

# Direct Nucleotide Sequencing of PCR-Amplified DNAs of the Closely Related Citrus Viroids IIa and IIb (Cachexia)\*

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**ABSTRACT.** Citrus viroids IIa (CVdIIa) and IIb (CVdIIb=cachexia), members of the hop stunt viroid (HSVd) group, can be distinguished by their biological properties in Etrog citron. CVdIIa and CVdIIb isolates from Florida were reverse transcribed and subsequently amplified *in vitro* from total nucleic acid extracts of infected citrus tissue using a reverse transcription-polymerase chain reaction assay (RT-PCR). The amplified products were gel purified, and subsequently sequenced using five distinct sequencing primers containing different segments of the HSVd prototype sequence. These products were sequenced without cloning or the generation of asymmetric PCR products using the *fmol*<sup>TM</sup> sequencing system. CVdIIa is 302 nucleotides in length. CVdIIb (cachexia) was determined to be 299 nucleotides in length and differs from CVdIIa by 3 nucleotide deletions and 2 nucleotide substitutions. CVdIIa and CVdIIb share 3 nucleotide substitutions which differed from the two published Japanese HSVd citrus variant sequences. CVdIIa shares greater than 99% sequence similarity with the Japanese HSVd variants. The HSVd variant associated with the grapefruit dwarfing agent from Israel is similar in size to CVdIIb, however these two viroids have very different biological properties and shares only 96% similarity with the published sequence.

*Index words:* citrus, viroids, cachexia, RT-PCR, sequencing.

Citrus trees are known to be naturally infected with five distinct viroid groups. These groups are characterized by molecular size, as determined by electrophoretic mobility and nucleotide sequencing, nucleic acid homology, as determined by hybridization with group-specific probes, and biological properties in various hosts, specifically Etrog citron (4, 5, 6, 7, 10). Presently, the best characterized citrus viroid is citrus exocortis viroid (CEVd), the causal agent of the exocortis bark shelling disease in some citrus species (9). In citron, exocortis induces severe stunting, leaf epinasty, and vein necrosis. During evaluation of citrus infected with CEVd in Japan, what was thought to be a mild strain of CEVd was found in citron. When mechanically transmitted to curcubits, this putative mild CEVd variant induced symptoms indistinguishable from hop stunt viroid (HSVd). Sequence analysis of this viroid confirmed it was a vari-

ant of HSVd (6). To date, nine HSVd variants exist, three of which infect citrus (4, 10).

HSVd variants infecting citrus possess physical and biological properties similar to the group II citrus viroids (CVdII) and are classified as such (8). These include CVdIIa, the causal agent of a mild exocortis disease of citrus, and CVdIIb the causal agent of the cachexia disease of citrus. Under field conditions, CVdIIa induces a mild bark cracking in trifoliolate orange and no reaction in mandarin or tangelo. CVdIIa induces leaf-tip browning, petiole wrinkling, and mid-vein necrosis in citron under controlled greenhouse conditions. CVdIIb causes the cachexia disease in citrus (8). In the field CVdIIb is symptomless in trifoliolate orange and induces gumming, wood pitting, and bark plugging in mandarin and tangelo. In citron, under greenhouse conditions, CVdIIb is characterized by a latent infection (7).

CVdIIa and CVdIIb differ in size by only a few nucleotides (7, 11), yet they cause two distinctly different diseases of citrus. Sequencing of CVdIIa and CVdIIb should establish their relatedness to each other, and to the Japanese HSVd citrus variants (10),

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and to the HSVd variant associated with the grapefruit dwarfing agent (HGda) (4). This paper describes the sequencing of purified reverse transcriptase-polymerase chain reaction (RT-PCR) products of CVdIIa and CVdIIb. The sequences are compared to each other and to existing HSVd variants from citrus. The implications of these findings in the rapid diagnosis of cachexia viroid at the nucleotide level is discussed.

