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RESEARCH PAPERS

Differentiation of *Citrus tristeza virus* (CTV) isolates by cleavase fragment length polymorphism (CFLP) analysis of the major coat protein gene

Natália T. MARQUES[1], Ana M. BAILEY[2], Charles L. NIBLETT[3] and Gustavo NOLASCO[1]

[1]Universidade do Algarve - Centro de Desenvolvimento de Ciências e Técnicas de Produção Vegetal, Campus de Gambelas, 8005-139 Faro, Portugal
[2]Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Irapuato, Apdo. Postal 629, 6500, Irapuato, Mexico
[3]Horticultural Sciences Department, University of Florida, P.O. Box 110780, Gainesville, FL 32611-0780 USA

Summary. A panel of *Citrus tristeza virus* (CTV, genus *Closterovirus*, family *Closteroviridae*) isolates of different origins and with different biological properties were compared for polymorphisms in the major coat protein (CP) gene by cleavase fragment length polymorphism (CFLP) and single stranded conformation polymorphism (SSCP) analysis. The similarity between the CFLP patterns, which consisted of 15 to 20 bands, was estimated by the Pearson coefficient. The clustering patterns from the CFLP data were very similar to those from sequence data in an experiment with 16 cloned standards of the CP gene. By SSCP analysis on the other hand, most of the clones were not clustered in the same way. To assess the ability of CFLP to analyse biological samples, which may consist of a mixture of genomic variants, the CP gene of 12 CTV isolates was obtained directly from infected plants by immunocapture/RT-PCR and analysed. With few exceptions, the isolates were correctly clustered according to the sequences of the variants composing the isolates. In artificial mixed infections of mild and severe isolates the patterns obtained were more closely related to the severe isolate. Thus the CFLP method was an accurate method for the identification, typing and clustering of CTV isolates. The usefulness of this technique as an alternative to SSCP analysis is suggested and discussed.

Key words: CFLP, genomic variability, typing.

Introduction

Citrus tristeza virus (CTV, genus *Closterovirus*, family *Closteroviridae*) is the causal agent of one of the most economically important diseases of citrus. The virus has a single-stranded positive-sense RNA genome varying from 19,226 (Mawassi *et al*., 1996) to 19296 (Karasev *et al*., 1995) nucleotides, containing 12 ORFs, potentially encoding at least 17 protein products (Karasev *et al*., 1995). The major capsid protein (here referred to as coat protein [CP]) has 25 KDa and covers about 95% of the virion (Febres *et al*., 1996). CTV isolates differ in the type and severity of the symptoms they induce in the various citrus hosts (Grant and Higgins,

Corrresponding author: G. Nolasco
Fax: +351 289818419
E-mail: gnolasco@ualg.pt

1956). It is generally accepted that CTV infection has caused the decline and death of millions of citrus trees grafted on sour orange rootstocks. Decline is rapid in certain cases, but slow decline over a period of months or years is most frequent. Strains causing quick decline or stem pitting on sweet orange or grapefruit are usually called severe strains. Those that do not cause these symptoms are mild strains. Attempts have been made to relate the biological properties of the virus to its biochemical characteristics (Niblett *et al*., 2000). The rapid identification of severe isolates is important to control the disease. CTV isolates have been characterized in a number of ways: by serological tests (Manjunath *et al*., 1993; Pappu *et al*., 1993), double-stranded RNA analysis (Moreno *et al*., 1990; Guerri *et al*., 1991; Moreno *et al*., 1993; Moreno *et al*., 1996), restriction fragment length polymorphism (Gillings *et al*., 1993; Nolasco and Sequeira, 1997), single stranded conformation polymorphism (SSCP) (Rubio *et al*., 1996; Nolasco and Sequeira, 1997; Ayllón *et al*., 1999; Gago-Zachert *et al*., 1999; Kong *et al*., 2000), hybridisation with complementary long DNA probes (Rosner *et al*., 1986; Narváez *et al*., 2000) or short DNA probes (Niblett *et al*., 2000; Zemzami *et al*., 2002), and by analysis of peptide maps (Guerri *et al*., 1990; Albiach-Martí *et al*., 2000).

Cleavase fragment length polymorphism (CFLP) analysis is based on the activity of the endonuclease cleavase, which recognizes the secondary structure of hairpins and cuts the DNA strand at the 5' end of the stem part. The DNA molecules to be analysed are thermally denatured and allowed to cool to a certain temperature so that on each DNA strand a certain amount of the secondary structure builds up by self-base pairing. Cleavase activity produces a collection of fragments that are resolved by denaturing gel electrophoresis. CFLP analysis has already been used to study variability in diverse genetic systems, such as human somatic genes (O'Connell *et al*., 1999), bacteria (Brow *et al*., 1996), plants (Fofana *et al*., 1998) and animal viruses (Marshall *et al*., 1997), but not plant viruses.

