Enzymatic cDNA Amplification of Citrus Exocortis and Cachexia Viroids from Infected Citrus Hosts

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ABSTRACT

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Reverse transcription-polymerase chain reaction (RT-PCR) assays were developed for the detection and identification of citrus exocortis viroid (CEV), citrus cachexia viroid (CCaV), and citrus viroid IIa (CVIIa) from nucleic acid extracts of infected sweet orange or Etrog citron. DNA primers (19–24 nucleotides in length) specific for CEV or hop stunt viroid (HSV) sequence were used for cDNA synthesis and specific amplifications of CEV and the HSV-related CCaV (or CVIIa), respectively. The size of the major RT-PCR product from CEV-infected tissue was the same as full length CEV (371 bp) and hybridized with a SP6-generated CEV cRNA probe. The size of the major RT-PCR product from CCaV or CVIIa-infected tissue was approximately 297 bp and 302–303 bp, respectively, and hybridized with a SP6-generated HSV cRNA probe. These products were absent from amplified extracts of uninfected tissue. The RT-PCR assay is more sensitive than existing detection methods and provides information about viroid detection from sweet orange or Etrog citron without requiring large samples or molecular hybridization.

Additional keyword: xyloporosis.

Citrus exocortis viroid (CEV), the causal agent of citrus exocortis disease, is an infectious single-stranded circular RNA (22,25) with a chain length of 371 nucleotides for the type isolate and 371–375 nucleotides for related variants (9,28-30). Citrus cachexia viroid (CCaV), the causal agent of cachexia (xyloporosis) disease of citrus, is also an infectious single-stranded circular RNA, but its chain length consists of approximately 300 nucleotides (19,23,24). CCaV (also known as citrus viroid IIb) is a few nucleotides shorter than the closely related citrus viroid IIa (CVIIa), which causes a mild form of the citrus exocortis disease (24). Both CCaV and CVIIa are variants of the hop stunt viroid (HSV) (3,4,24). The chain length for HSV variants is 297–303 nucleotides (17,18,26); the HSV type isolate and the plum dapple fruit viroid (PDFV) variant each consist of 297 nucleotides (26), and the cucumber pale fruit viroid (CPFV) variant consists of 301 or 303 nucleotides (17,26).

Exocortis and cachexia are two major viroid diseases in citrus (7,20,23). Exocortis is a bark-scaling and tree stunting disease that affects several citrus species and cultivars and some citrus relatives. The viroid also infects many other citrus hosts in a latent fashion. Several noncitrus hosts have also been reported (2,7,22). Symptoms of cachexia in reactive citrus hosts are discoloration and gum impregnation of the bark, stunting, chlorosis, and decline of diseased trees (20). Cachexia primarily affects tangelos, *Citrus reticulata* × *C. paradisi* Macfady; mandarins, *C. reticulata* Blanco; mandarin hybrids; kumquats, *Fortunella* sp.; and alemow, *C. macrophylla* P. J. Wester (23). Many citrus varieties, including grapefruit, *C. paradisi* Macfady; sweet orange, *C. sinensis* (L.) Osbeck; and lemon, *C. limon* (L.) N. L. Burm. are symptomless carriers of the agent (23). Biological indexing on indicator citrus plants requires 3–6 mo for CEV and 6–48 mo for CCaV. Field symptoms appear 2–8 yr after inoculation (7,20). Direct detection of nucleic acid extracts from diseased field trees by polyacrylamide gel electrophoresis and/or dot blot molecular hybridization with ^{32}P-labeled viroid cDNA probe has been reported for CEV (1,6,8). Direct detection of CCaV by polyacrylamide gel electrophoresis analysis in nucleic acid extracts from diseased field trees has been unsuccessful because of the low viroid concentration present. CCaV can be detected in infected Etrog citron, *C. medica* L, and inoculation to citron is commonly used to "amplify" CCaV for gel analysis (5).

Rapid amplification of viroid cDNA transcribed in vitro from viroid RNA in total nucleic acids from infected tissue is now possible with very high specificity and fidelity using *Taq* DNA polymerase and viroid-specific DNA primers in a reverse transcription (RT)-polymerase chain reaction (PCR) (14). The RT-PCR method has been valuable in improving the detection of viroids in the apple scar skin viroid (ASSV) group from pome fruit crops. Based on this finding, we have tested RT-PCR to amplify CEV and CCaV (or related CVIIa) from citrus extracts. This paper describes the detection and identification of CEV, a member of the potato spindle tuber viroid group (10), and CCaV and CVIIa, members of the HSV group, from total nucleic acids of infected Etrog citron and Madam Vinous sweet orange. A preliminary report of this work has been presented (31).

MATERIALS AND METHODS

Citrus and viroid sources. All tissues were collected from Etrog citron or Madam Vinous sweet orange plants, which were systemically infected with the viroids tested in Orlando, FL. The Etrog citron plants were rooted cuttings of selection Arizona 861, and the Madam Vinous plants were bud propagations on Carrizo citrange *C. sinensis* × *Poncirus trifoliata* (L.). Raf. seedlings. These source plants were maintained in a partially shaded greenhouse

with day temperatures ranging from 23 to 30 C and night temperatures ranging from 20 to 24 C.

