Identification and Preliminary Characterization of a Viroid-like RNA in *Atalantia citroides*

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ABSTRACT. *Atalantia citroides* propagated on rough lemon rootstock was graft-inoculated in the scion with an artificial mixture of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). Three years later, bioassays in Etrog citron and slot-blot hybridization with viroid specific probes showed that all the inoculated viroids were detected in the rootstock but none in the scion. However, sequential polyacrylamide gel electrophoresis (sPAGE) showed the presence in the *A. citroides* scion of a viroid-like RNA with an electrophoretic mobility between HSVd and CVd-III. Further analysis by denaturing PAGE of purified preparations thereof revealed two bands with the mobility expected for the circular and linear forms of viroids. Infectivity of the viroid-like RNA was demonstrated by graft transmission to Etrog citron as well as by slash inoculation with purified preparations. Sequencing of three partial-length cDNA clones of this new RNA species, obtained by retrotranscription and PCR amplification (RT-PCR) approach that does not require prior sequence knowledge showed that the sequences corresponded to the central conserved region characteristic of members of the genus *Apscaviroid.* Complete molecular characterization is in progress.

Index words. Citrus viroids, *Apscaviroid*.

Viroids are small circular RNAs of 246-401 nt with the ability to replicate autonomously in their hosts and in most cases induce specific diseases. Citrus species are natural hosts of five viroid species, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV), all belonging to the family *Pospiviroidae*. Members of this family lack the hammerhead-mediated self-cleavage activity characteristic of members of the family *Avsunviroidae,* replicate in the nucleus following an asymmetric variant of the rolling cycle mechanism (6), and adopt a rod-like or quasi-rod-like conformation with five domains and a central conserved region (CCR). In the left terminal domain CEVd, CBLVd and CVd-III have a terminal conserved region (TCR) which is characteristic of members of the genera *Pospiviroid* and *Apscaviroid*, whereas HSVd and CVd-IV contain a terminal conserved hairpin (TCH) characteristic of members of the genera *Hostuviroid* and *Cocadviroid* (6).

CEVd and certain specific variants of HSVd are the causal agents of exocortis and cachexia diseases, respectively (3, 5, 17, 19, 22). Symptoms resulting from infection with CBLVd, CVd-III and CVd-IV have been demonstrated in Etrog citron indicators (4), trifoliate orange seedlings (21) and Clementine trees grafted on trifoliate orange (22). Effects of viroids in other *Citrus* and

related species is limited to symptoms observed on trees infected with field isolates characterized by bioassay on exocortis and cachexia indicators. In 1998, a project was initiated to study the resistance or susceptibility to viroid infection of a number of citrus genotypes kept at the Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (IVIA) (1). In this study, *Atalantia citroides* was identified as an atypical species in which none of the five co-inoculated viroids was detected but showed the presence of a new viroid-like RNA. Here we report some of its biological and molecular properties.

MATERIALS AND METHODS

Plant materials and viroid inoculation. *A. citroides* propagated on Rough lemon rootstock was graft-inoculated in the scion with an artificial mixture of CEVd, CBLVd, HSVd, CVd-III and CVd-IV maintained in Fino lemon. The Fino lemon used as inoculum had been previously infected by graft inoculation with the following viroid sources: CEVd (E-117) (9), CBLVd (variant CVd-Ia) (7), HSVd (variants CVd-IIa-117 and X-707) (15), CVd-III (variant CVd-IIIb) (7) and CVd-IV (8). Infection of *A. citroides* was assessed by sequential polyacrylamide gel electrophoresis (sPAGE) and molecular hybridization (1).

Transmission assays on Etrog citron. Etrog citron plants were propagated by grafting on Rough lemon rootstock. Transmission assays were conducted by grafting a piece of bark from the *A. citroides* scion and the rough lemon rootstock on each of three Etrog citron plants. The graft-inoculated citrons were maintained under greenhouse conditions at 28-32°C. They were observed monthly for symptoms and viroid infection was determined every 6 mo by slot-blot hybridization using DIG-labeled viroid specific probes.

Viroid detection. Tissue samples (5 g) were homogenized in 5 ml of extraction buffer (0.4 M Tris-HCl (pH 8.9), 1% (w/v) SDS, 5 mM EDTA (pH 7.0), 4% (v/v) mercaptoethanol and 15 ml of water-satured phenol) (19). Total nucleic acids were partitioned in 2 M 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). These preparations were subjected to sPAGE and/or molecular hybridization with specific viroid probes.

For sPAGE analysis, aliquots (20 µl equivalent to 300 mg of fresh tissue) were subjected to electrophoresis in 5% gels (135 × 120 × 1.5 mm, 39:1 acrylamide to bis-acrylamide) under non-denaturing (2 h, 60 mA) and denaturing conditions (4 h, 18 mA) (18). Circular viroid forms were viewed by silver staining (11).

