Genomic Diversity Among Populations of Two Citrus Viroids from Different Graft-Transmissible Dwarfing Complexes in Israel

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Dedicated in memory of Schmuel Ashkenazi (1937–1993) a pioneer in the field of application of viroids for citrus intensification in Israel.

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ABSTRACT

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The nucleotide sequences of citrus bent leaf viroid (CBLVd) (formerly designated CV-Ib) and citrus exocortis viroid (CEVd), both found among the citrus viroid (CVd) populations from five graft-transmissible dwarfing complexes (GTDCs) originating from different source plants and geographical locations in Israel, were determined. The sequence homology varied only slightly among the CBLVd sequence variants, i.e., 0–7 nucleotides (nts); originating from a single GTDC and 2–8 nts between CBLVds that were obtained from different GTDCs. The lowest level of homology between CBLVd variants obtained from citrus was 97.5%. Considerably larger variation (8–15 nucleotide changes) was observed between CBLVd variants derived from citrus and the type strain that was passed through avocado seedlings. The type strain differed from all variants in six positions, located at 38, 62, 138, 179, 264, and 268 nts. The CEVd sequence variants showed considerable heterogeneity. Five variants derived from four GTDCs differed only in 2–9 nts. Five other variants derived from two GTDCs showed 27–50 nucleotide changes compared with the first group of CEVd variants. The largest variation within a single GTDC of up to 41 nucleotide changes was observed between CEVd variants derived from GTDC-G. Using CEVd #225 as a reference strain, most of the nucleotide changes occurred in the V, LT, and RT domains. Additional changes in the P domain were found only among CEVd isolates derived from GTDC-M and GTDC-G. The sequence homologies to CEVd #225 ranged from 89.2 (CEVd-G_2) to 99.0% (CEVd-NG_1).

Additional keywords: chimeric viroids, viroid pathogenicity.

Viroids are the smallest known agents of infectious diseases. Their genome size ranges from 246 to 600 nucleotides (nts) and consists of a highly structured single-stranded circular RNA molecule that lacks a capsid protein and detectable mRNA activity (5,6,8). At present there are about 20 known viroid species, grouped into two families according to the scheme of Koltunow & Rezaian (15).

Old-clone citrus trees from different geographical areas were found to host a range of citrus viroids (CVds), which were cataloged in five groups according to their molecular and biological properties (10). The type members of four of these groups have been characterized and found to consist of citrus exocortis viroid (CEVd) (368–371 nts) (13), hop stunt viroid (HSVd) (299 nts) (17), and two natural chimeric viroids: CVd-IV of 284 nts (18) and citrus bent leaf viroid (CBLVd, formerly designated as CV-Ib) of 318 nts (2). CVd-IV has been found to be composed of the right terminal (RT) domain and the central conserved (CCR) domain of CEVd and the left terminal (LT) domain of HSVd. CBLVd has been found to be composed of the LT and the pathogenic (P) domain of CEVd and the CCR domain of apple scar skin viroid (ASSVd).

This paper presents sequence analyses of five CEVd and five CBLVd variants originating from five different graft-transmissible dwarfing complexes (GTDCs) from Israel. Sequence homologies of 97.5–99.4% were found among the CBLVd isolates, and 89.2–99.0% among the CEVd isolates.

MATERIALS AND METHODS

GTDC isolates, propagation and isolation of CVds. Five GTDCs that have been previously used for experimental dwarfing of different citrus cultivars in Israel (1,4) were selected for this study.

GTDC #225 was obtained from an old-clone, dwarfed grapefruit (*Citrus* × *paradisi* Macfady) tree grafted on Troyer citrange rootstock at the 'Akko Experiment Station (northern coastal plain) (11).

GTDC-NG originated from a Shamouti sweet orange (SwO) (*C. sinensis* (L.) Osbeck) tree on Palestine Sweet Lime (PSL) rootstock at Nir Galim (southern coastal plain). Shamouti buds from GTDC-NG grafted on PSL rootstock seedlings in the nursery produce medium-size trees with very mild xyloporosis symptoms.

