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## Identification and Characterization of a Variant of *Citrus viroid V* (CVd-V) in Seminole Tangelo

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**ABSTRACT.** Previous studies on *Atalantia citroides*, a citrus relative that appeared to be immune to viroid infection, revealed the existence of a new viroid, which was designated tentatively as *Citrus viroid-V* (CVd-V) and has been proposed as a new member of the genus *Apscaviroid* within the family *Pospiviroidae*. Biological indexing of a Seminole tangelo tree on the Etrog citron indicator followed by sPAGE analysis revealed the presence of a viroid that has been characterized as a new variant of CVd-V. The variant has 97.6% sequence identity with the reference sequence of CVd-V and shows three characteristics: (i) two compensatory changes that modify an A-U base pair between the upper and lower “strands” into a G-C base pair, (ii) a U→A change located in a loop that does not seem to disturb the viroid secondary structure, and (iii) two sets of changes located in the upper and lower strands that result in a rearrangement of the base pairing between the upper and lower strands. Infectivity studies performed with an artificial mutant revealed the existence of structural constraints in the region in which major differences between CVd-V and the Seminole tangelo variant were identified.

*Index words:* Citrus viroids, *Apscaviroid*, structural motifs.

Studies performed to define the response of several species of citrus and citrus related genera to viroid infection showed that *Atalantia citroides* appears to be immune to infection with CEVd, HSVd, CBLVd, CVd-III and CVd-IV (1), and highlighted the presence of a viroid not described previously (2). This new viroid, tentatively named *Citrus viroid V* (CVd-V) has a GC-rich genome of 293-294 nucleotide residues, and its predicted secondary structure contains the central conserved region (CCR) characteristic of members of the genus *Apscaviroid*, and the terminal conserved region (TCR) present in this and other genera of the family *Pospiviroidae* (10).

Viroid indexing of a Seminole tangelo tree by graft-inoculation on citron Etrog followed by sequential polyacrylamide gel electrophoresis (sPAGE) (3) revealed the presence of a viroid-like RNA with a mobility similar to the circular forms of CVd-III. Molecular hybridization assays using viroid-specific probes failed to reveal a relationship between this viroid-like RNA and any of the viroids known at that time (unpublished results). Here, we describe

the characterization of this viroid-like RNA as a sequence variant of CVd-V.

### MATERIALS AND METHODS

**Extraction procedure and viroid analysis.** Tissue samples (5 g) of young leaves (Etrog citron) or bark (Seminole tangelo) were homogenized in 5 ml of extraction buffer (0.4M Tris-HCl pH=8.9; 1% (w/v) SDS; 5mM EDTA; 4% (v/v) 2-mercaptoethanol) and water-saturated phenol, and the total nucleic acids were partitioned in 2M LiCl (9). The soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1mM MgCl<sub>2</sub> pH 7.4). Aliquots of these preparations were used for sPAGE, Northern blot hybridization and RT-PCR analysis.

For sPAGE (5%, 39:1) analysis, aliquots (20 µl equivalent to 300 mg of fresh tissue) were subjected to electrophoresis under non-denaturing conditions (2 h, 60 mA) and 8M urea denaturing conditions (4 h, 18 mA) (7) and the circular forms of the viroid were

viewed by silver staining (4). For Northern blot hybridization, the nucleic acids were electrotransferred from the second denaturing gel to positively charged Nylon membranes (Roche) at 400 mA for 1 h, using TBE buffer (40 mM Tris; 40 mM boric acid; 1 mM EDTA pH 8.3). The membranes were UV cross-linked and hybridized with a DIG-labeled CVd-V probe synthesized by PCR using as template a full-length CVd-V sequence cloned into the pBluescript II KS (+) (Stratagene) (10). Prehybridization (at 50°C for 2-4 h) and hybridization (at 50°C overnight) were performed in 50% formamide and 6×SSPE as described by Sambrook *et al.* (8). After hybridization, the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1×SSC, 0.1% SDS at 60°C for 60 min, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminescent substrate CSPD (Roche Applied Science).

**RT-PCR amplification, cloning and sequencing.** For RT-PCR, two adjacent CVd-V-specific primers of opposite polarities were used: PI (5'-CGACGAAGGCCGGTGAGCA-3') and PII(5'-GACGACAGGTGAGTACTCTCTAC-3') homologous and complementary to positions 88-107 and 64-87, respectively, of the CVd-V reference sequence (10). First-strand cDNA was synthesized at 55°C using Thermoscript reverse transcriptase (Invitrogen®). Full-length viroid DNA, was recovered performing second-strand synthesis and DNA amplification in a 50- $\mu$ l reaction containing 1.5 mM MgCl<sub>2</sub>, 0.12 mM dNTPs, 0.5  $\mu$ M of each PI and PII primers and 1 U of *Taq* DNA polymerase. The cycling profile consisted of 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 10 min. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA product. The amplification

product was ligated in the pGEM-T vector (Promega) and the recombinant plasmids were used to transform DH5 $\alpha$  *E. coli* cells. Sequencing was performed automatically with an ABI PRISM 377 apparatus (Perkin Elmer).

