Molecular Variability of *Citrus Exocortis Viroid* in a Single Naturally Infected Citrus Tree

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Abstract

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We report the nucleotide sequence of *Citrus exocortis viroid* (CEVd) from a single natural infected citrus tree of the clementinier variety *(Citrus clementina)* in Tunisia. The sequence variability of this viroid from its natural host without using an alternative passage by an indicator host or an artificial inoculation was found to occur at 14 positions, giving a variability of 3.8%. This work confirms that naturally – occurring viroids belonging to the *Pospiviroid* genera contain a mixture of sequence variants. In addition, this study confirms the worldwide distribution of this viroid.

Keywords: nucleotide sequence; CEVd; genome; quasi-species

Viroids, small, single-stranded, circular RNAs molecules (246–400 nucleotides, nt) infect higher plants and cause significant losses in agriculture (Flores *et al*. 2000). They are the smallest nucleic acid-based pathogens known. These RNA species replicate through a rolling circle mechanism involving only RNA intermediates. Viroids do not code for proteins, therefore, these infectious agents must interact directly with the host factors in order to replicate and exert their pathogenic effects. Sequence comparisons of naturally occurring variants of the same viroid are of importance in defining the conserved and variable features and may be indicative of regions that have a role in either replication or symptom expression mechanisms.

The various viroids identified in citrus plants have been grouped into four genera based essentially on the type of the central conserved region (CCR) of the viroid and on biological properties (Flores *et al*. 2000). These are *Citrus exocortis viroid* (CEVd) (Semancik & Weathers 1972), *Hop stunt viroid* (HSVd) also known as citrus viroid II (CVd-II) (Semancik *et al*. 1988), *Citrus bent leaf viroid*, also known as citrus viroid I (CVd-I) (Ashulin *et al*. 1991), citrus viroid III (CVd-III) (Rakowski *et al*. 1994) and citrus viroid IV (CVd-IV) (Putcha *et al*.

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1991). CEVd belongs to *Pospiviroid* genus and its type species is PSTVd (*Potato spindle tuber viroid*). HSVd is the only member of *Hostuviroid* genus. Both the CVd-I and CVd-III belong to *Apsacaviroid* genus for which the type species is *Apple scar skin viroid* (ASSVd). Finally, the CVd-IV belongs to *Cocadviroid* genus for which the type species is CCCVd (*Coconut cadang-cadang viroid*).

Citrus exocortis, a worldwide distributed disease, could be the result of 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 on the rootstock and general stunting.

Until now the characterisation of the primary structure of most viroids, including those in citrus, has been accomplished after their purification from alternative hosts which bioamplify them providing high titres (Gross *et al*. 1982; Semancik *et al*. 1988). Since the viroid population of a given isolate can be modified as a result of host and tissue selection, its biological and molecular properties may also vary depending on the host chosen for its characterisation (Semancik *et al*. 1993). In the present study, the genetic variability of CEVd in a single infected field tree from the citrus cultivar clementinier (*Citrus reticulata*) was investigated by sequencing cloned cDNA copies of natural isolates. These are the first sequences of CEVd from the African continent. One question raised by this work is whether the observed genetic variability is due to the host (the indicator host), or to the viroid nature. Viroids offer unique opportunity for this type of study because of their small size allowing the complete analysis of their genomic full-length cDNA clones.

MATERIALS AND METHODS

Plant material. Only one infected citrus tree was used in this work. The vegetable material consisted of leaves from a 4 years old tree of the clementinier variety grafted on Citrus Volkamerina. This tree showed symptoms of the exocortis disease when grafted on the commonly used indicator host Ertog Citron S-861 *(Citrus medica).*

RNA extraction. The leaves were homogenised, and total RNA was isolated by phenol extraction and adsorption onto cellulose as described previously (Flores *et al*. 1985). The resulting RNA samples were fractionated by lithium chloride precipitation, quantified by UV spectroscopy and their quality was assessed by 1% agarose gel electrophoresis.

RT-PCR amplification. Template RNAs were denatured at 95°C for 5 min and chilled on ice for 2 min. First-strand cDNA of CEVd was synthesised using the corresponding antisense primer (5′ CCC GGG GAT CCC TGA AGG ACT TC 3′) and avian myeloblastosis virus reverse transcriptase (RT) according to manufacturer's recommended protocol (Roche Diagnostics, Indianapolis, IN). The resulting cDNA were then amplified by polymerase chain reaction (PCR) using the same anti-sense primer coupled with the corresponding sense primer (5′ GGA AAC CTG GAG GAA GTC GAG G 3′). In order to avoid PCR artifacts, PWO DNA polymerase (Roche Diagnostics) was used, and other precautions, including control experiments, were performed to confirm the authenticity of the DNA products (Pelchat *et al*. 2000). The amplifications were performed on a Perkin Elmer thermal cycler according to the following program of 30 cycles: 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. Then, a final extension of 5 min at 72°C was performed. PCR products were analysed by electrophoresis on 2.0% agarose gel.

