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Short communication

Sequencing of *Australian Grapevine Viroid* and *Yellow Speckle Viroid* isolated from a Tunisian grapevine without passage in an indicator plant

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Abstract

We report the nucleotide sequences of *Australian Grapevine Viroid* and *Grapevine Yellow Speckle Viroid* (type 1) isolated from grapevine trees in a Tunisian vineyard. Our data confirm the worldwide spread of these viroids and record their occurrence in Africa. This is the first description of *Australian Grapevine Viroid* sequences isolated from its natural host. Moreover, the sequences of these new natural variants suggests that the previous use of an indicator plant to amplify a viroid does not alter its nucleotide composition. In addition, we also present several new features of these two distinct quasi-species.

Abbreviations: AGVd – *Australian Grapevine Viroid*; CCR – central conserved region; CEVd – *Citrus Exocortis Viroid*; GYSVd – *Grapevine Yellow Speckle Viroid*; HSVd – *Hop Stunt Viroid*; nt – nucleotide; PCR – polymerase chain reaction; *Peach Latent Mosaic Viroid* – PLMVd; RT – reverse transcription.

Viroids, small (∼246–400 nucleotides), single-stranded, circular RNAs that infect higher plants and cause significant losses in agriculture, are the smallest known nucleic acid-based pathogens known (reviewed in Flores et al., 2000). They replicate through a rolling circle mechanism involving only RNA intermediates and do not encode any proteins. Consequently, the distinct biological properties of viroids, including the identification of their natural hosts, depends strictly on their RNA sequences and structures. Five viroids are known to infect grapevine trees (Flores et al., 2000): *Hop Stunt Viroid* (HSVd; Sano et al., 1986), *Citrus Exocortis Viroid* (CEVd; Garcia Arenal et al., 1987), *Australian Grapevine Viroid* (AGVd; Rezaian, 1990) and two *Grapevine Yellow Speckle Viroids* (GYSVd-1 and GYSVd-2; Koltunow and Rezaian, 1988; 1989). Viroids occurring in grapevine are classified into three genera based primarily on the type of central conserved region (CCR) and on the biological proprieties of the RNA species (Flores et al., 2000). GYSVd-1, GYSVd-2 and AGVd belong to genus *Apscaviroid*, CEVd to *Pospiviroid*, while HSVd is the only member of genus *Hostuviroid*. The primary aims of this study were to report the occurrence of AGVd and GYSVd in Africa, thereby confirming the worldwide spread of these viroids, and to characterize these infectious RNA quasi-species based on their nucleotide sequences.

The nucleotide sequences of GYSV-1 and -2 were originally determined after RNA isolation from leaves collected from naturally infected grapevine trees (Koltunow and Rezaian, 1988). In contrast, the only AGVd sequence reported is from viroid isolated from a cucumber plant mechanically inoculated at the cotyledon stage with viroid purified from infected grapevine

leaves (Rezaian, 1990). One of the main reasons for this procedure was the fact that vine tissue extracts contain notoriously high levels of phenolic compounds, polysaccharides and other complex substances known to have inhibitory effects on enzymes (Rezaian et al., 1992). AGVd is not the only viroid for which sequences were obtained after amplification in an indicator plant. For example, it was observed that HSVd isolated from grapevine exhibited similar host symptoms in cucumber plants (i.e. *Cucumis sativus* L. cultivar Suyo; Sano et al., 1986). Additionally, CEVd has been routinely propagated in either of the Compositae hosts *Gynura aurantiaca* and chrysanthemum, or in the Solonaceae tomato host (Sano et al., 1986; Semancik et al., 1993). Sometimes, the indicator host belongs to the same botanical family as the natural host, but not always (e.g. AGVd in cucumber). In order to avoid any potential alteration of the nucleotide sequence, we decided to sequence AGVd isolated from grapevine leaves without amplification through an indicator plant.

