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Short Communication

Sequence analysis of three citrus viroids infecting a single Tunisian citrus tree (Citrus, reticulata, Clementine)

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Abstract

We report the nucleotide sequences of three citrus viroids belonging to three different genera: *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus viroid-III* (CVd-III) isolated from a single natural infected *Citrus reticulata* var. *Clementine* tree growing in a tree nursery in Manouba (near Tunis Capital). We describe the sequence variability of these viroids from their natural host without using an alternative passage by an indicator host or an artificial inoculation. This work confirms that naturally occurring viroid infections contain a mixture of sequence variants. These are the first sequences of citrus viroids from Africa

Key words: CEVd, CVd-III, citrus, HSVd, quasi-species, sequence variability, viroid.

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Viroids are small, single-stranded, circular RNA molecules of about 246 to 400 nucleotides (nt) which infect higher plants and cause significant agricultural losses (see review by Flores *et al*., 2000) and are the smallest known nucleic acid-based pathogens. Sequence comparisons of naturally occurring variants of the same viroid are important for defining the conserved and variable features of the viroid genome and may indicate regions that have a role in replication or symptom expression mechanisms.

Most of the nearly 30 known viroid species belong to the family *Pospiviroidae*, the type species of which is *Potato spindle tuber viroid* (PSTVd) with five structural/functional domains: Central domain (C) containing a highly conserved region called Central Conserved Region (CCR), the pathogenic (P) domain, the variable (V) domain, the terminal left (TL) domain and the terminal right (TR) domain (Flores *et al*., 2000).

The various viroids identified in citrus plants have been grouped into four genera (*Apsacaviroid*, *Cocadviroid*, *Hostuviroid* and *Pospiviroid*) based essentially on the type of the central conserved region (CCR) of the viroid and on some biological properties. The five identified citrus viroids being: *Citrus exocortis viroid* (CEVd, a *Pospiviroid*: Semancik and Weathers, 1972); *Hop stunt viroid* (HSVd, also known as *citrus viroid II* (CVd-II), the sole *Hostuviroid*: Semancik *et al*., 1988); *Citrus bent leaf viroid* (CBLVd, also known as *citrus viroid I* (CVd-I), an *Apsacaviroid*: Ashulin *et al.*, 1991); *Citrus viroid III* (CVd-III), also an *Apsacaviroid*: Rakowski *et al*., 1994); and *Citrus viroid IV* (CVd-IV,), a *Cocadviroid*: Putcha *et al*., 1991).

Citrus exocortis, a citrus disease of worldwide distribution, could be the result of an infection either by CEVd alone or by a complex of the other citrus viroids mentioned above (Duran Vila *et al*., 1988). Infected susceptible plants show symptoms of bark scaling and general stunting. Symptoms are more frequently seen on the rootstock of susceptible species such as *Poncirus trifoliate*, citrange Troyer (*Citrus sinensis* * *Poncirus trifoliate*) Cv troyer , citrange Carrizo (*Citrus sinensis* * *Poncirus trifoliate*) Cv Carrizo and Rangpur lime (hybrid between mandarin orange and lemon) which are the widely used rootstocks. However, in the case of Tahiti lime (*Citrus latifolia* Tan) the symptoms are observed in the scion.

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Cachexia, called also xyloporosis, is a disease caused by a HSVd variant called CVd-IIb, infected susceptible plants showing symptoms of gumming, discoloration and stem pitting (Reanwarakon and Semancik, 1998). The other variant called CVd-IIa shows a 95% sequence identity with CVd-IIb (Reanwarakon and Semancik, 1999). The CVd-III variant consists of several types of sequence variants (CVd-III-a, -b, -c and -d; Rakwoski *et al*., 1994). The presence of some CVd-III variants has been shown to reduce the rate of tree growth without inducing disease symptoms. These variants show great promise as dwarfing agents (Owens *et al*., 2000).

Previous work has demonstrated viroid prevalence in Tunisian Citrus (Najar and Duran-Vila, 2004). In our present study, the sequence variability of natural variants of CEVd, HSVd and CVd-III from a single naturally infected tree were investigated. These are the first African CEVd and CVd-III sequences to be described.

The plant material used in this work consisted of leaves from a 4-year-old asymptomatic *Citrus reticulata* var. *Clementine* tree grafted on to *Citrus volkameriana* growing in a tree nursery in Manouba near Tunis capital.

