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The termini of a new citrus viroid contain duplications of the central conserved regions from two viroid groups

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Abstract A citrus viroid associated with dwarfing, CVdIIIA, has been sequenced and its 294 nucleotide residues can be arranged to form the typical rod-like secondary structure of other viroids with 71% of nucleotides base-paired. CVdIIIA has greatest sequence similarity with apple scar skin viroid (ASSVd; 69%) and has the central sequence which is conserved in the ASSVd group. CVdIIIA is the smallest member of the ASSVd group but contains the terminal conserved region shared by all viroids over 300 nucleotides. The two ends of CVdIIIA are highly unusual in that each end appears to be derived from the conserved central core region of a different viroid group.

Key words: Viroid; Citrus; CVdIIIA;
Conserved core sequence; Dwarfing; Nucleotide sequence

1. Introduction

Viroids are the smallest plant pathogens known. They consist of unencapsidated, circular, single-stranded RNA of 240–375 bases. Due to extensive intramolecular complementary base pairing, viroids form a rod-like structure of short helices and small internal loops. However, during thermal denaturation the rod-like native structure is disrupted and a metastable structure containing at least two large hairpins is formed [1]. Since their discovery 23 years ago [2], 21 viroids, excluding variants, have been characterised at the molecular level. They can be mechanically transmitted and multiple infections have been found in grape and citrus probably as a result of vegetative propagation in these crops. Fifteen viroid-like RNAs have been identified in citrus and placed into five groups based on infectivity of hosts, hybridization with nucleic acid probes, and estimated number of nucleotides [3]. The nucleotide sequence of at least one member of each group is known with the exception of group III which has at least four uncharacterised members. We were interested in characterising the largest member (approximately 295 nucleotides) of group III since it was strongly associated with dwarfing symptoms in citrus [4–6]. We have cloned and sequenced this viroid and in consideration of its prior classification [3] will refer to it in this report as citrus viroid group III, member A (CVdIIIA).

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Abbreviations: AGVd, Australian grapevine viroid; ASBVd, avocado sunblotch viroid; ASSVd, apple scar skin viroid; CBLVd, citrus bent leaf viroid; CBVd, *Coleus blumei* viroid; CCCVd, coconut cadang-cadang viroid; CCR, central conserved region; CEVd, citrus exocortis viroid; CLVd, *Columnea* latent viroid; CSVd, chrysanthemum stunt viroid; CTIVd, coconut tinangaja viroid; CVdIIIA, citrus viroid IIIA; CVdIV, citrus viroid IV; GYSVd1, grapevine yellow speckle viroid 1; GYSVd2, grapevine yellow speckle viroid 2; HLVd, hop latent viroid; HSVd, hop stunt viroid; PBCVd, pear blister canker viroid; PLMVd, peach latent mosaic viroid; PSTVd, potato spindle tuber viroid; TASVd, tomato apical stunt viroid; TCR, terminal conserved region; TPMVd, tomato planta macho viroid.

2. Materials and methods

2.1. Propagation of CVdIIIA

The isolate of CVdIIIA used in this study was originally isolated in New South Wales, Australia [6] from Allan Valencia and graft-inoculated to Etrog citron (*Citrus medica*). Cuttings from inoculated and non-inoculated Etrog citron plants were rooted and maintained in glasshouse conditions.

2.2. CVdIIIA extraction and purification

For viroid extraction, fresh leaf and stem tissues were frozen in liquid nitrogen, ground to powder and homogenised in an extraction medium previously described [7]. The viroid contained in the 2 M LiCl soluble fraction was concentrated by ethanol precipitation and isolated from other nucleic acids by sequential polyacrylamide gel electrophoresis (sPAGE) as described [8].

2.3. RNA sequencing

The sequence of 90% of CVdIIIA was obtained by direct RNA sequencing of fragments obtained by partial digestion with either RNase T_1 or RNase U_2 under non-denaturing conditions. The fragments were 5′-end labelled with [γ-^{32}P]ATP using polynucleotide kinase, fractionated on 8% polyacrylamide gels, eluted and sequenced [9].

