Arch Virol (2004) 149: 407–416
DOI 10.1007/s00705-003-0219-1

Archives of Virology
Printed in Austria

Variability of the progeny of a sequence variant of *Citrus bent leaf viroid* (CBLVd)

Brief Report

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Received February 14, 2003; accepted July 30, 2003
Published online October 30, 2003 © Springer-Verlag 2003

Summary. A field isolate of CBLVd was previously shown to contain two dominant subpopulations (I and II), which differed by the presence or absence of a *Sal* I restriction site in the PCR product [10]. Here we demonstrate the infectivity and symptom expression of subpopulation II by inoculating Etrog citron with a single representative haplotype. The resulting progeny was characterised as an heterogeneous population of closely related variants with a new fitness peak represented by an haplotype that was not identified in the original isolate. This demonstrates that CBLVd conforms a "quasispecies" model. The progeny shared features of the two subpopulations of the original isolate indicating that the original isolate probably arose from a single CBLVd ancestor.

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Citrus viroids were initially divided into five groups based on their electrophoretic mobility, sequence homologies determined by molecular hybridisation, host range, and symptom expression on Etrog citron (*Citrus medica* L.) and they were classified into five groups [6]. Sequencing studies subsequently demonstrated that the proposed groups were consistent with the viroid species concept proposed by the Executive Committee of International Committee on Taxonomy of Viruses [8, 9]. Recently, a sixth species termed CVd-OS viroid, has been reported to be an apscaviroid with 68% homology with *Citrus viroid* III (CVd-III) [15]. Only *Citrus exocortis viroid* (CEVd) and specific variants of *Hop stunt viroid* (HSVd) are disease-causing viroids on sensitive citrus hosts, inducing the exocortis and cachexia diseases on sensitive citrus hosts, respectively.

Citrus bent leaf viroid (CBLVd) initially described as Citrus viroid I (CVd-I) induces moderate epinasty and point necrosis of the mid vein of Etrog citron [5] and mild dwarfing in trees grafted on trifoliate orange rootstock (*Poncirus*

trifoliata (L.) Raf.) [22]. CBLVd is an apscaviroid belonging to the *Pospiviroidae* family, and appears to be a chimera containing parts of central domain (C) of *Apple scar skin viroid* (ASSVd) and the pathogenicity (P) and terminal left (T_L) domains of CEVd [2]. Two strains of CBLVd (namely CVd-Ia and CVd-Ib) with distinct electrophoretic mobilities were identified in citrus [6] and demonstrated to be highly homologous to the type strain which was sequenced after heterologous graft transmission to avocado [11]. Hataya et al., [12] have suggested that CVd-Ia arose by partial sequence duplications involving the right terminal region from CVd-Ib [12].

Viroids like other RNA replicons exist in susceptible hosts as complex populations of sequence variants or haplotypes, which follow the "quasispecies model" proposed by Eigen [7]. Foissac and Duran-Vila [10] have characterised a CBLVd (CVd-Ia) isolate which was composed of a population of 326–327 nt sequence variants containing two major subpopulations (I and II) that include the most divergent sequences and a continuum of additional variants, intermediate between the two major subpopulations. The two dominant sequences in subpopulations I and II differ by the presence or absence of a U to G mutation in position 246 which creates an additional *Sal* I restriction site in the corresponding cDNAs. In the present work, the dominant sequences of subpopulations I and II were selected to study their biological properties and to characterise their progeny on inoculated citrons.

Four CBLVd cDNA clones (clones 18 and 19, and clones 10 and 17 representing the dominant haplotypes of subpopulations I and II, respectively) [10] were used as a template for 30 cycles of PCR amplification, using 0.5 μM of primers CV-I-cp (5′-TTCGTCGACGACGACCAGTC-3′) complementary to bases 84–103 and CV-I-hm (5′-GGCTCGTCAGCTGCGGAGGT-3′) homologous to bases 104–123, in buffer containing 1 mM $MgCl_2$, 0.25 mM dNTPs and 1 unit of *Taq* DNA polymerase (94C/30 sec, 60C/1 min, 72C/1 min). Aliquots of PCR products were mixed with 27 μl of a denaturing solution (90% formamide; 25 mM EDTA; 0.05% xylene-cyanole and 0.05% bromophenol blue) heated for 10 min at 100 °C and cooled quickly. The denatured DNAs were subjected to SSCP analysis in 14% PAGE ($14 \times 11.5 \times 0.075$ cm gels) at 200 V constant voltage for 16 hr in TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 8.3). The DNA bands were visualised by silver staining [14]. Under these conditions, different DNA profiles were obtained (Fig. 1), indicating the suitability of this electrophoresis system to discriminate among closely related sequences. These conditions of SSCP analysis were further used as a tool for a preliminary screening of the viroid progeny from inoculated plants.

