The Use of RT-PCR in the Florida Citrus Viroid Indexing Program

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ABSTRACT. Reverse transcription polymerase chain reaction (RT-PCR) has been incorporated as an adjunct to Florida's biological indexing program for viroids, especially to test for Citrus viroid II (CVd-II) and all viroids where rapid results are needed. The sensitivity of RT-PCR for testing of composite samples was evaluated using 5, 10, 25, 50, 100 and 200 tree composites. Single positive trees were detected consistently in the 5- and 10-tree composite samples; these 5 and 10 tree composites are routinely used to test for CVd-II using Etrog citron indicators. When a composite sample is found to be positive, the individual trees comprising the composite are retested. Viroid infected source trees, identified by biological indexing, were tested by RT-PCR for CVd-II, *Citrus viroid III* (CVd-III) and *Citrus exocortis viroid* (CEVd) using tissue collected from both the citron indicators and the corresponding field trees to evaluate the validity of RT-PCR testing. Reliable detection of CVd-II, III and CEVd from field samples by RT-PCR is important for shortening testing time and reducing costs. RT-PCR also allows rapid diagnosis of field samples and testing of trees near a citrus canker quarantine zone without exposing our biological test facilities to potential sources of citrus canker inoculum. Agarose gel electrophoresis was adequate for visualization of CVd-II RT-PCR products, but the sensitivity of CEVd and CVd-III detection has been increased by visualization of RT-PCR products by using either polyacrylamide gels with GelStar stain or Metaphor agarose gels. Improved primers have increased our confidence in CEVd detection by RT-PCR. Screening PCR products from 32 Florida viroid isolates causing a mild citron reaction with oligonucleotide probes specific for CVd-IIIa or CVd-IIIb revealed that CVd-IIIa and IIIb occur both separately and as mixed infections.

Index words. Viroid, citrus, certification, biological indexing.

Citrus budwood certification programs must test for endemic graft-transmissible citrus viruses and viroids that cause economic damage to commercial citrus production. In the Florida Citrus Budwood Registration Program, the largest volume of testing is for *Citrus tristeza virus* (CTV) and citrus viroids (13). Testing to detect viroids in budwood source trees has increased in importance with an increasing reliance on rootstocks that are hybrids with *Poncirus trifoliata*, predominately Swingle citrumelo and Carizzo citrange that were 73% of the nursery propagations in Florida during the past decade (3). Viroids of concern to Florida are *Citrus exocortis viroid* (CEVd), Citrus viroid II (CVd-II) and *Citrus viroid III* (CVd-III) (12). CVd-III isolates were examined for variants; the nucleotide sequences of the two most common variants of CVd-III, CVd-III a (297 nt), and CVd-III b (294 nt) were first reported by Rakowski et al. (8).

The large number of budwood source trees in the Florida program has made pathogen testing difficult. In fiscal year 1996-1997, there were 15,583 scion trees and 3,375 foundation trees that required testing. In January 1997, when Florida's voluntary budwood program became mandatory (1), the number of trees registered with the program dropped dramatically. Three factors were responsible for this decrease: an annual charge of $2 to register each tree, the cost of an annual CTV test by a commercial testing laboratory for each active tree, and the loss of field trees dropped from the program after they tested positive for putative decline isolates of CTV, based on reactivity with the selective monoclonal antibody, MCA-13 (8). In fiscal year 2000-2001, the number of budwood source trees had dropped to 3,309 scion trees and 1,326 foundation scion trees, for a total of 4,635 trees that require routine viroid testing every 6 yr. Other

viroid testing requirements raise the annual viroid testing requirements to over 2,000 tests per year.

RT-PCR was added to the viroid-indexing program in 1998 (13). Etrog citrons inoculated for biological indexing are tested for CVd-II by RT-PCR. As the incidence of viroids in source trees is low (i.e., less than 1%), we have examined different sized composite samples to determine whether or not it is possible to maintain sufficient sensitivity, while reducing both the time needed, as well as the cost of reagents. The critical need for effective PCR diagnostics to complement biological indexing provides a strong incentive to improve PCR testing in the Florida Citrus Budwood Registration Program.