2.4. Cloning and sequencing of CVdIIIA

The RNA sequence data was used to design a thirty nucleotide primer (P1) (5′-TTTCCCTTTCCCTCTCTGCGTTTT(G/A)TTTTC-3′) for first strand cDNA synthesis. After confirmation of first strand cDNA synthesis, an adjacent twenty nucleotide primer (P2) (5′-CTTACCTGTCGTCGTCGACG-3′) was used to obtain a full-length PCR product of the circular RNA CVdIIIA molecule. Primer 3 (5′-CTTTCCGACTAGCGGAGACTCTCC-3′) and Primer 4 (5′-ACTCCGTGTGGTTCCTGTGG-3′) flanking the first set of primers were used to confirm the sequence bound by the first set. The position of all these primers on the CVdIIIA molecule is shown in Fig. 1. Reverse transcription and polymerase chain reaction (RT-PCR) were carried out as described [10]. 100 ng of CVdIIIA was mixed with a 100-fold molar excess of each primer and heated to 95°C for 2 min and quickly cooled on ice. RT-PCR was carried out in 100 μl reactions containing the template and primers, 50 mM Tris-HCl (pH 8.3 at room temperature), 50 mM KCl, 2.5 units AMV reverse transcriptase (Promega), and 0.5 unit of *Taq* DNA polymerase (Promega). The mixture was incubated at 42°C for 15 min followed by a 35 cycle PCR program of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C.

PCR fragments were cloned into a T-tailed Bluescript II SK(+) plasmid (Stratagene, La Jolla, CA) prepared as described [11]. Three independent RT-PCR clones were sequenced in both directions using the dideoxynucleotide chain termination method [12] and Sequenase Quick-Denature Plasmid Sequencing Kit (US Biochemical, Cleveland, OH).

3. Results and discussion

Fig. 2A shows the nucleotide sequence of CVdIIIA. It is a circular molecule of 294 nucleotide residues consisting of 72A

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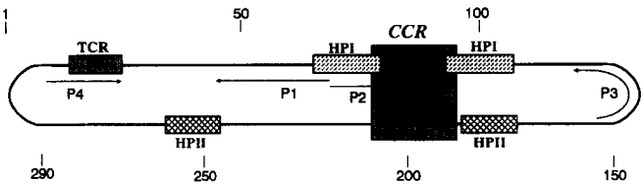

Fig. 1. The locations of conserved sequences and primers within the structure of CVdIIIA. The central conserved region (CCR), the terminal conserved region (TCR), hairpins I and II (HPI, HPII), and primer locations (P1, P2, P3, P4) are identified.

(24.5%), 74C (25.2%), 86G (29.2%) and 62U (21.1%). The secondary structure of CVdIIIA was determined with RNAFOLD (PC/GENE, Intelligenetics). The lowest ΔG structure (−167 kcal) is unbranched and linear like most other viroids. Overall, 71% of the bases are paired and consist of 61% GC, 35% AU and 4% GU pairs. The CVdIIIA sequence was analysed for the presence of open reading frames in both the plus and minus strand using AUG and GUG as possible initiation codons. The only AUG codon present is in the minus strand (residues 131–133) and is followed by 15 nucleotides before a stop codon. The largest GUG initiated reading frame in the plus strand (residues 211–48) and in the minus strand (residues 263–148) can theoretically encode peptides of 44 and 59 amino acid residues, respectively. Thus, like other viroids, CVdIIIA does not appear to encode any proteins.

Viroid classification based on conserved core sequences [13] places CVdIIIA in the ASSVd group. The CCR of CVdIIIA contains sixteen core nucleotides in the upper strand and seventeen core nucleotides in the lower strand which are identical with the core sequence of the other six members of the ASSVd group. CVdIIIA is the smallest group member but contains the highest percentage of complimentary base pairs (71% compared to 69% for AGV) with most being GC pairs (61% compared to 55% for AGV). In terms of overall sequence homology within the ASSVd group, CVdIIIA is most similar to ASSVd (69.4% identity) and least similar to GYSVd2 (56.6% identity).