In this work we describe the use of CFLP as an alternative to sequencing or SSCP for typing purposes.

Materials and methods

Virus isolates and clones of the CP gene

Two sources of the CP gene were used in the assays: the cloned CP gene and infected plant material. Sixteen CP gene clones used as standards were obtained from different sources as minipreps. The origin and the biological characterisation by

Table 1. Characterisation of the 12 *Citrus tristeza virus* isolates (Bonacalza, 1998).

Isolate	Origin	Biological characterisation		
		SP-SwO [c]	SP-ML [d]	SP-GF [e]
2	Madeira Island	+	+	+
8	Madeira Island	+	+	?
13	Madeira Island	+	+	-
14	Madeira Island	+	+	-
15	Madeira Island	+	+	-
19 [a]	Spain	-	+	-
25 [a]	Spain	-	+	-
28	Portugal	-	+	+
144	Reunion Island	-	+	n/a
196 [b] =25+15	Artificial mixed infection	+	n/a	n/a
200 [b] =25+8	Artificial mixed infection	+	n/a	n/a
206 [b] =25+212	Artificial mixed infection	+	n/a	n/a
212	Reunion Island	+	+	-

[a] Plants 19 and 25 were also tested for quick decline symptoms, with negative results.
[b] Plants double inoculated with different isolates.
[c] SP-SwO, stem pitting on sweet orange; [d] SP-ML, stem pitting on Mexican lime; [e] SP-GF, stem pitting on grapefruit.
n/a, not assayed.

means of the biological indexing of the isolates from which these clones were obtained are shown in Tables 1 and 2. A further twelve CTV isolates were taken from a collection maintained in insect-proof greenhouse-grown seedlings of sweet orange (*Citrus sinensis* [L.] Osb.) cv. Madam vinous that had been biologically characterised in an earlier study (Bonacalza, 1998). In some cases mixed infections were obtained by double inoculation in which the second inoculation was carried out after ELISA confirmed the establishment of the first infection.

Isolating and cloning the CP gene

The CP gene of the 12 CTV isolates was amplified by Immunocapture reverse transcriptional (RT) PCR, as described in Nolasco *et al*. (1993). Each RT-PCR reaction was done in 50 μl of a mixture containing: 4 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer, 3 units of RNase inhibitor (GE Healthcare, Chalfont St. Giles, UK, ref. 27-0815-01), 7.5 units of MuLV Rtase (Applied Biosystems, Foster City, USA, ref. N808-0018) and 1.25 units of *Taq* DNA polymerase (Fermentas Inc., Hanover, USA, ref. EPO402). The reaction tubes were incubated in a termocycler with the following cycling conditions: 38°C for 45 min, 94°C for 2 min, 35 cycles at 92°C for 30 s, 52°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 7 min. The primers used were: CTV 1 [5´–ATGGACGACGAAACAAAGAA–3´] (forward primer) and CTV 10 [5´–ATCAACGTGTGTTGAATTTCC–3´] (reverse primer), generating a 673 bp fragment corresponding to the whole CP gene, the stop codon and an extra nucleotide at the 3´ end. PCR amplifications using the cloned CP gene as template were done using 1 μl of a miniprep in the same conditions as above, except for the RT reagents and the incubation step, which were omitted. Usually, one-tenth of the amplified product was analysed in 1% agarose gel. When necessary, the PCR product was TA cloned in a pCRII vector (Invitrogen, Carlsbad, CA, USA) or a pGEM T-Easy vector (Promega, Madison, USA), according to manufacturer's instructions. Competent INVαF´ *Escherichia coli* cells were transformed and colonies containing the insert were selected in the presence of X-gal and confirmed by PCR. Minipreps were done according to standard procedures.

CFLP analysis

CFLP analysis was done using the CFLP" Scanning System (Takara Shuzo Co. Ltd., Otsu

Table 2. Description of 16 cloned coat protein gene standards.