GTDC-M was collected from an Interdonato lemon (*C. limon* (L.) N. L. Burm.) tree, originally introduced from Italy and grafted on a rough lemon (*C. jambhiri* Lush.) rootstock at Mesilot, in the Bet She'an Valley, the northern inland part of Israel. GTDC-M causes severe stunting, scaling, and gumming on young grapefruit trees grafted on Rangpur lime. GTDC-M inoculated trees tend, however, to recover and start to perform well about 2–3 yr after inoculation.

GTDC-G was collected from a nursery-grown grapefruit plant grafted on Rangpur lime rootstock that was inoculated with GTDC #225. Plants of this combination have shown excessive scaling and gumming, shortly after inoculation.

GTDC-K originated from a Temple orange tree on sour orange rootstock at Kefar Yona (central coastal plain). Several mandarin

trees that were inoculated with GTDC-K were found to be free of cachexia symptoms.

Buds from each source were chip grafted on Etrog citron scions grafted on Volkameriana rootstocks. The plants were maintained in a glasshouse with temperatures ranging between 25 and 35 C. Leaf tissue collected 4–6 mo after inoculation was used for viroid RNA extraction.

Viroid RNA extraction. RNAs were extracted according to Semancik et al (22) with a minor modification (13). The nucleic acid extracts were loaded on small CF-11 (Whatman) columns (prepared in 1-ml tips), washed three times, each with 1 ml of a solution containing 1× STE (50 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.0) and 30% ethanol, eluted with 400 μl of 1× STE and precipitated with ethanol. The RNA pellet was washed twice with 75% ethanol, dried, and resuspended in water; the presence of CVds was tested by sequential polyacrylamide gel electrophoresis (sPAGE) (20).

Infectivity assays on Rutgers tomato plants. The plants were mechanically inoculated at the cotyledonary stage by gently rubbing RNA extracts from each GTDC isolate. The inoculated plants were maintained in a temperature-controlled (30±5 C) cabinet. The presence of CEVd in the inoculated plants was assayed by sPAGE and the intensity of symptom reactions was monitored for several weeks.

cDNA synthesis and polymerase chain reaction. The phosphorylated primers 5-GCCTTCGTCGACGACGAC (oC-CBLVd) complementary to CBLVd (90–107) and the sense orientation 5-TCGTCAGCTGCGGAGGTT (oS-CBLVd-108-125) were used for synthesis of full-length cDNA to CBLVd. The primers 5-CGGGGATCCCTGAAGGACTT (oC-CEVd-77-96) and 5-GGGAAACCTGGAGGAAGTCG (oS-CEVd-97-116) were used for synthesis of cDNA to CEVd. The viroid RNA extracts (100 ng in 7 μl ddH_2O) were mixed with 2 μl (600 ng) of the complementary primer and denaturated by incubation for 10 min at room temperature with 100 mM methyl mercury (3).

cDNA synthesis was carried out for 60 min at 42 C, in a 25-μl reaction mixture containing reverse transcriptase buffer (50 mM Tris, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, dNTPs (Promega, Madison, WI) (1 mM each), 40 U RNase inhibitor (Boehringer, Mannheim, Germany) and 25 U AMV reverse transcriptase (Promega).

Two microliters of the cDNA synthesis mixture was transferred to a 100-μl reaction mixture containing 1× Vent DNA polymerase buffer (New England Biolabs, Beverly, MA), 100 μg bovine serum albumin, dNTPs (0.3 mM each), 600 ng oC. primer, 600 ng of the phosphorylated oS primer, and 2U Vent DNA polymerase (New England Biolabs). The cDNA was amplified using the Hybaid apparatus first for one cycle of 2 min at 96 C, 2 min at 58 C, and 2 min at 72 C, followed by 35 cycles each of 1 min at 96 C, 1 min at 58 C, and 2 min at 72 C. At the end of the reaction, a 10-μl aliquot of the polymerase chain reaction products was analyzed by gel electrophoresis in 1% agarose gel.