Promising initial results from a companion paper (4) describing the use of RT-PCR to detect viroids from field trees are further explored using data generated by the Florida Bureau of Citrus Budwood Registration. As described by Garnsey et al. (4), amplification of CEVd using primers designed to produce full-length amplification products often produces spurious bands with healthy extracts, making reliable detection of this viroid more difficult. Here, we describe the use of composite samples, improved PCR detection of CEVd, an approach for rapid profiling of populations of CVd-III, and easy practical detection of PCR products. These improvements have allowed the Florida Bureau of Citrus Budwood Registration to test large numbers of samples, while improving the reliability of test results.

MATERIALS AND METHODS

Composite sampling. Commercial cultivars of sweet orange and mandarin were inoculated with greenhouse material of CEVd (Isolate E9), CVd-II (Isolate X7) and CVd-III (Isolate E36) and grown in the field for one year before testing tissue. Hardened budwood was harvested during the summer and extracted using a Kleco Tissue Pulverizer (Kinetic Laboratory Equipment Co., Visalia, CA). To approximate 5, 10, 25, 50, 100 and 200 tree composite samples, viroid-infected and healthy tree sap extracted by the SDS-KAc method of Garnsey et al. (4) was mixed 1:0, 1:5, 1:10, 1:25, 1:50, 1:100 and 1:200 (v/v) just prior to ethanol precipitation.

RT-PCR. RT-PCR was performed using an Applied Biosystems RNA core kit according to the manufacturer's specifications. Large numbers of reactions were assembled using multi-pipetters to dispense master mixes. Primers and cycling times and temperatures for CVd-II, CEVd (14) and CVd-III (9) have been previously described and produced full-length double-stranded viroid cDNAs.

New primers for CEVd produced a product containing approximately 228 bp. CEV D-5 (5'-ACGAGCTCCT-GTTTCTCCGCTG-3') is complementary to nt 185 to 164, and CEV D-7 (5'-CCGGGCGAGGGTGAAAGCCC-3') is homologous to nt 329 to 348. For reverse transcription, the following temperatures and incubation times were used: 42°C (15 min), 99°C (5 min), and 5°C (5 min). RT-PCR reactions were carried out in an Eppendorf MasterCycler, and the annealing temperature was optimized for this machine. PCR was carried out for a total of 35 cycles of 94°C (1 min), 54°C (1 min), and 72°C (1 min).

Identification of CVd-III sequence variants. Forty isolates from the viroid collection at Winter Haven (believed to contain CVd-III) were selected for RT-PCR analysis. Total nucleic acids were extracted from samples of green bark tissues as described by Owens et al. (5). Bark samples from uninfected Etrog citron plants and citrons known to be infected with either CVd-IIIa or IIIb, were included as controls. Full-

length double-stranded CVd-III cDNAs were synthesized by RT-PCR using the Superscript II system (Invitrogen), primers C2' (5'-ACTCTCCGTCTTTACTCCAC-3', complementary to CVd-IIIb nt 138-119) and H2' (5'-CTCCGCTAGTCG-GAAAGACT-3', homologous to CVd-IIIb nt 139-158), and Amplitaq DNA Polymerase (Perkin-Elmer) essentially as described previously (6).

Two pairs of synthetic DNA probes, one specific for CVd-IIIa and the second specific for CVd-IIIb, were used for dot-blot hybridization analysis of CVd-III PCR products. Probes CVd-52,73A (5'-AATGCA-GAGAGGGGAAAGGGAA-3') and CVd-171,192A (5'-GCTGACCCTCCT [TC]AGCTC[G]CC-3') are specific for CVd IIIa; CVd-52,73B (5'-AACG-CAGAGAGGGAAAGGGAAA-3) and CVd-171,189B (5'-GCAGACCCTTC-TAGCTCCC-3') are specific for CVd-IIIb. In addition to several nucleotide substitutions (underlined), CVd-IIIa also contains two small insertions between positions 171 and 192 (in brackets). Oligodeoxynucleotides were labeled with [^{32}P] by incubation with [γ-^{32}P]ATP and polynucleotide kinase and purified by chromatography on Sephadex G-50 before hybridization analysis (11).

