described by Lynch and Crease (23). Data in bold in the diagonal are within-isolate genetic diversity and data under the diagonal are genetic diversity between pairs of isolates. Data within parentheses are standard errors.

divergent with respect to T308 and T36. Interestingly, isolates T397 and T398 showed sequence variants significantly more related to variants from other isolates than to variants from the same isolate. For example, the nucleotide distance between the two T398 variants was 0.080, whereas the distances between variants T398a and T373a, or between variants T398b and T300a, were 0.006 and 0.002, respectively.

DISCUSSION

RNA viruses have a high mutation rate, due to the absence of proofreading activity of RNA-dependent RNA polymerases, and they build up large populations. Therefore, virus isolates would be expected to consist of a predominant sequence and a population of closely related variants. This population structure, called a quasispecies, would result from mutations accumulating during virus replication (17, 36). Our SSCP analysis of gene p20 allowed detection of more than one sequence variant in isolates T308, T397 and T398, whereas only one variant was detected in isolates T300, T373 and T399. This indicates that the three latter isolates are likely more homozygous than T308, T397 or T398; however, a higher number of clones from each isolate should be analyzed to confirm this point. The aim of this analysis was to identify clones representing the most abundant sequence variants in the population of each isolate and this was achieved by comparing the SSCP profile of individual randomly selected clones with the profile of the corresponding RT-PCR product. For all isolates, the most frequent SSCP profile of the clones corresponded to the most intense DNA bands in the profile of the RT-PCR product. In previous work (41) we found that the SSCP profile of the p20 gene cDNA was indeed a faithful reflection of the RNA population.

Sequence analysis of the different variants detected by SSCP analysis indicated that, for the p20 gene, four of the isolates studied (T300, T308, T373 and T399) showed a typical quasispecies structure, similar to that observed in other CTV isolates (21) and in other viruses (38, 42, 43). Isolate T308, which appeared to be heterogeneous in the preliminary SSCP analysis, had low genetic diversity since the sequence variants

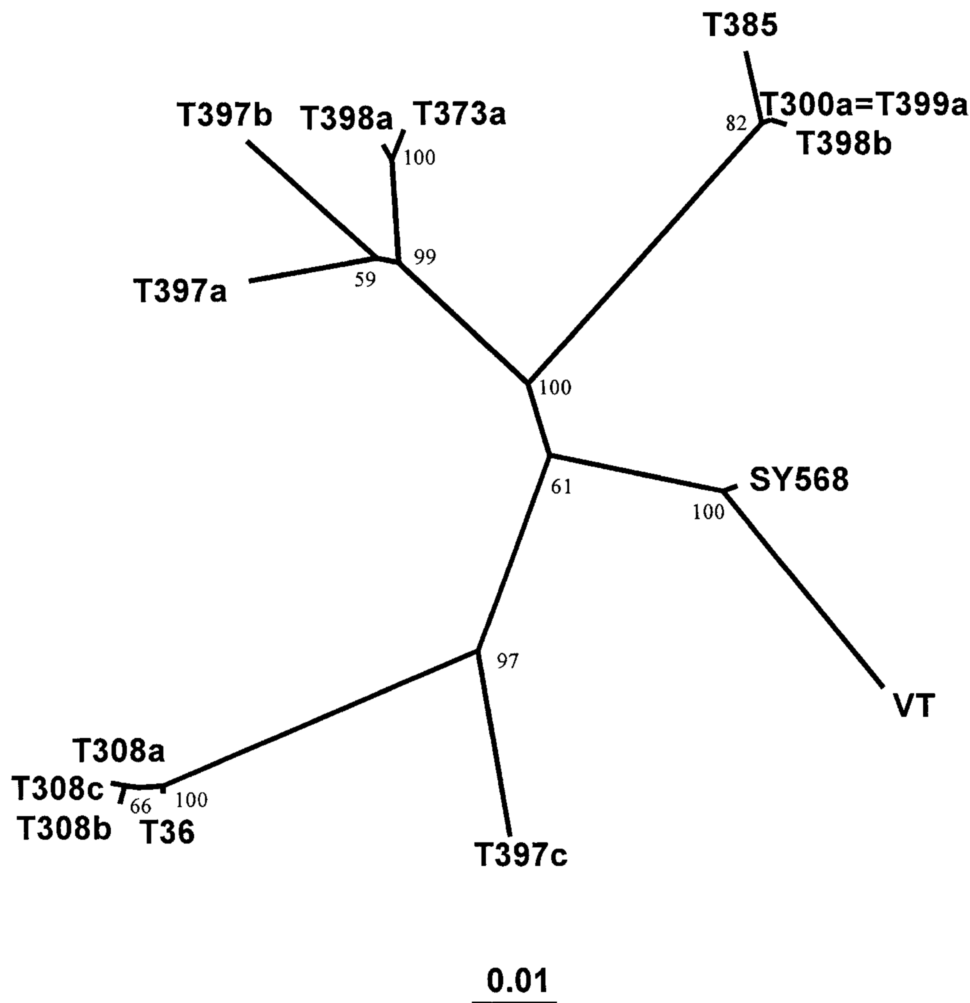

Fig. 2. Unrooted phylogenetic tree obtained with sequence variants of the *p20* gene from CTV isolates T300, T308, T373, T397, T398, T399, T385, SY568, T36 and VT, using the neighbor-joining method. Branch lengths are proportional to genetic distances and bootstrap values are indicated near the nodes. Sequence variants from the same isolate are indicated by letters, e.g. T397a, T397b and T397c are sequence variants from isolate T397. The major sequence of isolates T385, SY568, T36 and VT were obtained from GenBank (accessions Y18420, AF001623, U16034 and U16034).

detected in its population had high nucleotide identity. In contrast, isolates T397 and T398, which were also heterogeneous in the preliminary SSCP analysis, showed high genetic diversity because they had very divergent sequence variants within the same isolate. It is unlikely that these divergent variants originated from mutation accumulation during the virus replication within the plant. More likely, they have different origins and their presence in the same isolate probably results from double inoculation of one plant in the past. The fact that both T397 and T398 had sequence variants significantly more similar to variants from other isolates than to variants from the same isolate strongly support the diverse origin of these variants. Citrus trees which are maintained for decades in open groves are expected to become infected naturally by

aphids or artificially by topworking with different isolates.

The presence of divergent sequence variants within the same isolate opens the possibility of population shift to a distinct predominant sequence. This might explain the observed changes in biological and molecular characteristics in the transmission process of some CTV isolates (2, 5, 9, 28, 29, 41). Changes in the population structure could be due to factors like: i) irregular distribution of sequence variants within the infected plant and/or sorting of some variants during aphid acquisition (8), ii) aphid constraints to transmit certain variants, or host constraints to multiply them, iii) predominance of a new equally efficient variant by a founder effect. A potential result of these variations in the population structure is that minor sequence variants present in phenotypically mild isolates could become predominant under certain conditions and build up a population causing severe symptoms. This underlines the necessity of keeping mild isolates used for cross protection under insect-proof conditions and periodically monitoring their population structure.

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