Real-Time RT-PCR Based on SYBR-Green I for the Detection of Citrus Exocortis and Citrus Cachexia Diseases

M. Tessitori, S. Rizza, A. Reina, and A. Catara

DISTEF, Plant Pathology Section, University of Catania, Italy

ABSTRACT. *Citrus exocortis viroid* (CEVd) and Citrus viroid-IIb (CVd-IIb) are the etiological agents of citrus exocortis and citrus cachexia diseases, which cause economically important damage in citrus crops. For this reason they are both included in citrus certification programs. The need to speed up the detection of pathogens has stimulated progressive improvement in and application of appropriate technology. In the present study we have devised real-time RT-PCR protocols using the intercalating dye SYBR-Green I for fast detection of these two pathogens in field samples infected with a mixture of viroids. Primer pairs designed from highly conserved regions of the genome of different variants of each viroid amplified DNA fragments of 83-bp (CEVd) and 133-bp (CVd-II), which were detected by the increasing fluorescence observed during the reaction. The analysis of the melting temperatures (Tm) allowed for the discrimination of the amplified products generated from the two distinct viroid templates. The presence of products specific to CEVd, cachexia and non-cachexia CVd-II was always confirmed by visualization with polyacrylamide gel electrophoresis. The evidence indicates that real-time RT-PCR appears to be a useful tool for fast and reliable diagnosis of citrus viroids.

Index words: Viroids, citrus, real-time RT-PCR, SYBR-Green, melting temperature.

Viroids are transmitted mainly by vegetative propagation and the principal way of control is the use of viroid-free propagation material. For this purpose the availability of simple and rapid methods for their detection is critical. Currently, conventional techniques used for citrus viroid diagnosis include biological tests with indicator plants (Etrog citron and Parson's Special mandarin), sequential polyacrylamide gel eletrophoresis (sPAGE) (7) and reverse-transcription polymerase chain reaction (RT-PCR) (3, 8), but the need for decreasing the time needed for detection of these pathogens in field samples is still a primary focus of research in this branch of plant pathology. Recently real-time RT-PCR has been applied to the detection of *Potato spindle tuber viroid* (PSTVd) (1). In this paper we report the use of two protocols based on the use of a method employing SYBR-Green I real-time RT-PCR for the diagnosis of pathogenic citrus viroids. Real-time PCR allows, in contrast to conventional assays, the monitoring of *de novo* generation of PCR products by detecting the increase in fluorescence as the amplification progresses. As SYBR-Green I detects all dsDNA, including primer dimers and other undesired products, specific amplicons can be distinguished by their melting curve, which is dependent on GC content, length, and sequence (6).

For the current study, five citrus plants (trifoliate orange, Etrog citron, Orlando tangelo, Fino Iniasel and Kamarina lemons) infected with a mixture of different viroids and one healthy plant of Arizona 861-S1 Etrog citron were used as source material to establish the protocols. The viroid isolates were previously characterized by bioassay on Etrog citron and Parson's Special mandarin and by sPAGE analysis and found to contain CEVd, cachexia or non-cachexia CVd-II, as well as other viroids.

In order to define optimal conditions, total nucleic acids were phenol-extracted from 10 g of green bark and partially purified by CF11 cellulose column chromatography

(2), concentrated by ethanol precipitation and finally re-suspended in 100 µl sterile distilled H_2O. Primers were designed using the software FAST PCR (v.3.7 Inst. of Biotechnology, Univ. of Helsinki, Helsinki, Finland) (5, 9). Criteria chosen to design high efficiency primers using this program included amplification from the central conserved region of each viroid and similar annealing temperatures for primers, in order to perform both protocols contemporaneously in the same reaction. The primer pair designed for CEVd (Genbank Accession No: M34917) consisted of the 20-mer cev-a (5'-GGGAAACCTGGAGGAAGTCG-3'), homologous to nucleotides 98-117 and the 18-mer cev-b (5'-tgtttctc-cgctggacgc-3'), complementary to nucleotides 159-180. The primers for CVd-II (Genbank Accession No: AF213484, AF213503) were the 18-mer hsv-a (5'-GGCAACTCTTCTCA-GAATCC-3'), homologous to nucleotides 84-101 and the 19-mer hsv-b (5'-GTCTCACTCGAAGAGCCAG-3'), complementary to nucleotides 199-216. The effectiveness of the primer pairs was checked previously by RT-PCR (8).