Cloning. The polymerase chain reaction products were separated on a 1% agarose gel and eluted by using the Qiaex gel extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The separated DNA molecules were ligated to *Eco* R-V restricted pBluescript vector (Stratagene, La Jolla, CA).

TABLE 1. The distribution of citrus viroids (CVds) in five graft-transmissible dwarfing complexes (GTDCs) from Israel

CVd (nts[a]) GTDC	371	318	305[b]	299	295[b]	290[b]	284
#225	+	+		+	+		+
NG	+	+		+	+	+	+
G	+	+	+	+	+		+
K	+	+	+			+	+
M	+	+	+	+	+	+	+

[a] Nucleotides.
[b] Estimated by sequential polyacrylamide gel electrophoresis (sPAGE).

Transformation of *Escherichia coli* JM 101 cells and plasmid isolation were done according to standard procedures (16).

Sequencing and computer assistance. The DNA for the sequencing reaction was prepared by using the plasmid midi preparation kit columns (Qiagen). Sequencing of both strands was performed with the Sequenase version 2 kit (USB, Cleveland, OH) according to the manufacturer's instructions and with Applied Biosystems, Model 373A. Nucleotide sequences and the optimum secondary structures of lowest free energy of the five CBLVd and CEVd sequences were analyzed by the UWGCG programs SEQED, BESTFIT, PRETTYBOX, FOLD RNA, and PILEUP (7).

RESULTS

sPAGE analysis of the CVd composition of five GTDCs. The CVd composition of five GTDCs originating from old-clone citrus sources in Israel is shown in Table 1. All five GTDCs contained RNA bands corresponding to CEVd (371 nts), CBLVd (318 nts), and CVd -IV (284 nts). The CVds of 299 nts and CVds of approximately 305, 295, and 290 nts were present in only some of the five GTDCs. Interestingly, GTDC-G contained an extra band with an estimated size of 305 nts. Similar size bands were also found in plants inoculated with GTDC-M and -K.

Molecular cloning and sequence analyses of CBLVd strains. Figure 1 shows the sequences of 10 CBLVd clones from five GTDCs isolates from citrus, compared with the type strain of CBLVd (225A) that was previously isolated after transfer to avocado (12). The degree of sequence homology varied only slightly among the 10 citrus sequence variants. Table 2 shows that clones 225_1 and NG_1, clones NG_2 and M_2, and clones G_3 and K_1, differed in only 2 nts (99.4% homology). The largest variation of 8 nts was noted between NG_1 and 225_2 and between NG_1 and G_1 and G2 (97.5% homology). The variation among clones derived from a single GTDC ranged from 0 to 7 nts (Table 2, values in brackets). The largest variation (8–15 nucleotide changes) was observed between CBLVd variants derived from citrus and the type strain that was passed through avocado seedlings (12). The type strain CBLVd #225A differed from all other CBLVd isolates in six positions located at 38, 62, 138, 179, 264, and 268 nts.

Molecular cloning and sequence analysis of CEVd. Figure 2 shows the sequences of 13 CEVd clones that were obtained from five GTDCs. The degree of homology among the CEVd clones varied. Table 3 shows that clones 225, NG_1, K_1, K_2 and G_1 differed in only 2–9 nts, whereas clones derived from GTDC-M and clones G_2, G_3, and G_4 derived from GTDC-G differed from clones derived from GTDC #225, K, and NG by 27–50 nucleotide changes. The variation among clones derived from a single GTDC ranged from 1 to 41 (Table 3, values in brackets). The largest variation within a single GTDC of up to 41 nucleotide changes was observed between CEVd sequence variants derived from GTDC-G.