cDNA purification, cloning and sequencing. The strategy of cloning was described previously (Elleuch *et al*. 2002). Briefly, gel slices containing the full-size cDNA were isolated, extracted and precipitated with ethanol. An adenosine was then added to the 3′ ends of the PCR products using the Taq DNA polymerase so that the PCR-amplified fragments could be ligated in a 'sticky end' fashion to linearised pCR 2.1 vector with an overhanging 3′deoxythymidine (T) (TA cloning kit, Invitrogen, San Diego, CA). Recombinant clones were identified by restriction analysis releasing vector and insert fragments in the expected size. Four clones 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. Their accession numbers for CEVd tun/cl1, CEVd tun/cl2, CEVd tun/cl3 and CEVd tun/cl4 are AF540960, AF540961, AF540962, and AF540963, respectively.

Sequence analysis. Sequence analyses were carried out using ClustalW software (Thompson *et al*. 1994). The secondary structure was predicted with the aid of the *mfold* software (http://mfold2.wustl.edu/mfold/rna/form).

RESULTS

As the viroids are thought to be mixtures of RNA species, they could not be sequenced by direct methods. Therefore, in order to study the sequence variability of the citrus exocortis viroid, it was necessary to construct full-length cDNA clones from a naturally infected single citrus tree in Tunisia, i.e. from the cultivar clementinier. So, CEVd was amplified by RT-PCR, and the DNA products cloned into an appropriate vector. Finally, four complete sequences were determined.