In order to obtain infectious preparations of specific CBLVd haplotypes, clones 18 and 10 were subjected to 35 cycles of PCR amplification using *Pwo* polymerase (Roche) and CV-I-cp and CV-I-hm primers in buffer containing 2 mM $MgSO_4$, 0.13 mM dNTPs, 0.5 μM of each primer and 1 unit of *Pwo* DNA polymerase (94C/30 sec, 60C/30 sec, 72C/1 min). The PCR products were phosphorylated with 0.3 units of T4 polynucleotide kinase and ATP 10 mM and were self-ligated with 2 units of T4 DNA ligase (14C/16 h) to obtain dimeric molecules. Dimers were ligated into the pBS vector (Stratagene) and clones exhibiting the desired

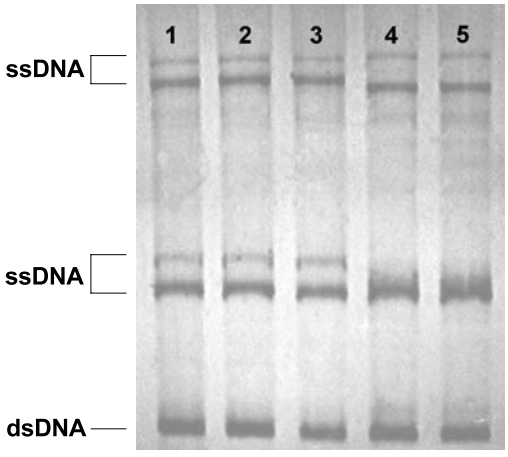

Fig. 1. SSCP analysis of haplotypes recovered from the original CBLVd isolate. From left to right (*1*) CBLVd positive control; (*2*) CBLVd cDNA clone 10; (*3*) cDNA clone 17; (*4*) cDNA clone 18; (*5*) cDNA clone 19

head-to-tail orientation were identified by restriction analysis and sequencing. Clones containing dimeric inserts were linearized and used as a template in a transcription reaction, with rNTPs 1 mM, DTT 1 mM and 50 units of T3-RNA polymerase or T7-RNA polymerase depending on the insert orientation to produce dimeric RNA transcripts homologous to the viroid sequence. Both, dimeric cloned cDNAs and the corresponding RNA transcripts, were used for infectivity assays.

The Etrog citron selection 861-S1 propagated by bud grafting onto the rough lemon (*C. jambhiri* Lush) rootstock, was used as host. Plants were slash inoculated with preparations of DNA (4 μg per plant and two plants per preparation) containing dimeric inserts of the two selected cloned cDNAs. Six months post inoculation, all the inoculated plants remained symptomless, and the viroid was undetectable by molecular hybridisation analysis. They were then further inoculated with 1 μg of RNA dimeric transcripts from the same clones. Inoculated plants were kept in the greenhouse at 28°–32 °C and 9 months later, the characteristic symptoms of CBLVd infection appeared in one of the plants inoculated with clone 10, belonging to subpopulation II (Fig. 2).

Samples (5g) of young leaf tissue from all the inoculated plants were homogenised in 5 ml of extraction buffer (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7; 4% (v/v) 2-mercaptoethanol) containing water-saturated phenol, and the total nucleic acids were partitioned with 2 M LiCl [21]. The soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM $MgCl_2$ pH 7.4). Aliquots of nucleic acid preparations from inoculated and non-inoculated citrons were subjected to sPAGE (Fig. 3) and slot-blot hybridisation using a DIG-labelled CBLVd specific probe [19] to confirm infection. The results confirmed that only one of the plants inoculated with the clone 10 was actually infected (Fig. 3).

