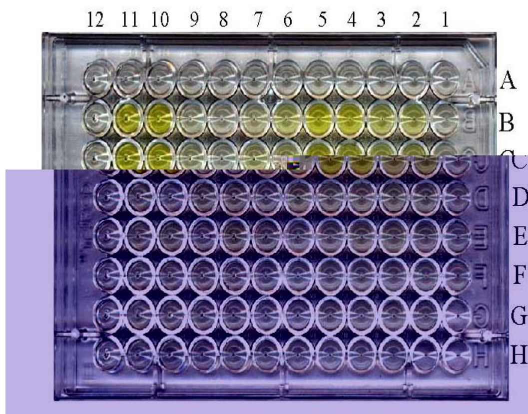
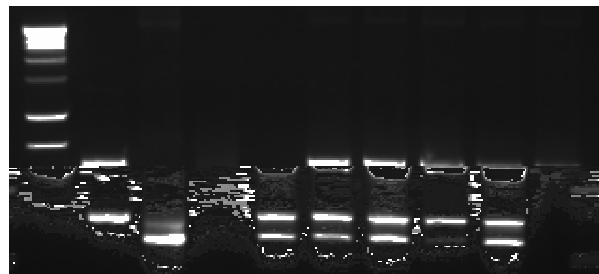
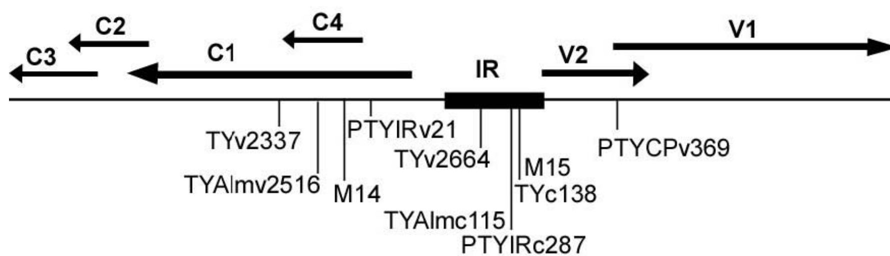
Detection Methods for Viruses of Banana, Citrus, Cucumber, Grape, Potato, Stone Fruits, and Tomato







Developed by MERC Scientists
2005

PREFACE

These methods for detection of plant viruses/viroids was compiled as part of the research output by scientists who participated in the U.S. AID MERC project entitled "Development of a Regional Viral Indexing and Certification Program for Plant Propagation Materials in the Middle East". The following scientists served as project leaders for their laboratories: Dr. Hanokh Czosnek, Hebrew University of Jerusalem, Israel, Dr. Ghandi Anfoka, Al-Balqa' Applied Univ., Jordan, Dr. Naim Iraki, Bethlehem University, Palestinian National Authority, Dr. Yusuf Abou Jawdah, American University of Beirut, Drs. Hamed Mazyad and Ahmed Shalaby, Agriculture Research Center, Cairo, Drs. Mohamed Marrakchi and Hatem Fakhfakh, University of Tunisia, Mr. Arifi Abdelaziz, Ministry of Agriculture, Morocco, Dr. A. Hatimi, Ibnor Zohr University, Agadir, Morocco, Dr. M. "Sid" Sedegui, Department of Agriculture, Oregon, Dr. Ahmed Hadidi, USDA/NIH, and Drs. Douglas P. Maxwell and Mark K. Nakhla, University of Wisconsin-Madison. These scientists were ably assisted by their students and technical support staff.

One objective of this grant was to provide methods for detecting the most important groups of viruses and viroids associated with these crops. These methods would then be used by scientists involved in certification programs and plant protection departments. Besides the development and testing of these methods, there was a substantial effort to train personnel within each country.

The MERC scientists hope that others will find these methods useful, and if there are questions about the methods, you (as the reader) should contact the individual scientists. You are free to make copies of these methods as our goal is to make virus detection methods more standardized among the various plant protection agencies.

4 February 2006

Douglas P. Maxwell and Christopher Martin
Department of Plant Pathology
1630 Linden Drive
University of Wisconsin-Madison
Madison, WI 53706 USA

GRANT INFORMATION

Development of a Regional Viral Indexing and Certification Program for Plant Propagation Materials in the Middle East

Project number: MERC, M20-023; Grant number: PEG-G-00-98-0009-00

U.S. Agency for International Development; Bureau for Global Programs, Field Support and Research; Center for Economic Growth and Agricultural Development

TABLE of CONTENTS

Page

Banana

Technical Sheet no. 1: *Banana bunchy top virus* DNA hybridization methods 2

Citrus

Technical Sheet no. 2: *Citrus tristeza virus*, DAS-ELISA 13

Technical Sheet no. 3: *Citrus tristeza virus*, DAS-ELISA 16

Cucumber

Technical Sheet no. 4: *Cucurbit yellow stunting disorder virus*, RT-PCR 19

Technical Sheet no. 5: *Cucumber mosaic virus*, DAS-ELISA 22

Technical Sheet no. 6: *Cucumber mosaic virus*, GroEL capture 24

Grapevine

Technical Sheet no. 7: *Grapevine fanleaf virus*, TR-PCR, IC-RT-PCR 28

Technical Sheet no. 8: *Grapevine leafroll virus* 1,3, IC-RT-PCR 32

Technical Sheet no. 9: *Grapevine leafroll virus* 1.3, IC-RT-PCR 35

Technical Sheet no. 10: *Grapevine virus A*, hybridization 39

Technical Sheet no. 11: *Grapevine virus A*, RT-PCR, IC-RT-PCR 42

Technical Sheet no. 12: *Grapevine virus A*, hybridization 46

Potato

Technical Sheet no. 13: *Potato leaf roll virus*, CP gene expression 49

Technical Sheet no. 14: *Potato leaf roll virus*, RT-PCR, PCR-ELISA 55

Technical Sheet no. 15: *Potato leaf roll virus* STRAINS, RT-PCR 61

Technical Sheet no. 16: *Potato leaf roll virus*, IC-RT-PCR 63

Technical Sheet no. 17: *Potato virus X*, IC-RT-PCR 65

Technical Sheet no. 18: *Potato virus X*, RT-PCR, PCR-ELISA 67

Technical Sheet no. 19: *Potato virus Y*, DAS-ELISA 74

Technical Sheet no. 20: *Potato virus Y*, tuber, hybridization 77

Technical Sheet no. 21: *Potato virus Y*, IC-RT-PCR, RFLP-IC-RT-PCR 80

Technical Sheet no. 22: *Potato virus Y*, RFLP, PCR, ELISA 85

Technical Sheet no. 23: *Potato virus Y*, necrotic strain, RT-PCR 89

Technical Sheet no. 24: PVX, PVY, PLRV, Multiplex RT-PCR 95

Stone Fruits

Technical Sheet no. 25: *Plum pox virus*, RT-PCR, CP expression 99

Technical Sheet no. 26: *Plum pox virus*, *Prune dwarf virus*, RT-PCR, PCR-ELISA 106

Technical Sheet no. 27: *Prune dwarf virus*, ELISA 112

Technical Sheet no. 28: *Prunus necrotic ringspot virus*, ELISA 114

Technical Sheet no. 29: *Potato spindle tuber viroid*, *Peach latent mosaic viroid*, RT-PCR probe capture hybridization – ELISA 116

Technical Sheet no. 30: General methods for detection of viroids 121

Tomato

Technical Sheet no. 31: *Tomato yellow leaf curl virus*, ELISA 129

Technical Sheet no. 32: *Tomato yellow leaf curl virus*, dot blot hybridization (non-radioactive) 131

Technical Sheet no. 33: *Tomato yellow leaf curl virus*, RFLP of CP gene dot blot hybridization 138

Technical Sheet no. 34: *Tomato yellow leaf curl virus*, squash blot hybridization 141
Technical Sheet no. 35: *Tomato yellow leaf curl virus*, non-radioactive hybridization 144
Technical Sheet no. 36: *Tomato yellow leaf curl virus*, IC-PCR 147
Technical Sheet no. 37: *Tomato yellow leaf curl virus*, multiplex PCR 150
Technical Sheet no. 38: *Tomato yellow leaf curl virus*, squash blot PCR 155

Banana

TECHNICAL SHEET No. 1

Virus Detection: *Banana bunchy top virus* (BBTV)

Method: Hybridization with radioactive and non-radioactive probes

General

Virus detection: BBTV from banana tissues
General methods: Hybridization methods, both radioactive and non-radioactive probes

Developed by

Name of researchers: Adel A. Rezk, Hamed Mazyad, and Ahmed Shalaby, Agriculture Research Center, Giza, Egypt; and M. K. Nakhla and D. P. Maxwell, University of Wisconsin-Madison
E-Mail address: adelrezk20@hotmail.com and aashalaby@link.net; mknakhla@plantpath.wisc.edu
Date: October, 2002

Goals

Develop rapid techniques for BBTV diagnosis in banana plants and banana aphids using radioactive and non-radioactive DNA probes.

Introduction

Banana (*Musa spp*.) is one of the world's largest and most important agricultural commodities. This tropical and subtropical crop contributes significantly to many national domestic and export incomes and in some regions is the principal source of carbohydrate. The success of virus disease control is dependent on the availability of accurate, sensitive, low cost and simple diagnostic techniques, which enable the early detection of virus infections in plant materials.

Information on *Banana bunchy top nanovirus* (6 cssDNA components) can be found on the WEB:
General descriptions:
http://life.bio2.edu/ICTVdB/index.htm
http://www.im.ac.cn/vide/descr056.htm
http://www.ictvdb.iacr.ac.uk/Ictv/fs_nanov.htm
http://www2.ctahr.hawaii.edu/oc/freepubs/pdf/PD-12.pdf (extension bulletin with images)
A list of some references:
http://www.bspp.org.uk/mppol/1997/0624karan/refs.htm

Material and Methods

DNA Extraction

DNA of BBTV was extracted from banana tissues using the Dellaporta technique (Dellaporta, et al., 1983) as following:

1. Grind 0.2 g from leaf midrib tissue or disk from infected plants with Kontes pestles in a 1.5 ml microfuge tube with 500 µl of Dellaporta buffer (100 mM Tris pH 8. 50 mM

ethylenediamine-tetraacetate EDTA, 500 mM NaCl, 10 mM beta mercaptoethanol (BME), Fisher Chemicals).

2. Add 33 µl of 20% sodium dodecyl sulfate (SDS, Fisher Chemicals) (w/v) and vortex and incubate the mixture for 10 min at 65°C.
3. Add 160 µl of 5 M potassium acetate KoAc (Sigma chemicals) and vortex.
4. Spin for 10 min at 10,000 rpm in a microfuge using (centrifuge 5415C).
5. Transfer 450 µl of supernatant to a new tube, avoiding the tissue debris.
6. Add 450 µl phenol, chloroform and isoamyl-alcohol (PCI) 25:24:1 and vortex for 5 min and then spin for 5 min at 10,000 rpm. Remove 400 µl of the upper phase to a clean microfuge tube and add 0.5 volumes of isopropanol, vortex and spin for 10 min at 14,000 rpm.
7. Remove the supernatant, the total nucleic acid was precipitated in the bottom of the tube.
8. Wash the pellet with 70% ethanol and spun 5 min at 10,000 rpm.
9. Resuspend the pellet in 200 µl of TE-RNase buffer (Tris EDTA-RNase buffer).

Designing the primers

Specific primers (A. Rezk and M.K. Nakhla, Univ. of Wisconsin-Madison) were designed to detect the presence of BBTV in infected banana tissues. for each component using Australian isolate sequence from Genbank no. L41574 to L41578. The primers, bbtv1v763, and bbtv1c768, were used to amplify component 1. The primer bbtv2v775 was designed as a general primer for components 2, 3, 5 and 6, and bbtv2c617, bbtv3c703, bbtv5c625 and bbtv6c549 were used as specific primers for components 2, 3, 5, and 6, respectively. The primers bbtv4v527 and bbtv4c526 were specific for component 4.

Table 1. Primers used in the PCR amplification of the six components of the isolated BBTV.

Comp. No.	Primer name	Sequences of primers	Expected size
		5---3	fragment
BBTV 1	bbtv1v763	AATTGTAGACTGTATAATTACGAGG	1111 bp
	bbtv1c768	AAATGTCTACATATATCCAATGATTTTCC	
BBTV 2	bbtv2v775	TACAAGACGCTATGACAAATGTACKGG	929 bp
	bbtv2c617	CTAAACACCTGTTAATCATGCTTCGCC	
BBTV 3	bbtv3c703	CTTACTCCAGAACTACAATAGAATGCC	973 bp
BBTV 4	bbtv4v526	AGATCAAGAACCGGCTGTGATACC	1050 bp
	bbtv4c525	CGTCTGTCTTCCACAATACCTCTGCC	
BBTV 5	bbtv5c625	ACTCCTACATCTTCTTCCTCTGTCG	801 bp
BBTV 6	bbtv6c549	CCGAATGGTACTATGAGTACTGGACGC	813 bp

Polymerase Chain Reaction (PCR) detection:

PCR Mixture

1. The PCR mixture contains 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 2.5 mM each of dNTPs, 10 pmoles each primer; 1.0 unit *Taq* DNA polymerase (Promega), and dH_2O to a final volume of 45 µl.
2. Add 5 µl extracted DNA from infected plant tissue to the mixture.

3. Cover the reaction mixtures with 50 µl of mineral oil and then subject to one cycle for denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 55°C for 2 min for annealing, 72°C for 2 min for extension, and finally 1 cycle at 72°C for 4 min, decrease the temperature then to 4°C until the reaction mixtures remove from thermocylcer.

Electrophoreses analysis

The PCR products in 0.7% agarose gel in 0.5% TBE buffer (Tris-Borate-EDTA electrophoresis buffer, 90 mM Tris acetate, 90 mM boric acid and 2 mM EDTA) and stained with ethidium bromide (10 µl/ml) (Sambrook et al., 1989). Visualize the bands by examination under a UV trans-illuminator and photograph using a (MP4 Polaroid camera).

DNA hybridization

A: preparation of the probes

(A-1): Preparation of digoxigenin-labeled BBTV-DNA probe

Incorporate Digoxigenin-11-dUTP (Dig-11-dUTP), (Boehringer Mannheim, Indianapolis, IN,) into the newly synthesized DNA during 30 cycles of PCR with melting, annealing and extension conditions of 1 min at 90°C, 2 min at 55°C and 2 min at 72°C and one cycle 5 min at 72°C (Saiki et al., 1988).

For each reaction a final concentrations of:

0.2 µM of each primer,
10 mM dATP, dCTP, dGTP; 0.65 mM dTTP;
0.35 mM Dig-11-dUTP and
1.2 U *Taq* DNA polymerase (Promega Corporation)
in 50 µl containing 200 ng target DNA.

Confirm the presence of the expected molecular weight PCR amplified product by agarose gel electrophoresis (Sambrook et al., 1989).

(A-2): Preparation of Biotin-Labeled Probes (Chemiluminescent Detection System)

1. Dissolve 100 ng from DNA-PCR product in 5-20 µl of distilled water in a microcentrifuge tube on ice.
2. Add 20 µl 2.5X Random Primers Solution (125 mM Tris-HCl pH 6.8, 12.5 mM $MgCl_2$, 25 mM 2-mercaptoethanol, 750 µg/ml oligodeoxyribonucleotide primers, random octamers) and denature by heating for 5 min in boiling water bath then immediately cool on ice.
3. Add 5 µl of 10X dNTP Mixture (1 mM biotin-14-dCTP, 1 mM dCTP, 2 mM dATP, 2 mM dGTP, 2 mM dTTP in 10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA) and add distilled water to a total volume of 48 µl and mix briefly.
4. Add 2 µl of Klenow Fragment (40 units/µl Klenow Fragment in 50 mM Potassium Phosphate pH 7.0, 100 mM KCl, 1 mM DTT and 50 % Glycerol) to the reactions and mix gently but thoroughly.
5. Centrifug for 15 – 30 sec and incubat at 37 °C for 60 min to over night in water bath and then add 5 µl Stop Buffer (0.5 M Na_2EDTA, pH 8.0).

Separate unincorporated nucleotides from biotinylated DNA probe by either of two methods:

(I)- Repeated ethanol precipitation:

1. Add sodium acetate 3M, 1/10 volume and two volumes cold ethanol absolute to the reaction tube and mix by inverting the tube.
2. Incubate the tube at -70 °C for 15 min and centrifuge at 15,000 rpm for 10 min.
3. Remove the supernatant carefully with a pipette, dry the pellet and resuspend in 50 µl distilled water.
4. Precipitate the probe again with sodium acetate and ethanol as described above.
5. Resuspend the probe in TE buffer (10 mM Tris-HCl pH 7.5 and Na_2EDTA) and store at –20°C till used.

(II)- Column chromatography:

Use one-ml column of Sephadex™ (Pharmacia LKB Biotechnology) with TE buffer (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984).

(A-3): Preparation of ^{32}P-labeled BBTV-DNA probe:

For the specific DNA probe, amplify the BBTV-DNA by PCR then ^{32}P labeled using Prime-a-Gene labeling system (Promega Corp.), which incorporated ^{32}P dCTP by random priming.

1. Denature DNA templates by boiling in a water bath (100°C) for 10 min.
2. Add the following to 50 ng of denatured DNA:
   1 µl each of dATP, dTTP, and dGTP,
   2 µl reaction mixture, and
   5 µl ^{32}P dCTP, adjusted the volume to 19 µl with water and add 1 µl of sequenase (Sequenase version 2.0 T7 DNA polymerase).
3. Incubate the reaction at 37°C for 30 min.
4. To stop the reaction, add 45 µl TE (10 mM Tris. HCl, 1 mM EDTA, pH 8.0 and 5 µl dye (4% dextran blue, 0.2% orange G).
5. Remove the unincorporated nucleotides using sephacryl-300 Spin column.
6. Pipette the probe into the middle of the column (75 µl) and centrifuge for 10 sec at 4,000 rpm in a 1.5-ml microfuge centrifuge, after which the probe is recovered from the bottom of the column in a microfuge tube (Technical Bulletin, Promega Corp, 1997).

B: Dot blot, squash blot and tissue blot hybridization using biotin-labeled Probe:

(B-1): Dot blot:

Extract the total nucleic acid from infected healthy banana tissues (frozen tissues or dry tissues) as described above and resuspend the pellets in 50 µl TE buffer. Dilute the extracted DNA and PCR product as follows:

DNA Dilution	1 normal	½,	¼	1/8,	1/16	1/32	1/64	1/128	1/256	1/512…

PCR product dilution	10 ng/µl	1 ng/µl	100 pg/µl	10pg/µl	1 pg/µl	1/10 pg/µl

Spot 5 µl of the extraction and different dilutions of DNA onto Hybond N+ nylon membrane (Qiabrane Nylon Plus, Qiagen). Allow the membrane to air dry before lysing.

(B-2): Squash blot and Tissue blot:

1. Excise disks (1 centimeter in diameter) from infected and healthy banana leaf tissues using a sterilized cork borer.
2. Place the leaf disks on Qiabrane Nylon Plus Membrane and crush the tissues on the membrane using a sterilized Kontes Pestles.
3. For Tissue Blot, cut the pseudostem or midrib of a small plant using a sterilized cutter and dot onto Qiabrane Nylon Plus Membrane.
4. Allow the membrane to air dry before lysing.

(B-3): Southern blot hybridization:

Southern blot hybridization was used to confirm that PCR-amplified fragments were BBTV using the non-radioactive (Biotin- Labeled Probes-Chemiluminescent and Dig-11-UTP) and radioactive labeled ^{32}P BBTV-DNA probe.

1. Run the samples on 0.7% agarose gel with loading dye and visualize using ethidium bromide and visualize the bands by examination under UV trans-illuminator and photograph using a (MP4 Polaroid camera).
2. Rinse the gel in distilled water with shaking to remove the loading dye and eithidium bromide.
3. Denature the DNA in the gel by shaking in 500 ml denaturation buffer (0.5 M NaOH and 1.5 M NaCl) for 10 minutes. At this point soak the membrane in distilled water for 5 min and then in denaturation buffer for 5 min.
4. Spread the saran wrap on top of a Pyrexglass plate, then transfer the denatured gel onto saran wrap and then the wetted membrane over the gel (avoid creating bubbles).
5. Place three wet 3MM Whatman paper over the membrane and a stack of the brown paper towels over the 3MM Whatman paper.
6. Cover all of these by plastic bag to keep the moisture, place Glass sheet over this set-up and put the weight (approximately 500 gm) on the glass sheet.
7. Blot overnight and remove the stack of paper.
8. Flip the right-side up of the membrane, rinse the membrane in 2X SSC twice for 15 min each time and dry the membrane on 3MM Whatman paper to be ready for hybridization.

Lysing the membranes:

1. Place the membranes in a large tray with 3 layers of Whatman 3MM paper saturated with 0.5 M sodium hydroxide for 5 minutes, for denaturation.
2. Transfer the membranes to 1 M Tris (pH 7.4) for 5 min, then transfer to 2X SSC (300 mM sodium chloride, 30 mM sodium citrate) for 5 min, followed by 95% ethanol for 5 min. All incubations with gentle shaking (50 rpm/min.) at R. T.
3. Allow the membranes to air-dry (Gilbertson et al., 1991) and then use directly for hybridization.

Non-radioactive methods:

Hybridization with Biotin-Labeled BBTV-DNA Probe:

1. Wet the membranes in 0.25 M disodium phosphate, pH 7.2, and prehybridize with Hybridization buffer (0.5 M disodium phosphate, pH 7.2, 50% formamide, 0.5 M EDTA and 7% SDS) for 60 minutes at 48°C in hybridization oven.

2. Denature the biotin-labeled BBTV DNA-1 probe by incubation on boiling water for 5 min and then place in ice for 2 min.
3. Dilute the probe in fresh Hybridization buffer (10-100 ng/ml) and add to membranes (10-100 µl per cm^2)
4. Incubate the membranes overnight at 48°C.
5. Wash the membranes 2 X 5 min at room temperature with 2X SSC, 1%SDS (1 ml/cm^2).
6. Wash 2 X 15 min at 65°C with 0.1X SSC, 1% SDS and wash again 2 X 5 min at room temperature in 1X SSC.
7. Wash the membranes 2 X 5 minutes in Blocking Buffer (0.5 ml/cm^2) and incubate for 10 min in Blocking Buffer (1 ml/cm^2).
8. Dilute the Avidx-AP conjugate 1:5,000 in Blocking Buffer.
9. Add 2 µl of Avidx-AP conjugate in 10 ml Blocking Buffer per 100 cm^2 and incubate for 20 min at room temperature with constant agitation in conjugate solution.
10. Wash the membranes in Blocking Buffer (0.5 ml/cm^2) for 5 min at room temperature, wash three times in Washing Buffer (0.5 ml/cm^2) for 5 min at room temperature and wash finally two times with 1X Assay Buffer (0.25ml/ cm^2) for 2 min at room temperature.
11. Drain the membranes by touching a corner on 3MM Whatman paper and place on Saran Wrap on a flat surface.
12. Pipette a thin layer of CDP-Star substrate solution onto the membranes (3 ml/100 cm^2) and incubate for 5 min at room temperature, and drain the excess CDP-Star solution.
13. Place the membranes in development folder, after removing anti-static sheet and expose to film (X-OMAT™ AR Kodak).

Radioactive method

1. Lyse the membranes as described for the non-radioactive with biotin-labeled method.
2. Prihybridize the membrane in the prehybridization buffer {0.125 M sodium phosphate dibasic salt (Na_2HPO_4), 7% SDS, and 1 mM EDTA, pH 8.0} in the hybridization oven.
3. Denature the probe by adding 0.15 volume of 2 M sodium hydroxide and incubate at room temperature for 30 min.
4. Replace the Prehybridization buffer with 10 ml of hybridization buffer with approximately 25 ng of denatured probe and hybridize the membranes overnight at 68C°.
5. Wash the membranes twice with 100 ml (0.25 M Na_2HPO_4, 1% SDS, and 1 mM EDTA, pH 8.0) and once with 100 ml (sodium phosphate monobasic salt (NaH_2PO_4), 1% SDS, 1 mM EDTA, pH 8.0).
6. Blot dry the membranes and expose at –80C° Kodak OG-1 X-ray film (Sigma Chemical Company, St. Louis, MO) with Lanex screens for 12-48 h.

Results

Total nucleic acids were extracted from BBTV-infected banana tissues using a modified Dellaporta extraction method (Dellaporta *et al*., 1983; and Rojas *et al*., 1993), and the pellets were resuspended in 200 µl of TE RNase buffer and 5 µl (250 ng) of total nucleic acid were added to PCR mixture. The polymerase chain reaction assay was successfully used with specific primers to amplify the six components of the isolated BBTV (Fig. 1).

Fig.1 Electrophoresis analysis of the PCR amplified the six components from banana plants infected with the BBTV using specific primers for each. M=1 kb DNA size marker; lane 1 = 470 bp amplified fragment of BBTV DNA using primer pair cBBTV1 and hBBTV1 for detection, lane 2 = 1.1 kb amplified fragment of BBTV DNA C1, lane 3 = 930 bp amplified fragment of BBTV DNA C2, lane 4 =970 bp amplified fragment of BBTV DNA C3, lane 5 = 1050 bp amplified fragment of BBTV DNA C4, lane 6 = 800 bp amplified fragment of BBTV DNA C5, and lane 7 = 810 bp amplified fragment of BBTV DNA C6.

DNA hybridization methods (squash blot, dot blot and Southern blot) using radioactive and non-radioactive probes were used to detect the presence of BBTV in BBTV-infected banana plant tissues and aphids. Comparison between the sensitivity and the reliabilities of the radioactive (^{32}P labeled DNA probe) and the non-radioactive (biotin labeled probe and digoxigenin labeled probe) were carried out.

The specific ^{32}P-dCTP labeled BBTV-DNA-1 probe was successfully used to detect the presence of BBTV-DNA in infected samples using dot blot hybridization method (Fig. 2-B), using different concentration of Dellaporta extracts from BBTV-infected tissues. The results showed that the sensitivity of the radioactive method was 10 pg of DNA and 1/256 with the dilution of the DNA extract.

Data in Fig.2-A showed that the dot blot of DNA-PCR product and DNA extraction using the Dellaporta method, gave a strong reaction with the specific probe of BBTV-DNA biotin-labeled probe. The results also illustrated that there was no reaction with the healthy plants, which was used as a negative control. The results showed that, the sensitivity of the non-radioactive method was 100 pg of DNA and 1/128 with the dilution of the DNA extract.

A **B**

a b c d e a b c d e

Fig. 2: Quantification of different concentration of total nucleic acid (TNA) extracted from banana tissue to determine the efficiency of the radioactive BBTV-C1 labeled probe with ^{32}P (B) and non-radioactive biotin-labeled probe (A). Lanes from up to down: Dilutions of TNA extraction from banana tissues, 1= original dilution, 2= 1/2, 3= 1/4, 4= 1/8, 5= 1/16, 6= 1/32, 7= 1/64, 8= 1/128, 9= 1/256, 10= 1/512. Lane (c,d) dilutions 1, 1/2, 1/4, 1/512, from TNA was extracted from healthy banana tissue. Lane (e), dilutions from BBTV-DNA component 1 PCR product, 1= 100 ng, 2= 10 ng, 3= 1 ng, 4= 100 pg, 5= 10 pg, 6= 1 pg.

Southern blot hybridization was used to confirm that PCR-amplified fragment was BBTV DNA C1 using radioactive labeled ^{32}P BBTV-DNA probe (Fig. 3-C) and non-radioactive with the biotin-labeled probe (Fig. 3-B). The samples were run on 1.0% agarose gel and the

fragments were transferred to nylon membrane. The results of the southern blot hybridization experiment showed that the reaction was strong with the BBTV-DNA (Fig. 3).

A **B** **C**

Fig. 3. Southern blot hybridization of BBTV-amplified DNA analyzed on 1% agarose gel (Fig 3-A) blotted onto nylon membrane and hybridized with the ^{32}P -dCTP labeled probe (Fig 3-C) and or hybridized with the biotin-labeled probe (Fig 3-B). Lane 1, 3 and 4 showed high levels of hybridization signal from PCR product amplified from infected banana tissue (leaf blade, leaf midrib and pseudostem, respectively) and no reaction in lane 2 where the non-infected banana tissue. On the other hand, lane 5, high levels of hybridization signal from *Eco*RI digestion of the potential recombinant plasmid from the cloning of the PCR fragment for BBTV-DNA component as a positive control.

Fig 4. Dot blot hybridization with specific non-radioactive BBTV-DNA probe labeled with digoxigenin.

A **B**

Fig 5: Squash blot hybridization with specific non-radioactive BBTV-DNA component 1 labeling with biotin (Chemeluminscent method). **(Fig. 5-A)** The membrane before developing, **1**: Infected tissues, **2**: Healthy tissues. **(5-B),** After developing, dark spots with infected tissue squashes and nothing with health tissue squashes (bottom squashes).

Discussion

The Dot blot technique was developed through rapid preparation of plant samples for hybridization based on detection and nucleic acid hybridization with cloned probes labeled with

^{32}P, Chemiluminescent–Biotin, and digoxigenin. In this study, the results showed that the radioactive method was more sensitive than Chemiluminescent–Biotin and digoxigenin methods. The sensitivity of radioactive methods (^{32}P labeled probe) was 10 pg of DNA PCR product and 1/256 with the dilution of the TNA extract, while the sensitivity of non-radioactive methods (Biotin-labeled probes) was 100 pg of DNA PCR product and 1/128 with the dilution of the TNA extract and 1/8 of TNA extract with Digoxigenin-labeled probe. From these results, we demonstrated that the non-radioactive with Biotin-labeled probe was more sensitive than Digoxigenin labeled probe and its sensitivity was almost like that of the radioactive method with ^{32}P -labeled probe, but the radioactive method is more hazardous than other techniques.

The squash blot procedure provides a specific rapid and simple means of using molecular hybridization techniques to detect infected plant tissues and insects. The diagnosis of BBTV in infected tissues and aphids by hybridization was performed directly on tissues squashed onto a nylon membrane where no pretreatment of the samples was necessary. Squash blotting was a tool for rapid, large scale diagnostic of BBTV with radioactive with ^{32}P - labeled probe and non-radioactive with biotin-labeled probe.

Southern blotting is a procedure developed for transferring dsDNA and denatured dsDNA from agarose gel to a nylon membrane or nitrocellulose membrane. The blotted DNA was successfully detected using radioactive with ^{32}P-labeled probe and non-radioactive biotin-labeled probe and the sensitivity was almost the same in both methods.

Table (2): Comparison between the sensitivity of radioactive and non-radioactive methods for rapid detection for BBTV of banana tissues using dot blot hybridization assay and three labeled probe, i.e., ^{32}P, Biotin, and Dig.

TNA dilutions	**Radioactive** ^{32}P labeled probe		**Non-radioactive**
Biotin labeled probe		Dig, labeled probe	
1	++++++	+++++	++++
½	+++++	+++++	+++
1/4	+++++	++++	++
1/8	++++	+++	+
1/16	+++	+++	-
1/32	+++	++	-
1/64	++	++	-
1/128	+	+	-
1/256	+	-	-
1/512	-	-	-

References

Feinberg, A. P. and Vogelstein, B. (1983) Anal Biochem. 132:6.
Feinberg, A. P. and Vogelstein, B. (1984) Anal Biochem. 137:266.
Gilbertson, R. L., Hidayat, S. H., Martinez, R. T., Leong, S. A., Faria, J. C., Morales , F., and Maxwell, D. P. (1991) Differentiation of bean-infecting geminiviruses by nucleic acid hybridization probes and aspects of bean golden mosaic in Brazil. Plant Disease 75: 336-342.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press.
Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503

Citrus

TECHNICAL SHEET No. 2

Virus Detection: *Citrus Tristeza Virus* (CTV)

Method: DAS-ELISA

General
Virus detected: CTV from citrus stems.
General Method: Double Antibody Sandwich - Enzyme Linked Immuno Sorbent Assay (DAS-ELISA).

Developed by
Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University. According to Sanofi Diagnostics Pasteur and BIO-RAD Service Phytodiagnostics Plantest ELISA kits.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: June. 28, 2004

Goals
To develop a rapid method for large scale detection of CTV.

Introduction

Citrus is a major fruit in Palestine. It is mainly cultivated in the Gaza strip, Qalqylia, Tulkarm, and Jericho. The major citrus cultivars in Palestine include; Valencia, Shammoty, Lemon, and Clement. One of the major constrains facing the citrus industry in Palestine is the Tristeza, a viral disease caused by Citrus Tristeza Virus (CTV), which threatens the citrus industry in the area. One of the first quarantine measures taken to prevent the spread of this disease should be the survey of nurseries that supply propagation material locally and to other countries. The technique of ELISA could be used for such large scale surveys.

The Double Antibody Sandwich ELISA method involves a capture step of the virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugated with an enzyme (e.g. Alkaline Phosphotase), and then a detection step uses a specific substrate of the chosen enzyme. The developed color is finally read by an ELISA Reader. This method was successfully applied in our lab for large scale detection of CTV and other viruses.

Methods

A- Coating wells with coating antibodies

1. Prepare the coating buffer: dilute the 5x stock solution 1:5 in distilled water.
2. Dilute the antibodies 1:100 in the 1x coating buffer and mix thoroughly.
3. Load the diluted antibodies in the plate wells (100 µl/ well) and cover with adhesive film.
4. Incubate the plate at 37 °C for two hours. (At the end of this incubation, plates can be stored at 4 °C if necessary).
5. Wash the wells three times with 1x washing buffer. For each wash, use 200 µl/ well of the washing solution. When pouring out the washing solution, shake the plate.
6. After the third wash, hit the plate gently on several layers of tissue paper until you remove all drops of liquid.

B- Sample preparation and deposition

Samples can be prepared during the incubation period of the coating step.

1. Dilute the 20x extraction buffer to 1x with distilled H_2O.
2. Peel the epidermis layer of the stem and discard it. Then take the tissues under the epidermis that include the phloem cells and grind them in the 1x extraction buffer at a ratio of: 1gram/ 5 ml extraction buffer. This ratio may be changed according to the season and the nature of the sample. Follow the recommended ratios described in the kit manual.
3. A clear extract could be obtained by either spinning the extract at 2000 rpm for 5 minutes or by incubating it for a few hours at 4°C.
4. Deposit 100 µl of the extract per each coated ELISA well (prepared in section A). Also, deposit 100 µl/well from the negative and positive controls (kit) in the appropriate wells. Controls should be rehydrated in 1ml of distilled water, stored at 4°C and used within 5 days.
5. Cover the plate with adhesive film and incubate at 2-8 °C for overnight.
6. Wash the plate twice with 1x washing buffer (200 µl /well), then wash the plate an additional two times, 3 minutes each.