For slot-blot hybridization, aliquots (10 µl equivalent to 150 mg of fresh tissue) were pre-treated in 6 × SSC with 8% formaldehyde for 15 min at 60°C and blotted onto positively charged nylon membranes (Roche Applied Science) using a Hybri-slot filtration manifold apparatus (BRL®). The samples were immobilized by UV cross-linking and hybridized against DIG-labeled viroid specific probes. Full length monomeric cRNA probes complementary to each viroid sequence were synthesized by a transcription with T7-RNA-polymerase in the presence of DIG-labeled UTP (14). Full-length DIG-labeled monomeric DNA probes were synthesized by PCR amplification (14). Pre-hybridization and hybridization were carried out in 50% formamide, and the DIG-labeled probes were detected with anti-DIG-alkaline phosphatase Fab fragments and visualized with the chemiluminescent substrate CSPD (Roche Applied Science). For Northern blot analysis, nucleic acids were electrotransferred from the second sPAGE gel to positively charged Nylon mem-

branes at 80 V for 1h using a modified TBE buffer (40 mM Tris, 40 mM boric acid, 1 mM EDTA, pH 8.3) for blotting. Hybridization was performed as described above.

Host range studies. Herbaceous hosts were mechanically inoculated with nucleic acid preparations from infected citron. Chrysanthemum and gynura were propagated as rooted cuttings and inoculated by stem slashing when they reached 10 cm in height. Cucumber, pepper, tomato, tobacco and *Tagetes patula* were propagated as seedlings and inoculated by stem puncture when they reached 5 cm in height. Plants were maintained in the greenhouse at 28-32°C and were tested for viroid infection six months after inoculation.

Purification of viroid circular forms and synthesis of a radiolabeled probe. Aliquots of the nucleic acid preparations from infected citrons were subjected to sPAGE. After ethidium bromide staining, the viroid circular forms were eluted 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. An aliquot of this purified preparation was treated with deionized formamide at 100°C for 15 min, cooled in ice, and then the partially hydrolyzed RNA was recovered by ethanol precipitation. The 5'-end labeling of the RNA fragments was performed for 30 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.

RT-PCR amplification, cloning and sequencing. cDNA clones were obtained by an approach that does not require prior knowledge of its sequence (13). In brief, first-strand cDNA was synthesized from purified circular forms of the target viroid with *Avian myeloblastosis virus* reverse transcriptase (AMV-RT) and an oligonucleotide containing a defined sequence at its 5' end and six degenerate nucleotides at its 3' end (5'-GCCCCATCACTGTCT-GCCCGNNNNNN-3'). Synthesis of second strand cDNA was primed by the same oligonucleotide using the Klenow fragment of the *Escherichia coli* DNA polymerase I. 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 without the six degenerate nucleotides at the 3' end. The PCR-amplified products were subjected to PAGE in 5% gels, stained with ethidium bromide and electro-blotted to nylon membranes that were hybridized at 54ºC in 50% formamide and then washed at 55°C in 0.1X SSC buffer (15 mM NaCl, 1.5mM sodium citrate, pH 7.0) containing 0.1% SDS.

The PCR-amplified product was ligated in the vector pTZ57R (Fermentas), and the recombinant plasmids were used to transform DH5α *E. coli* cells. The clones were digested with *Eco*RI and *Hind*III and analyzed by PAGE in 5% gels that were stained with ethidium bromide, electroblotted to nylon membranes and hybridized with the radiolabeled probe.

Sequence analysis. Sequencing was performed automatically with an ABI PRISM 377 apparatus (Perkin Elmer) and the sequences were aligned by using the Clustal W program (20).

RESULTS

Assessment of viroid infection on inoculated *A. citroides* and identification of a new viroid-like RNA. *A. citroides* plants inoculated with an artificial mixture of five viroid species were used as source plants for graft

transmission assays to Etrog citron indicators. Graft transmission was performed using bark tissue from the *A. citroides* scion and from the Rough lemon rootstock. The inoculated citrons were observed monthly for symptoms and tested by slot-blot hybridization with viroid-specific probes over a 24-mo period. Six months after inoculation, citrons inoculated with material from the Rough lemon rootstock developed characteristic symptoms of viroid infection. However, all five viroids were detected only after an incubation period of 24 mo (Table 1). It should be noted that three of the viroids (CBLVd, HSVd, CVd-III) were detected as early as 6 mo post-inoculation, and most by 18 mo post-inoculation in at least some plants. In contrast, no viroid was detected in citrons during the 24 mo after inoculation with material from the *A. citroides* scion (Table 1).