In order to characterise the progeny generated from the infectious sequence, the nucleic preparations were subjected to reverse transcription and PCR amplification using the same conditions described above. The PCR products were ligated into pGEM vector (Promega), and plasmids from transformed cells were subjected to restriction analysis to verify the presence of an insert of the expected size. The 26

Fig. 2. Citron inoculated with CBLVd cDNA clone 10 (subpopulation II). The arrow indicates the characteristic leaf bending resulting from point necrosis of the midvein induced by CBLVd

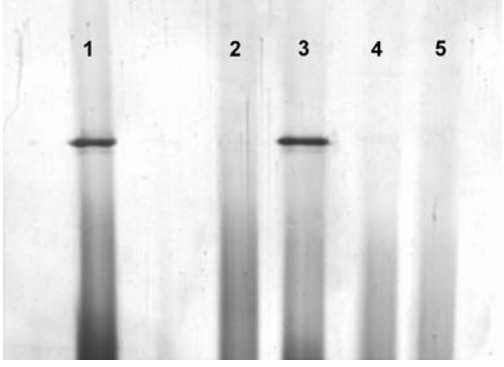

Fig. 3. sPAGE analysis of low molecular weight RNA extracted from inoculated citrons. From left to right: (*1*) CBLVd positive control; (*2*, *3*) citrons inoculated with cDNA clone 10; (*4*, *5*) citrons inoculated with cDNA clone 18

CBLVd cDNA clones obtained were subjected to SSCP analysis to identify clones containing the dominant sequence and to estimate the heterogeneity of the viroid population. Ten electrophoretic profiles were identified, and the corresponding clones were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with the program Clustal W [13] and secondary structure analyses were performed with the program MFOLD (circular version) from the GCG package [23] and RNAviz [4].

The progeny contained variants of 327–328 nt with a dominant haplotype (clone 14) representing 46.1% of the population, whereas the inoculated sequence was identified in only a single clone (clone 30), representing only 3.8% of the population. The dominant haplotype differed by 8 nucleotide changes from the inoculated one (Table 1). Five of these changes were characteristic of the

Table 1. Variation in the progeny generated after inoculation with clone 10 (subpopulation II) of the original CBLVd isolate

	Mutations[a]											Number of clones in the population
Clon	U to A (61)	A to U (272)	A to G (276)	U to G (247)	C to U (41)	A to U (65)	−U (269)	C to U (152)	U to A (179)	A to C (248)	Others	
10 (II)[b]				+	+	+	+	+	+			
30				+	+	+	+	+	+			1
20	+			+			+		+			3
12		+	+	+	+	+					A to C (243)	1
24	+	+	+	+							A to G (50)	2
5	+	+	+	+							A to G (304)	1
3	+	+	+	+							GUGGCG to UCGACA (143–148)	1
14	+	+	+	+								12
21	+	+	+	+							A to G (256) U to A (270)	2
17	+			*Sal* I						+	U to C (46) U to C (129)	2
7										+		1
18 (I)[b]												

[a]By comparison to the sequence of the inoculated haplotype (clon10 representative of subpopulation II of the original CBLVd isolate) according to Foissac and Duran-Vila [10]

[b]Haplotypes representatives of subpopulations I and II of the original CBLVd isolate according to Foissac and Duran-Vila [10]

