TECHNICAL SHEET No. 29

Virus Detection: Six viroids, including *Potato spindle tuber viroid* (PSTVd) and *Peach latent mosaic viroid* (PLMVd)

Method: RT-PCR probe capture hybridization (–ELISA)

General

Virus Detected: Six viroids, including *Potato spindle tuber viroid* (PSTVd) and *Peach latent mosaic viroid* (PLMVd)
Method: RT-PCR probe capture hybridization (ELISA)

Developed by

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Modified by Douglas P. Maxwell from the article published in Journal of Virological Methods 105:115-121 (2002).

Goals

This method was developed to allow the rapid detection of six viroids in four genera.

Introduction

Viroids can have major impact on potato and fruit tree production (Hadidi et al., 2002). Rapid, sensitive and specific protocols for detection of each viroid are important for management. Dr. Hadidi's laboratory has been developing methods for viroid detection for many years. His group was the first to introduce RT-PCR methods in 1990 (Hadidi and Yang, 1990) and RT-PCR-ELISA (Shamloul and Hadidi, 1999).

Material and Methods

Extraction of RNA from plant tissue

100 mg of viroid-infected tissues were powdered in liquid nitrogen in a mortar with a pestle. The total RNA was then extracted with Qiagen RNeasy Kit according to the instructions from the manufacturer.

cDNA synthesis and amplification

For each viroid, 1 µl (100 ng RNA) was mixed with 1 µg of the appropriate complementary primer – CPSTVd, 5' CCCTGAAGCGCTCCTCCGAG 3' and for CPLMVd, 5' AACTGCAGTGCTCCGT 3'. Water was added to bring volume to 15 µl. The RNAs/primer mixture was heated for 5 min at 100ºC, then chilled in ice and held at 37ºC for 15 min. 10 µl of reverse transcription reaction solution (Promega, Madison, WI), 1 µl of 2 mM dATP, dGTP, dCTP, dTTP each, and 2 µl of water was mixed with the annealing reaction mixture and held at 42ºC for 1 h.

PCR amplification-DIG-labeling

PCR-DIG-labeling mixtures each contained 5 µl of 10 x PCR buffer (1X 10 mM Tris-HCL, pH 8.3, 50 mM KCl, and 0.001% gelatin), 4 µl of 25 mM $MgCl_2$ 1 µl of 2 mM dATP, dCTP, dGTP, 5.7 mM dUTP and 0.3 mMDig-11-dUTP, 2 µl of uracil DNA glycosylase (1 U/µl), 1 µl each of 6 mM mixed complementary and homologous primers, 2.5 units AmpliTaq Gold DNA polymerase, and water to 48 µl. Homologous primers: HPSTVd, 5' ATCCCCGGGGAAACCTGGAGCGAAC 3'; HPLMVd, 5' CCCGATAGAAAGGCTAAGC ACCTCG 3'. 2 µl of the first-strand cDNA mixture was added to the PCR reaction, and incubated for 10 min at R T, then amplification in thermal cycler: 94ºC, 2 min for first cycle; 94ºC for 45 sec; primer annealing at 60ºC for 1 min, extension at 72ºC for 2 min, for 35 cycles, followed by 72ºC for 7 min.

Analysis of RT-PCR-DIG amplified product

5 µl of RT-PCR DIG labeled amplicons were separated on polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and 120 volts for 1-1.5 h.

Preparation of biotin-labeled viroid cDNA capture probes

The DNA of each capture probe was complementary to an internal nt sequence of one of the amplified viroid DNA strands. These probes were synthesized commercialy.
PSTVd-cap, 5'bio-AGGAGTAATTCCCGCCGAAAC3'
PLMVd-cap, 5' bioGATCCAGGTACCGCCGTAGAA 3'.

Microwell capture hybridization assay

The detection of the DIG-labeled amplified viroid DNA was achieved with the PCR-ELISA Detection System (Boehringer Mannheim Corp., Indianapolis, IN, USA). 5 µl of RT-PCR-DIG labeled amplified product was mixed with 20 µl of 0.25 N NaOH and kept at R.T. for 10 min, then 200 µl of hybridization solution, which contained 50 ng/ml biotinylated cDNA capture probe, was added. 200 µl of this mixture was added to an ELISA microtiter plate well coated with streptavidin and this plate was placed on a shaker at 50 C for 3 h. The hybridization solution was removed and the wells washed 6X with washing solution PBS-Tween (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.1 mM Na_2 EDTA, pH 6.8, 0.05% Tween 20). 200 µl of polycolonal anti-DIG Fab fragments conjugated with peroxidase diluted 1:100 in Tris-HCl, pH 7.5 buffer were added to each well and then the plates were shaken gently for 30 min at 37ºC. Next the wells were washed 6X with washing solution buffer, then 200 µl of substrate solution [100 mg mg/ml of 2,2%-azino-bix (3-ethylbenzthiazoline-6-sulfonic acid) diammonium] was added to each well and plates incubated for 30 min with shaking at 37ºC in the dark.