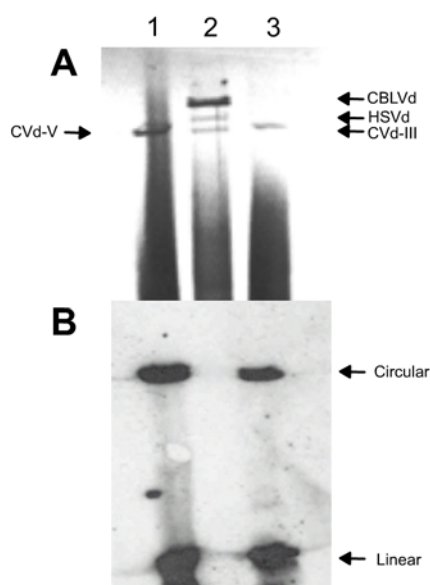
In order to verify the sequence of the primer region, a second RT-PCR reaction was performed using another set adjacent CVd-V-specific primers of opposite polarities: PIII (5'-TGTGGGTCACCCCGCCCC-3') and PIV (5'-GGAACCACAAGGTTGTTCAC-3') homologous and complementary to positions 24-41 and 4-23, respectively, of the CVd-V reference sequence. The uncloned DNA obtained was also sequenced.

**Infectivity assays with infectious clones.** Monomeric viroid-DNA inserts were recovered as blunt-end PCR products using phosphorylated primers PI and PII and *Pfu* DNA polymerase. The DNA product was subjected to ligation with 2 U of T4 DNA ligase (Gibco) and the dimeric molecules were cloned in pBluescript II KS (+) digested with *EcoRV*. Plasmids from transformed cells were sequenced to verify the desired head-to-tail orientation of the dimeric inserts. Clones with these inserts were linearized with *HindIII* and used as a template in a transcription reaction with 1 mM NTPs, 1 mM DTT and 50 U of T7 RNA polymerase to produce dimeric transcripts homologous to the viroid sequence. Three Etrog citron seedling plants were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28°-32°C.

## RESULTS

**Identification and characterization of a new variant of CVd-V in Seminole tangelo.** A Seminole tangelo tree from an orchard in Vall d'Uxó (Valencia) was selected as a candidate tree to be included in the Citrus Variety Improvement Program of Spain (CVIPS) (6) and was

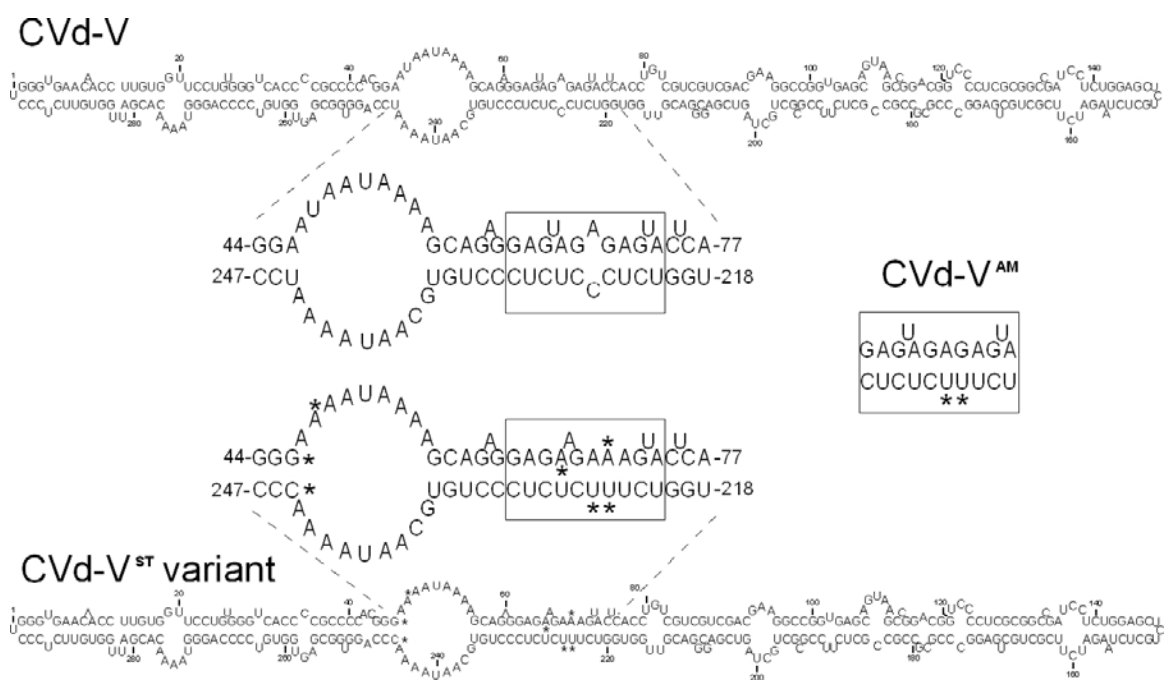
indexed on the Etrog citron indicator, followed by sequential PAGE (sPAGE) analysis for viroids. The sPAGE analysis revealed the presence of viroid-like RNA with the mobility of the circular forms of CVd-III and CVd-V (Fig. 1A). Northern blot hybridization analysis using a full-length CVd-V-specific probe revealed a relationship between the Seminole tangelo viroid-like RNA and the recently characterized CVd-V (Fig. 1B). RT-PCR analysis using two sets of CVd-V-specific primer pairs yielded a DNA of the expected size of CVd-V (data not shown).



