

## Sequence variability in p27 gene of Citrus Tristeza Virus (CTV) revealed by SSCP analysis

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Citrus tristeza closterovirus (CTV), is a phloem-limited virus transmitted by aphids in a semipersistent manner. The genome of CTV is composed of a ssRNA with two capsid proteins: CP, covering about 95% of the particle length, and a diverged coat protein (dCP), present only in one end of the particle, forming a rattlesnake structure. dCP is the product of p27 gene for which it is also postulated a function in the transmissibility by aphid vectors. Hybridization analysis showed a p27 gene region, which exhibits different patterns with two probes derived from two biological distinct CTV isolates. In an attempt to screen whether that gene region differs in mild and severe strains, six CTV isolates belonging to different biogroups were compared for variations in their p27 gene by analysis of single-strand conformation polymorphism (SSCP). The p27 gene was reverse transcribed and amplified by PCR and thirty clones of each isolate were obtained. From each clone, two fragments of the gene were amplified by PCR: fragment (a), 459 bp long, and fragment (b), 281 bp long. Sequence variations in both gene fragments were studied by SSCP analysis. A variety of SSCP patterns was obtained from each isolate, being isolates belonging to the groups II-IV and III those with the

higher and lower number of them. Moreover, SSCP analysis provided a rapid procedure to screen the genetic heterogeneity of the viral isolates reducing considerably the amount of nucleic acid sequencing necessary to gain that knowledge.

Citrus tristeza virus (CTV), a member of the closterovirus group is a phloem-limited virus transmitted by aphids in a semipersistent manner (Berlansky et al, 1988). The natural CTV host range is limited to large, woody plants of citrus and related genera. Because of the severe damage caused by individual citrus trees that can be productive for a century, this virus causes one of the most economically important diseases of plants.

CTV has a positive sense RNA genome of 19,3 nucleotides (Karasev et al., 1995) encapsidated in flexuous, rod-shaped particles of about 2000 nm long (Bar-Joseph et al., 1989), with two capsid proteins: a 25 kDa coat protein (Pappu et al., 1993) covering about 95% of the particle length, and a diverged 27 kDa coat protein (Pappu et al., 1994; Febres et al., 1994) that covers one end of the particle forming a rattlesnake structure (Febres et al., 1996). Four regions of sequence conservation (domains I, II, III and IV) exist between the

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two proteins of the Florida T36 isolate (Febres et al., 1994). As it was suggested in beat yellow closterovirus (Agranovsky et al., 1995), the semipersistent mode of CTV transmission may be due to the ability of the assembled 27 K protein to cling tightly to the cell membranes lining the aphid's alimentary tract. Another possibility, not necessarily alternative, may be that the p27 tail directs the closterovirus particle to the host (phloem) cell receptor. Experimentally, the bulk of p27 was found in the cell wall fraction of infected citrus leaves and it was also detectable in the soluble and membrane fractions (Febres et al., 1996).

A variety of CTV strains, differing by the symptoms induced on different host species, aphid transmissibility or capacity to interfere with other strains, has been described (Racah et al., 1980; Ballester-Olmos et al., 1993; Van Vuuren et al. 1993). Several approaches for strain identification and differentiation have been tested in an attempt to develop a quick and specific procedure that could be applied to eradicate a severe strain in early stages of field trees infection. Nucleotide sequencing of individual genes is the most accurate procedure for strain identification, but it is expensive and time consuming for routine purposes. Single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989; Yap and McGee, 1994) enables rapid identification of DNA fragments of the same size but with sequence variations. Sequence variations as small as single base point mutations can be identified in PCR products amplified from a variety of genomic or cDNA sources (Hayashi, 1991).

SSCP analysis is performed by denaturing dsDNA in the presence of formamide and fractioning both DNA strands by electrophoresis in a non-denaturing polyacrylamide gel. Under the appropriate conditions, the electrophoretic mobility of single-strand DNA (ssDNA) is dependent not only on its length and molecular weight, but also on its conformation. Small changes in the nucleotide sequence may alter conformation of ssDNA and, consequently, its electrophoretic mobility. SSCP analysis has been used to assign beet necrotic yellow vein virus to a known strain group (Koenig et al., 1995). More recently, using the same technique and combining two electrophoretic conditions and restriction of eight clones with Eco91I, Rubio et al. (1996) succeeded in the discrimination of 21 from the 22 CP gene clones selected from 17 CTV isolates.