Clone	Geographical origin	Biological characterisation of the isolate from which the CP clone was obtained[b]	GenBank accession number or bibliographic reference of sequence data
T36	Florida, USA	QD	M76485
B53	Spain / Japan[a]	QD, SP-GF[c], SP-SwO	Pappu *et al*., 1993; Akbulut *et al*., 1996
19.121	Spain	Isolate 19, Table 1	AF1841144
13C	Madeira Island	Isolate 13, Table 1	AF184113
B185	Japan	SP-GF, SP-SwO	Pappu *et al*., 1993
T3	Florida, USA	QD, SP-SwO	Pappu *et al*., 1993
15.118	Madeira Island	Isolate 15, Table 1	AY660009
T30	Florida, USA	SP-ML	AF260651
B274	Colombia	SP-ML	Acosta *et al*., 1994
B249	Venezuela	QD; SP-SwO	Peñaranda *et al*., 1996
2.93	Madeira Island	Isolate 2, Table 1	AF184116
2.98	Madeira Island	Isolate 2, Table 1	AF184117
25.120	Spain	Isolate 25, Table 1	AF184115
TR11a	Reunion Island	SP-GF	AY660010
28.121	Portugal	Isolate 28, Table 1	AF184118
B128	Colombia	QD, SP-GF, SP-SwO	Pappu *et al*., 1993

[a] The origin of this isolate is reported by several authors as Spain, by others as Japan.

[b] QD, quick decline; SP-GF, stem pitting on grapefruit; SP-SwO, stem pitting on sweet orange; SP-ML, stem pitting on Mexican lime.

Shiga, Japan, ref. 6627). PCR products were precipitated with ethanol and re-suspended in 15 μl of water. Depending on previous gel electrophoresis quantification, 1 or 2 μl of the PCR products was diluted to a final volume of 15 μl in 5 mM MOPS, pH 7.5, denatured at 95°C for 30 s, then cooled and maintained at the optimised reaction temperature (45°C). The pre-warmed cleavase enzyme solution (5 μl) was then added. Final composition of the reaction mix was 10 mM MOPS (pH 7.5), 0.2 mM of $MnCl_2$, and contained 0.05% (w:v) Tween 20, 0.05% (w:v) Nonidet P-40 and 1.25 U of cleavase. Cleavase digestion proceeded for 12 minutes until stopped by the addition of 16 μl of stop solution (95% formamide, 10 mM EDTA [pH 8], 0.05% xylene cyanol, 0.05% bromophenol blue). The DNA fragments were resolved by electrophoresis in 0.75 mm mini polyacrylamide gels (8% with 19:1 cross-linking) containing 7 M urea in 0.5×TBE buffer (Tris-borate 44.5 mM; EDTA 1 mM; pH 8.3). Before loading the gel the samples were denatured at 85°C for 1–2 min and the gel was pre-run for 30 min at 300 V. Electrophoretic separation was done for 45 min at 300 V. Silver staining and drying of the gels was done according to standard procedures. Digested DNA from CTV clone 15.118 was included in each assay as a reference sample. Analysis of band patterns was done with the help of the Bionumerics software package (Applied Maths, Kortrijk, Belgium) using the Pearson similarity coefficient and Unweighted Pair Group Average Method (UPGMA) clustering. Cophenetic correlation values were determined to assess the consistence of the clusters. To avoid interference caused by small variations in the length of the electrophoresis run, or problems caused by the software having difficulty in recognising faint bands at the lower end of the gel, only bands corresponding to more than 200 bases were compared. Sequence alignments and nucleotide distances were determined using the BioEdit sequence alignment editor software package (Hall, 1999).

SSCP analysis

The SSCP analysis was done on PCR products obtained from the 16 cloned standards (Table 2), as described in Rubio *et al*. (1996). The CP gene previously amplified by primers CTV1/CTV10 was reamplified with primers CTV 43 [5´–ATGTTGTTGCNGCNGAGTC–3´] (forward primer) and CTV 42 [5´–CTCAAATTGCGRTTCTGTCT–3´] (reverse primer), generating a fragment with 415 bp. Electrophoretic separation was done on 8% polyacrylamide gels run at 200 V for 100 min. Silver staining and drying of the gels was done according to standard procedures.

Results

Optimal temperature of cleavase digestion

Electrophoretic separation of the fragments produced by cleavase digestion of the CP gene PCR product caused patterns with several bands differing in signal intensity (Fig. 1). Reaction temperature may affect the number of DNA fragments produced. The optimal temperature for the cleavase reaction was found to be 45°C. At this temperature a greater number of bands (15–20) was obtained with the uppermost band present, signifying absence of over-digestion.

CFLP analysis of cloned CP gene standards

The 16 standards whose nucleotide sequence was known (sharing from 89.5% to 99.7% nucleotide identity) were assayed (Fig. 1), and the CFLP pattern similarities computed by the Pearson coefficient (Table 3). The ability of CFLP analysis to estimate the sequence similarity was inferred by comparing both sets of data (Fig. 2), showing a quadratic (second-degree polynomial) correlation with a statistically significant correlation coefficient ($P<0.01$). As can be seen in Fig. 2, CFLP was highly sensitive in detecting nucleotide differences above 90%: for instance, it was able to discriminate two clones, 2.93 and 25.120, which differed in only 2 positions. The two closest clones deduced by CFLP (2.93 and B274) differed in 10 nucleotides, and their patterns had a Pearson similarity coefficient of 98.1%.

