

# Molecular Detection and Prevalence of Citrus Viroids in Texas

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**Abstract.** Viroids are graft- or mechanically transmissible agents, disseminated through budding. Biological indexing of commercially important citrus cultivars grown in the Lower Rio Grande Valley of Texas showed that many are infected with citrus viroids. Most of these trees carried more than one viroid. In most cases, the infected trees are asymptomatic carriers because sour orange, the predominant rootstock used in Texas, does not show symptoms of viroid infection. Detection of viroids through biological indexing on sensitive indicator plants followed by sequential polyacrylamide gel electrophoresis (sPAGE) is the gold standard but is time-consuming and requires plants to be kept at optimum conditions. A conditional use of reverse transcriptase–polymerase chain reaction (RT-PCR) provides an efficient and alternative detection of viroids for use in the Texas virus-free citrus budwood certification program. RT-PCR could be useful in Texas to help expedite the evaluation for the presence of viroids before conducting the final biologic indexing. Using RT-PCR, we could detect, clone, and sequence full-length viroids of *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) (both cachexia and noncachexia variants), *Citrus viroid-III* (Citrus dwarfing viroid), and *Citrus viroid-IV* (Citrus bark cracking viroid) from a collection of viroid-inoculated grapefruit plants. The source plants were previously shown to be viroid-infected by biological indexing on Etrog citron plants. Based on our results, RT-PCR can be a conditional substitute for biological indexing of mother trees in foundation blocks and shoot tip-grafted trees in the virus-free budwood program. A positive RT-PCR result has a serendipitous value because those trees can be discarded from the pool before expensive biological indexing.

Viroids are small infectious agents of single-stranded, unencapsidated, nontranslated, circular RNA with self-complementary sequences (Flores et al., 1998). Citrus viroids have been classified into distinct groups based on their biological and physical properties: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CVD-III), and *Citrus bark cracking viroid* (CVD-IV). Recently, a new citrus viroid species, tentatively named Citrus viroid OS (CVD-OS), was reported (Ito et al., 2001). These viroids have small genomes ranging from 284 to 375 nucleotides (Duran-Vila et al., 1988). In citrus, viroids cause two economically important diseases, exocortis and cachexia (xyloporosis) (Roistacher, 1991). Other citrus diseases associated with viroids include wood pitting (Fernandez-Valiela et al., 1965), gum pocket, and gummy pitting in trifoliolate orange (Schwarz and McClean, 1969); yellow corky vein and gummy bark in sweet orange (Reddy et al., 1974); and kassala disease in grapefruit (Bové, 1995). Exocortis was first described in 1948 as a bark-shelling or scaling disorder in trees

grown on trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] and its hybrids (Fawcett and Klotz, 1948). Later, this disease was demonstrated to be graft-transmissible (Benton et al., 1949, 1950) and incited by CEVd (Semancik and Weathers, 1972). Similar disorders were also reported as scaly butt of trifoliolate orange in Australia (Benton et al., 1949) and in Rangpur lime (*Citrus limonia* Osbeck) in Brazil (Moreira, 1955, 1959). HSVd variants include CVD-IIa, CVD-IIb, and CVD-IIc (Foissac and Duran-Vila, 2000; Palacio-Bielsa et al., 2004; Reanwarakorn and Semancik, 1999a, 1999b; Semancik et al., 1988a, 1988b). CVD-IIb was shown to be the causal agent of cachexia disease, which induces severe gumming, stem pitting, and discoloration of the indicator plant, Parson's Special mandarin (PSL) (Duran-Vila et al., 1988; Semancik et al., 1988a, 1988b). It was also demonstrated that CVD-IIb or CVD-IIc can cause cachexia symptoms on Orlando tangelo and PSL (Reanwarakorn and Semancik, 1999a, 1999b).