## MATERIALS AND METHODS

**Viroid sources.** Viroid samples were collected from systemically infected Etrog citron or Madame Vinous sweet orange at Orlando, Florida. The Etrog plants were rooted cuttings of selection Arizona 861, and Madame Vinous plants were propagated on Carrizo citrange seedlings. Source plants were maintained in Florida in a partially shaded greenhouse with day temperatures from 23-30 C and night temperatures from 20-24 C. Florida viroid isolates used were X7 and E22. Sequential polyacrylamide gel electrophoresis (SPAGE) analysis revealed that X7 contained CVdIIb (cachexia), no CEVd or CVdIIa, and E22 contained CEVd, CVdIII, no CVdIIb, and was unknown for CVdIIa. Parsons Special mandarin test, a sensitive bioassay for the detection of cachexia (CVdIIb), showed X7 to be positive and E22 negative for the presence of CVdIIb (2).

**Nucleic acid extraction and purification.** Total nucleic acids (TNA) were extracted from shoot tissue as described (11). Briefly, 1 g of tissue was ground in liquid nitrogen and extracted with 5 ml of buffer consisting of 0.1M glycine-sodium hydroxide, pH 9.0, 50 mM sodium citrate, 1 mM disodium ethylenediamine tetraacetic acid (EDTA), 2% sodium lauryl sulfate (SDS), and 1% sodium lauryl sarcosine. Samples were further extracted with the addition of an equal volume of Tris-HCl buffered phenol, pH 7.6-8.0 containing 0.1% 8-hydroxyquinole and 0.2% 2-mercaptoethanol, and a sub-

sequent addition of an equal volume of chloroform. Nucleic acids were pelleted twice with 2.5 volume of ethanol plus 1/10 volume of 3M sodium acetate (pH 5.2) at -20 C. Nucleic acid preparations were further purified using RNase-free ELUTIP™-R minicolumns (Schleicher and Schuell, Keene, NH) according to the manufacturer with the following modification: low salt buffer contained 0.2M sodium chloride, 20mM Tris-HCl, pH 7.4, and 1.0mM EDTA.

**Viroid cDNA synthesis.** One g of TNA, determined spectrophotometrically, was added to 3µg of HSV<sub>1</sub> 19-mer primer (5'-GGCTCCTTTCTCAGGT-AAG-3') (synthesized by Synthecell Inc., Rockville, MD) complementary to HSVd variant nucleotides 61-79 of the upper central conserved region, or RAOH278 20-mer primer (5'-CGCGG-CAGAGGCTCAGATAG-3') complementary to the prototype HSVd nucleotides 259-278 of the lower left terminal domain (9). The primer-TNA mixture was added to 6 µl of 5X first strand cDNA buffer (250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM MgCl<sub>2</sub>, 15mM dithiothreitol) and deionized water to a final volume of 30 µl and denatured by heating at 100 C for 5 min, chilled on ice for 2 min, and annealed at room temperature for 1 hr. The following reagents were then added to the reaction mixture: 4 µl 5X first strand cDNA buffer, 5 µl 300mM 2-mercaptoethanol, 2.5 µl 10mM dNTP (2.5mM each of dATP, dGTP, dTTP, dCTP), 1 µl RNasin (40 units/µl, Promega Corp., Madison, WI), 2.0 µl of cloned Maloney murine leukemia virus reverse transcriptase (200 units/µl, GIBCO BRL Life Technologies, Inc., Gaithersburg, MD), and deionized water to a volume of 50 µl. The reaction was incubated at 42C for 2.5 hr.

**PCR amplification.** PCR reactions contained 5 µl of either the CVdIIa or the CVdIIb cDNA reaction added to a 45 µl reaction mixture containing 1X PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl<sub>2</sub>, and 0.001% gelatin), 200 mM dNTP(dGTP, dATP, dTTP, and dCTP), 1.2µM each

of either the HSV<sub>1</sub> 19-mer and the HSV<sub>2</sub> 25-mer primer (5'-CCGGGGCAACTC-TTCTCAGAATCCA-3'), homologous to HSVd variant nucleotides 80-104, or the RAOH278 20-mer and the RAOH279 20-mer primer (5'-GATCCTCTCTTG-AGCCCCTC-3') homologous to HSVd prototype nucleotides 279-299-1, 2 units of Taq DNA polymerase (Promega Corp.) and deionized water. The reaction was overlaid with 75  $\mu$ l of mineral oil to prevent evaporation during amplification. Cycling parameters were 1 min at 94 C, 2 min at 55 C, and 3 min at 72 C for 40 cycles with the final extension at 72 C for 7 min in a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT).

