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# Citrus viroid V: Molecular characterization and synergistic interactions with other members of the genus *Apscaviroid*

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**Abstract**

Studies on *Atalantia citroides*, a citrus relative, revealed the existence of a viroid not described previously. The new viroid has a GC-rich genome of 293–294 nucleotides and contains the central conserved region characteristic of members of the genus *Apscaviroid*, and the terminal conserved region present in this and other genera of the family *Pospiviroidae*. The secondary structure of minimum free energy predicted for the new viroid is a rod-like conformation with 68.7% paired nucleotides and showing sequence identities with other viroids always lower than 90%, the conventional limit that separates different species within a given genus. Infectivity assays showed that the new viroid induces mild but characteristic symptoms on the indicator Etrog citron. Co-inoculation of CVd-V with either *Citrus bent leaf viroid* or *Citrus viroid III*, two other members of the genus *Apscaviroid* infecting citrus, disclosed synergistic interactions manifested in enhanced leaf symptoms and very pronounced dwarfing. Viroid titers, however, remained unaltered in co-infected plants. Possible mechanisms underlying the observed synergistic effects are discussed. According to its molecular and biological properties and its unusual ability to replicate in *A. citroides*, the new viroid, tentatively named *Citrus viroid V* (CVd-V), should be considered a new species of the genus *Apscaviroid.*



*Keywords:* Citrus viroid V (CVd-V); Citrus viroids; CBLVd; CVd-III; Synergism

## Introduction

Viroids are small, infectious, circular RNAs, replicating independently in their host plants, in some of which may incite specific disease. They are classified within two families: *Pospiviroidae*, composed of species with a central conserved region (CCR) and without hammerhead ribozymes, and *Avsunviroidae*, encompassing four species lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes. Citrus are natural hosts of five viroid species, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Citrus viroid III* (CVd-III), *Hop stunt viroid* (HSVd), and *Citrus viroid IV* (CVd-IV), all belonging to the family *Pospiviroidae* whose type member is *Potato spindle tuber viroid* (PSTVd) (Duran-Vila et al., 1988; Flores et al., 2004). CEVd, CBLVd, and CVd-III possess in the left terminal domain of their proposed rod-like secondary structure, a terminal conserved region (TCR) characteristic of species of the genera *Pospiviroid* and *Apscaviroid*, whereas HSVd and CVd-IV present a terminal conserved hairpin (TCH) characteristic of species of the genera *Hostuviroid* and *Cocadviroid* (Flores et al., 1997).

In the frame of a study aimed at defining the response to viroid infection of several species in the genus *Citrus* and in citrus-related genera, *Atalantia citroides* was identified as an unusual viroid host (Barbosa et al., 2002). *A. citroides* plants, propagated on rough lemon (*Citrus jambhiri* Lush) rootstock and graft-inoculated with an artificial mixture of viroids, appeared to be immune to infection with CEVd, CBLVd, CVd-III, HSVd, and CVd-IV. Unexpectedly, sequential PAGE (sPAGE) analysis of RNAs extracted from the inoculated *A. citroides* scion revealed

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the presence of a viroid-like RNA with an electrophoretic mobility between those of HSVd and CVd-III that was absent from the non-inoculated controls. The viroid nature of this RNA was inferred from two lines of evidence: (i) denaturing PAGE of purified preparations showed two bands with the mobilities expected for the circular and linear forms characteristic of viroid RNAs, and (ii) the same purified preparations were infectious in Etrog citron (*C. medica* L.), the classical indicator of citrus viroids. Attempts to transmit this RNA to tomato, chrysanthemum, cucumber, pepper, tobacco, *Gynura aurantiaca*, and *Tagetes patula*, all of which support the replication of different viroids, failed, suggesting a restricted host range. Riboprobes specific for CVd-III gave a weak hybridization signal whereas riboprobes specific for CEVd, CBLVd, HSVd, and CVd-IV did not hybridize. These results, together with the restricted host range, suggested that the new viroid RNA was probably a novel member of the genus *Apscaviroid* (Barbosa et al., 2005). Here we report its molecular and biological characterization.

## Results

### *Molecular characterization*

To clone and sequence the new citrus viroid, nucleic acid preparations from infected citron plants were subjected to sPAGE, and the gel-eluted circular forms were used: (i) to produce a viroid-specific probe by 5′-end $^{32}$P-labeling the RNA fragments obtained by partial hydrolysis, and (ii) as template for cDNA synthesis.

The cDNAs obtained using an RT-PCR approach that does not require prior sequence knowledge (Navarro et al., 1998) were in the range of 100–300 bp (Fig. 1A), and included cDNAs of the target viroid as confirmed by hybridization with the viroid-specific probe (Fig. 1B). The PCR-amplified products were cloned in a plasmid vector and the resulting inserts analyzed by PAGE in 5% gels (Fig. 1C). Three plasmids containing viroid-cDNA inserts were identified by hybridization with the viroid-specific probe (Fig. 1D), the sequences of which were used to determine a consensus sequence of 205 nucleotides (Fig. 2, segment in red) that by comparisons with sequences in databases showed similarities with members of the genus *Apscaviroid*. In particular, the upper and lower CCR strands characteristic of this genus could be identified. However, while the sequence of the upper CCR strand was identical to that of the other members of the genus, the sequence of the lower CCR contained a C197→U transition (pointed by an arrow in Fig. 2).

To obtain the complete sequence of the viroid, two adjacent primers of opposite polarity PI and PII were designed from the 205-nucleotide sequence, and used in an RT-PCR in which purified circular forms of the viroid were included as templates.