The cDNA preparation for each viroid was synthesized using 1 µl of nucleic acid extracts in a 24 µl RT reaction containing RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 0.4 µM of complementary primers cev-b or hsv-b, 0.5 mM each of dATP, dTTP, dCTP and dGTP, 25 U of Rnasin (Promega) and 20 U of M-MLV reverse transcriptase (Promega). Reverse transcription reactions were conducted at 42°C for 30 min.

The threshold cycle (Ct), i.e. the cycle in which the level of fluorescence of the sample increases above the background, was chosen as one of the criteria to determine the presence of the desired target. Melting curve analysis was used as a second criterion to determine target presence or absence, since each PCR product exhibits a characteristic peak (Tm) at its maximum melting temperature. SYBR Green I-based PCR analyses were performed using the iCycler iQ Multicolor Real-time Detection System (BIO-RAD). Real time reactions were performed in a final volume of 25 µl containing 2 µl of cDNA and 1 µl of each primer at 0.4 µM final concentration and the iQ SYBR Green Supermix 2X (BIO-RAD Laboratories).

Real-time PCR used the following amplification parameters for both CEVd and CVd-II: 5 min of HotStar *Taq* polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. Melting curve analysis was conducted under the following conditions: 1 min denaturation at 95°C, 1 min annealing at 60°C, followed by 180 cycles with annealing temperatures increasing in 0.2°C increments (10 sec each) beginning at 60°C with continuous reading of fluorescence. Real-time PCR products were also analyzed by

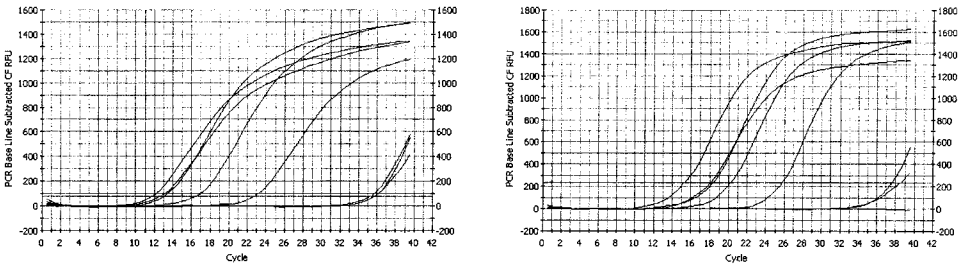

Fig. 1 Amplification plots generated in the real-time RT-PCR reactions with the primer pair cev-a/cev-b (left) and hsv-a/hsv-b (right).

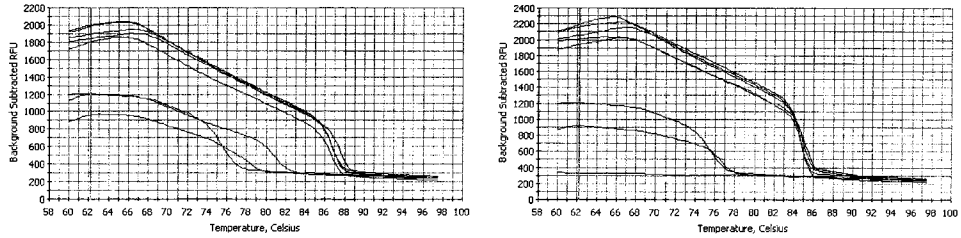

Fig. 2. Melting curves obtained for CEVd (left) and CVd-II (right).

6% PAGE to confirm the identity of the targets and show the absence of non- specific PCR products. CEVd as well as cachexia and non-cachexia CVd-II variants were detected by the Real-Time RT-PCR using SYBR green I intercalating dye and the indicated primer pairs. Ct values between 12.9 and 23.9 and between 14.5 and 24.9 were observed for CEVd and CVd-II, respectively (Fig. 1). Melting curve analysis performed at the end of amplification revealed Tm peaks of 87.26°C ± 0.10 for CEVd and 85.26 ± 0.09 for CVd-II respectively (Fig. 2). Healthy and no template controls did not show increased fluorescence before the 36th or 37th cycle, and melting curve analysis for these samples revealed a peak at about 75°C (Figs. 1, 2). Analysis by 6% PAGE showed amplicons of the expected size, 83 bp for CEVd and 133 bp for CVd-II without detectable non-specific products. No amplification products were observed from healthy and water controls (Fig. 3).

