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Characterisation of two citrus apscaviroids isolated in Spain

Brief Report

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Summary. Sequence variability in the PCR amplified cDNAs from two citrus apscaviroid isolates CVd-Ia and CVd-IIId from Spain, was analysed. CVd-IIId sequence was shown to be identical to previously described CVd-III sequences and no important variability was encountered within the viroid population. Conversely, CVd-Ia displayed population heterogeneity as shown by SSCP analysis, *Sal* I restriction site polymorphism and sequences of 27 CVd-Ia cloned DNAs. The CVd-Ia genomic heterogeneity is characterised by two major subpopulations with the most divergent sequences, and by the presence of individual variants, making a sequence continuum between the two major groups. Most sequence variations are clustered in the left part of the viroid molecule.

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Several viroids, i.e. unencapsidated, circular and single-stranded infectious small RNAs, have been described in citrus [3, 25]. In addition to the citrus exocortis viroid (CEVd), four viroid groups were initially established on the basis of electrophoretic and chromatographic properties, sequence homologies, host range and symptom expression [5]. After sequence analysis of field isolates from different countries, the citrus viroids have been classified into five species within the *Pospiviroidae* family [8]. The citrus exocortis viroid (CEVd) and specific variants of the hop stunt viroid (HSVd) are disease causing agents on sensitive citrus hosts [19, 22, 25] whereas citrus viroid I (CVd-I) and citrus viroid III (CVd-III), both belonging to the genus apscaviroids, have been proposed as dwarfing agents for commercial citrus grafted on the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock [21].

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Viroids may persist as a number of sequence variants in their hosts [10, 27] and therefore should be regarded as populations of closely related sequences or quasispecies [6]. Viroid population switches may also occur as a result of host and tissue selection affecting replication efficiency, host range and pathogenicity [7, 23]. The heterogeneity of CVd-I was initially ascertained as differences in migration observed on sPAGE analysis which allowed the discrimination of at least two types of variants (CVd-Ia and CVd-Ib) with distinct electrophoretic mobilities [5]. The molecular characterisation of an isolate of CVd-Ib, namely citrus bent leaf viroid (CBLVd) from an avocado plant (*Persea ameriacana* Miller) previously inoculated by heterologous grafting with a citrus source [1, 11], demonstrated the effect of the host selection process in the establishment of viroid variability [2]. Similarly, CVd-III was initially perceived as a heterogeneous population of variants by sPAGE and sequence analysis [5, 16] but later demonstrated to be a highly conserved viroid [21]. Here we describe the molecular characterisation of a CVd-I and a CVd-III sources recovered from the naturally occurring field isolate E-117 [4] originally found in a Nules clementine (*Citrus clementina* Hort. *ex* Tan) tree. This is a preliminary step towards the selection of specific variants to be further evaluated as dwarfing agents.

The E-117 isolate was inoculated on the viroid sensitive selection of Etrog citron 861-S1 (*Citrus medica* L.), and CVd-I and CVd-III were individually recovered by electoeluting the ethidium bromide stained sPAGE gel segments containing the circular forms of these viroids [5]. The electroeluted viroids were independently inoculated on Etrog citron plants, which have been maintained in the viroid collection of the Instituto Valenciano de Investigaciones Agrarias since 1984. Etrog citron inoculated with CVd-Ia showed an irregular pattern of symptom expression characterised by portions of the plant displaying the characteristic symptoms of epinasty and midvein necrosis alternating with symptomless flushes of tissue whereas CVd-IIId induced mild epinasty and petiole necrosis. These CVd-I and CVd-III sources had been categorised as CVd-Ia and CVd-IIId based on their electrophoretic mobility in sPAGE analysis [5].

Samples (5 g) of young leaves and stems from inoculated Etrog citron plants (a single plant infected with CVd-I and a single plant infected with CVd-III were used) were homogenised in 5 ml of extraction medium (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 saturated phenol [20]. The total nucleic acids were ethanol precipitated and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM $MgCl_2$; pH 7.4). After heat denaturation and slow cooling, the cDNAs were synthesised using 5 units of Avian Myeloblastosis Virus (AMV) reverse transcriptase in the presence of 2 mM dNTPs and 1 mM of primers CVd-I-cp (5′-TTCCGTCGACGACGACCAGTC-3′) and CVd-III-cp1 (5′-TTCGTCGACGACGACAGGTA-3′) complementary to basis 84–103 and 76–95 of the central conserved region (CCR) of CVd-I and CVd-III respectively [1, 16, 21]. Second strand synthesis and PCR amplification were performed using one tenth of the cDNA product and the proof-reading *Vent* polymerase (New England Biolabs)