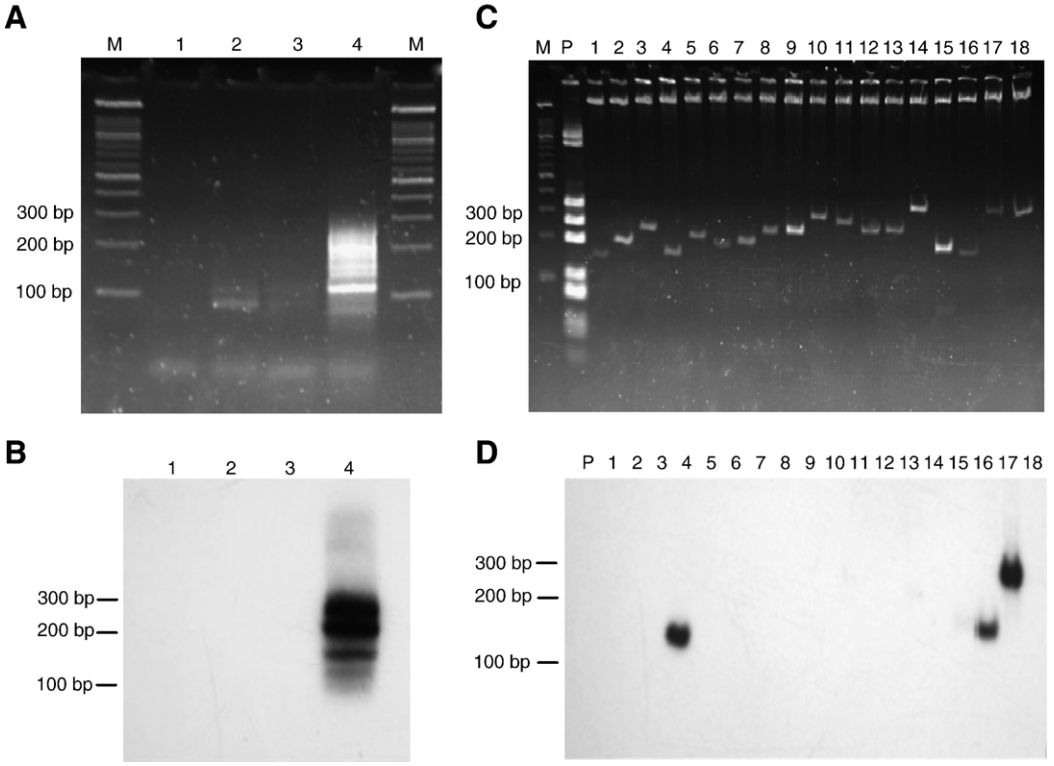


Fig. 1. (A) Analysis by PAGE and ethidium bromide staining of viroid cDNAs in the range of 100–300 nt obtained by RT-PCR following an approach that does not require prior knowledge of its sequence (Navarro et al., 1998): (1) PCR control without cDNA, (2) RT-PCR control devoid of AMV-RT, (3) RT-PCR control without RNA template, (4) RT-PCR in which all the ingredients were included, (M) 100-bp ladder. (B) Southern analysis of the viroid cDNAs electroblotted to membranes and hybridized with a viroid-specific radioactive probe synthesized by 5′-end labeling viroid RNA fragments obtained by partial hydrolysis. (C) Restriction analysis by PAGE and ethidium bromide staining of recombinant plasmids: (M) 100-bp ladder, (P) Bluescript KS+ plasmid digested with *Sau* 3A, (1 to 18) recombinant plasmids digested with *Eco*RI and *Hin*dIII. (D) Southern analysis of the plasmid inserts electroblotted to a membrane and hybridized with the viroid-specific radioactive probe.

