Biological and Molecular Characterization of Two Isolates of Citrus Viroids Recovered from Cuban Plantations

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ABSTRACT. Two field sources (PM 26-X and Cl 13-24) were subjected to biological and biochemical analysis for citrus viroids. PM 26-X was recovered from Frost Marsh grapefruit grafted on alemow showing chlorosis, stunting and gum impregnation of the bark, and Cl 13-24 was recovered from a symptomless Clementine tree grafted on Troyer citrange. Biological indexing on Etrog citron, Parson's Special mandarin and clemeline 11-20 showed symptoms characteristic of citrus viroids. Viroid infection was demonstrated by sPAGE and molecular hybridization analysis of nucleic acid preparations from the inoculated citrons. The nucleic acid extracts were subjected to retrotranscription, PCR amplification, cloning, SSCP analysis and sequencing. In all instances a "master sequence" was identified as the most frequent variant and was compared to other reported sequences of the same viroids.

Index words. Citrus viroids, cachexia.

Commercial citrus trees are frequently infected with citrus viroids unless they have been subjected to sanitation programs. Visual observation of field grown trees of sensitive rootstock/scion combinations indicate that they were probably infected with viroids. In the past, viroid detection was performed by biological indexing, but additional molecular tests are necessary to identify the viroid species present in a given field source. Here we report the biological and molecular characterization of two field isolates from Cuban plantations.

MATERIALS AND METHODS

Field sources. PM 26-X was recovered from a Frost Marsh grapefruit grafted on alemow showing chlorosis, stunting and gum impregnation of the bark, and Cl 13-24 was recovered from a symptomless Clementine tree grafted on Troyer citrange.

Biological indexing. Field sources PM 26-X and Cl 13-24 were indexed on Etrog citron, Parson's Special mandarin and clemeline 11-20 grafted on pummelo. Inoculation was performed by grafting onto four plants of each indicator species. The leaf symptoms on Etrog citron were recorded 6 mo after inoculation (2). Cachexia symptoms of gumming and wood staining symptoms at the bud union were recorded 12 mo after inoculation (15).

Identification of citrus viroids. Tissue samples (5 g) of young leaves and stems were collected from inoculated citrons and homogenized in 5 ml of extraction buffer (0.4M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) mercaptoethanol) and 15 ml of water saturated phenol (13). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM $MgCl_2$; pH 7.4). Aliquots of these nucleic acid preparations were used for sPAGE, molecular hybridization analysis, cloning and sequencing.

For sPAGE analysis, 20 µl aliquots (equivalent to 300 mg fresh weight tissue) were subjected to electrophoresis under non-denaturing and denaturing conditions (12) and the circular forms of the viroids were viewed by silver staining the second gel (4).

For molecular hybridization analysis, 10 µl aliquots (equivalent to 150 mg fresh weight tissue) of nucleic acid preparations were denatured in 20% formaldehyde and transferred to positively charged nylon (Boehringer Mannheim) membranes using a Hybri-slot filtration manifold (BRL), fixed by UV cross-linking and hybridized against DIG labeled viroid specific DNA probes. Probes were synthesized by PCR amplification of cloned monomeric viroid sequences (6). Prehybridization and hybridization were carried out in 50% formamide and 6×SSPE. The membranes were prehybridized at 42°C for 2-4 h and hybridized overnight at 50°C. After hybridization, they were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min, followed by another wash in 0.1× SSC, 0.1% SDS for 60 min at 60°C. The DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminescent substrate CSPD (Boehringer Mannheim®).

Cloning. Viroid DNAs were synthesized by retrotranscription and PCR amplification using specific primers for Citrus viroid II (CVd-II) (1), *Citrus viroid III* (CVd-III) (10) and *Citrus viroid IV* (CVd-IV) (9). First strand synthesis was performed using and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega). Second strand synthesis and DNA amplification were performed in 1mM $MgCl_2$, 0.25mM dNTPs, 0.5µM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of 30 cycles at 94°C for 40 sec, 60°C for 40 sec and 72°C for 1 min, with a final extension at 72°C for 5 min. The size of the DNA products was determined by electrophoresis in 2% agarose gels and its homology with the desired viroid was confirmed by slot blot hybridization. The purified CVd-II DNAs were digested with the endonuclease *Sma*I and the resulting DNA fragment was cloned at the *Sma*I restriction site of the commercially available digested and dephosphorilated pUC 18 plasmid (Pharmacia). The purified CVd-III and CVd-IV DNAs were ligated to the thymidylated *Eco*RV site of the pGEM®-T vector (Promega). Plasmids from transformed cells were subjected to restriction analysis to verify the presence of an insert of the expected size.