Table 1. Variability in the population of the CVd-Ia Spanish type isolate

Mutations[a]														*Sal*I group	SSCP group	Specific mutations	Potential representation in the viroid population
Clone number number	U→A (269)	U→A (61)	A→U (271)	A→G (275)	U→G (246)	ins A (290)	C→U (41)	A→U (65)	del U (296)	C→U (152)	U→A (179)	A→C (247)	Ins C (167)	–	–	–	–
18,19,20														I	1		13
30	- - - -													I	1	A→G (50)	1
																del A (59)	1
12	- - - -				*Sal*1									I	1	ins C (253)	
23	- - - -				- - - -									II	1		1
11		- - - -	- - - -	- - - -	- - - -									II	1	del U (265)	1
15		- - - -	- - - -	- - - -	- - - -									II	4		1
21		- - - -	- - - -	- - - -	- - - -	- - - -								II	3		1
26					- - - -	- - - -	- - - -	- - - -	- - - -					II	2		1
6					- - - -		- - - -	- - - -	- - - -					II	2		1
10,16,17					- - - -		- - - -	- - - -	- - - -	- - - -	- - - -			II	5	(152, 179)	6
Cal[b]	- - - -	- - - -	- - - -	- - - -								- - - -	- - - -	I	ND	del C (138)	
Jp[c]		- - - -	- - - -	- - - -								- - - -	- - - -	I	ND		
P2[c]		- - - -	- - - -	- - - -								- - - -	- - - -	I	ND	A→U (28) C→U (265) U→C (266) ins A (269)	

[a]By comparison to the sequence of clones 18, 19 and 20

[b]Isolate from California (Cal) according to Semancik et al. [21]

[c]Isolates from Japan (Jp) and Philippines (P2) according to Hataya et al. [12]

- - - - Presence of the mutation

ND Not done

during 35 cycles (92C/10 sec, 64C/10 sec, 72C/20 sec), using the complementary primers described above and the primers CVd-I-hm (5′-GGCTGCTCAGCTG-CGGAGGT-3′) and CVd-III-hm1 (5′-GGCAGCTAAGTTGGTGACGC-3′) homologous to basis 104–123 and 96–115 of CVd-I and CVd-III, respectively. A second set of primers designed from the sequence downstream of the CVd-III CCR, namely CVd-III-cp2 (5′-ACTCTCCGTCTTTACTCCA-3′) and CVd-III-hm2 (5′-CTCCGCTAGTCGGAAAGACTC CGC-3′) was used to obtain a PCR product overlapping the CCR.

Direct sequencing (fmol system, Promega) of the RT-PCR product of CVd-Ia gave an ambiguous gel pattern suggesting the existence of a heterogeneous population of sequence variants. Conversely, sequencing the RT-PCR product of CVd-IIId, a viroid which has been demonstrated to be highly conserved even when sources from different geographic locations were sequenced [21] gave an unambiguous sequence identical to CVd-IIIb from California [16] and CVd-IIIa from Australia [26]. The purified RT-PCR products from both viroids were A-tailed using a 20 min incubation at 72 C with 2.5 units of *Taq* polymerase and 200 μM dATP, before ligation into the thymidylated *Eco*RV site of the pT7-Blue vector (Novagen).

Twenty-seven clones carrying an insert of the expected size of CVd-Ia were selected for further analysis. *Sal*I restriction analysis indicated the existence of polymorphism within the cDNA population. All the clones had two *Sal*I sites in positions 95 and 128 near the borders of the cloned fragment (one included in the primer CVd-I-cp, and a second one 7 nucleotides downstream the CVd-I-hm), but twelve clones had an additional *Sal*I site at position 243. As shown in Table 1, this was used as a criteria to classify the clones into two distinct groups (*Sal*I group I; *Sal*I group II).

Additional heterogeneity was identified by single stranded conformation polymorphism (SSCP) analysis [14] modified to detect minor shifts in the mobility of the viroid cDNA strand. Full length cloned DNA were recovered by PCR amplification using the CVd-I-h primer described above and a digoxygenin-labelled CVd-I-c primer. Aliquots of the PCR products were diluted twenty times in 0.1 M NaOH and 0.1 mM EDTA, heated for 10 min at 50 °C and mixed with half the volume of formamide containing 0.05% xylene-cyanole and 0.05% bromophenol blue. The denatured DNAs were subjected to 5% PAGE (acrylamide:bisacrylamide ratio 29:1) containing 5% glycerol in 0.5×TBE buffer using the GATC 1500-system (GATC Gmbh, Germany). A non-denatured sample was also analysed as a control to monitor the migration of dsDNA. Electrophoresis was performed at constant voltage of 400 V for 1 h, followed by 1 h at 800 V and 6 h 1200 V. The samples were collected between 3 h and 8 h on a positively charged Nylon (Boehringer Mannheim) membrane running at 9 cm/h in the GATC 1500-system. The samples were fixed by UV crosslinking, and the digoxigenin labelled DNAs were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualised with the chemiluminiscence substrate CSPD (Boehringer Mannheim).