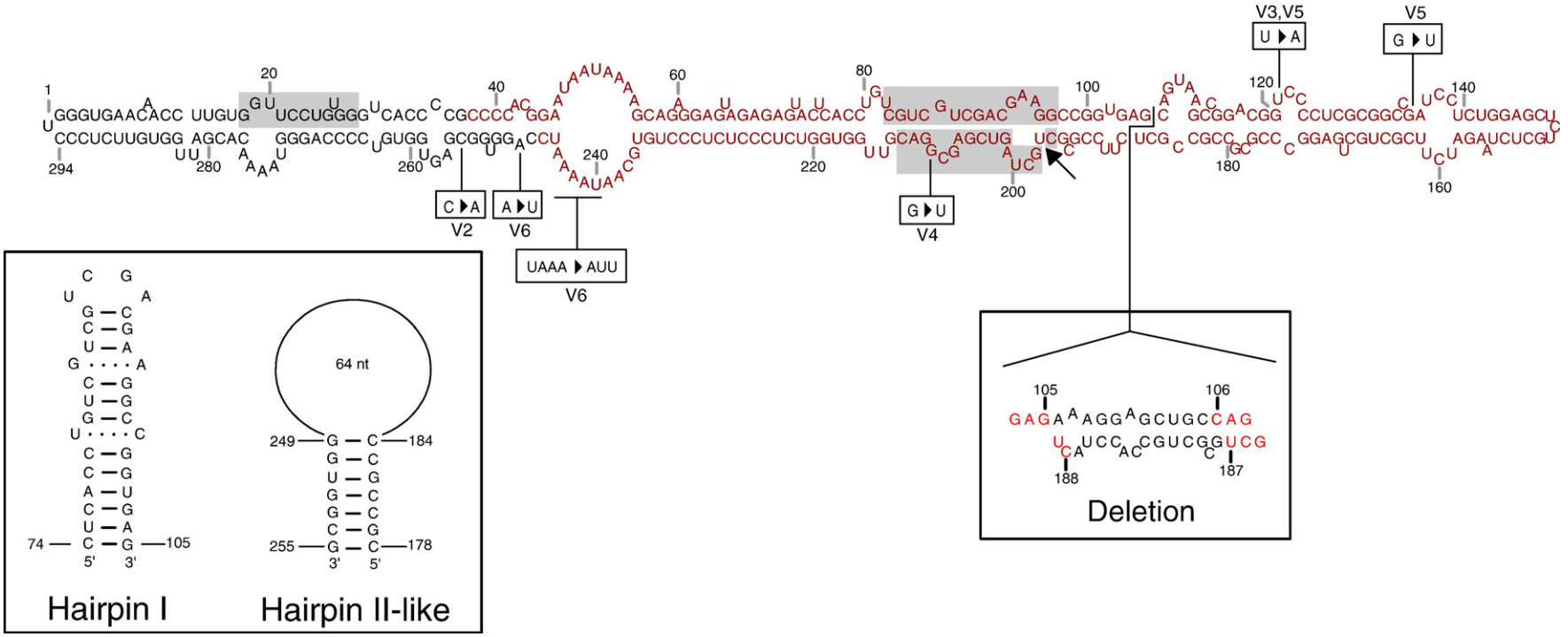


Fig. 2. Primary and proposed secondary structure of minimum free energy of variant V1 (the fragment of 205 nucleotides retrieved from the first cloning experiment is shown in red). The changes observed in five additional viroid variants (V2 to V6) are shown in boxes (V2: C254→A; V3: U121→A; V4: G209→U; V5: U121→A, G123→U; V6: UAAA[240–243]→AUU, A248→U). Variants were obtained with primers PI and PII (identical and complementary to positions 88–107 and 64–87, respectively). The sequence of the region covered by PI and PII was confirmed with additional variants obtained with primers PIII and PIV (identical and complementary to positions 24–41 and 4–23, respectively). Conserved regions (CCR and TCR) in members of the genus *Apscaviroid* are shaded. The arrow points at position 197, which is U instead of C as in all other members of the genus *Apscaviroid*. Left inset, hairpin I and hairpin II-like motifs are metastable structures that can be alternatively formed by sequences from the upper and lower strands of the rod-like structure. Right inset, fragment of ASSVd deleted in CVd-V that accounts for the difference in size between the two viroids.

The amplified cDNA product, which exhibited in non-denaturing PAGE the mobility expected for the size of the new viroid (data not shown), was cloned in a plasmid vector and 30 clones were analyzed by single-strand conformational polymorphism (SSCP). From the electrophoretic mobility of the ssDNAs, six different SSCP profiles or haplotypes were identified, one of which was clearly predominant (data not shown). Sequencing of 12 recombinant plasmids demonstrated that the population indeed contained a predominant variant (V1) of 294 nucleotides (nt) representing 80% of the overall population and five additional variants of 294 nt (V2, V3, V4, V5) and 293 nt (V6) differing from V1 by one to two changes (Fig. 2). Examination of their primary structure revealed the presence of the TCR characteristic of the genus *Apscaviroid* (Fig. 2, shaded nucleotides 18–28). To confirm the sequence of the region covered by primers PI and PII, a new RT-PCR was performed using a second pair of adjacent primers of opposite polarity (PIII and PIV) designed around the TCR. Sequencing of the inserts of four recombinant plasmids corroborated the existence of a dominant variant V1, with 88 G (29.9%), 90 C (30.6%), 53 A (18.0%), and 63 U (21.4%), having therefore a G+C content of 60.5%. All variants presented the C197→U change in the lower CCR strand (Fig. 2).

V1, selected as the reference variant of the new viroid, had a predicted rod-like secondary structure of minimal free energy (Fig. 2) with 68.7% of the nucleotides paired (71.3% G–C, 22.8% A–U and 5.9% G–U pairs). The transition C197→U in the lower CCR strand resulted in the change of a canonic base pair (G–C) between the upper and lower strands into a wobble base pair (G-U) (Fig. 2). The conserved nucleotides of the CCR upper strand and the flanking inverted repeats can form a thermodynamically stable hairpin (hairpin I in Fig. 2), which like in all members of the family *Pospiviroidae* includes a terminal tetraloop, an adjacent 3-bp stem and a long stem at the base. Its structure and nucleotide composition are identical to that proposed for the *Apple scar skin viroid* (ASSVd), the type species of the genus *Apscaviroid* (Koltunow and Rezaian, 1989; Flores et al., 1997). Moreover, the lower strand of the rod-like structure can alternatively form a stable hairpin (Fig. 2) with a GC-rich stem of 7 bp resembling the hairpin II detected, together with hairpin I, in PSTVd during thermal denaturation (Riesner et al., 1979; Loss et al., 1991).

### *Phylogenetic relationships*

Sequence alignment with the other members of the genus *Apscaviroid* revealed identities (in %) of 73.5 (ASSVd), 64.6 (*Apple dimple fruit viroid* and *Grapevine yellow speckle viroid-2*), 62.6 (CVd-III), 61.6 (*Grapevine yellow speckle viroid-1*), 59.9 (CBLVd), 49.3 (*Pear blister canker viroid*), and 39.1 (*Australian grapevine viroid*) (data not shown). These identities were always lower than 90%, the value adopted by convention to discriminate different species within a given genus. Following the nomenclature used to name citrus viroids (Duran-Vila et al., 1988), the new viroid has been tentatively designated as *Citrus viroid-V* (CVd-V) until more is known about its effects in different citrus hosts.

A consensus phylogenetic tree based on the multiple sequence alignment illustrates the relationship between CVd-V and the other members of the genus *Apscaviroid* (Fig. 3). Comparison between the primary and predicted secondary structures of CVd-V and its closest relative (ASSVd) revealed that nucleotide differences were scattered in distinct regions of the whole molecule including the lower CCR strand; whether this transversion is natural or a cloning artifact remains to be elucidated. Whereas all apscaviroids contain a U-rich segment in the lower strand of the pathogenicity domain (Koltunow and Rezaian, 1989), CVd-V has in the same region several U→A changes that result in a large loop of unpaired nucleotides (delimited by positions 46–56 and 235–245 in Fig. 2). In addition, CVd-V presents two compensatory deletions of 11 and 13 nt in the upper and lower strands of the viroid secondary structure, respectively, which account for the difference in size between ASSVd and CVd-V (Fig. 2).

### *Infectivity and symptom expression*

To obtain infectious preparations of CVd-V, a head-to-tail dimeric cDNA of the predominant variant V1 was synthesized and used as template to produce the corresponding *in vitro* transcripts that were inoculated mechanically to four citron plants. Analysis by sPAGE and Northern-blot hybridization confirmed infection of the four plants 6 months after inoculation, when no symptoms had yet developed (data not shown). However, 10 months after inoculation, the stems of the infected plants showed very small, necrotic, gum-filled lesions. The sequences of three cDNA clones, obtained by RT-PCR with primers PI and PII and viroid preparations from each of the four infected plants, were identical to that of variant V1.

