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.

The nucleotide sequences reported in this paper were submitted to EMBL, GenBank, and DDBJ databases and assigned the accession numbers U21125 and U21126.

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ABSTRACT


The nucleotide sequences of citrus bent leaf viroid (CBLVd) (formerly designated CV-1b) 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 CBLVd 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) to 99.0% (CEVd-NG).

Additional keywords: chimeric viroids, viroid pathogenicity.

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) (II).

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 xylemoporosis symptoms.

GTDC-M was collected from an Italian lemons (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 Be Sh'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 seedstock 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...
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 UWCGG 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 analysis of CBLVd strains. Figure 1 shows the sequences of 10 CBLVd strains from five GTDCs isolated 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, and NG1, clones NG2 and M2, and clones G1 and K1, differed in only 2 nts (99.4% homology). The largest variation of 8 nts was noted between NG1 and 225, and between NG2 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 #225 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, NG1, K1, K2, and G1 differed in only 2–9 nts, whereas clones derived from GTDC-M and clones G3, K3, and G4 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 changes (Table 3). These changes were predominantly in the V (125–152/217–245), LT (1–88/328–380) and RT (152–216) domains. Additional changes in the P (49–77/295–327) domain, including the central core region (P4), 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 P4 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-G4) and 99.0% (CEVD-NG). In the P domain of all 13 CEVD clones sequenced there is a 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-M4) when compared with CEV-A, and between 91.0%
(CEVd-G₂) and 95.9% (CEVd-K₁, NG₁) 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-clones 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

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**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**

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* CBLVd 225A isolate was obtained after transfer to avocado.
* 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

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*CEV-A  92.9  94.0  92.9  93.8  91.0  91.3  93.7  95.9  97.6  98.4  96.7  97.6  93.8
*CEV-DE26  95.0  95.9  95.6  95.9  91.0  92.9  95.6  91.6  92.9  93.5  94.3  93.2  93.8

*Values in brackets show differences among sequence variants derived from the same graft transmissible dwarfing complexes (GTDCs).

**Percentage of similarity to two previously characterized CEVs (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, and 225, and 9−15 nucleotide differences with the four other CBLVd. Changes in six base positions were unique for the avocado isolate and not found in the other CBLVd sequence variants maintained in Citrus. 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 grapesfrits 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 CEVd-M in class B (Table 3).

The P and LT domains of CBLVd #225 and -M₁ 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 Etrug citrus 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 CEVd 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 not 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.

LITERATURE CITED