**Analysis and isolation of PCR products.** RT-PCR products were analyzed by electrophoresis on 6% native polyacrylamide gels (11 x 14 x .12 cm) at 120 V for 2.5 hr in 1X TBE (89mM Tris, 89mM borate, and 2.5mM Na<sub>2</sub>EDTA, pH 8.3). Separated PCR products were visualized with silver nitrate (11). PCR products for isolation were stained with ethidium bromide. The major RT-PCR product for each viroid and primer combination were excised from the gel and isolated by the crush and soak method of Maniatis (3).

**Sequencing of isolated PCR products.** One-half to 1 ng of gel purified PCR products, determined spectrophotometrically, were sequenced using 5' end-labeled primer in the *fmol*<sup>TM</sup> sequencing system (Promega Corp., Madison, WI). The sequencing reaction was performed in a thermocycler following the recommended parameters: denaturation at 95 C for 30 sec, annealing at 42 C for 30 sec, and extension at 70 C for 1 min for a total of 30 cycles. The sequencing primers used and their position within the prototype HSVd secondary sequence are shown in Fig. 1.

## RESULTS

Fig. 2 shows the size determination of RT-PCR products of CVdIIa and CVdIIb prior to gel gel purification

used for sequencing. Only the lower RT-PCR product bands indicated by the arrows were excised from ethidium bromide stained gels and used for sequencing. The minor upper PCR products were not present in all samples containing CVdIIa or CVdIIb and, as we now know, are artifacts generated by high primer concentration (data not shown). CVdIIa was determined by nucleotide sequencing to be 302 nucleotides in length compared to CVdIIb (Cachexia) which is 299 nucleotides in length (Fig. 3). The nucleotide sequence of CVdIIa and CVdIIb varied from the HSVd variants from Japan (CV1 and CV2, 10) by the following three base substitutions: C - G at position 23; G - A at position 26; and an A - G at position 251. CVdIIb differs from CVdIIa by the deletion of a G at position 58, and an A at positions 109 and 122. CVdIIb also differs from CVdIIa by the following base substitutions: A - G at position 107; and a U - C at position 193. The HSVd variant associated with the grapefruit dwarfing agent (HGda) is reported to be 299 nucleotides in length (4) and also shares the same base substitution of a G - A at position 26 found in CVdIIa and CVdIIb. HGda differs from CVdIIa and CVdIIb, but is similar to the HSVd variants from Japan, by maintaining a C at position 23 and an A at position 251. HGda is similar to CVdIIb by maintaining the deletion of a G at position 58, but differs by maintaining an A at position 107, 109, and 122, and maintaining a U at position 193. HGda differs from all the above mentioned citrus viroids by a deletion of a C at position 246, a U at position 247, and a base substitution of A - G at position 271.

## DISCUSSION

Using RT-PCR we were able to amplify CVdIIa and CVdIIb from total nucleic acids, gel purify the major RT-PCR product (Fig. 2), and directly sequence the purified products using the *fmol*<sup>TM</sup> sequencing system without an intermediate cloning step. CVdIIa and