Biological indexing (Roistacher *et al.*, 1977) was performed using the viroid sensitive selection 861-S1 citron grafted on Volkamer lemon (C. *volkameriana)* rootstock and Parson's Special mandarin indicator (PSM) (Semancik *et al.*, 1988). Five, one year age plants of each indicator, were graft-inoculated with the clementine source and grown for several months at 28 to 30 °C. Nine months after graft-inoculation, stunting and epinasty were observed on the Ertog citron while the Parson´s Special mandarin was asymptomatic two years after inoculations.

Total RNA from the source tree was isolated by phenol extraction and adsorption onto cellulose as previously described (Flores *et al*., 1985). Template RNAs were denatured at 95 °C for 3 min and chilled on ice for 2 min. First-strand cDNA of CEVd, HSVd and CVd-III were synthesized using the corresponding anti-sense (AS) primer (Table 1) and avian myeloblastosis virus reverse transcriptase (AMV- RT) according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). The resulting cDNA were then amplified using the polymerase chain reaction (PCR) and the same AS primer coupled with the corresponding sense (S) primer (Table 1). In the case of CEVd and HSVd the primers hybridized to the upper CCR and adjacent sequence and to the terminal left domain in the case of CVd-III. In order to decrease PCR artifacts, Pwo DNA polymerase (Roche Diagnostics) was used. The amplifications were performed according to the following program of 30 cycles: 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C followed by a terminal extension of 10 min at 72 °C. PCR products were analyzed by electrophoresis on a 2% (2 g/100 mL) agarose gel.

Since viroids are thought to be mixtures of RNA species and cannot be sequenced directly we constructed full-length cDNA copies by reverse transcription using viroid specific primers and amplified CEVd, HSVd and CVd-III using specific PCR, the DNA products being cloned into an appropriate vector. Four complete sequences were determined for each viroid. The cloning strategy has been described previously (Elleuch *et al*., 2002). Briefly, four clones of each viroid were sequenced in both directions by the dideoxynucleotide chain termination method using the M13 universal and reverse primers (T7 DNA sequencing kit, United State Biochemical, Cleveland USA). The sequences were reported to GenBank and are archived under the following accession numbers: AF540960AF 540963 for CEVd tun/cl1 CEVd tun/cl4; AF 540964 AF540967 for CVdIII tun/cl1 CVd-III tun/cl4; and AY 143167 AY 143170 for HSVd tun/cl1 HSVd tun / cl4. Sequence analysis was carried out using ClustalW software (Thompson *et al*., 1994) with minor manual adjustments to optimize sequence homology. The secondary structures of the different variants were predicted using mfold software (Zuker, 2003).

Sequence analysis shows that they were more than 90% homologous with those previously reported, which according to the criterion of the International Committee for Taxonomy of Viruses (ICTV), indicates that they are variants of the three viroids rather than novel species (Flores *et al*., 2000). Nucleotide sequence analysis showed that the size of CEVd ranged between 370 and 371 nt (Figure 1-A), HSVd between 295 and 300 nt (Figure 1-B), and CVd-III between 293 and 297 nt (Figure 1-C). Various types of modifications were detected, including transition, transversion, insertion, rearrangement and deletion. Variability was as follows: CEVd, 14 positions out of 371 (3.8%); HSVd, 24 positions out of 295

Table 1 - Reverse transcriptase PCR primer sequences used for the variant viroids CEVd, HSVd and CVd-III.

Viroid	Primer sequence and function	Position	Reference
CEVd	CEV- AS 5'CCC GGG GAT CCC TGA AGG ACT TC 3'	78-101	(Semancik *et al*., 1993)
	CEV-S 5' GGA AAC CTG GAG GAA GTC GAG G 3'	100-122	
HSVd	VP 19- AS 5'GCC CCG GGG CTC CTT TCT CAG GTA AG 3'	60 -85	(Kofalvi *et al*., 1997)
	VP 20- S 5'C CCG GGG CAA CTC TTC TCA GAA TCC3'	78-102	
CVd-III	CVIII - AS 5'GG GGA AAC ACC AAT CGT G UG 3'	276-295	Our present paper
	CVIII- S 5'GGA GGA AAC TCC GTG TGG TTC 3'	1- 21	