Although these results suggested that *A. citroides* was immune to viroid infection, sPAGE analysis of nucleic acid extracts of the citrons inoculated with material from the *A. citroides* scion revealed an RNA migrating in the gel region characteristic of circular viroid forms. Intriguingly, this band had a mobility between HSVd and CVd-III and, therefore, it did not appear to be any one of the five co-inoculated viroids (Fig. 1A). When the viroid-like RNA was eluted and electrophoresed in a denaturing gel, two bands with mobilities characteristic of the circular and linear forms of viroids were observed (Fig. 1B). When slash-inoculated on Etrog citron, the inoculated plants became infected, thus demonstrating the infectious nature of this viroid-like RNA. No infectivity was achieved in chrysanthemum, cucumber, pepper, tomato, tobacco, gynura or *T. patula* by similar mechanical transmissions.

Previous analyses by sPAGE and molecular hybridization failed to reveal the presence of viroid-like RNAs in the inoculated *A. citroides* plants 24 mo after inoculation (1). However, further sPAGE analysis of the same plants 42 mo after inoculation revealed the presence of the new viroid RNA, whereas the five inoculated viroids remained undetected. This new viroid RNA was absent in both of the *A. citroides* plants in the Germplasm Bank used as budwood sources for propagation and from the inoculum sources.

Northern-blot analysis of the new viroid RNA using CEVd, CBLVd, HSVd, CVd-III and CVd-IV specific cDNA probes yielded negative results (results using a CVd-III-specific cDNA probe are shown in Fig. 2B). CVd-III-specific cDNA probes gave a positive signal only with the positive controls (Fig 2B, lanes 1 and 3). However, a weak hybridiza-

TABLE 1
GRAFT TRANSMISSION TO ETROG CITRON OF VIROIDS FROM *ATALANTIA CITROIDES* PROPAGATED ON ROUGH LEMON

Inoculum source	Incubation (months)	Viroid detection on Etrog citron[z]				
		CEVd	CBLVd	HSVd	CVD-III	CVd-IV
A. citroides	6	- - -	- - -	- - -	- - -	- - -
	18	- - -	- - -	- - -	- - -	- - -
	24	- - -	- - -	- - -	- - -	- - -
Rough lemon	6	- - -	+++	+++	+++	- - -
	18	- - -	+++	+++	+++	- - +
	24	+++	+++	+++	+++	+++

[z]Citron plants were analyzed by slot-blot hybridization using viroid-specific probes. ---: All citrons tested negative. +++: All citrons tested positive. --+: One out of three citrons tested positive.

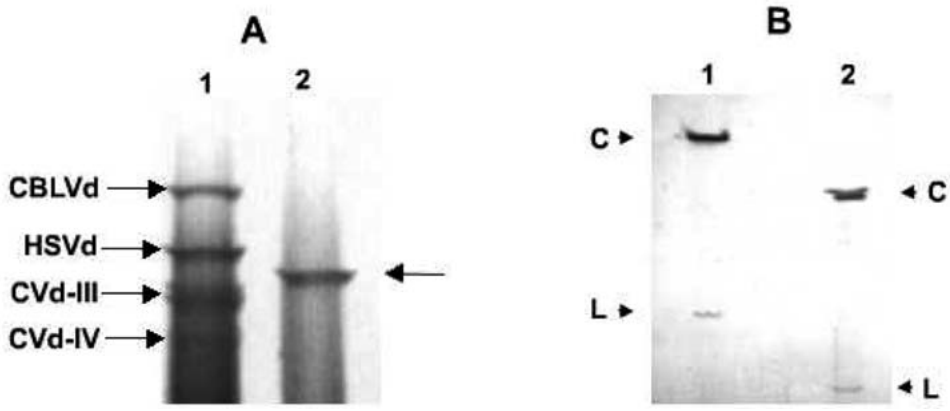

Fig. 1. A) sPAGE analysis of: (1) citron infected with CBLVd, HSVd, CVd-III and CVd-IV and (2) citron containing the viroid-like RNA identified in *Atalantia citroides*. B) Denaturing PAGE of purified preparations of the viroid-like RNA from CEVd (1) and *A. citroides* (2) showing their corresponding circular (C) and linear (L) forms.

tion signal was also observed in samples containing the new viroid RNA using a CVd-III specific cRNA probe (Fig. 2C, lanes 4 and 5). No hybridization signals were observed with CEVd, CBLVd, HSVd and CVd-IV specific cRNA probes (data not shown). Although the presence of low amounts of CVd-III contaminating the preparation of the new viroid RNA cannot be disregarded, this result suggests sequence similarity between CVd-III and the new viroid RNA.

