Nucleotide sequence and structural features of the Group III citrus viroids

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The nucleotide sequence and secondary structure of two representative variants from the Group III citrus viroids, CVd-IIIa (297 bases) and CVd-IIIb (294 bases) were determined. The variants are related to the apple scar skin viroid (ASSVd) family. Although smaller in size than any of the ASSVd-related viroids, the central conserved region as well as most of the terminal conserved region of ASSVd is retained. The rod-like structural configuration (characteristic of ASSVd) of the variants as predicted by minimum free energy analysis is presented.

Viroids identified from species in the family Rutaceae constitute the largest collection within any single plant group. With the exception of the avocado sunblotch and Coleus blumei viroid types, every related viroid family as well as chimeric derivatives are represented in the citrus viroids.

A cataloguing of the citrus viroids on the basis of physical and biological properties led to a division into five groups (Duran-Vila et al., 1988a, b; Semancik, 1988; Semancik & Duran-Vila, 1991). In addition to the well described citrus exocortis viroid (CEVd), four distinctly different groupings of citrus viroids were identified.

A variant of CVd-Ib, when sequenced from avocado and designated as citron bent leaf viroid (CBLVd), was found to contain part of the central conserved region of the apple scar skin viroid (ASSVd) (Ashulin et al., 1991). The Group II citrus viroids, including the causal agent of citrus cachexia disease (Semancik et al., 1988), have been described as part of the hop stunt viroid family (Sano et al., 1988; Puchta et al., 1989; Yang et al., 1992; Levy & Hadidi, 1993). The relationship of CVd-IV to CEVd as a ‘recombinant’ viroid was suggested by homology between the two viroids in the central conserved and right hand regions (Puchta et al., 1991).

The Group III citrus viroids have been poorly described due to a host range exclusive to citrus and the absence of a causal relationship to any citrus diseases. Nevertheless, distinct properties including a characteristic symptom expression in citron (Citrus medica L.) of ‘leaf-drooping’ resulting from petiole bending (Duran-Vila et al., 1988a), a relatively narrow size range of 280 to 292 nucleotides as estimated by sequential polyacrylamide gel electrophoresis (sPAGE), and sequence homology detected by hybridization with specific cDNA probes have been used to demonstrate the unique aspects of the Group III citrus viroids (Duran-Vila et al., 1988b; Semancik & Duran-Vila, 1991).

This report presents the nucleotide sequences of CVd-IIIa and CVd-IIIb, the most widespread viroid variants reported in the Group III citrus viroids. Structural features common to the Group III citrus viroids and relationships to other citrus viroid groups are discussed.

In order to culture viroids, citron (Citrus medica L.) seedlings or bud propagations of the CEVd sensitive selection Arizona 861-S1 were grown under high glasshouse temperatures (40 °C) to promote symptom expression. Viroid source materials were taken from the University of California Citrus Viroid Collection. These isolates are maintained in sweet orange [Citrus sinensis (L.) Osbeck] as a symptomless carrier. Inoculation was accomplished by razor slashing citron in the presence of inoculum consisting of ethanol concentrates or 2 M-LiCl-soluble fractions from phenol extracts (Semancik et al., 1988). Citron inoculated from the sweet orange source was the starting material for the work reported here. Tip tissues from infected plants consisting of apex, young leaves and stems were homogenized and nucleic acids extracted as previously reported (Semancik et al., 1988).

Viroid RNA was detected and isolated by sPAGE (Semancik & Harper, 1984; Rivera-Bustamante et al., 1986) followed by staining with either ethidium bromide before viroid purification or silver for greater sensitivity in viroid detection. Purified viroids were obtained by electrophoretion of the band which contained the circular viroid form from denaturing gels using an IBI model UEA unidirectional electrophorator.

Sequences of RNA fragments, generated from limited digestion of gel-purified circular CVd-IIIb with RNase A, were determined by partial enzymic hydrolysis as described by Haseloff & Symons (1981). The RNA
sequence obtained permitted the synthesis of oligonucleotide primers with sufficient homology or complementarity to synthesize a double-stranded (ds) DNA product from CVd-IIIb RNA with M-MLV reverse transcriptase (RT) and PCR by the method previously described by Semancik et al. (1993). From this partial CVd-IIIb sequence, primer C-2 (5' ACTCTACCGTTCTTTACTCCA 3') complementary to the nucleotide 120 to 138 region in the final sequence (Fig. 2) with the exception of the six ‘A’ nucleotides from the 5' end, and primer H-2 (5' CTCCGCTAGTCGGAAAGACTCCGC 3') homologous to nucleotides 139 to 162 were synthesized and used to produce full-length CVd-IIIb dsDNA. Full-length cDNA clones were ligated into the dsDNA of a SmaI-digested, dephosphorylated DNA of the plasmid vector pSP72 (Promega).

