

FIRST REPORT OF AUSTRALIAN GRAPEVINE VIROID FROM THE MEDITERRANEAN REGION

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SUMMARY

Grapevines can be infected by five different viroids, of which *Australian grapevine viroid* (AGVd) is the least studied. Here we report the use of RT-PCR for the detection of this viroid from infected grapevines in Tunisia. RT-PCR results were confirmed by cDNA sequencing. This is the first report of AGVd from the Mediterranean region. The data presented indicate a wider geographical distribution of this viroid than previously reported and record its occurrence in Africa. The molecular variability of AGVd from two different cultivars, Carignan and Syrah, was studied by restriction analyses and by cDNA sequencing. Sequence variability was not clustered in any specific domain or region of the genome.

Key words: Grapevine, Australian grapevine viroid, cloning and sequencing, molecular variability.

INTRODUCTION

Viroids are the smallest known plant pathogens, consisting solely of small circular single-stranded infective RNA molecules (Diener, 1991, 2001; Flores *et al.*, 2000). Their size ranges from 246 nucleotides (nt) in *Avocado sunblotch viroid* (ASBVd) (Symons, 1981) to 399 nt in *Chrysanthemum chlorotic mottle viroid* (ChCMVd) (Navarro and Flores, 1997). Most viroids characterized so far are grouped in the *Pospiviroidae* family. They contain five structural domains (Keese and Symons, 1985): two terminal regions, left (TL) and right (TR), a pathogenic (P), a variable (V) and a central (C) domain with a central conserved region (CCR). Viroids lacking a CCR possess hammerhead self-cleavage ability and belong to the *Avsunviroidae* family (Flores *et al.*, 2000).

Grapevine (*Vitis vinifera* L.), an important crop in the Mediterranean region, may be infected by five different viroids: *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), the two *Grapevine yellow speckle viroids*

(GYSVd-1 and GYSVd-2) and *Australian grapevine viroid* (AGVd) (Flores *et al.*, 1985; Sano *et al.*, 1985; García Arenal *et al.*, 1987; Rezaian, 1990; Wan Chow Wah and Symons, 1997; Szychowski *et al.*, 1998). These viroids belong to three different genera of the *Pospiviroidae*. GYSVd-1, GYSVd-2 and AGVd belong to the genus *Apscaviroid*, type species *Apple scar skin viroid* (ASSVd), whereas HSVd is the only member of the genus *Hostuviroid*. CEVd belongs to the genus *Pospiviroid*, type species *Potato spindle tuber viroid* (PSTVd). Of the five viroids infecting grapevine AGVd is the least studied. To date it has been recorded only from Australia (Rezaian, 1990) and California (Rezaian *et al.*, 1992). We now report the occurrence of AGVd in the Mediterranean region and in Africa. AGVd was detected in two different cultivars, Carignan and Syrah. We have also characterized this viroid using cDNA restriction profiles. The characterization and phylogenetic analysis of three new AGVd sequence variants from two different cultivars revealed that there is no relationship between AGVd variants and the host from which they were derived.

MATERIALS AND METHODS

Plant material. Twenty-three samples of young leaves from the grapevine cultivars Syrah Carignan, Cardinal, Muscat d'Italie (cv Italia) and Beldi were collected from a Tunisian vineyard in summer. As AGVd is not known to induce any symptom, the samples were collected without regard to symptom status.

RNA extraction. Two grams of young leaves were homogenized, and total RNA isolated by phenol extraction and adsorption onto cellulose as described by 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 content was determined by UV spectrophotometry and its quality assessed by electrophoresis in 1% agarose gels.