C- Deposition of conjugated antibodies

1. Dilute the 5x conjugate buffer to 1x in distilled water.
2. Dilute the conjugated antibodies to 1:100 with the 1x conjugate buffer.
3. Mix thoroughly before deposition.
4. Deposit the diluted conjugate 100 µl/ well.
5. Cover plates with adhesive film and incubate at 37°C for two hours (At the end of the incubation period samples can be stored at 4°C if necessary).
6. Wash three times with 200 µl/ well of washing buffer (1-2 min. each).

D- Deposition of substrate

1. Dilute the 5x pNPP buffer to 1x with distilled water.
2. For a plate dissolve one tablet of pNPP substrate in 12 ml of the 1x pNPP buffer (vortex for 30 seconds). Make sure that the material is dissolved.

Important: Do this step immediately before use (fresh preparation).

3. Deposit 100 µl/well of the prepared substrate.
4. Incubate wells at 37°C for 15 minutes. Leave at room temperature till reading O.D.s .

Buffers used-Dilute buffers as described in the protocol. The composition of the buffers as provided by the kit manufacturers:

Washing buffer, pH 7.4 (X20)	**Extraction buffer, pH 8.0 (X10)**	**Coating buffer, pH 9.6 (X5)**
PBS 20X	PBS X20	Na_2CO_3
Tween-20 (1%)	PVP 20%	$NaHCO_3$
NaN3 <1g/l	Tween-20 1%	NaN_3 <1g/l
	NaN_3 <1g/l	
Conjugate buffer, pH 7.05 (X5)	**Substrate buffer, pH 9.8 (X5)**	
PBS – Tween	Diethanolamine	
BSA	NaN_3 <1g/l	
NaN_3 <1g/l		

Dealing with the results

Read the Optical Density at wavelength 405: 30 minutes, 1 hour, and 2 hours after substrate deposit. Readings at times longer than two hours might be needed for better discrimination of the treatments, especially for poorly infected samples. However, if the OD reading was good after two hours then extra readings are unnecessary.

Samples OD = crude OD reading – average OD of substrate wells.

Detection threshold

It is recommended OD of the infected sample be set to twice that of the negative control. Samples of readings above this threshold are infected. Readings which are equal to the threshold are suspect. Also, a sample of a reading close to that of the positive control is infected.

References

SANOFI Diagnostics Pasteur, S.A
BIO-RAD Service phytodiagnostics
3, Boulevard Raymond Poincare
92430 Marnes la Coquette – France.
Tel: (33) 1 47956263/6265, Fax: (33) 1 47956264

TECHNICAL SHEET No. 3

Virus Detection: *Citrus tristesa virus* (CTV)

Method: DAS ELISA

General
Virus detected: Citrus Tristesa virus (CTV) from citrus green stems.
General method DAS ELISA

Developed by
Name of researcher: Fouad Akad
Address (Email): akad@agri.huji.ac.il
Date: May 1999

Goals
To develop a sensitive and easy method for CTV detection in infected stems.

Introduction

CTV is the most economically important virus problem affecting citrus worldwide and causes the death and decline of trees in sour orange rootstocks and a debilitating stem pitting disease in limes, grapefruit, and sweet oranges which affects trees on all rootstocks and reduces yield. CTV is today widespread in Israel, Morocco, India, China, Japan, Southern California, Florida, Argentina, Brazil, South Africa, Australia and southern Spain, and is moving into previously free, northern Spain. ELISA is the standard diagnostic method for CTV.

Materials and Methods

The anti-CTV antibody and the anti-CTV antibody alkaline phosphatase conjugate were from Bioreba

Coating

1. Dilute the CMV antibody 1:1000 in coating buffer. Coating buffer is for 1 liter (pH 9.6), in ddw: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.20 g.
2. Add 100- 200 µl to each well and cover plates tightly.
3. Incubate at 37°C for 4 hr or at 4-6 ^{o}C for 18 h.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper to wells. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween20 0.50 g, NaN_3 0.20 g.

Antigen extraction and binding

1. Add 100-200 µl extraction buffer per well. Extraction buffer is: 20 mM Tris buffer (pH 7.4) containing 137 mM NaCl, 3 mM KCl, 2 % PVP 24kD, 0.05 % Tween 20 and 0.02 % NaN_3.
2. Cut the citrus green stem into small peaces (1 mm^2); add 1-2 pieces to the well containing the extraction buffer. Cover the plate tightly and incubate at 4-6 ^{o}C for 18 h.

3. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Conjugate

1. Conjugate: Dilute anti-CTV alkaline phosphatase conjugate 1:1000 in conjugate buffer or in TBS buffer. Conjugate buffer is, for 1 liter, pH 7.4: Tris-(hydroxymethyl) amino-methane 2.40 g, NaCl 8.00 g, PVP (Polyvinyl-pyrrolidone) MW 24,000 20.00 g, Tween 20 0.50 g, BSA (bovine serum albumin) 2.00 g, $MgCl_2$-6 H_2O 0.20 g, KCl 0.20 g, NaN_3 0.20 g.
2. Add 200 µl per well and cover plates tightly.
3. Incubate at 37 ^{o}C for 3-5 hr.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels.

Color reaction

1. Dissolve p-nitrophenyl phosphate to 1 mg/ml in substrate buffer. Substrate buffer is for 1 liter (pH 9.8): in ddw, diethanolamine 97.00 ml, NaN_3 0.20 g (the substrate buffer available as 5x concentrate, Art. No. 110130).
2. Add 200 µl per well and incubate at ambient temperature in the dark.
3. Observe reaction and read yellow color development after 30-120 min.
4. Visually and/or read with an ELISA reader at 405 nm.

Cucumber

TECHNICAL SHEET No. 4

Virus Detection: *Cucurbit yellow stunting disorder virus* (CYSDV)

Method: RT-PCR

<u>General</u>

Virus detected: *cucurbit yellow stunting disorder virus* (CYSDV) from cucurbit leaves
Method: RT-PCR

Developed by

Name of researcher: Dr. Yusuf Abou-Jawdah, American University of Beirut, Lebanon
Address (email): abujawyf@aub.edu.lb
Date: October 26, 2002

Goals

To develop a reliable and fast detection system for CYSDV.

<u>Introduction</u>

Cucurbit yellow stunting disorder virus (CYSDV) is a member of the genus *Crinivirus* within the family *closteroviridae* (Fauquet and Mayo, 1999). CYSDV has a bipartite single-stranded plus-sense RNA encapsidated in long and flexuous particles. It is a whitefly-transmitted viral disease of cucurbits. It was first reported by Hassan and Duffus (1991) in the United Arab Emirates. This virus was also reported in Europe (Celix et al. 1996). Rubio et al. (2001) studied 71 isolates from collected from Jordan, Lebanon, Saudi Arabia, Spain, Turkey and North America. They found that these isolates could be divided into two subpopulations. Symptoms start as interveinal mottle on the middle leaves and develop into severe yellowing as leaves grow older. The fruits look normal but the fruit set is greatly reduced leading to a yield loss of about 50% (Abou-Jawdah et al., 2000). The development of an RT-PCR system would provide an easy, reliable and quick method to detect the disease.

<u>Materials and Methods</u>

Sample collection

Samples of infected cucurbit leaves (cucumber, squash, melon) were collected from greenhouses in the coastal area of Lebanon, at the end of the growing season, when symptoms are most apparent. Generally, the 4th or 5th yellow, papery leaf from the top of the plant was collected as is recommended in the literature.

Total RNA extraction

1. 0.2 g of leaf tissues are ground in liquid nitrogen
2. Add 400 µl of 100 mM Tris-HCl (pH 8) containing 0.2% sodium dodecyl sulfate (SDS) and 10 mM EDTA
3. Extract RNA with 1 volume of (1:1) phenol:chloroform
4. The aqueous phase is then adjusted to 2 M LiCl and incubated overnight at 4°C

5. Centrifuge at 12,000g for 15 min and dissolve the pellet in 100 µl RNAse-free-water

Primer design

Using the NCBI gene bank, the nucleotide sequence of the CYSDV HSP70 gene (AJ223619) was retrieved and used for primer design (Celix et al., 1996). In the sequence below, the bolded areas are the parts used for primer design.

1 atggcgaagg ctggtttgga gtttggtact actttctcta ctatcagcag ttatgttaat
61 ggtgttatga aagttttgaa attgaacgaa actgaattta tccctacctg tttagccata
121 acgtctaata atgatgtggt tgttggaggt cccgctcaag tattgtctaa tagtgacatg
181 cctaactgtt acttttatga cttgaaaagg tgggtaggtg ttgacagtat caattataat
241 gtgataaaaa ccaaaatcaa tccagcgtat gtcaccgagt tacgtggtaa cgacgtgtac
301 atcactggta tcgatagagg ttatacctgc acttacacag tcaaacaatt gatattgtta
361 tacattgaaa ctttagtaag attgttttct aaagttgagt ccataaccat aactagtctt
421 aacgtctctg ttccagctga ttataaatgt aagcagcgta tgtttatgaa atcagtttgt
481 gacagtctag gtttttcatt acgtagaatt ataaacgaac catctgcagc agctatatac
541 tttgtttcaa agtatccgca gtataacaac ttcctgatgt atgacttcgg aggaggaact
601 tttgattctt ctctaatagt c**agagacggt aagtat**gtca cagtggctga tactgaaggt
661 gattcgtttt tgggaggtag agacattgat aatgctatcg ctgactatat aacaacgacg
721 tatggtatga aaggtggttt gtccgctgac gtactggcgt ctataaagga ggattgtaat
781 tctaagggta gagagaattt caatgttata gattcatcag gcaaacttca taatgtgaaa
841 ttcacaagac aagatctgag tcgttgcatt gaacctttct ctaagaagag catagcactg
901 cttgataata tggtagtgcg taacataaca aaagattccg ctgtgtttat ggttggaggt
961 tcatcattgt tgaagaaagt tcaacatgat gtgatgaatt actgtgctag gacgaaacta
1021 gaatgcatca ttgataaaga ccttagatca gcagtgtcat ttggttgctc t**atgtcacat**
1081 **gcccaa**gaag acacaaaaaa tatgatatac atcgattgta attcacatcc cttaatgggc
1141 atatcatatt tttgttctcc aaagattata gttaggaaac ctgtggccat accttatact
1201 ggagtgcgag aagaaacctt aacaagacac tatacaattt tgaacgttta cgagggatca
1261 gatcccttcg ttttgaacaa cgattggttg attagtgcca atatgcagtc caacaagtac
1321 ggtgagatag gtgatacatt acaatatctt tacaaatata atgtagatgg catcttagag
1381 ttggttgtga ggaataaaag aacaggtaag gagacggtac ttccaattcc tttgcttttg
1441 actgagagta taaagaagct ggatgtaaat ctaactcaat tgtcaaatat tgatgaattg
1501 gcaactttgg tagctattat gagttactat aagcctgaat taaagtacct cctgacttat
1561 gtaaagacac caactatttt tgaaaatgaa attaagaaat ttggatctgg tgaagattta
1621 tataaatcct tagctgctct gaataaaaat ttcaagtaa

Primer 1: 5’ TTG GGC ATG TGA CAT 3’ (410L)
Primer 2: 5’ AGA GAC GGT AAG TAT 3’ (410U)

RT-PCR using the “Access RT-PCR system” (Promega)

PCR mix: 20 µl reaction mixture containing 80 ng of primers and 4 µl of RNA sample to each bead.

PCR cycles:

- 1 cycle at 48°C for 45 min
- 1 cycle at 94°C for 2 min
- 35-40 cycles at 94°C for 30 sec, 42°C for 30 sec, 68°C for 70 sec
- 1 cycle at 68°C for 7 min
- 4°C end of program

The PCR product was visualized by electrophoresis in 1.2% agarose gel in TAE.

Results

Electrophoresis from extracts of virus-infected cucumber, melon and squash plants showed a specific DNA band of about 460 bp that was absent in extracts of the respective healthy controls.

Discussion

This RT-PCR technique provides an easy and consistent procedure for CYSDV detection in plants with yellowing symptoms.

Figure 1. Gel electrophoresis of RT-PCR amplified products in 1.2% agarose gel. Lanes a-c: healthy cucumber, melon and squash, respectively; lane d: 100 bp ladder (Pharmacia); Lanes e & f: infected cucumber, lanes g & h: infected melon and squash, respectively.

References

Abou-Jawdah, Y., H. Sobh, A,. Fayyad, H., Lecoq, B., DelEcolle, and Trad-Ferre, J. 2000. Cucurbit yellow stunting disorder virus – a new threat to cucurbits in Lebanon. Journal of Plant Pathology 82: 55-60.

Célix, A., López-Sesé, A., Almarza, N., Gómes-Guillamón, M.L., Rodríguez-Cerezo, E. 1996. Characterization of cucurbit yellow stunting disorder virus, a *Bemisia tabaci* - transmitted closterovirus. *Phytophathology* 86:1370-1376.

Fauquet, M.C., and Mayo, M.A. 1999. Abbreviations for plant virus names. Virology Division News 144:1249-1273.

Hassan A.A., Duffus, J.E. 1991. A review of a yellowing and stunting disorder of cucurbits in the United Arab Emirates. *Emirates Journal of Agricultural Sciences* 2:1-16.

Rubio, L., Abou-Jawdah, Y**.,** Lin, H-X. and Falk, B.W. 2001. Geographically distant isolates of the *Crinivirus, Cucurbit yellow stunting disorder virus* (CYSDV)*,* show very low genetic diversity in the coat protein gene. Journal of General Virology 82:929-933.

TECHNICAL SHEET No. 5

Virus Detection: *Cucumber mosaic virus* (CMV)

Method: DAS ELISA

General

Virus detected: *Cucumber mosaic virus* was detected from cucumber, tobacco and watermelon leaves.
Method: DAS ELISA

Developed by

Name of researcher: Hanokh Czosnek, Hebrew University of Jerusalem, Israel
Address (email): czosnek@agri.huji.ac.il
Date: December 26, 2005

Goals

To develop a reliable and fast detection system for CMV.

Introduction

CMV is a well studied virus which infects many plant species.

Method

The CMV antibody is a gift from Prof. G. Loebenstein, The Volcani Center Israel

Coating of microtiter plate

1. Dilute the CMV antibody 1:1000 in coating buffer. Coating buffer is for 1 liter (pH 9.6), in ddw: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.20 g.
2. Add 100- 200 µl to each well and cover plates tightly.
3. Incubate at 37°C for 4 hr or at 4-6 ^{o}C for 18 h.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper to wells. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Antigen extraction and binding

1. Homogenize test sample 1:20 in either one of the extraction buffers (both extraction buffer are good). Extraction buffer I: for 1 liter, pH 8.5: Tris-(hydroxymethyl) aminomethane (Tris) 2.40 g, NaCl 8.00 g, Tween 20 0.50 g, KCl 0.20 g, NaN_3 0.20 g. Extraction buffer II: 0.5 M Citrate, 0.1% Thioglycolic acid, 0.1% Triton 100.
2. Add 200 µl per well. Cover plates tightly. Incubate in a moist chamber at 4-6 ^{o}C for 18 h.
3. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Conjugate

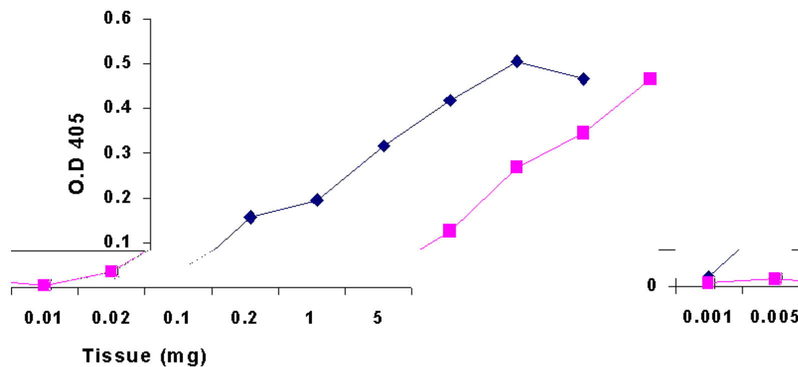
1. Conjugate: Dilute anti-CMV alkaline phosphatase conjugate 1:1000 in conjugate buffer or in TBS buffer. Conjugate buffer is, for 1 liter, pH 7.4: Tris-(hydroxymethyl) amino-methane 2.40 g, NaCl 8.00 g, PVP (Polyvinyl-pyrrolidone) MW 24,000 20.00 g, Tween 20 0.50 g, BSA (bovine serum albumin) 2.00 g, $MgCl_2$-6 H_2O 0.20 g, KCl 0.20 g, NaN_3 0.20 g.
2. Add 200 µl per well and cover plates tightly.
3. Incubate at 37°C for 3-5 hr.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels.

Color reaction

1. Dissolve p-nitrophenyl phosphate to 1 mg/ml in substrate buffer. Substrate buffer is for 1 liter (pH 9.8): in ddw, diethanolamine 97.00 ml, NaN_3 0.20 g (the substrate buffer available as 5x concentrate, Art. No. 110130).
2. Add 200 µl per well and incubate at ambient temperature in the dark.
3. Observe reaction and read yellow color development after 30-120 min.
4. Visually and/or read with an ELISA reader at 405 nm.

<u>Results</u>

Black line (upper line): the infected tissue was extracted with extraction buffer I containing uninfected plant tissue. Red line (lower line): the tissue was extracted with buffer II.



TECHNICAL SHEET No. 6

Virus Detection: *Cucumber mosaic virus* (CMV)

Method: Capture of plant viruses by GroEL

General
Virus detected: CMV from plants
General methods: Squash blot PCR

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad @agri.huji.ac.il; czosnek @agri.huji.ac.il
Date: December 26, 2005

Goals

Develop a rapid and very sensitive detection of CMV in tissues. This method is novel and not in general use.

Introduction:
See reference cited at end of methods for more details.

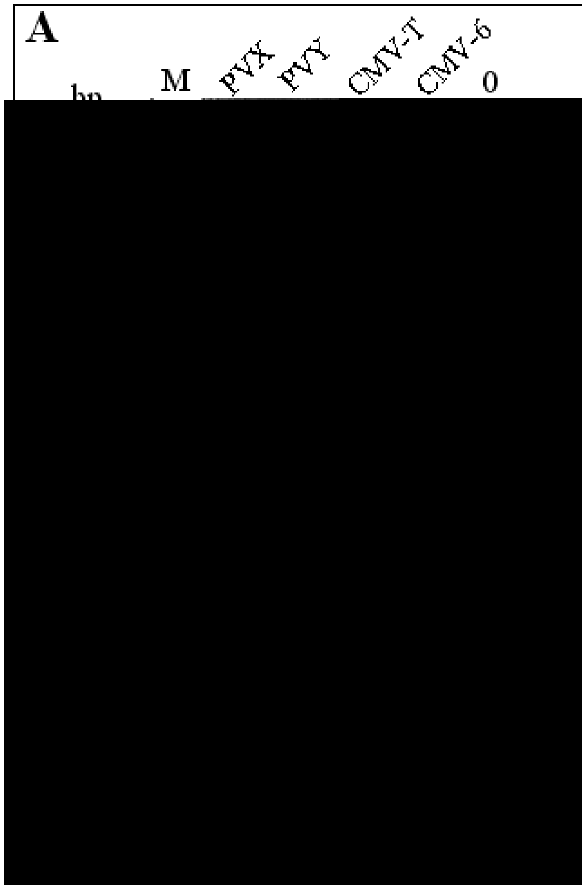
Methods:

1. PCR tubes or 96-well plates were filled with GroEL diluted in ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% NaN_3).
2. Incubate for 3 h at 37 °C and wash three times with TBST.
3. Add homogenates of infected material to the GroEL coated support, incubate for 18 h at 4 °C, and wash three times with TBST.
4. For PCR, reagents were added directly to the tube with primers specific for each virus. The PCR reaction contained: 0.25 µl of the four dNTP 25 mM, 1 µl each virus specific primers (10 pmoles), 2.5 µl Taq buffer (x10) and 1 unit Taq DNA polymerase. The reaction volume was adjusted to 25 µl with ddH_2O and subjected to cycling as follows. One cycle consisting of 3 min at 95 °C, 2 min at 50 °C, 2 min at 72 °C followed by 30 cycles consisting of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and terminated by 10 min at 72 °C.
5. The PCR products were subjected to electrophoresis in 1% agarose gel, in Tris-Phosphate- EDTA buffer (TAE) and stained with ethidium bromide (0.5 µg/ml) before photography.
6. For RT-PCR, 13 µl ddH_2O and 1 µl of the virus PCR complementary primer (10 pmoles) were added to the treated tubes. Following 10 min at 70 °C, the tubes were cooled in an ice bath for 10 min and 1µl of each of the four dNTP (25 mM) were added together with 4 µl of reverse transcriptase buffer (5X) and 10 units (1 µl) of AMV reverse transcriptase (Promega). After 1 h at 42 °C, the reaction was stopped by incubating the tube at 90°C for 10 min; the volume was then adjusted to 50 µl.
   One µl of the reverse transcriptase reaction was used for PCR. Reaction and cycling conditions were as above.

7. The following primers used to detect viruses by PCR were derived from the sequence published in GenBank. For TYLCV (accession No X15656) virion strand (position 61-80) 5'ATACTTGGACACCTAATGGC 3' and complementary strand (position 473-457) 5'AGTCACGGGCCCTTACA 3'. For PVX (accession No AF260641) virion strand (position (5643-5663) 5' CGAAAGATGTCAGCACCAGC 3' and complementary strand (position 6370-6350) 5' GACGTAGTTATGGTGGTGGT 3'. For PVY (accession No M95491) virion strand (position 102-120) 5' TCATCAAACAAACTCTTT 3' and complementary strand (position 937-918) 5' GGAAAGACACCAAAGCGGGA 3'. For CMV (accession No D10538) coat protein (CP) virion strand (position 115-133) 5' GGCATGGCTTTCCAAGGTA 3' and complementary strand (position 470-450) 5' GGAAAGACACCAAAGCGGGA 3'; for CMV movement protein (MP) virion strand (position 120-141) 5' ATGGCTTTCCAAGGTACCATG 3' and complementary strand (position 740-719) 3' TCTGTTGAAAGGCAGTACTAG 5'.

Results

Capture of CMV, but not of PVX and PVY, by GroEL, native and expressed in *E. coli*. (A): Tubes coated with native GroEL (1 µg protein/ml) were incubated with sap of potato leaves infected with PVX or PVY, or with sap of melon leaves infected with two CMV strains (0.1 g in 2 ml). After washing, the RT-PCR mixture was added together with primers specific of the virus CPs. The reaction products were subjected to gel electrophoresis and stained. Thick arrow shows amplified fragment. Thin arrow shows primers. 0: non-infected potato leaves. (B): Extracts of leaves of CMV-infected melon (0.1 g in 2 ml TBST, subsequently diluted 1:25) were incubated in tubes coated with recombinant GroEL (1 µg protein/ml). After washing, the RT-PCR mixture was added together with primers specific of the virus MP. The reaction products were subjected to gel electrophoresis and stained. 0: extract of non-infected melon leaf; P: plasmid containing a full-length clone of CMV RNA3. (C): Detection of PVX and PVY by immunocapture RT-PCR. Tubes were coated with commercial antibodies raised against PVX or PVY (IC), or with GroEL (1 µg protein/ml) and incubated with sap of infected potato leaves. After washing, the RT-PCR mixture was added together with virus-specific primers, the reaction products were subjected to gel electrophoresis and stained. M: molecular weight markers.



Reference

Akad F, Dotan N. and Czosnek H (2004). Trapping of *Tomato yellow leaf curl virus* (TYLCV) and other plant viruses with a GroEL homologue from the whitefly *Bemisia tabaci*. Archives of Virology 149:1481-1497.

Grapevine

TECHNICAL SHEET No. 7

Virus Detection: *Grapevine fanleaf virus* (GFLV)

Methods: RT-PCR, and IC-RT-PCR

General
Virus detected: GFLV from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription IC-RT-PCR.

Developed by
Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: Nov. 1, 2002

Goals
Sensitive methods for GFLV detection.

Introduction

Degeneration is a disease in grapevines caused by Nepoviruses. This disease is spread in many grapevine-growing areas of the world. Incidence of GFLV in Palestine is about 2% (1). European Nepoviruses, referred to as fanleaf, include distorting strains that induce malformation of leaves and canes, and chromogenic strains, which cause a yellow mosaic in the plant leaves (2). A well-known example of such viruses is Grapevine fanleaf (GFLV). The American Nepoviruses induce responses in grapevines that vary depending on the grapevine species and climatic condition. Grapevine decline, stunted growth, and low yield are the major symptoms of this disease (1). European and American Nepoviruses are both transmitted for long distance by infected propagating material and for short distance by nematodes (1).

Grapevine fanleaf virus is a single-stranded RNA virus belonging to the genus Nepovirus. Fanleaf disease was first reported in *Vitis vinifera*; from Austria; by Rathay in 1883 (3). The symptoms of this disease may vary according to different host species. For example, in *Vitis* spp. symptoms include green or yellow systemic mosaic, rings, line patterns and flecks, and leaf and nodal malformation. In other susceptible host species such as *Nicotiana clevelandii* the symptoms are systemic mottling and stunting. The virus is transmitted by a vector; a nematode; *Xiphinema index* and *X. italiae* (3). Virus does not require a helper virus for vector transmission. It could also be transmitted by mechanical inoculation or by grafting. The virus is neither transmitted by contact between plants; nor transmitted by pollen (although found in pollen of *Vitis*). Serology is the best tests used to identify fanleaf virus. Seedlings or rooted cuttings of *V. vinifera* and *V. rupestris* are good bait plants in studies with nematode vectors.

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set were used to detect GFLV in the tested samples:

Primers

1. RNA polymerase coding gene, 749 bp of RNA1.
H27: 5 TTATTTGCACGCATCGGATGCGC 3
C28: 5 CGACATCAGAGAGTTCACCTAAGCC 3

2. Coat protein coding gene, 605 bp of RNA2
H: 5 GTGAGAGGATTAGCTGGTAGAGG 3
C: 5 AGCACTCCTAAGGGCCGTGACC 3

RNA extraction from grapevine stems and petioles for RT-PCR

1. Cut the sample (0.5-0.7 g of the stems or petioles) into pieces with a scalpel and grind in liquid nitrogen with a mortar.
2. Add 5 volumes times the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudpmax till you get a green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80ºC till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1M DTT, dH_2O as requied).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H_2O).
13. Incubate at 37ºC for 1 h.
14. Store at –20ºC till used in the PCR.

IC-RT-PCR

Coating

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 ml in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extraction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborundum.
3. Collect the homogenate and centrifuge at 10,000 rpm for 10 min at 4ºC.

4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100 µl of the extract (supernatant) in each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, 1% Triton X-100, dH_2O as required) and heat at 65ºC for a few minutes.
7. Wash the plate 3 times with PBST.
8. Add 20 µl of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
9. Put the plate on ice and distrupt the virus particles by 5-10 sec by pipetting liquid in each well, then transfer the well content to a microfuge tube.
10. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5 µl of dH_2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37ºC for 1 h.
13. Store at –20ºC untill used for the PCR.

PCR conditions:

5 µl of the cDNA, 0.2 mM dNTPs, 1X Polymerase buffer including 2.5 mM $MgCl_2$, 100 ng of each primer, and 1 unit of the *Taq* DNA polymerase. Complete to 50 µl with dH_2O.

PCR cycles:

1. For RT-PCR

(Hot start): 94ºC for 5 min then 5 cycles of 94ºC for 45 sec, 40ºC for 1 min, and 72ºC for 2 min, then 30 cycles of 94ºC for 45 sec, 45ºC for 1 min, and 72ºC for 2 min. A final step of 72ºC for 10 min.

2. For IC-RT-PCR

(Hot start): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 40ºC for 1 min, and 72ºC for 1.5min. 30 cycles of 94ºC for 50 sec, 45ºC for 1 min, and 72ºC for 1.5 min. A final step of 72ºC for 10 min.

Results

RT-PCR detection of GFLV

Figure 1. (-) : negative control, M: 1 kb ladder, 1: leaf extract detected using the GFLV coat protein primer, 2: stem extract detected using GFLV coat protein primer, 1a: leaf extract detected using GFLV polymerase primer, 2a: stem extract detected using GFLV polymerase primer, 3 and 4: not related to the subject. The color of the GFLV leaf extract used for the RT-PCR was yellow, hence it might be the reason for the negative results from some of symptomatic samples.

IC-RT-PCR for GFLV

Figure 2. M: 1 kb ladder, 1 and 2: samples, (-): negative control.
Coat protien primers were used.

Discussion

There was no difference between the detection of GFLV using both methods, when the coat protein primers were used. On the other hand, the polymerase primer pair failed to detect the virus in RT-PCR reactions.

References

1. Ra'ed Al-Kowni. (1997) Institute agronomique Mediterraneen De Bari. M. Sc. Thesis
2. Hadidi, A., Khetarpal, R. and Koganezawa, H. (1998) Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society, St. Paul, MN, USA.
3. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 8

Virus Detection: *Grapevine Leafroll virus 1,3*

Method: IC-RT-PCR

General
Virus Detection: *Grapevine Leafroll virus 1,3*
Method: IC-RT-PCR

Developed by
Name of researchers: Ghandi Anfoka, Al-Balqa' Applied University, Al-Salt, Jordan
Address (Email): anfoka@joinnet.com
Date: Nov. 27, 2002

Goal
Detection of GLRV 1,3 by one step IC-RT-PCR.

Introduction

Leaf roll is a vitis disease with a very important economic impact throughout the world. It is characterized by a down rolling of leaves and reddening or yellowing of limbs and its occurrence causes significant reduction in crop quality and quantity. Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) is a powerful tool for diagnosis of grapevine viruses.

Materials and Methods

Tissue extraction

Leaf tissues from grape plants showing disease symptoms were collected from the University research center and kept at 4°C. Using pestle and mortar, 0.5 g of leaf petioles were cut into small pieces and grinded in 2.5 ml extraction buffer. The sap was transferred into an eppendorf tube and centrifuged at 14,000 rpm for 1 min and supernatants were kept on ice.

IC-RT-PCR

Coating with antibodies

- Polyclonal antibodies, developed by Sediag (France) were diluted using coating buffer (carbonate bicarbonate). Three antibody dilutions were tested (1:1000, 1:500 and 1:250).
- Two hundred microliters of diluted antibody were deposited into PCR tubes and tubes were incubated at 37°C for 4 h.
- Tubes were washed three times with PBS-T and briefly centrifuged to remove the remaining droplets of buffer.
- Two hundred microliters of leaf sap were dispensed into PCR tubes and incubated overnight at 4ºC.
- Tubes were washed three times with PBS-T.
- Tubes were incubated at 65°C for 10 min and used for RT-PCR.

One step RT-PCR

Primers

The primer set of GLRaV 3 designed by Saldarelli and Hadidi (1994) was used:
Downstream primer (C547): 5'- ATT AAC TTG ACG GAT GGC ACG C- 3'.
Upstream primer (H229): 5'-ATA AGC ATT CGG GAT GGA CC -3'.
Length of the predicted product is 340 bp.

RT-PCR kit

The access RT-PCR system kit developed by Promega (cat # A1260) was used in this study according to the manufacturer's instructions.

RT-PCR mixture:

- RT mixture per tube consisted of: 26 µl nuclease-free water, 10 µl AMV/TfI buffer (5X), 5 µl antisense primer (10 µM), 5 µl sense primer (10 µM), 1 µl dNTP (10 mM), 2 µl $MgSO_4$ (25 mM), 0.25 µl AMV reverse transcriptase (20 U/µl) 1 µl TfI DNA polymerase (5 U/µl). Tubes were vortex and then centrifuged for few seconds.

PCR cycles

The PCR cycle previously described by Acheche et al. (1999) was used. It consisted of: 46°C for 60 min, 94°C for 5 min then 30 cycles of 94°C for 30 sec, 56°C for 45 sec, and 72°C for 1 min, and a final step of 72°C for 10 min.

Agarose gel electrophoresis

PCR products (8 µl) were electrophoresed on 1% agarose gel and stained with ethidium bromide before gel was photographed under UV illumination. A lambda DNA *Eco*RI/*Hin*dIII digest was used to determine the size of amplified product.

Results

Part of the RNA polymerase gene (340 bp) of GLRV 1,3 could be amplified using one step IC-RT-PCR. The expected size of PCR (340 bp) could be only detected in tubes coated with antibodies diluted 1:250 and 1:500 (Fig. 1).

Figure 1. IC-RT-PCR of a grape sample. M: lambda DNA/*Eco*R1+*Hin*dIII marker. 1: tubes coated with antibodies diluted 1:1,000, 2: diluted 1:500, and 3: diluted 1:250.

Discussion

This technique is sensitive, saves time and effort and can be easly applied to detect GLRV 1,3 in large number of samples without the need of RNA extraction.

References

Acheche, H., Fattouch, S., M'Hirsi, S., Marzouki, N., and Marrakchi, M. (1999). Use of optimised PCR methods for the detection of GLRaV3: a closterovirus associated with grapevine leafroll in Tunisian grapevine plants. Plant Molecular Biology Reporter 17:31-42.

Minifra, A. and Hadidi, A (1994). Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. J. Virol. Meth 47:175-188.

TECHNICAL SHEET No. 9

Virus Detection: *Grapevine Leafroll Virus 1,3* (GLRV-1,3)

Methods: RT-PCR, and IC-RT-PCR

General

Virus detected: GLRV-1,3 from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription IC-RT-PCR.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: Nov. 1, 2002

Goals

To develop sensitive methods for GLRV-1,3 detection.

Introduction

Grapevine leafroll virus is a widespread disease caused by Closterviruses. Seven viruses of this genus have been detected in grapevines with leafroll symptoms (1). These viruses are called Grapevine leafroll associated viruses 1-7 (GLRaV 1-7). Leafroll is one of the most spread viral diseases in the Palestinian viticulture. Leafroll viruses are transmitted by infected propagating material and some of them by mealybugs (2). Since leafroll associated viruses multiply in the grapevine phloem, the leaves of infected vines are thicker than normal with discolored margins rolled downwards.