On the other hand, SSCP analysis has proven to be a very appropriate technique for the study of viral populations, because it allows the separation of complex mixtures of equal molecular weight DNA with different nucleotide sequence. Enomoto et al. (1994), using SSCP analysis of 5'-terminal region of the E2 gene of hepatitis C virus (HCV) in four patients with chronic hepatitis, observed that the HCV population was composed of viral quasispecies with different E2-hypervariable regions (HVR).

Hybridization analysis of a cDNA library obtained from dsRNA of the field CTV isolate C268-2 (Concordia, Entre Ríos) with two probes (Semorile et al., 1993), allowed the detection of a p27 gene region which showed differential hybridization with a severe probe (T387) and no hybridization with a mild probe (T312). In an attempt to establish if p27 gene differs in mild and severe strains, we compared six CTV isolates with different biological properties for variations in this gene region by SSCP analysis. Moreover, we have used this technique to analyze the structure of viral RNA population in the six different CTV isolates tested.

## Materials and Methods

**Virus Isolates.** The origins and biological characteristics of the CTV isolates used in this study are shown in Table 1.

**Isolation of double-stranded RNAs (dsRNAs).** CTV dsRNAs were purified from bark samples frozen with liquid nitrogen and pulverized in a mortar. Nucleic acids preparations enriched in dsRNAs were obtained by extraction with buffer-saturated phenol and fractionated by column chromatography on non-ionic cellulose (CF-11, Whatman) as previously described (Moreno et al., 1990).

**Cloning of PCR products of p27 gene.** First strand cDNA was synthesized using total CTV-specific dsRNAs as template. The dsRNAs were denatured by heating (100°C, 3min) and then by treatment with 2.4 mM methylmercuric hydroxide. The ssRNAs were reverse transcribed by incubation at 42°C for 120 min in a reaction mixture (10 µl) containing 1x AMV buffer (50 mM Tris-HCl pH 8.5, 30 mM KCl, 8mM MgCl<sub>2</sub>, 1mM DTT), 200µM each of the four dNTPs, 10 U RNAsin, 20 U avian myeloblastosis virus reverse transcriptase (AMV-RT) and 100 ng of antisense primer specific to the p27 ORF 3'Febres (Febres et al., 1994).

For the synthesis of second-strand cDNA an aliquot of 1µl of this preparation was PCR amplified in a reaction mixture (10 µl) containing 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 µM each of the four dNTPs, 0.02 U Taq DNA polymerase and 0.5 µM each of primers sense (5'Febres) and antisense (3'Febres) specific to the p27 ORF (Febres et al, 1994). The PCR cycling profile (35 cycles) was 94°C for 40 s, 50°C for 2 s and 72°C for 45 s, with a final extension step of 72°C for 4 min. All PCR-amplified products were 1/20 diluted and purified by 20% PEG 6000 / 2.5 M NaCl precipitation. Then, an aliquot of them was separated by 0.8% agarose-gel electrophoresis.

PCR products of p27 gene were cloned in pGEM-T vector (Promega) and 30 colonies containing recombinant plasmids were selected and controlled by PCR with specific p27 gene primers.

