TECHNICAL SHEET No. 26

Virus Detection: *Plum pox virus* (PPV) and *Prune dwarf virus* (PDV)

Method: RT-PCR, PCR-ELISA

General

Virus detection: PPV and PDV
General methods: RT-PCR, PCR-ELISA

Developed by

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Goals

Detection of PPV and PDV using RT-PCR
Detection of PPV and PDV using PCR-ELISA
Distinguish between the different strains (Isolates) of PPV and PDV

Introduction

Prune dwarf ilarvirus (PDV) and its various strains cause many types of stone fruit diseases, and are of considerable economic importance (Nemeth, 1986). The virus induces considerable damage in many hosts either by itself or in a mixed infection with other stone fruit viruses. PDV is transmitted naturally by infected pollen. PDV has worldwide distribution, especially where sweet and sour cherry are cultivated. Plum pox disease, Sharka, is caused by *Plum pox potyvirus* (PPV) (Dunez and Sutic, 1988; Hadidi and Candresse, 2001). Sharka is the most important viral disease of stone fruit diseases in Europe and the Mediterranean region because of reduced fruit quality, premature fruit drop, rapid natural spread by aphid vectors, and rapid decline and death of trees when co-infected with other viruses. During the last decade, PPV has been reported also from South and North America (Hadidi and Candresse, 2001).

Introduction of PDV and PPV through the international and national movement of stone fruit cultivars and germplasm to local stone fruit industry is of concern to federal and local governments. For this reason, effective control measures of PDV and PPV must be established to safely introduce stone fruit cultivars and/or germplasm that are free of PDV and/or PPV to prevent serious losses to the local stone fruit industry and significantly reduce PDV and/or PPV infection to stone fruits in countries where either virus is present.

Materials and Methods

Source of PDV and PPV infected tissues (Isolates):

PDV Isolates
Egyptian Isolates: PDV-B in peach and PDV-M 29 in plum
US isolates: PDV-cherry 37200 kindly provided by H.E. Waterworth;
PDV-Rainer cherry,
PDV-SIT 35 Bing cherry and PDV SIT 27 Bing cherry were kindly supplied by W. E. Howell and K. C. Eastwell. Virus infected and uninfected leaves and pollen were used.

PPV Isolates

The four standard PPV strains, whose nucleotide sequences had been published, were used: Egyptian El-Amar; the French D and M; and the Moldovian sour cherry (Kegler and Hartmann, 1998; Nemchinov et al., 1998). PPV-infected tissues of the four strains were kindly obtained from T. Candresse. Virus infected and uninfected leaves were used.

Total RNA extraction

In most cases, total RNA was extracted from virus-infected or uninfected leaf or pollen tissue using: BIO 101 FastRNA Green Protocol (BIO 101, Carlsbad, CA). and QIAGEN RNeasy plant mini kit (Qiagen Inc., Valencia, CA) as suggested by the manufacturer.

Gene Releaser treatment of total RNA

One µl of total RNA of each sample was placed in a thin-walled PCR tube containing 23 µl of freshly resuspended GeneReleaser (GR) (Bio Ventures, Inc., Murfreesboro, TN). The GR–RNA mixtures were vortexed at low speed for 30 sec and held in ice until all samples were prepared. Samples were then placed in a microwave-safe rack (polypropylene, Bio Ventures Inc.), overlaid with 50 µl of mineral oil, lids closed, and microwaved at a high power setting for 6 min.

Primer sequences and the expected size of amplified PDV cDNA or PPV cDNA

Primers for PDV were designed from the nucleotide sequence of the coat protein gene (Bachman et al., 1994) as previously described (Parakh *et al*., 1995). A 23-mer primer (5'-TAG TGC AGG TTA ACC AAA AGG AT- 3') complementary to nucleotides 1988-2010 and a 23 mer primer (5'-ATG GAT GGG ATG GAT AAA ATA AT- 3') identical to nucleotides 1838-1860 were designed to amplify a 172-bp cDNA fragment from PDV infected tissue. Primers for PPV were designed to amplify the whole 3' non-translated region of the viral genome (220 bp), as this region is conserved in all known strains of PPV (Hadidi and Levy, 1994; Levy and Hadidi, 1994; Nemchinov and Hadidi, 1996). A 24-mer complementary primer (5'-GTC TCT TGC ACA AGA ACT ATA ACC-3') and a 24-mer viral sense primer (5'-GTA GTG GTC TCG GTA TCT ATC ATA-3') were used for amplification. Primers were synthesized by Life Technologies, Inc., Gaithersburg, MD