Grapevine leafroll associated viruses 1-7, which belong to the genus Closterovirus, consist of a filamentous particle and a single-stranded RNA genome. The disease caused by these viruses is a widely distributed and affecting grapevine in all viticulture. It causes significant reduction in yield and quality of the crop. Preliminary survey in Palestine showed that about 30% of the trees are infected by leafroll viruses (2). The major symptoms include down rolling of leaves and inter-veinal chlorosis. Leaf rolling begins at the base of the cane and spreads to younger leaves during midsummer. Discoloration of leaves (reddish-purple in red-fruited, see figure 3, and yellowish in white-fruited cultivars) is conspicuous in late summer and early autumn in intolerant cultivars (3). Leafroll is symptomless in some American *Vitis* spp. and hybrids (3). These viruses are transmitted by mealy bugs (some viruses) and grafting (1).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set was used to detect GLRV 1,3 in the tested samples:

Primers

1. Designed by Hadidi and Minafra for part of the viral RNA polymerase gene, amplified fragment of 340 bp:

C547 (22 bases): 5 ATTAACTTGACGGATGGCACGC 3
H229 (20 bases): 5 ATAAGCATTCGGGATGGACC 3

2. We use another primer set which was design by the Tunisian MERC team, M. Marrakchi and Hatem Fakhfakh and associates at University of Tunisia, for the coat protein gene. Amplified fragment of 945 bp.

C50 (27 bases): 5 CGTAGGCTACTTCTTTGCAATAGTTGG 3
H49 (25 bases): 5 ATGGCATTTGAACTGAAATTAGGGC 3

RNA extraction from grapevine stems and petioles for RT-PCR.

1. Cut the sample (0.5-0.7 g of the stems or petioles) into pieces with a scalpel and place in liquid nitrogen and grind with mortar.
2. Add 5 volumes the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudpmax until you get green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80ºC till used.
6. Dilute the extract to 40% in distilled water containing 1% Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, qsp with dH_2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H2O).
13. Incubate at 37ºC for 1 h.
14. Store at –20ºC till used in the PCR.

IC-RT-PCR

Coating

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 µl in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extaction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborudpmax.
3. Collect the homogenate and centrifuge at 10000 rpm for 10 min at 4ºC.
4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100 μl of the extract (supernatant) per each well.

Results:

RT-PCR detection of GLRV1,3

1Kb 1 2 + -

Figure 1. L: Lambda ladder, 1,2: the sample amplified with polymerase primers. +: positive control (340 bp), -: negative control using the polymerase primer.

IC-RT-PCR for GLRV

1Kb 1 2

Figure 2: IC-RT-PCR of a grapevine sample: 1-Kb: ladder, 1: the sample amplified using polymerase primer (340 bp), 2: the same sample amplified using the coat protein primer (about 940 bp).

Comments

The virus was detected by both sets of primers. However, the coat protein primer was better, since it didn't produce non-specific bands. Both the RT-PCR and the IC-RT-PCR were successfully applied for the detection of GLRV 1,3 in total RNA extracts of the tested samples.

References

1. Hadidi, A.H., Khetarpal, R. and Koganezawa, H. 1998. Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, Minnesota.
2. Ra'ed Al-Kowni. 1997. Institute agronomique Mediterraneen De Bari. MSc Thesis
3. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 10

Virus Detection: *Grapevine virus A* (GVA)

Methods: Non-radioactive hybridization (Digoxigenin-labeled probes)

General
Virus detected: GVA from grapevine leaves
Methods: Non-radioactive hybridization (Digoxigenin-labeled probes)

Developed by
Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: November 10, 2002

Goals
Sensitive method for GVA detection

Introduction

Several methods have been developed for the detection of grapevine viruses. These methods include the use of monoclonal and polyclonal antibodies, i.e ELISA (1), molecular hybridization (2) and PCR (3). The hybridization methods involved either radioactive or digoxignin-labeled probes. The Digoxignin system is an easy, safe, and effective non-radioactive method for labeling and detecting nucleic acids. This method involve the use of Digoxigenin-labeled probes for Southern, Northern, and dot-blot hybridization. In the case of Dig DNA labeling, Digoxigenin-11-dUTP could be incorporated by the random-primed labeling method. This labeling reaction contains a template DNA, random hexa-nucleotides mixture, dNTP labeling mixture with Dig-dUTP, and Klenow enzyme, H_2O, and EDTA. The hexa-nucleotides are randomly annealed to the denatured template DNA. Then the Klenow enzyme catalyzes the labeling reaction, which is then terminated by addition of EDTA. The resultant labeled probe is then used for hybridization with the target DNA template. The probe template is detected by adding Anti-Dig-Alkaline Phosphatase antibody followed by the specified substrate. Color is precipitated in few minutes.

Materials and Methods

Extraction of nucleic acids with PEX

1. Weigh 0.1 g of grapevine leaves and freeze at -80 ºC.
2. Incubate the frozen leaf with 500 µl of 70% ethanol for 10 min.
3. Discard the ethanol and add 500 µl of PEX solution (PEX: 6.25 mM potassium ethyl xanthogenate, 100 mM Tris-HCL, 700 mM NaCl, 10 mM EDTA, pH 8) then incubate at 65 ºC for 5 min.
4. Centrifuge the samples at 14,000 rpm for 5 min.
5. Remove the tissue by toothpicks, then add 1 ml of ethanol and incubate at -80 ºC for 30 min.
6. Centrifuge the mixture at 14,000 rpm for 10 min. Dry the pellet at 65ºC, then dissolve

it with 25 µl d.d. water.

7. Incubate the dissolved pellet at 65 ºC for 15 min.

Probe Preparation

Heat 10 µl of GVA coat protein clone at 100 ºC for 10 min, then put on ice. Add the following components to the heated clone: (2 µl of hexa-nucleotide mixture, 2 µl of dNTPs, and 1 µl Klenow enzyme) and mix them. Incubate the mixture at 37 ºC for 20 h.

Molecular Hybridization and chemiluminescent detection

1. Dot blot 1 µl of the extracted RNA on nylon-membrane, and then expose the membrane to U.V. light for 5 min.
2. Prehybridize the dot blots for 2 h with pre-hybridization solution: (2.5 ml 50X SSC, 2.5 ml 10% SDS, 3.24 ml 1 M NaH_2PO_4, 1.76 ml 1 M Na_2HPO_4, 1 µl of Denhards solution and adjust the volume to 40 µl with d.d.water. Add 0.7 ml of boiled Salmon Sperm to the previous components, then boil the whole mixture for 10 min).
3. Remove the pre-hybridization solution and incubate blots with boiled probe for 16 h at 42 ºC.
4. Washing: Wash the blots twice for 5 min each with 2X SSC containing 0.1% SDS at room temperature. Then another two washes for 15 min each in 0.1 SSC containing 0.1% SDS.
5. Detection procedure: Incubate the blots at 42 C for 1 h with blocking solution: (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 4% blocking reagent). We use this solution as substitute for the malic acid buffer indicated in the kit manual.
6. Wash the blots with previous washing buffers as in step 4.
7. Incubate blots with 1:5000 diluted anti-dig conjugate in blocking solution for 1 h.
8. Incubate the mem0001 T2(on 92 0 0 12BT/TT0 1 ing buffe8ckin9m1h0o(solut2ac)7J0.o Tw 19.5

References

1. Martelli, G.P., Saldarelli, P. and Boscia, D. (1997). Filamentous viruses of the grapevine: closteroviruses. In P.L.Monette (Ed.) Filamentous viruses of woody plants. Research Signpost, Trivandpmax, India.

2. Saldarelli, P., Guglielmi-Montano, H. and Martelli, G.P. (1994). Detection of three grapevine closterolike viruses by non-radioactive molecular probes. Vitis 33, 157-352.

3. Minafra, A. and Hadidi, A. (1994). Sensitive detection of grapevine virus A, B and Leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. J. Virol. Methods 47, 175-188.

TECHNICAL SHEET No. 11

Virus Detection: *Grapevine virus A* (GVA)

Methods: RT-PCR, and IC-RT-PCR

General

Virus detected: GVA from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription PCR.

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology Center at Bethlehem University.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: Nov. 1, 2002

Goals

To develop sensitive methods for GVA detection in grapevines.

Introduction

Grapevine, which belongs to Vitidaceae family, is considered the second important fruit crop, after olives in Palestine. Based on the Palestinian Central Bureau of Statistics (1), the total fruit-trees cultivated area in Palestine is 1,118,075 dunums, of which 83,174 dunums are grapevine yards (7.4%). About three quarters of the grapevine area is located in Bethlehem and Hebron districts (1).

Until recently, 44 different viruses had been identified in grapevines worldwide, but not all of them cause serious diseases (2). Viruses causing the most important grapevine diseases in Palestine belong to three genera; Nepovirus, Closterovirus, and Trichovirus. Another disease detected in Palestine (grapevine fleck) is caused by a virus that has not been grouped yet (2).

A preliminary work conducted in 1995 (3) in an attempt to determine incidence of viral infections in viticulture in Palestine has shown that infection ranges between 50-98%. The most prevalent virus was GVA followed by Grape leaf roll association virus (GLRaV) 1,3, grapevine fleck virus, GLRaV-2, GVB, Grape fanleaf virus, and GLRaV-7. The highest infection with most viruses was in Bethlehem, Jenin, and Hebron areas. The GFLV and GLRaV-7 were the least common viruses among those detected in Palestine, and were restricted to Bethlehem, Jenin, and Hebron areas.

Grapevine A is a single stranded RNA virus belonging to the Trichovirus genus. GVA is one of the most common causes of the Rugose wood disease, particularly the syndrom known as Kober stem grooving disease (3). This disease is highly distributed in Bethlehem and Hebron areas, where infections reach 85% (3). The virus was first reported in *Vitis vinifera*; from Taranto, Italy; by Ciccarone. The symptoms caused by GVA include pits and grooves in the trunk. GVA is transmitted by a vector (very rarely); an insect; Pseudococcidae. The virus is also transmitted by mechanical inoculation (only to *Nicotiana glutinosa*); or by grafting but not transmitted by contact between plants; neither by seed or pollen. Grapevine A susceptible host species are *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Vitis labrusca*, *Vitis rupestris, Vitis rupestris var. Rupestris, and Vitis vinifera* (4).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set was used to detect GVA in the tested samples:

Primers

PCR Product: 430 bp of the coat protein

Primer 1, complementary sense primer: C995: 5'AAGCCTGACCTAGTCATCTTGG 3'.

Primer 2, antisense primer: H587: 5'GACAAATGGCACACTACG 3'.

RNA extraction from grapevine stems and petioles for RT-PCR

1. Cut the sample (0.5-0.7 g of the stems or petioles) into pieces by a scalpel and grind in liquid nitrogen with a mortar.
2. Add 5 volumes the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudpmax till you get green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80ºC till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, qsp with dH_2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate for 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H_2O).
13. Incubate at 37ºC for 1 h.
14. Store at –20ºC till used in the PCR.

IC-RT-PCR

Coating

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 µl in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extraction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and grind in the presence of carborundum.
3. Collect the homogenate and centrifuge at 10,000 rpm for 10 min at 4ºC.

4. Transfer the supernatant into a new tube.
5. At the end of the coating period, deposit 100 µl of the extract (supernatant) in each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, 1% Triton X-100, enough dH_2O as required) and heat at 65ºC for a few min.
8. Wash the plate 3 times with PBST.
9. Add 20 ml of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
10. Put the plate on ice and disrupt the virus by 5-10 sec pipetting liquid in each well, then transfer the well content to a microfuge tube.
11. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
12. Add 5 µl of dH_2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
13. Incubate the tubes at 37ºC for 1 h.
14. Store at –20ºC till use for the PCR.

PCR conditions

5 µl of the cDNA, 0.2 mM dNTPs, 1X Polymerase buffer including 2.5 mM $MgCl_2$, 100 ng of each primer, and 1 unit of the *Taq* DNA polymerase. Complete to 50 µl with dH_2O. However, amounts less than 1.25 units of the *Taq* DNA polymerase failed to detect GVA. Hence we used 1.25 units per reaction for detection GVA.

PCR cycles

(Hot start: Add the *Taq* DNA polymerase while the reaction is heated at 94ºC): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 50ºC for 1 min, and 72ºC for 1 min. 30 cycles of 94ºC for 50 sec, 51ºC for 1 min, and 72ºC for 1 min. A final step of 72ºC for 5 min.

Results

M 1 2 3

Figure 1.
M: molecular weight marker.
1: IC-RT-PCR product of tested sample.
2,3: RT-PCR products of tested samples.

Discussion

The detection of GVA using the IC-RT-PCR method was much sensitive than the RT-PCR method. The results showed very faint bands in some of the tested sample using the RT-PCR method compared to the bands detected using the IC-RT-PCR method. Hence, we recommend the later technique for the detection of GVA especially in samples containing low titer of the virus, which could be previously determined by ELISA or other tests.

References

1. Palestinian Central Bureau of Statistics. Agricultural Statistics 1995/1996.
2. Hadidi, A., Khetarpal, R. and Koganezawa, H. (1998) Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society.St. Paul, MN
3. Ra'ed Al-Kowni. (1997) Institute agronomique Mediterraneen De Bari. M. Sc Thesis.
4. http://life.anu.edu.au/viruses/Ictv/

TECHNICAL SHEET No. 12

Virus Detection: *Grapevine virus A* (GVA)

Methods: RNA dot blot hybridization

General
Virus detected: GVA from grapevine leaves, petioles, and stems.
General Methods are RNA dot blot hybridization.

Developed by
Name of researchers: Hanokh Czosnek, The Hebrew University of Jerusalem.
Address (Email): czosnek @agri.huji.ac.il
Date: December 26, 2005

Goals
To develop sensitive methods for GVA detection in grapevines.

Introduction

See other technical sheets on *Grapevine virus A* for information about this virus and references.

Methods

RNA purification

1. Cut a leaf or stem grape tissue and transfer it to a microfuge tube, freeze in liquid nitrogen, and add 500 µl 70% ethanol for 10 min.
2. Discard the ethanol and add 500 µl of PEX solution; incubated at 65 ^{o}C for 5 min. (PEX solution: 6.25 mM potassium ethyl xanthogenate, 100 mM Tris- HCl pH 7.5, 700 mM NaCl, 10 mM EDTA pH 8.0).
3. Centrifuge the samples for 6 min at 14,000 rpm (12,000 x g) and incubate for an additional 15 min at 65 ^{o}C.
4. Remove the piece of tissue using a toothpick and add 1 ml ethanol and incubate for 30 min at –80 ^{o}C.
5. Collect nucleic acids by centrifugation for 10 min at 14,000 rpm, discard ethanol and dry gently under vacuum.
6. Dissolve the pellet of nucleic acid with 30 µl ddH_2O, incubate at 65 °C for 15 min and store at –80 °C.

Dotting RNA on membrane

1. Spot 5 µl from each RNA sample onto a nylon-membrane.
2. Cross-link the nucleic acid to the membrane by exposure to U.V. for 3 min.

Preparation of ^{32}P labeled probes by Random priming method (kit from Amersham)

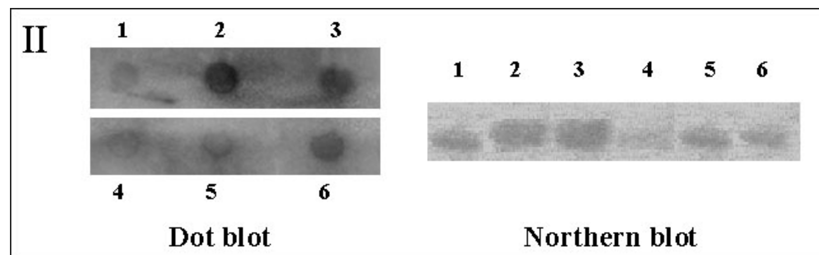
1. In a total reaction volume of 50 µl add: 5µl of 0.3 µg/µl plasmid pGEM/GVA, 5µl 10 x hexanucleotide buffer, and 22 µl ddH_2O.
2. Heat for 10 min at 100 °C then cool on ice for 15 min.
3. Add 10 µl of dNTPs (0.5 mM each) without dCTP and 3 µl of the Klenow fragment of DNA polymerase I.
4. Add 5 µl of [^{32}P] dCTP (3000 Ci/mmole) and incubate at 37 °C for 3 h.
5. Boil for 10 min and immediately put in ice bath.

Hybridization

1. Membranes are prehybridized at 42 °C with a solution that contains, for 50 ml: 12.5 ml 50 x SCC, 5 ml Denhardt solution x 50, 2.5 ml 10 % SDS, 3.2 ml NaH_2PO_4, 1.7 ml Na_2HPO_4, 0.7 ml boiled salmon sperm DNA (50 µg/ml) and ddH_2O up to 50 ml.
2. After 2 h, add the radiolabeled probe and hybridize for 18 h at 42 °C.
3. Wash the blots with 1xSSC - 0.1% SDS for 20 min at 65°C, twice. If background is dirty, wash with 0.1 x SSC - 0.1% SDS at 65 ^{o}C.
4. Expose the membrane to X-ray film or to a Phosphor imager.

Results

The same samples are compared using dot blot hybridization and northern blot hybridization.



Potato

TECHNICAL SHEET No. 13

Virus Detection: *Potato leaf roll virus* (PLRV) CP Gene Expression

Method: RT PCR

General
Virus under study: PLRV from potato leaves.
General method for detection: RT-PCR
Gene expression strategy: Coat Protein (PLRV CP) gene based strategy.

Developed by
Name of researcher: Ahmed Kamal, Hamed Mazyad, and Ahmed Shalaby, Agriculture Research Center, Giza, Egypt
E-mail address: elprince4@hotmail.com and aashalaby@link.net
Date: November 4, 2002

Goals

Expression of the PLRV coat protein for production of ELISA kits that may use for the serological detection of PLRV in infected potato plants.

Materials and Methods

Isolation of total RNA from plant tissue

Total RNA was isolated from the infected potato plants using RNeasy® plant Mini Kit obtained from QIAGEN as the following:

1. Grind a small part of the top leaf of the infected potato to a fine powder using a sterile mortar and pestle and liquid nitrogen.
2. Transfer the tissue powder and liquid nitrogen to microfuge tube and allow the liquid nitrogen to evaporate.
3. Transfer the flow-through fraction from the QIA shredder to a new microfuge tube without disturbing the cell-debris in the collection tube, then add 225 µl ethanol (96%) to the cleared lysate and mix well by pipetting.
4. Apply the total mixture, including any precipitate, onto an Rneasy mini spin column sitting in a 2-ml collection tube and centrifuge for 15 sec at 12,000 rpm.
5. Add 700 µl of RW1 buffer onto the Rneasy column and centrifuge for 15 sec at 12,000 rpm then discard flow-through and transfer the column into a new collection tube.
6. Add 500 µl of buffer RPE containing ethanol onto the column and centrifuge for 15 sec at 12,000 rpm then discard the flow-through.
7. Add additional 500 µl of buffer RPE to the column and centrifuge at maximum speed (13,000 rpm) for 2 min to dry the column.
8. Transfer the Rneasy column into a new 1.5-ml collection tube. To elute the RNA, pipette 40 µl of RNase-free water directly onto the RNeasy membrane and centrifuge for 1 min at 12,000 rpm.

Oligonucleotide primers design and synthesis

A pair of degenerate oligodeoxynucleotide primers specific for PLRV coat protein gene were designed (M.K. Nakhla, Univ. of Wisconsin-Madison), synthesized and used for isolation, detection and gene expression of the coat protein gene. The forward primer (PLRVCPv) sequence was: 5`- AAT AGA ATT CTA ATG AGT ACG GTC GTG GTT ARA GG 3` with *Eco*RI restriction enzyme site at the 5` end, and the complementary primer (PLRVCPc) sequence was: 5`- AAA ACC ATG GCT ATY TGG GGT TYT GCA RAG CTA C – 3`, with *Nco*I restriction enzyme site at the 5` end. PCR amplification of the coat protein gene using these primers generated *Eco*R*I* and *NcoI* compatible ends (5` and 3`, respectively). The expected size of the PCR product was approximately 548 bp. The oligodeoxynucleotides were synthesized at the 50 nM scales in GIBCOBRL life technologies.

One step RT-PCR

Total RNA extracted from infected potato plants was used as templates for one-tube RT-PCR amplification reactions (one step RT-PCR system, QIAGEN). One step RT-PCR amplification was performed in a reaction volume of 50 µl.

RT-PCR mix:

10 ng/µl of total RNA
10 µl of 5x buffer
2 µl of dNTPs mix
10 µl of 5x Q-solution
3 µl of 10 µM of each primer
2 µl of QIAGEN enzyme mix
0.2 µl of Rnase inhibitor
12.8 µl DEPC treated water.

RT reaction started with incubation at 50°C for 30 min followed by denaturation at 95°C for 15 min. PCR amplification was performed by 35 cycles in thermal cycler Bio-Metra starting with denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, lastly, extension at 72°C for 1 min. and an additional extension at the end of 35_{th} cycle at 72°C for 7 min.

Electrophoresis analysis

Agarose gel electrophoresis was performed in DNA electrophoresis sub-cells (Bio-Rad). Nuseive/Seakem (3:1) molecular biology grade agarose. Agarose concentration was selected according to DNA size to be 1% and electrophoresis was performed in 1X TBE buffer. DNA samples were mixed with gel loading buffer. DNA was stained with ethidium bromide added both to the gel and the buffer at a concentration of 0.5 µg/ml. DNA was visualized on an UV transilluminator (λ = 254 nm).

Molecular cloning

The fresh PCR product was molecularly cloned in *E. coli* into pBAD TOPO-TA Expression Kit vector system from Invitrogen.

Cloning reaction:

The ligation reaction mix contained: 2 µl fresh PCR product (insert) 1 µl salt solution DEPC treated water up to 5 µl, and 1 µl vector (pBAD-TOPO). The ligation mixture was

incubated 30 min at room temperature. **Note:** The uncut control plasmid was used to check the transformation efficiency of the competent cells.

Bacterial transformation

The *E. coli* XL1-Blue competent cells were transformed by the standard method.

1. Add 5 µl of pBAD cloning reaction or 1 µl uncut control plasmid to each 50 µl competent XL1-Blue cells, and mix gently by tapping.
2. Incubate tubes on ice for 30 min then heat shock at 42ºC for 45-50 sec and place again on ice for 2 min.
3. Add 950 µl of room temperature SOC medium to each transformation mix, and incubate the tubes at 37ºC for one hour with 250 rpm shaking. From the transformation tube of the ligation mix, and 1:10 dilution of the uncut control plasmid in SOC medium, plate 100 µl on LB plates containing 50 mg/ml ampicillin.
4. Invert Plates and place them in a 37ºC incubator overnight.
5. Positive clones were identified by blue/white selection, and restriction endonucleases digestions, after plasmid purification.

Isolation of Recombinant Plasmids

Recombinant plasmids were extracted using the standard Alkaline Lysis Miniprep method.

1. Inoculate 1.5 ml of 2XYT medium containing ampicillin (60 µg/ml) with a single colony and grow overnight in shaking water bath at 37°C.
2. Centrifuge one and half milliliters of overnight culture in a 1.5-ml microcentrifuge tube at 4,000 rpm for 5 min.
3. Decant the supernatant and re-suspend the pellet in 100 µl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM Glucose).
4. Add 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS [sodium dodecyl sulphate] w/v) to lyse the cells
5. Add 150 µl of solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of water) to precipitate chromosomal DNA and the large macromolecules.
6. Centrifuge the precipitated DNA at 10,000 rpm for 5 min and transfer the supernatant into a clean microfuge tube.
7. Add an equal volume of PCI to 400 µl of the supernatant and vortex for 5 min then centrifuged as in step 6, then remove the top aqueous phase to a new 1.5 ml microfuge tube
8. To precipitate the DNA add 2.5 volume (1 ml) of absolute ethanol and incubate at -80ºC for 2 h then centrifuge the precipitated DNA at maximum speed (14,000 rpm) for 15 min.
9. Wash the DNA pellet with 70% ethanol and centrifuge at 1000 rpm for 5 min then dry the pellet at the DNA vacuum concentrator.
10. Resuspend the DNA in 30 µl of TE-RNase (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 20 µg/ml RNase A) to remove about 80% to 90% of RNA from the DNA minipreparation.

Restriction digestion of plasmid DNA

Recombinant plasmids thought to contain cloned viral cDNA were digested using restriction endonuclease *Nco*I to confirm insert size.

Digestion mix:

1 µl of restriction enzyme (*Nco*I)
1 µl of 10x buffer
6 µl of DNA (mini-prep)
2 µl DEPC treated water

Mix the reaction mixture by pipetting and incubate in water bath at 37°C for 2 h. The digested DNA was electrophoresed using agarose gel (1%) and the band of interest appeared at the expected size (~580 bp).

Procedure of coat protein gene expression:

To express a gene in *E. coli* liquid LB medium was inoculated with one colony, and incubated overnight at 37 ºC to obtain a saturated culture.

1. Inoculate 50 ml of LB medium containing ampicillin with 1 ml of saturated cultures and incubate for 2 h at 37ºC.
2. Remove 1 ml of the uninduced culture and place immediately in a centrifuge tube, then spin at 12,000 rpm for 1 min at room temperature.
3. Remove the supernatant. Resuspend the pellet in 100 µl of 1x SDS gel loading buffer (50 mM TrisCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and heat to 100ºC for 3 min and store at -20ºC until all of the samples have been collected and are ready to be loaded onto the PAGE.
4. Induce the remaining culture [from step 2] by adding (L)-Arabinose.
   Note: to optimize the inducer concentration; 5 different concentrations were tested in a pilot experiment. (L)-Arabinose was used at final concentrations of: 0.00002%, 0.0002%, 0.002%, 0.02%, and 0.2% [see Fig. 1].
5. Remove 1-ml aliquots of the induced culture at 2, 3, and 4 h after induction, and immediately centrifuge at 12,000 rpm for 1 min at room temperature, and discard supernatants.
6. Centrifuge the induced culture and store the bacterial pellets at -20ºC.

Protocol for purification of 6xHis-Tagged Fusion Protein Using B-PER 6xHis Spin Column Kit

1. Thaw pellets on ice before starting protein extraction. Resuspend the cell pellet in 10 ml B-PER reagent by pipetting up and down until the cell suspension is homogeneous. Once a homogenous mixture is established, shake gently at RT for 10 minutes.
2. Separate soluble from insoluble proteins by centrifugation at 14,000 rpm for 15 min.
3. Transfer the supernatant (soluble fraction) from step 2 to a 15-ml capped conical centrifuge tube. Add 1 ml the Nickel-Chelated agarose to the soluble fraction. Shake for 10 min at RT and spin at 2,500 rpm.
4. Remove supernatant and resuspend the resin with 0.25 ml wash buffer. The total volume should be about 0.75 ml.
5. Transfer the resin-bound, 6xHis fusion protein to one of the B-PER spin columns provided. Centrifuge at 10,000 rpm for 2 min.
   Note: Transfer the B-PER spin column to a new collection tube after each spin.
6. Add 0.5 ml of wash buffer to the B-PER spin column and incubate for 5 min.

7. Centrifuge at 10,000 rpm for 2 min.
8. Elute the 6xHis fusion protein by adding 0.5 ml of the elution buffer and incubating for 5 minutes. Centrifuge at 10,000 rpm for 2 min.
9. Repeat step eight 3 times for a total 4 fractions [collect each fraction into a separate collection tube]
10. The eluted 6xHis fusion protein is then assayed by SDS-PAGE [see Fig. 2].

SDS-PAGE Analysis of the expressed protein

The separating gel contains:

12.5% (w/v) acrylamide-bis acrylamide (37.5:1)
0.375 M Tris-HCl (pH 9.0)
0.1% (w/v) SDS
0.05% (w/v) ammonium persulphate
0.1% (v/v) TEMED.

The stacking gel contains:

4.0% (w/v) acrylamide-bis acrylamide
0.126 M Tris-HCl (pH 6.8)
0.1 % (w/v) SDS
0.05% (w/v) ammonium persulphate
0.1% (v/v) TEMED.

For obtaining the best results, the ammonium persulphate was prepared freshly.

1. Thaw the samples and spin down at 12,000 rpm for 1 min at RT.
2. Load 15 µl of each suspension onto 12% SDS polyacrylamide gel.
3. Stain the gel with Coomassie blue R-250 and destained using destaining solution (50% methanol and 10% acetic acid).

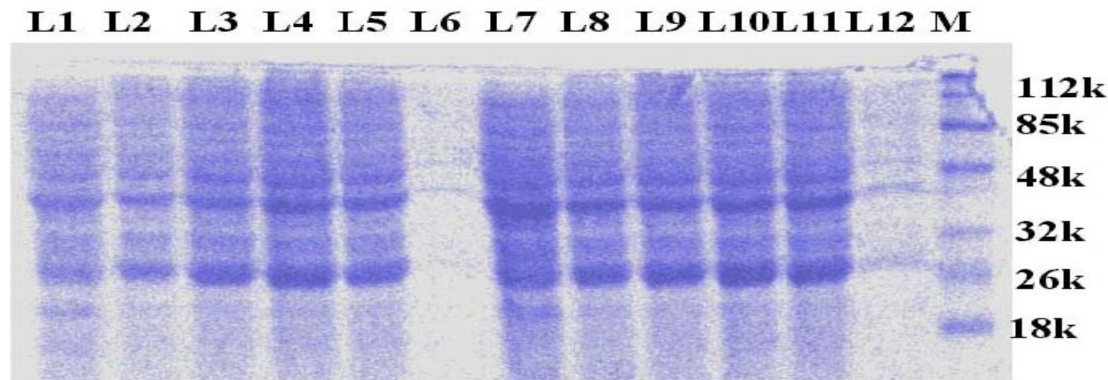


Fig 1. SDS-PAGE for induced and uninduced bacterial cells expressing PLRV CP. L1, 2, 3, 4, 5; induced cultures of the same clone at five different inducer [(L) Arabinose] concentrations; L6 is the uninduced culture of the same clone. L7, 8, 9, 10, 11 induced cultures of another clone; L12 is the uninduced culture of the same clone. The (L) Arabinose final concentrations are; 0.00002% in L5 and L11, 0.0002% in L4 and L10, 0.002% in L3 and L9, 0.02% in L2 and L8, and 0.2% in L1 and L7. M is the blue ranger prestained protein marker. Expected size was about 24KD.

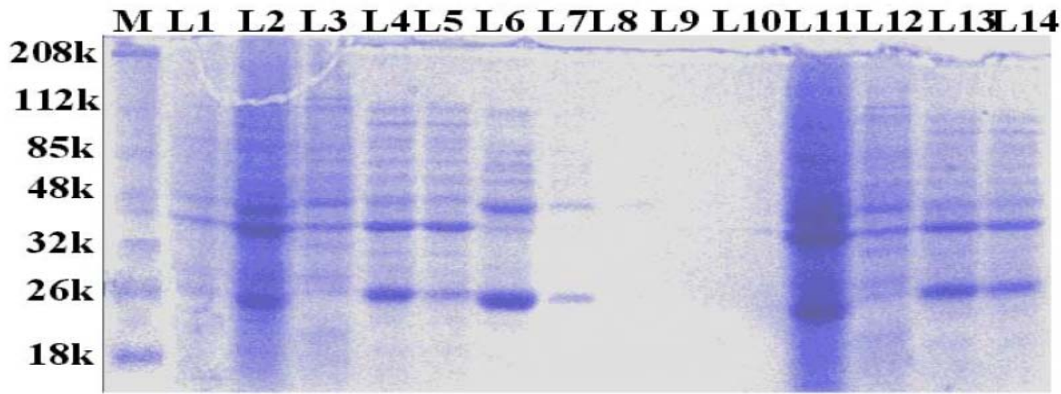


Fig 2: SDS-PAGE for induced, uninduced bacterial cells expressing PLRV CP and the purified PLRV CP. M is the blue ranger prestained protein marker, L1 uninduced bacterial culture; L2 induced bacterial culture; L3 pellet collected after chemical lysis of the induced culture (insoluble proteins); L4 Bacterial cell lysate; L5 supernatant collected after resin purification; L6 purified protein (1st elution); L7 purified protein (2nd elution); L8 purified protein (3rd elution); L9 purified protein (4th elution). Expected size of the purified protein was about 24 KD.

Conclusion

The above results demonstrated that; the best inducer concentration is 0.002% of Arabinose. The 1st elution contains the higher purified protein concentration. Elutions 3 and 4 didn't contain any proteins. Western blotting should be performed before injecting rabbits.

TECHNICAL SHEET No. 14

Virus Detection: *Potato leaf roll virus* (PLRV)

Method: RT-PCR and PCR-ELISA

General
Virus detected: PLRV from potato leaves.
General method: RT-PCR-ELISA.

Developed by
Name of researchers: Ahmed M. Soliman, Hamed Mazyad and Ahmed Shalaby, Agriculture Research Center, Giza, Egypt; M. K. Nakhla and D. P. Maxwell, University of Wisconsin-Madison
Address (E-mail): amsma33@hotmail.com and aashalaby@link.net; mknakhla@plantpath.wisc.edu
Date: November 8, 2002

Goals

Describe the feasibility of reverse transcription-polymerase chain reaction-enzyme linked immunosorbant assay (RT-PCR-ELISA) for diagnosis of potato leaf roll virus infection in infected potato plants.

Introduction

A rapid and sensitive assay for the specific detection of plant viruses, using reverse transcription-polymerase chain reaction-capture probe hybridization (RT-PCR-ELISA) (Shamloul and Hadidi, 1999; Shamloul et al., 2002), was applied successfully for the detection and characterization of an isolate of potato leaf roll virus from infected potato tissues collected in Egypt. No more than 10 hours are needed to complete the RT-PCR-ELISA for PLRV detection from infected potato tissue.

More information of PLRV can be found at the web sites:
htm.644descr/vide/cn.ac.im.www://http
htm.12390100/ICTVdB/edu.2bio.life://http

Materials and Methods

I. Extraction of PLRV Viral RNA

RNA was extracted from either frozen (–80°C) or fresh samples using RNAgents Total RNA Isolation System (Promega cat. # Z5110) as follows:

1. Grind 50 mg of tissues in liquid nitrogen then transfer to a sterile tube and homogenize with 600 µl of the denaturing solution.
2. Disrupt tissues with Kontes pestles and add 60 µl of 2 M sodium acetate (pH 4.0) then mix thoroughly by inverting the tubes 4-5 times.
3. Add 600 µl of PCI to the tube, carefully mix by inversion 3-5 times then shake vigorously for 10 sec, chill on ice for 15 min, centrifuge at 10,000 rpm for 20 min at

4°C, remove carefully the top aqueous phase, and transfer to a fresh DEPC-treated tube.

4. Add an equal volume of isopropanol (~ 600 µl) to the aqueous phase and incubate at 20°C for 30 min, then centrifuge at 10,000 rpm for 10 min at 4°C.
5. Wash the pellet by adding 1 ml of 70% ice-cold ethanol, break with a sterile RNase-free pipette tip, centrifuge at 10,000 rpm for 10 min at 4°C, and finally dry in speed vacuum for 5-20 min.
6. Resuspend the pellet in nuclease-free water (50-200 µl) and keep at –20°C for long-term storage.