**Table 1. Origins and biological properties of CTV isolates analyzed in this work.**

Isolate	Origin	Biological properties
T385	Alicante, Spain	This isolate was collected from a healthy looking tree in a 80-year-old citrus planting showing severe decline caused by tristeza. Inoculated on Mexican lime induced only very mild vein clearing and no stem pitting (Moreno et al., 1993)
T312	Valencia, Spain	Induces mild to moderate symptoms in Mexican lime but did not cause seedling yellows or stem pitting on grapefruit and sweet orange (Ballester-Olmos et al., 1993)
T300	Valencia, Spain	Induces mild to moderate symptoms in Mexican lime but did not cause seedling yellows or stem pitting on grapefruit and sweet orange (Ballester-Olmos et al., 1993)
T388	Valencia, Spain	Induces seedling yellows on grapefruit and sour orange and severe stem pitting in various citrus species including Mexican lime and sweet orange seedlings (Ballester-Olmos et al., 1988)
C-269-6	Jujuy, Argentina	This field isolate was collected from a grapefruit tree showing severe decline. Inoculated on Mexican lime induced a mild stem pitting. Also induced seedling yellows and stem pitting on grapefruit but did not cause stem pitting on sweet orange seedlings (Costa, 1995)
C-268-2	Entre Ríos, Argentina	This field isolate was collected from a sweet orange Valencia Frost tree. Inoculated in Mexican lime induced stem pitting. Also induced seedling yellows on grapefruit but did not cause stem pitting on grapefruit and sweet orange seedlings (Costa, 1995).

The dsRNA of the Spanish isolates were kindly provided by Dr. P. Moreno and Dr. J. Guerri, Inst. Valenciano de Investigaciones Agrarias, Valencia, Spain. The Argentine isolates were biologically characterized by Eng. Norma Costa, EEA-INTA Concordia (Entre Ríos, Argentina).

**PCR amplification of p27 gene fragments.** From each of the 30 recombinant plasmids, p27 gene fragments (a)(459 bp long) and (b)(281bp long) were amplified with primers sense 5'Febres and antisense p27-P-3 (5'ACTTACGTAGAGCGTTTGG3') in the first case and primers sense p27-E-3 (5'CAAACGCTCTACGTAAGT3') and antisense 3'Febres in the second case. Primers p27-P-3 and p27-E-3 were designed according to sequences of CTV isolates from Data Bases and from C268-2 cDNA library clones of p27 gene region. An aliquot of PCR products was analyzed in 1.5% agarose-gel electrophoresis and the remains of the samples were purified by phenol extraction and ethanol precipitation.

**SSCP analysis.** SSCP analysis was performed directly on PCR products of fragments (a) and (b) of p27 gene. Usually, PCR products were extracted twice, first with phenol-

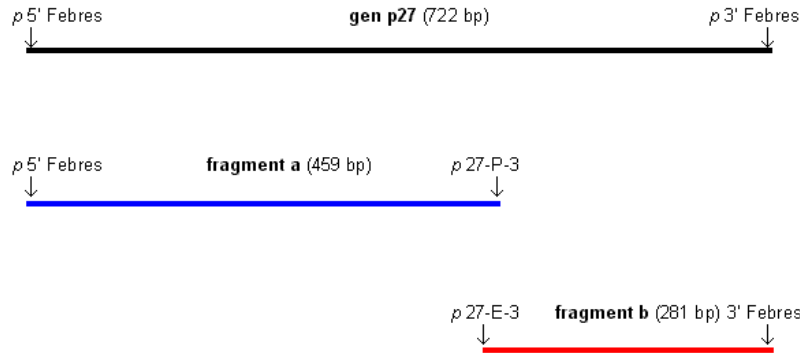
chloroform and then with chloroform, precipitated and resuspended in 10 µl of bi-distilled water. A fraction of 1 µl was mixed with 9 µl of the denaturing solution (950 ml/L) formamide, 20 mM EDTA and 500 mg/L bromophenol blue, heated by 10 min at 100°C, and chilled on ice.

Denatured DNA of p27(a) fragment was subjected to electrophoresis in a non-denaturing 12% polyacrylamide minigel (Mighty Small II, SE 250/SE 260, Hoefer Scientific, 10 x 8 cm), using 1x TBE as electrophoresis buffer, 200 V, 3 hs 30 min, 22°C. The electrophoretical conditions for denatured DNA of p27(b) fragment were 15% polyacrylamide, 1x TBE buffer, 200 V, 3 hs 30 min, 25°C. During electrophoresis, temperature was maintained constant by water circulation. The gels were stained with silver nitrate according to the procedure of Beidler et al (1982).