Generally, old-line red grapefruit (*C. paradisi* Macf.) trees in Texas carried at least cachexia, and most sweet orange (*C. sinensis*) trees carried both cachexia and exocortis viroids (Olson, 1952; Olson and Shull, 1955; Olson et al., 1958; Sleeth 1959). Moreover, susceptibility of 25 cultivars of mandarin (*C. reticulata* L.) and mandarin hybrid rootstocks to cachexia and its occurrence in commercial red grapefruit prompted

intensive rootstock studies through viroid indexing in Texas (Olson, 1954). It was also shown that 12-year-old, viroid-free Valencia orange trees on Cleopatra mandarin, rough lemon, and sour orange rootstocks generally grew faster and yielded more compared with trees infected with exocortis and cachexia (Olson and Shull, 1962). Also, inoculation of Star Ruby grapefruit on Troyer citrange [*P. trifoliata* (L.) Raf. × *c. sinensis* (L.) Osbeck] rootstock with a severe strain of exocortis reduced trunk diameter and canopy volume (Timmer, 1978). Viroid indexing studies on Etrog citron showed symptoms resembling those induced by CEVd, CVD-II, CVD-III, or CVD-IV (Miao et al., 1996). They observed a few plants showed petiole wrinkle or discoloration or tip browning of the leaves, which may be associated with CVD-II. Identification of citrus viroids based only on the symptomatology in Etrog citron (*C. medica* L.) has many disadvantages because the symptom expression requires a long waiting period and can be affected by environmental conditions.

The objectives of this study were to evaluate the reverse transcriptase–polymerase chain reaction (RT-PCR) technique as a quick method to screen a large number of trees for the presence of viroids in the virus-tested budwood program to improve the budwood program and help expedite introduction of new germplasm into the program and to identify which viroids occur in Texas. Biological indexing, the gold standard in virus-free citrus production, is expensive, time-consuming but a necessity (Skaria et al., 1997). However, there may be potential for a conditional replacement if a rapid, sensitive, and reliable technique is available as is used in other programs.

## Materials and Methods

*Plant materials.* Viroid-infected Rio Red grapefruit (*C. paradisi* Macf.) plants on sour orange rootstock were selected based on results from previous biological indexing (Miao et al., 1996). All original viroid source plants were initially indexed on Etrog citron 861-S-1. They produced symptoms of leaf epinasty, plant stunting, leaf drooping, leaf browning, petiole necrosis, petiole twisting, and petiole wrinkle. Sets of five Rio Red grapefruit trees were inoculated with different viroid sources from commercial grapefruit and orange cultivars in the field and maintained at the Texas A&M University–Kingsville Citrus Center. One tree from each source group was selected for analysis. Healthy, viroid-free plants were used as controls. Bark tissue from mature stems and leaves of these plants were used for nucleic acid extraction. Very tender shoot tips or mature leaves were avoided because they were reported to be unsuitable sources for CEVd and CVD-II detection (Garney et al., 2002). Tissues collected were either used immediately for nucleic acid extractions or stored at –20 °C until extracted. The test plants were harvested during the summer

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Table 1. The oligonucleotide primer pairs used for various citrus viroids in reverse transcriptase–polymerase chain reaction and polymerase chain reaction.