Reverse transcription (RT)

A 20-µl aliquot of GR matrix containing RNA was removed immediately after microwaving and added to a primer annealing reaction mixture containing: 6 µl of 5x first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 3 µl of 0.1 M dithiothreitol (DTT), and 1 µg complementary primer. The mixture was vortexed briefly and denatured by heating at 100°C for 5 min, chilled on ice for 2 min and annealed at 37°C for 5-30 min or at room temperature for 45 min to allow primer annealing to the viral RNA template. The annealed reaction was added to 20 µl of a cDNA reaction mixture containing: 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl Rnasin (40 units, Promega Corp., Madison, WI), 2 µl of 10 mM dNTPs (2.5 mM each of dGTP, dATP, dTTP and dCTP), and 1 µl of Maloney murine leukemia virus reverse transcriptase (200 U/µl; Promega Corp.). Reactions were mixed briefly, and incubated for 1-1.5 h at 42°C.

Polymerase chain reaction (PCR)

Amplifications were performed in thin - walled PCR tubes and contained the following reaction mixture: 5 µl of 10 x PCR buffer (1x =10 mM Tris- HCl, pH 8.3, 50 mM KCl, and 0.001% gelatin), 3 µl of 25 mM $MgCl_2$ (1.5mM final concentration), 1 µl of 10 mM dNTPs, 1 µl each of 6 µM complementary and homologous DNA primers, 2.5 units of AmpliTaq Gold™ DNA polymerase (Perkin- Elmer Cetus Corp., Norwalk, CT), and sterile H_2O to a volume of 45 µl and 5 µl of cDNA mixture. Each reaction mixture was overlaid with two drops of mineral oil to prevent evaporation during amplification.

Cycling parameters were 13 min at 95°C at the first cycle to activate AmpliTaq Gold™ DNA polymerase, 30 sec at 94°C, 30 sec at 62°C and 45 sec at 72°C for 30 cycles with final extension at 72°C for 7 min in a DNA thermal cycler (Pekin- Elmer Cetus Corp).

Cycling parameters for multiplex PCR for amplification of both PDV cDNA and PPV cDNA were similar to standard PCR except that the DNA polymerase was activated at 94ºC for 12 min. In some experiments with multiplex PCR, a gradient of different annealing temperatures (60, 59, 57, 55, or 53ºC) were used. These experiments were conducted in a Hybaid thermal cycler (Hybaid Inc., Franklin, MA).

PCR amplification – DIG labeling of PDV cDNA and / or PPV cDNA

PCR-DIG labeling mixtures each contained 5 µl of 10 x PCR buffer, 3 µl of 25 mM $MgCl_2$, 5 µl of 2 mM dATP, dCTP, dGTP, 5.7 mM dUTP and 0.3 mM DIG-11-dUTP, 2 µl of uracil DNA glycosylase (1U/µl), 1 µl of 6 µM complementary and viral sense primers, 2.5 units of AmpliTaq Gold™ DNA polymerase, and sterile water to a volume of 48 µl. Two microliters of cDNA mixture were added to the PCR reaction and the mixture was covered with 50 µl of mineral oil. The mixtures were amplified with the following cycling parameters: 95°C for 14 min at first cycle, 94°C for 1 min, 60°C for 1 min, 72°C for 2 min for 35 cycles with a final extension at 72°C for 7 min. The PCR cycling parameters for multiplex DIG-labeling of PDV cDNA and PPV cDNA were: 94°C for 12 min at first cycle, 94°C for 45 sec, 60°C or (60, 59, 57, 55, or 53°C) for 1 min, 72°C for 2 min for 35 cycles with final extension at 72°C for 7 min.

Electrophoretic analysis of amplified products

Aliquots (5 µl each) of amplified products were analyzed by electrophoresis on 5% polyacrylamide gels at 100-120 V for 1.5 h in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3) and visualized by staining with silver nitrate. BioLow DNA molecular weight marker (Bio Ventures, Inc.) was used to determine the size of amplified products.