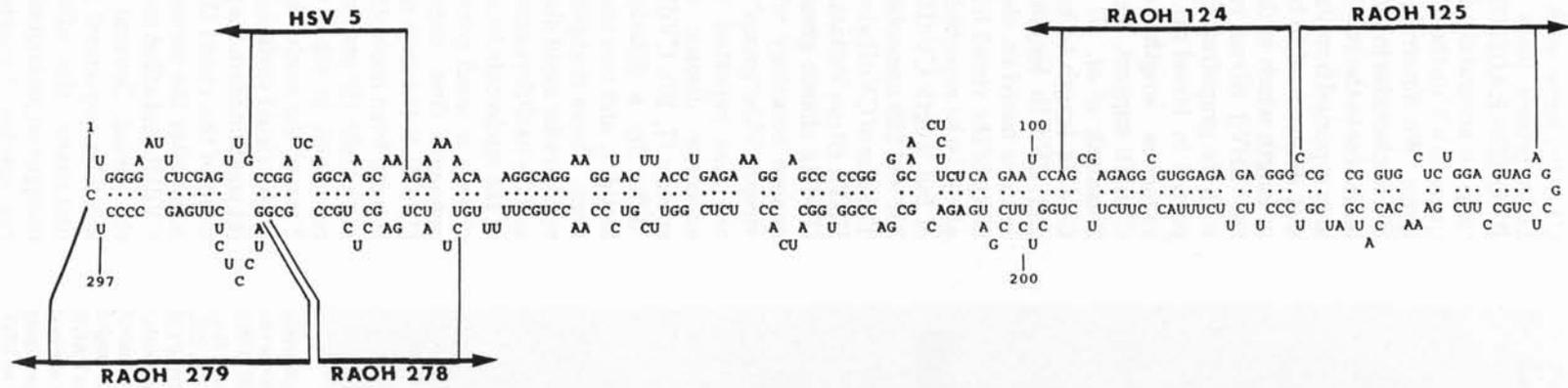


Fig 1. The sequence and secondary structure of the prototype hop stunt viroid. This figure shows the position and orientation of primers RAOH 124, RAOH 125, RAOH 278, RAOH 279, and HSV 5 used to sequence CVIIa and CVIIb. The primer sequences were RAOH124 (5'-CCCTCTCTCCACGCCTCTCGCT-3'), RAOH125 (5'-CCGCGGTGCTCTGGAGTAGA-3'), RAOH278 (5'-CGCGGCAGAGGCTCAGATAG-3'), RAOH279 (5'-GATCCTC-TCTTGAGCCCCTC-3'), and HSV<sub>5</sub> (5'-TTTGCT TGCCTGATCCGGC-3').

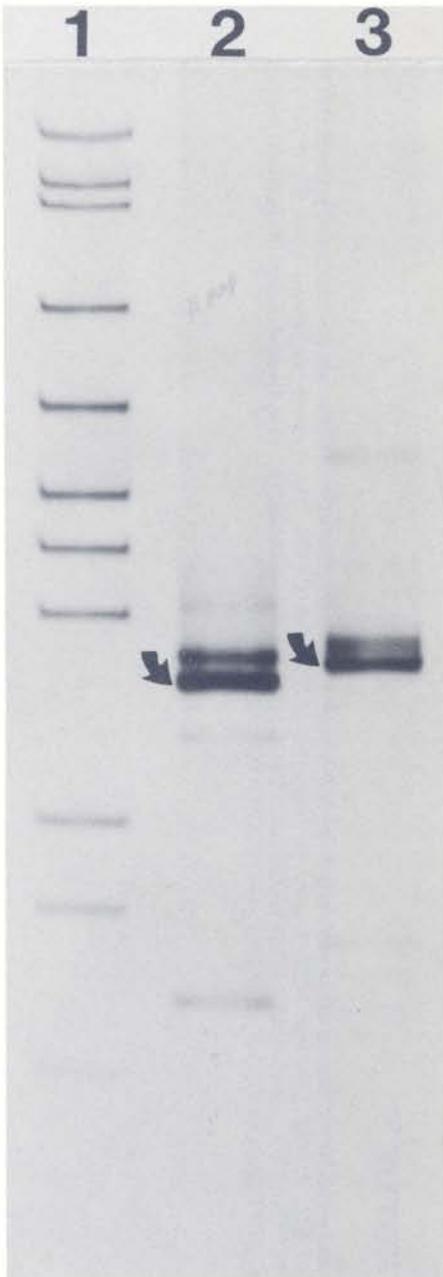


Fig 2. Polyacrylamide gel electrophoretic analysis of the RT-PCR products of citrus viroid group II-infected citrus. PGEM DNA molecular marker with fragment sizes (bp) 2,645, 1,605, 1,198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36 (lane 1). RT-PCR product (299 nucleotides) of CVIIB (cachexia) (lane 2). RT-PCR product (302 nucleotides) of CVdIIa (lane 3). Arrows indicate the major RT-PCR product (subsequently run in PAGE and stained with ethidium bromide) excised and used for sequencing. Amplified products were analyzed on 6% polyacrylamide at 120V for 2.5 hr and stained with silver nitrate.

