An Improved Protocol for Extraction and RT-PCR Detection of Citrus Viroids

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ABSTRACT. The great sensitivity of RT-PCR makes this technique a desirable alternative to other diagnostic methods for detection of viroids. In previous studies, difficulties have been encountered in obtaining amplicons from samples known to contain viroids. The aim of the present study was to minimize extraction procedures and to design RT-PCR protocols based on the use of thermostable reverse transcriptases. The use of 27-nucleotide primers and reverse transcription at 60°C minimized re-naturation of the viroid template and consistently yielded the expected PCR products for each viroid. The protocol has been tested with samples from a large number of viroid-host combinations grown under greenhouse conditions as well as with samples from the field.

Viroids are small (246-401 nucleotides) covalently closed single-stranded RNAs that replicate in their host plants in which they may elicit disease symptoms. Citrus is a natural host of five viroid species all belonging to the *Pospiviroidae* family: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV). The greater sensitivity of reverse transcription polymerase chain reaction (RT-PCR) for viroid detection is regarded as a desirable alternative to other diagnostic methods. Several approaches have been reported demonstrating its potential for detection of citrus viroids (2, 4, 6). However, a number of assays conducted in our laboratory showed that in many instances, samples known to contain viroids yielded negative results.

This probably was due to i) the presence of inhibitors that could interfere with the reactions, or ii) inefficient synthesis of cDNA of a suitable size to be amplified by PCR. The aim of the present study was to optimize current protocols by:

1. Modifying extraction procedures to minimize tissue damage in order to avoid the release of putative inhibitors and to prevent cross-contamination among samples.
2. Designing primers for optimization of RT-PCR protocols.
3. Verifying the suitability of the method for viroid detection in different citrus species and cultivars.

Extraction procedures. Nucleic acid preparations obtained with standard extraction procedures using a harsh homogenization in an extraction medium containing phenol followed by partition of nucleic acids in 2M LiCl (5) (Fig. 1A) were compared to preparations obtained using a simplified method based on a gentle homogenization in sealed plastic bags followed by alkaline denaturation (1, 3) (Fig. 1B). The simplified extraction method yielded low viroid titers (data not shown) but the preparations were suitable for RT-PCR analysis. The method has several advantages: i) no phenol is used, ii) mild extraction with hand-model homogenizer results in release of few inhibitors, iii) minimal manipulation of samples by homogenization in sealed plastic bags minimizes cross contamination risks, and iv) there is the possibility of handling large numbers of samples in a short period of time.

Primer design and RT-PCR protocol. The protocol is based on the utilization of a reverse transcriptase (ThermoScript™ RNase H-) which is active at temperatures as

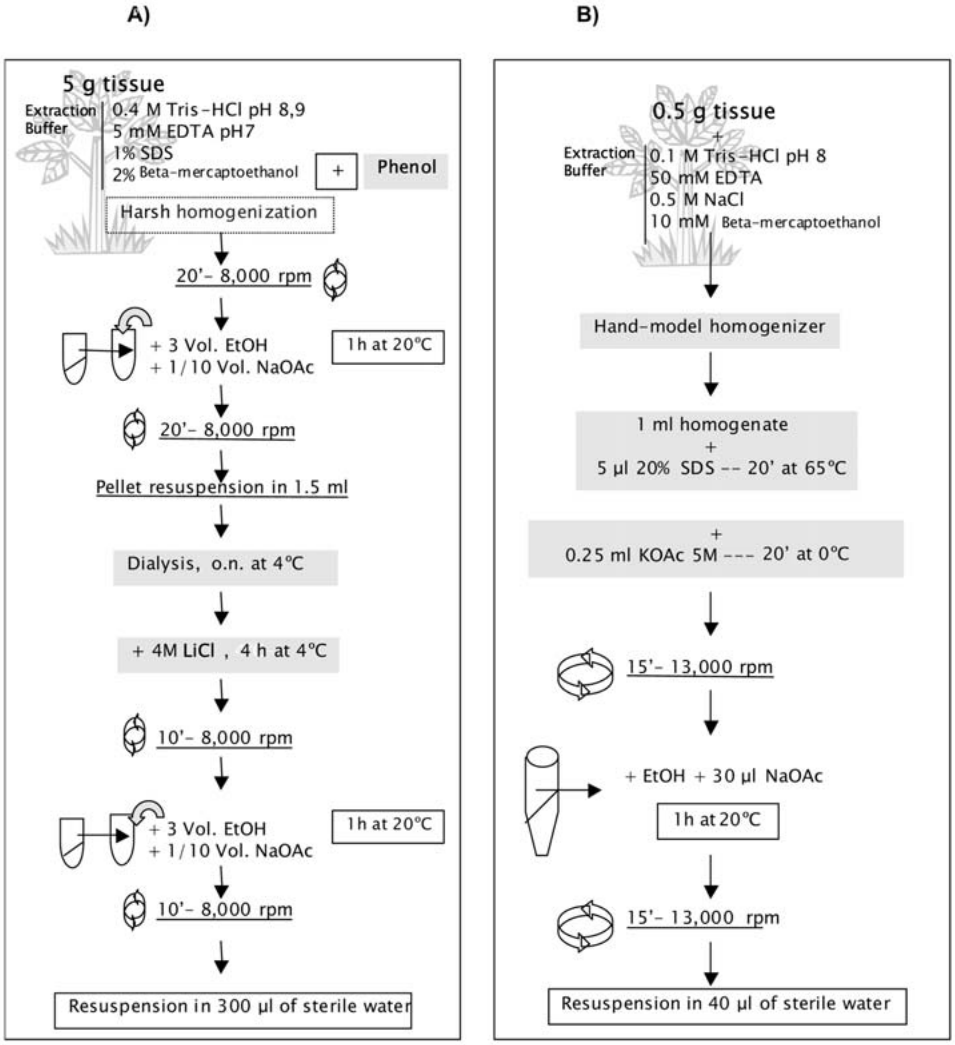

Fig. 1. Extraction procedures tested for RT-PCR analysis of citrus viroids. A) Standard protocol according to Semancik et al. (5) that yields high viroid titers suitable for sequential polyacrylamide gel electrophoresis. B) Simplified protocol according to Astruc et al. (1) and Pallás et al. (3).