Most tests were done with seven viroid isolates. X-7 is an isolate of cachexia that gives a diagnostic reaction for cachexia in Parson's Special mandarin grafted on rough lemon *C. jambhiri* Lush. and produces no symptoms in Etrog citron. E9 is a standard source of CEV used in numerous studies and deposited in the American Type Culture Collection as PV194. It produces typical symptoms in Etrog citron. E22 was obtained by graft inoculation of Etrog citron from a Temple orange tree (Te 20-3-5) that indexed positively for CEV bark scaling when grafted on trifoliate orange *P. trifoliata* (L.) Raf. rootstock. It was subsequently transmitted by slash cut inoculation to Etrog citron and produced typical CEV symptoms in this host. E11 was obtained by graft inoculation from a source of Te 20-3-5; however, it produces a mild reaction on Etrog citron. Sequential polyacrylamide gel electrophoresis analysis of E9, E11, and E22 indicated that E9 contains only CEV, E11 contains CVIIa and citrus viroid III (CVIII) but no CEV, and E22 contains CEV and CVIII (S. M. Garnsey et al, *unpublished*). E8, E25, and E28 are viroid isolates originally obtained from different field trees in Florida. All produce typical CEV symptoms in Etrog citron, but have not been fully characterized for other viroids.

Sources of plum dapple fruit viroid (PDFV) and cucumber pale fruit viroid (CPFV). Plum budwood infected with PDFV (21,26) was obtained from Y. Terai, Yamanashi Fruit Tree Experiment Station, Yamanashi, Japan. Total nucleic acid extracts from tomato leaves infected with CPFV (17) were obtained from H. L. Sänger, Max-Plank-Institute for Biochemistry, Martinsried, Germany.

Tissue and nucleic acid extraction. Total nucleic acids from CEV-, CCaV-, or CVIIa-infected as well as uninfected bark tissue were isolated as described (12), except that the extraction buffer contained 50 mM NaCl. Briefly, 1–2 g of bark tissue were cut into small pieces, triturated with liquid nitrogen, and extracted for 20–30 min in 6 ml of extraction buffer that contained 0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM ethylenediaminetetra acetic acid, disodium salt (Na_2EDTA), 2% sodium dodecyl sulfate (SDS), and 1% sodium lauryl sarcosine. Each sample extract was mixed with 8 ml of Tris-HCl-buffered phenol, pH 7.6–8.0 (16) containing 0.1% 8-hydroxyquinoline and 0.2% 2-mercaptoethanol for 15 min, then mixed after adding 8 ml of chloroform for an additional 15 min. Samples were centrifuged in a GH-3.7 horizontal rotor at 3,008 *g* for 15 min in a tabletop, refrigerated Beckman GPR centrifuge (Beckman Instruments, Fullerton, CA). The aqueous phase of each sample was recovered, and the nucleic acids were precipitated by the addition of 2.5 vol of absolute ethanol and 0.10 vol 3 M sodium acetate, pH 5.2, while keeping the mixture at −70 C for at least 2 h. The nucleic acids were pelleted by centrifugation at 12,000 *g* for 10 min in a low-speed, refrigerated Sorvall RC5C centrifuge. Each pellet was dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then reprecipitated with ethanol and sodium acetate as described above. Following centrifugation, pellets were again redissolved in TE buffer, and the nucleic acid concentration was determined spectrophotometrically. An optical density of 1 at wavelength of 260 nm corresponds to approximately 50 μg/ml for double-stranded DNA and 40 μg/ml for single-stranded DNA and RNA (16).

Purification of host total nucleic acids. Total nucleic acids were further purified on RNase-free ELUTIP-r minicolumns containing RNA binding matrix (Schleicher and Schuell, Keene, NH) as suggested by the manufacturer with some modification. The minicolumns were hydrated and equilibrated at room temperature for 2 h with 5 ml of "low salt" buffer containing 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 1.0 mM Na_2EDTA. Total nucleic acids obtained from 0.5 to 1.0 g of tissue were dissolved in 2–3 ml of the low salt buffer and then passed slowly through the minicolumn. The minicolumn was washed once with 5 ml of the low salt buffer, and then total nucleic acids were eluted with 0.4 ml of "high salt" buffer (1.0 M NaCl, 20 mM Tris-HCl, pH 7.4, and 1.0 mM EDTA). Nucleic acids were precipitated by the addition of 1 ml of absolute ethanol at −70 C for at least 30 min followed by centrifugation in an Eppendorf microcentrifuge at 12,000 *g* for 15 min at 4 C. Each pellet was washed once with cold 70% ethanol to remove any traces of salt, dried in vacuo, and then dissolved in deionized water.