SSCP analysis and sequencing. Cloned viroid DNAs were recovered from the plasmids by PCR amplification using the same conditions described above and subjected to SSCP analysis (5). The partially denatured PCR products were subjected to 14% PAGE in TBE buffer (89 mM Tris-Borate, 2mM EDTA, pH 8.3) at 200V constant voltage. For analysis of CVd-III and CVd-IV clones, the gel dimensions were 14 × 11.5 × 0.075 cm and electrophoresis run 16 h. For analysis of CVd-II clones, the gel dimensions were 8.5 × 7 × 0.1 cm electrophoresis run for 3 h. The DNA bands were visualized by silver staining (4).

Clones showing the most frequent SSCP profiles were selected for sequencing using the ABI PRISM DNA sequencer 377 and program (Perkin-Elmer). Alignment of multiple sequences was performed using the program "ABI PRISM DNA Sequencing Analysis". The sequences were aligned with reported sequences of the same viroid using the program Clustal V (3). Secondary structure analysis was obtained with the program MFOLD (circular version) from the GCG package (16).

RESULTS

Biological indexing of PM 26-X and Cl 13-24 on Etrog citron, Parson's Special mandarin, and clemeline 11-20 indicated that both isolates contained viroids (Table 1). The positive reaction of PM 26X on the two cachexia indicators is compatible with the symptoms observed

TABLE 1
BIOLOGICAL INDEXING ON VIROID-SENSITIVE INDICATORS AND IDENTIFICATION OF VIROID SPECIES.

	Biological indexing			Citrus viroids (sPAGE, molecular hybridization)				
Isolate	Citron	Parsons Special	Clemeline 11-20	CEVd	CVd-I	CVd-II	CVd-III	CVd-IV
PM 26-X	—	+	+	—	—	+	—	+
Cl 13-24	+	—	—	—	—	+	+	—

in the rootstock of the original source tree and with the identification of CVd-II by sPAGE and molecular hybridisation analysis. Cl 13-24 induced on citron, moderate dwarfing, epinasty and petiole necrosis, characteristic of CVd-III infection which was confirmed by sPAGE and molecular hybridisation. The identification of CVd-II in isolate Cl 13-24 even in the absence of symptoms on the two cachexia indicators suggested that this isolate contains a non-cachexia variant with the characteristics of CVd-IIa (11).

When the nucleic acid preparations from inoculated citrons were subjected to retrotranscription and PCR amplification, monomeric DNAs which showed a positive hybridization against viroid specific probes were obtained (data not shown). The DNA products obtained from each isolate were ligated to suitable cloning vectors. Clones containing inserts of the expected viroid sizes were consistently obtained. SSCP analysis of the clones recovered from each isolate showed differences in the electrophoretic mobility of the ssDNAs, thus illustrating the heterogeneity of the viroids present in each isolate (Fig. 1). This analysis allowed the