CVdIIb were each amplified using DNA primer pairs HSV<sub>1</sub>/HSV<sub>2</sub> and RAOH278/RAOH279 in separate reactions to accurately determine the sequences within the RT-PCR primer regions. We determined CVdIIa to be 302 nucleotides in length. This is similar in size to the two HSVd citrus variants reported from Japan (10). CVdIIb was determined to be 299 nucleotides in length which is the same length as the HSVd citrus variant associated with the grapefruit dwarfing agent reported in Israel (4). The determined sequence lengths of CVdIIa and CVdIIb support the earlier work by Semancik *et al.* (7) who determined CVdIIa length to be 302 nucleotides and CVdIIb length to be 299 nucleotides based on electrophoretic mobility of the viroid RNAs, and Yang *et al.* (11) who reported the amplification of full length CVdIIa and CVdIIb of 302 and 299 nucleotides, respectively. The size of CVdIIa is similar to the two HSVd citrus variants from Japan and CVdIIa shares greater than 99% sequence homology with the Japanese viroids. This "group" of HSVd variants are also reported to cause a mild exocortis disease on some citrus species (7, 10). CVdIIa and CVdIIb differ by a deletion of three nucleotides, and two nucleotide substitutions. These changes could be used as markers for rapid diagnosis of CVdIIb and for its differentiation from CVdIIa at the nucleotide level by amplification of only a small portion of the viroid genome. One such region within CVdIIb is the area containing a five G stretch from nucleotide number 106 to nucleotide 110 provided this change is consistent in other CVdIIb isolates. Four of the nucleotide changes occur in the viroid variable domain, and one (at position 58) in the pathogenicity domain of the viroid (1). These changes could alter the secondary structure of CVdIIb and affect the pathogenicity of the viroid. Several CVdIIb isolates must be sequenced and compared to determine the affect of sequence changes on pathogenicity in similar citrus species. Sequence manipulations