Both healthy and infected leaves from three different *Vitis Vinifera* L. cultivars of both Syrah and Carignan were collected from a Tunisian vineyard in the hotter months of the summer. The leaves were homogenized, and total RNA isolated by phenol extraction and adsorption onto cellulose (Flores et al., 1985). The resulting RNA samples were fractionated by lithium chloride precipitation and the polysaccharides removed by methoxyethanol precipitation (Satub et al., 1995). The RNA samples were quantified by UV spectroscopy and their quality assessed by 1% agarose gel electrophoresis. First-strand cDNA of AGVd was synthesized using an antisense primer (5′-GTCGACGACGAGTCGCCAGGTGAG-3′) and avian myeloblastosis virus reverse transcriptase (RT) according to manufacturer's recommended protocol (Roche Diagnostics, Indianapolis, IN). The resulting cDNA was amplified by polymerase chain reaction (PCR) using the same antisense primer coupled with a sense primer (5′-GTCGACGAAGGGTCCTCAGCAGAG-3′). These primers hybridize to the CCR and adjacent sequence. In order to avoid PCR artefacts PWO DNA polymerase (Roche Diagnostics) was used, and other precautions, including control experiments, were performed in order to confirm the authenticity of the DNA products (Pelchat et al., 2000). The amplification consisted of thirty cycles (1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C), after which the mixtures were extracted with phenol/chloroform and 20 µl aliquots analyzed on 2.0% agarose gels (Figure 1). A band of ∼370 bp

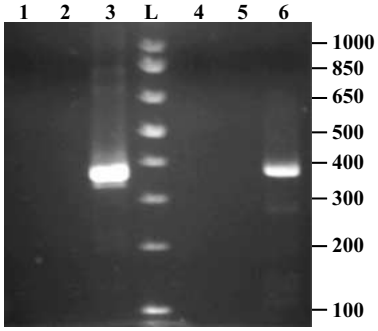

Figure 1. Analysis of RT-PCR amplifications on a 2% agarose gel. Lanes 1–3 and 4–6 are RT-PCR amplifications with AGVd and GYSVd, respectively. RT-PCR were performed using RNA isolated from either healthy (lanes 1 and 4) or infected plants (lanes 2, 3, 5 and 6). The RT step was omitted in the experiments in lanes 2 and 5. Lane L is the 1 kb plus DNA size marker (Invitrogen). The size of the DNA fragments is indicated adjacent to the gel.

corresponding to full size AGVd was only detected in RT-PCR amplifications using RNA samples isolated from infected leaves (compare lane 3 with lanes 1 and 2).

Subsequently, gel slices containing the full-size AGVd DNA were isolated, and the DNA extracted, precipitated and washed with ethanol. An adenosine was 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 linearized pCR 2.1 vector (which possesses an extra thymidine residue at 5′ end) as recommended by the manufacturer (TA cloning kit, Invitrogen, San Diego, CA). Three of the resulting clones were sequenced in both directions, using the M13 universal and reverse primers, by the dideoxyribonucleotide chain termination method (T7 DNA sequencing kit, United State Biochemical, Cleaveland USA). Two different nucleotide sequences from the grapevine Carignan cultivar, and one from the Syrah cultivar, were obtained (Figure 2). The two variants from the Carignan cultivar were 368 and 364 nt in size (Cari-1 and -2, respectively), while the one from the Syrah cultivar was 361 nt. The sequence retrieved after propagation in cucumber was 369 nt in size (Rezaian, 1990). None of these new variants show any important differences in either size or composition when compared to that previously reported (Figures 2 and 3A). Minor differences are observed throughout the molecules, with no clustering of the changes. We observed both the same types (i.e. deletion, insertion and mutation) and numbers of