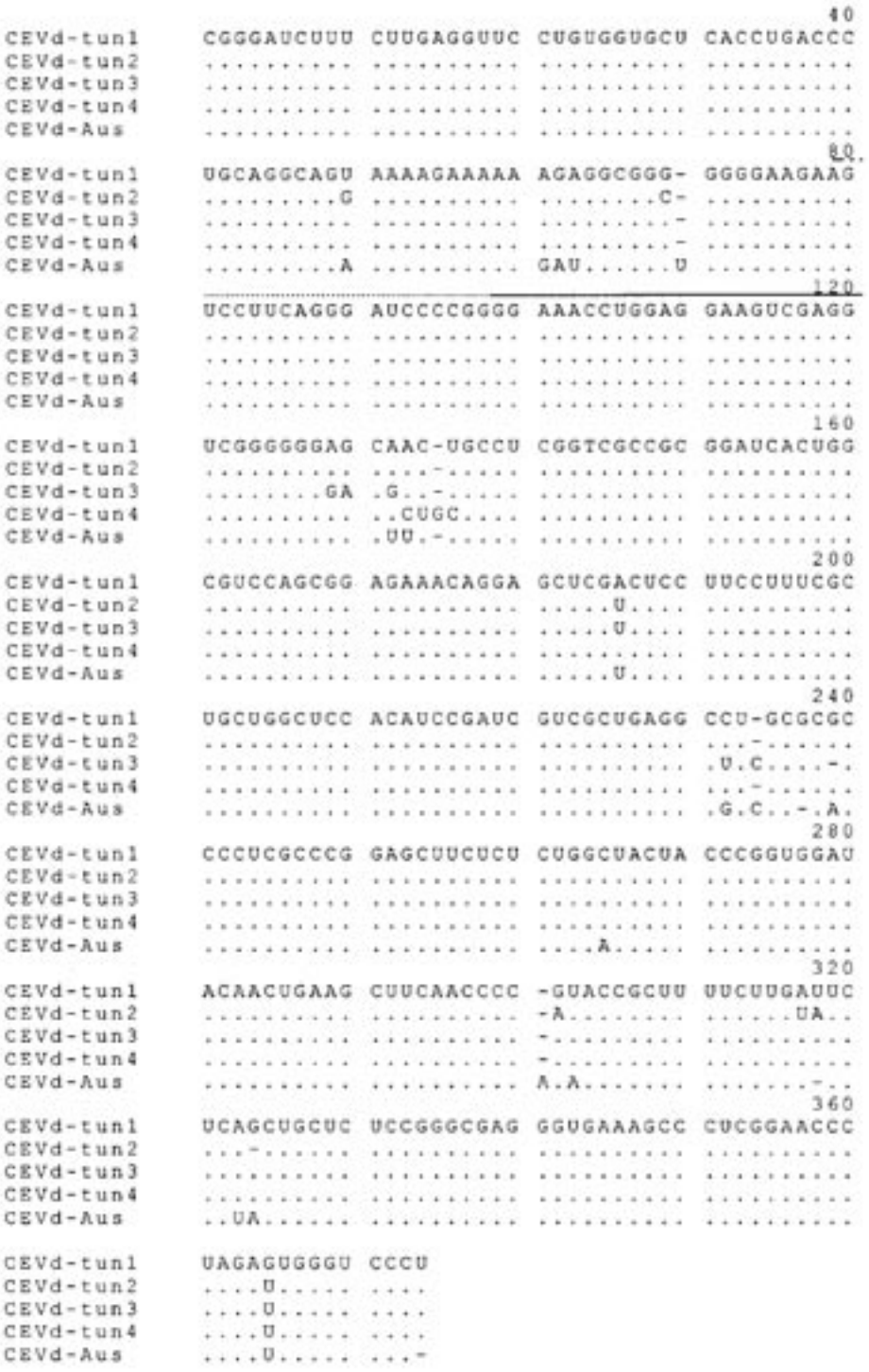

Figure 1. Sequence alignment of the CEVd variants. The sequence of the variants from the clementinier cultivar from Tunisia, and that of a sequence reported previously from Australia (Visvader & Symons 1985), are aligned

Analysis of the new sequences between themselves

In order to analyse the new nucleotide sequences, they were aligned using the ClustalW software; then minor manual adjustments were introduced in order to optimise sequence homology. Nucleotide sequence analysis showed that CEVd ranged in size between 370 and 371 nt (Figure 1). Modifications of various types including transition, transversion, insertion, rearrangement and deletion were detected. Differences between the four sequences were found to occur at 14 positions out of 371. This gives a variability of 3.8%.

In order to allow a comparative study, the new RNA sequences were presented according to typical rod-like secondary structures. Figure 2 illustrates the rod-like secondary structure using the nucleotide sequence of the CEVd variant Tun/cl1. Only the mutations observed in the other three variants were indicated. We observed that the mutations were clustred in the pathogenicity and variable domain.

The secondary structures of the different variants were predicted using the *mfold* software (data not shown). This computer tool allows the prediction of the most stable secondary structure with the minimum free energy. The most stable secondary structure of CEVd was a classical rod-like structure for each variant. The mutations retrieved in CEVd variants do not alter significantly its model rod-like structures, as a consequence they should not affect the general function like its replication.

Comparison of the new variants with those already known

In order to compare the sequences characterised in this work with those already known, all natural variants of CEVd were retrieved from the subviral RNA database (Pelchat 2003), and then an alignment was performed. The novel sequences showed the presence of only minor modifications compared to those described previously. We did not find any new polymorhic position in the new sequences.

Finally a blast (*blastn*) search using the NCBI server (data not shown) revealed that the closest sequences to the new variants are those reported from a multiple citrus infection that has the ability to produce exocortis-like symptoms in Japanese citron (Ito *et al*. 2002).

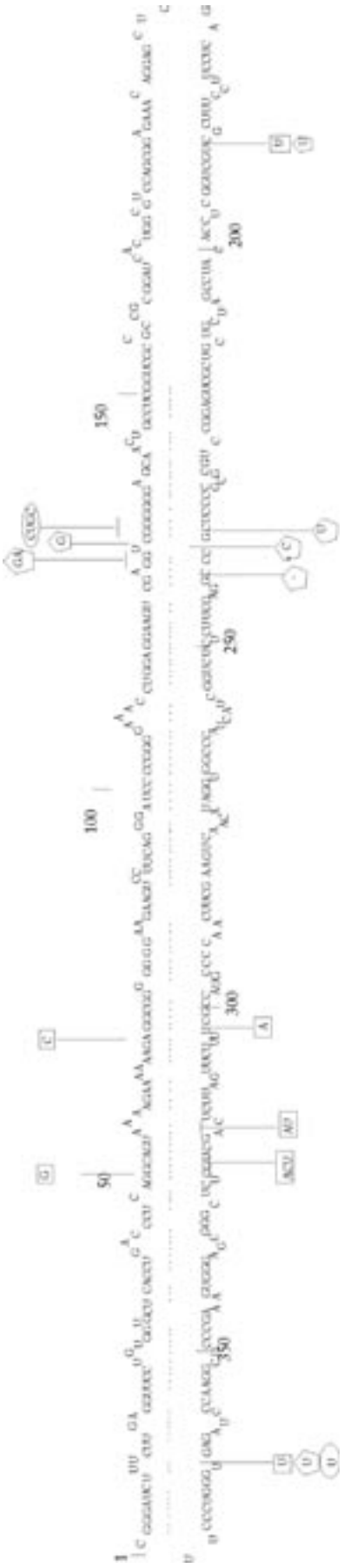

Figure 2. Potential rod-like secondary structures for CEVd variant CEVd tun/cl1. The variations found in the other sequences are indicated by □ for CEVd tun/cl2, by △ for CEVd tun/cl3 and by ○ for CEVd tun/cl4