Prior to hybridization analysis, PCR products (9 µl containing approx. 100 ng DNA) were denatured by addition of 1 µl 3 M NaOH and 5 min incubation at room temperature. Denaturation was terminated by addition of 1.1 µl 2N HCl and 1.85 µl 1 M Tris-HCl, pH 7.5. After denaturation, a 2 µl aliquot of the resulting single-stranded CVd-III DNAs was diluted 5-fold with 10× SSC. Aliquots (2 µl) of the denatured DNAs were then spotted onto a positively charged nylon membrane (Boehringer Mannheim) and auto-crosslinked in the presence of 10× SSC buffer. Hybridization reactions and subsequent washes were carried out in the presence of 6× SSC–1× Denhardt's solution–100 µg/ml yeast tRNA–0.05% sodium pyrophosphate as described (2). Assays containing probes CVd-171,192A or -171,189B were pre-hybridized for 30 min at 42°C, hybridized overnight at 42°C, and washed the next day at room temperature. Those containing probes CVd-52,73A or -52,73B were washed for 30 min at 60°C. Washing temperatures were empirically chosen so as to provide maximum discrimination between control samples known to contain only CVd-IIIa or CVd-IIIb. Images were recorded using either Kodak X-AR film or a BioRad GS363 phosphoimager.

Automated sequence analyses of uncloned PCR products derived from Winter Haven isolates E-21a and E-21b (GenBank accession numbers AF447788 and AF447789) were carried out using Perkin-Elmer/Applied Biosystem's AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP. Primers C2' and H2' were used as sequencing primers, and analyses were carried out on an ABI Model 373A sequencer. Double-stranded cDNAs from two Florida isolates that failed to hybridize with probes specific for CVd-IIIa or IIIb were cloned using the Topo TA Cloning Kit (Invitrogen) and sequenced using dye-labeled M13 forward and reverse sequencing primers.

Detection of PCR Products. PCR products from CEVd and CVd-III were run on 1.5% agarose gels, stained with ethidium bromide, and the resulting patterns compared to those from 6% non-denaturing polyacrylamide gels stained with a silver salts kit (BioRad). Silver stained polyacrylamide gels were compared to acrylamide gels stained with GelStar nucleic acid stain (BioWhittaker Molecular Applications). Large agarose (20 × 20 cm) gels were stained with 50 ml of a Gelstar solution in gallon ziplock bags to reduce the volume of stain required. The sensitivity of GelStar stained acrylamide gels was also compared to 3% Metaphor (BioWhittaker Molec-

ular Applications, Rockland, Maine, USA) agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Composite sampling. The sensitivity of testing of composite samples by RT-PCR was evaluated by mixing extracts from viroid infected and healthy trees at 1:5, 1:10, 1:25, 1:50, 1:100 and 1:200 ratios. Tests were conducted with four sweet orange and one Satsuma mandarin source. Results are summarized in Table 1.

CVd-II was detected consistently at all dilutions from all samples. CVd-III could be detected at dilutions ranging from 1:10 to 1:25. For CEVd in sweet orange, the detection limit ranged from 1:25 to 1:200. Consistent with results reported in a companion paper (4), CVd-III and CEVd were not detected from the Satsuma mandarin source at any dilution. Ten-tree composites were chosen for routine use for RT-PCR testing for CVd-II from Etrog citron.

Composite testing is effective only when the rate of positive infection in the populations sampled is low and retesting of individuals in the composite sample is infrequent. Our viroid infection rate is less than one percent for routine indexing of program trees. Samples thought to have a good chance of viroid infection (for example, problematic trees or parent trees) are not composited, but are tested as single-tree samples.