Sequence comparisons among the 13 CEVd clones using CEVd #225 as a reference isolate showed several base exchanges (Table 3). These changes were predominantly in the V (123-152/217-245), LT (1-48/328-380) and RT (152-216) domains. Additional changes in the P (49-77/295-327) domain, including the central core region (P_L), which was previously found to be responsible for modulating symptoms on sensitive tomato plants, were found only in CEVd-M and three isolates from CEVd-G. Changes in the P_L region of CEVd-M that are expected to affect the secondary structure of this molecule were indicated by computer analysis. Similar changes have been previously recorded for other CEVd isolates causing severe symptoms on tomato plants (26).

Sequence homologies to CEVd #225 ranged between 89.2% (CEVd-G_2) and 99.0% (CEVd-NG_1). In the P domain of all 13 CEVd clones sequenced there is a two base variation (positions 61 and 64) that had no effect on CEVd-sensitive tomato plants.

The nucleotide sequences of the 13 CEVd strains were compared with the published sequence of a CEVd strain showing severe (CEV-A) and mild effects on tomato plants (CEV-DE26) (24). Sequence homologies were between 92.9% (CEVd #225) and 98.4% (CEVd-M_1) when compared with CEV-A, and between 91.6%

(CEVd-G_2) and 95.9% (CEVd-K_1, NG_1) when compared with CEV-DE26 (Table 3). Only CEVd-M was found to cause severe symptoms on Rutgers tomato plant.

DISCUSSION

Previous sPAGE analyses of CVds in GTDC #225, the main source of inoculum for grapefruit dwarfing in Israel (4,13), indicated the presence of five CVds with estimated molecular sizes of 284, 295, 300, 318, and 371 nts (13). sPAGE analyses of four additional GTDCs originating from different old-clone plants from several locations in Israel indicate that CEVd, CBLVd, and CVd-IV were present in each of the main natural GTDCs currently used for experimental citrus dwarfing. Three other CVds with estimated molecular sizes of 305, 295, and 290 nts, and the