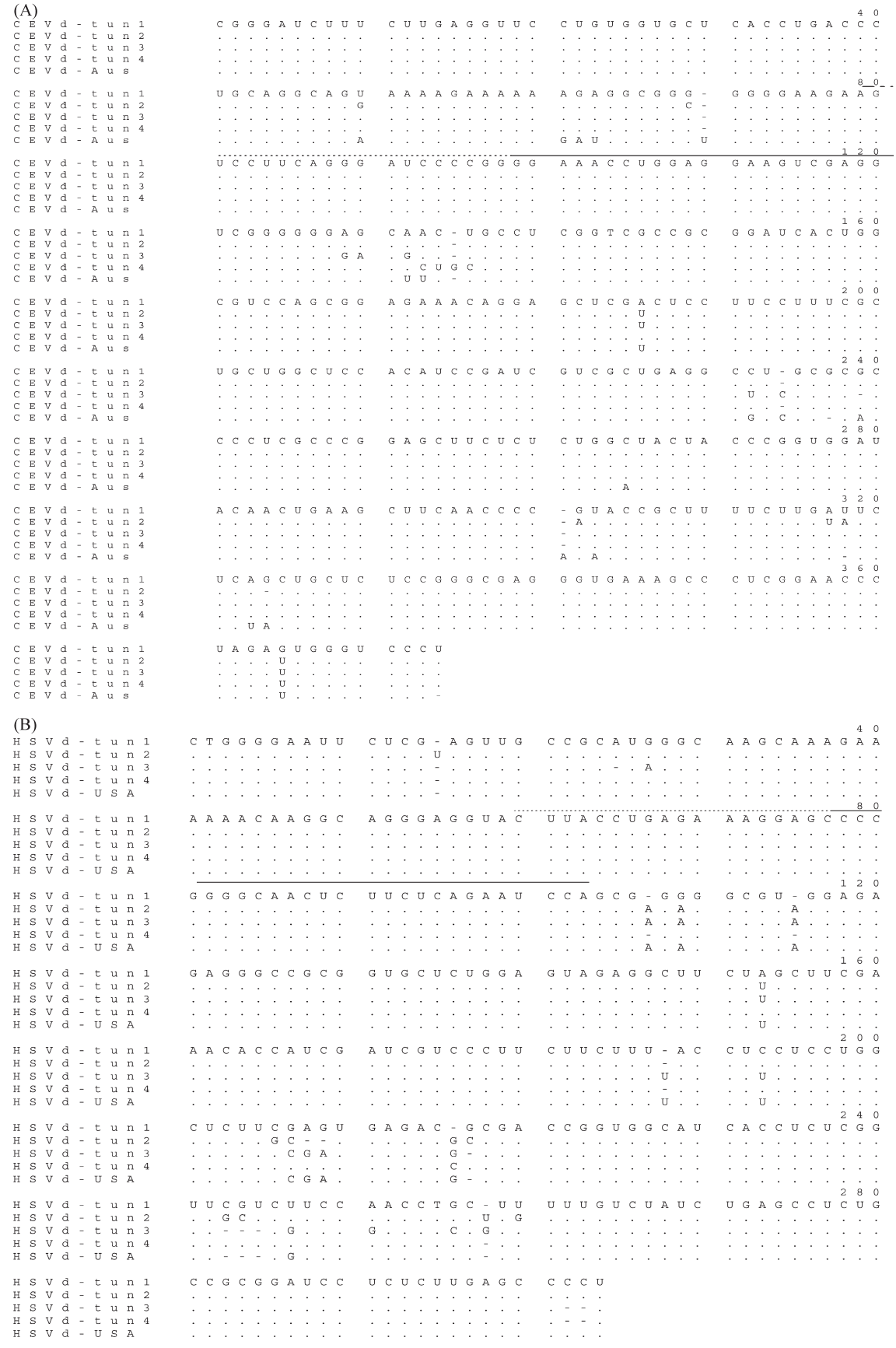

Figure 1 - (A) Alignment of the CEVd variants from Tunisian *clementinier* cultivars and the Australian sequence reported by Visvader *et al*. (1982). (B) Alignment of the HSVd variants from Tunisian *clementinier* cultivars and the sequence from the USA reported by Reanwarakron and Semancik (1998).