Biotin-labeled PDV cDNA and PPV cDNA capture probes

Biotin-labeled PDV cDNA, 27 oligonucleotides in length, (5'-BIO-TGATTGTGCTTCCACTATGAGTATTCC-3') was used as a capture probe for products amplified from PDV-infected tissue. PPV cDNA, 23 oligonucleotides in length, (5'-BIO-AGG CCC TTG TAT CTG ATG TAG CG-3') was used as the capture probe for products amplified from PPV-infected tissue. Probes were synthesized and biotinylated at Life Technologies, Inc. The sequence of each probe was selected by using the primer analysis software (rawprimer) from University of Wisconsin, Madison.

Microwell capture hybridization assay

The detection of DIG-labeled amplified DNA was carried out using the PCR-ELISA Detection System (Boehringer Mannheim Corp., Indianapolis, IN) essentially as described by Shamloul and Hadidi, 1999. Briefly, five microliters of RT-PCR-DIG labeled amplified product were mixed with 20 µl of 0.25 M NaOH then chilled on ice for 2 min. The mixtures were kept at room temperature for 10 min, and then 200 µl of hybridization solution containing 50 ng/ml 5'-biotinylated DNA capture probe were added. Two hundred microliters of each mixture were pipetted into an ELISA microtiter plate well coated with streptavidin, then the plate was covered with self adhesive tape (3M Scotch™, St. Paul, MNn. ThMN

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Detection and Analysis of DIG-Labeled PPV cDNA by Probe Capture Hybridization Assay Using Biotinylated PPV cDNA Probe.

All known four subgroups of PPV were detected by this method. Table 2 shows that RT-PCR-ELISA of PPV - El Amar subgroup was at least 100 fold more sensitive than analysis of amplified products by polyacrylamide gels. Similar results were also obtained with PPV-D, PPV-M, and PPV-C subgroups.

Specificity of biotin-labeled PDV cDNA and PPV cDNA capture probes.

Amplified product	Capture probe	Hybrid formation	
Source of Plant Material	Absorbance at 405 nm	Color Development	
PDV - infected tissue	PDV	2.672	+
PPV - infected tissue	PDV	0.021	-
Uninfected tissue	PDV	0.024	-
PDV - infected tissue	PPV	0.018	-
PPV- infected tissue	PPV	3.536	+
Uninfected tissue	PPV	0.035	-

Absorbances of the above readings were measured at 405 nm. Absorbance for H_2O was 0.000; PDV in the presence of PPV capture probe = 0.081; PPV in the presence of PDV capture probe = 0.021

Fig. 1-Gel electrophoretic analysis of multiplex RT-PCR products amplified from GeneReleaser - treated total RNA mixture from PDV-infected and PPV-infected tissues. Molecular DNA marker (M), arrows indicate PPV cDNA (220 bp) and PDV cDNA (172 bp) amplified products.

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PDV,Peach,USA St. ATGGATGCGATGGATAAAATAGTCAGTGGATGACTATATGATCCATCATTTGATTGTGCTTCCACTATGAGTATTCCTAG     80
PDV, Peach, EGYPT -------g-----------------------------------------------a----------------------     80
PDV, Plum, EGYPT  -------g---------n-------------------------g---------------------------------a     80
PDV, Cherry, USA1 -------g-----------------------------------g---------------------------------a     80
PDV, Cherry, USA2 -------g---------t-------------------------------------------------------------a     80

PDV,Peach,USA St. GAATATTCGTAGTTGGAAATGCTGCTTTTGCAACAGAATCCACCATTCAGAGTTTGTCACTGAATGTTAAATCCTTTTGG    160
PDV, Peach, EGYPT ----------------------------------g----------------t-----------------------------    160
PDV, Plum, EGYPT  ---------------------------------t-----------------------------------------------    160
PDV, Cherry, USA1 ------------------------t--------t-----------------------------------------------    160
PDV, Cherry, USA2 ---------------------------------t-----------------------------------------------    160

PDV,Peach,USA St. TTAACCTGCACTA                                                                      173
PDV, Peach, EGYPT -------------
173
PDV, Plum, EGYPT  -------------
173
PDV, Cherry, USA1 -------------
173
PDV, Cherry, USA2 -------------
173
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Fig. 2. Multiple alignment of the nucleotide sequence of several clones of amplified PDV cDNA with the corresponding region of published PDV standard sequence (Bachman et al., 1992). Nucleotide sequences of PDV isolates were compared with that of the coat protein gene of PDV

RNA 3. The percentage identity of PDV peach or plum isolate from Egypt and cherry isolates from the US was 97% - 98% to that of the US PDV peach standard.

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