Partial characterization of RNA of the new viroid. Purified preparations of this RNA from citron were subjected to an RT-PCR approach designed to clone small circular RNAs of unknown sequence from minimal amounts of template (13). The cDNAs obtained by applying this methodology were in the range of 100-300 nt (data not shown) and the presence of cDNAs of the target viroid was confirmed by hybridization with 5'-end labeled RNA fragments of the target viroid. The cDNAs were then cloned in a plasmid vector and several recombinant plasmids were obtained. Following hybridization, three plasmids containing viroid-cDNA inserts were identified.

With the sequences of these three inserts, a consensus sequence of 205 nt was obtained (data not shown). Alignment with the sequences of the five citrus viroids used as inoculum showed that the closest similarity was with CVd-III. Like CVd-III, the 205 nt sequence contained two segments corresponding to the upper and lower CCR strands of members of the genus *Apscaviroid*.

DISCUSSION

Previous work conducted to determine the effect of viroid infection in a number of citrus and citrus relatives showed that citrus viroids had a very wide host range but their titers varied considerably depending on specific viroid-host combinations (1). The work presented here confirms the absence of the five co-inoculated viroids in *A. citroides* suggesting that this species may be immune to viroid infection. However, the positive detection of the five viroids in the Rough lemon rootstock indicates that the inoculated viroids were able to move from the inoculation site of the *A. citroides* scion to the rootstock where they replicated and accumulated at detectable titers. The phenomenon of long-distance transport of viroids through the phloem was first estab-

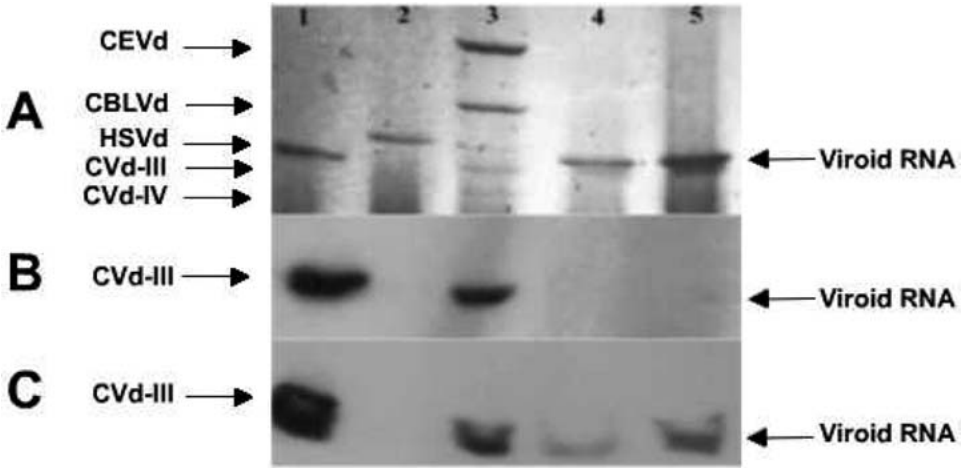

Fig. 2. A) sPAGE analysis of citron plants infected with: (1) CVd-III; (2) HSVd; (3) CEVd, CBLVd, HSVd, CVd-III and CVd-IV; (4, 5) viroid-like RNA identified in *Atalantia citroides*. B) and C) Northern blot hybridizations to viroid RNAs in (A) using a CVd-III-specific DIG-labeled cDNA probe and a CVd-III-specific DIG-labeled cRNA probe respectively.

lished for PSTVd (16). It was demonstrated that viroids move and first become detected in the shoot tip and in the leaves close to the tip and eventually down to the roots (16), probably through the formation of a ribonucleoprotein complex (10). Another important component of the viroid infection process is cell-to-cell movement via plasmodesmata which may be mediated by a specific sequence or structural motif (2). The positive detection of the five co-inoculated viroids in the Rough lemon rootstock suggests that the long-distance transport in *A. citroides* functions normally whereas the infection process via cell-to-cell movement is impaired. Additional work is being conducted to demonstrate if this genotype is actually immune to viroid infection. To our knowledge there is no information regarding the long distance movement of viroids through a non-host, however a similar situation has been described for *Citrus tristeza virus* (CTV) that is able to move with phloem flux from inoculated resistant genotypes up to a susceptible scion cultivar (12).

In spite of the evidences suggesting that *A. citroides* may be immune to the five co-inoculated viroids, the present study revealed the presence of a viroid-like RNA. This viroid RNA is infectious and able to replicate and accumulate to detectable titers in *A. citroides* as well as in the Etrog citron indicator. Like the members of the genus *Apsacaviroid* this viroid RNA has a restricted host range and some sequence identity with CVd-III. Partial sequencing confirmed that it contained two segments corresponding to the upper and lower CCR strands of members of the genus *Apscaviroid* and therefore it is probably a new member of this genus, most likely emerging from recombination events. Complete characterization of what seems to be a new viroid will allow to better trace its origin and relationships with other known viroids, and to synthesize probes for its specific detection.

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