To better compare the symptoms induced by CVd-V with those characteristic of the other two citrus apscaviroids (CBLVd and CVd-III), three buds from a citron plant infected with CVd-V and three buds from each of two citron plants infected with CBLVd or CVd-III, were graft-propagated on rough lemon seedlings. Three buds from a viroid-free citron plant were similarly propagated as controls. The new growing material of the grafted plants was observed over a 4-month period and showed the symptoms summarized in Table 1. CBLVd-infected citron plants presented the typical "variable syndrome" characterized by flushes of tissue showing mild leaf epinasty alternating with flushes of symptomless leaves (Fig. 4A). The

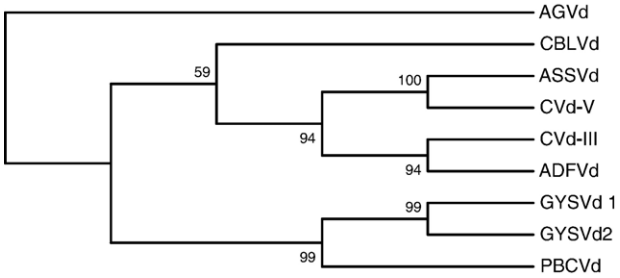


Fig. 3. Consensus phylogenetic tree (based on 10,000 bootstrap replicates) obtained for all members of the genus *Apscaviroid*. Bootstrap values (in %) are indicated in the nodes.

Table 1
Symptoms induced by citrus apscaviroids and their synergistic interactions

| Viroid | Plant growth | | | Leaf symptoms | | | Stem symptoms | | |
|---|---|---|---|---|---|---|---|---|---|
| | Variable syndrome[a] | Branching | Stunting | Midvein necrosis | Petiole necrosis | Leaf epinasty | Necrotic lesions | Cracking | Gum exudates |
| Uninfected | – | – | – | – | – | – | – | – | – |
| CBLVd | + | + | Mild | Local | – | Mild | Severe | Severe | Severe |
| CVd-III | – | – | Mild | General | + | Moderate | – | – | – |
| CVd-V | – | – | Mild | – | – | – | Mild | Mild | Mild |
| CVd-V+CBLVd | – | + | Severe | General | – | Severe | – | Severe | – |
| CVd-V+CVd-III | + | – | Severe | General | + | Severe | Moderate | – | – |

[a] Variable syndrome refers to the presence in the same shoot of flushes with leaves showing epinasty alternating with flushes of symptomless leaves, as first described by Schlemmer et al. (1985).

mild epinasty or bending of the leaves (Fig. 4B) was the result of local midvein necrosis on the underside of the leaf (Fig. 4C). The stems presented severe necrotic lesions and cracks releasing gum exudates (Figs. 4D and E). Eventually, the main shoot lost apical dominance, stopped growing, and the plants underwent an unusual branching pattern (Figs. 4A and E). CVd-III-infected citron plants presented the "dropping leaf" pattern (Fig. 4A) due to a moderate epinasty resulting from petiole and midvein necrosis (Fig. 4F) and no stem symptoms. CVd-V-infected citron plants showed the mildest symptoms, with only very small necrotic lesions and cracks, sometimes filled with gum, being observed in the stems (Fig. 4G).

*Synergistic effects with other apscaviroids*

To see the effect of CBLVd or CVd-III co-infecting CVd-V-infected plants, two additional treatments were carried out. Six buds from a CVd-V-infected citron were each graft-propagated on rough lemon seedlings and, concurrently, three of these plants were graft-inoculated with bark from a CBLVd-infected citron, and the other three with bark from a CVd-III-infected citron. Co-infection was verified by dot-blot hybridization (data not shown). Periodical examination of plant growth showed that, in contrast to the mild stunting observed in plants singly-infected with CBLVd, CVd-III or CVd-V, both sets of doubly-infected plants (CBLVd and CVd-V, and CVd-III and CVd-V) were very stunted (Fig. 4A), thus revealing a synergistic effect between CVd-V and either CBLVd or CVd-III. Fig. 5 displays data on plant growth over a 4-week period for the three sets of treatments (non-inoculated controls, infection with a single viroid and co-infection with two viroids). Comparisons of the average height values, the linear components and the quadratic components using the orthogonal contrast ANOVA confirmed that: (i) plant growth was affected as a result of viroid infection, (ii) growth of plants co-infected with two viroids were more affected than growth of plants infected with a single viroid, and (iii) plants infected with a single viroid presented similar growth patterns, and plants co-infected with two viroids also presented similar growth patterns but distinct from that of plants infected with a single viroid.

As shown in Table 1, all doubly infected plants also expressed severe epinasty (Fig. 4H) associated with multiple lesions in the midvein (Fig. 4I), a symptom not observed in plants singly-infected with CBLVd, CVd-III, or CVd-V. Regarding stem symptoms, plants co-infected with CBLVd and CVd-V presented the severe cracking characteristic of CBLVd but cracks were devoid of gum exudates (Fig. 4J), whereas plants co-infected with CVd-III and CVd-V presented necrotic lesions and the variable syndrome normally associated with CBLVd infection.

*Viroid titer remains unaffected in co-infected plants*

To find out whether there was any correlation between the dramatic effect of double infection on symptom expression and the titer of the co-infecting viroids, plants were analyzed by sPAGE and Northern-blot hybridization 6 months post inoculation. Total RNA levels in the preparations were comparable, as revealed by ethidium bromide staining of the first non-denaturing gel (Figs. 6A and A′). In plants singly infected with CBLVd or CVd-V, or co-infected with both, silver staining of the second denaturing gel showed that the titer of CBLVd was always higher than that of CVd-V (Fig. 6B), and Northern-blot hybridization with specific probes failed to detect difference in the titter of these two viroids in singly or doubly infected plants (Figs. 6C and D).