Viroid cDNA synthesis and amplification. CEV cDNA, CCaV cDNA, or CVIIa cDNA was synthesized and amplified from the minicolumn-purified total nucleic acids by the following procedure. For each sample, deionized sterile water was added to a mixture of total nucleic acids (0.01–1 μg), 1–3 μg of a 20-mer primer (5′-CCCTGAAGGACTTCTTCCCC-3′, complementary to CEV-A nucleotides 71–90 in the central conserved region [28]) or a 19-mer primer (5′-GGCTCCTTTCTCAGGTAAG-3′, complementary to HSV variant nucleotides 60–78 or 61–79 in the central conserved region [26]), 6 μl of 5× first strand cDNA buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) to yield a final volume of 30 μl. The resulting mixture was heated for 5 min at 100 C, chilled on ice for 2 min, and then stored at room temperature for 1 h to allow the viroid RNA and primer to anneal. Primers were synthesized by Synthecell Inc., Rockville, MD. Eighteen microliters of reaction solution (containing 4 μl of 5× first strand cDNA buffer, 5 μl of 0.3 M 2-mercaptoethanol, 2.5 μl of 10 mM dNTP, 1 μl of RNasin (40 units/μl, Promega Corp., Madison, WI), and 5.5 μl of deionized water) and 2 μl of cloned Moloney murine leukemia virus reverse transcriptase (200 units/μl, GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) were mixed with the 30 μl of annealing mixture and incubated at 42 C for 2.5 h.

Aliquots (5 μl) of the resulting specific CEV cDNA, CCaV cDNA, or CVIIa cDNA were transferred to tubes each containing 45 μl of 1× polymerase chain reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [w/v] gelatin), 200 μM dNTP, 1.2 μM of the CEV 20-mer primer, 1.2 μM CEV 24-mer primer (5′-ATCCCCGGGGAAACCTGGAGGAAG-3′ homologous to CEV-A nucleotides 91–114 [28]) for the tubes containing CEV cDNA or 1.2 μM of the HSV 19-mer primer, 1.2 μM of HSV 19-mer primer (5′-CCGGGGCAACTCT-TCTCAGAATCCA-3′, homologous to HSV variant nucleotides 79–103 or 80–104 [26]) for the tubes containing CCaV or CVIIa, and 0.5 μl (2.5 units) of Ampli Taq DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT). Each mixture was overlayed with 50 μl of mineral oil to prevent evaporation. Polymerase chain reactions were done for 40 cycles using a programmable DNA thermal cycler (Perkin-Elmer Cetus Corp.). Denaturation was at 94 C for 1 min., annealing of the primers was at 55 C for 2 min, and extension of primers was at 72 C for 3 min. Heat denaturation started the cycle again. The final primer extensions were for 7 min.

Analysis of RT-PCR amplified products. Aliquots of the RT-PCR products were analyzed by electrophoresis on 6% polyacrylamide slab gels (11 × 14 × 0.15 cm) at 100 V for 3 h in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3). Separated nucleic acids were either stained with silver (14) or denatured with 0.4 M NaOH/0.6 M NaCl for 15 min and then transferred electrophoretically to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH) at 0.6 mA for 16 h at 4 C. Transferred nucleic acids were cross-linked to membranes by irradiation with 1,200 μJ for 45 s in a UV crosslinker (Stratagene, La Jolla, CA). Membranes were prehybridized and then hybridized with a SP6-generated ^{32}P-labeled CEV- or HSV-specific cRNA probe, washed, and exposed to X-ray film with intensifying screen at −70 C as previously described (13).

Return gel electrophoresis and dot blot hybridization. Return gel electrophoresis analysis was done as described by Singh and Boucher (27). For dot blot hybridization assay, total nucleic acids of plant tissue in 12× standard saline citrate (SSC) (1× SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0) containing 6% formaldehyde were vacuum-blotted on Nytran membranes that had been saturated with 20× SSC. Nucleic acids were then cross-

linked to membranes by irradiation as described above. Prehybridization, hybridization with a SP6-generated ^{32}P-labeled CEV or HSV cRNA probe, washing, RNase A treatment, and autoradiography were carried out as described by Hadidi et al (13).

RESULTS

Requirements for reverse transcriptase and primers. Hadidi et al (11) reported that viroid replication does not involve the synthesis of DNA intermediates and that no major portion of viroid sequences exist in host nuclear DNA before or after infection. Thus, reverse transcription of viroid RNA to viroid cDNA in vitro, in the presence of viroid-specific primers, is required for its amplification with PCR. Figure 1 shows gel electrophoresis analysis of RT-PCR-amplified products of CEV cDNA that were reverse-transcribed from total nucleic acids of CEV-infected tissue with different amounts of reverse transcriptase. In the absence of RT, no CEV size molecules were detected, but products of less than 175 base pairs (bp) were observed (lane 2). In the presence of RT, a product in the size range of CEV (371 bp) was synthesized (lanes 3–6). The amount of this product increased by increasing the amount of reverse transcriptase from 100 to 200 units (lanes 3 and 4, respectively) but then leveled off at higher amounts of enzyme.