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          1
Cari-1 UGGGCACCAA CUAGAGGUUC CUGUGGUACU CACCGAAGGC -GCGAAGCUA
Cari-2 .......... .......... .......... .......... -.........
Syrah  .......... .......... .......... .......... -.....CG.U
indic  .......... .......... .......... .......... C.....CG..
          51
Cari-1 GGAAAGGAAA AGAUAGAAAA GCUGGGUAAG ACUCACCUGG CGACUCGUCG
Cari-2 ......-... .......... .......... .......... ..........
Syrah  .....A.... ..-.......- .......... .......... ..........
indic  ......A... .......... .......... .......... ..........
          101
Cari-1 UCGACGAAGG GUCCUCAGCA GAGCUCACCG GCAGAGGCGC UAUGCAGGAA
Cari-2 .......... .......... ...--..... ..C-...... ..........
Syrah  .......... .......... ...--..... ..C-..-... ..........
indic  .......... .......... ...--..... ..C-...... ..........
          151
Cari-1 CGCUAGGGGU CCUCCAGCGG AG-ACUGAAG AAACUCCGGU UUCUUCUUUC
Cari-2 ........A. .......... ..-....... ......-... ..........
Syrah  ........A. ...U...... ..G....... .......... ..........
indic  .......... .......... ..G....... .......... ..........
          201
Cari-1 ACUCUGUAGC UGGAAUCCUU GUUGCGCUUG CUGGCGAAAC CUCGAGGGAA
Cari-2 .......... .........- .......... .......... ..GC......
Syrah  .......... .......... .......... .......... ..GC....-.
indic  .......... ........C. .......... .......... ..GC......
          251
Cari-1 GCUAGCUGGG UCCCGCUAGU CGAGCGGACU CGUCCCAGCG GUCCCAACCA
Cari-2 .......... .......... .......... .......... ..........
Syrah  .......... ...-...... ......-... .......... -.........
indic  .......... .......... .......... .......... ..........
          301
Cari-1 GUUUUCUUUA UCCUAUUUUU CCU-GCGGGC GCCCGGUCGU GGUUACCCUG
Cari-2 .......... .......... ...-...... .......... ..........
Syrah  .......... .......... ...C....A. .......... .-.......A
indic  .......... .......... ...-...... .......... ..........
          351
Cari-1 GAGCUCCCUG UUUGGAGGCC C
Cari-2 .......... .......... .
Syrah  .......... .......... .
indic  .......... .......... .
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Figure 2. Sequence alignement of the AGVd variants. The sequence of the variants from both the Carignan (Cari-1 and Cari-2) and the Syrah (Syrah) grapevine cultivars, and of the original variant obtained after passage in the cucumber indicator (Rezaian, 1990), are aligned. The sequences are reported in the GenBank Nucleotide Sequence Database under accession nos. AF462154 (Syrah), AF462155 (Cari-2), and AF462156 (Cari-1). The sequences corresponding to both the antisense and sense primers are identified by the upper and lower lines, respectively (minor sequence variation(s) may occur in these regions).

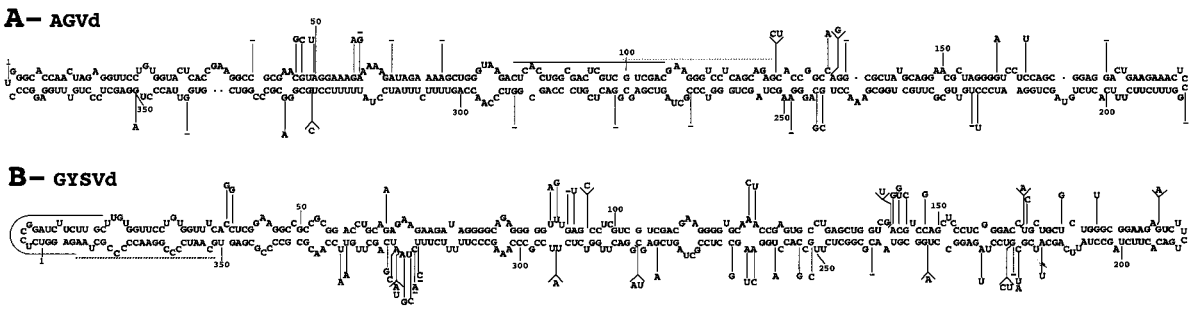

Figure 3. Sequence variations and a potential rod-like secondary structures for both AGVd and GYSVd. (A) The original AGVd variant, which was obtained after passage in the cucumber indicator (Rezaian, 1990). (B) The original GYSVd sequence reported previously (Polivka et al., 1996). The sequence variations reported in Figures 2 and 4 are indicated. The sequences corresponding to the sense and antisense primers are indicated by the fulled and dashed lines, respectively.