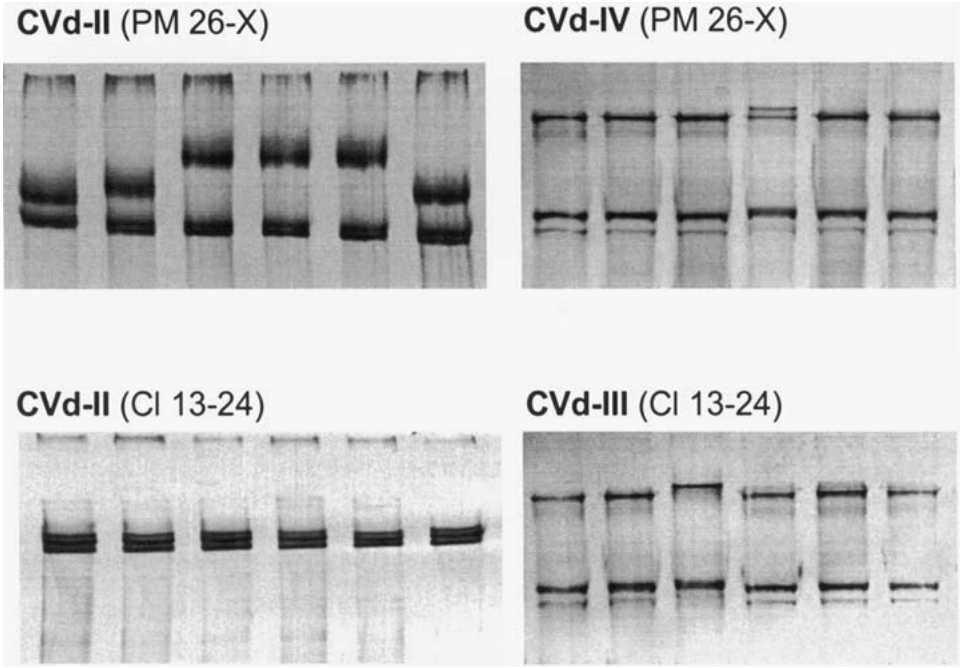

Fig. 1. Example of the SSCP analysis results of cloned monomeric Citrus viroid sequences from isolates PM 26-X and Cl 13-24.

TABLE 2
VARIABILITY OF CITRUS VIROIDS ESTIMATED BY SSCP ANALYSIS

Isolate	Viroid	Clones analyzed	SSCP profiles	Frequency of dominant profile[z]
PM 26-X	CVd-II	48	16	18/48
PM 26-X	CVd-IV	18	4	13/18
Cl 13-24	CVd-II	50	6	42/50
Cl 13-24	CVd-III	30	3	26/30

[z]Expressed as number of clones showing the most frequent profile over the total number of clones analyzed.

identification of the most frequent SSCP profile to select those clones representing the most frequent variants (master sequences) for sequencing (Table 2).

Sequence analysis of CVd-II (PM 26-X) confirmed that it contained the nucleotide motif characteristic of cachexia variants (shaded in Fig. 2), as described by Reanwarakorn and Semancik (11) for CVd-IIb and other cachexia inducing variants. Since SSCP analysis indicated that the population of CVd-II variants in PM 26-X was very heterogeneous (Table 2), additional less frequent variants were also sequenced. All of them were similar to the "master sequence" shown in Fig. 2 except for a few single nucleotide changes affecting all the regions of the secondary structure of the viroid molecule, except the central domain (data not shown).

Sequence analysis of the non-cachexia CVd-II source from Cl 12-24 showed that, from the six nucleotide changes reported to discrimi-

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CVd-IIb     1 CUGGGGAAUU CUCGAGUUGC CGCAUAAGGC AAGCAAAGAA AAAACAAGGC AGGAAGGAGA
PM 26X        .......... .......... ......-... .......... .........G CA.G..-...
Cl 13-24      .......... .......... .....G-... .......... .......... ...G......
CVd-IIa       .......... .......... .....G-... .......... .......... ...G......


CVd-IIb    61 CUUACCUGAG AAAGGAGCCC CGGGGCAACU CUUCUCAGAA UCCAGCGGG- GGCGU-GGAG
PM 26X        .......... .......... .......... .......... .......... ..........
Cl 13-24      .......... .......... .......... .......... .......A.A ..........
CVd-IIa(2)    .......... .......... .......... .......... .......A.A .....A....


CVd-IIb   121 AGAGGGCCGC GGUGCUCUGG AGUAGAGGCU -UCUGCUUCG AAACACCAUC GAUCGUCCCU
PM 26X        .......... .......... .......... .CUA...... .......... ..........
Cl 13-24      .......... .......... .......... .......... .......... ..........
CVd-IIa(2)    .......... .......... .......... UCUA...... .......... ..........


CVd-IIb   181 UCUUCUUU-A CCUCCUUCUG GCUCUUCCGA UGAGACGCGA CCGGUGGCAU CACCUCUCGG
PM 26X        .........- .......... .......... .......... .......... ..........
Cl 13-24      .........- ...U..C... .......GAG .......... .......... ..........
CVd-IIa(2)    ........U. ...U..C... .......GAG .......... .......... ..........