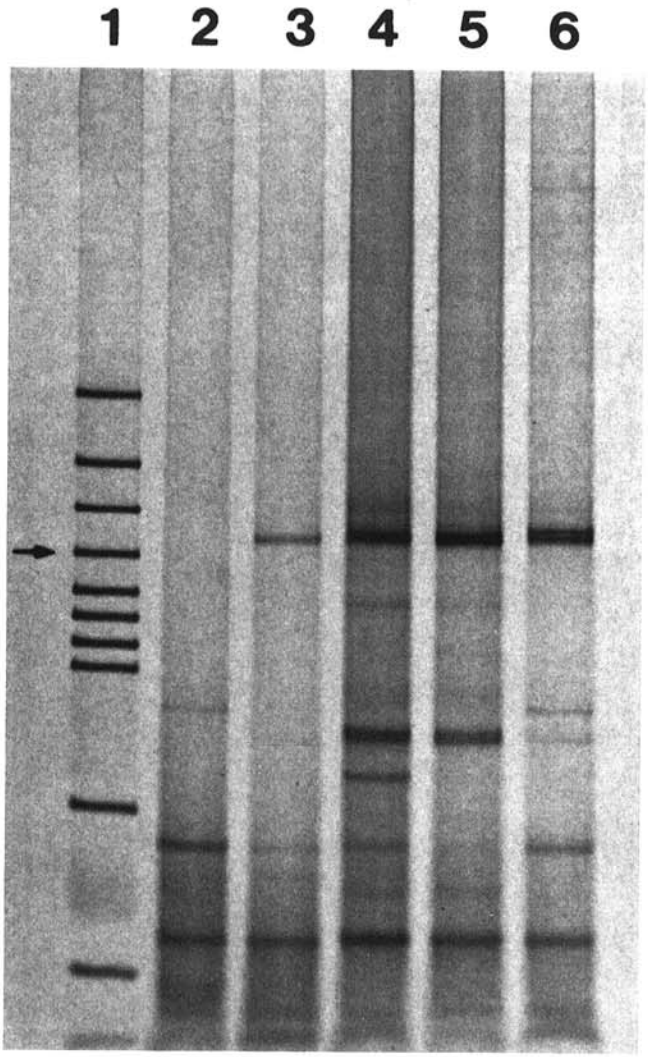

Fig. 1. Polyacrylamide gel electrophoresis analysis of reverse transcription-polymerase chain reaction (RT-PCR) products amplified from citrus exocortis viroid (CEV)-infected tissue at different amounts of reverse transcriptase. Molecular DNA marker of *Hae*III-digested PST B14 dimer with fragment sizes (bp) of 587, 454, 434, 359, 320, 275, 201, 173, 119, and 80 (lane 1); the arrow indicates 359 bp. RT-PCR products amplified from CEV cDNA and synthesized by the following amounts (in units) of reverse transcriptase: 0 (lane 2), 100 (lane 3), 200 (lane 4), 400 (lane 5), 800 (lane 6). Five microliters of each amplified DNA product was analyzed on 6% polyacrylamide gel at 100 V for 3 h in 89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3, buffer.

When CEV primer was omitted during reverse transcription, only products with sizes less than 175 bp were observed. Products in the size range of CEV were amplified when 0.1, 0.5, 1.0, 3.0, or 5.0 μg of CEV primer was added during transcription. The amount of amplified products increased gradually with increases in the amount of primer from 0.1 to 3.0 μg but then leveled off at 5.0 μg (results not shown).

Reverse transcriptase and HSV primers were also required for reverse transcription of CCaV or CVIIa. The sizes of the RT-PCR amplified products, however, were different from that of CEV transcripts (see below).

Specificity of RT-PCR products. The amplification of CEV cDNA and CCaV cDNA from CEV- and CCaV-infected tissues, respectively, by RT-PCR was specific (Fig. 2). A major DNA product of 371 bp (full length) (A, lanes 9 and 10) that hybridized with CEV cRNA probe (B, lanes 9 and 10) was observed after amplification of total nucleic acids from CEV-infected Etrog citron (A,B, lane 9) and sweet orange, (A,B, lane 10). This product did not hybridize with HSV cRNA probe (C) and was not detected in total nucleic acids from uninfected citrus (A,B, lanes 7 and 8). A major DNA product of approximately 300 bp (full length) was amplified in nucleic acids from CCaV-infected Etrog citron, (A, lane 4) and sweet orange, (A, lane 5) but not from uninfected citrus (A, lanes 2 and 3). This product hybridized with HSV cRNA probe but not with CEV cRNA probe (C and B, respectively).

In Figure 3, the electrophoretic mobility of the RT-PCR amplified products of CCaV transcript (lane 2) was compared with that of CVIIa (lane 3) and the 301 nucleotides long CPFV variant of HSV (lane 4). RT-PCR major product of CCaV cDNA has an electrophoretic mobility slightly but distinctly faster than that of CVIIa or CPFV. In addition to the major full length product, a product of approximately 600 bp, probably a dimer, was also observed for each viroid. The amplified cDNA products of the three viroids hybridized with the ^{32}P-labeled HSV cRNA probe (data not shown).