Viroid	Name	Sequence	Target (product size)
CEVd	CEVd-RT <sup>z</sup>	5'-CTTCCTCCAGGTTTCCCCGGGGATCCC-3'	371 bp
	CEVd-R1 <sup>z</sup>	5'- CCGGGATCCCTGAAGGA-3'	
	CEVd-F1 <sup>z</sup>	5'-GGAAACCTGGAGGAAGTCG-3'	
	CEVd-H <sup>y</sup>	5'-ATCCCCGGGGAACCTGGAGGAAG-3'	
	CEVd-C <sup>b</sup>	5'-CCCTGAAGGACTTCTCCCC-3'	
HSVd	HSVd-RT <sup>z</sup>	5'-GTGTTGCCCGGGGCTCCTTCTCTGG-3'	299 bp
	HSVd-R1 <sup>z</sup>	5'-GGGGCTCCTTTCTCAGGTAAGTC-3'	
	HSVd-F1 <sup>z</sup>	5'-GGGGCAACTCTTCTCAGAATCC-3'	
	HSVd-Ca <sup>z</sup>	5'-GAATCCAGCGGGGGCGTG-3'	
Cachexia variants	HSVd-A <sup>z</sup>	5'-TTTTACCTTCTCTGGCTTTCGAGTG-3'	283 bp
	Noncachexia variants	CBLVd-A <sup>z</sup>	
CBLVd	CBLVd-RT <sup>z</sup>	5'-TTCGTCGACGACGACCAGTC-3'	327 bp
	CBLVd-R1 <sup>z</sup>	5'-GGCTCGTCAGCTGCGGAGGT-3'	
	CBLVd-F1 <sup>z</sup>	5'-CCAACCTAGCTGCCTTCGTGACGACG-3'	
	CBLVd-H <sup>y</sup>	5'-TTCGTCGACGACGAGGTA-3'	
CVd-III	CVd-III-RT <sup>z</sup>	5'-GGCAGCTAAGTTGGTGACGC-3'	294 bp
	CVd-III-R1 <sup>z</sup>	5'-CTCCGCTAGTCGAAAGACTCCGC-3'	
	CVd-III-F1 <sup>z</sup>	5'-CTCCGCTAGTCGAAAGACTCCGC-3'	
	CVd-III-C <sup>y</sup>	5'-GTCTGAAGAGATTTCCCGGGATCCC-3'	
CVd-IV	CVd-IV-RT <sup>z</sup>	5'-GGGGATCCCTCTTCAGGT-3'	284 bp
	CVd-IV-R1 <sup>z</sup>	5'-GGGGAAATCTCTTCAGAC-3'	
	CVd-IV-F1 <sup>z</sup>	5'-ACGACAGGTGAGTTCTCCTT-3'	
CVd-OS	CB3-CM <sup>a</sup>	5'-CGTCGACGAAGGCATGTGAGCT-3'	330 bp
	CB3-CP <sup>x</sup>		

<sup>z</sup>Primers described previously by Bernad and Duran-Vila (2006).

<sup>y</sup>Primers defined by Garnsey et al. (2002).

<sup>x</sup>Primers defined by Ito et al. (2001).

months when growth conditions are optimum for viroids (Roistacher, 1991).

**Preparation of nucleic acid extracts with viroid RNA targets.** The method used for extraction of dsRNAs from plant tissue was an adaptation of the sodium dodecyl sulfate-potassium acetate (SDS-KAc) method (DePaulo and Powell, 1995). Tissue (0.25 g) was pulverized with a mortar and pestle in the presence of liquid nitrogen and the tissue was immediately transferred to a 2.0-mL centrifuge tube. Thawing of the pulverized tissue was avoided. To this powder, 750 µL of the extraction buffer (0.1 M Tris, pH 8.0, 50 mM EDTA, 500 mM NaCl, and 10 mM 2-mercaptoethanol) and 100 µL of 10% SDS were added. The preparation was vortexed and incubated for 30 min at 65 °C, 500 µL of 5 M KAc was added, mixed thoroughly, and incubated on ice for 20 min. After centrifugation (≈17,000 × g), the supernatant was transferred to a clean 2.0-mL centrifuge tube. To the supernatant, 0.1 vol of sodium acetate (NaOAc) and 3 vol of ethanol were added, mixed, and incubated for 5 h at –20 °C. The nucleic acids were recovered as a pellet by centrifugation at 9000 × g for 20 min, air-dried, resuspended in 100 µL sterile water, and stored at –70 °C.

**Reverse transcriptase–polymerase chain reaction.** First-strand viroid cDNA was synthesized using 15 U of ThermoScript Reverse Transcriptase (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Reverse transcription of 0.5 µg of total RNA was performed in a 20-µL reaction including 4 µL of 5× buffer (250 mM Tris acetate, 375 mM potassium acetate, 40 mM magnesium acetate), 200 µM of each dNTPs, 1 µM of reverse specific primer (Table 1), 40 U of RNase Out, 10 mM DTT, and 15 U of ThermoScript Reverse Transcriptase. The reaction was incubated at 60 °C for 1 h and