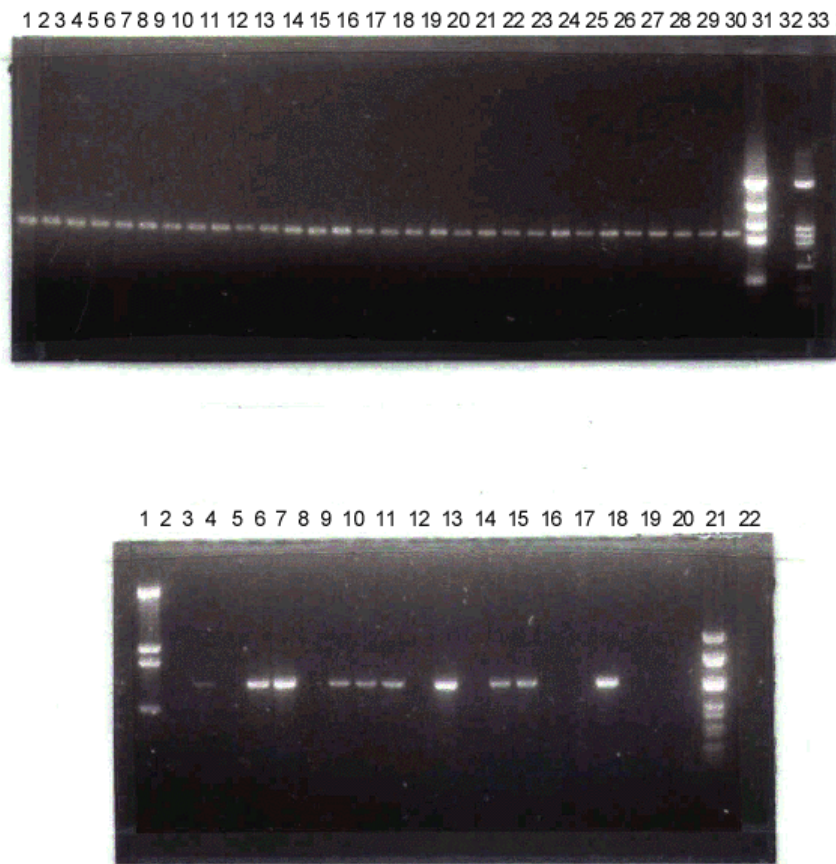
**Results**

The p27 gene of six CTV isolates with different biological properties was reverse transcribed and amplified by PCR, and twenty seven-thirty clones of each isolate were obtained. In all cases, a single DNA fragment of 722 bp was produced.

From each clone, two fragments of p27 gene were amplified by PCR: one of them, 459 bp long (a), includes domains I and II, and the other, 281 bp long (b), includes domains II, III and IV, according to the scheme showed in Figure 1. In both cases, only one PCR fragment of the expected size was obtained for each clone (Figure 2).