The similarity data from CFLP (Table 3) were used to construct a dendrogram (Fig. 1) in which several clusters could be recognised. The 16 cloned standards analysed are represented on clusters B to G. The most striking aspect here was that in general the standards were grouped according to the biological properties of the isolates from which they were obtained. Standards obtained from severe sweet orange stem-pitting isolates (B128, 13C, B185) clustered in group C or in group F (15.118, 2.98, B249), while clones from mild isolates (T30,

2.93, 25.120, B274) clustered in group E. Clones from severe quick-decline isolates were clustered in a less distinct mode in groups G and D. A very similar dendrogram was obtained when the standards were clustered according to sequence similarity (Fig. 3), the only exception being standards T3 and Tr11a, which now appeared separated. To test reproducibility of CFLP, the set of 16 clones of the CP gene was re-analysed at two dates about 6 months apart and the dendrogram from the CFLP patterns was mostly identical at both dates.

CFLP analysis targeted to the CTV CP gene of infected plant material

Infected plant material from biologically characterised sources (Table 1) was analysed by immu-

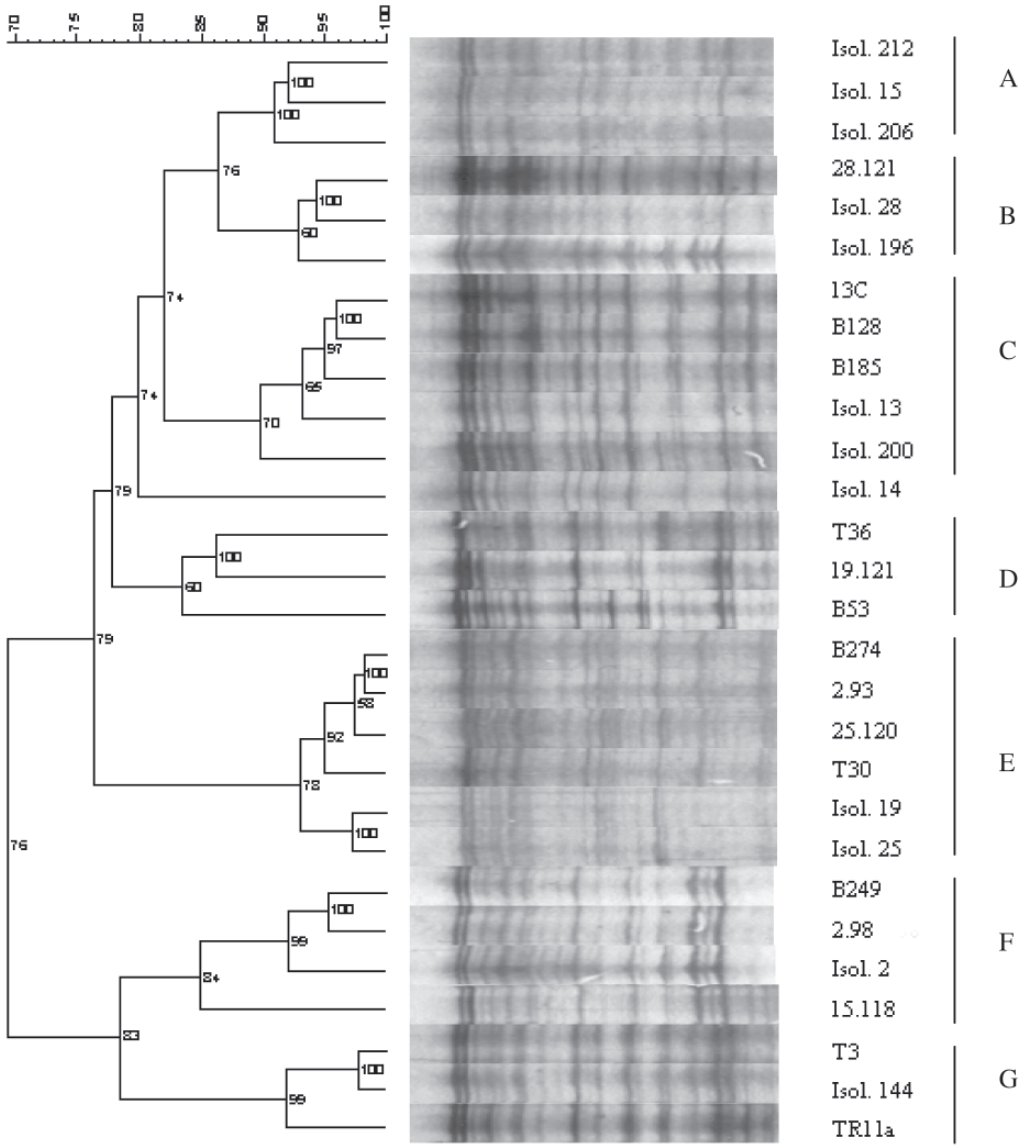

Fig. 1. Dendrogram and respective CFLP patterns of the 12 isolates and 16 cloned standards. Major clusters are indicated on the right (A, B, C, D, E, F and G). The rule-bar at the top-left represents the similarity (%) between patterns. Numbers in the dendrogram refer to the cophenetic correlation values. Isolates 19 and 25 were the only ones that did not induce stem pitting in sweet orange (SP-SwO) or grapefruit, nor quick decline, thus being considered mild ones.