Incorporation of RT-PCR for CVd-II into the Citron Indexing Program. To coordinate with the natural seasons and to take advantage of superior bud live during summer months, seeds of vigorous rootstocks (including Palestine sweet lime, rough lemon, Volkamer lemon and Kinkoji) are planted in equal numbers during the months of July through November. These are budded March through July using commercial budders. Test sets are inoculated with one budstick from

TABLE 1
CITRUS VIROID DETECTION BY RT-PCR FROM COMPOSITE MIXTURES OF EXTRACTS FROM HEALTHY AND VIROID-INFECTED PLANTS

	Dilution																	
	1:1			1:5			1:10			1:25			1:50			1:100			1:200		
Variety	II [1]	III [2]	CEV[3]	II	III	CEV	II	III	CEV	II	III	CEV	II	III	CEV	II	III	CEV	II	III	CEV
Hamlin	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	—	+	+	—	+
Navel 1	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	—	+
Navel 2	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+	+	—	+	+	—	+
Midsweet	+	+	+	+	+	+	+	+	+	+	—	+	+	—	—	+	—	+	—	—	—
Satsuma	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—

[1]CVd-II.
[2]CVd-III.
[3]CEVd. Primers CEV D-5 and D-7.

each of six sectors of the tree collected from foundation trees at Winter Haven, Dundee and Immokalee, or field and screen house-grown scion trees from around the state. Samples comprising six budsticks from distinct sectors of the tree are collected and inoculated into the Etrog citron from June through October. We have increased the number of inoculated plants per test set from four to six to eliminate the need to recollect material for re-budding. Test sets are considered to be valid as long as three of the original six inoculated plants have viable inoculum at 6 weeks. Any test set not meeting this criterion is discarded and restarted the following year.

Samples for extraction for CVd-II RT-PCR are taken after the second readings. Second readings start in October for plants inoculated in May, and run through June for plants inoculated in October. Although minimum temperatures in the greenhouses may be as low as 22°C during the winter months, Garnsey et al. (4) have shown that CVd-II can be detected even in cool weather. Test sets are run as 10 tree composites, with 0.1 g of bark collected from all of the indicators per each of the 10 test sets. RT-PCR is run before the final reading when the plants are to be discarded. When a positive is found, either the tissue is recollected from the 10 field trees making up the composite, or the 10 test sets in the greenhouse are flagged and tested individually at the final reading.

Positive Source Trees. When positive samples are found by composite testing of the Etrog citron indicators with RT-PCR, the sources comprising the composite are retested individually. In cases where the citron indicators did not show the characteristic CVd-III and CEVd symptoms of leaf epinasty and petiole necrosis, routine testing of the citron indicators by RT-PCR revealed that five sweet orange and one lemon source trees contained CVd-II (Table 3).

Five tree composites were also used to survey a block of trees exposed to viroid infection for the presence of CVd-II, III and CEVd. Although Garnsey et al. (4) found that there might be some difficulty in detecting viroids from some varieties growing in the field, we were able to detect one or more viroids from field trees of sweet orange, mandarin, lime, pummelo and *Microcitrus* (Table 3). These included not only CVd-II, but also CVd-III and CEVd. Note, however, that transmission of mixed infections from source trees to citron indicators frequently resulted in the loss of one or more viroid species when four budwood sticks from different sectors of the tree are used for inoculation.

Identification of CVd-III sequence variants. Thirty-five of 40 viroid isolates subjected to RT-PCR analysis using primers C2' and H2' yielded the ~294-297 bp product characteristic of CVd-III (results not shown). Of the 31 isolates examined by oligonucleotide hybridization, five were shown to contain solely IIIa, 17 solely IIIb, 7 both IIIa and IIIb, and two (Winter Haven isolates E-21a and E-21b) failed to hybridize with any of the four probes. Five of 40 isolates tested failed to amplify by RT-PCR, and product yields from four additional isolates were too low for analysis without re-amplification.

To confirm the presence of sequence changes in portions of molecule complementary to the probes used for hybridization analysis, uncloned PCR products derived from isolates E-21a and 21b were subjected to automated sequence analysis. The nucleotide sequences of these two isolates proved to be identical. As shown in Fig. 1, this variant contains only 292 nt and differs from all other CVd-III sequences in GenBank at several positions between nucleotides 180 and 225. E-21a/b is most closely related to three variants recovered from the Israeli navelate isolate (6).