In plants infected with CVd-III or CVd-V, or co-infected with both, silver staining of the second denaturing gel showed that the titer of CVd-III was lower than that of CVd-V in singly-infected plants, but the resolution of sPAGE did not allow to discriminate between the two viroids (which moved as a single band) in the co-infected plants (Fig. 5B′). However, Northern-blot hybridization showed that the titer of each of these two viroids was the same irrespective of whether the plants where singly infected with any of these two viroids or doubly-infected with both (Figs. 5C′ and D′).

## Discussion

Citrus plants are natural hosts of several viroids, all of which belong to the family *Pospiviroidae* (Duran-Vila et al., 1988; Flores et al., 2004). Except in countries where sanitation programs have been implemented, viroids are widespread in commercial plantations that have been propagated from symptomless but viroid-infected budwood. All citrus viroids induce specific symptoms on the Etrog citron indicator but only two, CEVd and HSVd, have other sensitive citrus hosts in which they cause exocortis and cachexia diseases, respectively

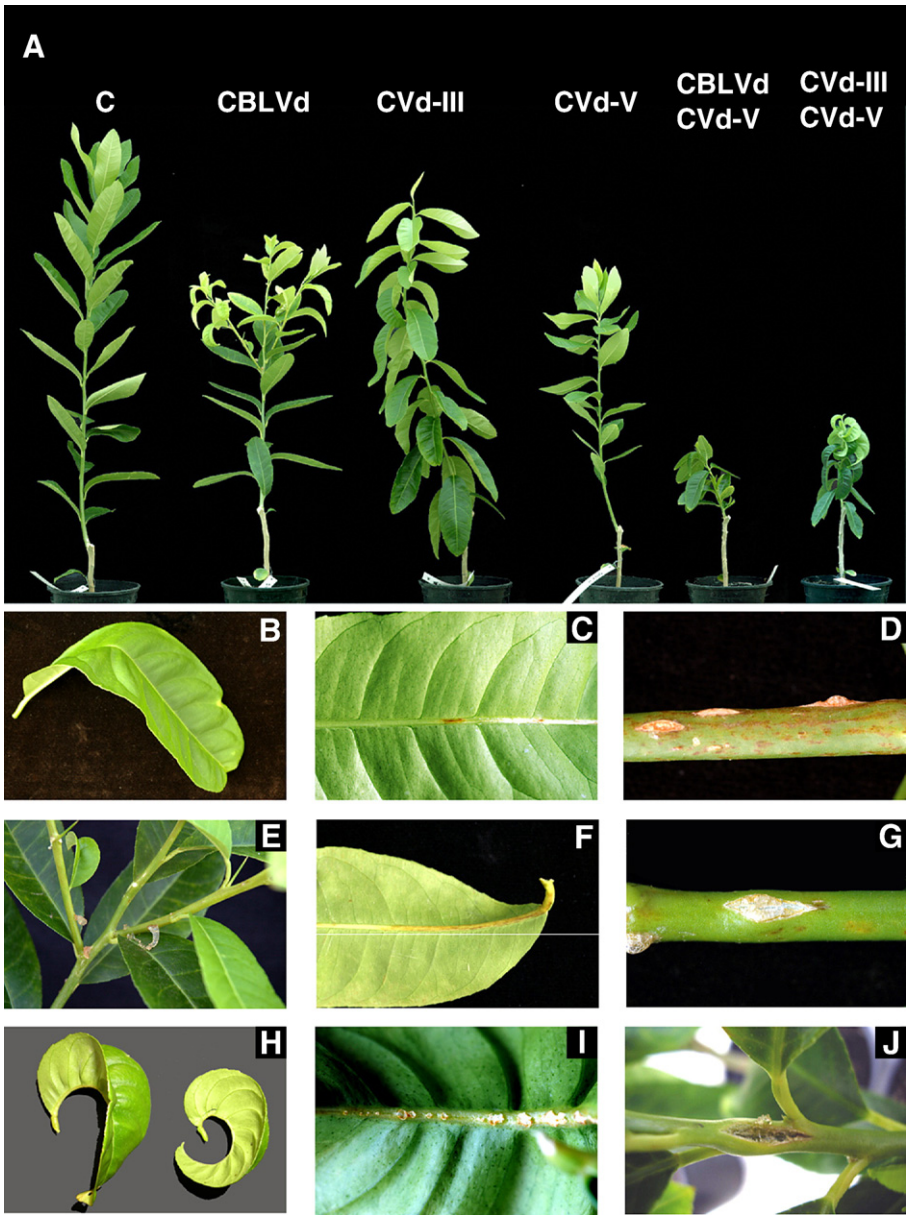


Fig. 4. Symptoms of viroid infection in Etrog citron plants, 4 months post inoculation. (A) General aspect of plants non-inoculated (c), infected with a single viroid (CBLVd, CVd-III, or CVd-V), or co-infected with two viroids (CBLVd and CVd-V; CVd-III and CVd-V). (B) Bending of the leaves of CBLVd-infected plants. (C) Local midvein necrosis on the underside of the leaf blade of CBLVd-infected plants. (D) Cracks releasing gum exudates in CBLVd-infected plants. (E) Branching pattern and gum exudates in CBLVd-infected plants. (F) Petiole and midvein necrosis of CVd-III-infected plants. (G) Cracks in the stem of CVd-V-infected plants. (H) Leaf curling of plants co-infected with CVd-V and CBLVd (left), or with CVd-V and CVd-III (right). (I) Lesions in the midvein of plants co-infected with CVd-V and CBLVd, or with CVd-V and CVd-III. (J) Severe cracks devoid of gum in plants co-infected with CVd-V and CBLVd.

(Semancik and Weathers, 1972; Semancik et al., 1988; Reanwarakorn and Semancik, 1998). Other citrus viroids, particularly CBLVd and CVd-III of the genus *Apscaviroid*, do not induce easily recognizable effects in citrus used commercially as scions or as rootstocks, but infection may cause reduction in tree size and yield (Semancik et al., 1997; Vernière et al., 2004; Vidalakis et al., 2004).