**Figure 1.** Scheme of size and position of the p27 gene fragments generated for SSCP analysis.



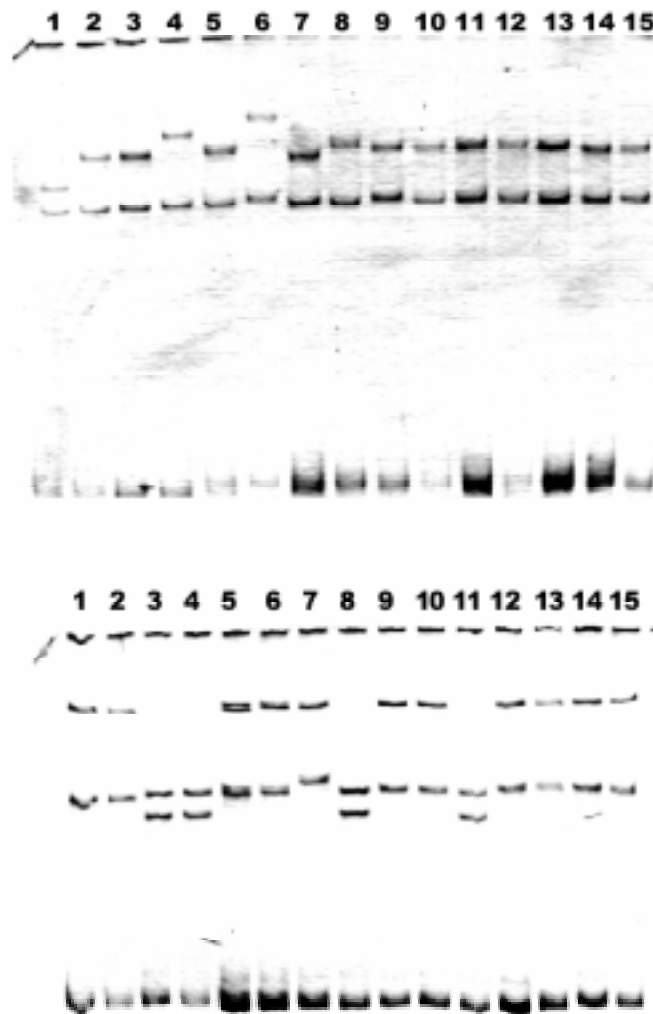
**Figure 2.** PCR amplifications of p27 gene fragments in two CTV isolates .

**Upper panel:** 0.8% agarose gel electrophoresis of PCR products of p27 (a) gene fragment (459 bp) in 30 clones of T-300 CTV isolate (lanes 1 to 30). Lane 31: molecular weight marker (pcDNAII/ *DdeI*/ *XhoI*: 1140, 758, 540, 409 and 166 bp, respectively). Lane 32: negative control PCR reaction. Lane 33: molecular weight marker (pcDNAII/ *HinfI*/ *HindIII*: 1129, 517, 453, 396, 236, 120, 75, 65 and 22 bp, respectively).

**Lower panel:** 0.8% agarose gel electrophoresis of PCR products of p27 (b) gene fragment (281 bp) in 10 clones of T-312 CTV isolate (lanes 3, 5, 6, 8, 9, 10, 12, 14, 15, and 18). Lane 1: molecular weight marker (pcDNAII/ *DdeI*: 1898, 540, 409, and 166 bp, respectively). Lane 21: molecular weight marker (pcDNAII/ *HaeIII*: 657, 458, 434, 290, 272, 174, 142, 102, 80, 44, 40, 29 and 24, respectively). Lane 22: negative control PCR reaction.

Nucleotide sequence variation in both gene fragments was studied by SSCP analysis. In preliminary experiments, several electrophoretical conditions (acrylamide concentration, temperature, presence or absence of glycerol, time, buffer concentration) were assayed. It was established that electrophoresis at a constant voltage of 200 V, for 3.5 h, in 12% polyacrylamide gels, at 22°C, provided adequate resolution of the two strands of PCR products of p27(a) gene fragment. For PCR products of p27(b) gene fragment, the best discrimination of the two strands was obtained with the same conditions mentioned above, except that acrylamide gel concentration was 15% and the temperature during electrophoresis was 25°C.

SSCP showed, for each CTV isolate analyzed and for each p27 gene fragment, a variable number of patterns and clones in each pattern (Figure 3, Table 2). In a high percentage of the clones, only two intense bands, corresponding to only one stable molecular conformation of each DNA strand, were obtained. For p27 (a) gene fragment the exceptions were: ten clones of C269-6, two clones of T385 and two clones of T300 CTV isolates, that showed three intense bands. For p27 (b) gene fragment the exceptions were: one clone of C269-6 and one clone of T388 that showed only one SSCP band; one clone of C269-6, one clone of T388 and one clone of T300, that showed three intense bands, and one clone of T388 CTV isolate, that showed four SSCP bands.



**Figure 3.** SSCP patterns of CTV p27 gene.

**Upper panel:** Single-strand conformation polymorphism of 15 clones of the fragment (a) of the p27 gene of the T312 CTV isolate (lanes 1 to 15). Electrophoresis under non-denaturing conditions was performed at 22°C, 200 V for 3.5 h in 12% acrylamide gels. Gels were stained with silver nitrate.