**Fig. 1. Sequential PAGE (A) and Northern blot hybridization (B) analysis of: (1) Etrog citron infected with CVd-V; (2) Etrog citron co-infected with CBLVd, HSVd and CVd-III; (3) Etrog citron graft-inoculated with a Seminole tangelo source.**

To obtain the complete sequence of the Seminole tangelo viroid-like RNA, the amplified cDNA obtained using two adjacent primers of opposite polarity (PI and PII) was cloned in a plasmid vector.

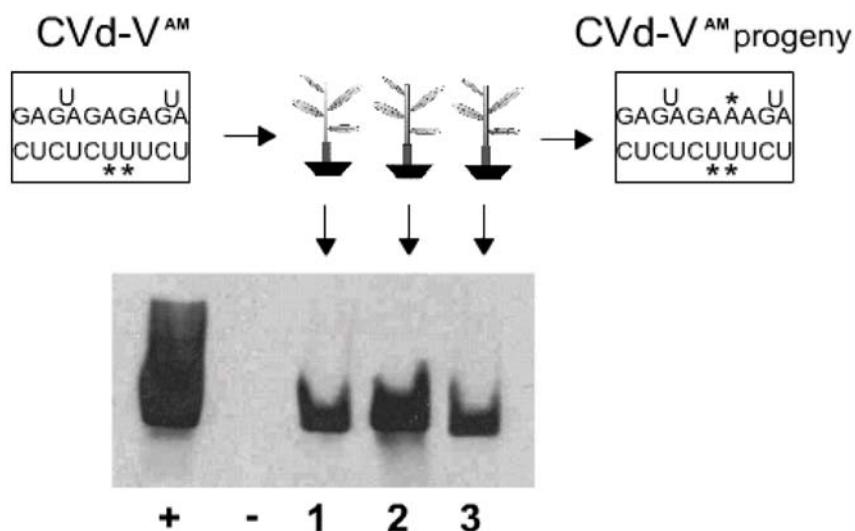
Sequencing of three recombinant plasmids showed that the viroid-like RNA was indeed a variant of CVd-V (CVd-V<sup>ST</sup>) with five changes (A46→G, U48→A, C224→U, C225→U and U245→C) located in the right hand side of its predicted rod-like secondary structure (Fig. 2). In order to confirm the sequence of the region covered by primers PI and PII, the amplified cDNA obtained using another set of adjacent primers of opposite polarity (PIII and PIV) was sequenced. The sequence unequivocally revealed two additional changes (U65→A, G69→A) located in the upper strand of the central region of the viroid secondary structure (Fig. 2). This CVd-V<sup>ST</sup> variant had a GC-rich genome of 294 nucleotide residues, a sequence identity of 97.6% with the reference sequence of CVd-V, and a predicted rod-like secondary structure of minimal free energy (Fig. 2). Two of the changes (A46→G and U245→C) were compensatory and resulted in a G-C base pair between the upper and lower “strands” instead of the A-U base pair of the CVd-V reference sequence. The U48→A change, located in a loop, did not seem to disturb the secondary structure of the viroid molecule. The four remaining changes, two in the upper strand (U65→A, G69→A) and two in the lower strand (C224→U, C→225U), resulted in a rearrangement of the base pairing of the viroid secondary structure. More specifically, the rearrangement yielded a set of five base pairs that lacked the small AC loop present in the CVd-V reference sequence (Fig. 2). The nucleotide sequence of CVd-V<sup>ST</sup> reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases (EU433392).