Sensitivity of CEV and CCaV detection by RT-PCR. Figure 4 shows the sensitivity of CEV and CCaV detection from infected tissue by gel electrophoresis analysis of RT-PCR products. A full length CEV cDNA product was detected from 10 ng to as little as 0.001 ng of total initial nucleic acids (lanes 1–5). Maximum CEV cDNA amplification was obtained at 10, 1, and 0.1 ng. The amount of amplified CEV cDNA decreased as total initial nucleic acids decreased to 0.01 and 0.001 ng. No amplified CEV cDNA product was detected at 0.0001 ng. Full length and dimer CCaV cDNA products were detected in samples transcribed and amplified from 10 to 0.1 ng of total initial nucleic acids from CCaV-infected tissue. No amplified CCaV cDNA product was detected from 0.01 ng of total nucleic acids.

Comparison of RT-PCR, return gel electrophoresis, and hybridization assays for citrus viroids. Table 1 shows a comparison between RT-PCR, return gel electrophoresis, and dot blot hybridization assays for the detection of CEV, CCaV, and CVIIa from infected Madam Vinous sweet orange and Etrog citron. An excellent correlation was observed between known biological properties and RT-PCR assays for CEV in the two hosts. A very good relationship was also observed between biological properties and RT-PCR assays for CCaV and CVIIa. The diagnosis of CCaV and CVIIa by the RT-PCR assay is presented in Figure 5. CCaV cDNA has an identical electrophoretic mobility with that of PDFV cDNA (lanes 1–3), however, CVIIa cDNA has an electrophoretic mobility slightly slower than that of CPFV cDNA (lanes 4–10) Sources E8, E25, and E28 tested positive for CVIIa and negative for CCaV by RT-PCR assay. Confirmation by biological assay for CVIIa is difficult because of co-infection with CEV (Table 1). Results of RT-PCR, return gel electrophoresis, and dot blot

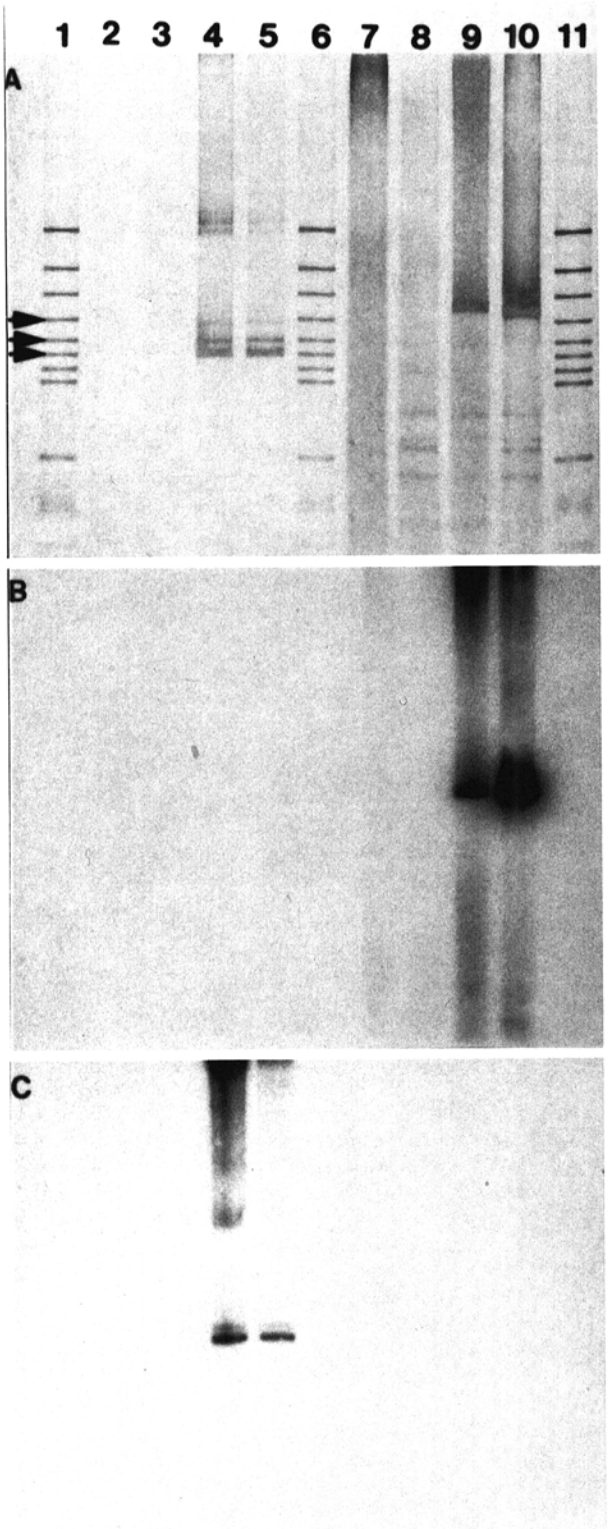

Fig. 2. Specificity of reverse transcription-polymerase chain reaction to amplifiy citrus exocortis viroid (CEV) cDNA or citrus cachexia viroid (CCaV) cDNA from total nucleic acids of infected citrus tissue. **A,** polyacrylamide gel electrophoresis analysis; **B** and **C**, autoradiograph of Southern blot hybridization analysis with ^{32}P-labeled CEV cRNA (B) and ^{32}P-labeled HSV cRNA (C) probes. Molecular DNA marker of *HaeIII*-digested PST B14 dimer (lanes 1, 6, and 11); the arrows indicate 359, 320, and 275 bp. Total nucleic acids from Etrog citron: uninfected (lanes 2 and 7), CCaV-infected (lane 4), CEV-infected (lane 9). Total nucleic acids from Madam Vinous sweet orange: uninfected (lanes 3 and 8), CCaV-infected (lane 5), CEV-infected (lane 10). Four to 6 μl of each amplified DNA product were analyzed.