II. Design of Primers: (A. Soliman and M.K. Nakhla, University of Wisconsin-Madison)
Three primers were used in order to detect PLRV as follows:
[a] Two specific primers for a part (420 bp) of the coat protein gene of PLRV were designed using lineup of published sequences for PLRV from GenBank (The accession numbers were: Y07496, X74789, X14600, D13954, D13953, D00734, D00733 and D00530), these primers were called PPLRVv1 and PPLRVc2 (Table, 1).
[b] One capture probe primer, PPLRVp4 Biotin, (which was used in PCR-ELISA) was synthesized and biotinylated at Life Technologies, Inc. The DNA sequence of capture probe was complementary to the internal nucleotide sequence of amplified DNA. The sequence of the capture probe primer was selected manually (Table 1).

Table.1. Primers for RT-PCR-amplification and capture cDNA probe.

Primers' Name	Nucleotides Sequence	Polarity	Fragment size
PPLRVv1	5'GTNCARCCNGTNGTNATGGTNAC3'	sense	420 bp
PPLRVc2	5' RTGCCAYTCNACNCCRTTDATCAT 3'	comp. sense	420 bp
PPLRVp4 Biotin	5' Bio GAYTGYCCNGCNTTYAARGAYGG 3'	sense	

P = primer; v1 = viral sense primer (anneals to complementary sense DNA); c2 = complementary sense primer (anneals to viral sense DNA); p4 Biotin = biotin labelled viral cDNA capture probe; PLRV = potato leaf roll virus. Nucleotide at degenerate positions are represented by a single letter of the IUPAC ambiguity code; D = A, G, T; H = A, C, T; K = G, T; M = A, C; N = A, C, G, T; R = A, G; W = A, T; Y = C, T.

III. RT-PCR method

RT-PCR was done with Access RT-PCR Introductory System (Promega cat. # A1260) as follows.

1. Prepare the reaction mix by combining 21 µl of Nuclease-Free Water, 10 µl of AMV/Tfl 5X reaction buffer, 1 µl of dNTP mix, 5 µl of both of the specific upstream and downstream primers (PPLRVv1 and PPLRVc2, final concentration 1 µM) (Table 1), and 2 µl of 25 mM $MgSO_4$ (final concentration is 1 mM) in 0.5 ml tube on ice. Mix the components by pipetting. Add 1 µl of the AMV reverse transcriptase and 1 µl of Tfl-DNA polymerase (final concentration of both is 0.1 unit/µl) to the reaction. Gently vortex the tube for 10 sec to mix the components. Initiate the reaction by adding 10 µl of RNA template.
2. Overlay the reaction with one or two drops of mineral oil.
3. Use the following parameters for the first strand cDNA synthesis: 48°C for 45 min at 1 cycle, 94°C for 2 min at 1 cycle.

4. Use the following cycling parameters for the second strand of cDNA synthesis and PCR amplification: 94°C for 30 sec, 60°C for 1 min, 68°C for 2 min (40 cycles), 68°C for 7 min at 1 cycle.
5. PCR amplified DNA fragments were separated by agarose gel electrophoresis in 1.5 % agarose "minigels" in 0.5X TBE buffer using 100 bp DNA ladder.

IV. RT-PCR-ELISA Method

(A) Extraction of Total RNA and cDNA Synthesis and Amplification

They were done with RNAgents Total RNA Isolation System (Promega cat. # Z5110), and Access RT-PCR Introductory System (Promega cat. # A1260) as described above.

(B) PCR Amplification-DIG Labelling

DIG-labelled DNA was prepared with Dig Labelling kit (Boehringer Mannheim Corp., Indianapolis, IN, USA, cat. # 1636120) as follows:

1. All reagents except *Taq* DNA polymerase should be thawed, mixed thoroughly (vortex) and shortly centrifuged before use.
2. To prepare a reaction mixture for amplification, add the following reagents in the same order as described in the table below. Place the tubes on ice during pipetting.
3. Mix the reagents thoroughly and centrifuge to collect at the bottom of the tube, then overlay the reaction with 50 µl of mineral oil.
4. Place the samples in a thermocycler and use the following cycling program: 94°C for 1 min, 55°C for 2 min, 72°C for 2 min (30 cycles); 94°C for 1 min, 55°C for 2 min, 72°C for 4 min (one cycle).
5. Keep the samples at –20°C for long-term storage.

Reagents	Volume	Final conc.
Sterile water	29.75 µl	-
PCR buffer without $MgCl_2$	5 µl	1X
$MgCl_2$-stock solution	3 µl	0.5-2.5 mM
PCR Dig labelling mix	5 µl	200 µM
Viral sense primer (PPLRVv1)	1.25 µl	250 nM
Complementary sense primer (PPLRVc2)	1.25 µl	250 nM
Taq DNA polymerase	0.25 µl	2.5 U
DNA	2.5 µl	1 fg to 500 ng

(C) Analysis of RT-PCR-DIG Amplified Products

Five microliter aliquots of RT-PCR-DIG labelled amplified products were analyzed on 1.5% agarose gels in TBE buffer using DNA molecular weight marker to determine the size of RT-PCR unlabelled or DIG-labelled amplified product of PLRV. Gels were stained with ethidium bromide and visualized by UV illuminator.

(E) Preparation of Biotin-Labelled cDNA Capture Probe

DNA oligonucleotide (23 nucleotides in length) (capture probe primer, PPLRVp4 Biotin, Table. 1) was synthesized and biotinylated at Life Technologies, Inc. The DNA sequence of capture probe is complementary to the internal nucleotide sequence of amplified DNA. The sequence of the capture probe primer was selected manually.

(F) Microwell Capture Hybridization Assay (DIG Detection)

The detection of DIG-labelled amplified DNA was carried out using the PCR-ELISA Detection System (Boehringer Mannheim cat. # 1636111).

1. Mix 5 µl of RT-PCR-DIG labelled amplified product with 20 µl of 0.25 M NaOH or heat at 100°C for 5 min, then chill on ice for 2 min.
2. Keep the mixture at room temp for 10 min, and then add 200 µl of hybridization solution containing 50 ng/ml of DNA capture probe.

3. Pipette 200 µl of each mixture into an ELISA microtiter plate well coated with streptavidin, and then cover the microtiter plate with self adhesive tape and keep in a water bath shaker at 55°C for 3 h, remove the hybridization solution and wash the wells six times with washing solution PBS-Tween.
4. Add 200 µl of polyclonal anti-DIG Fab fragments, conjugated to peroxidase diluted 1:100 in Tris-HCI (pH 7.5) buffer, to each well and shake the microtiter plates gently at 37°C for 30 min, then wash the wells six times with the washing solution.
5. Add 200 µl of substrate solution (100 µg/ml) to each well and incubate the microtiter plates for 30 min at 37°C in the dark with agitation. Solutions containing hybridized products were green in color.

The absorbencies of hybridized products were measured at 405 nm in an ELISA reader. Results were expressed as net absorbance, after the optical density of the blank solution was automatically subtracted for each well.

Results

I. Detection of PLRV using RT-PCR

Electrophoresis analysis of RT-PCR product showed that an amplified fragment of 420 bp was obtained from the coat protein gene of PLRV and no fragments were amplified from the RNA extracted from symptomless plants. (Fig. 1).

M L1 L2 L3 L4 L5

Fig.1. Agarose gel electrophoresis analysis of RT-PCR amplified products. M: 100 bp DNA ladder, L1 & L2: *Physalis floridana* suspected to be infected with PLRV and showing PLRV symptoms; L3 & L4: *Datura stramonium* infected with Egyptian isolate of PLRV; and L5: healthy *Physalis floridana*.

II. RT-PCR-ELISA

(A) Detection of DIG-Labelled PLRV Amplified Product

To detect DIG-labelled PLRV cDNA product dilutions of PCR products of PLRV were done (Fig. 2).

M L1 L2 L3 L4 L5 L6

Fig. 2. Agarose gel electrophoresis analysis of DIG-labelled RT-PCR products of PLRV. M: 100 bp ladder; L1: 250 ng total of Dig-labelled RT-PCR product; L2: 25 ng total of Dig-labelled RT-PCR product; L3: 250 pg total of DIG-labelled RT-PCR product; L4: 25 pg total of DIG-labelled RT-PCR product L5: 2.5 pg total of DIG-labelled RT-PCR product.

(B) Detection of DIG-Labelled RT-PCR Products using Specific Capture Probe in a Microwell Capture Hybridization Assay

DIG-labelled cDNA was analyzed by probe capture hybridization assay. The colorimetric (visual, not shown; absorbance, Table. 2) dilution end point for the detection of DIG-labelled PLRV cDNA product was 10^{-4}, when a biotinylated PLRV cDNA was used as the capture probe. Results showed that using PCR-ELISA we can detect up to 2.5 pg of cloned DNA. The method proved very sensitive compared to ELISA and the reaction could be completed in 10 hrs.

Table.2. Colorimetric detection of DIG-labelled PLRV cDNA product as shown by absorbance values of each hybridization assay.

Sample	Absorbance	Visual
Healthy potato	0.033	_
Undiluted product	1.887	++++
10^{-1} dilution	1.493	+++
10^{-2} dilution	1.319	+++
10^{-3} dilution	0.698	++
10^{-4} dilution	0.39	+
10^{-5} dilution	0.311	±
buffer control	0.035	-

Discussion

The results obtained in this study show the successful use of RT-PCR-ELISA to directly detect PLRV from infected leaves of potato plants and indicate its feasibility as a rapid assay for detecting PLRV. RT-PCR-ELISA has advantages of speed, sensitivity, suitability for a large number of samples tested, safety, and visual examination.

Applications of RT-PCR-ELISA technique should be especially useful to clean stock programs and regulatory agencies worldwide. With the ability to run large numbers of samples, from diverse tissue types and in all seasons. it should be possible to improve the reliability of

current pathogen testing protocols. Ultimately, this could lead to significant improvements in the quality of certified potato stock, streamlining of importation and quarantine programs, and facilitate international trade in plant materials.

References

Shamloul, A.M., and Hadidi, A. 1999. Sensitive detection of potato tuber and temperate fruit tree viroids by reverse transcripition-polymerase chain reaction-probe capture hybridization. J. Virol. Methods 80:145-155.

Shamloul, A.M., Faggioli, F., Keith, J.M., and Hadidi, A. 2002. A novel multiplex RT-PCR probe capture hybridization (RT-PCR-ELISA) for the simultaneous detection of six viroids in four genera: *Apscaviroid*, *Hostuviroid*, *Pelamoviroid*, and *Pospiviroid*. J. Virol. Methods 105:115-121.

TECHNICAL SHEET No. 15

Virus Detection: *Potato leaf roll virus* STRAINS

Method: RT-PCR

General
Virus detected: PLRV strains from potato samples
Method: RT-PCR

Developed By
Name of researchers**:** Fattouma DJILANI and Hatem FAKHFAKH, University of Tunisia
Adresses (e-mail): Hatem.fakhfakh@fsb.rnu.tn or hafakhfakh@voila.fr
Date: October 24, 2002

Goals
To develop a method for rapid detection of PLRV strains.

Materials and Methods

Nucleic acid extraction

Sample leaves were ground (1/5, w/v) in PBS-Tween buffer (8 g of NaCl; 0.2 g of KH_2PO_4; 2.9 g of Na_2HPO_4 ($12H_2O$) and 0.5 ml of Tween 20 per liter) containing 2% (w/v) polyvinylpyrrolidone K25 and 20 mM DIECA. After centrifugation at 13,000 rpm for 10 min, 200 µl of supernatant were transferred to a microfuge tube, mixed with SDS to 1% (w/v) final concentration and incubated at 55°C for 15 min. One hundred microliters of 3 M potassium acetate were added, the mixture vigorously vortexed and incubated on ice for 5 min. After centrifugation (5 min, 13,000 rpm, 4°C), the supernatant was adjusted to 4.2 M NaI. Five microliters of a suspension of silica particles (Sigma) were added, carefully mixed by low speed vortexing and the mixture incubated at room temperature for 5 min. After a brief centrifugation (1 min, 5,000 rpm at room temperature) the supernatant was discarded and the pellet gently resuspended in 500 µl of washing buffer (20 mM Tris-HCl pH 7.5; 1 mM EDTA; 100 mM NaCl; 50% (v/v) ethanol). The centrifugation was repeated and the pellets of silica particles similarly washed twice more before being resuspended in 400 µl sterilized water. After incubation at 55°C for 5 min and centrifugation at 13,000 rpm for 2 min, 300 µl of the supernatant were transferred to a new microfuge tube. Total nucleic acid extracts were then used directly for RT-PCR amplification or stored at –20°C until used.

RT-PCR Amplification of a part of the coat protein region

a- Reverse Transcription

- 13 µl of total RNA
- 1 µl dNTP (10 mM each)
- 1 µl DTT (0.1 M)
- 4 µl transcriptase buffer (5X)
- 0.5 µl antisense primer (25 µM)
- 0.1 µl of MMLV Reverse Transcriptase (200 U/µl)
- H_2O 20 µl

The reaction is incubated for one hour at 37°C.

b-Amplification PCR

- 2.5 µl of Reverse Transcription reaction (see above)
- 2.5 µl of 10X *Taq* DNA polymerase buffer without $MgCl_2$
- 1 µl MgCl2 (25 mM)
- 0.5 µl dNTP (10 mM each)
- 0.75 µl sens primer (10 µM)
- 0.75 µl antisens primer (10 µM)
- 1 µl de *Taq* DNA polymerase (1 U/µl)
- H_2O 25 µl

PCR Program: 35 cycles of 5 min 94°C, 1 min 94°C, 1 min 50°C, 1 min 72°C, 10 min 72°C

Sequence of primers

TECHNICAL SHEET No. 16

Virus Detection: *Potato leaf roll virus* (PLRV)

Method: Immunocapture RT-PCR

<u>General</u>
Virus detected: PLRV from potato leaf and potato tubers.
General method was immunocapture RT-PCR

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad@agri.huji.ac.il and Czosnek@agri.huji.ac.il
Date: December 26, 2005

Goals
To develop a sensitive method for PLRV detection based on immunocapture RT-PCR.

<u>Methods</u>
Immunocapture IC

1. Add 200 µl of anti-PLRV antibody (diluted 1:1000 in coating buffer) to each ELISA plate well or to PCR tube and incubate at 37°C for 3 h. We used a commercial antibody (Bioreba).
2. Empty the ELISA well/ PCR tube and wash 3 times with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20).
3. Homogenize 25 mg of plant leaf or tuber with 5 ml of suitable extraction buffer.
4. Add 200 µl of the homogenate to the coated ELISA well/ PCR tube and incubate for 18 h at 4 ^{o}C.
5. Empty the ELISA well/ PCR tube and wash 3 times with TBST.
6. Dry the ELISA well/ PCR tube, add 5 to10 µl ddH2O and heat at 70 ^{o}C for 15 min.

RT-PCR
cRNA synthesis

1. To 5 µl IC add 8 µl ddH$_2$O and 1 µl primer C582 (100 pmoles); incubate at 70 ^{o}C for 10 min, followed by 30 min in and ice bath.
2. Add 1 µl of each dNTPs (25mM each), 4 µl reverse transcriptase buffer 5X, 1 µl AMV reverse transcriptase (Promega); incubate at 42 ^{o}C for 1h.
3. Heat for 10 min at 90 ^{o}C; adjust the volume to 50 µl with ddH$_2$O

PCR
The primers used were as follows (derived from sequence GenBank accession numbers D13954 and D13953):

C582: 5'TATCGTCCATGGGTACGGTCGTGGT3'
R732: 5'TCTAGATCTTTGGGTTTTGCAAAGC3'

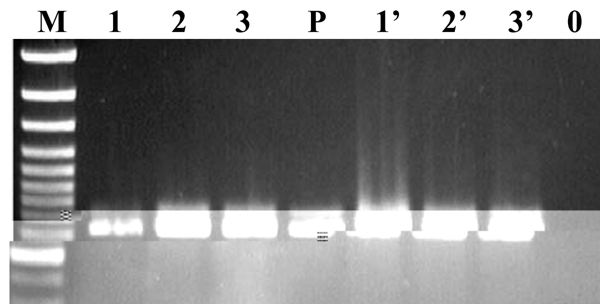
Following PCR, a 640 bp band is obtained.

1. The PCR reaction contains 5 µl from the reverse transcriptase reaction, 0.25 µl 25mM dNTPs, 1 µl each primers 1 (100 pmoles each), 2.5 µl Taq 10 x buffer and 1 unit *Taq* polymerase; add ddH$_2$O to a final volume of 25 µl.

2. Cycling: One cycle: 95 ^oC for 3 min, 50 ^oC for 2 min, 72 ^oC for 2 min. Thirty cycles: 95 ^oC for 1min, 55 ^oC for 1min, 72 ^oC for 1 min. One additional cycle: 72 ^oC for 10 min.
3. Analysis of reaction products: Subject the reaction products to 1% agarose gel electrophoresis.

Results

The following figure shows the detection of PLRV by IC-RT-PCR and by RT-PCR
For RT-PCR (lanes 1 – 3), RNA was from a *Datura stramonium* infected plant
For IC-RT-PCR (lanes 1' - 3'), samples were from a *Datura stramonium* infected plant.
M: molecular weight marker; P: cloned PLRV DNA; 0: no template.



TECHNICAL SHEET No. 17

Virus Detection: *Potato Virus X* (PVX)

Method: Immunocapture RT-PCR

General
Virus detected: PVX from potato leaves.
General method: Immunoicapture RT-PCR, PCR-ELISA.
Date: December 26, 2005

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad@agri.huji.ac.il and Czosnek@agri.huji.ac.il

Introduction

For animation of RT-PCR (need shockwave downloaded):
http://www.bio.davidson.edu/courses/Immunology/Flash/RT_PCR.html

For WEB information on PVX see:
http://www.im.ac.cn/vide/descr651.htm
http://life.bio2.edu/ICTVdB/56010018.htm

Information on RT-PCR-ELISA can be found in Shamloul and Hadidi (1999), Shamloul et al. (2002) and at the Web site:
http://biochem.boehringer-annheim.com/prod_inf/manuals/pcr_man/Chapter10/CHAP10-Seite254.htm

Materials and Methods

Immunocapture

1. Add 200 µl of anti-PVX antibody (diluted 1:1000 in coating buffer) to each ELISA plate well or to PCR tube and incubate at 37°C for 3 h. We used a commercial antibody (Bioreba) and an anti-PVX antibody, a gift from Prof. Gad Loebenstein.
2. Empty the ELISA well/ PCR tube and wash 3 times with TBST.
3. Homogenize 25 mg of plant leaf or tuber with 5 ml of suitable extraction buffer.
4. Add 200 µl of homogenate to the coated ELISA well/PCR tube and incubate for 18h at 4°C.
5. Empty the ELISA well/ PCR tube and wash 3 times with TBST.
6. Dry the ELISA well/ PCR tube, add 5 to10 µl ddH2O and heat at 70 ^{o}C for 15 min.

RT-PCR
a) cRNA synthesis

1. To 5 µl IC add 8 µl ddH$_2$O and 1 µl primer 1(100 pmoles); incubate at 70 ^{o}C for 10 min, followed by 30 min in and ice bath.
2. Add 1 µl of each dNTPs (25 mM each), 4 µl reverse transcriptase 5 x buffer, 1 µl AMV reverse transcriptase (Promega); incubate at 42 ^{o}C for 1h.

3. Heat for 10 min at $90^{\circ}C$; adjust the volume to 50 µl with ddH_2O

b) PCR. The following two primers allowed amplifying a 713-bp fragment from the plasmid pGEM/PVX which contains the cloned virus.

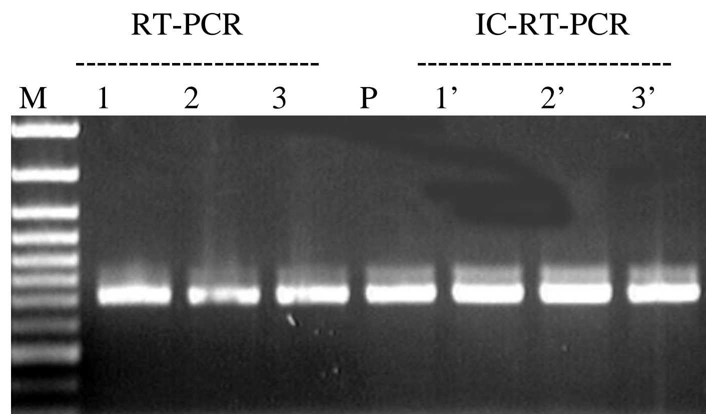
R823: 5'ATGTCAGCACCAGCTAGCAC3'
C382: 5'TTATGGTGGTGGTGAAGTGAC3'

1. The PCR reaction contains 5 µl from the reverse transcriptase reaction, 0.25 µl 25mM dNTPs, 1 µl each primers 1, 2 and 3 (100 pmoles), 2.5 µl Taq 10 x buffer and 1 unit *Taq* polymerase; add ddH_2O to a final volume of 25 µl.
2. Cycling: One cycle: $95^{\circ}C$ for 3 min, $50^{\circ}C$ for 2 min, $72^{\circ}C$ for 2 min. Thirty cycles: $95^{\circ}C$ for 1min, $55^{\circ}C$ for 1min, $72^{\circ}C$ for 1 min. One additional cycle: $72^{\circ}C$ for 10 min.
3. Analysis of reaction products: Subject the reaction products to 1% agarose gel electrophoresis.

Results

The figure shows the detection of PVX by immunocapture RT-PCR using three primers. The RNA was extracted from infected *Nicotiana glutinosa*.

M: molecular weight markers; 1-3: RT-PCR; 1'-3': IC-RT-PCR; P: cloned virus



TECHNICAL SHEET No. 18

Virus Detection: *Potato Virus X* (PVX)

Method: RT-PCR and PCR-ELISA

General

Virus detected: PVX from potato leaves.
General method: RT-PCR, PCR-ELISA.

Developed by

Name of researchers: Ahmed M. Soliman, Ahmed Shalaby, and Hamed Mazyad, Agriculture Research Center, Giza, Egypt; M. K. Nakhla and D. P. Maxwell, University of Wisconsin-Madison
Address (E-mail): amsma33@hotmail.com, aashalaby@link.net, mknakhla@plantpath.wisc.edu
Date: August 2002

Goals

Describe the feasibility of reverse transcription-polymerase chain reaction-enzyme linked immunosorbant assay (RT-PCR-ELISA) for diagnosis of *Potato virus X* infection in infected potato plants.

Introduction

Potato virus X (PVX) is widely recognized as a serious threat to potato production in Egypt and several countries. This has increased the need for accurate identification of this virus. A rapid and sensitive assay for the specific detection of plant viruses using reverse transcription-polymerase chain reaction-capture probe hybridization (RT-PCR-ELISA) was applied successfully for the detection and characterization of an isolate of PVX from infected potato tissues collected in Egypt. No more than 10 hours are needed to complete the RT-PCR-ELISA for PVX detection from infected potato tissue.

For animation of RT-PCR (need shockwave downloaded):
http://www.bio.davidson.edu/courses/Immunology/Flash/RT_PCR.html

For WEB information on PVX see:
http://www.im.ac.cn/vide/descr651.htm
http://life.bio2.edu/ICTVdB/56010018.htm

Information on RT-PCR-ELISA can be found in Shamloul and Hadidi (1999), Shamloul et al. (2002) and at the Web site:
http://biochem.boehringer-annheim.com/prod_inf/manuals/pcr_man/Chapter10/CHAP10-Seite254.htm

Materials and Methods

I. Extraction of PVX Viral RNA:

RNA was extracted from either frozen (–80°C) or fresh samples using RNAgents Total RNA Isolation System (Promega cat. # Z5110) as follows:

1. Grind 50 mg of tissues in liquid nitrogen then transfer to a sterile tube and homogenize with 600 µl of the denaturing solution.
2. Disrupt tissues with Knotes pestles and add 60 µl of 2 M sodium acetate (pH 4.0) then mix thoroughly by inverting the tubes 4-5 times.
3. Add 600 µl of PCI to the tube, carefully mix by inversion 3-5 times then shake vigorously for 10 sec, chill on ice for 15 min, centrifuge at 10,000 x rpm for 20 min at 4°C, remove carefully the top aqueous phase, and transfer to a fresh DEPC-treated tube.
4. Add an equal volume of isopropanol (~ 600 µl) to the aqueous phase and incubate at 20°C for 30 min, then centrifuge at 10,000 x rpm for 10 min at 4°C.
5. Wash the pellet by adding 1 ml of 70% ice-cold ethanol, break with a sterile RNase-free pipette tip, centrifuge at 10,000 x rpm for 10 min at 4°C, and finally dry in speed vacuum for 5-20 min.
6. Resuspended the pellet in nuclease-free water (50-200 µl) and keep at –20°C for long term storage.

II. Design of Primers:

Three primers were used in order to detect PVX as follows:

[a] Two specific primers for a part (360 bp) of the coat protein gene of PVX were designed using lineup of published sequences for PVX from GenBank (The accession numbers were: X88781, X88783, X88784, X88786, X88788 and Z23255), these primers were called PPVXv1 and PPVXc2 (Table 1).

[b] One capture probe primer, PPVXp3 Biotin, (which was used in PCR-ELISA) was synthesized and biotinylated at Life Technologies, Inc. The DNA sequence of capture probe was complementary to the internal nucleotide sequence of amplified DNA. The sequence of the capture probe primer was selected manually (Table 1).

Table.1. Primers for RT-PCR-amplification and capture cDNA probe

Primer name	Nucleotide sequence	Polarity	Expected size
PPVXv1	5\`GAYACNATGGCNCARGCNGCNTGG3\`	sense	360 bp
PPVXc2	5\`YTGNGCNGCRTTCATYTCNGCYTC 3\`	comp.	
PPVXp3 Biotin	5\`Bio GCNCCNGTNGTNTGGAAYTGG 3\`	sense	

p = primer; v1 = viral sense primer (anneals to complementary sense DNA); c2 = complementary sense primer (anneals to viral sense DNA); p3 Biotin = biotin labelled viral cDNA capture probe; PVX = potato virus X. Nucleotide at degenerate positions are represented by a single letter of the IUPAC ambiguity code; D = A, G, T; H = A, C, T; K = G, T; M = A, C; N = A, C, G, T; R = A, G; W = A, T; Y = C, T.

III. RT-PCR method:
RT-PCR was done with Access RT-PCR Introductory System (Promega cat. # A1260) as follows:

1. Prepare the reaction mix by combining 21 µl of Nuclease-Free Water, 10 µl of AMV/Tfl 5X reaction buffer, 1 µl of dNTP mix, 5 µl of both of the specific upstream and downstream primers (PPVXv1 and PPVXc2, final concentration 1 µM) (Table 1), and 2 µl of 25 mM $MgSO_4$ (final concentration is 1 mM) in 0.5 m1 tube on ice. Mix the components by pipetting. Add 1 µl of the AMV reverse transcriptase and 1 µl of Tfl-DNA polymerase (final concentration of both is 0.1 unit/µl) to the reaction. Gently vortex the tube for 10 sec to mix the components. Initiate the reaction by adding 10 µl of RNA template.
2. Overlay the reaction with one or two drops of mineral oil.
3. Use the following parameters for the first strand cDNA synthesis: 48°C for 45 min at 1 cycle, 94°C for 2 min at 1 cycle.
4. Use the following cycling parameters for the second strand cDNA synthesis and PCR amplification: 94°C for 30 sec, 60°C for 1 min, 68°C for 2 min (40 cycles), 68°C for 7 min at 1 cycle.
5. PCR amplified DNA fragments were separated by agarose gel electrophoresis in 1.5% agarose "minigels" in 0.5X TBE buffer using 100 bp DNA ladder as the DNA marker.

IV. RT-PCR-ELISA Method

(A) Extraction of Total RNA and cDNA Synthesis and Amplification

They were done with RNAgents Total RNA Isolation System (Promega cat. # Z5110), and Access RT-PCR Introductory System (Promega cat. # A1260) as described above.

(B) PCR Amplification-DIG Labeling

DIG-labelled DNA was prepared with Dig Labelling kit (Boehringer Mannheim Corp., Indianapolis, IN, USA, cat. # 1636120) as follows:

1. All reagents except *Taq* DNA polymerase should be thawed, mixed thoroughly (vortex), shortly centrifuged before use.
2. To prepare a reaction mixture for amplification, add the following reagents in the same order as described in the table below. Place the tubes on ice during pipetting.

Reagents	Volume	Final conc.
Sterile water	29.75 µl	-
PCR buffer without $MgCl_2$	5 µl	1X

$MgCl_2$

3. Mix the reagents thoroughly and centrifuge to collect at the bottom of the tube, then overlay the reaction with 50 µl of mineral oil.

(C) Analysis of RT-PCR-DIG Amplified Products

Five microliter aliquots of RT-PCR-DIG labelled amplified products were analyzed on 1.5% agarose gels in TBE buffer using DNA molecular weight marker to determine the size of RT-PCR unlabelled or DIG-labelled amplified product of PVX. Gels were stained with ethidium bromide and visualized by UV illuminator.

(D) Preparation of Biotin-Labelled cDNA Capture Probe

DNA oligonucleotide (21 nucleotides in length) (capture probe primer, pPVXp3 Biotin, Table. 1) was synthesized and biotinylated at Life Technologies, Inc. The DNA sequence of the capture probe is complementary to the internal nucleotide sequence of amplified DNA. The sequence of the capture probe primer was selected manually.

(F) Microwell Capture Hybridization Assay (DIG Detection)

The detection of DIG-labelled amplified DNA was carried out using the PCR-ELISA Detection System (Boehringer Mannheim cat. # 1636111).

1. Mix 5 µl of RT-PCR-DIG labelled amplified product with 20 µl of 0.25 M NaOH or heat at 100°C for 5 min, then chill on ice for 2 min.
2. Keep the mixture at room temp for 10 min, and then add 200 µl of hybridization solution containing 50 ng/ml of DNA capture probe.
3. Pipette 200 µl of each mixture into an ELISA microtiter plate well coated with streptavidin, and then cover the microtiter plate with self adhesive tape and keep in a water bath shaker at 55°C for 3 h, remove the hybridization solution and wash the wells six times with washing solution PBS-Tween.
4. Add 200 µl of polyclonal anti-DIG Fab fragments, conjugated to peroxidase diluted 1:100 in Tris-HCI (pH 7.5) buffer, to each well and shake the microtiter plates gently at 37°C for 30 min, then wash the wells six times with the washing solution.
5. Add 200 µl of substrate solution (100 µg/ml) to each well and incubate the microtiter plates for 30 min at 37°C in the dark with agitation. Solutions containing hybridized products were green in color.

The absorbencies of hybridized products were measured at 405 nm in an ELISA-reader. Results were expressed as net absorbance after the optical density of the blank solution was automatically subtracted for each well.

Results

I. Detection of PVX using RT-PCR

Electrophoresis analysis of RT-PCR product showed that an amplified fragment of 360 bp was obtained from the coat protein gene of PVX from infected plants and no fragments were amplified from the RNA extracted from symptomless plants (Fig. 1).

Fig. 1. Agarose gel electrophoresis analysis of RT-PCR amplified products. M: 100-bp DNA ladder, L1: healthy *Nicotiana benthamiana*, L2: healthy *Nicotiana tabacum*, L3: *N. benthamiana* inoculated with sap from potato field samples with viral symptoms from Egypt, L4: *N. tabacum* inoculated with sap from potato field samples with viral symptoms from Egypt, L5: *N. benthamiana* infected with Egyptian isolate of PVX, and L6: *N. tabacum* infected with Egyptian isolate of PVX.

II. Nucleotide Sequence Analysis

Sequencing of the RT-PCR amplified fragment in the recombinant plasmid for the PVXEG1 was completed to determine if this PCR fragment was from potexvirus group or not and to compare the sequence from this isolate with those of other potato-infecting potexvirus groups available in GenBank (Fig. 2). Percent nt identity of the Egyptian isolate was 96% with PVX from United Kingdom (accession no. X88788).

```
PPVXv1
1     GATACTATGGCCCAGGCGGCGTGGGACTTAGTCAGACACTGCGCTGATGTGGGCTCATCTGCT
1         D  T  M  A  Q  A  A  W  D  L  V  R  H  C  A  D  V  G  S  S  A

64    CAAACAGAAATGATAGACACAGGCCCCTATTCCAACGGCATCAGCAGAGCCAGACTGGCAGCA
       T  E  M  I  D  T  G  P  Y  S  N  G  I  S  R  A  R  L  A  A
22    Q PPVXp3.Biotin
127   GCAATTAAAGAGGTGTGCACACTTAGGCAATTTTGCATGAAGTATGCCCCAGTGGTATGGAAC
43        A  I  K  E  V  C  T  L  R  Q  F  C  M  K  Y  A  P  V  V  W  N

190   TGGATGCTGACTAACAACAGTCCACCTGCTAACTGGCAAGCACAAGGTTTCAAGCCTGAGCAC
64        W  M  L  T  N  N  S  P  P  A  N  W  Q  A  Q  G  F  K  P  E  H

253   AAATTCGCAGCATTTGACTTCTTCAATGGAGTCACCAACCCAGCTGCCATCATGCCCAAAGAG
85        K  F  A  A  F  D  F  F  N  G  V  T  N  P  A  A  I  M  P  K  E
PPVXc2
316   GGGCTCATCCGGCCACCGTCTGAGGCTGAAATGAACGCAGCCCAA
106       G  L  I  R  P  P  S  E  A  E  M  N  A  A  Q
```

Fig. 2. Partial nucleotide sequence of the coat protein gene of the Egyptian isolate of PVX (PVXEG1) and the predicted amino acids below. Underlined sequences indicate the locations of the primers.

II. RT-PCR-ELISA

(A) Sensitivity of DIG-Labelled PVX Amplified Product

To determine the sensitivity of DIG-labelled PVX cDNA product, dilutions of PCR products of PVX were done (Fig. 3).

Fig. 3. Agarose gel electrophoresis analysis of DIG-labelled RT-PCR products of PVX. M: 100 bp ladder; L1: 250 ng total of DIG-labelled RT-PCR product; L2: 250 ng total of unlabelled RT-PCR product; L3: 25 ng total of DIG-labelled RT-PCR product; and L4: 2.5 ng total of DIG-labelled RT-PCR product. Note that the Dig labeled probe is slightly larger than the unlabeled probe.

(B) Detection of DIG-Labelled RT-PCR Products using Specific Capture Probe in a Microwell Capture Hybridization Assay

DIG-labelled cDNA was analyzed by probe capture hybridization assay. The colorimetric (visual, not shown; absorbance, Table. 2) dilution end point for the detection of DIG-labelled PVX cDNA product was 10^{-4} when a biotinylated PVX cDNA was used as the capture probe. Results showed that using PCR-ELISA we can detect up to 25 fg of cloned DNA. The method proved very sensitive compared to ELISA and the reaction could be completed in 10 hrs.