RT-PCR amplification. First-strand AGVd cDNA was synthesized using an anti-sense primer (5'-GTC-GACGACGAGTCGCCAGGTGAG-3') and *Avian*

myeloblastosis virus reverse transcriptase (RT) according to the manufacturer's recommendations (Roche Diagnostics, Indianapolis, USA). The resulting cDNA was then amplified by polymerase chain reaction (PCR) using the same anti-sense primer coupled with a sense primer (5'-GTCGACGAAGGGTCCTCAGCAGAG-3'). These primers hybridize to the CCR and adjacent regions. To avoid PCR artefacts PWO DNA polymerase (Roche Diagnostics, town country) 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 mixture was extracted with phenol/chloroform and 20 µL aliquots analysed on 2% agarose gels.

cDNA cloning. Gel slices containing the full-size AGVd cDNA were isolated, and the cDNA extracted, precipitated and washed with ethanol. An adenosine residue 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 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, USA).

cDNA restriction analyses. Clones carrying an insert of the size expected for AGVd were identified by restriction analysis using *EcoRI*. Three positive clones were restricted using *BamHI*, *DraI*, *NcoI*, *NdeI* and *XbaI*. Digestion was carried out under the conditions described by the manufacturer (Gibco-BRL, town country). The products were analysed by electrophoresis on 2% agarose gels.

cDNA sequencing. The clones analysed by restriction were then sequenced in both directions, using the M13 universal and reverse primers, by the dideoxyribonucleotide chain termination method (T7 DNA sequencing kit, United State Biochemical, Cleveland, USA). Two different nucleotide sequences were obtained from cv Carignan, and one from cv Syrah.

Phylogenetic analysis. Multiple alignments of AGVd sequences were obtained using ClustalW (Thompson *et al.*, 1994). The alignments were corrected manually to maximize sequence homology. Phylogenetic analyses were performed using the neighbour-joining method (Saitou and Nei, 1987) using ClustalX.

RESULTS AND DISCUSSION

AGVd was detected in only six samples of the 23 analysed, and was found in cvs Carignan and Syrah. This is the first report of AGVd from the Mediter-

anean region. This may be because it was not looked for earlier. GYSVd-1, GYSVd-2 and HSVd have been identified in Italy (Minafra *et al.*, 1990) and in Spain (Duran-Vila *et al.*, 1990).

When using AGVd-specific primers, a band of *ca* 370 bp corresponding to the full length genome of AGVd was detected in RT-PCR amplicons from cvs Carignan and Syrah. RT-PCR can sometimes give false positives due to the presence of host RNAs with partial similarity to primer sequences. In order to demonstrate unequivocally the presence of AGVd in the samples that tested positive, the cDNAs obtained were cloned. Two clones obtained from cv Carignan and one clone from cv Syrah were further analysed for sequence variation using several restriction enzymes. Polymorphism was observed only with *BamHI* (Fig. 1 and data not shown), which gave two distinct restriction patterns. In the first, no band was obtained; this profile was observed in the clones from cv Syrah. In the second pattern, an additional band about 100 nt in size was observed, this pattern was obtained from clones of cv Carignan (Cari-1 and Cari-2) (Fig. 1). *BamHI* restriction analysis suggested the existence of polymorphism in the cDNA coming from different cultivars.

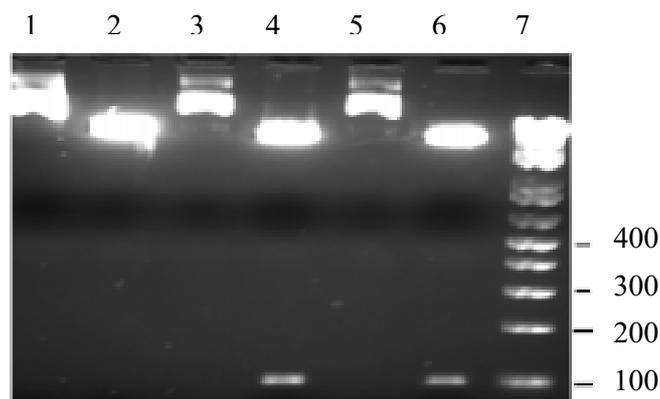


Fig. 1. 2% agarose gel analyses of *BamHI* restriction products. Lanes 1, 3 and 5, clones of Syrah, Cari-1, and Cari-2, respectively, that were not submitted to restriction. Lanes 2, 4, and 6, the same clones submitted to restriction. Lane 7: 1 Kb DNA ladder.

In order to test this hypothesis, to conclusively demonstrate AGVd infections in Tunisia, and study possible relationships between the viroid genomes and their hosts, the clones used in restriction analysis were sequenced.