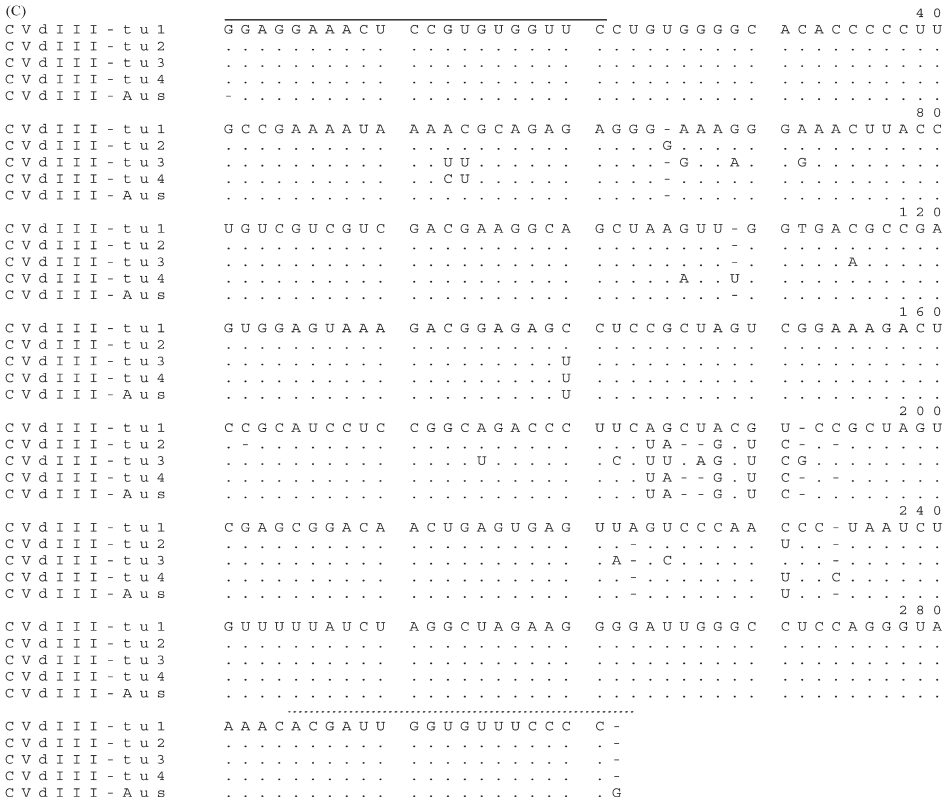

Figure 1 (cont) - C) Alignment of the CVd-III variants from Tunisia *clementinier* cultivars and the Australian sequence reported by Rakowski *et al*., 1994). Dots denote identity and slashes indicate deletions. Antisense primer sequences are indicated by dashed lines while the sense primer sequences are indicated by solid lines.

(8.1%); and CVd-III, 26 positions out of 297 (8.8%). The CVd-III sequences were somewhat more variable than those of HSVd and CEVd.

We observed that the Tunisian CEVd mutations were widespread throughout the entire RNA genome (Figure 1-A) and that the variants belonged to class B (mild) (Gross *et al*., 1982), although one variant (CEVd tun/cl2) presented a characteristic class A (severe) mutation at position 311 (G to A) (Visvader and Symons, 1985). Furthermore it has been shown by Vernière *et al*., (2004) that Class A and Class B strains cause severe and mild exocortis symptoms in clementine trees grafted onto *P. trifoliate*. However, since our clementine source tree was grafted onto *C. volkameriana* we did not observed any symptoms.

In contrast, the mutations observed for the HSVd variants were clustered in the left terminal loop, the lower portion of the putative pathogenicity domain, the lower portion of the central domain, and within both strands of the variable domain. The terminal right domain of HSVd contains only one mutation. One HSVd sequences (HSVd tun/cl3) contained the cachexia pathogenicity motif defined by Reawankaron and Semancik (1998). In the case of the CVd-III variant, the mutations observed in the new sequences were essentially clustered in both strands of the variable and pathogenicity domains. We also noted that CVd III Tun/cl3 had all the characteristic mutations of the variant CVd-IIIa while the other variants were very similar to the CVd-IIIb variant.