CVd-IIb   241 UUCGUCGUCC AACCUGC-UU UUUGUCUAUC UGAGCCUCUG CCGCGGAUCC UCUCUUGAGC CCCU
PM 26X        .---...... G......C.. .......... .......... .......... .......... ....
Cl 13-24      ......U... .......... .......... .......... .......... .......... ....
CVd-IIa(2)    ......U... .......... .......... .......... .......... .......... ....
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Fig. 2. Sequence alignment of two Citrus viroid II (CVd-II) variants against reported sequences of cachexia (CVd-IIb) and non-cachexia (CVd-IIa) variants. The characteristic six-nucleotide motif discriminating cachexia and non-cachexia variants (11) is shown shaded.

nate cachexia and non-cachexia variants, it contained only G → A, +A, C → U and U → C at positions 108, 110, 194 and 197 respectively, whereas positions 116 and 189 were identical to those of cachexia variants. As a result, the secondary structure of the V domain shares similarities with both cachexia and non-cachexia variants (Fig. 3). Sequencing of additional clones confirmed the homogeneity of this viroid source. The sequence of the infrequent variants identified by SSCP analysis contained the same composition as the master sequence (Fig. 1) in the five-six positions defined as discriminating cachexia and non-cachexia variants (7, 11).

SSCP analysis of CVd-III (Cl 13-24) and CVd-IV (PM 26-X) indicated that they were very homogeneous, observation that was confirmed by sequencing. The sequence of CVd-III was identical to that of CVd-IIIb reported by Rakowski et al., (10). This is in agreement with a previous observation showing that the sequence of CVd-IIIb was highly conserved in sources from different citrus growing areas (14). Similarly, the sequence of CVd-IV (PM 26-X) was identical to the sequence previously reported for this viroid (9).

DISCUSSION AND CONCLUSIONS

The viroid content of the two field isolates is consistent with the field symptoms and the biological indexing results. Both field isolates contained variants of CVd-II (HSVd) with a characteristic nucleotide composition in the V domain. As previously reported by Palacio and Duran-Vila (7), the isolate CVd-II (PM 26-X) is very heterogeneous and contains in the V domain the motif characteristic of cachexia inducing variants (7, 11). The non-cachexia isolate CVd-II (Cl 13-24) is more conserved and its V domain has a primary and secondary structure intermediate between those reported as being characteristic of cachexia and non-cachexia variants. This isolate contains two compensatory deletions which were reported only in cachexia variants, thus suggesting that the differences in this nucleotide motif and the pathogenicity of citrus variants of HSVd are even more subtle than initially reported (7, 11).

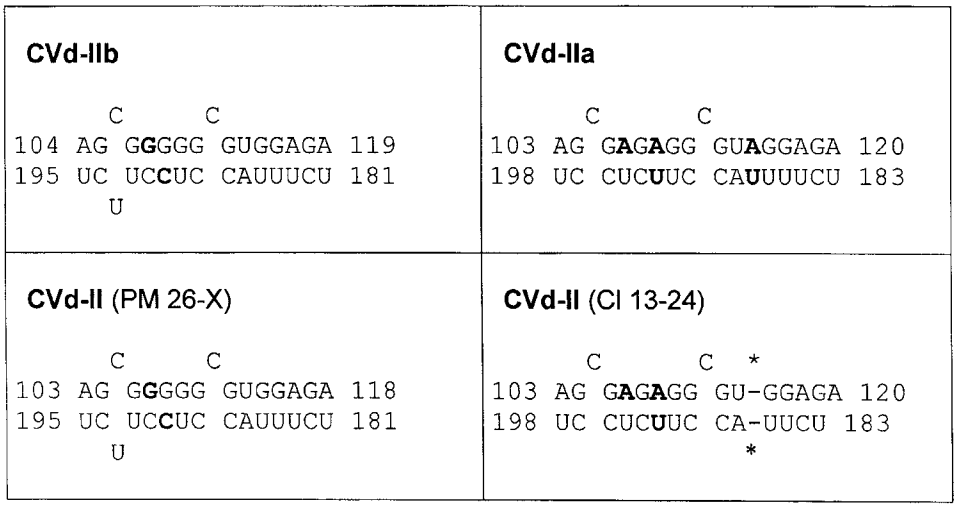

Fig. 3. Sequence and secondary structure of the V domain region in which changes (shown in bold) discriminating cachexia and non-cachexia variants are located. * = Compensatory deletions.

The sequence of CVd-III (Cl 13-24) and CVd-IV (PM 26-X) were virtually identical to those reported from other sources (9, 10) suggesting that these viroids are highly conserved.

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