TABLE 2
VIROIDS DETECTED BY RT-PCR IN FIELD TREES AND ETROG CITRON INDICATORS IN SINGLE TREE AND 5 AND 10 TREE COMPOSITE SAMPLES

Variety	Source	Number of trees[1]	Sampling	RT-PCR
Sweet Orange	Citron	4	5 tree composite	II[2]
Sweet Orange	Citron	1	10 tree composite	II
Sweet Orange	Field tree	1	5 tree composite	II, III[3], CEV[4]
Sweet Orange	Field tree	1	5 tree composite	CEV
Sweet Orange	Field tree	4	Single tree	II
Sweet Orange	Field tree	5	Single tree	II, III
Mandarin	Field tree	1	Single tree	II, III
Grapefruit	Field tree	1	5 tree composite	II
Lemon	Citron	1	5 tree composite	II
Lemon	Field tree	8	Single tree	II
Lime	Field tree	3	Single tree	II, II, CEV
Lime	Field tree	1	Single tree	III
Pummelo	Field tree	1	Single tree	II, III
Microcitrus	Field tree	1	Single tree	CEV

[1]Number of times positive(s) detected in that type of sample (single tree or 5- or 10-tree composite) for that variety.
[2]CVd-II.
[3]CVd-III.
[4]CEVd. Primers CEV-20 and CEV-24.

Improved Primers for CEVd. CEVd detection was improved with the new primers. Eleven viroid isolates causing severe leaf epinasty in Etrog citron, were tested with the new primers, CEV D-5 and CEV D-7, and the results compared to those with the primers CEV-20 and CEV-24 (13). Ten tested positive for CEVd with both sets of primers, and one was negative with both sets of primers. Twelve other isolates were tested with the new primers, five of which tested negative and seven positive; these results were consistent with the corresponding citron reaction. Primers CEV D-5 and CEV D-7 which were designed to produce full-length amplification products, often produced spurious bands with healthy extracts, making reliable detection of this viroid difficult (4).

TABLE 3
VIROIDS DETECTED BY RT-PCR IN FIELD TREES AND CITRON INDICATORS

			RT-PCR					
			Citron			Field		
Variety	Number	Citron reaction	II	III	CEVd	II	III	CEVd
Pummelo	1	Mild	+	+	—	+	+	—
Orange	3	Moderate	+	+	—	+	+	—
Mandarin	1	Moderate	+	+	—	+	+	—
Lime	1	Severe	+	+	+	+	+	+
Lime	1	Severe	—	—	+	+	+	+
Lime	1	Moderate	—	—	—	—	+	—
Lime	1	Severe	—	—	+	+	+	+
Microcitrus	1	Severe	—	—	+	—	—	+

[1]CVd-II.
[2]CVd-III.
[3]CEVd. Primers CEV-20 and CEV-24.