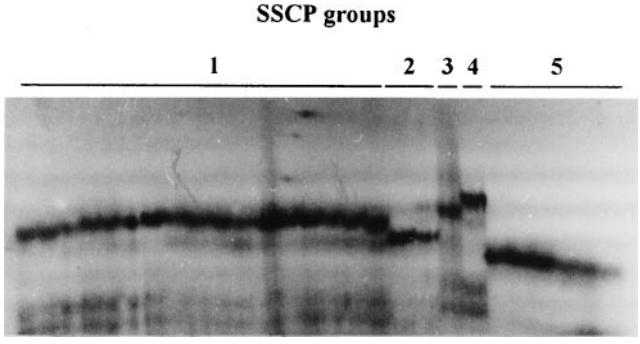

Fig. 1. SSCP analysis of the digoxigenin labelled complementary strand from CVd-Ia cDNAs. The differences in electrophoretic migration of the cDNAs were used as a criteria for the classification of clones in five groups. From left to right clones number 2, 12, 30, 1, 3, 5, 7, 9, 13, 14, 18, 19, 20, 24, 25, 23, 11 (*1*); 6, 26 (*2*); 21 (*3*); 15 (*4*); 10, 16, 17, 22, 27 and 29 (*5*)

Five different SSCP profiles were clearly identified (Fig. 1) illustrating the existence of additional variability among CVd-I clones. The 15 clones that contained only two *Sal*I sites (group I) presented identical SSCP profiles (Fig. 1, lanes 1 to 15) but in two of them (clones number 12 and 30) the digoxigenin-labelled insert recovered by PCR presented a slightly different electrophoretic mobility than the rest (data not shown). The 12 clones that contained an additional *Sal*I site (group II) could be grouped accordingly with the four distinct SSCP profiles observed (Fig. 1, lanes 16 to 27). This variability was confirmed by sequence analysis of 14 selected clones (fmol system, Promega), demonstrating that the CVd-Ia source studied contained a heterogeneous population of sequence variants ranging from 326 to 327 nucleotides. The results are summarised in Table 1. When the same strategy of cloning and sequencing was used to characterise the CVd-IIId source, a sequence identical to that obtained by direct sequencing of the RT-PCR product was consistently obtained, thus confirming that the sequence variants identified in CVd-Ia were not likely introduced during the RT-PCR or cloning processes.

Two "master" sequences of CVd-Ia representing group I (13 clones) and group II (6 clones) were identified as fitness peaks of the viroid quasispecies distribution [6]. The eight additional sequence variants represent a continuum or valley around and between these "master" sequences. The distribution pattern of the sequence variants identified (Table 1), with a small number of clones representing convergent sequences, indicates that the generation of viable variants is highly restricted, probably due to constrains imposed to preserve the viroid structure. Indeed, many of the changes observed do not disturb the viroid overall secondary structure: insertions and deletions occur in loops and changes appear to be highly compensatory (Fig. 2A). Most of the nucleotide changes observed are clustered in the left of the viroid secondary structure (Fig. 2A), which as a consequence should be regarded as a variable region. This region corresponds to the pathogenic (P) domain of potato spindle tuber viroid (PSTVd) in which variations affect the pathogenicity [13]. However, since the determinants of pathogenicity of CVd-I