substitutions between the variants isolated from the Syrah and Carignan cultivars as was observed between the grapevine and cucumber isolates. Moreover, our results suggest that the passage of AGVd through cucumber did not significantly modify the nucleotide sequence.

The most stable secondary structures, in terms of energy, were predicted for all new sequences using

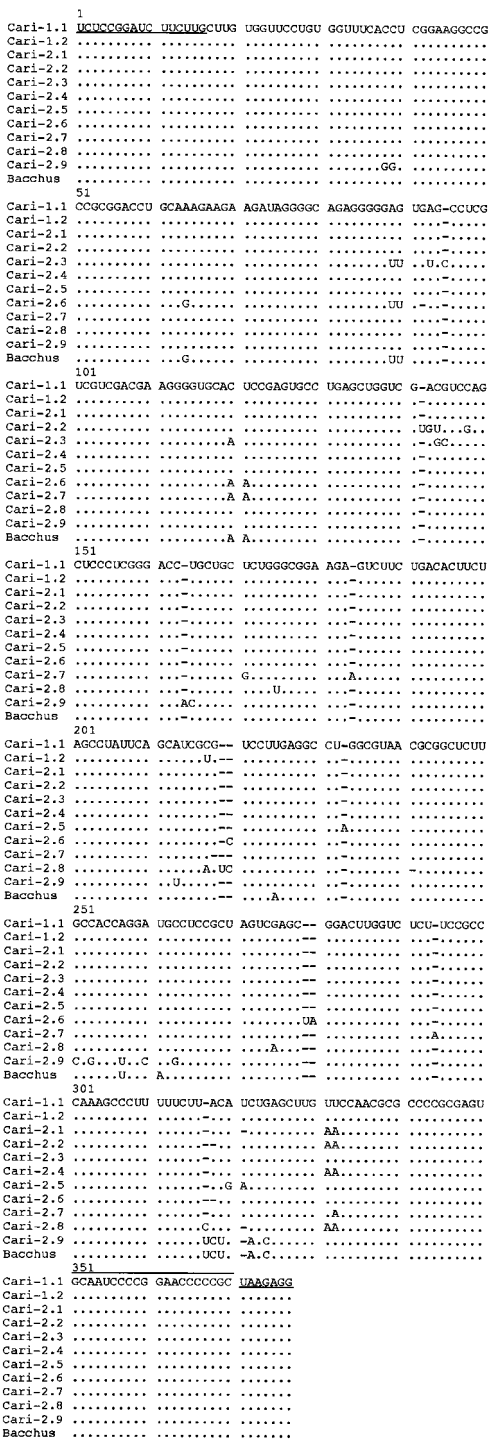

Figure 4. Sequence alignment of the GYSVd variants. The sequences of the variants isolated from the two trees (Cari-1 and Cari-2), and that of a sequence reported previously (Polivka et al., 1996), are aligned. The sequences are reported in the GenBank Nucleotide Sequence Database under accession nos. AF462157–AF462165, for variants Cari-2.9 to Cari-2.1, AF462166 for variant Cari-1.2 and AF462167 for variant Cari-1.1. The sequences of both the antisense and sense primers are identified by the upper and lower lines, respectively (minor sequence variation(s) may occur in these regions).