**Lower panel:** Single-strand conformation polymorphism of 15 clones of the fragment (b) of the p27 gene of the C268-2 CTV isolate (lanes 1 to 15). Electrophoresis under non-denaturing conditions was performed at 25°C, 200 V for 3.5 h in 15% acrylamide gels. Gels were stained with silver nitrate.

Four to nine SSCP patterns could be distinguished for fragment (a) p27 gene, depending on the CTV isolate (Table 2). T388 isolate showed the lowest number of SSCP profiles (4), whereas the C269-6 isolate exhibited the highest number of them (9) (Table 2).

On the other hand, p27(b) gene fragment (Table 2) yield a higher number of different SSCP patterns: eleven different

patterns in the most variable isolate (T-388) and three distinct patterns in the less variable isolate (C268-2). The number of clones included in each of the less represented individual SSCP profiles varied from one to a maximum of seven (Table 2). From 11 to 23 clones were included in the main SSCP pattern of each p27 gene fragment of the CTV isolates analyzed, depending on the viral isolate (Table 2).

**Table 2. Summary of SSCP analysis of all clones of the p27 (a) and (b) gene fragments for the six CTV isolates studied.**

Isolates	p27 (a) gene fragment				p27 (b) gene fragment			
	analyzed clones (qty.)	different patterns (qty.)	distribution of clones in SSCP patterns (qty.)	major clone (%)	analyzed clones (qty.)	different patterns (qty.)	distribution of clones in SSCP patterns (qty.)	Major clone (%)
<b>T-385</b>	27	7	(17,3,2,2,1,1,1)	63	27	8	(18,3,1,1,1,1,1,1)	67
<b>T-300</b>	29	7	(21,3,1,1,1,1,1)	72	28	10	(14,4,2,2,1,1,1,1,1,1)	50
<b>T-312</b>	30	8	(21,2,2,1,1,1,1,1)	70	30	8	(18,4,2,2,1,1,1,1)	60
<b>C268-2</b>	30	6	(22,3,2,1,1,1)	73	30	3	(24,5,1)	80
<b>C269-6</b>	30	9	(11,7,4,2,2,1,1,1,1)	37	30	8	(19,4,2,1,1,1,1,1)	63
<b>T-388</b>	30	4	(23,4,2,1)	77	30	11	(15,3,2,2,2,1,1,1,1,1,1)	50

**Table 3. Summary of SSCP analysis of all clones of p27 complete gene for the six CTV isolates analyzed.**

Isolates	biogroup (type)	Clones with sequence variations only in p27 (a) fragment (qty.) <sup>(a)</sup>	Clones with sequence variations only in p27 (b) fragment (qty.) <sup>(a)</sup>	Clones with sequence variations in both fragments (qty.) <sup>(a)</sup>	Clones without sequence variations (qty.) (main SSCP pattern)	major clone (%)
<b>T-385</b>	I	6	5	4	11	41
<b>T-300</b>	II	6	12	2	7	24
<b>T-312</b>	II	4	7	5	14	47
<b>C268-2</b>	III	4	2	4	21	70
<b>C269-6</b>	IV	11	4	9	9	30
<b>T-388</b>	V	4	11	3	11	37