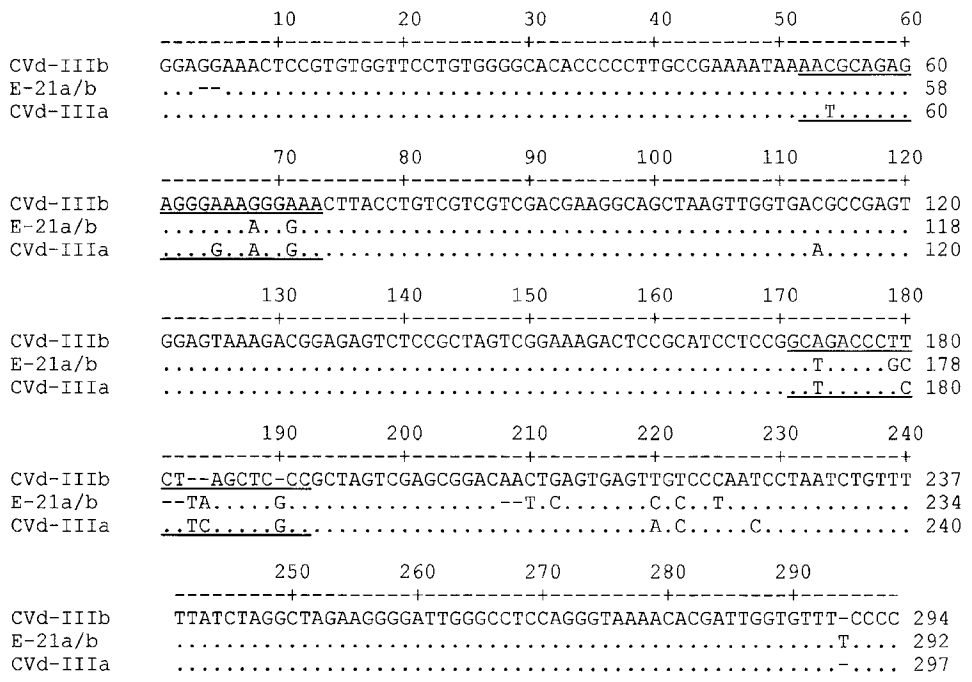

Fig. 1. Nucleotide sequence of CVd-III isolates E-21a and 21b. Underlined nucleotides are complementary to the DNA probes used to distinguish CVd IIIa from CVd-IIb.

Repeated attempts with single-tree samples were often necessary to have confidence in the results, with all controls working properly. Dilutions were not attempted with this set of primers. The sensitivity of detection with the primers CEV-20 and CEV-24 was as low as a 1:200 dilution for many trees (Table 1). However, with the increased sensitivity, greater care was required in the setting up of reactions. It was necessary to dispense the PCR master mix individually with new filter pipet tips to avoid contamination.

Improved Detection of PCR Products. Improved detection of CEVd and CVd-III requires a sensitive, convenient method to visualize their respective amplification products after electrophoresis in either polyacrylamide or Metaphor agarose gels. Silver staining of polyacrylamide gels was a lengthy procedure (i.e., overnight plus half of the next day); however, GelStar staining required only 30 minutes with a brief rinse. As shown in Fig. 2, the sensitivities of these methods are very similar. After a 3-day drying period, silver stained dried polyacrylamide gels were kept as documentation instead of using photography. GelStar stained gels were photographed with UV illumination and a GelStar filter. Metaphor agarose gels were visualized with ethidium bromide and photographed with UV illumination. GelStar staining of polyacrylamide gels was more rapid than silver staining, the timing was not as critical, and there was less background. By staining in ziplock bags, we were able to keep the cost of GelStar low by minimizing the volume of staining solution used. The cost to stain a 20 cm × 20 cm gel with GelStar was $1.20 versus $21 for the silver staining with the kit from BioRad. GelStar was clearly the preferred method to stain polyacrylamide gels. Its reduced background gave the clearest results, and it was the fastest, easiest and cheapest method to use. Metaphor agarose gels also gave good visual-