As expected we obtained positive results for all the infected samples tested, with a useful increase of fluorescence and consequently low Ct values. In contrast, healthy controls gave a very delayed Ct, a common problem associated with the use of SYBR-Green which may be due to the formation of primer dimers (4), thus providing a clear distinction of positive from negative samples. The synthesis of amplicons from both cachexia and non-cachexia CVd-II isolates indicates that we can amplify different variants of CVd-II but we cannot distinguish pathogenic from non-pathogenic forms due to a low annealing temperature used in the reaction. Designing primers from sequence in the vari-

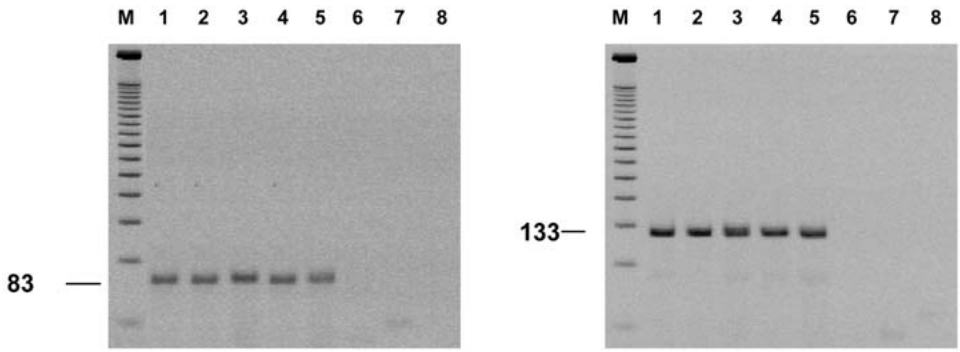

Fig. 3. Polyacrylamide gel electrophoresis (5%) analysis of real-time RT-PCR products of CEVd (on the left) and CVd-II (on the right) compared with a DNA molecular size marker (50bp DNA Step Ladder, Promega). Isolates were: 1. trifoliate orange; 2. Etrog citron; 3. Fino iniasel lemon; 4. Kamarina lemon; 5. Orlando tangelo; 6. healthy control; 7. RT H_20; 8. H_20.

able domain (which contains five mutations which distinguish the two groups of variants) could resolve the two groups, but also could give erratic results in RT-PCR. The primer pairs used in this protocol in conjunction with a specific probe designed from sequence in the variable domain could allow differentiation of those mutations, coupling high efficiency of amplification with increased specificity.

In conclusion, these results show that real-time RT-PCR can be a useful tool for fast and reliable detection of CEVd and CVd-II, providing the possibility to run both reactions simultaneously using the same annealing temperature. The method is rapid and easy to perform since, in routine analyses, it does not require electrophoresis, which is time-consuming and uses the mutagenic agent ethidium bromide.

LITERATURE CITED

1. Boonham N., L. Gonzàlez Perez, M. S. Mendez, E. Lilia Peralta, A. Blockley, K. Walsh, I. Barker, and R. A. Mumford
2004. Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. J. Virol. Methods 116: 139-146.
2. Flores, R. and G. Llácer
1988. Isolation of a viroid-like RNA associated with peach latent mosaic disease. Acta Hort. 235: 325-332.
3. Garnsey, S. M., D. L. Zies, M. Irey, P. J. Sieburth, J. S. Semancik, L. Levy, and M. E. Hilf
2002. Practical field detection of citrus viroids in Florida by RT-PCR. In: *Proc.15th Conf. IOCV*, 219-229. IOCV, Riverside, CA.
4. Gibson, U. E., C. A. Heid, and P. M. Williams
1996. A novel method for real time quantitative RT-PCR. Genome Res. 6: 995-1001.
5. Palacio-Bielsa, A., J. Romero-Durbàn, and N. Duran-Vila
2004. Characterization of citrus HSVd isolates. Arch. Virol. 149: 537-552.
6. Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer
1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analyt. Biochem. 245: 154-160.
7. Rivera-Bustamante, R., R. Gin, and J. S. Semancik
1986. Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous pH system. Analyt. Biochem. 156: 91-95.
8. Tessitori, M., R. La Rosa, G. Albanese, and A. Catara
1996. PCR diagnosis of citrus viroids in field samples. In: *Proc. 13th Conf. IOCV*, 230-235. IOCV, Riverside, CA.
9. Visvader, J. E., A. R. Gould, G. E. Bruening, and R. H. Symons
1982. Citrus exocortis viroid: nucleotide sequence and secondary structure of an Australian isolate. FEBS Lett. 137: 288-292.