Table 3. Percent nucleotide identity obtained from sequence data (above diagonal) and percent similarities as estimated by CFLP (below diagonal) of the 16 cloned standards of the coat protein gene.

												Percent nucleotide identity				
T36	*	94.7	94.4	93.3	92.2	93.4	93.3	91.6	92.1	92.2	91.6	91.3	93	93	92.8	93
19.121	86.1	*	97.3	93.4	92.4	93.7	92.8	92.4	92.5	92.7	92.1	91.6	92.5	92.5	92.4	92.7
B53	81.3	85.5	*	93.1	91.9	93.6	93.0	92.5	92.8	93.3	92.4	91.8	92.2	92.2	92.4	92.5
13C	78.3	82.4	76.4	*	96.7	96.4	97.3	93.6	92.2	92.4	91.8	91.2	92.7	92.7	92.5	92.7
B185	78.6	83.6	72.5	94.8	*	94.7	94.3	92.5	90.7	91.0	90.3	89.5	91.9	91.9	92.2	92.1
28.121	80.3	82.7	76.1	89.9	86.3	*	94.6	93.8	92.4	92.5	91.9	91.6	93.0	93.0	92.8	93
B128	75.7	79.3	73.2	95.8	95.1	87.6	*	92.2	93.1	93.6	92.7	91.8	91.5	91.5	91.6	91.8
T3	81.2	84.6	68.8	69.7	75.7	74.1	70.2	*	91.6	91.8	91.2	91.8	92.5	92.5	92.2	92.7
15.118	74.4	79.6	63.1	64.5	69.6	75.8	62.3	82.8	*	99.5	99.1	96.8	91.6	91.6	91.0	91.5
B249	80.3	86.5	77.4	73.3	71.2	85.9	69.6	80.0	87.4	*	98.9	96.7	91.5	91.5	91.5	91.6
2.98	77.2	80.9	70.5	67.1	66.5	80.6	63.9	82.6	87.6	95.2	*	96.2	91.2	91.2	90.6	91
TR11a	76.7	80.8	66.2	66.7	73.8	66.6	66.5	92.2	79.9	69.2	71.6	*	91.0	91.0	90.4	90.9
2.93	75.7	69.5	73.3	78.5	69.7	83.4	72.4	60.6	64.0	72.9	63.7	54.9	*	99.7	98.6	99.1
25.120	77.2	68.6	72.1	80.2	73.6	85.1	75.6	58.3	62.8	68.5	60.2	53.0	98.0	*	98.6	99.1
B274	76.7	68.0	72.3	78.6	71.4	81.1	73.8	58.5	63.2	71.9	62.1	53.7	98.1	96.7	*	98.9
T30	73.4	68.9	69.5	74.9	69.5	81.7	68.3	61.0	66.3	70.9	60.8	56.3	95.0	94.2	95.6	*
	T36	19.121	B53	13C	B185	28.121	B128	T3	15.118	B249	2.98	TR11a	2.93	25.121	B274	T30
	Percent similarity by CFLP															