+ Presence of the mutation

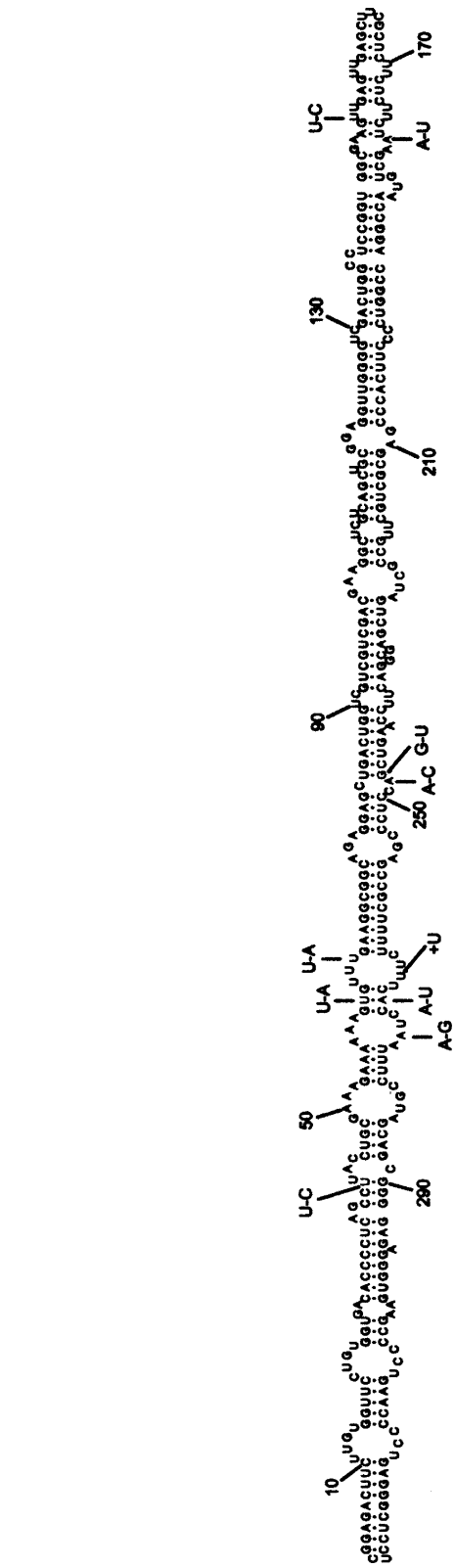

Fig. 4. Secondary structure of an infectious CBLVd variant (clone 10) that induced symptoms in citron. Positions of the sequence changes observed in the progeny generated by this haplotype are marked

subpopulation I (i. e., positions 41, 65, 152, 179, 269) but the *Sal* I restriction site characteristic of the subpopulation II was preserved. All but two other variants also contained changes specific to subpopulation I as well as the *Sal* I restriction site characteristic of subpopulation II. (Fig. 4). Changes specific to group I were:

– U to C in position 41;
– U to C in position 152 (all variants except clone 30);
– A to U in position 179;
– U to A in position 65 (clones also containing A change AT position 41);
– G to U in position 247 (destroys *Sal* I restriction site specific to group II);
– +U in position 269 (specific to group I)

Most of these changes are located in the left part of the secondary structure of the molecule (Fig. 4) where the pathogenicity domain of PSTVd is located. As also found for the original isolate [10], most changes appear to be clustered in the left of the viroid secondary structure, and therefore this should be regarded as a variable region. Additional changes also have been found in the lower strand of the Central and the Right Terminal domains. As shown by Ambrós et al., with *Peach latent mosaic viroid* (PLMVd), the generation of new variants is limited to conserve the viroid structure and therefore its properties [1]. The variants recovered in the progeny of CBLVd present changes that do not affect the viroid secondary structure (change A to G in position 276) and compensatory mutations (U to A in position 61 and A to U in position 272) that were not identified in the two subpopulations of the original isolate. The variants found in the progeny presented changes affecting 1–8 nucleotides indicating the existence of lower variability than found by Ben-Shaul et al., [3]. However, this progeny differed in 4–13 nucleotides from the inoculated haplotype, indicating a quick evolution of the viroid population.

The phylogenetic tree obtained from the analysis of the progeny illustrates the relationship among the recovered haplotypes (Fig. 5) that appear to be clustered in two main branches depending on whether or not they retained the *Sal* I restriction site. The parental haplotype (CBLVd-10) and two haplotypes recovered form the progeny (CBLVd-30, CBLVd-12), all containing the *Sal* I restriction site, are located in a central position from which two groups of divergent sequences arose. The upper branch of the phylogenetic tree contains a cluster of the 5 haplotypes that retained the *Sal* I restriction site characteristic of the inoculated haplotype and 4 changes in positions 61, 247, 272 y 276 (Table 1). CBLVd-12 represents the evolutionary link between this group and the parental haplotype. The lower branch contains the two haplotypes (CBLVd-7, CBLVd-17) that lost the *Sal* I restriction site and as a consequence are clustered together with the representative of subpopulation I (CBLVd-18). CBLVd-20 which retained *Sal* I restriction site but contained changes characteristic of the two major groups can be considered as the evolutionary link between both subpopulations.