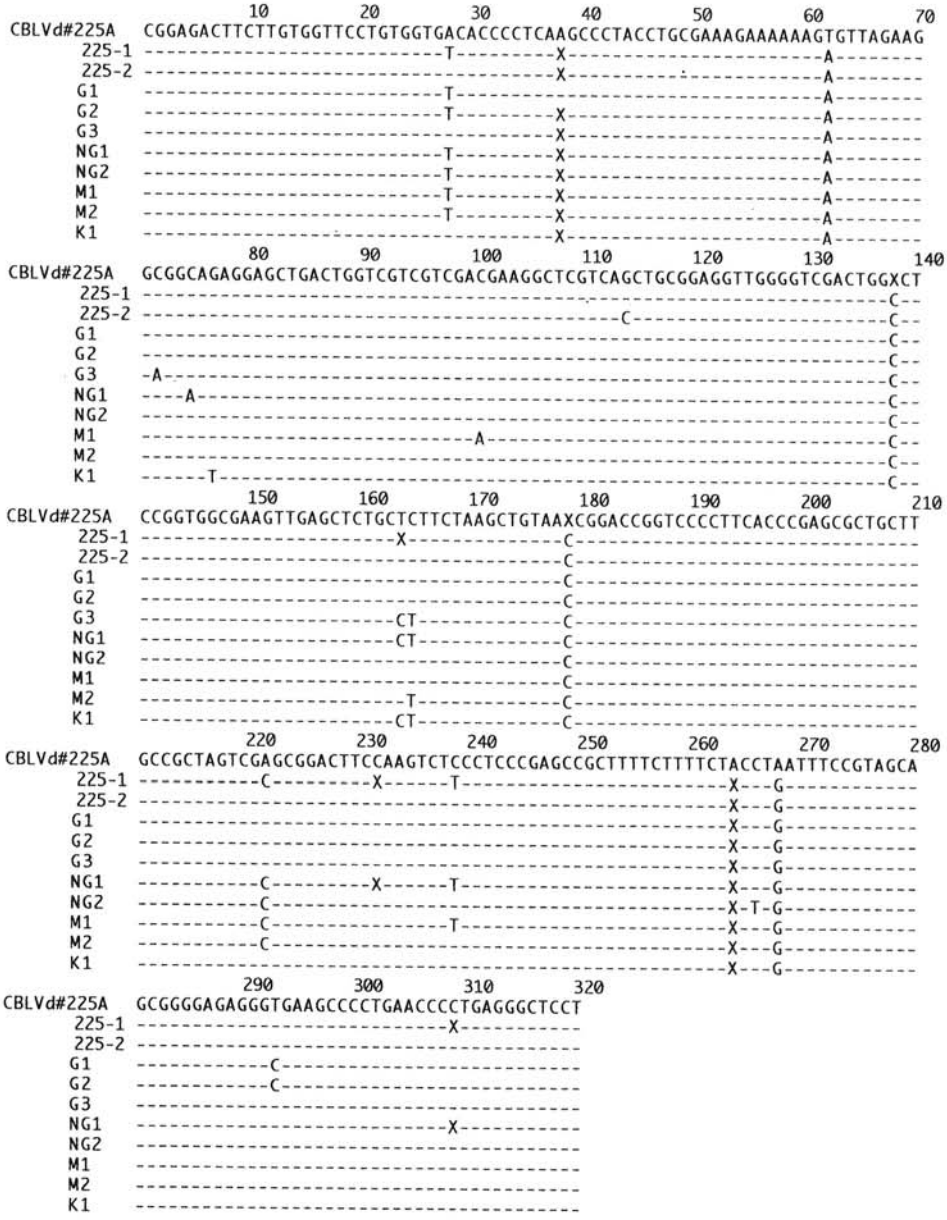

Fig. 1. Sequence analyses of ten citrus bent leaf viroid (CBLVd) sequence variants compared with a CBLVd-A (2). x = missing nucleotides.

TABLE 2. Number of nucleotide differences among citrus bent leaf viroid (CBLVd) sequence variants

	225A[a]	225_1	225_2	G_1	G_2	G_3	NG_1	NG_2	M_1	M_2	K_1
225A[a]	—	13	8	9	9	10	15	10	11	10	10
225_1		—	[7][b]	5	6	7	2	5	4	5	6
225_2			—	3	3	4	8	4	5	4	4
G_1				—	[0]	5	8	3	4	3	5
G_2					—	[5]	8	3	4	3	5
G_3						—	7	6	7	4	2
NG_1							—	[6]	6	5	7
NG_2								—	3	2	6
M_1									—	[3]	7
M_2										—	4
K_1											—

[a]CBLVd 225A isolate was obtained after transfer to avocado.
[b]Values in brackets show differences among sequence variants derived from the same graft transmissible dwarfing complexes (GTDCs).