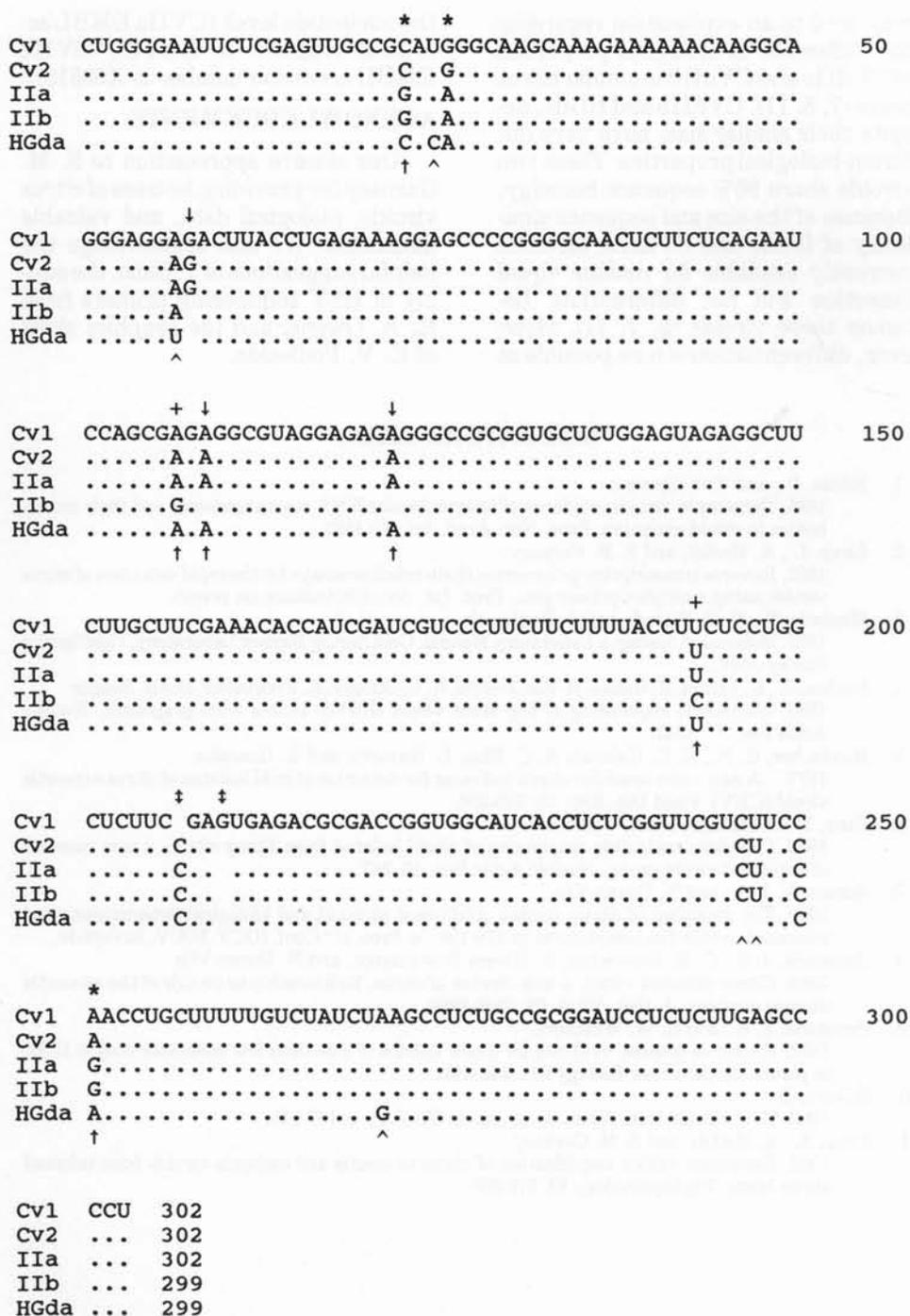


Fig 3. The complete nucleotide sequences of citrus viroid IIa and IIb (cachexia). Hop stunt citrus variant 1 and 2 (Cv1 and Cv2, respectively) are from published sequence data (8). The hop stunt variant from grapefruit associated with the grapefruit dwarfing agent (HGda) from Israel is also from published sequence data (3). (‡) indicates published differences between CV1 and CV2 sequences. (\*) indicates CVdIIa and CVdIIb (cachexia) sequence differences when compared to the sequences of CV1 and CV2. (↓) indicates deletions in the CVdIIb sequence when compared to CVdIIa. (+) indicates substitutions in the CVdIIb sequence when compared to CVdIIa. (†) indicates differences in the published HGda sequence when compared to CVdIIb. (^) indicates differences in the HGda sequence which are not present in the other four viroids.

may lead to an explanation regarding the difference in biological properties of CVdIIa and CVdIIb in similar citrus hosts (7, 8, 11). CVdIIb and HGda, despite their similar size, have very different biological properties. These two viroids share 96% sequence homology. Because of the size and sequence similarity of HGda and CVdIIb, methods currently available for routine viroid detection will not differentiate between these viroids (2, 7, 11). However, differentiation is now possible at

the nucleotide level. (CVIIa EMBL accession number is X69518. CVIIB EMBL accession number is X69519).

#### ACKNOWLEDGEMENTS

Our sincere appreciation to S. M. Garnsey for providing isolates of citrus viroids, biological data, and valuable discussion. We also acknowledge the helpful suggestions of T. Sano, the supply of HSV sequencing primers from R. A. Owens, and the graphics skills of E. V. Podleckis.

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