We also compared the sequences characterized by us in this study to those already known from the subviral RNA database (Pelchat *et al*., 2003). Our novel sequences showed only minor modifications compared to those described previously. For example, our CVd-III variants showed only 6 new polymorphic positions: U or C instead of A at position 53; a G -insertion at position 64; A instead of G at position 105; a C-deletion at position 156; C instead of G at position 188; and C-deletion at position 190. Even so, our results show that CVd-III has considerably more sequence variability than previously thought (Owens *et al*., 2000). Variability remains concentrated within the lower portion of the central conserved and variable domains, but some changes affecting the right terminal loop of CVd-III were also identified. Only one change (the deletion of two C's at positions 295-296) that has never been described previously for HSVd variants was observed in HSVd tun/cl3 and HSVd tun/ cl4. This mutation may be a PCR artifact because this region, the Terminal conserved region (TCR), is known to be highly conserved. For the CEVd variants no new polymorphic positions were found.

The secondary structures of the different variants predicted using the mfold software (data not shown) showed that the most stable secondary structure of CEVd was a

classical rod-like structure for each variant while both the HSVd and CVd-III adopted cruciform structures including various additional small hairpins. The mutations in the CEVd variants do not significantly alter their model rod-like structure and consequently should not affect the general functions such as replication. In contrast, the mutations found in the HSVd and CVd-III variants have the potential to alter the conformation model and produce different branched structures for each variant.

A blast search using the NCBI server revealed that the closest sequences to the new variants reported in this work are those reported from multiple citrus infection with the ability to produce exocortis-like symptoms in the Japanese citron (*Citrus sinensis*) Cv Allanncio Belladonna and (*C limon)* Cv Feminello Apireno (accession numbers AB054592 to AB054599 for CEVd; AB0546051 to AB0546054 for HSVd; and AB054622 to AB054623 for CVd-III. See Ito *et al*., 2002). Our sequences also showed a strong homology with those reported from a study of variability of citrus viroids in Uruguay (unpublished data) (accession numbers AF458771 to AF458776 for CEVd; AF416557 to AF416554 for HSVd; and AF416552 to AF416374 for CVd-III).

Previous studies have demonstrated that CEVd, HSVd and CVd-III (Visvader et *al.*, 1985; Palacio-Bielsa *et al.*, 2004; Owens et *al.*, 2000) are present as populations in citron**.** By getting sequences of three different citrus viroids species belonging to different genera (i.e. *Apscaviroid*, *Hostuviroid* and *Pospivroid*) we confirm that the same is true for naturally infected clementine trees. Citrus viroids, like many other RNA pathogens, propagate in their hosts as populations of similar, but not identical, sequences, fitting the quasi-species concept defined by Eigen (1983).

The demonstrated sequence heterogeneity may result from co-infection and co-propagation of different variants of the viroid studied or from the accumulation of mutants appearing *de novo* during the replication of the parental genome. This latter possibility is supported by the error-prone nature of RNA replication and by isolated case of phenotype conversion upon propagation (Domingo and Holland, 1994). It seems that CVd-III and HSVd may be more tolerant to sequence variation than CEVd, the stability of which could be due to strong structural constraints limiting variability. The lower diversity of CEVd may also be the result of a later infection with this variant compared to the other variants, resulting in less time for the generation of mutants.

The presence of a cachexia-inducing variant (HSVd Tun/cl3) in the initial source did not induce onset of the typical symptoms in the indicator host three years after inoculation. This can be interpreted by assuming that the effects of the cachexia-inducing variant can be moderated by the other accompanying HSVd variants. Therefore, by itself, the presence of a cachexia-inducing variant may not necessarily lead to the expression of symptoms, although the possibility that symptoms may eventually appear cannot be excluded.

Most viroid sequence variability studies have been performed using experimental hosts. Since the sequences reported in this paper were obtained from a tree growing in the field, without using alternative passage through an indicator host, they can be considered to reflect the diversification of the encountered viroids under similar selective pressures. The sequence variants observed could be accounted for by either a high rate of copy-error by an RNA polymerase replicating a single RNA species or by infection of one plant by several sequence variants during the propagation of citrus varieties by grafting or during normal agricultural practices such as pruning. The long potential life of citrus trees (over 60 years in the field) would allow the accumulation of sequence variants in each tree by either route.

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