The lower left terminal region of CVdIIIA contains a twenty-eight nucleotide sequence (residues 266–293) with twenty-six bases identical in sequence and located in the same relative position in ASSVd.

ASSVd group and PSTVd group viroids have inverted repeat sequences that can potentially form the stem structures of viroid hairpins I and II. Hairpin I includes the upper CCR while hairpin II includes the lower CCR in a much larger loop. Diener [14] has proposed a model in which formation of hairpin I is necessary for processing viroid replication intermediates into monomers. Owens et al. [15] and Loss et al. [16] have shown that the formation of hairpin II is necessary for infection. CVdIIIA has an inverted repeat of 12 bases, with one mismatch, that flanks the upper CCR (residues 72–83 and 96–107) and can potentially form hairpin I while an inverted repeat of 11 bases flanks the lower CCR (residues 176–186 and 245–255) and can potentially form hairpin II. The relative position of these conserved structures in CVdIIIA is shown in Fig. 1.

Viroids are known to have prominent polypurine and polypyrimidine tracts of undetermined biological significance [17]. CVdIIIA has a seventeen nucleotide cluster of polypurines starting at residue 57 (Fig. 2A). This compares with a polypurine cluster of the same length in GYSVd1 and one of fifteen nucleotides in ASSVd which are located in the same relative position. The polypurine symmetry (GGGAAAGGG-AAA) in the CVdIIIA cluster is unique. It may have resulted from a duplication of the sequence GGGAAA which is part of the extended upper CCR sequence in nine of eleven PSTVd group members but does not occur in any ASSVd group viroids.

To date, the eleven nucleotides (GGTTCCTGTGG) of the TCR [18] located in the upper left terminal region of viroids (Fig. 1) correlates with viroid size for all ASSVd group and PSTVd group viroids. Twelve viroids greater than 300 nucleotides contain the element and five viroids less than 300 nucleotides do not contain the sequence. CVdIIIA with 294 nucleotides contains the TCR and is therefore the first exception to the association of TCR with size. CVdIIIA also shares an