Results of a previous study revealed that *A. citroides* is an unusual viroid host because it is resistant to all previously known citrus viroids, yet capable of replicating a viroid not reported earlier (Barbosa et al., 2005). Even though the origin of this new viroid (CVd-V) is still uncertain, it was probably present, but overlooked, in the inoculum sources of HSVd or CVd-III, two viroids with very similar electrophoretic mobility in sPAGE. Molecular characterization of CVd-V showed that: (i) it contains the characteristic CCR of members of the genus *Apscaviroid*, as well as the TCR present in this and other genera of the family *Pospiviroidae*, (ii) its most stable secondary structure is a rod-like conformation with 68.7% of paired nucleotides, (iii) its sequence identity with other viroids is lower than 90%, the conventional limit used to separate different species within a given genus, and (iv) it induces

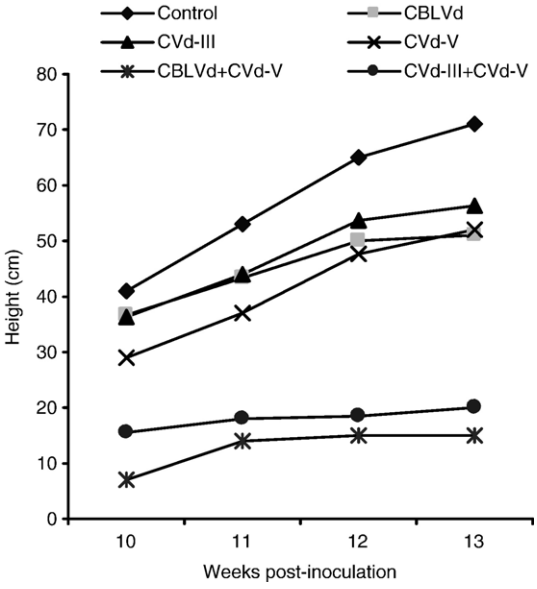


Fig. 5. Growth curves of citron plants infected with a single viroid (CBLVd, CVd-III, or CVd-V), co-infected with two viroids (CBLVd and CVd-V, CVd-III and CVd-V) and non-inoculated controls. Data are the means of the height values determined over a 4-week interval. Orthogonal contrast ANOVA revealed: (i) significant differences among treatments in the average height ($P$-value=0.0001<0.05) and the linear component ($P$-value=0.0008<0.05), but not in the quadratic component ($P$-value=0.7333>0.05). (ii) significant differences between infected and non-inoculated plants in average height ($P$-value=0.0001<0.05) and linear component ($P$-value=0.0007<0.05); (iii) significant differences between plants infected with a single viroid and plants co-infected with two viroids in average height ($P$-value=0.0001<0.05) and linear component ($P$-value=0.0005<0.05); and (iv) no significant differences in average height ($P$-value=0.3135>0.05) and linear component ($P$-value=0.2812>0.05) in the remaining variability.

specific symptoms in citron. From the above properties, we propose that CVd-V should be considered as a new species in the genus *Apscaviroid*. CVd-V propagates *in vivo* as a population of closely related variants with changes distributed throughout the viroid rod-like secondary structure, as also happens with CVd-III and CBLVd (Owens et al., 1999; Foissac and Duran-Vila, 2000). However, a unique feature of CVd-V is that, unlike the other members of the genus *Apscaviroid*, it presents in the lower CCR strand the transition C197→U 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. This change C197→U was retained in all CVd-V variants identified, therefore discarding that it could be an artifact.

Interactions between co-infecting agents, today known to be viroids, were described long ago. The best example is cross-protection, in which a plant first infected with a mild strain of a viroid and then challenge-inoculated with a severe strain of the same viroid, continues expressing only mild symptoms, with the severe strain usually accumulating to low levels. Cross-protection between mild and severe strains of PSTVd, *Peach latent mosaic viroid* (PLMVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd), was exploited for biological indexing even before viroids were discovered (Fernow, 1967; Horst, 1975; Desvignes, 1976). These early observations were extended in further studies to strains of PSTVd, CEVd, HSVd, and CChMVd (Khoury et al., 1988; Duran-Vila and Semancik, 1990; Semancik et al., 1992; De la Peña et al., 1999). In addition, cross-protection was shown to also occur between different but closely related viroids (Niblett et al., 1978; Pallás and Flores, 1989). Even though the effect is less dramatic, co-inoculations with two strains of the same viroid or with two closely related viroids also result in modulation of symptom expression and changes in the titer of one of the viroids (Branch et al., 1988; Pallás and Flores, 1989; Semancik et al., 1992; De la Peña and Flores, 2002). Although cross-protection seems to be a general phenomenon in viroids of both families, intriguingly it has never been described between members of the genus *Apscaviroid.*

In addition to cross-protection, several observations indicate the existence of a second class of interactions in plants co-infected with mixtures of distantly related viroids. They can result either in no effect or in symptoms being much more severe than those expected for purely additive effects. More specifically, enhancement of symptom expression in citron was described with viroid mixtures containing CBLVd and CVd-III (Semancik and Duran-Vila, 1991; Duran-Vila et al., 1988), but no further study of this apparently synergistic interaction was conducted. We report here a detailed examination and evaluation along time of the synergistic effects between two pairs of viroids (CBLVd and CVd-V, and CVd-III and CVd-V), manifested in enhanced leaf symptoms and pronounced dwarfing without discernible changes in viroid titers. A similar synergistic effect was observed in a separate assay in which citron plants were co-inoculated with CBLVd and CVd-III (data not shown), thus confirming previous results and showing that effects of this kind are not restricted to mixtures in which one of the components is CVd-V.