hybridization assays for viroids from Etrog citron were very similar. Return gel electrophoresis analysis, however, was less sensitive than RT-PCR or dot blot hybridization assay for detecting CEV or hop stunt-related viroids from Madam Vinous sweet orange. RT-PCR is superior to dot blot hybridization assays as the latter failed to detect the X7 isolate of CCaV and the E25 isolate of CEV from infected sweet orange.

DISCUSSION

The present study demonstrates successful use of RT-PCR to directly detect CEV, CCaV, and CVIIa in total nucleic acids from infected citrus trees and indicates its feasability as a rapid laboratory assay for detecting CEV, CCaV, and CVIIa from field samples. Only a few days are required for positive identification of the viroid from infected citrus trees. The specificity of the reverse-transcribed amplified products for each viroid was established as follows: the reverse transcriptase and DNA primers were required for viroid cDNA synthesis from total nucleic acids of infected tissue; the size of the major product from CEV-infected tissue was identical to that of the 371-bp full length CEV, whereas that from CCaV-infected tissue was very similar to the 297-bp full length of PDFV, and that from CVIIa was a few nucleotides larger than the 301-bp full length of CPFV; absence of these major products in total nucleic acids from uninfected tissue; and

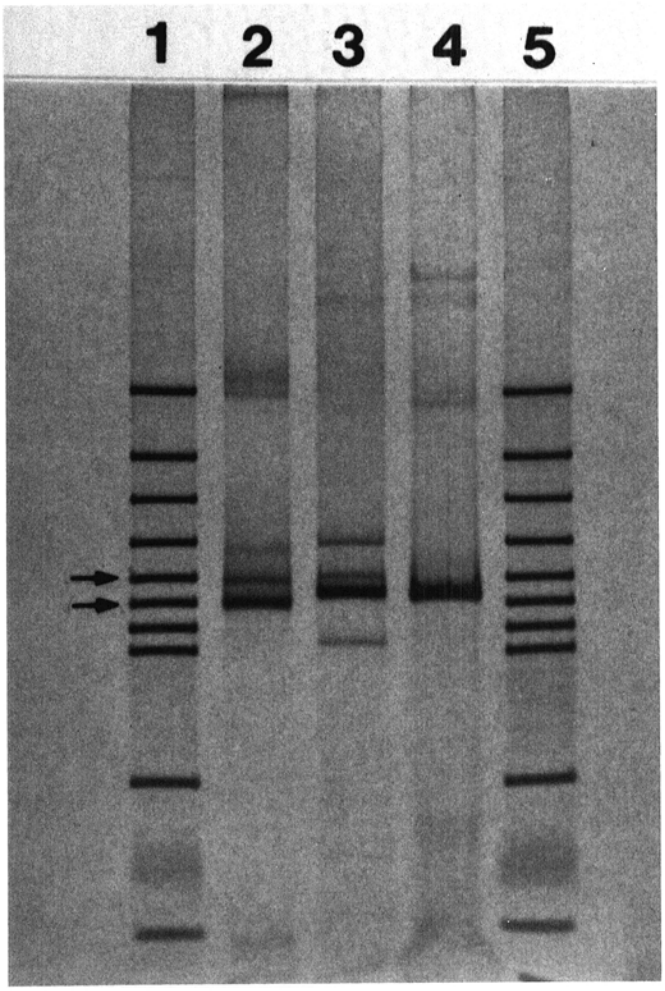

Fig. 3. Polyacrylamide gel electrophoresis analysis of reverse transcription-polymerase chain reaction-amplified citrus cachexia viroid (CCaV) cDNA (lane 2), citrus viroid IIa (CVIIa) cDNA (lane 3), cucumber pale fruit viroid (CPFV) cDNA (lane 4) from total nucleic acids of infected tissue. Molecular DNA marker (lanes 1 and 5); arrows indicate 320 and 275 bp. One microgram of nucleic acids was reverse-transcribed and amplified as described in Materials and Methods. Three to 5 μl of amplified DNA/sample were analyzed.

specific hybridization of amplified CEV cDNA with a CEV cRNA probe and CCaV cDNA as well as CVIIa cDNA with a HSV cRNA probe.

The detection of full length products of CEV cDNA, CCaV cDNA, and CVIIa cDNA indicated the successful isolation of undergraded viroids from citrus tissue using the nucleic acid extraction method described, as well as specific priming of an efficient RT reaction. The amplification of the dimeric forms of CCaV cDNA and CVIIa cDNA suggests that CCaV and CVIIa are better templates than CEV for dimer formation in vitro and/or in vivo. The formation of viroid dimeric forms by RT-PCR has been recently reported for ASSV (14).