**Fig. 2.** Primary and proposed secondary structure of minimum free energy of the reference sequence of CVd-V and the Seminole tangelo variant CVd-V<sup>ST</sup>. The inset highlights the seven changes (\*) discriminating CVd-V and CVd-V<sup>ST</sup> and their effect on the secondary structure of the viroid. CVd-V<sup>AM</sup> shows the changes introduced in the artificial mutant used for infectivity assays.

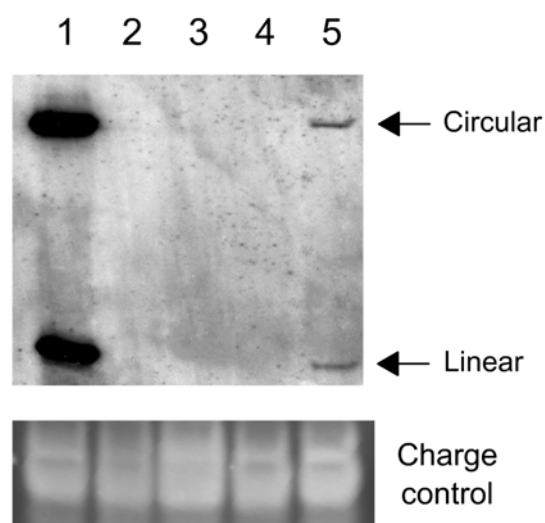
**Properties of an artificial mutant of CVd-V.** In order to establish if there was any interdependence between the two changes (U65→A, G69→A) in the upper strand and the two changes (C224→U, C→225U) of the lower strand, an artificial mutant (CVd-V<sup>AM</sup>) recovered during the cloning strategy was chosen for infectivity assays. The cloned, monomeric viroid-DNA obtained using primers PI and PII in the RT-PCR reaction possessed nucleotides U65 and G69 characteristic of CVd-V due to the composition of PII, whereas the rest of the DNA insert had the sequence of the Seminole tangelo variant CVd-V<sup>ST</sup>. To obtain infectious preparations, a head-to-tail dimeric cDNA was synthesized and used as template to

produce the corresponding *in vitro* transcripts that were inoculated mechanically to three Etrog citron seedling plants. Analysis by sPAGE and Northern-blot hybridization confirmed infection of the three plants 12 months after inoculation (Fig. 3). Infected plants displayed extremely mild symptoms. Nucleic acid preparations from each of the three infected citron plants were subjected to RT-PCR using primers PIII and PIV. Sequencing the amplified cDNA products showed that even though the inoculated artificial mutant was infectious, its progeny only retained the change introduced in position 65 whereas the change introduced in position 69 reverted (G69→A) (Fig. 3).



**Fig. 3.** Northern blot hybridization analysis of three citron seedlings inoculated with the artificial mutant CVd-V<sup>AM</sup>. Analysis of the progeny showed that only the change introduced in position 65 was retained, whereas the change introduced in position 69 reverted (G69→A).

**Availability of viroid-free Seminole tangelo.** The Seminole tangelo plant issued from the CVIPS after shoot-tip grafting *in vitro* (6) using as a source of tissue the CVd-V<sup>ST</sup> infected plant, was analyzed by Northern blot hybridization to confirm its viroid-free status. The results showed that the Seminole tangelo available in Spain (IVIA-348) is free of the CVd-V<sup>ST</sup> present in the original source (Fig. 4). It should be noted that its titer is very low compared to that of the infected Fino lemon used as a control (Fig. 4). It should be noted that the charge control that was similar in all the samples tested (Fig. 4), thus confirming that indeed CVd-V accumulates at relatively low titers in Seminole tangelo. Because of the low titer reached in Seminole tangelo and the mild symptoms induced in Etrog citron, CVd-V and its variants may be overlooked during routine viroid indexing.



**Fig. 4.** Northern blot hybridization analysis using CVd-V specific probes showing the circular and linear forms of CVd-V. (1) Fino lemon infected with CVd-V; (2) Non-inoculated Etrog citron; (3) Seminole tangelo issued from the CVIPS; (4) Etrog citron infected with CVd-III; (5) Seminole tangelo infected with variant CVd-V<sup>ST</sup>. Charge control shows the host RNAs visualized in the ethidium bromide stained gel.