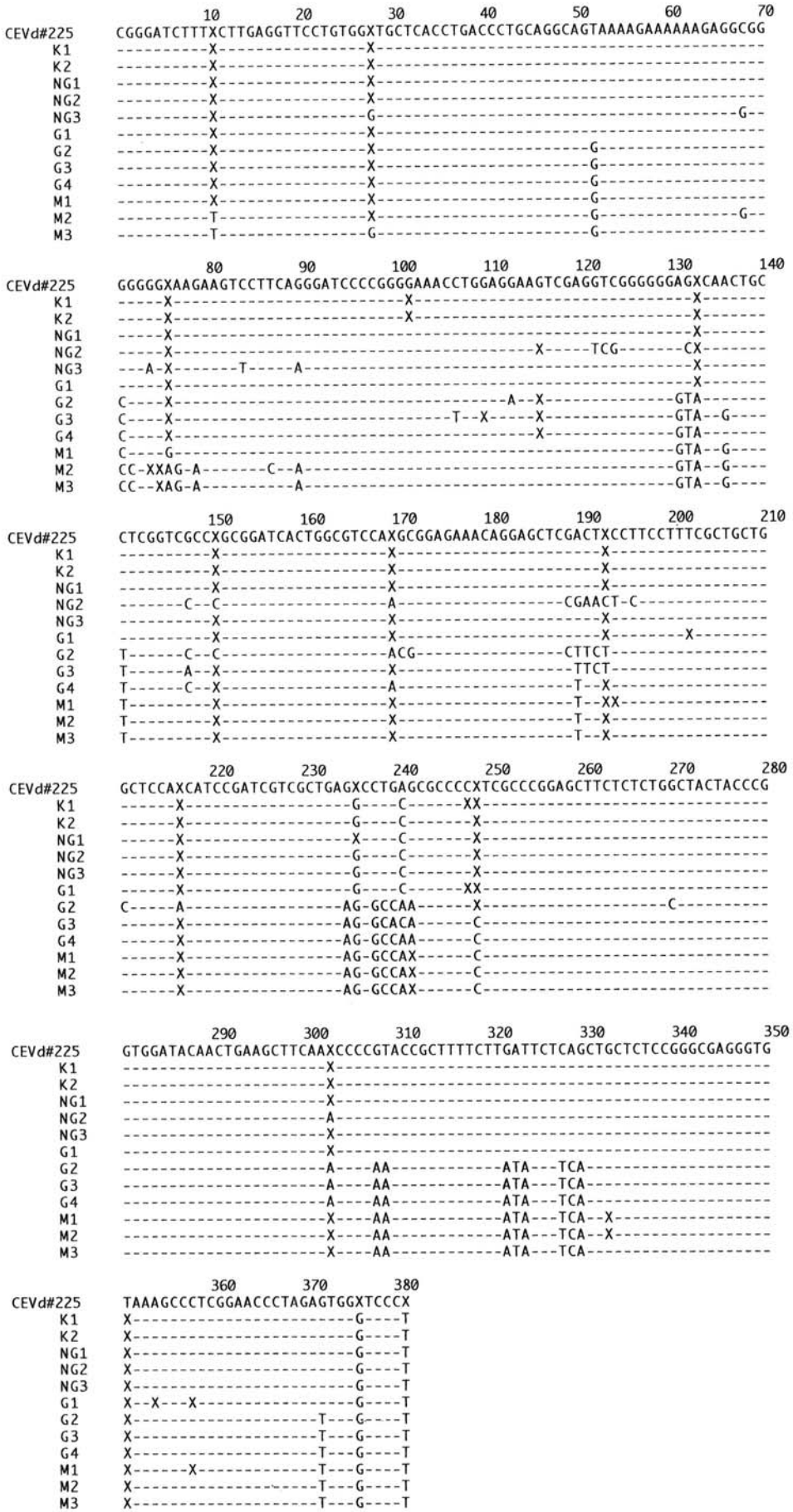

Fig. 2. Sequence analyses of twelve citrus exocortis viroid (CEVd) variants compared with a CEVd isolate from GTDC #225. x = missing nucleotides.

TABLE 3. Number of nucleotide differences among citrus exocortis viroid (CEVd) sequence variants

	225	K_1	K_2	NG_1	NG_2	NG_3	G_1	G_2	G_3	G_4	M_1	M_2	M_3
225	—	7	6	4	21	10	9	40	36	30	32	39	35
K_1		—	[1][a]	3	18	7	4	37	33	29	30	38	34
K_2			—	2	17	6	5	38	32	28	29	37	33
NG_1				—	[17]	6	5	37	30	27	30	37	33
NG_2					—	[21]	19	36	38	34	42	50	46
NG_3						—	9	41	36	32	34	36	33
G_1							—	[41]	34	31	30	40	36
G_2								—	[16]	12	23	30	26
G_3									—	[10]	16	23	19
G_4										—	10	18	13
M_1											—	[12]	12
M_2												—	[6]
M_3													—
[b]CEV-A	92.9	94.0	92.9	93.8	91.0	91.3	93.7	95.9	97.6	97.8	98.4	96.7	97.6
[b]CEV-DE26	95.0	95.9	95.6	95.9	91.9	92.9	95.6	91.6	92.9	93.5	94.3	93.2	93.8

[a]Values in brackets show differences among sequence variants derived from the same graft transmissible dwarfing complexes (GTDCs).
[b]Percentage of similarity to two previously characterized CEVds (A and CEV-DE26).

