Variability of p27 Gene from Several Isolates of Citrus Tristeza Virus as Analyzed by Single-Strand Conformation Polymorphism

S. Gago-Zachert, L. Semorile, N. Costa, and O. Grau

ABSTRACT. Hybridization analysis showed a region of the citrus tristeza virus (CTV) p27 gene which exhibits different hybridization patterns using probes derived from 10 biological distinct CTV isolates. In an attempt to determine if that gene region differs between mild and severe strains, 10 CTV isolates representing different biogroups were compared for nucleotide sequence variation by single strand conformation polymorphism (SSCP) analysis. The p27 gene was reverse transcribed, amplified by PCR and 30 clones of each isolate were obtained. From each clone, two gene fragments were amplified by PCR, (a) 459 bp and (b) 281 bp. Sequence variations in both gene fragments were studied by SSCP analysis. The p27 b gene region showed a higher rate of sequence variation than the p27 a region. On the other hand, the intrapopulation variability for the p27 gene showed no correlation with the biological characteristics of the viral isolates or the environmental conditions. The analysis of SSCP profiles of the majority of the clones suggests that the mild CTV isolates (biogroups I and II) are more homogeneous than the severe isolates (biogroups III, IV and V) for the p27 gene region.

The citrus tristeza virus (CTV) genome is composed of a single stranded RNA with two capsid proteins (CP) with the p25 CP encapsidating about 95% of the particle length, and a p27 diverged (d)CP present only on one end of the particle, forming a "rattlesnake" structure (5, 6). Moreover, the dCP may have a function in the transmission by aphid vectors as was suggested in beet yellows closterovirus (1).

CTV populations in citrus trees are unusually complex mixtures of viral genotypes and defective RNAs developed during long-term vegetative propagation and by additional mixing by aphid transmission (14). A variety of CTV strains, differing by symptoms induced on different host species, aphid transmissibility or capacity to interfere with other strains have been described (2, 10, 13). Several approaches for CTV isolate identification and differentiation have been tested in an attempt to develop a quick and specific procedure that could be applied to routine purposes.

Single-strand conformation polymorphism (SSCP) (9) is a method for detecting sequence differences of ssDNA by non-denaturing polyacrylamide gel electrophoresis (PAGE). Partially denatured DNA in the gel matrix has a sequence-specific three dimensional conformation having a characteristic mobility. Even a single nucleotide difference can produce a mobility shift. SSCP analysis has been shown to be an appropriate technique for the study of viral populations. Complex mixtures of the same size, such as viral quasispecies, can be separated into bands of different mobility (4). Rubio et al. (11) applied this technique to CTV isolates and compared them for variations in their CP gene.

From dsRNA isolated from field isolate C268-2 (from Concordia, Entre Ríos, Argentina), we obtained a cDNA library composed of 30 clones (12). Southern blot hybridizations of these clones with short copy cDNAs probes generated from the dsRNAs from 10 CTV isolates, representing biogroups I to V, allowed us to select 11 genomic regions, including the p27 gene, usable to discriminate among biologically different viral isolates (7).

With the aim of screening for variability of the p27 gene among CTV isolates, we compared 10 CTV isolates for sequence variations in

the p27 gene region by SSCP analysis. Moreover, we used SSCP to analyze the variability of viral RNA populations in the CTV isolates studied. The origins and biological characteristics of the CTV isolates used in this study are shown in Table 1.

Bark from young shoots infected with CTV was pulverized in a mortar while frozen in liquid nitrogen. Nucleic acid preparations enriched in dsRNAs were obtained by extraction using buffered-saturated phenol and fractioned by column chromatography on non-ionic CF-11 cellulose (Whatman) as previously described (8).

The p27 gene was reverse transcribed with the antisense p27 ORF primer (5), amplified by PCR with the sense and antisense p27 ORF primers (5) and 30 clones of each isolate were obtained. SSCP analyses were performed on PCR products of the p27 gene split into two fragments of about 460 and 280 bp in size. Usually, PCR products were extracted twice, first with phenol-chloroform and then with chloroform, precipitated and resuspended in 10 μl of double distilled water. A fraction of 1 μl was mixed with 9 μl of the denaturing solution (95% formamide, 20 mM EDTA and 500 mg/liter bromophenol blue), heated for 10 min at 100°C, and chilled on ice. Denatured DNA was electrophoresed in a non-denaturing polyacrylamide 10 × 8 cm minigel (SE 250/SE 260, Hoefer Scientific), using 1× TBE (90 mM Tris borate, 2 mM EDTA) as electrophoresis buffer, 200 V, 3.5 h. During the electrophoresis, temperature was maintained constant by water circulation. The gels were stained with silver nitrate (3).