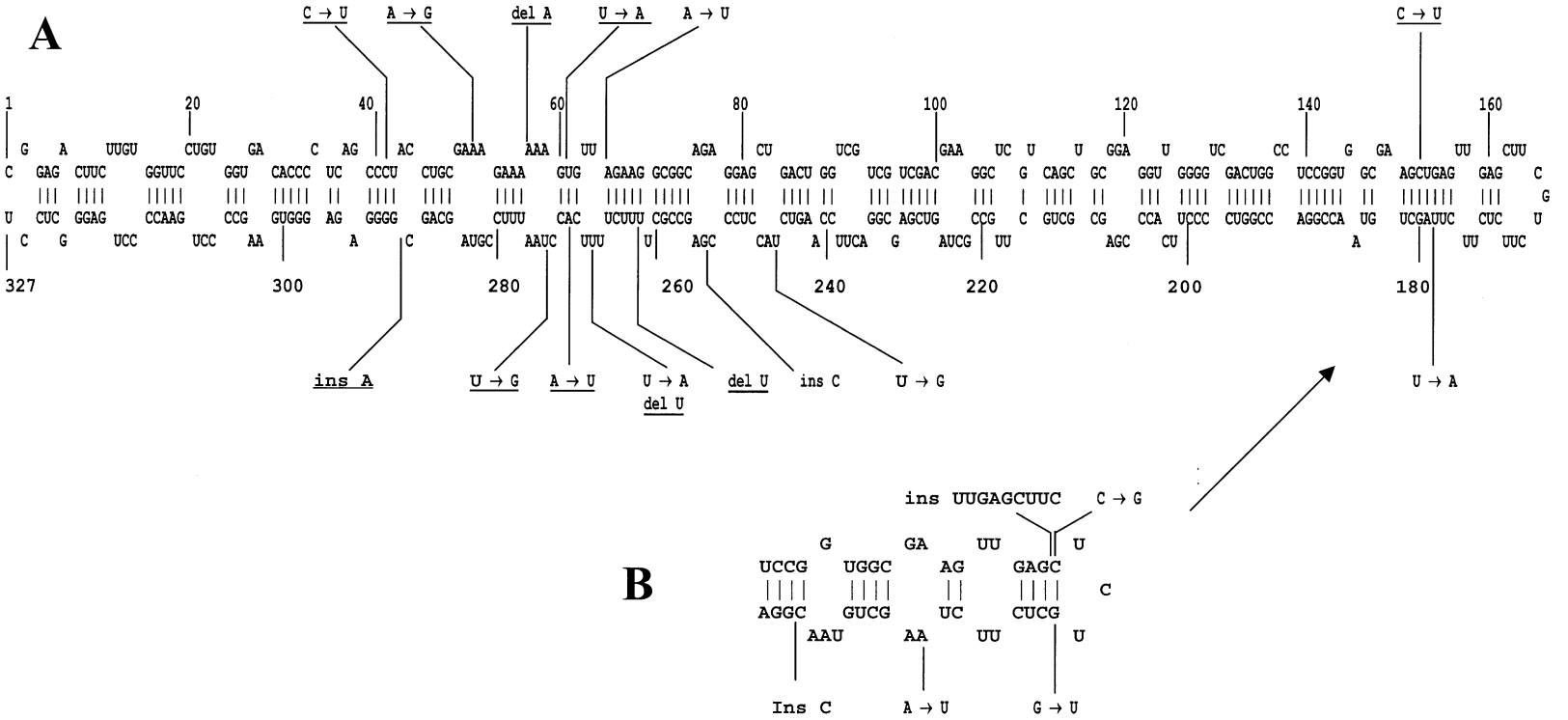

Fig. 2. Location of sequence variations on the CVd-Ia minimum free energy secondary structure. **A** Secondary structure of the viroid molecule is according to the minimum free energy analysis [29] of the clone 18 sequence. Underlined nucleotide differences indicate changes that do not affect the overall viroid structure. For corresponding clones see Table 1. **B** CBLVd (CVd-Ib) right terminal structure. Variations indicated are by comparison to CVd-Ia clone 18

and other apscaviroids have not been studied, the involvement of other parts of the molecule, as demonstrated with artificial chimeras of other viroids [18], cannot be ruled out. Only two nucleotide changes have been observed in the right part of the secondary structure of the molecule where a variable (V) domain has been established for pospiviroids [13].

Some apscaviroids have been regarded as recombinants sharing sequence homology not only with other apscaviroids but also with pospiviroids [15, 17]. CBLVd (CVd-Ib), an apscaviroid with 95% sequence homology with CVd-Ia, has been considered as a chimeric viroid with left hand part of the molecule like CEVd [1]. Although this remains an untested hypothesis, the possibility that CVd-Ia and CVd-Ib have arisen from recombination events should be entertained. Recently, Hataya et al. [12] have proposed that CVd-Ia could be a derivative of CBLVd (CVd-Ib) by partial sequence duplications taking place in the right terminal region. One could argue that CBLVd (CVd-Ib) could be a derivative of CVd-Ia by partial 5 nucleotide deletion naturally stabilised by a complementary 5 nucleotide deletion, as this phenomenon has been shown to restore infectious property of viroid RNA from a non-infectious in vitro-deleted PSTVd [28]. CVd-Ia and CBLVd (CVd-Ib) could have thus evolved one from another by a single nine-nucleotide insertion or deletion in the right terminal region (Fig. 2B). Indeed, the involvement of the terminal loop in viroid enlargement has been illustrated in the case of CEVd [24]. However, the epidemiological information accumulated to date on the natural occurrence of viroids in citrus [4, 9] indicates that CVd-Ia and CVd-Ib: a) are less widespread in citrus than CEVd, HSVd and CVd-III; b) have a restricted host range; c) have never been found co-infecting the same plant; and d) have only been found co-infecting the host plants with other citrus viroids. These observations indicate that CVd-Ia and CVd-Ib probably arose independently by similar recombination events in citrus that were already infected with other viroids.

Genbank accession numbers

Genbank accession numbers for CVd-Ia clones 17 and 18 are AF040721 and AF040722, respectively.

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