the reverse transcriptase was inactivated by heating the reaction mixture to 85 °C for 5 min followed by rapid cooling on ice. Second-strand cDNA synthesis and subsequent polymerase chain reaction (PCR) amplification were performed on 1.5 µL of first-strand cDNA mixture using 1 U of HotStarTaq DNA polymerase (Qiagen, Valencia, Calif.) in a 25-µL reaction including 2.5 µL of 10× PCR buffer [Tris. HCl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 15 mM MgCl<sub>2</sub>; pH 8.7], 0.2 mM each of dNTPs, and 0.25 µM each of selected forward and reverse primers (Table 1). The PCR conditions were optimized (Bernad and Duran-Vila, 2006) as 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR parameters for CVd-OS detection were the same except that the annealing temperature was 55 °C. The PCR products were run on 2% agarose gels in Tris-Acetate-EDTA buffer, stained with ethidium bromide, and visualized under ultraviolet light to confirm the synthesis of amplicons of expected size.

**Sequencing and analysis of viroid cDNA.** Full-length viroid amplicons of expected size were gel-purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into PCR 4-TOPO vector (Invitrogen). Plasmids from transformed cells were purified by Qiaprep Miniprep kit (Qiagen) and sequenced at MWG-Biotech DNA sequencing facility (MWG-Biotech, High Point, N.C.). The DNA sequence was compared for homology to sequences deposited at the GenBank database.

## Results and Discussion

Results from RT-PCR assay of test plants are shown in Table 2, Figure 1, and Figure 2. The full-length cDNAs of CEVd, HSVd, CVd-III, and CVd-IV consisting of 370 bp, 303 bp, 295 bp, and 284 bp, respectively, were sequenced. Because source plant 12E gave negative results (Fig. 1), the other four trees in the group inoculated from the same field source (a, b, c, d) were analyzed by RT-PCR and were found to contain cachexia variants of HSVd (Table 2). It is therefore

Table 2. Results of reverse transcriptase–polymerase chain reaction assay of viroid-infected grapefruit plants.

Field source of viroids	Tested plants	Viroid presence
Henderson grapefruit G	1A	CEVd, HSVd (cachexia variants of HSVd), and CVd-III
Valencia orange A	2E	CEVd, HSVd (cachexia variants of HSVd), and CVd-III
N-33 Navel orange A	3E	CEVd, HSVd (cachexia variants of HSVd), and CVd-III
N-33 Navel orange B	4D	CEVd, HSVd (noncachexia variants of HSVd), and CVd-III
Everhard Navel orange C	6E	CEVd, HSVd (cachexia variants of HSVd), CVd-III, and CVd-IV
Marrs orange E	12E	Negative
Marrs orange E	12A, B, C, and D	HSVd (cachexia variants of HSVd)
Henderson grapefruit 1	13E	CEVd, HSVd (cachexia variants of HSVd), and CVd-III
Control, not inoculated	44D	Negative
Rio red grapefruit	Healthy	Negative

possible that 12E was not successfully infected during the original inoculation. PCR amplification using strain-specific primers of HSVd (Table 1) resulted in a 220 bp amplicon only for noncachexia variants and 279 bp amplicon only for cachexia variants (Fig. 3). Homology searches for these nucleotide sequences at the GenBank database showed 98% to 100% identities to previously established, respective, viroid sequences. A similarity search for full-length cDNA of CVd-III showed 100% identity to a citrus dwarfing viroid from Cuba and a citrus dwarfing viroid, CVd-IIIa, from Australia (accession nos. AJ630358 and S75465). Also, cDNA nucleotide sequence of CVd-IV showed 100% identity with CVd-IV sequences reported from Cuba (accession no. AJ630360) and Japan (accession no. AB054633). Alignment of CEVd, HSVd, cachexia variants of HSVd, CVd-III, and CVd-IV cDNA nucleotide sequences as described by Corpet (1988) revealed some conserved regions.