## DISCUSSION

Studies performed using *Atalantia citroides*, an unusual viroid host, revealed the existence of a viroid not described earlier (2). This viroid tentatively named CVd-V has been fully characterized as a new member of the genus *Apscaviroid*. The new viroid induces mild, but characteristic, symptoms on the Etrog citron indicator, and displays synergistic effects when co-inoculated with other members (CBLVd or CVd-III) of the same genus (10). CVd-V has sequence identities with other members of the genus *Apscaviroid*, ranging from 39.1% with *Australian grapevine viroid* (AGVd) to 73.5% with *Apple scar skin viroid* (ASSVd).

Even though it was speculated that CVd-V might have emerged from recombination events (2), its origin is still uncertain. It is likely that CVd-V was present, but overlooked, in the inoculum sources of HSVd or CVd-III, since these two viroids and CVd-V having very similar electrophoretic mobility in sPAGE. The identification of CVd-V<sup>ST</sup>, a variant of CVd-V, indicates that CVd-V has not emerged recently, and that it may also be present in other citrus sources.

CVd-V and CVd-V<sup>ST</sup> have a predicted rod-like secondary structure of minimal free energy, which is characteristic of most members of the family *Pospiviroidae*. Following the general scheme for dividing the rod-like structures into five domains (5), efforts have been made to adjust newly described viroids into such schemes. However, whereas the structural and biological properties of viroids of the genus *Pospiviroid* fit the proposed scheme, viroids of other genera either present important deviations (genus *Hostuviroid*) from the scheme or insufficient information is available on a relationship between structural features and biological properties (genus *Apscaviroid*). The secondary structure of CVd-V and other members of the genus *Apscaviroid*

contains two conserved regions, the central conserved region (CCR), characteristic of members of the genus *Apscaviroid*, and the terminal conserved region (TCR), present not only in the genus *Apscaviroid*, but also in other genera of the family *Pospiviroidae* (10). In viroids of the genus *Apscaviroid*, the strictly conserved nucleotides of the upper and lower CCR strands cover relatively small stretches of 16 and 17 nucleotides, respectively, that are not located in the center of the rod-like structure. The upper strand of the CCR is flanked by inverted repeats and consequently can form a thermodynamically stable hairpin (hairpin I), characteristic of the members of the family *Pospiviroidae* (10). It should also be noted that the sequences of the upper CCR strand of CVd-V as well as of variant CVd-V<sup>ST</sup> are identical to those of the other members of the genus. However, the sequence of the lower CCR strand contains a C197→U transition that results in the change of a canonic C-G base pair between the upper and lower strands into a non-canonic G-U base pair. In addition to the fact that CVd-V and the other viroids of the genus *Apscaviroid* have their CCR displaced towards the right of the rod-like structure, the putative boundaries for the so-called pathogenic (P) and variable (V) domains have not been defined. Unfortunately, the biological properties of these viroids have been poorly studied because they are restricted to woody plants that require long-term assays, and as a consequence information regarding the relationship between structure and biology is still lacking or extremely scarce.

One of the features of the CVd-V<sup>ST</sup> variant reported here is the presence of two sets of changes (U65→A, G69→A) and (C224→U, C→225U) in the upper and lower strands that result in a rearrangement of the base pairing of the viroid secondary structure. Surprisingly, the results of infectivity assays conducted with the artificial mutant CVd-V<sup>AM</sup> with a predicted structure very similar to that of

CVd-V<sup>ST</sup>, resulted into a progeny in which one of the changes that imposed a non-canonic G-U base pair reverted to form a canonic A-U base pair. This indicates that in spite of the differences observed in the relevant region between the structures of CVd-V and the CVd-V<sup>ST</sup> variant, there are nevertheless restrictions regarding the variability of this region of the viroid molecule. It should be noted that these differences and in particular those of the upper strand, are located close to the inverted repeat on the right of the CCR involved in the formation of hairpin I. It can be speculated that there are constraints regarding the secondary or even the tertiary structure of this region.

In summary, CVd-V is a newly described citrus viroid and, as reported here, a variant of this new viroid has now been identified and characterized, suggesting that CVd-V and its variants may be more widespread than initially thought. They must be taken into consideration by those agencies involved in implementation of sanitation, variety improvement, and certification programs (6).

## ADDENDUM

Since this information was presented at the 17<sup>th</sup> IOCV Conference held in 2007, the International Committee on Taxonomy of Viruses (ICTV) has accepted some changes regarding viroid nomenclature. The new names for CVd-III and CVd-IV are *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd), respectively.

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