Results and Discussion

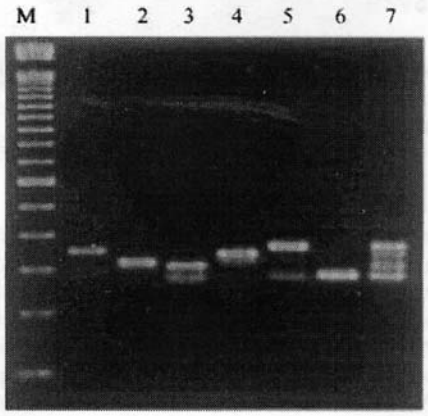

Fig. 1. Polyacrylamide gel electrophoretic analysis of RT-PCR full-length products of six viroids amplified from total RNA of viroid infected tissues. Molecular DNA marker (100 bp DNA ladder, GIBCO BRL, Gaithersburg, MD) with fragment sizes (bp) of 2072, 1500, 600, 500, 400, 300, 200, 100 (M). Standard RT-PCR products amplified from: ASSVd-infected apple (lane 1), PBCVd-infected quince (lane 2), ADFVd-infected apple (lane 3), PLMVd-infected peach (lane 4), PSTVd-infected tomato (lane 5), HSVd-infected peach (lane 6). Multiplex RT-PCR products amplified from all the above samples (lane 7).

Fig 1. Complementary and homologous primers for each viroid were used to amplify a full-length unit of each viroid.

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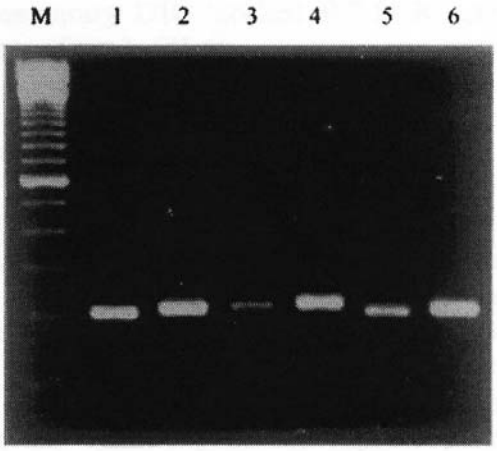

Fig. 2. Polyacrylamide gel electrophoretic analysis of RT-PCR (lanes 1, 3, and 5) and DIG-labelled RT-PCR (lanes 2, 4, and 6) products synthesized with cAPSCAVd and hAPSCAVd primers and amplified from: ASSVd-infected apple (lanes 1 and 2), PBCVd-infected quince (lanes 3 and 4), and ADFVd-infected apple (lanes 5 and 6). The size of the amplified product of each viroid is about 200 bp, which is less than viroid full length. Molecular DNA marker (M) (see Fig. 1).

Fig 2. The unlabeled and DIG-labeled viroid products were amplified with complementary and homologous primers and were analyzed by 6% polyacrylamide gel electrophoresis.

Copied from J. Virol. Methods 105:119 (2002).

The RT-PCR-ELISA was used to detect viroid in RNA extracts from infected tissues. First the complementary primers were used to synthesize the cDNAs, then the complementary and the homologous primers were used for DIG-PCR amplification of viroid cDNAs. The DIG-labeled amplified product was hybridized with the capture probe (Fig 3). In this case, the RT-PCR-ELISA method was used effectively to detect and differentiate six viroids at the same time.

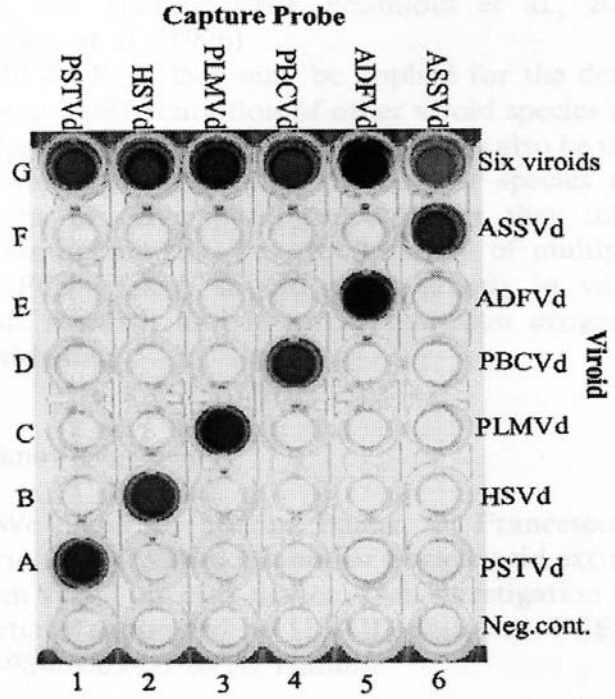

Fig. 3. Specificity of biotin-labelled capture probe for the colorimetric detection of homologous and heterologous DIG-labelled amplified viroid cDNA product. The DIG-labelled cDNA amplified product of each viroid was placed in six wells in the hybridization plate as indicated in the figure. Each viroid capture probe was hybridized to amplified product from: PSTVd-infected tomato (A1, PSTVd), HSVd-infected peach (B2, HSVd), PLMVd-infected peach (C3, PLMVd), PBCVd-infected quince (D4, PBCVd), ADFVd-infected apple (E5, ADFVd), and ASSVd-infected apple (F6, ASSVd), and all of the above samples (G1-6, six viroids). Standard RT-PCR-ELISA (A-F, 1-6), positive samples: A1; B2; C3; D4; E5; F6; multiplex RT-PCR-ELISA (G, 1–6), six positive samples: G 1–6. Negative controls for standard RT-PCR-ELISA: uninfected tomato, peach, quince, and apple leaves (wells 1–4) labelled negative controls; negative controls for multiplex RT-PCR-ELISA: a mixture of uninfected tomato, peach, quince and apple leaves (well 5), and buffer control (well 6) labeled negative controls.

Copied from J. Virol. Methods 105:120 (2002).

References

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