Regarding the underlying mechanism, we can only advance some ideas derived from recent studies on interactions between co-infecting viruses. RNA-mediated cross-protection between viruses has been shown to be mechanistically equivalent to post-transcriptional gene silencing (PTGS) (Ratcliff et al., 1999). This mechanism, mediated by the small interfering RNAs (siRNAs) generated by one or more dicer-like enzymes, could also operate in cross-protection between viroids, with the siRNAs from the first inoculated strain loading the RNA induced silencing complex and targeting the RNA of the challenging strain for degradation (Flores et al., 2005). Because PTGS additionally regulates plant development, and because the defensive and the developmental PTGS pathways share common components, co-infection by two distinct viruses may result in enhanced symptom expression as a result of their silencing suppressors acting at distinct sites of the RNA silencing pathways (Pruss et al., 1997; MacDiarmid, 2005). A parallel interpretation cannot be extrapolated to explain synergism between viroids because, lacking any messenger RNA activity, they do not encode silencing suppressors. However, new data indicate that a plant RNA virus suppresses RNA silencing as a consequence of sequestering for its replication enzymes involved in the biogenesis of the siRNAs and the microRNAs, the final effectors of PTGS (Takeda et al.,

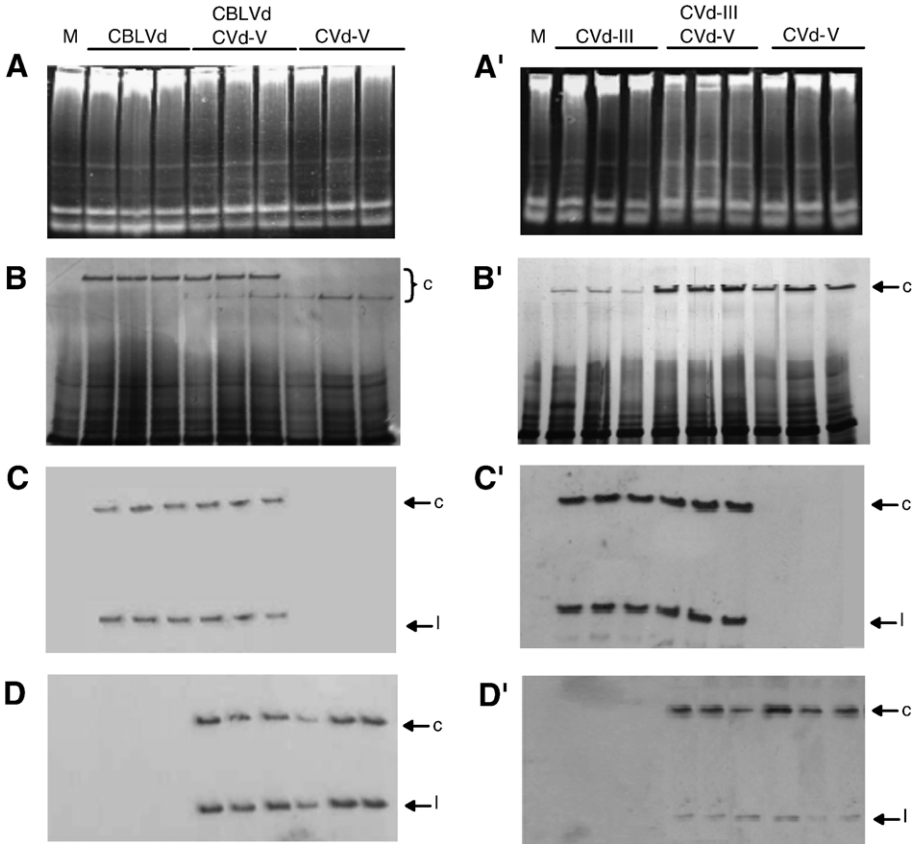


Fig. 6. Analysis by sPAGE and Northern-blot hybridization of citron plants infected with CBLVd, CVd-V, or co-infected with both (A) and (D), and citron plants infected with CVd-III, CVd-V, or co-infected with both (A′) and (D′). M, mock-inoculated control. (A and A′) Ethidium bromide staining of the first non-denaturing gel of sPAGE showing that RNA levels in all preparations were comparable. (B and B′) Silver staining of the second denaturing gel of sPAGE. (C and C′, D and D′) Northern-blot hybridizations with DIG-labeled probes specific for CBLVd (C), CVd-III (C′) and CVd-V (D and D′). Positions of the viroid circular (c) and linear (l) forma are indicated at the right.

2005). It is possible that viroids could also interfere with the RNA silencing machinery of their hosts through a similar mechanism, and that the synergistic effects between distantly related pairs of co-infecting apscaviroids could result from affecting more than one component of this machinery.

## Materials and methods

### *RNA analysis by sPAGE and purification of viroid circular forms*

Aliquots of the nucleic acid preparations from viroid-infected citrons were examined by two consecutive polyacrylamide gel electrophoreses (sequential PAGE, sPAGE), the first under non-denaturing and the second under denaturing conditions (Rivera-Bustamante et al., 1986). The denaturing gel was stained with ethidium bromide and the viroid circular forms were eluted overnight with TEP buffer (0.1 M Tris–HCl, pH 9.0, containing 0.1 M, 2-mercaptoethanol, 10 mM EDTA, and 1% SDS) in the presence of phenol/chloroform. The RNA was recovered by ethanol precipitation and resuspended in water. For analytical purposes, the denaturing gel was stained with silver (Igloi, 1983) or electroblotted to nylon membranes for Northern hybridization.

### *Northern and slot-blot hybridization*

For Northern-blot hybridization, the RNAs separated by PAGE or sPAGE were electroblotted (400 mA for 2 h) to positively charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA). RNAs were immobilized by UV cross-linking and hybridized with $^{32}$P- or DIG-labeled probes. 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. (1989). After hybridization, the membranes were washed twice in 2× 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 by autoradiography (radiolabeled probes) or with an anti-DIG alkaline phosphatase conjugate and the chemiluminescence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

### *Preparation of viroid-specific probes*

Viroid-specific probes were synthesized essentially as described earlier (Negruk et al., 1980) with minor modifications. A preliminary time-course experiment, conducted to optimize the hydrolysis treatment with deionized formamide at

100 °C prior to 5′-end labeling with polynucleotide kinase and [γ-$^{32}$P]ATP, showed that hydrolysis for 15 min yielded the highest amount of labeled full-length linear RNAs. Therefore, an aliquot of the purified viroid preparation was treated with deionized formamide at 100 °C for 15 min and, after cooling on ice, the partially hydrolyzed RNA was recovered by ethanol precipitation. RNA fragments were 5′-end labeled for 60 min at 37 °C with 10 U of T4 polynucleotide kinase, 20 U of RNase inhibitor, and 10 μCi of [γ-$^{32}$P]ATP (3000 Ci/mmol) in 20 μl of 50 mM Tris–HCl, pH 8.3, containing 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 7% glycerol, and 1 mM spermidine. The non-incorporated [γ-$^{32}$P]ATP was removed by Sephadex G-50 chromatography. DIG-labeled viroid probes were synthesized by PCR using as a template plasmids containing full-length viroid sequences (Palacio et al., 2000).