Sample	Absorbance	Visual
Healthy potato	0.039	–
Undiluted product	1.767	++++
10^{-1} dilution	1.393	+++
10^{-2} dilution	1.279	+++
10^{-3} dilution	0.718	++
10^{-4} dilution	0.31	+
10^{-5} dilution	0.171	±
buffer control	0.037	-

Table. 2. Colorimetric detection (ELISA) of DIG-labelled PVX cDNA product as shown by absorbance values of each hybridization assay.

Discussion

The results obtained in this study show the successful use of RT-PCR-ELISA to directly detect PVX from infected leaves of potato plants and indicate its feasibility as a rapid assay for detecting PVX. RT-PCR-ELISA has advantages of speed, sensitivity, suitability for testing a large number of samples, safety, and visual examination.

Applications of RT-PCR-ELISA technique should be especially useful to test "mother plants" in certification programs and regulatory agencies worldwide. With the ability to run large numbers of samples, from diverse tissue types and in all seasons, it should be possible to improve the reliability of current pathogen testing protocols. Ultimately, this could lead to significant improvements in the quality of certified potato stock, streamlining of importation and quarantine programs, and facilitate international trade in plant materials.

References

Shamloul, A.M., and Hadidi, A. 1999. Sensitive detection of potato tuber and temperate fruit tree viroids by reverse transcripition-polymerase chain reaction-probe capture hybridization. J. Virol. Methods 80:145-155.

Shamloul, A.M., Faggioli, F., Keith, J.M., and Hadidi, A. 2002. A novel multiplex RT-PCR probe capture hybridization (RT-PCR-ELISA) for the simultaneous detection of six viroids in four genera: *Apscaviroid*, *Hostuviroid*, *Pelamoviroid*, and *Pospiviroid*. J. Virol. Methods 105:115-121.

Soliman,A.M., Shalaby, A. A., Barsoum, B. N., Mohamed, G. G., Nakhla, M. K., Mazyad, H. M. and Maxwell, D. P. 2000. Molecular characterization and RT-PCR-ELISA detection of a potato virus X (PVX) isolate from Egypt. Annals Agric. Sci., Sp. Issue 4: 1791-1804.

TECHNICAL SHEET No. 19

Virus Detection: *Potato virus Y* (PVY)

Method: DAS-ELISA

General
Virus detected: PVY from *Nicotiana glutinosa*, potato leaf and potato tubers.
General method DAS ELISA

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad@agri.huji.ac.il and Czosnek@agri.huji.ac.il
Date: October 3, 2002

Goals
To develop a sensitive method for PVY detection based on DAS-ELISA.

Introduction

PVY strains are pest quarantine in Israel, and therefore development of sensitive methods for their detection is pivotal for establishing virus free potato seeds. Israel imports all potato seeds for planting from Europe, where the PVY^N strain is present. The current method routinely used for PVY detection is ELISA.

PVY is a member of the *Potyviridae*, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm). Currently, three main strains of PVY are known. (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^O. (iii) PVY^N was recognized in the 1950's as causing severe veinal necrosis in tobacco. It was originally imported from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^C strains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. There is, currently, considerable efforts being made to better understand the relationship between the different strains of PVY, specifically their genomes and biological properties.

Materials and Methods

Two sources of anti-PVY antibody and conjugates were used: from Bioreba, and from a gift from Prof. G. Loebenstein, The Volcani Center, Israel.

Coating

1. Dilute the PVY antibody 1:1000 in coating buffer. Coating buffer is for 1 liter (pH 9.6), in ddw: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.20 g.
2. Add 100-200 µl to each well and cover plates tightly.
3. Incubate at 37°C for 4 h or at 4-6ºC for 18 h.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Antigen extraction and binding

1. Homogenize test sample 1:20 in either one of the extraction buffers: For leaves use "General extraction buffer": 20 mM Tris buffer (pH 7.4) containing 137 mM NaCl, 3 mM KCl, 2 % PVP 24 kD, 0.05% Tween 20 and 0.02% sodium azide. For tubers, use "tuber extraction buffer": for 1 liter, dissolve 10 g of egg albumin first in 20 ml using a spatula in a small cup, then make up to 1000 ml with "General extraction buffer" (Prepare freshly before use).
2. Add 200 µl per well. Cover plates tightly. Incubate in a moist chamber at 4-6 °C for 18 h.
3. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Conjugate

1. Conjugate: Dilute anti-PVY alkaline phosphatase conjugate 1:1000 in conjugate buffer or in TBS buffer. Conjugate buffer is for 1 liter, pH 7.4: Tris-(hydroxymethyl) amino-methane 2.40 g, NaCl 8.00 g, PVP (Polyvinyl-pyrrolidone) MW 24,000 20.00 g, Tween 20 0.50 g, BSA (bovine serum albumin) 2.00 g, $MgCl_2$-$6H_2O$ 0.20 g, KCl 0.20 g, NaN_3 0.20 g.
2. Add 200 µl per well and cover plates tightly.
3. Incubate at 37 °C for 3-5 h.
4. Wash: Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels.

Color reaction

1. Dissolve p-nitrophenyl phosphate to 1 mg/ml in substrate buffer. Substrate buffer is for 1 liter (pH 9.8): in ddw, diethanolamine 97.00 ml, NaN_3 0.20 g (the substrate buffer available as 5x concentrate, Art. No. 110130).
2. Add 200 µl per well and incubate at ambient temperature in the dark.
3. Observe reaction and read yellow color development after 30-120 min.
4. Visually and/or read with an ELISA reader at 405 nm.

References

Background on PVY: http://life.anu.edu.au/viruses/ICTVdB/57010001.htm

Carnegie, S.F, and van de Haar, H. 1999. http://www.unece.org/trade/agr/meetings/ge.06/1999_09.r/pvyntn.doc

Ellis, P., R. Stace-Smith, G. Bowler, and D. J. Mackenzie. 1996. Production of monoclonal antibodies for detection and identification of strains of potato virus Y. Canad. J. of Plant Pathol. 18:64-70.

Glais, L., M. Tribodet, and C. Kerlan. 2002. genomic variability in *Potato potyvirus Y* (PVY): evidence that $PVY^{N}W$ and PVY^{NTN} variants are single to multiple recombinants between PVY^{O} and PVYN isolates. Arch. Virol. 147:363-378.

Nie, X., and R.P. Singh. 2002a. A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RT-PCR. J. Virol. Methods 104:41-54.

Nie, X., and R.P. Singh. 2002b. Probable geographical grouping of PVYN and PVYNTN based on sequence variation in P1 and 5'-UTR of PVY genome and methods for differentiating North American PVYNTN. J. Virol. Methods 103:145-156.

TECHNICAL SHEET no. 20

Virus Detection: *Potato virus Y* (PVY)
(PVY detection in tubers)

Method: Dot Blot Hybridization

General
Virus detected: PVY from potato tubers
General method: RNA extraction from tubers using the PEX method, RNA dot blot hybridization

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew Univeristy and Mouhammed Zeidan, Plant Protection and Inspection Services, Bet-Dagan, Israel
Address (Email): akad@agri.huji.ac.il and Czosnek@huji.ac.il
Date: Oct. 8, 2002

Goals

To extract RNA from large numbers of tuber samples in a short time, using a simple and economic method. This RNA is used for virus detection by RNA dot blot hybridization with a radiolabeled probe.

Introduction

PVY strains are pest quarantine in Israel, and therefore development of sensitive methods for their detection is pivotal for establishing virus free potato seeds. Israel imports all potato seeds for planting from Europe, where the PVY^Nstrain is present. The method routinely used for PVY detection is ELISA.

We wanted to develop another method for confirming ELISA or to replace ELISA as the main method for virus detection from potato tubers, in a large-scale potato virus detection scheme. The method is applied for the detection of PVY^N and includes the cloning N-terminus of PVY^N, and development of RNA purification procedures suitable for dot-blot hybridization.

The advantages of this method are:

a) Simplicity and rapid extraction of nucleic acids.
b) No need of RNAase inhibitors, which may increase the costs (the PEX solution by itself is an RNAase inhibitor).
c) The extraction steps are carried out in one tube, minimizing manipulations and allowing the simultaneous handling of a large number of samples at one time.

Materials and Methods

RNA purification from potato tubers using PEX
The procedure was modified from:
Nakahara K, Hataya T, and Uyeda I. (1999) A simple, rapid method of nucleic acid extraction without tissue homogenization for detecting viroids by hybridization and RT-PCR. Journal of Virological Methods 77:47-58.

1. Cut a potato eye (0.05 to 0.1 g) and transfer it to a microfuge tube, freeze in liquid nitrogen; add 500 µl 70% ethanol and let sit for 10 min.
2. Discard the ethanol and add 500 µl of PEX solution; incubated at 65°C for 5 min. (PEX solution: 6.25 mM potassium ethyl xanthogenate, 100 mM Tris- HCl pH 7.5, 700 mM NaCl, 10 mM EDTA pH 8.0).
3. Centrifuge the samples for 6 min at 14,000 rpm and incubate for an additional 15 min at 65°C.
4. Remove the piece of tissue using a toothpick and add 1 ml ethanol and incubate for 30 min at - 80 ^{o}C.
5. Collect nucleic acids by centrifugation for 10 min at 14,000 rpm, and discard ethanol; dry gently under vacuum.
6. Dissolve pellet of nucleic acid with 30 µl ddH_2O, incubate at 65 °C for 15 min and store at -80 °C.

Dot blot hybridization

a) Preparation of membrane

1. Spot 5 µl from each RNA sample onto a nylon-membrane.
2. Cross-link the nucleic acid to the membrane by exposure to U.V. for 3 min.

b) Preparation of ^{32}P labeled probes

1. In a total reaction volume of 50 µl add: 5 µl of 0.3 µg/µl pGEM/PVY, 5 µl of 10 x hexanucleotide buffer, and 22 µl ddH_2O (plasmid pGEM/PVY: contains cloned PVY DNA obtained from PVY TNT leaf tissue supplied with the ELISA kit, cloned by immunocapture RT-PCR using the primers described in the technical sheet allowing to distinguish between the different PVY strains).
2. Heat for 10 min at 100°C, then cool on ice for 15 min.
3. Add 10 µl of dNTPs (0.5 mM each) without dCTP and 3 µl of the Klenow fragment of DNA polymerase I.
4. Add 5 µl of [^{32}P] dCTP and incubate at 37°C for 3 h.
5. Boil for 10 min and immediately put in ice bath.

c) Hybridization

1. Membranes are prehybridized at 42°C with a solution that contains: 12.5 ml 50 x SCC, 5 ml Denhardt solution x 50, 2.5 ml 10% SDS, 3.2 ml NaH_2PO_4 , 1.7 ml Na_2HPO_4 , 0.7 ml boiled salmon sperm DNA (50 µg/ml) and ddH_2O up to 50 ml (final pH should be 6.8). Denhardt solution x 50: 2% bovine serum albumin (BSA), 2%, Ficoll 400, 2% Polyvinylpyrolidone.
2. After 2 hr, add the radiolabeled probe and hybridize for 18 h at 42°C.
3. Wash the blots with 1 x SSC - 0.1% SDS for 20 min at 65 ^{o}C, twice. If necessary, wash again with 0.1 x SSC - 0.1% SDS at 65 oC. SSC x 20 is: 3 M sodium chloride, 0.3 M Tri-sodium citrate.
4. Expose the membrane to X-ray film or to a Phosphor imager.

Results

Figure 1. Four different unrelated potato batches (A, B, C and D) were analyzed. The blots were hybridized with radiolabeled cloned PVY^N.

TECHNICAL SHEET No. 21

Virus Detection: *Potato virus Y* (PVY)

Method: Immunocapture RT-PCR, RFLP Immunocapture RT-PCR

General
Virus detected: PVY from potato
General method: IC-RT-PCR, RFLP-IC-RT-PCR

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University Address (Email): akad@agri.huji.ac.il and Czosnek@agri.huji.ac.il
Date: October 8, 2002

Goals

To develop a sensitive and specific method for PVY detection based in serology and PCR, easily and reliably method to distinguish between necrotic PVY^N and common PVY^C strains.

Introduction

PVY strains are pest quarantine in Israel, and therefore development of sensitive methods for their detection is pivotal for establishing virus free potato seeds. Israel imports all potato seeds for planting from Europe, where the PVY^N strain is present. The current method routinely used for PVY detection is ELISA.

PVY is a member of the Potyviridae, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm). Currently, three main strains of PVY are known: (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^O. (iii) PVY^N was recognized in the 1950's as causing serve veinal necrosis in tobacco. It was originally imported from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^Cstrains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. Currently, there are considerable efforts being made to better understand the relationship between the different strains of PVY, specifically their genomes and biological properties.

Materials and Methods

Design of primers

The figure below shows the partial sequence of the p1 protease gene of PVY-Hungarian N-strain (M95491) and the location of the primers used to discriminate between PVY strains:

```
1     AAATTAAAAC AACTCAATAC AACATAAGAA AATCAACGCA AAAACACTCA CAAAAGCTTT
      TTTAATTTTG TTGAGTTATG TTGTATTCTT TTAGTTGCGT TTTTGTGAGT GTTTTCGAAA
                                                            primer 2
61    CAACTCTAAT TCAAACAATT TGTTAAGTTT CAATTTCGAT CTTCATCAAA CAAACTCTTT
      GTTGAGATTA AGTTTGTTAA ACAATTCAAA GTTAAAGCTA GAAGTAGTTT GTTTGAGAAA

121   CAATTTCAGT GTAAGCTATC GTAATTCAGT AAGTTATTTC AAACTCTCGT AAATTGCAGA
      GTTAAAGTCA CATTCGATAG CATTAAGTCA TTCAATAAAG TTTGAGAGCA TTTAACGTCT

181   AGATCATCCA TGGCAACTTA CACATCAACA ATCCAGATTG GTTCCATTGA ATGCAAACTT
      TCTAGTAGGT ACCGTTGAAT GTGTAGTTGT TAGGTCTAAC CAAGGTAACT TACGTTTGAA

241   CCATACTCAC CCGCTCCTTT TGGGCTAGTT GCGGGGAAAC GAGAAGTTTC AACCACCACT
      GGTATGAGTG GGCGAGGAAA ACCCGATCAA CGCCCCTTTG CTCTTCAAAG TTGGTGGTGA

301   GACCCCTTCG CAAGTTTGGA GATGCAGCTT AGTGCGCGAT TACGACGGCA AGAGTTTGCA
      CTGGGGAAGC GTTCAAACCT CTACGTCGAA TCACGCGCTA ATGCTGCCGT TCTCAAACGT
                                                            primer 3
361   ACTATTCGAA CATCCAAGAA TGGTACTTGC ATGTATCGAT ACAAGACTGA TGCCCAGATT
      TGATAAGCTT GTAGGTTCTT ACCATGAACG TACATAGCTA TGTTCTGACT ACGGGTCTAA

421   GCGCGCATTC AAAAGAAGCG CGAGGAGAGA GAAAGAGAGG AATATAATTT CCAAATGGCT
      CGCGCGTAAG TTTTCTTCGC GCTCCTCTCT CTTTCTCTCC TTATATTAAA GGTTTACCGA

481   GCGTCAAGTG TTGTGTCGAA GATCACTATT GCTGGTGGAG AGCCACCTTC AAAACTTGAA
      CGCAGTTCAC AACACAGCTT CTAGTGATAA CGACCACCTC TCGGTGGAAG TTTTGAACTT

541   TCACAAGTGC GGAAGGGTGT TATCCACACA ACTCCAAGGA TGCGCACAGC AAAAACATAT
      AGTGTTCACG CCTTCCCACA ATAGGTGTGT TGAGGTTCCT ACGCGTGTCG TTTTTGTATA

601   CGCACGCCAA AATTGACAGA GGGACAAATG AACCACCTTA TCAAGCAGGT GAAGCAAATT
      GCGTGCGGTT TTAACTGTCT CCCTGTTTAC TTGGTGGAAT AGTTCGTCCA CTTCGTTTAA

661   ATGTCAACCA AAGGAGGGTC TGTTCAACTG ATTAGCAAGA AAAGTACCCA TGTTCACTAT
      TACAGTTGGT TTCCTCCCAG ACAAGTTGAC TAATCGTTCT TTTCATGGGT ACAAGTGATA

721   AAAGAAGTTT TGGGATCACA TCGCGCAGTC GTTTGCACTG CACACATGAG AGGTTTACGA
      TTTCTTCAAA ACCCTAGTGT AGCGCGTCAG CAAACGTGAC GTGTGTACTC TCCAAATGCT

781   AAGAGAGTGG ACTTTCGGTG TGATAAATGG ACCGTTGTGC GCCTACAGCA TCTCGCCAGG
      TTCTCTCACC TGAAAGCCAC ACTATTTACC TGGCAACACG CGGATGTCGT AGAGCGGTCC

841   ACGGACAAGT GGACTAACCA AGTTCGTGCT ACTGATCTAC GCAAGGGCGA TAGTGGAGTT
      TGCCTGTTCA CCTGATTGGT TCAAGCACGA TGACTAGATG CGTTCCCGCT ATCACCTCAA
                                    primer 1
901   ATATTGAGTA ATACTAATCT CAAAGGACAC TTTGGAAGAA GCTCGGAGGG CCTATTCATA
      TATAACTCAT TATGATTAGA GTTTCCTGTG AAACCTTCTT CGAGCCTCCC GGATAAGTAT
```

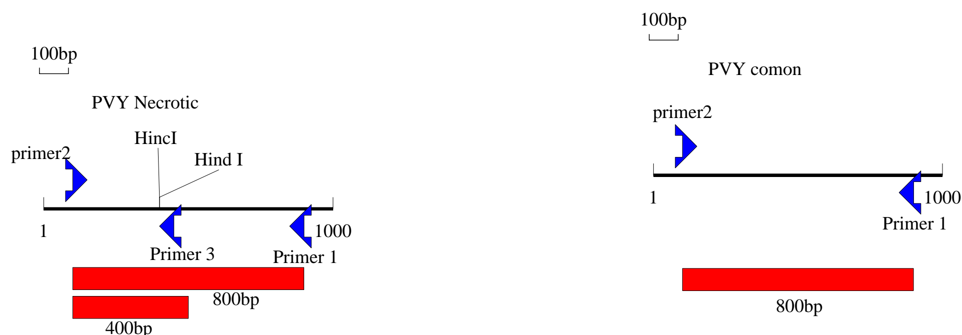
In order to detect PVY^N, we carried out a sequence comparison between PVY strains. The sequences were retrieved from GenBank and compared. Multiple alignments did not show many differences between the strains. Minor differences were found in restriction enzyme site (such as an *Hin*dII site) that allowed us to use the polymorphism PCR method (or RFLP-PCR).

RFLP-PCR is based on the different patterns obtained after the PCR products are incubated with restriction enzymes and separated by agarose gel electrophoresis. These polymorphisms were characteristics of the different PVY strains sequences.

Three primers were designed to amplify PVY strains and to allow the detection of the *Hin*dII polymorphic site of PVYN. The location of the primers on the sequence of PVY is indicated above. Primer 1, which is a complementary sense primer (identical in all strains), was used for first strand cDNA synthesis (from viral RNA purified): 5'TTCCAAAGTGTCCTTTGAG3'. Primer 2, which is a sense primer (identical in all the strains), was used to amplify the first cDNA strand: 5'CTTCATCAAACAAACTCTTT3'. Primer 3, is a second complementary sense primer, which is located between primer 1 and primer 2. The sequence of primer 3 is specific to PVY^N strains and is different from all other PVY groups (PVY^C strains, PVY^O): 5'ATCTGGGCATCAGTCTTG3'.

Discrimination between PVY strains using RT followed by PCR with three primers.

In the case of PVY^N infection, PCR amplification results in the production of two DNA fragments (one migrates at ~ 800bp and the other at ~ 400bp). In contrast, only one DNA fragment amplified (~ 800bp) with PVY^C infected material.



Immunocapture

1. Add 200 µl of anti-PVY (diluted 1:1000 in coating buffer) to each ELISA plate well or to PCR tube and incubate at 37°C for 3 h. We used both a commercial anti-PVY antibody (Bioreba) and an antibody from Prof. Gad Loebenstein, The Volcani Center.
2. Empty the ELISA cells/ PCR tube and wash 3 times with TBST.
3. Homogenize 25 mg of plant leaf or tuber with 5 ml of suitable extraction buffer.
4. Add 200 µl of the homogenate to the ELISA well/ PCR tube and incubate for 18 h at 4 °C.
5. Empty the ELISA well/ PCR tube and wash 3 times with TBST.
6. Dry the ELISA well/ PCR tube, add 5 to10 µl ddH2O and heat at 70 °C for 15 min.

RT-PCR polymorphism

The IC-PCR procedure was tested for the detection of various PVY strains. This method was based on PCR amplification of viral cRNA using three primers.

a) cRNA synthesis

1. To 5 µl IC add 8 µl ddH_2O and 1 µl primer 1 (100 pmoles); incubate at 70 °C for 10 min, followed by 30 min in an ice bath.
2. Add 1 µl of each dNTPs (25 mM each), 4 µl reverse transcriptase 5X buffer, 1 µl AMV reverse transcriptase (Promega); incubate at 42°C for 1h.
3. Heat for 10 min at 90 °C; adjust the volume to 50 µl with ddH2O.

b) PCR

1. The PCR reaction contains 5 µl from the reverse transcriptase reaction, 0.25 µl 25 mM dNTPs, 1 µl each primer 1, 2 and 3 (100 pmoles), 2.5 µl Taq 10x buffer and 1 unit *Taq* DNA polymerase; add ddH_2O to a final volume of 25 µl.
2. Cycling: One cycle: 95°C for 3 min, 50°C for 2 min, 72°C for 2 min. Thirty cycles: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min. One additional cycle: 72°C for 10 min.
3. Analysis of reaction products. Subject the reaction products to 1% agarose gel electrophoresis.

c) RFLP PCR

1. Incubate the PCR products with *Hin*dIII for 3 h.
2. Subject the reaction products to agarose gel electrophoresis.

<u>Results</u>

The figure below shows the pattern obtained with PVY^N and PVY^C

Lane 1 and 2: IC-RT-PCR using the two primers P1 and P2. The RT PCR products were incubated with *Hin*dII and subjected to agarose gel electrophoresis. In the case of PVY^N, (lane 2) only the 400-bp fragment will appear (Cleaved with *Hin*dII). In the case of PVY^C, only the 800-bp fragment will appear (lane1) (not cleaved with *Hin*dII). Lane 3 and 4: IC-RT-PCR using three primers P1, P2 and P3. The IC-RT-PCR products were subjected to agarose gel electrophoresis. In case of PVY^N (lane 4) two bands will appear: 400 and 800 bp. In the case of PVY^C (lane 3) only the 800-bp fragment will appear.

References

Background on PVY: http://life.anu.edu.au/viruses/ICTVdB/57010001.htm

Carnegie, S.F, and van de Haar, H. 1999. http://www.unece.org/trade/agr/meetings/ge.06/1999_09.r/pvyntn.doc

Ellis, P., R. Stace-Smith, G. Bowler, and D. J. Mackenzie. 1996. Production of monoclonal antibodies for detection and identification of strains of potato virus Y. Canad. J. of Plant Pathol. 18:64-70.

Glais, L., M. Tribodet, and C. Kerlan. 2002. genomic variability in *Potato potyvirus Y* (PVY): evidence that $PVY^{N}W$ and PVY^{NTN} variants are single to multiple recombinants between PVY^{O} and PVYN isolates. Arch. Virol. 147:363-378.

Nie, X., and R.P. Singh. 2002a. A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RTPCR. J. Virol. Methods 104:41-54.

Nie, X., and R.P. Singh. 2002b. Probable geographical grouping of PVYN and PVYNTN based on sequence variation in P1 and 5'-UTR of PVY genome and methods for differentiating North American PVYNTN. J. Virol. Methods 103:145-156.

TECHNICAL SHEET no. 22

Virus Detection: Variability analysis of PVY STRAINS

General
Virus detected: PVY strains from tomato, potato and pepper samples

Developed by
Name of researchers: -Hatem FAKHFAKH and Faten GORSANE, University of Tunisia

Adresses: Hatem.fakhfakh@fsb.rnu.tn or hafakhfakh@voila.fr
Faten.gorsane@fss.rnu.tn or fatgorsane@voila.fr
Date: October 25, 2002

Goals

Rapid detection of PVY strains from large number of samples, and analysis of the molecular variability of full genome.

Introduction

PVY is a member of the Potyviridae, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm). Currently, three main strains of PVY are known. (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^C. (iii) PVY^N was recognized in the 1950's as causing serve veinal necrosis in tobacco. It was originally imported from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^C strains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. There is, currently, consider efforts being made to better understand the relationship between the different strains of PVY, their genomes and biological properties.

Materials and Methods

Tissue Blotting

PVY in field samples was primarily detected by tissue blotting (Lin et al., 1990). Leaf samples were directly imprinted on a nitrocellulose membrane (Protran, Schleicher et Schuell) by pressing a freshly cut section of the leaf mid-rib. The membrane was then air dried at room temperature and, if needed, stored at room temperature until processed. After saturation of the membrane for 30 min at room temperature in blocking buffer (0.35 M NaCl, 10 mM Tris-HCl pH 7.4, 1% (w/v) gelatin), the membrane was incubated for 2 h at room temperature with alkaline

phosphatase-conjugated PVY-specific IgG's derived from a polyclonal antiserum, diluted in RIA buffer (10 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.1% (w/v) SDS and 1% (w/v) Triton X-100) (conjugate diluted 1/1000, similar to the concentration used for a DAS-ELISA assay). After three 10 min washes in RIA buffer, the membrane was incubated in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 9.5) supplemented with NBT and BCIP. Color development was stopped by washing the membrane in tap water and drying. Tissue blots were individually scored by eye or by observing the membrane under a stereo-microscope at low magnification.

Nucleic acid extraction

Method 1.

Sample leaves were ground (1/5, w/v) in PBS-Tween buffer (8 g of NaCl; 0.2 g of KH_2PO_4; 2.9 g of Na_2HPO_4 (12 H_2O) and 0.5 ml of Tween 20 per litre) containing 2% (w/v) polyvinylpyrrolidone K25 and 20 mM DIECA. After centrifugation at 13,000 rpm for 10 min, 200 µl of supernatant were transferred to a microfuge tube, mixed with SDS to 1% (w/v) final concentration and incubated at 55°C for 15 min. One hundred microliters of 3 M potassium acetate were added, the mixture vigorously vortexed and incubated on ice for 5 min. After centrifugation (5 min, 13,000 rpm, 4°C), the supernatant was adjusted to 4.2 M NaI. Five microliters of a suspension of silica particles (Sigma) were added, carefully mixed by low speed vortexing and the mixture incubated at room temperature for 5 min. After a brief centrifugation (1 min, 5,000 rpm at room temperature) the supernatant was discarded and the pellet gently resuspended in 500 µl of washing buffer (20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 100 mM NaCl; 50% (v/v) ethanol). The centrifugation was repeated and the pellet of silica particles similarly washed twice more, before being resuspended in 400 µl sterile water. After incubation at 55°C for 5 min and centrifugation at 13,000 rpm for 2 min, 300 µl of the supernatant were transferred to a new tube. Total nucleic acid extracts were then used directly for RT-PCR amplification or stored at –20°C until used.

Method 2.

Total RNA extractions from PVY-infected plants were carried out with the basic phenol/detergent mixture (Robaglia et al., 1993). Leaf tissue (200 mg) was ground in a microcentrifuge tube with 0.3 ml of TE3D buffer/water saturated phenol mixture (1:2) containing 2% of beta-mercaptoethanol. [TE3D buffer: 2M Tris (pH not adjusted), 200 mM EDTA, 15% lithium dodecyl sulfate, 10% sodium deoxycholate, 10% Nodidet P40]. 0.4 ml of chloroform-isoamyl alcohol and 0.25 ml of 3 M ammonium acetate were added and the tube was vortexed for 1 min, then centrifuged for 15 min at 12-15 krpm. Then, 0.2 ml of supernatant was collected and mixed with 0.8 ml of 3.6 M LiCl, 10 mM EDTA, RNA was allowed to precipitate on ice for 1 h. The RNA was centrifuged (30 min at 12 krpm) washed with 70% ethanol and resuspended in 0.1 mM EDTA, pH 8, or DEPC-treated water.

RT-PCR

Method 1: amplification of Coat protein region

PVY-specific primers used were the sense primer corresponding to nucleotides 8504-8522 of the PVY genome (Robaglia et al., 1989) and the antisense primers complementary to nucleotides 9400-9418 of the PVY genome. RT-PCR was performed using the one buffer, one

tube format. Briefly, 3 µl of total RNA extract were submitted to amplification in a 50 µl RT-PCR reaction mix [10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 50 mM KCl; 0.3% (v/v) Triton X100; 250 µM of each dNTPs; 1 µM of each primer pair; 2.5 µl of formamide; 0.25 units of AMV reverse transcriptase (Stratagene) and 0.5 units of Taq DNA polymerase (Appligen-Oncor). The mix was overlaid by 50 µl of mineral oil and the tubes were incubated for 45 min at 42°C for reverse transcription, followed by incubation for 5 min at 95°C for denaturation of RNA-DNA hybrids and reverse transcriptase. 35 cycles of amplification with the following thermal profile were then performed: 2 cycles (50 sec at 92°C, 50 sec at 37°C and 1min at 72°C) followed by 33 cycles (50 sec at 92°C, 50 sec at 45°C and 1 min at 72°C). Finally 10 min at 72°C were performed. PCR products were finally analyzed by electrophoresis in 0.8% agarose gels in TBE buffer and visualized under UV light following ethidium bromide staining.

Method 2: Full-length cDNA synthesis

The first-strand cDNAs were synthesized with AMV reverse transcriptase (promega) using viral RNA or total RNAs as templates. 1-5 µg viral RNA were mixed with 40-100 ng of antisense primer: 5'GCGTTAATTAATTTTTTTTTTTTTTTTTT-TTTTTTTTTTTGTCTCCTGATTGAAGTTTACAGTC3' in water and heated for 2 min at 70°C. Nucleotides (1 mM Final concentration of each), 20 units of RNAse inhibitor and 40 units of ANV-RT (Promega) were added to the 20 µl reaction containing 5X RT-Buffer. After 1 h at 42°C, the samples were diluted 20-100 times with water. The cDNAs were amplified using the following polymerase mixes: rTth and Vent DNA Polymerase (XL-PCR Kit, Perkin Elmer). Reaction conditions were as recommended by the suppliers: 0.2 mM dNTPs, 200 ng primers, 1.1 mM $Mg(OAC)_2$, 1X buffer, 4 units of enzyme mix. 1 µl of diluted cDNA template was used with antisense and sense primer: 5' CTGCCGCGGATTTAGGTGACACTATAGAAATTAAAACAACTCAA-ACAACATAA 3'. PCR was run using the following program: initial denaturation at 94°C for 1 min, 16 cycles of 15 sec at 94°C, 10 min at 65°C and 12 cycles of 15 sec at 94°C and 10 min at 65°C with and added 15 sec at each cycle, followed by final 10 min elongation at 72°C.

An aliquot of the PCR product was analyzed by 0.8% agarose gel electrophoresis.

Restriciton Fragment Length Polymorphism Analysis of cDNA

The amplified cDNA was purified from the reaction mixture either by phenol-chloroform extraction and ethanol precipitation or by gel electrophoresis excision of the appropriate band and isolation of the cDNA using the Gene Clean Kit. One µg of cDNA was digested with one restriction enzyme of choice: BstYI, HaeIII, HincII, PvuII. The digestion products were analyzed on 1% agarose gel.

Results and Discussion

The tissue-blotting assay proved simple, fast, reliable and very economical. As previously reported this simple technique offers numerous advantages when a large number of samples need to be processed rapidly. In this respect, this technique should prove, in the future, very useful for epidemiological studies of PVY. The only key parameter seems to be the quality of the antiserum, which causes interpretation problems, if it gives a significant cross-reaction to healthy plant components.

Coat Protein gene amplification by RT-PCR (fragment of 870 bp) is a reliable and very rapid protocol for detection of most of the PVY strains spreading in the different hosts: pepper, tomato and potato.

RFLP scanning of the whole genome could be seen as a fast highly informative and relatively simple supplement for the established methodologies. Such a fast method will be of interest for analyzing viral population structures and their evolution under different pressures created by local production practices.

References

Carnegie, S.F, and van de Haar, H. 1999. http://www.unece.org/trade/agr/meetings/ge.06/1999_09.r/pvyntn.doc

Ellis, P., R. Stace-Smith, G. Bowler, and D. J. Mackenzie. 1996. Production of monoclonal antibodies for detection and identification of strains of potato virus Y. Canad. J. of Plant Pathol. 18:64-70.

Glais, L., M. Tribodet, and C. Kerlan. 2002. genomic variability in Potato potyvirus Y (PVY): evidence that PVY^{N}W and PVY^{NTN} variants are single to multiple recombinants between PVY^{O} and PVYN isolates. Arch. Virol. 147:363-378.

Lin, N.S., Hsu, H.Y., Hsu, H.T. 1990. Immunological detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.

Nie, X., and R.P. Singh. 2002a. A new approach for the simultaneous differentiation of biological and geographical strains of Potato virus Y by uniplex and multiplex RT-PCR. J. Virol. Methods 104:41-54.

Nie, X., and R.P. Singh. 2002b. Probable geographical grouping of PVYN and PVYNTN based on sequence variation in P1 and 5'-UTR of PVY genome and methods for differentiating North American PVYNTN. J. Virol. Methods 103:145-156.

Robaglia, C., Bruening, G., Haseloff, J., Gerlach, W.L. 1993. Evolution and replication of tobacco ringspot virus satellite RNA mutants. EMBO J. 12:2969-2976.

Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., Casse-delbart, F. 1989. Nucleotide sequence of Potato virus Y (strain N). J. Gen. Virol 70:935-947.

TECHNICAL SHEET No. 23

Virus Detection: *Potato virus Y* (PVY) and PVY^N

Method: RT-PCR

General
Virus detected: PVY from potato tubers and leaf.
General method is reverse transcription PCR (RT-PCR).

Developed by
Name of researchers: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad@agri.huji.ac.il; czosnek@agri.huji.ac.il
Date: Oct. 8, 2002

Goals
Sensitive method for PVY detection based on RT-PCR.

Introduction

PVY strains are pest quarantine in Israel, and therefore development of sensitive methods for their detection is pivotal for establishing virus free potato seeds. Israel imports all potato seeds for planting from Europe, where the PVY^N strain is present. The current method routinely used for PVY detection is ELISA.

PVY is a member of the Potyviridae, the largest plant virus family. For general information on PVY see the Web Site (life.anu.edu.au/viruses/ICTVdB/57010001.htm).