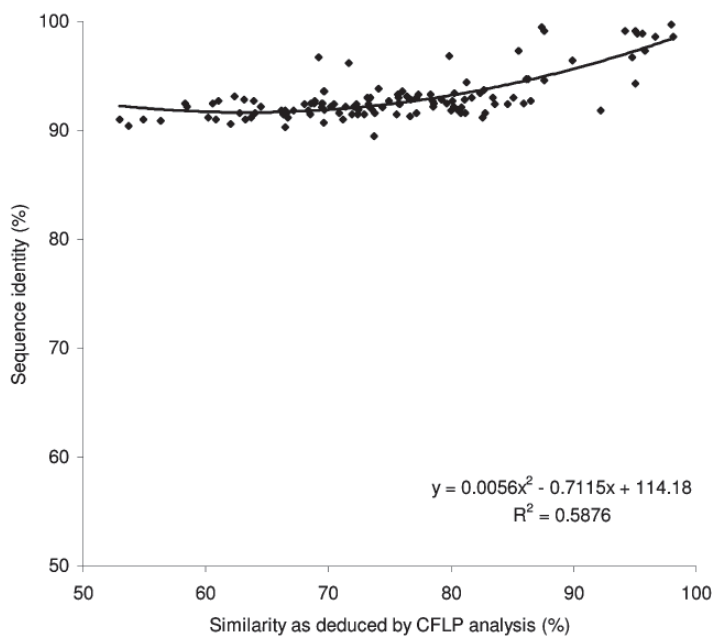

Fig. 2. Comparison of sequence identity and similarity between patterns generated by CFLP analysis.

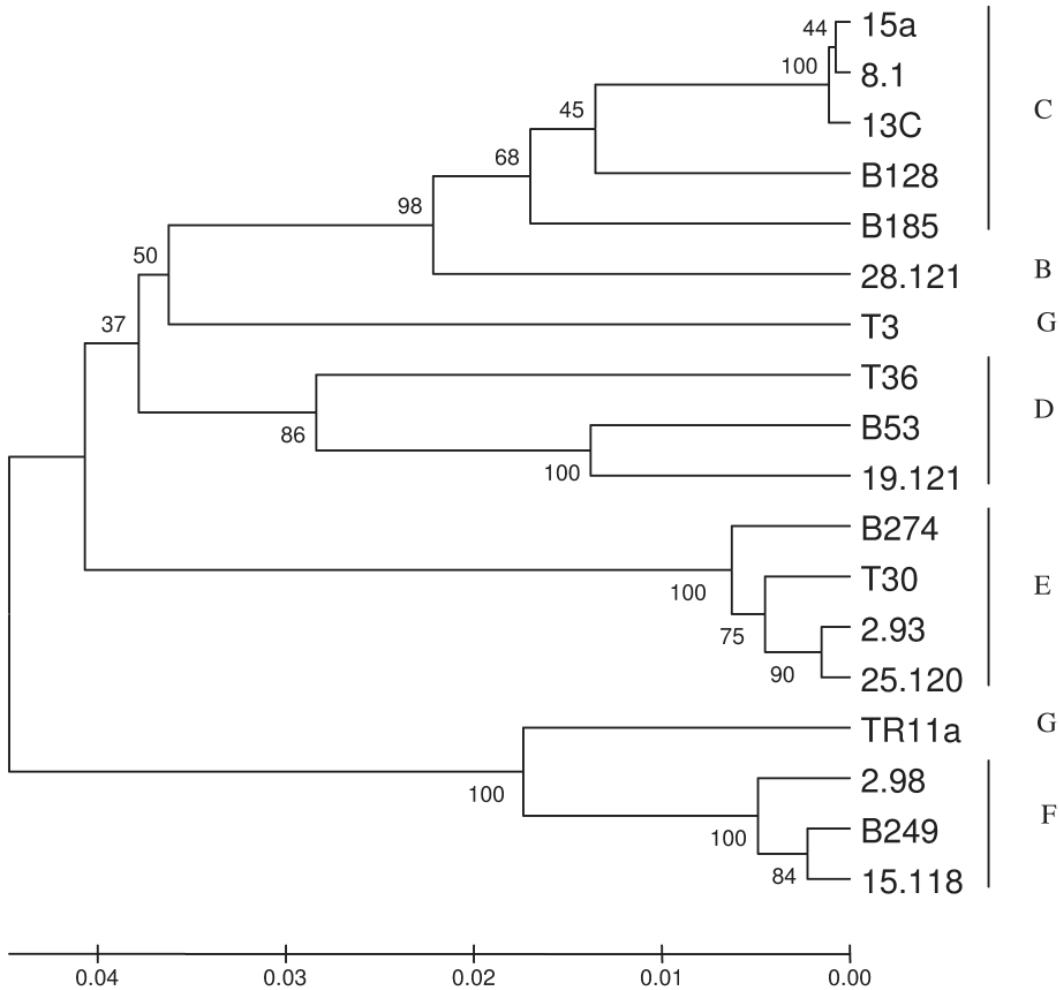

Fig. 3. Dendrogram showing UPGMA clustering of the coat protein (CP) gene sequences of the standards used. CP clones 15a and 8–1 were not analysed by CFLP. Letters on the right refer to the cluster as determined by CFLP.