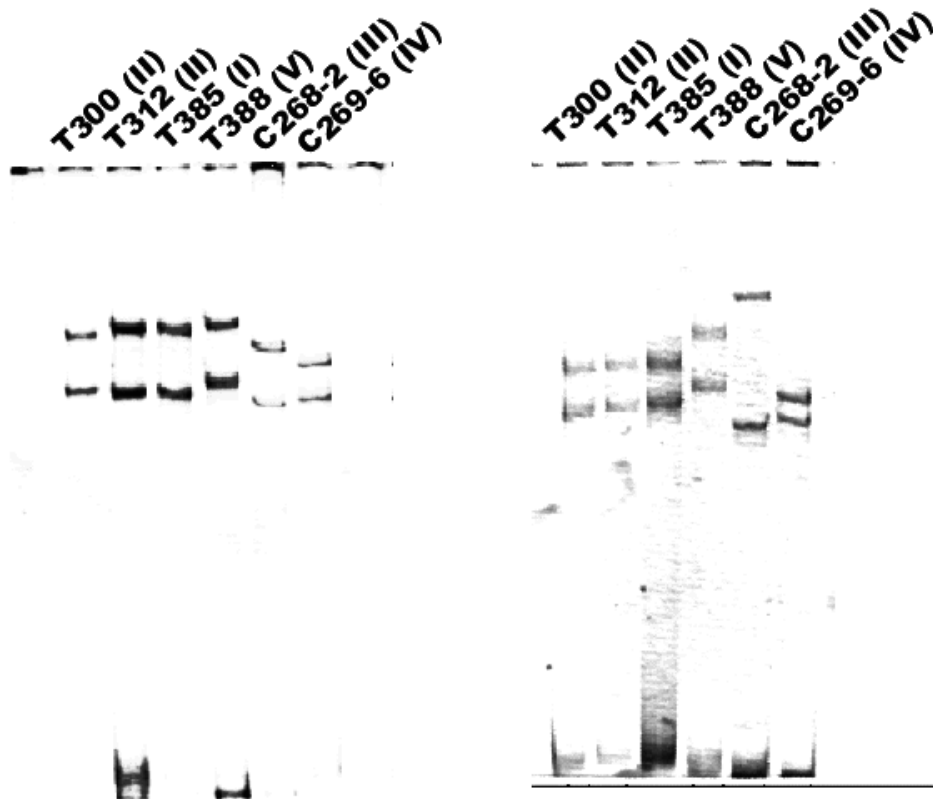
<sup>(a)</sup>These numbers of clones does not include the quantity of them that showed the main SSCP pattern.

The number of clones that showed sequence variation in both p27 gene fragments varied between two (T300) and nine (C269-6). Only the CTV field isolate C269-6 exhibited a higher number of those clones (Table 3). On the other hand, the number of clones that showed any sequence variation in p27 gene varied from 21, for C268-2 CTV isolate, to 7, for T-300 CTV isolate (Table 3).

The percentage of the major clone was calculated for (a) and (b) p27 gene fragments of each isolate analyzed (Table 2). The percentage of principal clone for the complete p27 gene without sequence variations in both (a) and (b) gene fragments was also estimated (Table 3). C268-2 field CTV isolate exhibited the higher value (70%) for the major clone, being the most homogeneous isolate for this gene region.

On the contrary, T300 CTV isolate showed the lowest value (24%) for the major clone, being the most heterogeneous for p27 gene region among the CTV isolates analyzed. The C269-6 CTV field isolate exhibited a similar value than T300 (30%), whereas T312 isolate showed an intermediate value for the p27 majority clone percentage (47%). Percentages for the other two CTV isolates (T385 and T388) were similar (41 and 37%, respectively) and 15% higher than the lowest value (24%). According to the data observed in Tables 2 and 3, p27 (b) gene fragment showed a higher rate of sequence variation than p27 (a) fragment.

When the SSCP profiles of one representative of the majority clone for each CTV isolate were compared in a unique polyacrylamide gel, results obtained were those presented in Figure 4.



**Figure 4:** Scheme of SSCP analysis of one representative majority clon of each CTV isolate studied.

**Left panel:** Lanes 1 to 6: one clone of p27 (a) fragment corresponding to CTV isolates T-300, T-312, T385, T388, C-268-2 and C-269-6.

**Right panel:** Lanes 1 to 6: one clone of p27 (b) fragment of the same CTV isolates above mentioned. In both gels, electrophoretical conditions were the same that established in Figure 3.

For p27 (a) gene fragment (Figure 4, left), T-385 and T-312 CTV isolates exhibited the same SSCP profile, but T300, T388, C268-2 and C269-6 CTV isolates exhibited a different SSCP profile between them and with respect to the above mentioned isolates.