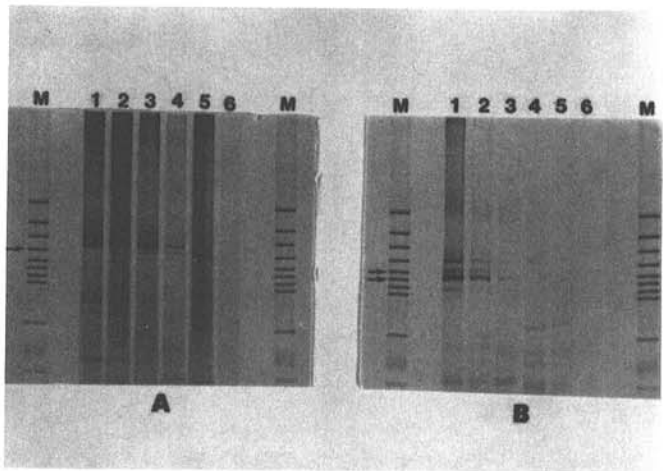

Fig. 4. Polyacrylamide gel electrophoresis analysis of reverse transcription-polymerase chain reaction-amplified citrus exocortis viroid (CEV) cDNA (A) and citrus cachexia viroid (CCaV) cDNA (B) synthesized from different initial amounts of total nucleic acids from CEV- or CCaV-infected tissue, respectively. Several amounts of total nucleic acids (0.01–1 ng) from infected tissue were reverse-transcribed and amplified as described in Materials and Methods. Five microliters of each amplified product (obtained from 0.0001–10 ng of total nucleic acids of infected tissue) were then analyzed on polyacrylamide gels. Molecular DNA marker (lanes M); the arrows indicate 359 (A), 320, and 275 bp (B). Amplified CEV cDNA or CCaV cDNA products from the initial amounts of total nucleic acids from infected tissue were analyzed 10 ng (lane 1), 1 ng (lane 2), 0.1 ng (lane 3), 0.01 ng (lane 4), 0.001 ng (lane 5), 0.0001 ng (lane 6).

The extremely high fidelity of RT-PCR is illustrated by the polyacrylamide gel electrophoresis analyses of amplified transcripts of CCaV and CVIIa. The electophoretic mobility of CCaV cDNA was identical to PDFV cDNA marker (Fig. 5, lanes 1–3). CCaV must contain approximately 297 nucleotides, as the chain length of PDFV cDNA is 297 nucleotides (26). The electrophoretic mobility of CVIIa cDNA was similar but distinctly slower than that of CPFV cDNA marker (Figs. 3,5). The chain length of CPFV used in this investigation is 301 nucleotides (17), thus the CVIIa chain length is very similar to the 302 nucleotides of the HSV variants isolated from Etrog citron in Japan (26).

The minimum amount of initial total nucleic acids required to detect CEV and CCaV by RT-PCR amplification and polyacrylamide gel electrophoresis analysis was 0.001 and 0.1 ng, respectively. Thus, the amount of CEV in infected tissue is at least 100-fold higher than that of CCaV in the same citrus cultivar, assuming similar transcription and amplification efficiency. Previous studies, using polyacrylamide gel elctrophoresis analysis, have indicated that CEV occurs in much higher amount than CCaV in citrus (23). The limits of detection of the two citrus viroids by RT-PCR flank the 0.01 ng of total nucleic acids required to detect ASSV from infected apple trees (14).

The detection of citrus viroids from Madam Vinous sweet orange by gel electrophoresis analysis of the RT-PCR amplified products indicates that RT-PCR is potentially useful for direct detection of CEV, CCaV, and CVIIa from sweet orange trees. Currently, return gel or sequential gel analysis for citrus viroids requires inoculation to Etrog citron or processing a large sample of the field variety. RT-PCR would increase the speed of detection without the need for large samples or molecular hybridization. Because the chain length of CCaV cDNA and CVIIa cDNA RT-PCR amplified products differs by only a few nucleotides (Figs. 3,5), biological assays may be needed to confirm the identity of these two viroids.

Primers selected for the detection of CEV were specific for the upper central conserved region (nucleotides 71–90) and its adjacent segment (nucleotides 91–114). This nucleotide sequence, or a very similar sequence, is unique to members of the potato spindle tuber group. Similarly, primers selected for the detection of CCaV or CVIIa were specific for the upper central conserved region and its adjacent segment of the HSV group. Thus the CEV primers used in this investigation (or primers with a very similar sequence), as well as the HSV primers should detect members of the PSTV group and HSV group, respectively. Indeed,

TABLE 1. Comparison of the results[a] obtained with reverse transcription-polymerase chain reaction (RT-PCR), return-gel electrophoresis, and dot blot hybridization assays for the detection of citrus exocortis viroid (CEV), citrus cachexia viroid (CCaV), and citrus viroid IIa (CVIIa) in total nucleic acids of infected bark tissue[b]