nocapture RT-PCR followed by CFLP analysis targeted to the CP gene. The similarities between band patterns were computed and used to cluster the isolates in the same dendrogram as for the standards (Fig. 1). With the exception of isolate 14, which was not clearly assigned to any group, the remaining 11 isolates appeared in different clusters, frequently grouped with isolates or standards with which they shared some kind of geographic or biological relationship. For instance, the mild isolates 19 and 25 (from Spain) were grouped with clones of cluster E, which came from plants described as having mild symptoms. Isolate 144 from Reunion Island was grouped in cluster G, together with a clone from Reunion Island. Isolate 28 and one clone originated from it, clone 28.121, were grouped closer in the same cluster B. Isolate 13 was grouped in cluster C along with standards obtained from plants which caused stem pitting on sweet orange (SP-SwO), and with a standard (13C) that was obtained from itself. Isolate 2, which is a SP-SwO isolate from Madeira Island, produced two different clones, clone 2.98 and clone 2.93. Clone 2.98 was grouped together with isolate 2 and other clones that were produced by SP-SwO isolates. Clone 2.93 was grouped in cluster E with mild isolates and mild-origin clones (in accordance with the sequence clustering in Fig. 3).

As it was clear that some isolates harboured a mixture of sequence variants, we used CFLP to screen isolates that had been subjected to inoculations of known origin. Plants 196, 200 and 206 were inoculated with a mixture of mild and stem pitting isolates and showed SP-SwO symptoms. Both mild and SP-SwO components were later retrieved by cloning and sequencing the genomic variants, thus confirming the mixed character of the infection (results not shown). A new cluster (A) appeared, which did not contain any of the previous cloned standards. In this cluster some isolates were grouped known to harbour a mixture of haplotypes: isolate 206 which is an artificial mixture of isolates 25 and 212; isolate 15 (from which were obtained the haplotypes 15.118, group F, and 15a, group C - see Fig. 1). Isolate 212 was also in this group. All the isolates from this cluster caused SP-SwO. Isolate 200, which was an artificial mixture of isolates 25 and 8, was clustered in group C. Although isolate 8 was not analysed by CFLP, the sequence of a haplotype obtained from this isolate clustered in group C (Fig. 3). The only isolate which clustered in an unrelated group was isolate 196.

SSCP analysis of cloned CP gene standards

The SSCP analysis of the 16 cloned standards is shown in Fig. 4. Only conspicuous bands were considered to group the patterns. Six standards had unique patterns that could not be grouped. The remaining ten standards fell into three different patterns, named 1 to 3. Only group 2 had a close relationship to group E as defined by CFLP, the other two did not.

Discussion

Optimising the CFLP technique and cleavase digestion

CFLP analysis usually relies on the radioactive labelling of one DNA strand. However, O'Connell *et al*. (1999) obtained discriminating patterns by labelling both strands. Using the band patterns generated by both strands makes it possible to silver stain the gels, thus avoiding radioactive labelling, and this was done in this work. In this way the protocol is much simpler and more user-friendly. Silver staining was tried in an earlier study (Rossetti *et al*., 1997) but without much success because of the limited number of bands produced with the CFLP protocol used. In the present study the CFLP patterns for DNA fragments with 673 bp consisted of 15 to 20 bands. Different patterns were formed by sets of bands differing in signal intensity. When establishing the optimum conditions for the CFLP reaction it is desirable to obtain patterns with a maximum number of bands. According to the manufacturer, a major parameter that determines the number of bands is the temperature of the cleavase digestion. Temperatures that are too low do not produce enough bands, while temperatures that are too high lead to over-digestion and the disappearance of the slower migrating bands. Avoiding over-