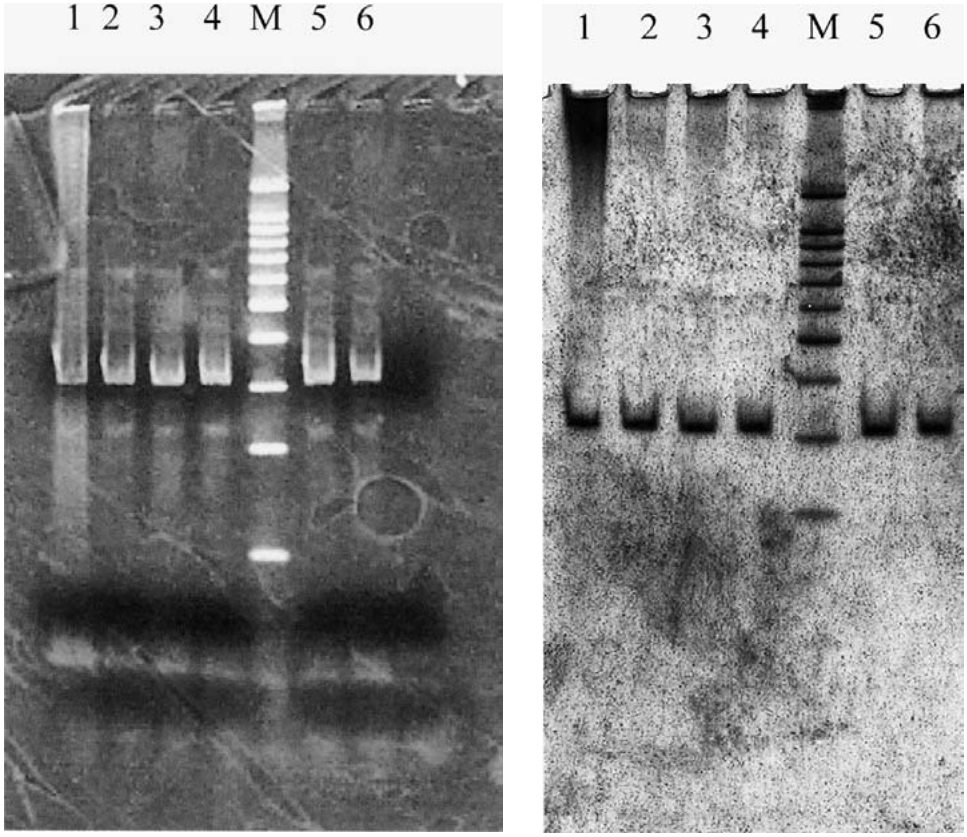

Fig. 2 RT-PCR products of CVd-III from isolate E-11 from different tissues of Etrog citron. Acrylamide gels stained with Gelstar stain and visualized with UV on the left and a silver stained dried gel on the right. Samples of trees 1-6 amplified with primers for CVd-III. M: molecular marker.

ization of PCR products, and the gels were easier to prepare than polyacrylamide gels.

CONCLUSIONS

Comprehensive testing programs use both biological indexing and laboratory testing to screen for the same pathogens (10). Greenhouse biological indexing ensures that a broad range of strains is picked up; laboratory testing gives quicker results that are more cost effective than biological indexing. For programs where large numbers of source trees need to be tested, the use of composite samples for PCR make it possible to test all budwood source trees at the proper time interval. Viroid titer is thought to be higher in Etrog citron indicators than in field trees (14); by testing composite samples from citron for CVd-II, the Florida Citrus Budwood Registration Program has a good confidence level for detection of all positives. However, composite samples are only practical when the rate of infection in the test population is low. RT-PCR also allows rapid diagnosis via direct testing of field trees, although these may need to be tested as single tree samples depending on the rate of infection in the population. Imprint hybridization has previously been found to be a fast, effective and cost effective method for screening for citrus viroids, but it cannot be used to test field trees (7). The ability to detect CVd-II, CVd-III and CEVd from field samples by RT-

PCR is important for decreasing testing time and reducing costs. As PCR is only used routinely for detection of CVd-II, and PCR for all viroids is mainly used when a positive is found, multiplex PCR has not been utilized. However multiplex PCR would be a viable option for testing large numbers for multiple viroids. Direct RT-PCR testing of field trees also allows testing of trees that could pose a risk if material were brought into the certification and testing area for biological indexing; e.g., citrus canker or other diseases not endemic to the area.

Viroids were detected by RT-PCR from pummelo and *Microcitrus* in addition to hosts for which detection by RT-PCR has been previously reported (4). It is very important to know what varieties are or are not amenable (Satsuma mandarin for CVd-III) to RT-PCR analysis (4). CVd-II and CVd-III were recovered more frequently than CEVd from budwood source trees, but all three viroids do occur in trees in Florida. Agarose gel electrophoresis and ethidium bromide staining is adequate for visualization of RT-PCR products derived from CVd-II. The sensitivity of CEVd and CVd-III detection has been increased by visualization of RT-PCR products by either GelStar stained polyacrylamide gels or Metaphor agarose gels visualized with ethidium bromide. Based on initial tests, improved primers for CEVd have increased our confidence for detecting this viroid by RT-PCR and decreased problems noted by Garnsey et al. (4).

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