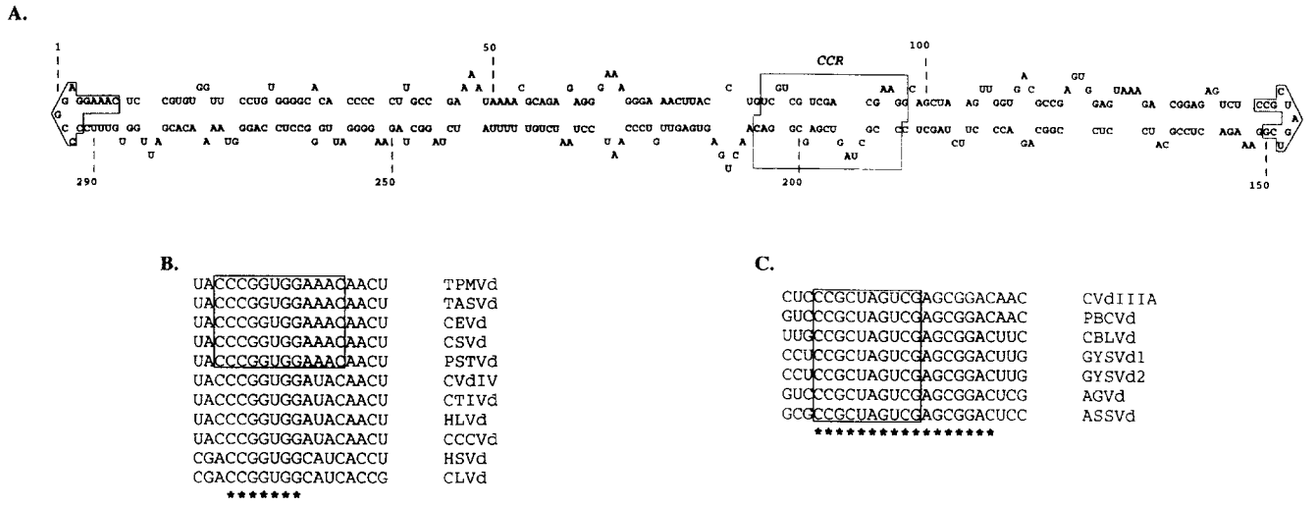

Fig. 2. (A) Nucleotide sequence and proposed secondary structure of CVdIIIA. The upper and lower strands of the CCR and important terminal sequences are outlined. (B) Sequence alignment of the lower CCR of the PSTVd group showing the seven conserved core nucleotides (*). The left-hand terminus of CVdIIIA contains a twelve nucleotide duplication, with one change (U-to-A), of the boxed area. (C) Sequence alignment of the lower CCR of the ASSVd group showing the seventeen core nucleotides (*). The right-hand terminus of CVdIIIA contains a ten nucleotide duplication of the boxed area.

additional three bases (UGU) at the 5′ end of the TCR with CBLVd, GYSVd1 and GYSVd2 which probably indicates that its TCR has a common origin with these viroids.

Keese and Symons [19] have previouly proposed that RNA exchanges between viroids occurs in modules or domains which contain both the upper and lower strands of RNA. In CVdIIIA, the extended upper CCR is most related to ASSVd with 81% of the nucleotides being identical in a forty-seven nucleotide overlap (residues 60–105). In contrast, the sequence of the extended lower CCR is most related to PBCVd with 80% of the nucleotides being identical in a thirty-nine nucleotide overlap (residues 177–211). Thus, in the case of CVdIIIA, it appears that the CCR sequences were exchanged as two individual RNA strands from two different viroids.

The sequences of the terminal regions of CVdIIIA are highly unusual in that they derive from the conserved sequences of two different CCRs. The left terminus contains twelve nucleotides (with one mismatch) from the lower CCR of the PSTVd group (Fig. 2B) and the right terminus contains ten nucleotides from the lower CCR of the ASSVd group (Fig. 2C). The upper CCR which normally base pairs with the lower CCR may, in the case of CVdIIIA, form an alternative secondary structure by base pairing with the right terminal region. This would not be as stable as the native state but may occur during replication of the RNA strand as an intermediate conformation or may be facilitated by binding of host cell proteins. Studies of other viroids have shown the ends do not fold back into a globular structure [1]. The biological significance of different conformations, if they occur, is unknown but as viroids do not encode any translation products, their capability to interact with host cell factors for replication and pathogenesis depends on their RNA structure and the ability of that structure to undergo structural transitions [1]. The single-stranded loop outs of the two termini may be prominently positioned for such putative interactions. This is the first report of a viroid containing CCR nucleotide sequences at both ends.

The ability of the CCR to form a palindromic structure [14] raises the possibility that the right terminal of CVdIIIA may base pair with the upper CCR of another CVdIIIA molecule. Alternatively, the right terminus of CVdIIIA may base pair with the sequence of the upper CCR in a CVdIIIA replication intermediate. While palindromic structures formed by intermolecular upper CCR base pairing are believed to be critical to processing of viroid replication intermediates into monomers [14] the role of such putative additional palindromes in viroid replication and processing remains unknown.

The surprising presence of a second CCR duplication in the left terminus of CVdIIIA, which contains the partial duplication of the lower PSTVd group CCR (Fig. 2B), may suggest CVdIIIA interacts with the PSTVd group of viroids or alternatively represents a link in the evolution of these viroids. The apparent flexibility observed in the sequence of CVdIIIA termini suggests CVdIIIA may be tolerent to other changes introduced through in vitro mutagenisis.

CVdIIIA is the second characterised member of the ASSVd group infecting citrus and has a history of being strongly associated with a citrus dwarfing phenotype [4–6]. It is the same size as an unidentified viroid in the Graft Transmissible Dwarfing (GTD) agent 225T [20] used in Israel to deliberately dwarf grapefruit trees for greater economy of grove management. All our glasshouse Etrog citron plants infected with CVdIIIA are dwarfed to varying degrees. The use of a descriptive viroid name linking CVdIIIA and the citrus dwarfing character would be more appropriate, but this will await the confirmation of dwarfing effects from current infectivity tests using cloned CVdIIIA inocula to rule out the presence of other viroids.

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