				Assay						
	Viroids[c]			RT-PCR-gel electrophoresis			Return-gel electrophoresis		Dot blot hybridization	
Host and source code	CEV	CCaV	Others[d]	CEV	CCaV	CVIIa	CEV	Others[e]	CEV	Hop stunt viroid-related
Sweet orange, H	−	−	−	−	−	−	−	−	−	−
Sweet orange, E9	+	−	−	+	−	−	+[f]	−	+	−
Sweet orange, X7	−	+	−	−	+	−	−	−	−	−
Sweet orange, E22	+	−	+	+	−	+	+[f]	+[f]	+	+
Sweet orange, E28	+	ND	ND	+	−	+	−	−	+	+
Sweet orange, E8	+	ND	ND	+	−	+	−	−	+	+
Sweet orange, E25	+	ND	ND	+	−	+	−	−	−	−
Citron, H	−	−	−	−	−	−	−	−	−	−
Citron, E9	+	−	−	+	−	−	+	−	+	−
Citron, E11	−	−	+	−	−	+	−	+	−	+
Citron, X7	−	+	−	−	+	−	−	+	−	+
Citron, E9	+	−	−	+	−	−	+	−	+	−

[a] A plus sign indicates a positive assay; a minus sign indicates a negative assay; ND indicates not determined. Assays were repeated at least once.
[b] Total nucleic acids were extracted by the phenol/chloroform method from 1–2 g of bark tissue, then purified on RNase-free ELUTIP-r minicolumns as described in the Materials and Methods section.
[c] Viroids were previously identified by biological indexing on citrus indicator plants.
[d] CVIIa, CVIII, and CVIV.
[e] CCaV, CVIIa, CVIII, and/or CVIV.
[f] Faint silver-stained viroid bands were always obtained.

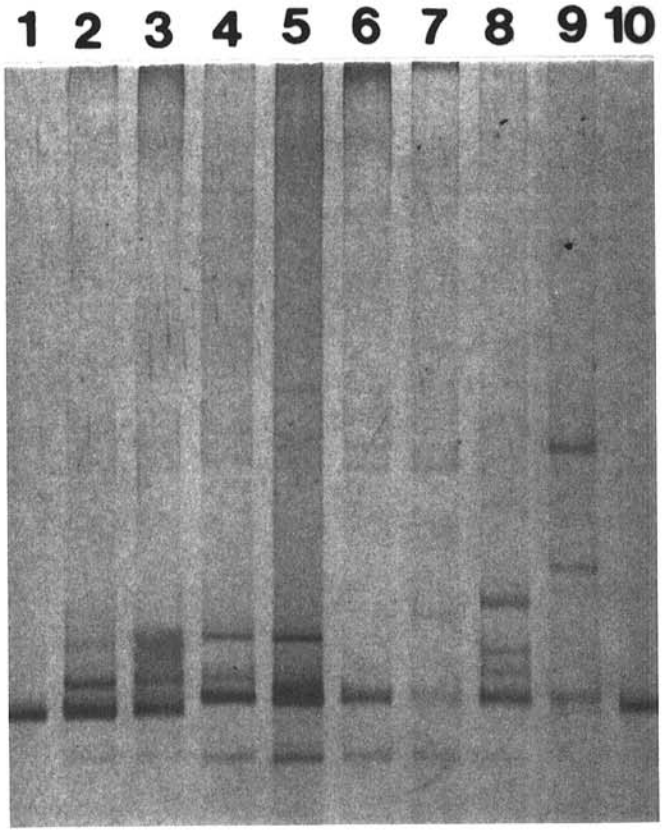

Fig. 5. Diagnosis of citrus cachexia viroid (CCaV) and citrus viroid IIa (CVIIa) by polyacrylamide gel electrophoresis analysis of reverse transcription-polymerase chain reaction (RT-PCR) products amplified from total nucleic acids of infected citrus tissue. RT-PCR products of the 297-bp plum dapple fruit viroid (PDFV) cDNA and 301-bp cucumber pale fruit viroid (CPFV) cDNA markers (lanes 1 and 10, repectively). RT-PCR products of CCaV-infected tissue: citron, X7 (lane 2); sweet orange, X7 (lane 3). RT-PCR products of CVIIa-infected tissue: citron, E11 (lane 4); sweet orange, E22 (lanes 5 and 6); sweet orange, E28 (lane 7); sweet orange, E8 (lane 8); sweet orange, E25 (lane 9). Aliquots (5–20 μl) of the RT-PCR products were analyzed by electrophoresis on 6% polyacrylamide slab gel at 100 V for 6–7 h in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3) buffer until the xylene cyanol FF dye reached the end of the gel.

the HSV primers have been used in our laboratory to detect HSV in infected plum, peach, and grapevine (A. Hadidi et al, *unpublished*).

Other viroids in addition to CEV, CCaV, and CVIIa occur in citrus. These may require additional specific primers for their detection. Isolates E11 and E22 used in this study carry CVIII, and there was no evidence of cross priming in the RT-PCR assays (Table 1). The lack of hybridization of CVIII with CEV or CCaV probes (4) supports these results. Recently, the simultaneous detection of CEV and CCaV in a single reaction by multiplex RT-PCR has been reported (15), and the simultaneous detection of additional citrus viroids in a single sample should be feasible with appropriate primers.

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