*RT-PCR amplification, cloning, and sequencing*

Viroid cDNA clones were obtained by an approach that uses minimal amounts of template and does not require prior knowledge of its sequence (Navarro et al., 1998). In brief, first strand cDNA was synthesized at 42 °C for 30 min with avian myeloblastosis virus reverse transcriptase (AMV-RT) and an oligonucleotide containing a defined sequence at its 5′ moiety and six randomized positions at its 3′ end (5′-GCCCCAT-CACTGTCTGCCCGNNNNNN-3′). Synthesis of second strand cDNA was primed by the same oligonucleotide and catalyzed by the Klenow fragment of the *Escherichia coli* DNA polymerase I at 37 °C for 30 min. The resulting DNA was subjected to PCR amplification with *Taq* DNA polymerase (Roche Applied Science) and a primer having the same sequence as that used for cDNA synthesis with the exception of the six degenerated positions at the 3′ end. The cycling profile consisted of 30 cycles of 40 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C, with an initial denaturation at 94 °C for 2 min and a final extension at 72 °C for 10 min. The PCR-amplified product was ligated in the vector pTZ57R/T (Fermentas) with protruding 3′-terminal Ts, and the recombinant plasmids were used to transform DH5α *E. coli* cells. The resulting plasmids were digested with *Eco*RI and *Hin*dIII and analyzed by PAGE in 5% gels and hybridization to identify those containing viroid-cDNA inserts.

From the consensus sequence of three partial-length viroid-cDNA clones, a pair of adjacent viroid-specific primers of opposite polarities PI (5′-TCGACGAAGGCCGGTGAGCA-3′) and PII (5′-CGACGACAGGTGAGTACTCTCTAC-3′) homologous and complementary to positions 88–107 and 64–87, respectively, of the viroid reference sequence (see Fig. 2), was designed and applied for RT-PCR amplification of the complete viroid sequence with *Pfu* DNA polymerase (Stratagene) using the same conditions described above. The amplification products were cloned in pBluescript II KS (+) (Stratagene) digested with *Eco*RV. From the sequence of the first series of full-length viroid cDNA clones, another pair of adjacent viroid-specific primers of opposite polarities PIII (5′-TGTGGGTCACCCCGCCCC-3′) and PIV (5′-GGAACCA-CAAGGTTGTTCAC-3′) homologous and complementary to positions 21–41 and 4–23, respectively, was synthesized and used for generating a second series of full-length viroid cDNA clones with the same protocol. Sequencing was performed automatically with an ABI PRISM 377 apparatus (Perkin Elmer).

*Single-strand conformation polymorphism (SSCP) analysis*

PCR amplifications of full-length viroid-cDNA clones with primers PI and PII were carried out in a final volume of 50 μl containing 4 μl of the corresponding overnight cultures. Aliquots (3 μl) of the amplified products were mixed with 2 μl of denaturing solution (90% formamide, 25 mM EDTA [pH 7], 0.05% xylene cyanol and 0.05% bromophenol blue), heated at 95 °C for 10 min, and cooled immediately on ice. The denatured DNA strands were separated by PAGE in 14% gels and visualized by silver staining (Igloi, 1983). Under these electrophoretic conditions, the DNA migrates as two partially denatured single strands (hDNA and cDNA) homologous and complementary to the viroid sequence, respectively (Palacio and Duran-Vila, 1999).

*Sequence analysis and prediction of RNA secondary structure*

Alignment of multiple sequences was performed using the program Clustal W (Thompson et al., 1994). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on 10,000 replicates. Genetic distances were calculated following the method of Jukes and Cantor (1969) after manual adjustment for keeping aligned the CCR and TCR. All these analysis were conducted using the MEGA 3.1 program (Kumar et al., 2004). The most stable secondary structure analysis was obtained with the MFOLD program (circular version) from the GCG package (Zuker, 1989), and with RNAviz program (De Rijk and De Wachter, 1997). The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases (EF617306).

*Infectivity assays*

Monomeric viroid-DNA inserts were recovered as blunt-end PCR products using phosphorylated primers PI and PII and *Pfu* DNA polymerase. The DNA products were subjected to ligation with 2 U of T4 DNA ligase (Gibco) and the dimeric molecules were cloned in pBluescript II KS (+) digested with *Eco*RV. 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 *Hin*dIII and used as a template in 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. Four Etrog citron (selection 861-S1) plants graft-propagated on rough lemon rootstocks were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28–32 °C.

Viroid infected plants were obtained with two strategies: (i) graft-propagation of buds from viroid-infected citron plants, or

(ii) graft-inoculation of bark from viroid-infected citron plants on rough lemon seedlings.

*Statistical analysis*

Plant growth data were subjected to orthogonal contrast ANOVA (Bewick et al., 2004) taking into consideration for each plant and treatment: (i) the average height values (mean of the measurements made at weakly intervals) as an indicator of plant size, (ii) the linear component that refers to the slope of the growth curve, and (iii) the quadratic component that refers to the non-linear growth. In addition, also using the orthogonal contrast ANOVA, the average height values and the linear components were compared for: (i) infected plants versus non-inoculated controls, (ii) plants infected with a single viroid versus plants co-infected with two viroids, and (iii) the remaining variability accounting for differences among plants infected with a single viroid and differences between the plants co-infected with two viroids.

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