RNAfold. A typical example using the original AGVd variant, which was obtained after passage in the cucumber indicator, is illustrated in Figure 3A. Overall, this new secondary structure prediction of AGVd is similar to that reported previously (Rezaian, 1990), with only minor and local differences between the two. The use of the original AGVd variants offers the advantage that all mutations of the new variants are illustrated. All AGVd variants folded into a rod-like shape in which approximately 70% of the residues are basepaired, suggesting that the sequence variations do not affect the most stable secondary structure. In addition, no covariation is observed.

One possible explanation for AGVd sequence variability might be the existence of a host pressure. Therefore, the sequence of another viroid (i.e. GYSVd) infecting grapevine was analyzed. RT-PCR using primers that correspond to the sequence of the right terminal conserved region of GYSVd-1 (sense 5′-TAAGAGGTCTCCGGATCTTCTTGC-3′ and antisense 5′-GCGGGGGTTCCGGGGATTGC-3′; Polivka et al., 1996) were performed. A typical RT-PCR amplification of GYSVd-1 is presented in Figure 1 (lanes 4–6). Note that the same RNA sample was used for both the GYSVd and AGVd amplifications (i.e. a tree of the Carignan cultivar that was infected by both viroids). The cDNA products were cloned and sequenced as described above. Sequencing of four GYSVd clones gave only two different variants 366 nt in size (Figures 3B and 4). These sequences differ by only one residue at position 217 (i.e. substitution of a T for a C). Subsequently, using an RNA sample that was extracted from another tree of cultivar Carignan, one infected solely by GYSVd-1, nine other sequence variants were isolated. These variants ranged in size from 365 to 367 nt, and increased the sequence variability known for GYSVd-1 (Rigden and Rezaian, 1993; Polivka et al., 1996; Szychowsk et al., 1998). In contrast to AGVd, the variations appear to be primarily clustered into small domains, including the variable and pathogenecity domains (Figure 3B). When compared

to all GYSVd-1 sequences reported to date, the new sequence variants cannot be differenciated from those from Australia, Japan or Europe.

The most stable secondary structures, in terms of energy, were also predicted for all new GYSVd sequences using RNAfold. In contrast to AGVd, the most stable secondary structures of the GYSVd-1 sequences varied considerably. Among the 11 new sequence variants, only one adopted a rod-like structure similar to that of one of the orginal variant (Figure 3B). The other variants adopted a rod-like shape that always included at least two additional small hairpins. However, no covariation supporting a rod-like shape or an alternative structure was detected. Clearly, the future description of the secondary structure of GYSVd-1 in solution will be interesting. In addition, it is surprising that two closely-related viroids are predicted to fold in such different ways (based on a computer-aided approach).

More generally, comparison of Figures 2 and 4 suggest that GYSVd-1 exhibited less genome variability (i.e. elasticity) than the AGVd variants isolated from the same. Thus, the variability observed for AGVd is not the result of a pressure exerted by the host. The observed sequence variability was restricted to a relatively small number of positions within both AGVd and GYSVd. This limited variability strongly suggests that constraints exist to limit the heterogeneity of both viroids. The hypothesis of the existence of constraints to limit the heterogeneity of nucleotide sequence was recently postulated based on the characterization of many natural variants of *Peach Latent Mosaic Viroid* (PLMVd) and HSVd (Ambros et al., 1998; Pelchat et al., 2000; Amari et al., 2001). Even so, it seems that each isolate is composed of a mixture of RNA species, suggesting that both AGVd and GYSVd are quasi-species.

Aside from answering an important question with regard to a procedure used for validating the sequences of many viroids, we also report the first sequences of AGVd and GYSVd RNA species from the African continent, and confirm the worldwide spread of these viroids. The reduced frequency of sequence variation observed for AGVd has been proposed to be the result of a filtering effect due to its passage in cucumber (Rezaian, 1990). According to our data, which shows that the frequency of variation is not significantly higher in the natural grapevine host, this is not the case. Finally, we suggest that the use of an indicator plant in order to propagate a viroid, specifically cucumber in the case of AGVd, does not necessarily significantly alter the sequence.

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