The two variants from cv Carignan were 361 and 364 nt in size (Cari-1 and Cari-2, respectively), while the clone from cv Syrah was 368 nt. These new variants showed some differences in size when compared to the sequence (Pelchat *et al.*, 2003) reported from Australia (369 nt). This latter sequence was isolated from a cucumber plant mechanically inoculated at the cotyledonary stage with a viroid preparation purified from infected grapevine leaves (Rezaian, 1990). One of the

main reasons for this procedure was the fact that vine tissue extracts contain high levels of phenolic compounds, polysaccharides and other complex substances known to have inhibitory effects on enzymes (Rezaian *et al.*, 1992). So this is the first description of AGVd sequences isolated from their natural host.

Comparison between the known sequence variant isolated from cucumber and the sequence variant reported in this work shows modifications of various types including transition, transversion, insertion, rearrangement and deletion (Fig. 2). The number of polymorphic positions taking in account all the AGVd sequences variants is 27 out of 372 positions. So the variability is either 7.2% or 8.1% depending on whether the primer regions are considered or not. These point mutations were distributed throughout the molecules, with no clustering of the changes. We also noted that the G deletion at position 278 fell in the lower strand of the CCR. Mutation in the lower strand of central domain but not in the CCR has previously been reported from other members of the genus *Apscaviroid* (Ambros *et al.*, 1995; Owens *et al.*, 1999; Di Serio *et al.*, 2002). Mutation in the lower and upper strand of *Apscaviroid* central domain has been reported only for CVd-III (Owens *et al.*, 2000). It cannot be excluded that the mutation we detected in the CCR of AGVd could be an artefact. A conclusive proof that a variant containing the same mutation is viable can be obtained only by recovering several variants containing the same mutation and demonstrating the stability of such a mutation in the progeny of infectious *in vitro* transcripts of the mutated variants.

The most stable secondary structures, in terms of energy, were predicted for all new sequences at a folding temperature of 37°C using the program mfold Version 2.3, from <http://mfold2.wustl.edu/mfold/rna/form1-2-3.cgi>. All AGVd variants folded into a similar rod-like shape in which approximately 70% of the residues are base paired, suggesting that the sequence variations did not affect the most stable secondary structure. Overall, this secondary structure prediction of AGVd is similar to that reported previously (see Rezaian, 1990), with only minor and local differences. This strongly suggests that structural constraints exist to limit sequence heterogeneity of AGVd. The hypothesis of the existence of constraints to limit the heterogeneity of nucleotide sequences 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).

When the first sequence of AGVd from cucumber was reported, a single deletion at position 55 was detected in a cDNA clone. The low frequency of sequence variation observed for AGVd was proposed to be the result of a filtering effect due to its passage through cucumber (Rezaian, 1990). Our results show that the AGVd sequence variability is higher in the natural than in the experimental host.

	1			
cucumber	UGGGCACCAA	CUAGAGGUUC	CUGUGGUACU	CACCGAAGGC
cari1
cari2
syrah
	41			
cucumber	CGCGAACGUA	GGAAAGAAAA	AGAUAGAAAA	GCUGGGUAAG
cari1	-.....UAG
cari2GC
syrahGCG
	81			
cucumber	ACUCACCUGG	CGACUCGUCG	UCGACGAAGG	GUCCUCAGCA
cari1
cari2
syrah
	121			
cucumber	GAGC--ACCG	GCAGGAGGCG	CUAUGCAGGA	ACGCUAGGGG
cari1A
cari2A
syrahUC
	161			
cucumber	UCCUCCAGCG	GAGGACUGAA	GAAACUCCGG	UUUCUUCUUU
cari1U
cari2
syrah
	201			
cucumber	CACUCUGUAG	CUGGAAUCCG	UGUUGCGCUU	GCUGGCGAAA
cari1U
cari2U
syrahU
	241			
cucumber	CCUGCAGGGA	AGCUAGCUGG	GUCCCGCUAG	UCGAGCGGAC
cari1
cari2
syrahCG
	281			
cucumber	UCGUCCAGC	GGUCCCAACC	AGUUUUCUUU	AUCCUAUUUU
cari1
cari2
syrah
	321			
cucumber	UCCU-GCGGG	CGCCCGGUCG	UGGUUACCCU	GGAGCUCCCU
cari1C...AA
cari2
syrah
	361			
cucumber	GUUUGGAGGC	CC		
cari1		
cari2		
syrah		

Fig. 2. Variation of nucleotide sequences in the three isolates of AGVd from grapevine (Cari-1, Cari-2 and Syrah) compared to the type AGVd-cucumber (Rezaian, 1990).