The initial full-length CVd-IIIb sequence permitted the synthesis of an additional primer pair: C-3 (5' CAGTCGACGACGACAGGTAAGTTTC 3') complementary to nucleotides 70 to 92, and H-3 (CTGTCGACAGGCAGCTAAGTT 3') homologous to nucleotides 87 to 107 with the addition of two nucleotides on the 5' ends non-specific to the CVd-IIIb sequence. These primers were used to verify the viroid sequence in the region of the C-2 and H-2 primers.

The dsDNA PCR products as well as cDNA clones were sequenced using 5' end-labelled primer in the Promega fmol sequencing system. For sequencing the plasmid dsDNA, primers were used that annealed to the promoter region of the T7 and SP6 promoters within the sequence of the plasmid DNA.

Employing the [C-2,H-2] and [C-3,H-3] primers, PCR products with overlapping sequences suggested the presence of circular template molecules of the expected size for Group III viroids in extracts of infected citron and sweet orange. Three CVd-IIIb isolates (E805, 806, 807) as well as the CVd-IIa isolate (E830), all previously reported as pure isolates of Group III citrus viroids, yielded a similar size PCR product (Fig. 1).

Under the conditions described, no PCR products were obtained when extracts were used from healthy and CVd-IIa-infected citron and sweet orange (Fig. 1). With the similarity in size, the Group II citrus viroids can easily be confused with Group III viroids in sPAGE analysis. This emphasizes the importance of the oligonucleotide primers reported here with specificity for detection of Group III citrus viroids by the RT-PCR protocol.

The four reported members of the CVd-III Group were previously estimated to be in the size range 280 to 292 nucleotides by relative migration in sPAGE (Semancik & Duran-Vila, 1991). The two largest viroids in Group III, CVd-IIla and CVd-IIlb, are 297 and 294 nucleotides, respectively, as determined from fmol sequence analyses of PCR products and full-length cDNA clones of CVd-IIlb. The only exchange detected in three full-length cDNA clones of CVd-IIlb is A→G at nucleotide 203 found in one of the clones.

With the widespread occurrence of CVd-IIlb throughout citrus-growing regions of the world, this CVd-III variant was designated as the type sequence for the Group III citrus viroids. Specific differences in the sequence of CVd-IIla from CVd-IIlb are indicated in Fig. 2. The 297 nucleotide sequence of CVd-IIla is composed of 88 G (29.6%), 76 C (25.6%), 72 A (24.2%) and 61 U (20.5%) with a G+C content of 55.2%. The 294 nucleotide sequence of CVd-IIlb is composed of 86 G (29.3%), 74 C (25.2%), 72 A (24.5%) and 62 U (21.1%) with a G+C content of 54.5%. The overall sequences of CVd-IIla and CVd-IIlb share 96.6% identity. This level of relationship suggests that the two viroids sequenced here should be considered as the ‘a’ and ‘b’ variants of the single viroid, CVd-III.

The similarity of the CVd-III viroids to representative viroids from the other citrus viroid groups is presented in Table 1. Among the different viroid families or subgroups represented in the citrus viroids (Elena et al., 1991), the percentage similarity values indicate the closest relationship between the CVd-III viroids and CVd-Ib, a reported member of the ASSVd group (Ashulin et al., 1991). The percentage similarity between ASSVd and CVd-IIla (56.8%) or CVd-IIlb (57.4%) supports this relationship.

The CVd-IIIb sequence was analysed for the minimum free energy secondary structure according to the FOLD
Fig. 2. Nucleotide sequence of CVd-IIIa and CVd-IIIb presented as the rod-like secondary structure of the ASSVd group. Changes from the CVd-IIIb model are indicated in the CVd-IIIa sequence.

Table 1. Sequence homology between Group III citrus viroids and representative isolates from the other citrus viroid groups

<table>
<thead>
<tr>
<th>Citrus viroid*</th>
<th>Related viroid family</th>
<th>CVd-IIIa</th>
<th>CVd-IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEVd</td>
<td>PSTVd</td>
<td>47-3</td>
<td>43-4</td>
</tr>
<tr>
<td>CVd-1b</td>
<td>ASSVd</td>
<td>54-6</td>
<td>52-4</td>
</tr>
<tr>
<td>CVd-IIa</td>
<td>HSVd</td>
<td>34-3</td>
<td>35-8</td>
</tr>
<tr>
<td>CVd-IV</td>
<td>CEVd-chimera</td>
<td>38-4</td>
<td>40-9</td>
</tr>
</tbody>
</table>

* Nucleotide sequences were determined from isolates in the University of California-Riverside Viroid Collection from citrus.

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References


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