From each p27 clone, two gene fragments were amplified: Fragment a, 459 bp long and including domains I and II, and Fragment b, 281 bp long and including domains II, III and IV. To obtain good discrimination of SSCP profiles of p27 gene Fragment a, we had to use a 12% polyacrylamide gel concentration and a lower temperature (20°C); whereas for Fragment b, we used 15% acrylamide and a temperature of 22°C. In most clones, only two intense bands, corresponding to only one stable conformation of each DNA strand, were obtained. More than one SSCP profile was observed in the 30 clones analyzed in each of the p27 gene fragments. These results indicate the heterogeneity of the population.

According to the results, the p27 Fragment b showed a higher rate of sequence variation than p27 Fragment a. These changes may be related eventually with the biological properties of the virus considering that p27 Fragment b, which contains domains II, III, and IV that are involved in protein folding, showed the highest number of variants. The number of SSCP patterns that could be distinguished for p27 Fragment a varied between four (for the biogroup V isolates) to 11 (for Pera GS of biogroup III). On the other hand, p27 Fragment b yielded a higher number of different SSCP patterns: 12 patterns in the most variable isolates (Pera GS, biogroup III; Baraõ B, biogroup IV and, T388, biogroup V), and three distinct patterns in the less variable isolate (C268-2, biogroup III). The clone number that showed variation in both p27 gene fragments varied between one (T32, biogroup I) to 12 (Pera GS, biogroup III). On the other hand, the quantity of clones for each isolate that show any variation in p27 gene varied from 21 (C268-2) to six (Pera GS).

SSCP has been shown to be a powerful alternative for detection of variation within a virus population, therefore, it was applied to study the intrapopulation variability for the p27 gene of CTV. With this purpose, the percentage of principal clones for the complete p27 gene without sequence variations was

TABLE 1
ORIGIN AND BIOGROUP INFORMATION FOR THE CITRUS TRISTEZA VIRUS ISOLATES USED IN THIS STUDY

Isolates	ML[z] (SP[y])	SwO[z]/SO[z] (decline)	Gpft[z] or SO (SY[y])	Gpft (SP)	SwO (SP)	Biogroup (type)
T385 (Valencia, Spain), T32 (Valencia, Spain)	+	—	—	—	—	I
T300 (Valencia, Spain), T312 (Valencia, Spain)	+	+	—	—	—	II
C268-2 (Argentina), Pera GS (Brazil, isolate used in cross-protection assays)	+	+	+	—	—	III
C269-6 (Star Ruby grapefruit, Argentina), Baraõ B (Brazil)	+	+	+	+	—	IV
T388 (Valencia, Spain), Capaõ Bonito (Brazil)	+	+	+	+	+	V

[z]Indicator species: ML = Mexican lime; SwO = sweet orange; Gpft = grapefruit; SO = sour orange.
[y]Symptoms: SP = stem pitting; SY = seedling yellows.

also estimated. C268-2 exhibited the higher percentage (66.7%) for the whole gene, being the most homogeneous isolate for this gene region. In contrast, the Pera GS isolate had the lowest percentage (20%) of clones without nucleotide changes and was the most heterogeneous isolate for the p27 gene region among the CTV isolates analyzed. The results obtained showed any correlation between biological characteristics of the viral isolates or environmental conditions and the variability of the viral population for this gene region.

The SSCP profiles of one representative majority clone for each CTV isolate were compared by PAGE. The p27 Fragment a of the isolates in biogroup I, and one isolate (T312) from biogroup II exhibited the same SSCP profile, but T300 (biogroup II) and the other six isolates compared exhibited different SSCP profiles among themselves and with the fore-mentioned isolates. For p27 Fragment b fragment, the four isolates of biogroup I and II shared the same SSCP pattern while all the other isolates showed a different SSCP profile among themselves and with biogroups I and II. These results suggest that if the p27 CTV gene region is considered, the mild isolates (biogroups I and II) are more homogeneous in nucleotide sequence. The more severe isolates (biogroups III, IV, and V) showed more nucleotide sequence heterogeneity in both intragroup and intergroup comparisons. Moreover, no correlation was found between geographical origin and SSCP pattern for the analyzed gene region.

ACKNOWLEDGMENTS

We are grateful to Dr. P. Moreno and Dr. J. Guerri for providing the dsRNAs of Spanish CTV isolates and, to Dr. M. A. Machado for providing dsRNAs of Brazilian CTV isolates. This work was supported by grants of SECYT/CONICET-BID802-OC/AR PID332 and the European Union, Contract EBBICI8CT960044. L. S. and O. G. are recipients of the Research Career award from Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina. S. Gago-Zachert is a fellow of the CONICET-Argentina.

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