DISCUSSION

The genome sequences of four different CEVd sequence variants were determined, confirming the worldwide distribution of this viroid. These sequences are more than 90% homologous with those previously reported. According to the criterion suggested by the International Committee for Taxonomy of viruses (Flores *et al*. 2000), less than 90% sequence similarity indicates that the two genomes are distinct species rather than strains of one species. Therefore, the new sequences are clearly variants of the three viroids rather than novel species.

Four different sequences were obtained. Such observation indicates that citrus viroids like many other RNA pathogens, propagate in the host as a population of similar but non identical sequences, fitting the quasi-species concept defined by Eigen (1993). Field and greenhouse viroid isolates from indicator host often have been found to be in fact a population of molecular variants. This was documented for example with CEV (Visvader & Symons 1985), PSTVd (Gora-Sochacka *et al*. 1997), *Grapevine yellow speckle viroid* (GYSVd-1) (Elleuch *et al*. 2002) and *Peach latent mosaic viroid* (PLMVd) (Ambros *et al*. 1998). It has been demonstrated that indicator host may induce sequence variability (Semancik *et al*. 1993). Another study has shown that inoculating a RNA species having a unique sequence yields several variants after a period of time (Gora-Sochacka *et al*. 1997), thereby suggesting that sequence variability was not restricted to the passage by an indicator plant. Clearly, the results present in this work confirm that viroids are quasi-species and that sequence variation occurs naturally without passage in an indicator plant. The selection pressures introduced by different host species and further complicated by environmental factors may also result in a drift in the population of quasi-species and the emergence of a new master sequence. Furthermore, since the viroid is characterised by a total dependence on host processes, a corresponding vulnerability to these host-mediated pressures must result and potentially affect all cooperative activities between any specific host and viroid variant including replication and pathogenicity (Skoric *et al*. 2001).

The demonstrated heterogeneity may result from co-infection and co-propagation of different versions of the viroid studied or from the accumulation of mutants appearing de novo during the replication of the parental genome. This latter possibility is suggested by the error-prone nature of RNA replication and by isolated case of phenotype conversion upon propagation (Domingo & Holland 1994). The considerable stability of CEVd variants could be due to strong structural constraints limiting variability. Genetic diversity of a viroid population is limited by the need to maintain functional secondary and tertiary structures (Aranda *et al*. 1997). Therefore, the hypothesis of the existence of constraints to limit the heterogeneity of nucleotide sequence was recently postulated based on the characterisation of many natural variants of HSVd and PLMVd (Ambros *et al*. 1998; Amari *et al*. 2001). The smaller diversity of CEVd may be also the result of a later infection with this pathogen, so it would have less time to generate mutant and its population would be less diverse.

To conclude, while most of the viroids sequence variability studies were performed using experimental hosts, the sequences reported here were detected from a field tree without using alternative passage by an indicator host. The large number of sequence variants occurring could be accounted for either a high copy error rate of the RNA polymerase replicating a single RNA species, or to the infection of one plant by several sequence variants during propagation of citrus varieties by grafting or during regular practices such as pruning. The long potential life of citrus trees in the fields (over 60 years) would allow the accumulation of sequence variants in each tree by either route.

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Elleuch A., Marrakchi M., Lévesque D., Bessais N., Perreault J.P., Fakhfakh H. (2003): **Molekulární variabilita viroidu *Citrus exocortis viroid* z jednoho přirozeně infikovaného citrusového stromu.** Plant Protec. Sci., **39**: 139–145.

Studovali jsme sekvenci *Citrus exocortis viroid* (CEVd) izolovanou z jednoho přirozeně infikovaného citrusového stromu variety *Citrus clementina* v Tunisku. Sekvenční variabilita tohoto viroidu z přirozeného hostitele bez použití alternativní pasáže indikátorovou hostitelskou rostlinou nebo umělé inokulace se vyskytuje ve 14 pozicích, což odpovídá variabilitě 3,8 %. Potvrdilo se, že přirozeně se vyskytující viroidy rodu *Pospiviroid* obsahují směs sekvenčních variant. Studie rovněž potvrdila celosvětové rozšíření tohoto viroidu.

Klíčová slova: *Citrus exocortis viroid;* nukleotidová sekvence; genom; kvazi druh

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