Studies on mechanical transmission of cachexia and exocortis showed that these viroids can be transmitted by slashing with a knife blade (Garnsey and Jones, 1967; Garnsey and Whidden, 1973; Roistacher, 1983). In a greenhouse transmission study, it was also reported that CBLVd and all strains of HSVd, CVd-III, and CVd-IV were mechanically transmissible by a single slash (Barbosa et al., 2005). Transmission of viroids in trees growing in the field was also demonstrated with different transmission efficiencies in different host plants (Barbosa et al., 2002). Thus, there is a great possibility for spreading of these viroids by propagation of infected budwood and contaminated pruning tools (Roistacher, 1991). The Texas citrus budwood certification program was initiated to produce virus-free budwood regulated by the Texas Department of Agriculture (Skaria et al., 1996, 1997). Budwood sources were subjected to biological indexing to test for the presence of viroids and viruses, shoot tip-grafted to produce virus- and viroid-free budwood sources, reindexed, and planted in a foundation block (Skaria et al., 1996). Budwood from these plants is used to produce increased block trees that supply budwood to nurseries. There are over 80 varieties in the foundation block and  $\approx 280,000$  buds have been supplied to nurseries since 1998 (Kahlke et al., 2005). For viroids, biologic indexing is being performed every 4 to 5 years (Kahlke et al., 2005). Citrus viroids can be detected by indexing on sensitive indicators such as Etrog citron for exocortis (Calavan et al., 1964) and PSL for cachexia (Roistacher et al., 1973; Vogel and Bové, 1976) followed by sequential polyacrylamide gel electrophoresis (sPAGE). This is a standard, sensitive method but there are some disadvantages, for example, biological indexing on Etrog citron requires 3 to 6 months and indexing on PSL for cachexia requires at least 1 year. Maintaining index plants at 28 to 30 °C for several months and requiring expertise in diagnosis of symptoms make this

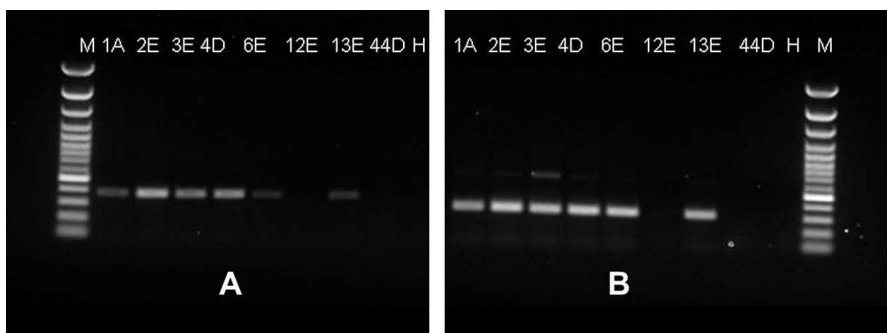


Fig. 1. Agarose gel showing reverse transcriptase–polymerase chain reaction products amplified with specific primers for (A) CEVd and (B) HSVd from viroid-infected grapefruit samples. 44D = noninoculated control; H = healthy (-); M = 100 bp DNA ladder.

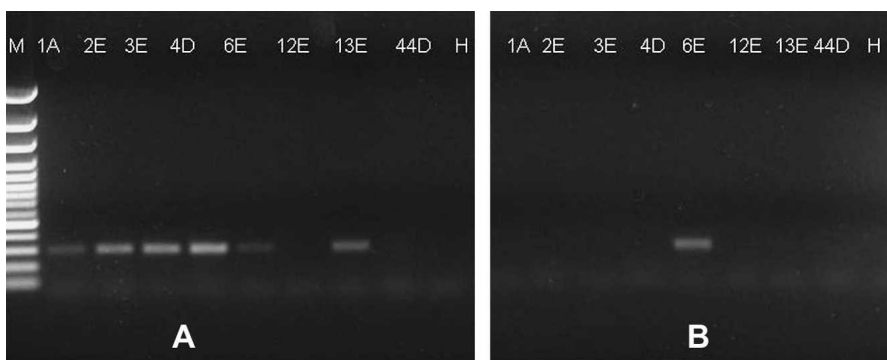


Fig. 2. Agarose gel showing reverse transcriptase–polymerase chain reaction products amplified with specific primers for (A) CVd-III and (B) CVd-IV from viroid-infected grapefruit samples. H = healthy (-); 44D = noninoculated control; M = 100 bp DNA ladder.