For p27 (b) fragment, T300, T312 and T385 CTV isolates shared the same SSCP pattern; meanwhile, C268-2, C269-6 and T-388 CTV isolates showed a different SSCP profile (Figure 4, right). These results suggest that, when considering the complete p27 gene region, the CTV isolates belonging to the biogroups type III, IV, and V could be distinguished from the CTV isolates belonging to the biogroups type I and II, and between them, at least for the CTV genome region analyzed.

## Discussion

SSCP analysis was applied to p27 gene of six CTV isolates belonging to five different biogroups in an attempt to screen if that gene region differs in isolates with distinct biological properties. These CTV biogroups were defined on the basis of the symptoms induced on different greenhouse indicator plants. The isolates belonging to biogroup type I are the mildest ones and practically asymptomatic. There is an increase in the severity of the symptoms up to biogroup type V, that contains the most severe isolates causing pitting in grapefruit and sweet orange seedlings.

On the other hand, since the SSCP technique has shown to be a powerful alternative to detect the extent of variation within a virus population, it was also applied to know the intrapopulation variability for p27 gene in the CTV isolates tested.

Since the DNA conformation can be altered by several factors (i.e. electrophoresis buffer, voltage, temperature, acrylamide concentration), these factors will alter the electrophoretic mobility. According to that, the conditions to optimize the discrimination capacity of SSCP analysis were adjusted in each particular case. In this work, several experimental conditions were assayed and it was established that, for a good discrimination of SSCP profiles of p27(a) gene fragment, a lower polyacrylamide concentration (12%) and a lower temperature (22°C) was required than for p27(b) gene fragment (15% acrylamide, 25°C).

Different authors (Orita *et al.*, 1989; Hayashi, 1991; Sheffield *et al.*, 1993) have recommended to use DNA fragments from 135 to 400 bp to be able to detect single base mutations by SSCP analysis. For this reason, in this work, the p27 gene region was analyzed in two sub-regions.

For the twenty seven to thirty clones analyzed for each p27 gene fragments (a and b), more than one SSCP profile was observed in the six CTV isolates studied. This result is an

indirect way of assessing the heterogeneity of the population. It was surprising that the T300 CTV isolate, that has been purified by aphid's passage and maintained under greenhouse conditions, showed a similar or higher number of SSCP profiles than field CTV isolates C269-6 and C268-2. Under field conditions, it is reasonable to suppose a higher degree of heterogeneity in viral population because successive infections by different CTV inocula could explain the presence of sequences from more than one viral variant in the same isolate. The reason for this discrepant finding could be the RT-PCR approach used to obtain the clones of p27 gene fragments. This approach may favour the amplification of some virus variants because the set of primers used was designed according to p27 gene sequence of the severe T-36 CTV isolate and three clones of cDNA library of the severe C268-2 CTV field isolate belonging to the same gene region. It could also indicate the exclusion of some virus strains by others *in vivo*. The results obtained, applying the SSCP analysis with the RT-PCR approach described, permit us to discern subpopulations of the CTV genome present in a proportion as low as 3%. The relative amounts of viral subpopulations detected by us using SSCP analysis, were similar to the result obtained by Enomoto *et al.* (1994) using the same experimental procedure. These authors could detect minor variants of E2 -HVR gene of HCV populations representing 5% of the whole population.

Sequencing studies of different p27 gene clones are now under way to determine the percentage of nucleotide exchanges among the majority clone and each of the minority ones.

Biological properties of an isolate may depend on the structure of viral RNA populations. The majority SSCP profiles obtained for the CTV isolates analyzed, which represent the predominant sequence variant observed, allowed us to discriminate four of the five biogroups defined for CTV strains, suggesting a discrimination power of 80% for this procedure. SSCP analysis, which is less time consuming than nucleotide sequencing studies, seems a convenient tool for assigning CTV isolates to a certain biogroup as well as for detecting mixed infections. To confirm this result, we are now conducting SSCP analysis of p27 gene in other CTV isolates belonging to the different biogroups mentioned above. Moreover, nucleotide sequence analysis allow to detect changes in p27 gene sequence. These changes may be eventually related with the biological properties of the virus considering that p27 (b) gene fragment (which contains domains II, III and IV involved in protein folding) showed the higher number of virus variants.

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