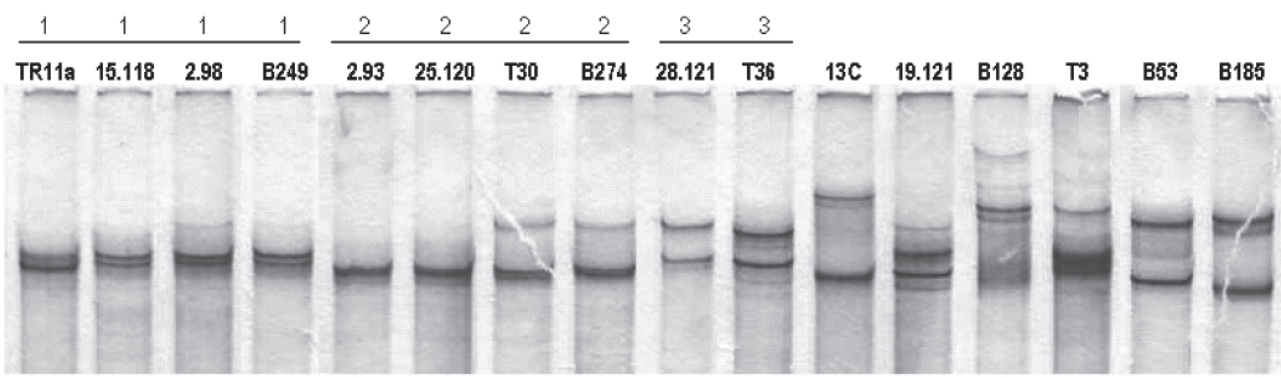

Fig. 4. SSCP patterns of the 16 cloned standards. SSCP groups are marked 1 to 3.

digestion is important since it ensures that the whole set of fragments will be present. In this work results obtained at 45°C were deemed to be adequate.

Comparative ability of CFLP and SSCP to distinguish and cluster genomic variants

The similarity of cloned standards obtained by CFLP analysis is in close relationship with similarities computed at the nucleotide level. This resulted in very similar dendrograms obtained with both methods. Moreover, as can be seen in Fig. 2, the CFLP technique was sensitive enough to detect minor changes at the nucleotide level even between very similar sequences (e.g. those with a nucleotide similarity greater than 90%). As a result, all the 16 clones showed different patterns with CFLP and in the pool of sequences tested, the smallest difference detected was of only 2 nucleotides. This sensitivity makes CFLP a good method to compare viral genes, as for instance the coat protein gene, in which variants of the same species usually have a greater than 85% nucleotide identity.

SSCP is also suitable for the detection of polymorphisms in CTV (Rubio *et al*., 1996; Nolasco *et al*., 1997) and other RNA viruses and is now very commonly used. A short report on the SSCP analysis of the 16 standards used in this study presented at an earlier date (Marques *et al*., 2002) showed that this technique had only a limited ability to group genomic variants. Of the three groups that could be formed, only one was in agreement with the clusters formed by CFLP and nucleotide sequence analysis. With SSCP the emphasis is usually on its capacity to distinguish between sequence variants. For instance, Rubio *et al*. (1996) used this method to discriminate between certain CTV variants that differed in only 1 nucleotide. However, CTV has a high capacity for genomic variation, with each isolate being associated with genetically related variants (Rubio *et al*., 2001). Thus the main aim when using SSCP to compare two CTV isolates should be to ascertain their degree of similarity, rather than to determine whether the isolates are different or not (which however they usually are). SSCP is therefore a very limited technique since it does not relate the migration of molecular conformations to the nucleotide sequence (Orita *et al*., 1989). Thus the information obtained with SSCP is less useful than that obtained with CFLP when the objective is to determine the relationships between sequences. Another advantage of the CFLP technique, reported also by O'Connell *et al*. (1999) and Brow *et al*. (1996), is that it yields reproducible results, which SSCP does not always do (O'Connell *et al*., 1999).

CFLP and the typing of CTV isolates

Cost-effective molecular methods to characterise CTV variants and establish their relation to already characterised strains are vital for early CTV control. An important aspect of CFLP analysis is that it clusters the majority of CTV isolates together with cloned standards of the same origin and also with isolates having similar biological properties. This characteristic seems to be the direct result of the ability of CFLP to cluster isolates according to the nucleotide sequence relationship, which makes this technique very promising for CTV typing. Under natural conditions trees are frequently infected with mixtures of CTV strains, which may be distributed irregularly (Grant and Higgins, 1956). When isolates are composed of a mixture of sequence variants, the results need to be interpreted carefully. For instance, isolate 2 is composed of a mixture of sequence variants 2.98 and 2.93 which cluster in different groups; in this case the CFLP pattern of isolate 2 clustered close to the pattern obtained from clone 2.98 in a group of severe isolates. On the contrary, the CFLP pattern from isolate 19 clustered according to biological properties, far from the only clone from this isolate (19.121) that was analysed by CFLP. However, further work (not presented) showed that clone 19.121 is a minor variant present in isolate 19. In the other cases, when mixtures of mild and SP-SwO components were studied, the CFLP patterns clustered close to the stem-pitting component. Since sequencing showed both variants were present, probably the SP-SwO component multiplied at a higher rate, being easily detected. Further studies using artificial mixtures of cloned standards should be carried out to estimate the proportion of components that will cause the pattern to shift from one type of cluster to another. From a practical point of view, being able to detect the severe components so that they can be eradicated is very important.

CFLP thus is an alternative to SSCP analysis, providing useful information that enables the relationships between isolates to be established according to their degree of sequence similarity, and, indirectly, according to their biological activity.

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