The results of the present study confirms that CBLVd follows the quasispecies model [7], and that a population of closely related variants is quickly generated upon infection with a single infectious variant. The heterogeneity of the progeny ($H = 0.7785$), estimated according to the Nei's formula [18] ($H = n/(n-1)$

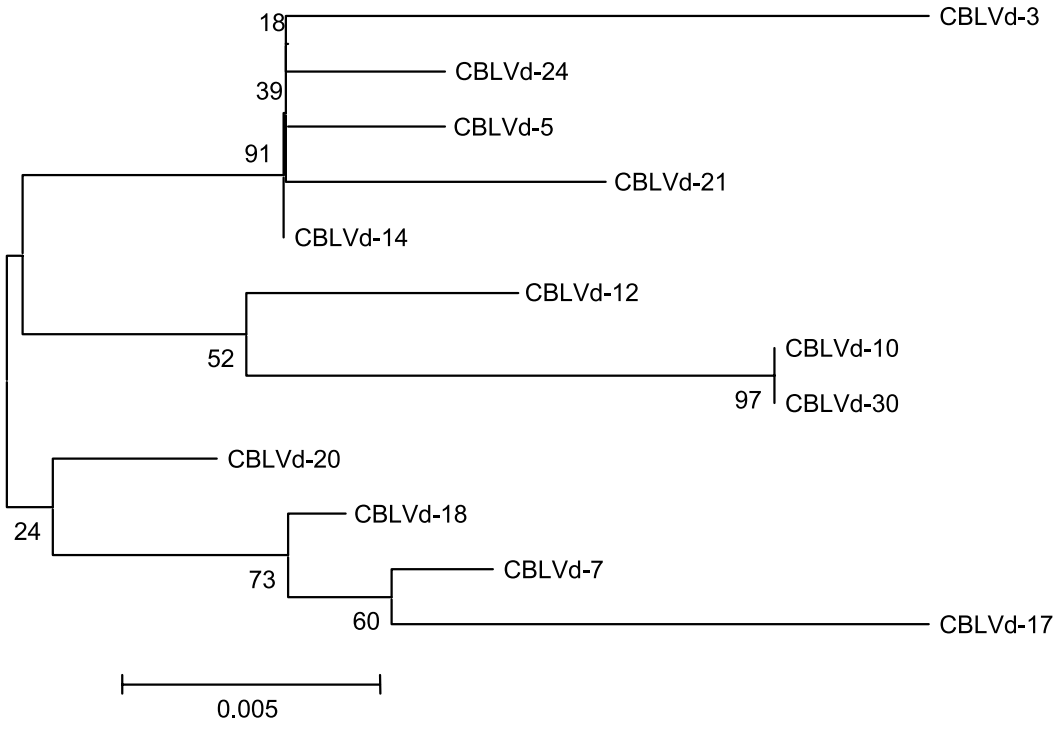

Fig. 5. Phylogenetic tree of CBLVd variants using MEGA 2.1 program (17). Distances were calculated with Jukes-Cantor method (16) and phylogenetic tree was obtained with Neighbor-joining method (20). The analysis was made 1000 times and the numbers refer to bootstrap confidence level

$(1-\sum Xi^2)$, where n is the number of clones analysed and Xi is the population frequency of each variant), was similar to that of the original source ($H = 0.735$) characterized by Foissac and Duran-Vila [10]. However, whereas the original source contained two major subpopulations with two fitness peaks connected by a continuum around them, after infection the progeny generated with a single haplotype presented a single peak sharing features of both subpopulations.

Given the small number of inoculated plants, differences in infectivity and symptom expression between haplotypes cannot be inferred with the results of the present study. However, the generation of a progeny sharing features of subpopulations I and II contained in the original isolate indicates that infection with variants from subpopulation I is not required to produce a population with the characteristics found in the natural isolate. In contrast to the original isolate that requires an incubation period of only 3 months, the infectious variant required a longer incubation period before the viroid could be detected and symptoms observed. This observation indicates that individual variants may not exhibit the same biological properties as the isolate as a whole. In fact, the analysis of the progeny demonstrated that symptom expression was associated with a new heterogeneous population of closely related variants with a new fitness peak represented by a new haplotype that was not identified in the original isolate but appears to

have displaced the inoculated haplotype. The overall progeny population shares characteristics of the two subpopulations identified in the original isolate with most of them retaining the *Sal* I restriction site.

Genbank accession numbers

Genbank accession numbers for variants generated by CVd-I clone 10 are AY226156 to clone 20; AY226157 to clone 12; AY226158 to clone 24; AY226159 to clone 5; AY226160 to clone 3; AY226161 to dominant variant clone 14; AY226162 to clone 21; AY226163 to clone 17 and AY226164 to clone 7 respectively.

Acknowledgements

The authors thank Carmen Vives and Susana Martin for their help on computer analysis. This work was supported by an IVIA predoctoral fellowship to the first author and projects SC97-108 and RTA01-119 provided by MCyT.

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