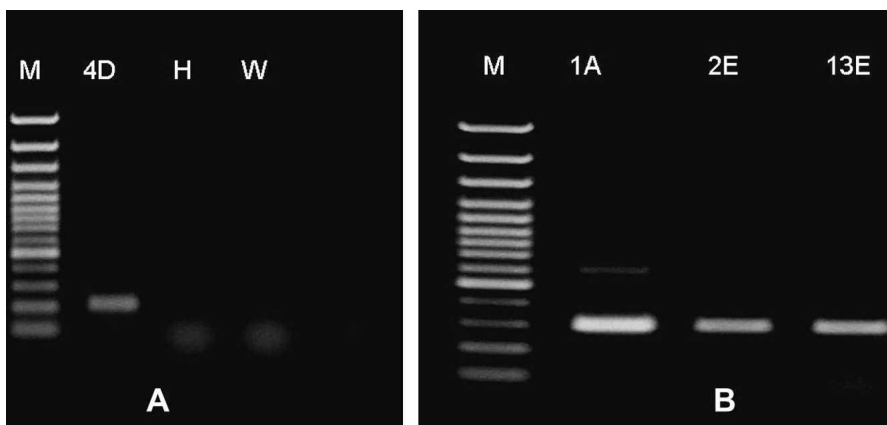


Fig. 3. Agarose gel electrophoresis of reverse transcriptase–polymerase chain reaction products amplified with specific primers for noncachexia variants of (A) HSVd and (B) cachexia variants from viroid-infected grapefruit samples. H = healthy (-); W = water (-) controls; M = 100 bp DNA ladder.

an expensive process. Conversely, RT-PCR became a useful and popular method for practical detection of viroids because of its simplicity and sensitivity when compared with conventional bioassays and sPAGE. However, presence of inhibitors in the plants such as polyphenolics, polysaccharides, and endogenous ribonucleases drastically decreases the detection sensitivity of the RT-PCR reactions (Gibb and Padovan, 1994; Singh et al., 2002). Moreover, detection of viroids through RT-PCR is limited

by inefficient synthesis of viroid full-length cDNA during the PCR reaction (Bernad and Duran-Vila, 2006). Additionally, it was reported that some sequence variants of CVd-III may escape the detection by RT-PCR; if proper sequence primers are not used (Garnsey et al., 2002). Highly purified viroid preparations, achieving viroid full-length cDNA by optimizing amplification conditions and careful selection of primers, are prerequisite in using RT-PCR detection of viroids (Ragozzino et al., 2004). Use of the

RT-PCR method in detection and characterization of citrus viroids is well established (Bernad and Duran-Vila, 2006; Ito et al., 2002; Sieburth et al., 2002).

This is the first report of the detection and characterization of citrus viroids by RT-PCR in Texas confirming the presence of CEVd, HSVd, and CVd-III commonly in all the trees. CVd-IV was found in only one tree in our study. CVd-IV was previously reported from California (Duran-Vila et al., 1988), Israel (Puchta et al., 1991), Japan (Hataya, 1997), Turkey (Önelge et al., 2000), Cuba (Velázquez et al., 2002), Tunisia (Najar et al., 2002), and Italy (Malfitano et al., 2005). CVd-IV was not detected in Uruguay (Pagliano et al., 2000) or Mexico (Almeyda-León et al., 2007). CBLVd and CVd-OS (Ito et al., 2001) were not detected in the sampled trees. RT-PCR is highly useful in routine diagnosis of citrus viroids and it can selectively replace some of the need for biological indexing of RT-PCR-positive trees in the citrus budwood program. In the virus-free budwood program, all test plants from the foundation block or shoot tip grafting with a positive RT-PCR test can be discarded from the budwood program without an expensive biological indexing with substantial saving of money and time.

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