Currently, three main strains of PVY are known: (i) PVY^O (ordinary or common strain) is widely distributed throughout the potato growning areas of the world. This strain induces mild to severe mosaic and can cause stem necrosis in some cultivars. (ii) The PVY^C is the stipple streak strain and produces systemic mosaic in some cultivars and a hypersenstive reaction in other cultivars. In tobacco, it produces the same symptoms as the PVY^O (iii) PVY^N was recognized in the 1950's as causing serve veinal necrosis in tobacco. It was originally imported from Europe and South America. A new sub-group of PVY^N that causes superficial necrotic ring spots on tubers was first described in 1980. It is called potato tuber necrotic ringspot strain. The tuber symptoms develop during storage (Carnegie and van de Haar, 1999).

A monoclonal antibody assay was reported by Ellis (1996) for distinguishing the PVY^O and PVY^Cstrains from PVY^N. An ELISA assay is commonly used in the USA for detection of PVY^N. Recently, RT-PCR assays have been developed for detection of PVY^N (Nie and Singh, 2002a and 200b). The existence of recombinants between strains has been suggested (Glais et al., 2002), which makes for difficulties in detection and characterization. Currently, there are considerable efforts being made to better understand the relationship between the different strains of PVY, specifically their genomes and biological properties.

Materials and Methods

Design of primers

The figure below shows the partial sequence of p1 protease gene of PVY-Hungarian N-strain (M95491) and the location of the primers used to discriminate between PVY strains:

```
1      AAATTAAAAC AACTCAATAC AACATAAGAA AATCAACGCA AAAACACTCA CAAAAGCTTT
       TTTAATTTTG TTGAGTTATG TTGTATTCTT TTAGTTGCGT TTTTGTGAGT GTTTTCGAAA
                                                      primer 2
61     CAACTCTAAT TCAAACAATT TGTTAAGTTT CAATTTCGAT CTTCATCAAA CAAACTCTTT
       GTTGAGATTA AGTTTGTTAA ACAATTCAAA GTTAAAGCTA GAAGTAGTTT GTTTGAGAAA

121    CAATTTCAGT GTAAGCTATC GTAATTCAGT AAGTTATTTC AAACTCTCGT AAATTGCAGA
       GTTAAAGTCA CATTCGATAG CATTAAGTCA TTCAATAAAG TTTGAGAGCA TTTAACGTCT

181    AGATCATCCA TGGCAACTTA CACATCAACA ATCCAGATTG GTTCCATTGA ATGCAAACTT
       TCTAGTAGGT ACCGTTGAAT GTGTAGTTGT TAGGTCTAAC CAAGGTAACT TACGTTTGAA

241    CCATACTCAC CCGCTCCTTT TGGGCTAGTT GCGGGGAAAC GAGAAGTTTC AACCACCACT
       GGTATGAGTG GGCGAGGAAA ACCCGATCAA CGCCCCTTTG CTCTTCAAAG TTGGTGGTGA

301    GACCCCTTCG CAAGTTTGGA GATGCAGCTT AGTGCGCGAT TACGACGGCA AGAGTTTGCA
       CTGGGGAAGC GTTCAAACCT CTACGTCGAA TCACGCGCTA ATGCTGCCGT TCTCAAACGT

                                                    primer 3
361    ACTATTCGAA CATCCAAGAA TGGTACTTGC ATGTATCGAT ACAAGACTGA TGCCCAGATT
       TGATAAGCTT GTAGGTTCTT ACCATGAACG TACATAGCTA TGTTCTGACT ACGGGTCTAA

421    GCGCGCATTC AAAAGAAGCG CGAGGAGAGA GAAAGAGAGG AATATAATTT CCAAATGGCT
       CGCGCGTAAG TTTTCTTCGC GCTCCTCTCT CTTTCTCTCC TTATATTAAA GGTTTACCGA

481    GCGTCAAGTG TTGTGTCGAA GATCACTATT GCTGGTGGAG AGCCACCTTC AAAACTTGAA
       CGCAGTTCAC AACACAGCTT CTAGTGATAA CGACCACCTC TCGGTGGAAG TTTTGAACTT

541    TCACAAGTGC GGAAGGGTGT TATCCACACA ACTCCAAGGA TGCGCACAGC AAAAACATAT
       AGTGTTCACG CCTTCCCACA ATAGGTGTGT TGAGGTTCCT ACGCGTGTCG TTTTTGTATA

601    CGCACGCCAA AATTGACAGA GGGACAAATG AACCACCTTA TCAAGCAGGT GAAGCAAATT
       GCGTGCGGTT TTAACTGTCT CCCTGTTTAC TTGGTGGAAT AGTTCGTCCA CTTCGTTTAA

661    ATGTCAACCA AAGGAGGGTC TGTTCAACTG ATTAGCAAGA AAAGTACCCA TGTTCACTAT
       TACAGTTGGT TTCCTCCCAG ACAAGTTGAC TAATCGTTCT TTTCATGGGT ACAAGTGATA

721    AAAGAAGTTT TGGGATCACA TCGCGCAGTC GTTTGCACTG CACACATGAG AGGTTTACGA
       TTTCTTCAAA ACCCTAGTGT AGCGCGTCAG CAAACGTGAC GTGTGTACTC TCCAAATGCT

781    AAGAGAGTGG ACTTTCGGTG TGATAAATGG ACCGTTGTGC GCCTACAGCA TCTCGCCAGG
       TTCTCTCACC TGAAAGCCAC ACTATTTACC TGGCAACACG CGGATGTCGT AGAGCGGTCC

841    ACGGACAAGT GGACTAACCA AGTTCGTGCT ACTGATCTAC GCAAGGGCGA TAGTGGAGTT
       TGCCTGTTCA CCTGATTGGT TCAAGCACGA TGACTAGATG CGTTCCCGCT ATCACCTCAA
                                  primer 1
901    ATATTGAGTA ATACTAATCT CAAAGGACAC TTTGGAAGAA GCTCGGAGGG CCTATTCATA
       TATAACTCAT TATGATTAGA GTTTCCTGTG AAACCTTCTT CGAGCCTCCC GGATAAGTAT
```

In order to detect PVY^N, we carried out a sequence comparison between PVY strains. The sequences were retrieved from GenBank and compared. Multiple alignments did not show many differences between the strains. Minor differences were found in restriction enzyme site (such as an *Hin*dII site) that allowed us to use the polymorphism PCR method (or RFLP-PCR).

RFLP-PCR is based on the different DNA fragment patterns obtained, after the PCR products are incubated with restriction enzymes and separated by agarose gel electrophoresis. These polymorphisms were characteristic of the different PVY strains sequences.

Three primers were designed to amplify PVY strains and to allow the detection of the *Hin*dII polymorphic site of PVY^N. The location of the primers on the sequence of PVY is indicated above.

Primer 1, which is a complementary sense primer (identical in all strains) was used for first strand cDNA synthesis (from viral RNA purified. 5'TTCCAAAGTGTCCTTTGAG3'

Primer 2, which is a sense primer (identical in all the strains), was used to amplify the first cDNA strand. 5'CTTCATCAAA CAAACTCTTT3'

Primer 3, which is a second complementary sense primer, is located between primer 1 and primer 2. The sequence of primer 3 is specific to PVY^N strains and is different from all other PVY groups (PVY^C strains, PVY^O). 5'ATCTGGGCATCAGTCTTG3'

RNA purification from potato tubers and from leaves using the Tri-REAGENT method

1. Homogenize 0.2 g of plant tissues with 750 µl Tri-reagent (Molecular Research Center, Inc.); incubate for 5 min at room temperature.
2. Add 200µl chloroform, shake the sample vigorously for 15 sec and incubate the mixture for 15 min at ambient temperature.
3. Centrifuge at 14,000 rpm for 15 min and collect the aqueous phase in a new tube.
4. Add 0.5 volume of isopropanol and incubate at –20 °C for 30 min.
5. Centrifuge at 14,000 rpm for 10 min, discard the supernatant.
6. Wash the pellet with 70% ethanol and dry under vacuum.
7. Dissolve the pellet with 20 µl ddH_2O, incubate at 70°C for 15 min, store at –80 °C.

cRNA synthesis

1. To 5 µl RNA extract add 8 µl ddH_2O and 1 µl primer 1 (100 pmoles); incubate at 70°C for 10 min, then incubate in ice bath for 30 min.
2. Add 1 µl of each dNTPs (25 mM each), 4 µl reverse transcriptase 5X buffer, 1 µl AMV reverse transcriptase (Promega); incubate at 42°C for 1h.
3. Heat for 10 min at 90°C; adjust the volume to 50 µl with ddH_2O

PCR

1. The PCR reaction contains 5 µl from the reverse transcriptase reaction, 0.25 µl 25 mM dNTPs, 1 µl each primers 1, 2 and 3 (100 pmololes each), 2.5 µl *Taq* polymerase 10 x buffer and 1 unit *Taq* DNA polymerase; add ddH_2O to a final volume of 25 µl.
2. Cycle: 1 cycle of 95°C for 3 min 50°C for 2 min, 72°C for 2 min; then 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; additional cycle of 72°C for 10 min.
3. Subject the reaction products to 1% agarose gel electrophoresis.

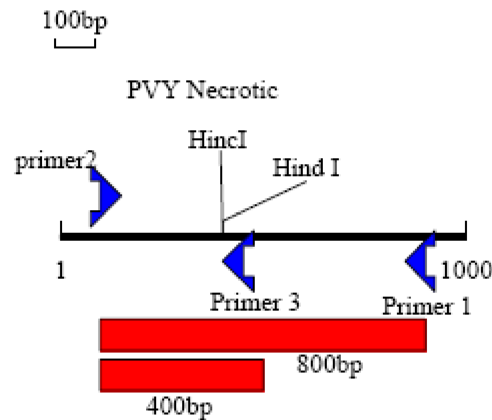
One step RT-PCR

We are now carrying out the RT-PCR reaction in one step using the Access RT-PCR system (Promega). The advantage of this method is that the reverse transcription and the PCR steps are all done in one tube, cutting down manipulations, time and price.

1. To 5 µg total RNA disolved in 10µl ddH_2O and 1µl from each one of the three primers (final concentration 10 µM).
2. Transfer the solution to PCR tubes, heat at 70°C for 15 min; cool on ice for an additional 10 min.
3. Add the reaction mix, which contain 5µl AMV/T*FI* reaction buffer 5X, 2 µl 25 mM $MgSO_4$, 2 µl dNTP mix (10mM each dNTP), 0.5 µl T*FI*-DNA polymerase (5 U/µl), 0.1µl AMV reverse transcriptase (5U/µl); adjust the volume to 25 µl with ddH_2O.
4. PCR conditions: first strand synthesis: 1 cycle at 48°C for 1 hr followed by one cycle 94°C for 3 min. Second strand cDNA synthesis and PCR amplification: 35 cycles at 94°C for 45 sec, 58°C for 1 min and 68°C for 2 min.

Discrimination between PVY strains using RT followed by PCR with three primers

In the case of PVY^N infection, PCR amplification results in the production of two DNA fragments (one migrates at ~ 800 bp and the other at ~ 400 bp). In contrast, only one DNA fragment amplified (~ 800 bp) with PVY^C infected material.



Results

RT-PCR discrimination between PVY strains

In the case of PVY^N infection, PCR amplification results in the production of two DNA fragments (one migrates at ~ 800 bp and the other at ~ 400 bp). In contrast, only one DNA fragment amplified (~ 800 bp) with PVY^C infected material.

M PVY^N PVY^C ?

M: molecular weight marker.

PVY and $PVY^{C:}$ leaves infected with the relevant viruses.

?: unknown sample.

Discrimination between PVY strains by RFLP-RT-PCR

The RT PCR product were cut with *Hin*dII and subjected to PCR using the three primers. In the case of PVY^N, only the P2-P3 400 bp will appear. In the case of PVY^C, only the P2-P1 800 bp will appear. The figure below shows the pattern obtained with PVY^N and PVY^C.

M PVY^C PVY^N

Discussion

The strain PVY^N does not exist in Israel, therefore we cloned this fragment from infected plant samples supplied as positive control with the ELISA detection kit (Bioreba). This allowed us to have a positive control for our tests and for preparing PVY-specific probes. Following RT-PCR with the primers 1 and 2, the 800-bp amplified fragment was cut out from the gel, cleaned and cloned into the $pGEM^R$ –T Easy plasmid, using the $pGEM^R$ –T Easy system protocol (Promega). A unique restriction site (*Hin*dII site) is present only in the PYV^N PCR fragment and allows discrimination between virus strains.

References

Background on PVY: http://life.anu.edu.au/viruses/ICTVdB/57010001.htm

Carnegie, S.F, and van de Haar, H. 1999. http://www.unece.org/trade/agr/meetings/ge.06/1999_09.r/pvyntn.doc

Ellis, P., R. Stace-Smith, G. Bowler, and D. J. Mackenzie. 1996. Production of monoclonal antibodies for detection and identification of strains of potato virus Y. Canad. J. of Plant Pathol. 18:64-70.

Glais, L., M. Tribodet, and C. Kerlan. 2002. genomic variability in *Potato potyvirus Y* (PVY): evidence that PVY^NW and PVY^{NTN} variants are single to multiple recombinants between PVY^O and PVYN isolates. Arch. Virol. 147:363-378.

Nie, X., and R.P. Singh. 2002a. A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RT-PCR. J. Virol. Methods 104:41-54.

Nie, X., and R.P. Singh. 2002b. Probable geographical grouping of PVYN and PVYNTN based on sequence variation in P1 and 5'-UTR of PVY genome and methods for differentiating North American PVYNTN. J. Virol. Methods 103:145-156.

TECHNICAL SHEET No. 24

Virus Detection: PVY, PVX and PLRV

Method: MULTIPLEX – RT-PCR

General

Virus detected: PVY, PVX and PLRV in one reaction
General method: multiplex-RT-PCR

Developed By

Name of researcher: A. Shalaby and Hamed Mazyad, Agricultural Research Center, Giza, Egypt
Address (E-mail): aashalaby@intouch.com
Date: April 11, 2002

Goals

The m-RT-PCR assays developed is a reliable, rapid, and sensitive method for the detection of these three viruses in one reaction. The use of the m-RT-PCR assays are recommended for applications where improved sensitivity over standard RT-PCR is necessary for the early detection of infection and for quarantine and breeding programs.

Introduction

Potato *(Solanum tuberosum* L.) often becomes infected with two to three different viruses. The most common viruses affecting potato crops throughout the world are *Potato virus Y* (PVY, potyviruses), *Potato virus X* (PVX, potexvirus) and *Potato leaf roll virus* (PLRV, luteovirus). Egypt imports all potato seeds for planting from Europe, where theses viruses are present.

The current method routinely used for detection of these viruses is ELISA and there are no procedures to detect these three viruses in one step reaction. Multiplex-RT-PCR for PVY strains has been reported by Nei and Singh (2002).

Materials and Methods

Viral RNAs were extracted from potato samples infected with potato viruses (PLRV, PVX and PVY) using RNeasy Plant Mini kit (QIAGEN® cat # 74903) as follows:

1. Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer to an appropriately sized tube.
2. Add 450 µl of either Buffer RLC to a maximum of 100 mg of tissue powder. Vortex vigorously. A 1-3 min incubation at 56 ºC may help to disrupt tissue.
3. Apply lysate to the QIAshredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer the flow-through fraction from QIAshredder to a new tube without disturbing the cell-debris pellet in the collection tube.
4. Add 0.5 volumes (usually 225 µ1) ethanol (96-100%) to the cleared lysate and mix well by pipeting.
5. Apply sample (usually 675 µ1), including any precipitate, which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at 10,000 rpm.

6. Pipet 700 µ1 Buffer RWI onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow-through and collection tube.
7. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500 µ1 Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm. Discard flow-through and reuse the collection tube in step 8.
8. Add 500 µl Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane.
9. Transfer RNeasy column into a new 1.5-ml collection tube, and pipet 30-50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 10,000 rpm to elute the RNA. Repeat if the expected RNA yield is >20 µg.

Design of Primers

In order to detect PVY, PVX and PLRV in one-step reaction, we along with M. K. Nakhla (University of Wisconsin-Madison), designed three specific primers for each virus to amplify the full-length of the coat protein gene. The viral sequences were retrieved from GenBank and compared.

Table (1) The oligonucleotide sense and complementary sense primer pairs used for m-RT-PCR and RT-PCR

a- The oligonucleotide sense and complementary sense primer pairs used for m-RT-PCR

Primer name	Nucleotide sequence (5` to 3`)	Polarity	Expected amplicon size (bp)
Potato virus Y PVYCPv*BamH1* PVYCPc*EcoR1*	5' TCAAGGATCCGCAAATGACACAATTGATGCAGG 3' 5' AGAGAGAATTCATCACATGTTCTTGACTCC 3'	sense comp.	801 bp
Potato virus X PVXCPv*EcoRI* PVXCPc*NcoI*	5`-GATA<u>GAATTC</u>AGATGACTACACCAGCCAACACC-`3 5`-TACGCGTCGGTT<u>CCATGG</u>ACGTAGTTATGG TGG-`3	sense comp.	700 bp
Potato leaf roll virus PLRVCPvEcoR1 PLRVCPcNco1	5'AATA<u>GAATTC</u>TAATGAGTACGGTCGTGGTTARAGG 3 5'AAAA<u>CCATGG</u>CTATYTGGGGTTYTGCARAGCYAC-3	sense comp.	548 bp

Multiplex reverse transcription polymerase chain reaction (m RT-PCR)

Three primer mixes (Table 1) were made up with each primer at a final concentration of 0.45 µM. The primer mixes could be stored frozen at -20°C, or freeze-dried for resuspension to the same concentration at a later date.

The one step RT-PCR kit and protocol (QIAGEN) was used according to Manufacturer's instructions as follows:

Fifty nanograms of viral RNA was mixed with 10 µl of 5X buffer, 2 µl of dNTPs mix, 10 µl of 5X Q-solution, 3 µl of 10 µM of each viral sense primer, 3 µl of 10 µM of each complementary sense primer, 2 µl of QIAGEN enzyme mix, 0.2 µl of RNase inhibitor and water up to 50 µl. The one step RT-PCR mixture varied depending on the number of intended viruses to be detected and the number of primers involved. The PCR parameter starting with 30 min at 50°C and 15 min at 95°C (for RT Reaction) followed by 30 cycles, each cycle consisted of denaturation at 94°C (1 min), primer annealing at 55°C (1 min) and extension at 72°C (1 min),

with final step for 10 min at 72°C. The reaction products were subjected to agarose gel electrophoresis.

Results

Fig. (1.): Detection of PVY, PVX and PLRV by RT-PCR and multiplex-RT-PCR from cDNAs primed by specific sense and complementary primers for each virus as shown in Table 1. Lane M, DNA leader molecular size markers; the sizes in bp are indicated on the left hand margin. Lane 1, amplified PVY by RT-PCR, lane 2, multiplex RT-PCR in which two pairs of viral specific primers for PVY and PVX were present in the reaction and two viral RNAs, and lane 3, multiplex RT-PCR in which three pairs of viral specific primers for PVY, PVX and PLRV were present in the reaction and three viral RNAs. The amplified products were: PVY (800 bp), PVX (700 bp) and PLRV (550 bp).

Discussion

Multiplex PCR is increasingly used because it improves the efficiency of diagnostic PCR (Johnson, 2000). In the near future multiplex PCR will probably be adapted for the simultaneous detection of viruses of one particular crop and for the simultaneous detection of other major plant pathogens such as viruses, viroids, bacteria, and fungi in the same reaction, as already demonstrated for viruses and viroids (Nie and Singh, 2001). An increase in sensitivity would probably be achieved if multiplex PCR in a single closed tube were developed based on this technology.

These techniques demonstrate the feasibility of multiplex RT-PCR based on specific primer design for the identification of several potato viruses i.e., PVY, PVX and PLRV, in a single step reaction. Such a method should increase both the sensitivity and specificity of the diagnosis and thus reduce the possibility of false negatives.

References

Nie, X., and Singh, R.P. 2001. A novel usage of random primers for multiplex RT-PCR detection of virus and viroid in aphids, leaves, and tubers. J. Virol. Methods 91:37-49.

Nie, X., and Singh, R.P. 2002. A new approach for the simultaneous differentiation of biological and geographical strains of *Potato virus Y* by uniplex and multiplex RT-PCR. J. Virol. Methods 104:41-54.

Stone Fruits

TECHNICAL SHEET No. 25

Virus Detection:*Plum pox virus* (PPV)

Method: RT-PCR

General

Virus under study: PPV from peach leaves.
General method for detection: RT-PCR
Gene expression strategy: Coat Protein (PPV- CP) gene expression strategy.

Developed by

Name of researcher: Sahar A. Youssef, Hamed Mazyad, and Ahmed Shalaby, Agriculture Research Center, Giza, Egypt
E-mail address: saharyoussef@link.net and aashalaby@link.net
Date: Nov. 6, 2002

Goals

Detection of *Plum Pox Potyvirus* infected leaves and the production of polyclonal antibody specific against PPV- El Amar strain based on coat protein gene expression.

Information

Plum pox disease, Sharka, is caused by *Plum pox potyvirus* (PPV) (Dunez and Sutic, 1988; Hadidi and Candresse, 2001). Sharka is the most important of the viral diseases that affect stone fruit in Europe and the Mediterranean region because of a resultant 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 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 PPV must be established to safely introduce stone fruit cultivars and/or germplasm that are free of PPV to prevent serious losses to the local stone fruit industry and significantly reduce PPV infection to stone fruits in countries where either virus is present.

Materials and Methods

Isolation of the PPV nucleic acid:

RNeasy Plant Mini Protocol (Qiagen) for Isolation of Total RNA from Plant Tissues:

1. Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer to an appropriately sized tube.
2. Add 450 µl of either Buffer RLC to a maximum of 100 mg of tissue powder. Vortex vigorously. A short (1-3 min) incubation at 56ºC may help to disrupt tissue.
3. Apply lysate to the QIA shredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer flow-through fraction from

QIA shredder to a new tube without disturbing the cell-debris pellet in the collection tube.

4. Add 0.5 volumes (usually 225 µ1) ethanol (96-100%) to the cleared lysate and mix well by pipetting.
5. Apply sample (usually 675 µ1), including any precipitate, which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at 10,000 rpm.
6. Pipet 700 µ1 Buffer RWI onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow-through and collection tube.
7. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500 µ1 Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm. Discard flow-through and reuse the collection tube in step 8.
8. Add 500 µl Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane.
9. Transfer RNeasy column into a new 1.5-ml collection tube, and pipet 30-50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 10,000 rpm to elute the RNA. Repeat if the expected RNA yield is >20µg.

RT-PCR

The RT–PCR was done by using Access RT-PCR system (Promega) with a modified PCR file according to the primer specification. The PPV coat protein primer is as follows:

PPV-CPcNcoI
ATA TCC ATG GCA TCA CAC TCC CCT CAT ACC GAG GAG G

PPV-CPvBamHI
AAY RTW GTT RTV GGA TCC GCT GAY GAR ARR GAR GAC

Electrophoretic analysis of PCR products

Following amplification, RT-PCR products were electrophoresed in agarose gels at 120V for 45 min in 0.5X TBE and visualized by staining with ethidium bromide. Product sizes were determined using the 1 kb DNA Ladder molecular weight marker (GiBCO BRL, Inc.).

Addition of 3′ A-Overhangs Post – Amplification

1. After the amplification using the RT-PCR Access System that has *Pfu* polymerase, add 1 unit of *Taq* polymerase to each tube and mix well.
2. Incubate at 72°C for 10 min.
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pBAD- TOPO ® Expression vector.

TOPO Cloning Reaction

Reagent	Chemically Component *E. coli*
Fresh PCR product	3 µl
Salt Solution	1 µl
Sterile Water	1 µl
TOPO vector	1 µl

1. Mix the reaction gently and incubate at RT for 5 min.
2. Place the reaction on ice.

TOPO Transformation

1. Add 2 µl of the TOPO Cloning reaction into a vial of XL1 Blue Competent *E. coli* and mix gently.
2. Incubate on ice for 5- 30 min.
3. Heat shock the cells for 30 sec at 42°C.
4. Immediately transfer the tube to ice.
5. Add 1 ml of room temperature LB medium.
6. Shake the tube at 37 °C for 1 h.
7. Spread 50-200 µl from each transformation on a selective plate and incubate overnight at 37°C.

Mini Prep Protocol for isolation of the recombinant DNA

Mini prep was done as described in the laboratory protocol of the Geminivirus team in UW- Madison, which is a standard mini-preparation procedure for recombinant plasmids.

Evaluation of the recombinant DNA

Two methods were used for the evaluation

1. Digest using N*co*I.
2. PCR Colony with specific PCR primers for coat protein gene.

Transformation into BL21 Competent *E. coli*

The transformation was done as described above for the TOPO transformation into XL1 blue cells.

Induction Protocol - Pilot Expression Protocol

1. For each transformation inoculate 2 ml of LB containing 50 µg/ml ampicillin with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking (250 rpm) to OD 600=1-2.
3. The next day, label five tubes 1 through 5 and add 10 ml LB containing 50 µg/ml ampicillin.
4. Inoculate each tube with 0.1 ml of the overnight culture.
5. Grow the cultures at 37°C with vigorous shaking (250 rpm) to an OD 600= ~0.5.
6. While the cells are growing, prepare four 10-fold serial dilutions of 20% L- arabinose with sterile water using aseptic technique (e.g. 2%, 0.2%, 0.02% and 0.002%).
7. Remove a 1 ml aliquot of cells from each tube, centrifuge at maximum speed in a microcentrifuge for 30 sec, and aspirate the supernatant.
8. Freeze the cell pellet at – 20°C. This is the zero time point sample.

9. Use the stock solution prepared in step 6 and add L-arabinose to the five 9 ml cultures as described in the table below.

Tube	Stock Solution	Volume (ml)	Final Concentration
1	0.002%	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2%	0.09	0.02%
5	20%	0.09	0.2%

10. Grow at 37°C with shaking for 4 h.
11. Take a 1 ml sample at 4 h and treat as in step 7 and 8.

Preparing Samples for SDS-PAGE gel

1. When all the samples have been collected from Step 8 and 11, above, resuspend each pellet in 100 µl of 1X SDS-PAGE sample buffer.
2. Heat 5 min at 70°C and centrifuge briefly.
3. Load 15 µl of each sample onto an SDS-PAGE gel and separate by electrophoresis. Store the rest of the samples at -20°C.

Scale-up of Expression for Purification of coat protein

The conditions determined in the pilot expression protocol were used to grow and induce large amounts of recombinant protein.

1. Inoculate 2 ml of LB containing 50 µg/ ml ampicillin with single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking.
3. The next day, inoculate 50 ml of LB containing 50 µg/ml ampicillin with 1 ml of the overnight culture.
4. Grow the culture at 37°C with vigorous shaking to an OD 600 = ~0.5.
5. Add the optimal amount of L-arabinose to induce expression.
6. Grow at 37°C with shaking until the optimal time point is reached. Harvest the cells.
7. Store at –80°C for future use.

Purification of 6xHis-Tagged Fusion Protein Using B-PER 6xHis Spin Column Kit

1. Prepare the bacterial lysates using either frozen or fresh bacteria from a 250 ml culture (OD 600 = 1.5-3.0). If frozen bacteria are used, thaw pellets on ice before starting protein extraction. Resuspend the cell pellet in 10 ml B-PER™ Reagent either by vortexing or pipetting up and down until the cell suspension is homogeneous. Once a homogenous mixture is established, shake gently at room temp. for 10 min. Separate soluble from insoluble proteins by centrifugation at 14,000 rpm for 15 min.
2. Transfer the supernatant (soluble fraction) from step 1 to a 15 ml capped conical centrifuge tube. Add 1ml the Nickel-Chelated agarose to the soluble fraction. Shake for 10 min at RT and spin at 2,500 rpm.

3. Remove supernatant and resuspend the resin with 0.25 ml wash buffer. The total volume should be about 0.75 ml.
4. Transfer the resin-bound, 6xHis fusion protein to one of the B-PER™ Spin Columns provided. Centrifuge for 2 min at 10,000 rpm.
5. Add 0.5 ml of wash buffer to the B-PER™ Spin Column and incubate for 5 min. Centrifuge at 10,000 rpm for 2 min.
6. Elute the 6xHis fusion protein by adding 0.5 ml of the Elution Buffer and incubating for 5 min. Centrifuge at 10,000 rpm for 2 min.
7. Repeat 3 times for a total 4 fractions (collect each fraction into a separate collection tube)
8. The eluted 6xHis fusion protein assayed by SDS-PAGE.

Results

Fig 1. Agarose gel of RT-Polymerase Chain Reaction (PCR) amplified 900-bp fragment for *Plum pox potyvirus* (PPV) El Amar strain using primer pair (PPV-CP3 *Nco*I and PPV-CP5 *Bam*HI) to amplify the coat protein gene.

Fig 2. Agarose gel electrophoresis for *Nco*I digest of the potential recombinant plasmids from the cloned PCR fragment in Fig. 1.

Note. The *Nco*I digestions of the recombinant plasmid always give a fragment less in size than the expected one. The insert will be sequenced to provide an explanation for this *Nco*I restriction site.

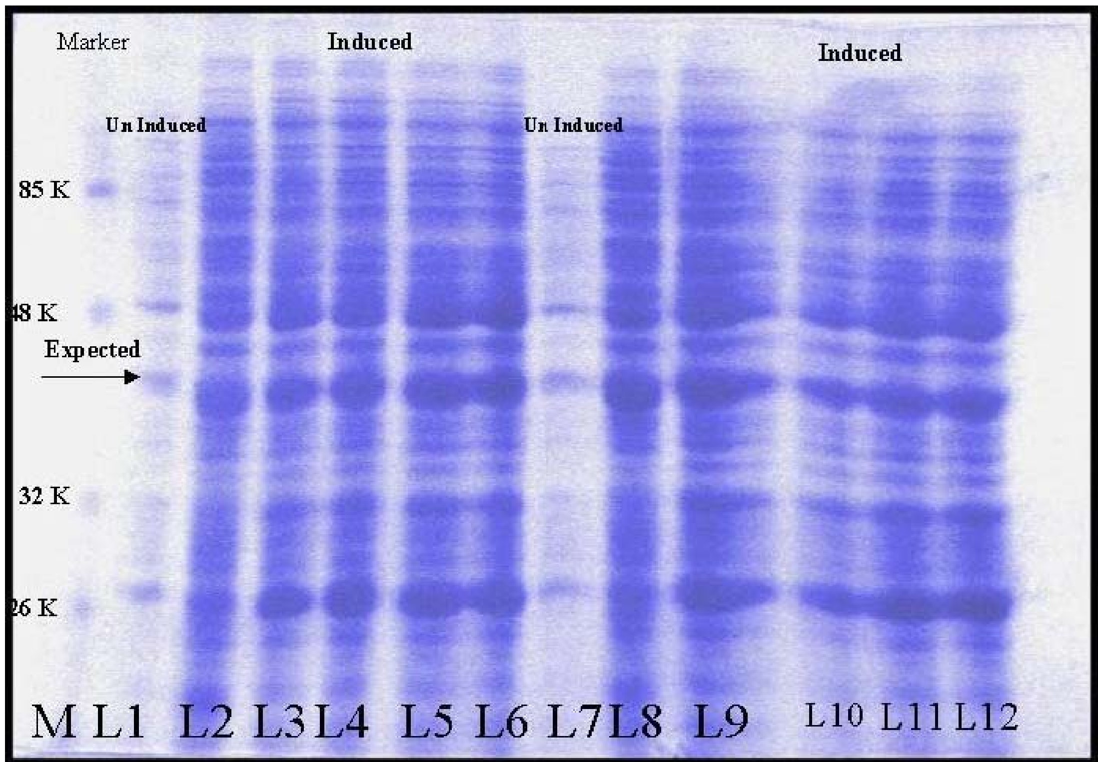


Fig 3. Marker, L1 uninduced sample, L2 induced with 20% L-Arabinose, L3 induced with 2% L-Arabinose, L4 induced with 0.2% L-Arabinose, L5 induced with 0.02% L-Arabenose,L6 induced with 0.002% L-Arabinose, L7 Uninduced, L8, 9, 10, 11, 12 induced sample using different concentrations of L- Arabinose as described before.

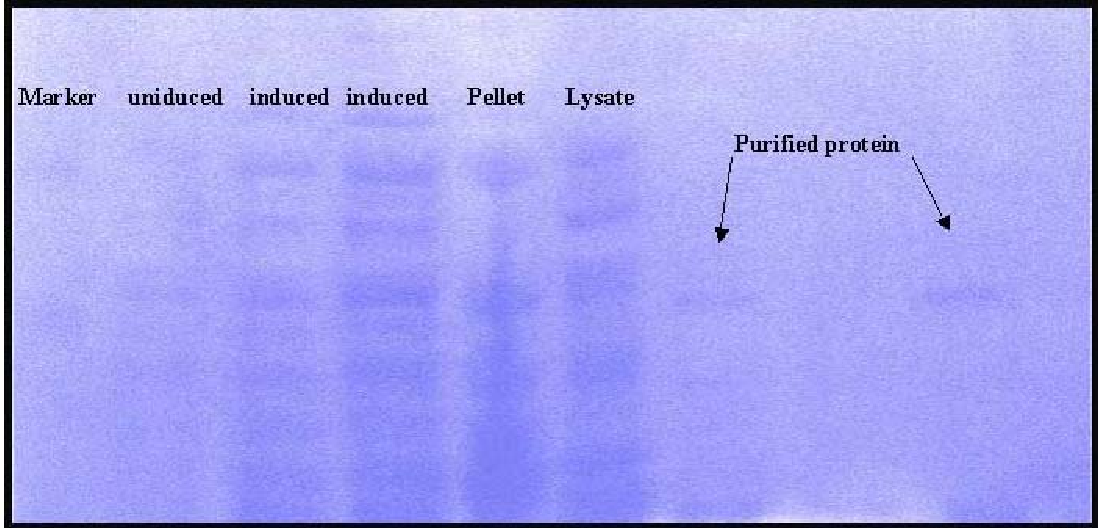


Fig 4. Polyacrylamide gel has the uninduced, induced, pellet, lysate, and purified protein in the expected size.