HSVd-like CVd of 299 nts, were present in some of the GTDCs. Similar populations of CVds have been previously described from other geographical areas (9–11).

The genomic sequences of 10 CBLVd and 13 CEVd cDNA clones that we obtained from five different GTDCs showed a high degree of homology, ranging between 97.5 and 99.4% for the CBLVd isolates and 89.2 and 99.0% for the CEVd isolates. Interestingly, CBLVd #225 A, which was passed through an avocado plant, shows 8–13 nucleotide differences with 225_1 and 225_2 and 9–15 nucleotide differences with the four other CBLVds. Changes in six base positions were unique for the avocado isolate and not found in the other CBLVd sequence variants maintained in citron. This variation might have been caused by a specific selection process for a given sequence variant in the avocado host. A similar situation for host selection had been previously reported for CEVd isolates passed through tomato (23). Additional trials to transfer other CBLVd sequence variants to avocado will be needed in order to establish the role of alternating host on CBLVd genomic diversity.

Sequencing four CEVd sequence variants derived from GTDC-G showed the presence of a variant closely related to CEVd #225 as well as three variants that closely resembled CEV-M. This information, taken together with the CVd picture presented in Table 1, suggests the possibility that GTDC-G originated from the double inoculation of GTDC #225 and GTDC-M on a common host. The unusually severe reaction of grapefruits grafted on Rangpur lime to GTDC-G in the nursery was thus caused by additional factors originating in GTDC-M or from the possible combined effects of the two GTDCs. Attempts are being made to study whether the contamination resulted from double infection of the GTDC-G budwood source.

Field isolates of CEVd were previously grouped into two classes—A (pathogenic) and B (mild)—according to their pathogenic effect on tomato plants (25). Comparing the sequences of the presently isolated CEVd clones with two previously studied CEVds, CEVd-A (the representative of class A) and CEVd-DE26 (class B), further suggests placing all three CEVd-M isolates and three of four CEVd-G isolates in class A and the other CEVds in class B (Table 3).

The P and LT domains of CBLVd #225 and -M_1 were compared with the same domains in their respective CEVd strains. The P domain of CBLVd was found to have a high degree of sequence homology with the P domain of CEVd. It is interesting to note, however, that the presence of such a P domain in CBLVd had little if any effect on the pathogenic nature to Etrog citron or to its avocado hosts (13). This is consistent with the findings of Sano et al (21), who have reported that, in addition to the P domain, the LT loop and the RT loop also make a significant contribution to viroid pathogenicity.

It was apparent that the common region of CEVd and CBLVd molecules originating from a single GTDC source did not show greater similarity than those found among CEVd and CBLVd molecules, from different GTDCs. This probably suggests that neither of these CEVds was the original source for the recombination event leading to the construction of the chimeric CBLVd molecule.

The possibility of recombination among viroid (14,19) and virus (5,27) RNAs is now the focus of much attention. It is interesting to note that the presently known chimeric viroids in citrus appear to cause milder symptoms than their parent viroids. This is consistent with the possible evolutionary advantage for viroid molecules not to cause severe debilitation of their respective host plants (4,26).

Sequencing data offers a useful means for locating the possible sources of plant epidemics. Thus, besides its present use for research, sequencing is expected to become in the future an important tool for enforcing quarantine vigilance measures against the introduction of novel strains of plant pathogens. Similar technologies are already applied for forensic medicine.

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