To study the relationship between the AGVd variant from grapevine (Cari-1, Cari-2, Syrah), and from cucumber (Rezaian, 1990) and other viroids, we produced an alignment using CLUSTAL-X (Thompson *et al.*, 1997) using these sequences and others of viroids either from the same genus, *Apscaviroid* (GYSVd.27, ASSVd.3, CVd-III.2 and CBLVd.2) or from a different genus, *Pospiviroid* (CEVd.16, PSTVd.1 and HSVd.h1). We have also used a sequence variant of a viroid from a different family *Avsunviroidae* (PLMVd.1).

The phylogenetic tree (Fig. 3) derived by the neighbour-joining method from the alignment of the complete sequences reproduces the same distribution in families and genera obtained previously by Flores *et al.* (2000) and Elena *et al.* (2001). It also illustrates the relationship between the AGVd variant and other viroids. Grapevine viroids AGVd and GYSVd-1 form a single cluster suggesting that they share the same origin.

Some *Apscaviroid* species have been regarded as recombinant, sharing sequence homology not only with

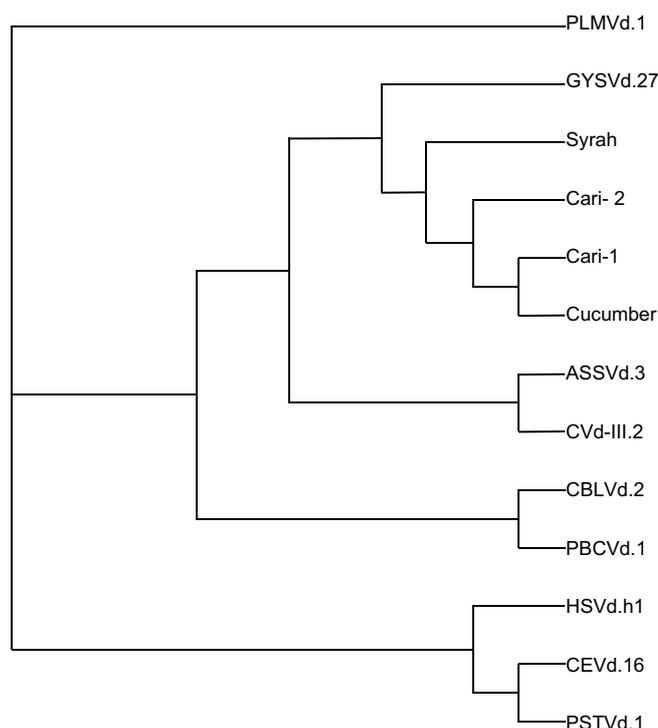


Fig. 3. Phylogenetic analysis of the AGVd isolates from Tunisia (Cari-1, Cari-2 and Syrah) compared with the AGVd sequence from cucumber and with sequences of other viroids from the same genus (GYSVd.27, ASSVd.3, CVd-III.2 and CBLVd.2) and from a different genus (CEVd.16, PSTVd.1 and HSVd.h1). The Neighbor-joining method described by Saitou and Nei (1987) was used, employing in ClustalX (Thomson *et al.*, 1994). The tree, rooted using PLMVd-1 as an outgroup, was drawn using Tree View (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

other *Apscaviroid* species but also with *Pospiviroid* species. On the basis of sequence homology, AGVd has been proposed to be the result of extensive recombination between four viroids, GYSVd-1, ASSVd, CEVd and PSTVd (Rezaian, 1990). Although this hypothesis remains untested, it seems plausible. Recombination depends on replication of different viroids in the same host, and grapevine is a suitable host because it can support the replication of at least five viroids concurrently. The vegetative propagation of grapevines over many years has probably provided an ideal environment of multiple viroid infections, allowing the possibility for RNA exchange (Rezaian, 1990).

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