high as 65°C, temperatures which diminish renaturation of the target viroid molecule. The higher temperature requires the use of reverse primers that will anneal to the target viroid at the temperature chosen for reverse transcription. Suitable primers make it possible i) to increase the specificity of the reaction by using annealing temperatures up to 60°C, ii) to avoid renaturation of the viroid template, resulting in an increased yield and size of amplified viroid cDNAs (Fig. 2). Following reverse transcription using 27-nucleotide primers complementary to the central conserved region (CCR) sequence, second strand synthesis and PCR amplification can be successfully performed using contiguous primer sets derived from the sequence of the C region of the indicated viroids (Fig. 2).

Reverse transcription parameters consisted of a denaturation step at 95°C for 5 min, followed by sequen-

RT reverse primers	
CEVd-RT	5' CTTCCTCCAGGTTTCCCCGGGGATCCC 3'
CBLVd-RT	5 GCTGACGAGCCTTCGTCGACGACGACC 3'
HSVd-RT	5' GTGTTGCCCCGGGGCTCCTTTCTCTGG 3'
CVd-III-RT	5' CCAACTTAGCTGCCTTCGTCGACGACG 3'
CVd-IV-RT	5' GTCTGAAGAGATTTCCCCGGGGATCCC 3'
PCR reverse primers	
CEVd-R	5' CCGGGGATCCCTGAAGGA 3'
CBLVd-R	5' TTCGTCGACGACGACCAGTC 3'
HSVd-R	5' GGGGCTCCTTTCTCAGGTAAGTC 3'
CVd-III-R	5' TTCGTCGACGACGACAGGTA 3'
CVd-IV-R	5' GGGGATCCCTCTTCAGGT 3'
PCR forward primers	
CEVd-F	5' GGAAACCTGGAGGAAGTCG 3'
CBLVd-F	5' GGCTCGTCAGCTGCGGAGGT 3'
HSVd-F	5' GGGGCAACTCTTCTCAGAATCC 3'
CVd-III-F	5' GGCAGCTAAGTTGGTGACGC 3'
CVd-IV-F	5' GGGGAAATCTGTTCAGAC 3'

Amplicon expected size
CEVd: 371bp
CBLVd: 325-331 bp
HSVd: 299-301 bp
CVd-III: 294-297 bp
CVd-IV: 284-286 bp

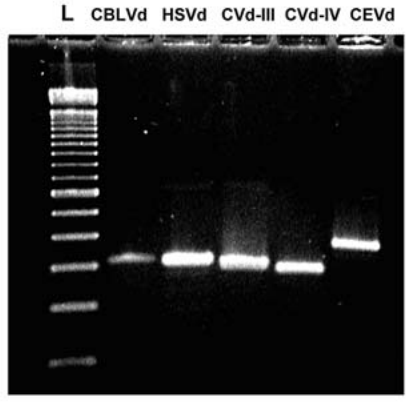

Fig. 2. Primers designed for the RT and PCR reactions and the expected sizes of amplicons obtained from samples infected with CEVd, CBLVd, HSVd, CVdIII and CVdIV. The gel shows amplicons amplified from tissue containing the viroid indicated at the top of each lane (L = 100 bp ladder).

tial first-strand cDNA synthesis steps at 60°C for 1 h and 85°C for 5 min. The 20 µl reaction volume contained 0.75 µM of the 27-mer primer specific for each viroid, 1 mM dNTPs, 0.5 M DTT, 40 U of RNase Out (Invitrogen®) and 7.5 U of Thermoscript reverse transcriptase (Invitrogen®). In order to recover full-length viroid DNA, second strand synthesis and DNA amplification were performed using a set of two contiguous forward and reverse primers specific for each viroid in a 50 µl reaction volume containing 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of a denaturation step at 94°C for 5 min, followed by 35 cycles (94°C for 30 s, 60°C for 30 s and 72°C for 1 min), concluding with an extension step at 72°C for 5 min. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of a DNA product of the expected size (Fig. 2).

Suitability of the method for viroid detection in different citrus species and cultivars. This revised protocol has been successfully tested with several viroid/host combinations. In Etrog citron, we successfully assayed for CEVd (84 tests), HSVd (39 tests), CBLVd (21 tests), CVd-III (30 tests) and CVd-IV (17 tests). In sour orange, we assayed for CEVd (14 tests) and HSVd (24 tests). In trifoliate orange, assays for CEVd (19 tests) and HSVd (26 tests) were successful. Tests in Orlando tangelo for HSVd (18 tests) also were positive, as were tests in Washington navel sweet orange for CEVd (3 tests), HSVd (2 tests) and CVd-III (4 tests). Tests in Verna and Fino lemons found HSVd (2 tests) and CEVd (3 tests) respectively. Assays in *Gynura aurantiaca* for CEVd (9 tests) and in Mexican lime, calamondin, *Citrus depressa*, *Citrus karna*, *Citrus bergamia*, *Citrus pyriformis* and eggplant (three tests each) were also successful. (Data not presented).

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