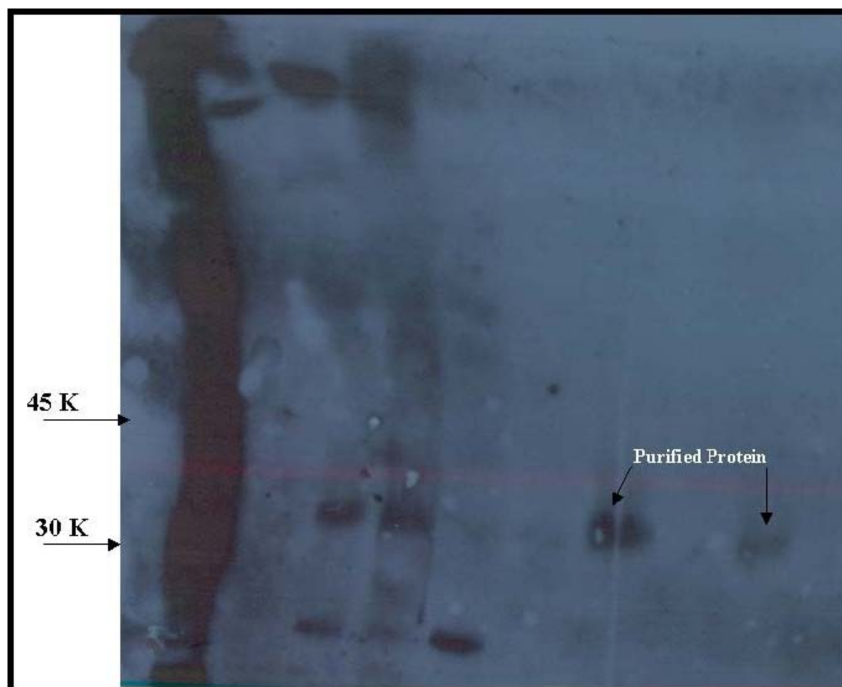


Fig 5. Western blot for the purified protein, the polyclonal antibody against PPV used to detect the expressed protein.

Discussion

The results indicated that the PPV-CP gene was isolated, cloned and expressed in *E. coli*. The expressed protein was tested by the polyacrylamide gel, and then the expressed protein was purified and tested using western blotting, which reacted positively with PPV antiserum.

References

Dunez, R. P., and Stutic, D. 1988. *Plum pox virus*. Pages 44-46. In: European Handbook of Plant Disease, I. M. Smith, J. Dunez, R.A. Lelliot, D.H. Philips, and S.A. Archer eds., Blackwell Scientific, Oxford, U.K. Pp. 44-46.

Hadidi, A., and Candresse, T. 2001. Plum pox. Pages 788-791. In: Encyclopedia of Plant Pathology, O.C. Maloy and T.D. Murray, eds. John Wiley and Sons, Inc., New York.

Nemeth, M. 1986. Virus, mycoplasma and rickkettsia diseases of fruit trees. Akademic Kiado, Budapest. 841 pages.

Sahar A. Youssef, A.A. Shalaby, H.M. Mazyad and A. Hadidi. 2002. Detection and identification of *prune dwarf virus* and *plum pox virus* by standard and multiplex RT-PCR probe capture hybridization (RT-PCR-ELISA). JPP, 84 (2):113-119.

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
Name of researcher: Sahar A. Youssef, Hamed Mazyad, and Ahmed Shalaby, Agriculture Research Center, Giza, Egypt and A. Hadidi, NIH/USDA
Address (E-Mail): saharyoussef@link.net and aashalaby@link.net

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, MN) and kept in a shaker at 50°C for 3 h. The hybridization solution was removed and the wells were washed five times with washing PBS-Tween solution (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.1 mM Na_2EDTA, pH 6.8, 0.05% Tween-20). Two hundred microliters of polyclonal anti-DIG Fab fragment conjugated to peroxidase diluted 1:100 in Tris-HCl, pH 7.5 buffer were added to each well and the microtiter plates were shaken gently at 37°C for 30 min. The Tris-HCl buffer was included in the Detection System obtained from Boehringer Mannheim Corp. Wells were then washed five times with the washing solution. Two hundred microliters of substrate solution (100 µg/ml of 2,2-azino-bis {3-ethylbenzthiazoline-6-sulfonic acid} diammonium) were added to each well and microtiter plates were incubated for 0.5-1.5 h at 37°C in the dark with agitation. Solution containing hybridized products was green in color. The absorbency of hybridized products was measured at 405 nm in an ELISA- reader (Multiskan Plus-MK II314). Results were expressed as net absorbance after the optical density of the blank solution was automatically subtracted for each well.

Cloning and nucleotide sequencing of RT-PCR amplified PDV cDNA and PPV cDNA products

The 172-bp PDV cDNA and 220-bp PPV cDNA amplified products were directly cloned into the pCR™ vector using the TA™ Cloning system (Invitrogen, Carlsbad, CA). The ligation mixtures were then used for electroporation of *E.coli* BL21 cells. Recombinant plasmids were selected and sequenced. Both strands of each DNA fragment were sequenced by ABI- PRISM™ 373A Genetic Analyzer (Perkin-Elmer) by using dye-primer and dye-terminator methods at University of Maryland College Park, MD (DNA Sequencing Facility, Center for Agricultural Biotechnology).

Results

Detection of PDV DIG - Labeled RT-PCR Products Using PDV - Specific Capture Probe in a Microwell Capture Hybridization Assay.

DIG-labeled PDV cDNAs were analyzed by capture hybridization assay using biotinylated PDV cDNA probe (Table 1). All infected tissue samples showed positive RT-PCR-ELISA assay with absorbance values at 405 nm ranging from 2.908 to 0.459. All infected samples were green in color. Color development was absent with products from healthy tissue or buffer control samples and their absorbance values were less than 0.025. The sensitivity of detection of PDV DIG-labeled RT-PCR product by probe capture hybridization as compared to gel electrophoretic analysis was 10 - 100 fold (data not shown).

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.

```
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
```

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.

References

Bachman, E.J., Scott, S.W., Xin, G.E., and Vance, V.B. 1994. The complete nucleotide sequence of prune dwarf ilarvirus RNA3: implication for coat protein activation of genome replication in ilarviruses. Virology 201:127-131.

Parakh, D.R., Shamloul, A.M., Hadidi, A., Scott, S.W., Waterwarth, H.E., Hawell, W.E., and Mink, G.I. 1995. Detection of prune dwarf ilarvirus form infected stone fruit using reverse transcription – polymerase chain reaction. Acta Hort. 386:421 – 430.

Hadidi, A., and Candresse, T. 2001. Plum pox. Pages 788-791. In: Encyclopedia of Plant Pathology, O.C. Maloy and T.D. Murray, eds. John Wiley and Sons, Inc., New York.

Dunez, R. P., and Stutic, D. 1988. Plum pox virus. Pages 44-46. In: European Handbook of Plant Disease, I. M. Smith, J. Dunez, R.A. Lelliot, D.H. Philips, and S.A. Archer eds., Blackwell Scientific, Oxford, U.K. Pp. 44-46. Public.

Nemeth, M. 1986. Virus, mycoplasma and rickkettsia diseases of fruit trees. Akademic Kiado, Budapest. 841 pages.

Sahar A. Youssef, A.A. Shalaby, H.M. Mazyad and A. Hadidi. 2002. Detection and identification of prune dwarf virus and plum pox virus by standard and multiplex RT-PCR probe capture hybridization (RT-PCR-ELISA). JPP, 84 (2):113-119.

TECHNICAL SHEET No. 27

Virus Detection: *Prune dwarf virus*

Method: ELISA

General
Virus detected: *Prune Dwarf Virus* (PDV)
General method: ELISA.

Developed by
Name of researcher: Hana Sobh and Yusuf Abou-Jawdah, American University of Beirut, Lebanon
Address (email): hs05@aub.edu.lb and abujawyf@aub.edu.lb
Date: November 26, 2002

Goals
Identification of PDV

Introduction

Prune Dwarf virus belongs to the Genus Ilarvirus family Bromoviridae. Virus particles of this genus are isometric to short bacilliform and labile. It is a single-stranded RNA virus composed of three RNAs (but four RNAs are needed for infection to occur). The two largest RNAs code for the RNA polymerase enzyme (2 proteins, 120 and 100 Kd), the third RNA codes for a 34 Kd protein, the movement protein. RNA 4 codes for the coat protein but the genetic code for this gene is also present in RNA 3. The capsid is composed of one type of protein subunits, with a MW between 24 to 30 Kd, depending on virus isolate.

PDV is transmitted by seed, pollen, and grafting. PDV causes a yield reduction of 50% and the remaining fruits are large and firm. In chronically infected trees, the reduced numbers of fruit spurs give a bare wood appearance. PDV-infected plums and prunes develop narrow, strap-like leaves that are thicker than normal and the internodes tend to be rosette. In most peach cultivars, PDV produces mild stunting and no leaf symptoms while in the Muir peach cultivar, PDV infections produce a dense canopy due to the shortening of the internodes. PDV is considered by Martelli and Savino (1997) to be the most damaging and widespread Ilarvirus.

Materials and Methods

A. ELISA: The standard double-antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA) was used for the detection of PNRSV. IgGs and conjugated IgGs were purchased from Sanofi, France or Agdia, USA. Extraction and detection of the infected leaves by ELISA are as follows:

Phosphate Buffer Saline (PBS)	**Washing Buffer pH 7.4**	**Coating Buffer pH 9.6**
Distilled water 1.0 l	PBS 1.0 l	Distilled water 1.0 l
NaCl 8.0 g	TWEEN 20 0.5 ml	Na_2CO_3 1.6 g
$Na_2HPO_4.12H_2O$ 2.9 g		$NaHCO_3$ 3.0 g
KH_2PO_4 0.2 g		
KCl 0.2 g		

Conjugate Buffer pH 7.1-7.3	Substrate Buffer pH 9.6 adjusted by concentrated HCl
PBS TWEEN 100 ml	Distilled water 80.0 ml
PVP 2 g	Diethanolamine 9.7 ml
Ovalbumine 0.2 g	

Coating of ELISA plates

1. Dilute antibodies in coating buffer as recommended by the supplier
2. Add 100 µl to each well, cover the plate
3. Incubate for 3-4 h at 37ºC
4. Wash three times with PBS-TWEEN

Antigen extraction and binding

1. Samples were either stored at 4ºC until processed, normally 3-4 days, or stored at -80ºC for few weeks.
2. Leaf tissues (1:10 w/v) were extracted in 0.1 M phosphate buffer Saline-Tween-Polyvinylpyrolidone (PBST-PVP), pH 7.4 containing 2% PVP 0.05% Tween-20, 0.15 M NaCl, and 4 mM KCl.
3. Add 100 µl extracted sample/well; and two wells were used per sample
4. Incubate overnight at 4ºC
5. Wash three times with PBS-TWEEN

Conjugate

1. Dilute conjugated antibodies in conjugate buffer as recommended by the supplier and add 100 µl to each well
2. Incubate for 3-4 hr at 37ºC
3. Wash three times with PBS-TWEEN

Color reaction

1. Dissolve 10 mg pNPP in 10 ml substrate buffer just before use, transfer 100 µl to each well, incubate for 1 h
2. After incubation, the reaction may be detected visually or at A_{405} nm using an ELISA reader.
3. The test was considered positive when the mean absorbance value of a sample was over twice that of healthy controls

References

Martelli, G.P. and Savino V. 1997. Infectious diseases of almond with special reference to the Mediterranean area. EPPO Bulletin. 27 (4) 525-534.

TECHNICAL SHEET No. 28

Virus detection: *Prunus necrotic ringspot virus*

Method: ELISA

General

Virus detected: *Prunus necrotic ringspot Ilarvirus* (PNRSV)
General method: ELISA

Developed by

Name of researcher: Hadia Kawtharani and Yusuf Abou-Jawdah, American University of Beirut, Lebanon
Address (email): haddoush21@hotmail.com and abujawyf@aub.edu.lb
Date: November 23, 2002

Goals

Identification of PNRSV strains and production of PNRSV ELISA Kits.

Introduction

Prunus necrotic ringspot virus belongs to the genus *Ilarvirus*, family *Bromoviridae.* Virus particles in this genus are isometric to short bacilliform and labile. It is an RNA virus composed of 3-4 RNAs. The two largest RNAs code for the RNA polymerase enzyme (2 proteins, 120 & 100 Kd), the third RNA code for a 34 Kd protein, the movement protein. RNA 4 codes for the coat protein but the genetic code is also present in RNA 3. PNRSV occurs in all temperate regions, the disease affects most stone fruits including sour cherry, cherry, almond, peach, apricot, plum, their wild and flowering counterparts and also some ornamental species like roses. PNRSV is the most widespread virus disease of stone fruit trees. Symptoms vary according to species infected and strain of the virus. The yield of virus infected trees may be 20 – 60 % lower than that of healthy trees.

PNRSV is transmitted by grafting. It is also mechanically transmitted to cucumber and several other herbaceous plants. Seed transmission rates vary between 5-70%. Pollen transmission is reported to seeds and to the mother plants. No vectors are reported. Therefore, the control of PNRSV is based almost exclusively on starting with virus free nursery stock and on eliminating PNRSV-infected *Prunus* trees from the area. Therefore, sensitive, reliable diagnostic tests are needed to make sure that locally produced or imported seedlings or seeds are free from PNRSV and other viruses like *Prune dwarf virus* (PDV), *Plum pox virus* (PPV), etc.

Materials and Methods

Leaf samples were collected randomly from symptomatic as well as asymptomatic shoots. Samples were either stored at 4ºC until processed, normally one week, or stored at - 80ºC for several weeks.

The standard double-antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA) was used for the detection of PNRSV. IgGs and conjugated IgGs were purchased from Sanofi, France or Agdia, USA. Extraction and identification of the infected leaves by ELISA are as follows:

Phosphate Buffer Saline (PBS)	Washing Buffer pH 7.4	Coating Buffer pH 9.6
Distilled water 1.0	PBS 1.0 l	Distilled water 1.0 l
NaCl 8.0 g	TWEEN 20 0.5 ml	Na_2CO_3 1.6 g
$Na_2HPO_4.12H_2O$ 2.9 g		$NaHCO_3$ 3.0 g
KH_2PO_4 0.2 g		
KCl 0.2 g		

Conjugate Buffer pH 7.1-7.3	Substrate Buffer pH 9.6 adjusted with concentrated HCl
PBS TWEEN 100 ml	Distilled water 80.0 ml
PVP 2 g	Diethanolamine 9.7 ml
Ovalbumine 0.2 g	

Coating of ELISA plates

1. Dilute antibodies in coating buffer as recommended by the supplier.
2. Add 100 µl to each well, cover the plate.
3. Incubate for 3-4 hr at 37ºC.
4. Wash three times with PBS-TWEEN.

Antigen extraction and binding

1. Leaf tissues (1:10 w/v) were extracted in 0.1 M phosphate buffer Saline-Tween-Polyvinylpyrolidone (PBST-PVP), pH 7.4 containing 2% PVP, 0.05% Tween-20, 0.15 M NaCl, and 4 mM KCl.
2. Add 100 µl extracted sample/well; two wells were used per sample.
3. Incubate overnight at 4ºC.
4. Wash three times with PBS-TWEEN.

Conjugate

1. Dilute conjugated antibodies in conjugate buffer as recommended and add 100 µl to each well.
2. Incubate for 3-4 h at 37ºC.
3. Wash three times with PBS-TWEEN.

Color reaction

1. Dissolve 10 mg pNPP in 10 ml substrate buffer just before use.
2. Incubate for 1 h.
3. After incubation, the reaction was detected calorimetrically at A_{405} nm using an ELISA reader.
4. The test was considered positive when the mean absorbance value of a sample was over twice that of healthy controls.

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
Name of researchers: A. M. Shamloul, f. Faggioli, J. M. Keith, A. Hadidi, NIH/USDA
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

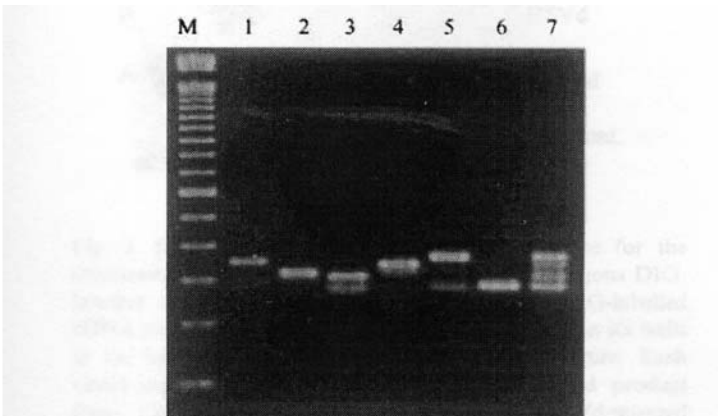


Fig. 1. Polyacrylamide gel electrophoretic analysis of RT-PCR full-length products of six viroids amplified from total RNA of viroid infected tissues. Molecular DNA marker (100 bp DNA ladder, GIBCO BRL, Gaithersburg, MD) with fragment sizes (bp) of 2072, 1500, 600, 500, 400, 300, 200, 100 (M). Standard 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.

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

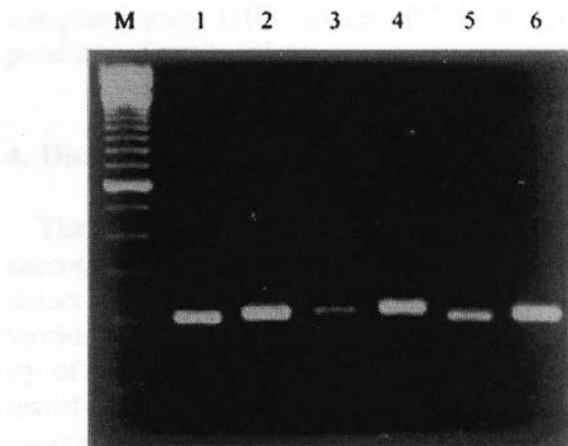


Fig. 2. Polyacrylamide gel electrophoretic analysis of RT-PCR (lanes 1, 3, and 5) and DIG-labelled RT-PCR (lanes 2, 4, and 6) products synthesized with cAPSCAVd and hAPSCAVd primers and amplified from: ASSVd-infected apple (lanes 1 and 2), PBCVd-infected quince (lanes 3 and 4), and ADFVd-infected apple (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.

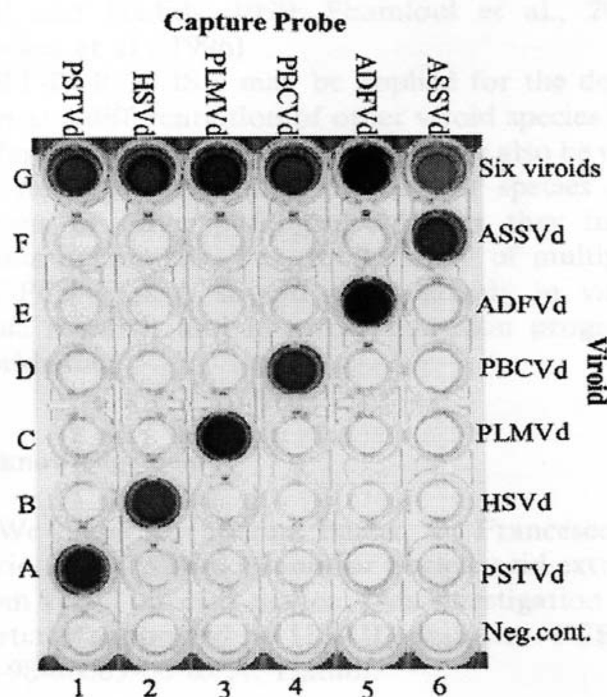


Fig. 3. Specificity of biotin-labelled capture probe for the colorimetric detection of homologous and heterologous DIG-labelled amplified viroid cDNA product. The DIG-labelled cDNA amplified product of each viroid was placed in six wells in the hybridization plate as indicated in the figure. Each viroid 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

Hadidi, A., R. Flores, J.W. Randles, and J.S. Semancik (eds.). 2002. Viroids. CSIRO Publishing, Collingwood, Victoria, Australia.

Shamloul, A.M., and A. Hadidi. 1999. Sensitive detection of potato spindle tuber and temperate fruit tree viroids by reverse transcription-polymerase chain reactin-probe capture hybridization. J. Virol. Methods 80:145-155.

TECHNICAL SHEET No. 30

General Detection of Viroids

Methods: RT-PCR, Electrophoresis

General
Viroids detected: All viroids or viroids-like pathogens from infected plants (citrus, grapevine, stone fruits)
General method: Electrophoresis, RT-PCR

Developed by
Amine Elleuch, Hatem Fakhfakh, and Marrakchi Mohamed, Laboratory of Molecular Genetics, Immunology and Biotechnology. Faculty of Sciences of Tunis, Campus Universitaire 2092 Elmanar Tunis, Tunisia. (Email): hatem.fakhfakh@fsb.rnu.tn

Goals

To develop sensitive and specific method for viroid detection based electrophoresis and RT-PCR amplification.

Introduction

Yield and quality losses are caused by viroids in vegetable, horticultural, field and ornamental crops. The *Potato spindle tuber viroid* (PSTVd) could cause 17 to 65% yield losses depending on the viroid strain and potato cultivar. The losses could reach 100% by the third generation of growth. Up to 1982, in the Philippines, over 30 million coconut trees had been killed by cadang-cadang disease.

Viroids are composed only of a circular, single-stranded RNA molecule of 240-400 nucleotides. Unlike viruses, they do not code for any protein and therefore must rely on host enzymes for their replication. In general, it is believed that they elicit their pathogenic effects by direct interaction between either the viroid RNA itself, or other viroid-specific RNAs generated in the course of the infection, and one or more cellular targets (Flores, 2001). Viroids can be classified into two separate families based on comparative sequence analyses and whether or not they possess a central conserved region (CCR) (Flores et al., 2004). There are currently only four members in the *avsunviroidae* family, whose type member is *Avocado sunblotch viroid* (ASBVd), in which both plus (+) and minus (-) RNAs have the ability to self-cleave their RNA multimers. All other viroids identified to date fall into the *pospiviroidae* family, whose type member is PSTVd. The latter family possesses a highly conserved central region (CCR), and has not been shown to be capable of any specific self-cleavage reaction. Viroids are transmitted mechanically. This mechanism of transmission is wide-spread among plant pathogens where the infectious agent is transmitted following contact between an infected plant (or infested tool, carrier insect) and a wounded, non-infected plant.

Materials and Methods

Viroid RNA isolation and fractionation from plant tissue with phenol

1. Homogenate 10 g of plant tissue in a Polytron for 3 min with:
   - 40 ml of water-saturated phenol (neutralized)
   - 10 ml of Tris-HCl 0.2 M pH 8.9
   - 2.5 ml EDTA 0.1 M pH 7.0
   - 2.5 ml SDS 5%
   - 1.25 ml β-mercaptoethanol
2. Centrifuge the mixture for 15 min at 8,000 rpm.
3. Remove aqueous phase and re-extract with 0.5 volumes of water-saturated phenol.
4. Centrifuge the mixture for 15 min at 8,000 rpm.
5. Remove aqueous phase, bring it to a final volume 20 ml with water, and add:
   - 3.7 ml 10X STE (1 M NaCl, 0.5 M Tris, 10 mM EDTA, pH 7 with HCl)
   - 13.4 ml of EtOH little by little while shaking
   - 1.25 g of non-ionic cellulose (CF-11, Whatman)

   Shake it at room temperature for at least 1 h
6. Centrifuge the mixture for 5 min at 3,000 rpm and discard the supernatant.
7. Wash the pellet three times with 30 ml of 35% EtOH in 1X STE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.2 [adjust pH with HCl]); each time the mixture is centrifuged for 5 min at 3,000 rpm at room temperature. (To prepare 1 liter 10X STE: 60.57 g Tris, 58.44 g NaCl and 3.72 g EDTA, and bring it to pH 7.2 with HCl).
8. To elute the nucleic acids, resuspend the CF-11 pellet in 3.3 ml 1X STE, centrifuge the mixture for 5 min at 3,000 at room temperature, and recover the supernatant. Repeat this step two more times.
9. Add to the combined supernatant 2.5 vol of EtOH and mix. Keep the sample for at least 2 h at -20 °C.
10. Centrifuge the sample at 8,000 rpm for 30 min and discard the supernatant.
11. Air-dry the pellet for 30 min, and resuspend it in water. Add 0.25 ml of water per 10 g of fresh tissue.

Viroid RNA isolation and fractionation from plant tissue without phenol

1. Homogenate the plant tissue (20 g) in a Polytron for 3 min in the presence of extraction buffer (50 mM EDTA pH 7.0, 500 mM NaCl, 100 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol). Normally, 5 ml of extraction buffer per gram of fresh tissue should be used.
2. Add 5 ml of 20% SDS followed by an incubation of 20 min at 65°C.
3. Add 25 ml of sodium acetate 3 M, pH 5.5 and incubate the suspension at 4°C for 20 min.
4. Centrifuge the mixture for 15 min at 8,000 rpm.
5. Collect the supernatant carefully (125 ml) and transfer it to a centrifuge tube.
6. Add 23 ml of 10X STE (1 M NaCl, 0.5 M Tris, 10 mM EDTA, pH 7, adjusted with HCl).
7. Add 85 ml of EtOH little by little while shaking.
8. Add 2.5 g of non-ionic cellulose (CF-11, Whatman), and shake it at room temperature for at least 1 h.
9. Centrifuge the mixture for 5 min at 3,000 rpm and discard the supernatant.
10. Wash the pellet three times with 30 ml of 35% EtOH in 1X STE % (10 mM Tris, 100 mM NaCl, 1 mM EDTA pH 7.2 con HCl); each time the mixture is centrifuged for 5

min at 3000 rpm at room temperature. (To prepare 1 liter 10X STE: 60.57 g Tris, 58.44 g NaCl and 3.72 g EDTA, and bring it to pH 7.2 with HCl).

11. To elute the nucleic acids, resuspend the pellet in 3.3 ml 1X STE, centrifuge the mixture for 5 min at 3000 at room temperature, and recover the supernatant. Repeat this step two more times.
12. Add to the combined supernatant 2.5 vol of EtOH and mix. Keep the sample for at least 2 h at -20°C.
13. Centrifuge the sample at 8000 rpm for 30 min and discard the supernatant.
14. Air-dry the pellet for 30 min, and resuspend it in water. Add 0.5 ml of water per 20 g of fresh tissue.

Treatment with methoxyethanol to remove polysaccharides (optional)

1. Mix on ice in a 15 ml Corex tube:
   1 vol of extract
   1 vol of K_2HPO_4 2.5 M, pH 8.0 (for 2 ml of extract, add 2 ml of K_2HPO_4 2.5 M and 40 µl of H_3PO_4 85%)
   1 vol of methoxiethanol
2. Mix by vortexing and keep on ice for 5 min.
3. Centrifuge the suspension at 3,000 rpm for 3 min. Recover the aqueous phase and transfer it to a new tube.
4. Add per volume of the aqueous phase,
   0.05 vol of sodium acetate 3 M, pH 5.5
   0.5 Volume CTAB 1% (CTAB is cetyl trimethyl ammonium bromide)
5. Mix by shaking and keep on ice for 5 min.
6. Centrifuge the mixture at 8,000 for 20 min and 4 °C.
7. Discard the supernatant and dry the pellet.
8. Resuspend the pellet in 2 ml of 1 M NaCl.
9. Add 6 ml of cold EtOH and mix.
10. Keep the sample at -20 °C for at least 2 h.
11. Centrifuge the sample at 8,000 rpm for 20 min and discard the supernatant.
12. Dry the pellet and resuspend in water (200 µl aprox.)

Fractionation with high salt (optional)

If the extract is not clean, this extra step can be done.

1. Add to 500 µl of extract:
   55 µl 10X STE
   140 µl LiCl 10 M
2. Vortex and keep the mixture overnight at 4 °C.
3. Centrifuge for 20 min at 10,000 rpm and collect the supernatant.
4. Add to the collected supernatant:
   70 µl sodium acetate 3 M pH 5.5
   3 vol of EtOH
5. Keep the sample at -20 °C for at least 2 h.
6. Centrifuge the sample at 10,000 rpm for 20 min and discard the supernatant.
7. Dry the pellet and resuspend in water (500 µl aprox.).

Viroid analysis by electrophoresis polyacrylamide gels (12 cm X 14 cm X 2 mm)

PAGE 5% (Non-denaturing conditions)

H_2O	28.6 ml
10X TAE	4.0 ml
Solution A	6.7 ml
TEMED	60.0 µl
Ammonium persulfate (10%)	0.6 ml

Load samples in 1X TAE with glycerol. Gels are run for 2.5 hours in1X TAE at constant intensity (70 mA) (corresponding approximately to 200 V).

PAGE 5% (Denaturing conditions, 8 M urea, 1X TBE)

Urea	16.8 g
H_2O	8.0 ml
5X TBE	7.0 ml
Solution A	5.8 ml
TEMED	30.0 µl
Ammonium persulphate (10%)	0.5 ml

The electrophoresis buffer is 1X TBE and gels are run for 1.5 h at constant voltage (200 V), corresponding to an approximate intensity of 50 mA.

Before loading the samples, remove the undissolved urea from wells with some buffer. Add 1 mg urea/µl of sample (and 0.2 µl of a solution of xylene cyanol and bromophenol blue). Dissolve with a vortex, heat the samples at 65 °C for 10 min, cool them down in ice, and load (avoid a long time in ice to prevent urea precipitation).

PAGE 5% (Denaturing conditions, 8 M urea, 0.25X TBE)

Urea	16.8 g
H_2O	11.7 ml
5X TBE	1.75 ml
Solution A	5.8 ml
TEMED	30.0 µl
Ammonium persulphate (10%)	0.5 ml

The electrophoresis buffer is 0.25X TBE and gels are run for 3 h at constant intensity (17 mA), setting a maximum voltage of 350 V.

Before loading the samples, remove urea from wells with some buffer. Add 1 mg of urea/µl of sample (and 0.2 µl of a solution of xylene cyanol and bromophenol blue). Dissolve with a vortex, heat the samples at 65°C for 10 min, cool them down in ice, and load (avoid a long time in ice to prevent urea precipitation).

SOLUTIONS AND BUFFERS

SOLUTION A

Acrylamide	15 g
Bis-acrylamide	0.38 g
H_2O	50 ml (final volume)

Staining of Polyacrylamide gels with silver

Solution I (100 ml):

10 ml ethanol
0.5 ml acetic acid (glacial)
89.5 ml water

- 3 minutes with solution I
- 2 washes with distilled water

Solution II: $AgNO_3$ (0.2%)

- 15 minutes with 100 ml of Solution II
- 2 washes with distilled water

Developing

Solution III (100 ml):

3 g NaOH
96.5 ml distilled water
0.5 ml formaldehyde (37%)

Amplification of CEVd by RT-PCR

Reverse Transcription

5 µl Plant extract
2 µl (200 ng) reverse primer
heat 3 min at 95°C and directly transfer on ice for 5 min
Add: 4 µl MMLV-RT buffer 5X
2 µl dNTP (10 mM each)
2 µl DTT
1 µl MMLV (200 U), MMLV-RT (Murine molony lococyte virus-reverse transcriptase)
4 µl H_2O
Incubate for 1 h at 37°C

Polymerase chain reaction

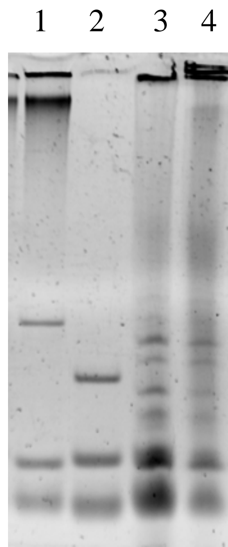
5 µl cDNA (reverse transriptin reaction, first strand complementary DNA)
1 µl reverse primer (100 ng) 5' CCCGGGGATCCCTGAAGGACTTC 3'
1 µl forward primer (100 ng) 5' GGAAACCTGGAGGAAGTCGAGG 3'
2 µl Taq polymerase buffer 10X
0.5 µl *Taq* DNA polymerase 2.5 U)
12.5 H_2O

PCR Program

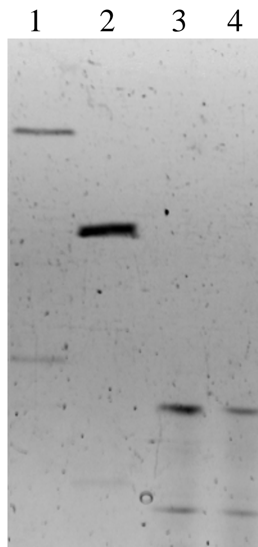
2 min at 94°C, 1 time, Denaturation 1 min at 94 °C, Hybridation 1 min at 56°C, Elongation 1 min at 72°C, repeat 35 times, then 10 min at 72 °C, final extension,1 time, and hold at 4°C or RT.

The reaction products are analyzed by 1.5% agarose gel electrophoresis.

Results



Native Gel



Denaturated gel

1. Extract from Gunyra infected with CEVd (*Citrus exocortis viroid*)
2. Extract from avocado infected with ASBVd (*Avocado sunblotch viroid*)
3. Extract from healthy Gunyra
4. Extract from healthy avocado

References

Flores R. 2001. A naked plant-specific RNA ten-fold smaller than the smallest viral RNA: the viroid. *C R Acad Sci.*; 324: 943–52.

Flores R., Randles J.W., Bar-Joseph M., Owens R.A., Diener T.O. 2004. Viroidae. In *Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses*, ed. CM Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, A.L. Ball, pp. 1145–1159. London: Elsevier/Academic.

Tomato

TECHNICAL SHEET No. 31

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV)

Method: Direct Elisa

General
Virus detected: TYLCV from tomato leaf.
General method: Direct ELISA

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad@agri.huji.ac.il and czosnek@agri.huji.ac.il
Date: Oct. 8, 2002

Goals
TYLCV detection based on direct ELISA

Introduction

TYLCV is the name given to a large number of genetically diverse whitefly-transmitted viruses infecting tomato. TYLCV infection reduces yields considerably; losses may reach 100% of the crop.

Information about geminivirus can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae).

Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998, 1) from the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia, 2) from Southwest Europe (Italy, 3) from tropical Africa (Senegal, Tanzania, 4) from Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begamoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002).

The coat proteins of the tomato begomoviruses have a high degree of homology in their amino acid sequences. Hence capsids have common serological determinants and a polyclonal antibody raised against one begomovirus may detect the presence of others.

We have used a polyclonal antibody raised against the TYLCV (Israel) coat protein expressed in *E. coli*, a gift from Dr. R. L. Gilbertson, University of California-Davis.

Materials and Methods

Antigen extraction and coating

1. Homogenize test sample 1:20 in coating buffer. Coating buffer is (1 liter, pH 9.6), in ddw: Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.20 g.
2. Add 100-200 µl per well. Cover plates tightly. Incubate at 4-6ºC overnight.

Alternatively: cut tomato leaf and/or stem and place them in ELISA well containing 100-200 µl coating buffer. Incubate at 4-6ºC overnight.

3. Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels. Washing buffer is (for 1 liter, pH 7.4): in ddw, NaCl 8.00 g, KH_2PO_4 0.20 g, Na_2HPO_4 1.15 g, KCl 0.20 g, Tween 20 0.50 g, NaN_3 0.20 g.

Conjugate

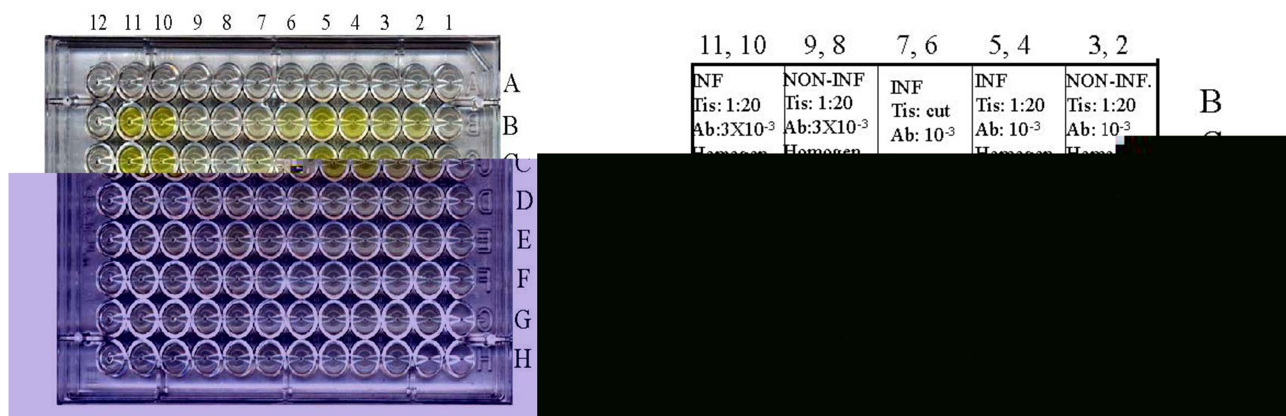
1. Dilute anti-TYLCV antibody (e.g. 1: 1000 in conjugate buffer or TBS buffer) and add 200 µl per well. Conjugate buffer is for 1 liter, pH 7.4: Tris-(hydroxy-methyl) aminomethane 2.40 g, NaCl 8.00 g, PVP (Polyvinyl-pyrrolidone) MW 24,000 20.00 g, Tween 200.50 g, BSA (bovine serum albumin) 2.00 g, $MgCl_2$-6 H_2O 0.20 g, KCl 0.20 g, NaN_3 0.20 g.
2. Cover the plates tightly and incubate at 37ºC for 3-5 h or 18 h at 4 to 6ºC.
3. Empty the wells and wash 3-4 times with washing buffer, remove any liquid by blotting the plate on paper towels.
4. Conjugate: Dilute anti-rabbit alkaline phosphatase conjugate 1:1000 in conjugate buffer or in TBS buffer, and add 200 µl per well.
5. Cover the plates tightly and incubate at 37 ºC for 3-5 h.
6. Wash 3-4 times with washing buffer, and then remove any liquid by blotting the plate on paper towels. It is important to remove all liquid by blotting the plate on paper towels.

Color reaction

1. Dissolve p-nitrophenyl phosphate to 1 mg/ml in substrate buffer. Substrate buffer is for 1 liter (pH 9.8): in ddw, diethanolamine 97.00 ml, NaN_3 0.20 g (the substrate buffer available as 5x concentrate, Art. No. 110130).
2. Add 200 µl per well and incubate at ambient temperature in the dark.
3. Observe reaction and read yellow color development after 30-120 min.
4. Visually and/or read with an ELISA reader at 405 nm.

<u>Results</u>

Effect of homogenization, virus and antibody dilutions, on the detection of TYLCV by direct ELISA



Discussion

This antiserum against TYLCV (Israel) should be tested against other begomoviruses.

References

Chatchawankanphanich, O. and D.P. Maxwell. 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant *Begomovirus*. Phytopathology 92:637-645.

Navas-Castillo, J., Sánchez-Campos, Noris, E., Louro, D., Accotto, G. P., and Moriones, E. 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. J. Gen. Virol. 81:2797-2801.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. *Tomato yellow leaf curl virus*: A whitefly-transmitted geminivirus with a single genomic component. Virology 185:151-161. (GenBank no. X15656)

Zeidan, M., Green, S. K., Maxwell, D. P., Nakhla, M. K., and Czosnek, H. 1998. Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia. Trop. Agr. Res. Exten. 1:107-115.

TECHNICAL SHEET No. 32

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV) species

Method: Non-Radioactive Technique for Dot Blot Hybridization

General
Virus Detected: *Tomato yellow leaf curl virus* (TYLCV) species
Method: non-radioactive technique for dot blot hybridization

Developed by
M. "Sid" Sedegui, Ministry of Agriculture, Morocco, (MohamedSedegui@hotmail.com**),** Mark K. Nakhla (mknakhlaakhla@plantpath.wisc.edu) and Douglas P. Maxwell (dpmax@plantpath.wisc.edu), University of Wisconsin-Madison
Date: November 2, 2002

Goals
To detect and distinguish between TYLCV species by a non-radioactive hybridization.

Advantages
The method can be used in many laboratories, since it is a non-radioactive technique. Also, it is a quick and reliable method.

Introduction
Tomato-infecting whitefly transmitted geminiviruses (begomoviruses) are a complex of strains and species. Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998). 1. From the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991). 2. From Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSarV) (Kheyr-Pour et al., 1991). 3. From tropical Africa (Senegal, Tanzania), and 4. From Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002; Navas-Castillo et al., 2000).

Information about geminiviruses can be found on the Web:
http://www.danforthcenter.org/iltab/geminiviridae
http://gemini.biosci.arizona.edu/
http://life.bio2.edu/ICTVdB/29030043.htm
http://www.im.ac.cn/vide/descr840.htm

Materials and Methods

Summary

1. Tissue samples are collected and stored in manner so that they don't decompose (E.G., cut young leaves in very small pieces (1 mm strips) and then let dry for at least 48 hours on filter paper).
2. Membrane spotting: Sample is ground in a buffer and debris removed by an appropriate method (see Dellaporta/heat technique). Small amount (5 µl) is spotted on a neutral nylon membrane, and then dried for about a half hour.
3. Lysing: prior to hybridization, membranes are treated with denaturing solution to form ssDNA, which binds to the nylon membrane and will be available for hybridization with a nucleic acid probe.
4. Fixing: UV-cross linker can be used to cross link the DNA to the membrane.
5. Preparing the probe and adding it to the membrane: This is the hybridization step. The membrane and labeled probe are placed in a hybridization solution. The hybridization conditions can vary so that the DNA probe will hybridize with the DNA of the test organisms when the percent DNA similarity is 65-75% (low stringency) or greater than 90% (high stringency). Low stringency would be used to detect any begomovirus in the sample and high stringency conditions would be used if differences in geminiviral species were being detected.
6. Washing: membranes are washed in a mild salt/detergent solution to remove any probe that was not hybridized to the test DNA.
7. The film is exposed for a time period from hours to days.

DNA Extraction Sardinian – Species Specific Primer (J. Potter et al., 2003)

1. Extract DNA from sample in extraction buffer: Plant tissues to be tested are: TYLCV positive control, unknown sample, tissue from non-infected tomato.
2. Obtain a small amount of tissue (2 cork borer for fresh or frozen tissue, 5 mg for dry tissue) and place in a microfuge tube.
3. Resuspend sample in 500 µl of Dellaporata buffer.
4. For dry tissue, leave on ice for 20 min with 500 µl Dellaporta.
5. Grind using a Kontes pestle.
6. Incubate at 65ºC for 5-10 min.
7. Spin for 10 min at 14,000 rpm.
8. Remove supernatant to new tube amd discard tissue debris.
9. Dilute 1:10 and 1:100 for PCR. Use without dilution for hybridization.
10. Store at –20ºC.

Dellaporta Buffer: total of 50 ml	
150 µl BME (beta mercaptoethanol)	Fisher BP 176-100
5 ml 1 M Tris pH 8.0	Fisher BP 152-5
5 ml 0.5 EDTA pH 8.0	Fisher BP 120-500
5 ml 5 M NaCl	Fisher S 271-10
34 ml dds H_2O	

Membrane Lysing

1. In a large glass tray, saturate 3 layers of Whatman (Cat# 3030917) 3 mm paper with 0.5 N NaOH. (20 ml 5M NaOH +180 ml H_2O). Remove any excess.
2. Lay membranes DNA side up on saturated paper, create no bubbles or folds. Leave for 5 min (saturate paper again before lysing another set of membranes).
3. Transfer membrane to a tray with 1 M Tris pH 7.4, leave for 5 min with agitatation.
4. Transfer membranes to a tray with 2X SSC (20 ml 20X SSC + 180 ml H_2O) (20X SSC: dissolve 175.3 g of NaCl + 88.2 g sodium citrate in 800 ml H_2O, adjust pH to 7.0 with 10 N NaOH. Adjust the volume to 1 liter with H_2O, autoclave).
5. Leave for 5 min agitating occasionally.
6. Transfer membranes to tray with 95% ethanol. Leave for 5 min, agitating occasionally. Ethanol will get cloudy, replace if lysing many membranes.
7. Air dry membranes (1/2 hour).

DNA Fixation on Membrane

There are two methods:

1. UV cross-link DNA by selecting optional cross-link on machine or by exposing to UV light with trans-illumination device for 3 min.
2. Bind the DNA to the nylon membrane by baking for 2 h at 80ºC.

Preparation of DNA probes

One of the easiest ways to prepare a DNA probe is to use the viral amplified fragment from a PCR reaction with appropriate primers and target DNA.

General Probe: For a general probe (about 500 bp) for Eastern Hemisphere begomoviruses, sequences of several viruses were aligned and the most conserved region of the coat protein (*CP*) gene determined. Primers were designed that would amplify this highly conserved region, which corresponds to the 3' end of the *CP* gene, from TYLCV (Israel) isolate (Navot et al., 1991):
1. PTYCPv (PTYv369) (viral sense) 5'- ACGCCCGCTCGAAGGTTCG-3'
2. PTYCPc (PTYc1023) (comp. sense) 5'-CGTCAGCCATATACAATAACAAGGC-3'

A full-length, infectious clone (pEG1) of the TYLCV from Egypt was generally used as the target DNA.

TYLCV (Israel)-specific probe: The viral species-specific probe was the intergenic region (common region, ori region), which is the most variable among different begomovirus species. Two different sets of primer pairs could be used for TYLCV (Israel) (Navot et al., 1991):

One primer set was designed by Navas-Castillo et al. (1999):
1. MA-30 (viral sense): 5' GAGCACTTAGGATATGTGAGG 3' (anneals at nt 2566-2586)
2. MA-31(comp. sense): 5' AGTGGATCCCACATATTGC 3' (anneals at nt 152-170)

One primer set was designed by M. K. Nakhla (University of Wisconsin-Madison):
1. PTYv21 (viral sense): 5' GTTGAAATGAATCGGTGTCCC 3'
2. PTYc287 (comp. sense): 5' TTGCAAAGACAAAAACTTGGGACC 3'

(Number in primer name corresponds to nt position in genome.)

TYLCSarV-specific probe: The primer set was designed by Navas-Castillo et al. (1999) for the Sardinian species of TYLCV, TYLCSardiniaV (Kheyr-Pour et al., 1991):
1. MA15: 5'-GGATCCCACATATTG-3'
2. MA14: 5'-TGCATTTATTTGAAAACG-3'

PCR Conditions

The thermocycler used was Thermocycle PTC-0200 DNA Engine (Alpha unit). Program: (94ºC, 45 sec, 55ºC,1:00 min, 72ºC, 1:45 min) x 30 cycles, followed by (94ºC, 1:00 min, 55ºC, 1:00 min, 72ºC, 5:00 min) x 1 cycle, then 4ºC.

PCR reaction mixture

H_2O 12.9 µl	Primer(+) 10 µM 1.0 µl
Buffer (Taq) 10X 2.5 µl	Primer (-) 10 µM 1.0 µl
Mg++ (Taq) 10 X 2.5 µl	dNTP's 10X 2.5 µl (each dNTP at 2.5 mM)
Taq DNA polymerase 0.1 µl	DNA sample 2.5 µl
Total: 25 µl	

Target DNA could be either DNA extracted from infected plants as describe above or plasmid DNA with an appropriate insert. Other DNA extraction methods outlined in Rojas et al. (1993) could be used.

Cleaning the DNA - DNA precipitation from PCR reaction

Combine the volume from two 50-µl PCR reactions, measure the volume and adjust to 400 µl. Add 0.1 volume of 3 M sodium acetate, (about 40 µl in 400 µl), add 2.5 volume 100% ETOH (about 1 ml), vortex and incubate at – 80ºC for 30 min or –20ºC for 4 h, spin at 14K for 15 min, keep pellet and add 70% ETOH spin at 14K remove ETOH, dry in speed vac, resuspend in 50 µl H_2O, shake for 5 min, run argarose gel to see how much DNA you have.

Considerations:
- The concentration of salt in the probe DNA to be labeled should be as low as possible and not exceed 50 mM. This is the reason for the precipitation step above.
- The DNA concentration should be accurately determined and adjusted to 10 ng/µl before labeling.
- Maintaining the DNA in single-stranded form is required to ensure a good labeling efficiency.
- Enzyme labeled probes should not be denatured prior to addition to the hybridization buffer.

Preparation of Labeled Probe

KIT: AlkaPhos Direct labeling reagents from Amersham Pharmacia Biotech UK limited, Cat number: RPN 3680. CRR-star detection reagent Cat number: 3682 from Amersham.

1. Preparation of cross-linker working solution from kit: 10 µl of cross-linker working solution are need for each probe; thus for 3 probes, the total is 30 µl. Take an 8 µl cross-link tube (black tube in kit) + 32 µl H_2O supplied = 40 µl cross link working solution. (This cross-linker working solution can be saved at 2-8ºC for later use).
2. Dilute the DNA to a concentration of 10 ng/µl using the water supplied (10 µl = 100 ng DNA).

3. Place 10 µl of the dilute DNA sample in a microfuge tube and denature by heating for 5 min in a vigorously boiling water bath.
4. Immediately cool the DNA on ice for 5 min. Spin briefly in microfuge to collect the contents at the bottom of the tube.
5. Add 10 µl of reaction buffer (red tube in kit) to cooled DNA. Mix thoroughly, but gently.
6. Add 2 µl labeling reagent (yellow tube). Mix thoroughly, but gently.
7. Add 10 µl of the cross-linker working solution. Mix thoroughly. Spin briefly in microfuge to collect the contents at the bottom of the tube.
8. Incubate the reaction for 30 min at 37ºC.
9. The probe can be used immediately or kept on ice for up 2 h. For long-term storage probes may be stored in 50% (v/v) glycerol at –15ºC for up to six months.

Hybridization

1. Pre-heat the required volume of prepared Alk. Phos direct hybridization buffer to 55ºC. The volume of buffer should be equivalent to 0.25 ml/cm^2 of membrane (generally, about 23-25 ml per hybridization tube).
2. Place the blot (membrane) into the hybridization buffer and pre-hybridize for at least 15 min at 55ºC in shaking water bath or hybridization oven.
3. Add the labeled probe to the buffer used for the pre-hybridization step (directly to the hybridization tube). Typically use 5-10 ng of probe/ml of buffer. (100-200 ng of probe for 20 ml of hybridization solution).
4. Hybridize at 55ºC overnight in shaking bath or hybridization oven.

Post-Hybridization Stringency Wash

1. Primary wash in hybridization oven:
   a. Pre-warm the primary wash buffer at 55ºC for general probe (low stringency) or 60-65ºC for species-specific probe (high stringency).
   b. Place blots in 1-2 ml/cm (30-40 ml) wash buffer.
   c. Wash for 2X 20 min.
2. Second wash:
   a. Prepare second wash buffer (100 ml in tray: 5 ml of 20X stock + 95 ml H_2O + 200 µl $MgCl_2$)
   b. Wash bath at room temperature for 2X 10 min, gently agitating.

Solutions for Hybridization

A) Hybridization Buffer: Store in aliquots at -20ºC (Add NaCl to Hyb buffer to give final solution of 0.5 M. Add blocking reagent to give a final concentration of 4 %. Mix at room temp for 1-2 hours on magnetic stirrer until clear.)

- Hyb Solution 500 ml bottle (in the kit).
- NaCl 14.61 g
- Blocking reagent 20 g (Kit)

B) Primary Wash: Make to a volume of 500 ml, can be stored 1 week at 4ºC.

- Urea 60 g
- SDS 0.5 g
- 0.5 M NaH_2PO_4 50 ml*
- NaCl 4.35 g
- 1.0 M $MgCl_2$ 0.5 ml
- Blocking reagent 1 g (kit)

* monobasic sodium phosphate, adjust to pH 7.0 with NaOH.

C) Secondary Wash: Add above to 450 ml dH_2O, adjust pH to 10.00 with NaOH. Store up to 4 months at 4ºC.

500 ml 20X Stock:

- Tris base 60.5g
- NaCl 56 g

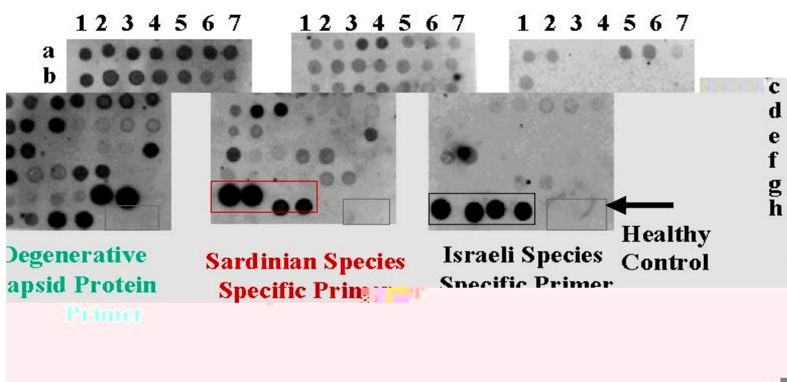
Secondary Wash, 500 ml Working Solution:

- Dilute 1:20, (20X stock, secondary wash stock) 25 ml
- dH_2O 475 ml
- 1M $MgCl_2$ 1 ml

Signal Detection with CDP-Star

Note: wear powder-free gloves or rinse gloved hands with water.

1. Drain excess secondary wash buffer from the blots by touching the corner of the blots against a clean surface, place the blots (sample side up) on clean non-absorbent, flat surface, eg, a tray. Do not allow the blots to dry out.
2. Pipette detection reagent (in the kit, CDP star) on to the blots, about 30-40 $\mu l/cm^2$ (2-4 ml/blots, enough to cover the whole blot) and leave for 2-5 minutes at room temperature. Drain excess reagent from membranes before next step.
3. Wrap the blots in Saran Wrap or place in detection bag. Place the blots DNA side up in the film cassette.
4. Switch off the lights and place a sheet of autoradiography film (Ex. ECL film, Hyper-film) on the top of the blots (shiny side of the film up). Close the cassette and expose for 4 h at room temperature. The DNA side of the filter (wrapped in saran Wrap) must be placed next to the film for maximum sensitivity.
5. Remove the film and develop, you can also expose a second film for 24 hours.



Results

The example to the right is from tomato samples collected in Morocco and hybridized with the general probe (CP gene), TYLCSarV-species probe, and TYLCV (Israel)-species probe (PTYv21/PTYc287, primers). Three identical membranes were prepared. None of the probes hybridized with the DNA extracted from symptomless (non-virus infected) tomatoes, and the general probe hybridized with most samples.

Many tomato samples had mixed infections and more tomatoes were infected with TYLCSarV than TYLCV.

Discussion

This non-radioactive method has been tested in laboratories in San Carlos University, Guatemala, Agricultural Research Center, Giza, and Egypt by our group and has worked very well. This method offers several advantages. Many samples can be tested and the species-specific probes are effective in distinguishing between TYLCV and TYLCSarV, where as the general probe hybridizes effectively with these two viruses. Similar methods have been developed by Potter et al. (2003) for detection of bean-infecting begomoviruses.

References

Chatchawankanphanich, O. and D.P. Maxwell. 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant *Begomovirus*. Phytopathology 92:637-645.

Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1(4):19-21.

Kheyr-Pour, A. M., Bendahmane, V. M., Accotto, G. P., Crespi, S., and Gronenborn, B. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res. 19:6763-6769.

Potter, J.L., Nakhla, M.K., Mejía, L., and Maxwell, D.P. 2003. PCR and DNA hybridization methods for specific detection of bean-infecting begomoviruses in the western hemisphere. Plant Dis. 87:1205-1212.

Navas-Castillo, J, Sanchez-Campos, S., Diaz, J.A., Saez-Alonso and Moriones, E. 1999. Tomato yellow leaf curl virus-IS causes a novel disease of common bean and severe epidemics in tomato in Spain. Plant Disease 83:29-32

Navas-Castillo, J., Sánchez-Campos, Noris, E., Louro, D., Accotto, G. P., and Moriones, E. 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. J. Gen. Virol. 81:2797-2801.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. Tomato yellow leaf curl virus: A whitefly-transmitted geminivirus with a single genomic component. Virology 185:151-161. (GenBank no. X15656)

Rojas, M. R., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis. 77:340-347.

Wyatt, S. D., and Brown, J. K. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. Phytopathology 86:1288-1293.

Zeidan, M., Green, S. K., Maxwell, D. P., Nakhla, M. K., and Czosnek, H. 1998. Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia. Trop. Agr. Res. Exten. 1:107-115.

TECHNICAL SHEET No. 33

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV)

Methods: Variability analysis of TYLCV species (strains)

General
Virus detected: TYLCV species (strains) from tomato, pepper and bean samples
Methods: Variability analysis of TYLCV species (strains)

Developed By
Name of researchers: Faten GORSANE, University of Tunisia, Hatem FAKHFAKH, University of Tunisia.
Adresses (email): Faten.gorsane@fss.rnu.tn or fatgorsane@voila.fr
Hatem.fakhfakh@fsb.rnu.tn or hafakhfakh@voila.fr
Date: October 27, 2002

Goals

To develop a method for the rapid detection of TYLCV species (strains) from a large number of samples; and assignment of isolates into TYLCV (Israel) or TYLCSardiniaV species.

Introduction

Tomato-infecting whitefly transmitted geminiviruses (begomoviruses) are a complex of strains and species. Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998). 1. From the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991). 2. From Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSarV) (Kheyr-Pour et al., 1991). 3. From tropical Africa (Senegal, Tanzania), and 4. From Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002; Navas-Castillo et al., 2000). Information about geminiviruses can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae).

Materials and Methods

Nucleic acid extraction

5 mg of tomato leaf tissue was ground with 500 µl of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 0.5 M NaCl, 10 mM Beta-mercaptoethanol) vortexed and allowed to stand at 65°C for 10 min. After adding potassium acetate (5 M, pH 8), the mixture was incubated on ice for 10 min and clarified by centrifugation at 13,000 rpm for 20 min at 4°C. An equal volume

of isopropanol was added to the supernatant, and then the solution was incubated for 10 min at –20°C and centrifuged for 10 min at 13,000 rpm. The pellet was resuspended in sterilized water and subjected to RNase treatement followed by phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol extraction. After precipitation with three volumes of absolute alcohol for 30 min at –20°C and centrifugation for 10 min at 13,000 rpm, DNA was washed with 70% ethanol and resuspended in steriled water.

Dot blot hybridization

200 ng of extracted DNA from each sample was first added to an equal volume of formaldehyde (15%) then heated at 65°C for 20 min. Using a dot blot apparatus (BioRad) under vacuum, DNA was transferred to a nylon filter (membrane) previously washed in 20X SSC (150 mM NaCl, 15 mM sodium citrate). The membrane was air dried at room temperature and stored until used.

A random primed digoxygenine-dUTP DNA probe spanning a TYLCV genome region of choice was used. The labelling reaction was carried out according to manufacturer's instructions (Bohringer). 1 µg of DNA was denatured at 100°C for 10 min, then 2 µl of hexanucleotides, 2 µl of dNTP (10 mM each) and 1 µl of Klenow DNA polymerase fragment were added. The mixture was incubated for one hour at 37°C and 2 µl of a stopping reaction (200 mM EDTA, pH 8) added to stop the reaction. The labelling probe was precipitated using 2.5 µl of 4 M LiCl, 1 µl of glycogene and 75 µl of absolute ethanol.

The membrane was incubated for one hour at 42°C, then the denatured probe (100°C for 10 min) was added and both were incubated at 65°C overnight. The membrane was then washed twice with 2X SSC, 0.1% SDS for 5 min at room temperature and twice with the same solution for 15 min at 42°C. After another wash with buffer I (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 min, the membrane was saturated for 30 min with buffer II (buffer I plus 0.5% blocking agent), washed again with buffer I and incubated for 30 min with buffer I containing an alkaline phosphatase-conjugated dUTP-specific IgG diluted at 1/5000. The membrane was washed twice for 15 min with buffer I, then for 2 min with buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and finally incubated in buffer I supplemented with NBT and BCIP according to manufacturer's instructions. Color development was stopped by washing the membrane in 50 ml Tris-EDTA and drying.

PCR - RFLP of the coat protein gene of TYLCV (species/strains)

Degenerate primers allowing the amplification of the coat protein gene were designed by analyzing sequences of TYLCV (Israel) from Egypt (NC 001996.1), Portugal (AF105975), Spain (AF071228), and the sequence of TYLCSardiniaV from Sardinia (X61153) (M. K. Nakhla, University of Wisconsin-Madison). These primers should thus anneal with both types of genomes: TYLCV (Israel) and TYLCSarV.

PCPv (PTYCPv369) (viral sense)
5'ACGCCCG(T/C)CTCGAAGGTTCG 3'
PCPc (PTYCPc1023) (complementary sense)
5' GTACA(T/A)GCCATATACAATAACAAGGC 3'

PCR amplification was done with 150 ng of total extracted DNA of each selected sample. PCR conditions were: one cycle of 3 min at 94°C followed by 35 cycles of 50 sec at 94°C, 50 sec at 55°C and 1 min at 72°C, then a final extension step of 10 min at 72°C.

Amplified fragments of about 673 bp were subjected to electrophoresis in a 1% agarose gel and stained with 0.5 µg/ml of ethidium bromide.

PCR fragments were column purified according to Qiagen Kit. DNA was then digested with the restriction endonuclease *Ava*II, which was expected to cut TYLCSarV differently from TYLCV (Israel) (Accotto et al., 2000). Digestion products were analyzed by electrophoresis on a 1.6% agarose gel.

Results and Discussion

Analysis of samples by dot blot hybridization under high stringency provided a reliable tool for both identification and quantification of TYLCV. The intensity of the signal is positively correlated with the virus accumulation in the infected plant. This assay offers a reliable tool to test a large number of samples rapidly.

Using degenerate primers of the coat protein gene, PCR fragments can be amplified from either Israeli or Sardinian viral genome. Analysis by PCR-RFLP using AvaII allows the assignment of TYLCV isolates into one of the two species: TYLCV (Israel) isolates produce a restriction pattern of about 370 and 300 bp while that of the TYLCSarV isolates consistently have three fragments with approximate sizes of 360, 160 and 150 bp.

Our ultimate goal is to develop a simple, rapid and reliable method for diagnosis and typing of TYLCV isolates.

References

Accotto, G.P., Navas-Castillo, J., Noris, E., Moriones, E., Lourou, D. 2000. Typing of tomato yellow leaf curl viruses in Europe. European Journal of Plant Pathology 106: 179-186.

Chatchawankanphanich, O. and D.P. Maxwell. 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant *Begomovirus*. Phytopathology 92:637-645.

Kheyr-Pour, A. M., Bendahmane, V. M., Accotto, G. P., Crespi, S., and Gronenborn, B. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res. 19:6763-6769. (GenBank no.

Navas-Castillo, J., Sánchez-Campos, Noris, E., Louro, D., Accotto, G. P., and Moriones, E. 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. J. Gen. Virol. 81:2797-2801.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. Tomato yellow leaf curl virus: A whitefly-transmitted geminivirus with a single genomic component. Virology 185:151-161. (GenBank no. X15656)

Zeidan, M., Green, S. K., Maxwell, D. P., Nakhla, M. K., and Czosnek, H. 1998. Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia. Trop. Agr. Res. Exten. 1:107-115.

TECHNICAL SHEET No. 34

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV)

Method: Squash Blot Hybridization with Radiolabled Probe

General
Detection from tomato plants and from the whitefly vector
General methods: Squash blot hybridization with radiolabeled probe

Developed by
Name of researcher: Nir Navot, Fouad Akad, and Hanokh Czosnek, The Hebrew University
Address (Email): akad @agri.huji.ac.il; czosnek @agri.huji.ac.il
Date: Oct. 8, 2002

Goals

To develop a rapid, sensitive method that allows detection TYLCV in a large number of samples. Easy sampling in field conditions. Samples are stable for several months at ambient temperature.

Introduction

Tomato yellow leaf curl virus, TYLCV, is the name given to a large number of genetically diverse whitefly-transmitted viruses infecting tomato. TYLCV infection reduces yields considerably; losses may reach 100% of the crop. Information about geminivirus can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae).

Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998). 1. From the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991). 2. From Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSV) (Kheyr-Pour et al., 1991). 3. From tropical Africa (Senegal, Tanzania), and 4. From Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002).

Materials and Methods

Tomato squashes were done using symptomatic leaves from the field, inoculated in the lab with viruliferous whiteflies or by agroinoculation. The method is based on hybridization method described by Navot and Czosnek (1989).

Squashing and Sampling

Squash tomato leaves (young leaf from the shoot apex, or individual whitefly) on a dry nylon membrane (Qiabrane. Biolabs) using a glass rod, an Ependorf tube or a pen cover. Use an X-ray film matrix in which 5 mm diameter holes have been drilled.

Fixation

This stage is not necessary; however nucleic acids can be fixed by exposing the membrane to UV for 3 min.

Prehybridization

Squash blots are prehybridized using a pre-hybridization solution containing: 12.5 ml 50X SCC, 5 ml Denhardt's solution x 50, 2.5 ml 10% SDS, 3.2 ml NaH_2PO_4, 1.7 ml Na_2HPO_4 and double distilled water up to 50 ml (final pH should be 6.5 to 6.8). Add 0. 7 ml of salmon sperm DNA (1µg/ml) after boiling for 10 min. Keep the mixture for 10 min at 65ºC. Add to the membrane and incubate for at least 3 h at 65 ºC. SSC x 20 is 3M sodium chloride, 0.3 M tri-sodium citrate; Denhardt solution x 50 is 2% bovine serum albumin (BSA), 2% Ficoll 400, 2% Polyvinylpyrolidone.

Preparation of TYLCV- DNA ^{32}P labeled probe

The probe used is a full length clone of TYLCV (plasmid pTYH20.7) as in Navot et al., (1991). Labelling (random priming, Amersham) was done as follows:

1. In a volume of 50 µl add: 5µl of 0.3 µg/µl plasmid, 5 µl of 10x hexanucleotide buffer, and 22µl ddH_2O.
2. Heat for 10 min at 100°C.
3. Keep the mixture in ice for 15 min.
4. Add 10 µl of (0.5mM) of dNTP without dCTP and 3 µl of the Klenow DNA polymerase.
5. Mix. Add 5 µl of the radiolabeled [^{32}P]dCTP. Incubate at 37°C for 3 h.
6. Heat for 10 min at 100°C.

Hybridization and washes

To the pre-hybridization solution, add the boiled labeled probe and incubate for 18 h at 65°C with shaking. The blots are washed with 0.1% SDS and 1x SSC for 20 min, twice. Monitor radioactivity with a Geiger counter. Wrap the membrane in Saran Wrap. Expose the membrane to X-ray film at –80°C for 18 h before development, or expose the membrane to a Phosphor imager screen for 2-3 h.

Results

Figure 1. Squash blot hybridization. Upper panel: squashes; Lower panel: hybridization.

Figure 2. Squash blot hybridization for the screening of resistant plants (e.g. 84-2, 84-10).

Figure 3. Squash blot of whiteflies. Each spot is an individual insect *.
The amount of virus is quantified using standards of cloned TYLCV DNA.

Figure 4. Squashes of organs from infected tomato *

* From Navot N, Ber R and Czosnek H (1989) Rapid detection of *tomato yellow leaf curl virus* in squashes of plants and insect vectors. Phytopathology 79: 562-568.

References:

Chatchawankanphanich, O. and D.P. Maxwell. 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant *Begomovirus*. Phytopathology 92:637-645.

Navas-Castillo, J., Sánchez-Campos, Noris, E., Louro, D., Accotto, G. P., and Moriones, E. 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. J. Gen. Virol. 81:2797-2801.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. Tomato yellow leaf curl virus: A whitefly-transmitted geminivirus with a single genomic component. Virology 185:151-161. (GenBank no. X15656)

Navot N., Ber, R., and Czosnek, H. 1989. Rapid detection of tomato yellow leaf curl virus in squashes of plants and insect vectors. Phytopathology 79: 562-568.

TECHNICAL SHEET No. 35

Virus Detection: *Tomato Yellow Leaf Curl Virus* (TYLCV)

Method: Non-radioactive hybridization using the Enhanced Chemi Luminescence labeling system (ECL).

General

Virus detected: TYLCV from tomato leave and whiteflies.
Method: Non-radioactive hybridization using the Enhanced Chemi Luminescence labeling system (ECL).

Developed by

Name of researchers: Omar Dar-Issa, and Naim Iraki, UNESCO Biotechnology, Center at Bethlehem University.
Address (Email): omari@bethlehem.edu; niraki@behtlehem.edu
Date: Nov. 10, 2002

Goals

To develop a sensitive method for TYLCV detection.

Introduction

Tomato (*Lycopersicon esculentum)* is one of the most important vegetable crops of the family Solanaceae, which includes about 1,500 tropical and subtropical species. The genus *Lycopersicon* consists of only eight species and it is subdivided into two subgenera: *Eulycopersicon* and *Eriopersicon*. Fruits of plants of *Eulycopersicon* are usually red or yellow in color when ripe. This genus includes the cultivated tomato (1). Fruits of the *Eriopersicon* on the other hand, remain green or purple green throughout development. Tomato fruits normally contain 5-10% dry matter, of which 1% is skin and seeds. Reducing sugars such as glucose and fructose constitute nearly 50% of the dry matter. In addition to these sugars, tomato fruit also contains potentially toxic glyco-alkaloids, such as tomatine and solanine. These alkaloids protect the tomato plant against microorganisms and pests. Tomato is native to South America and Mexico (1). Large-scale cultivation of tomato did not begin until about a century ago then it became generally cultivated only after World War I. Now it is consumed all over the world and is the second largest vegetable crop in terms of dollar value (1). A report from Food and Agriculture Organization (FAO) 1993, shows that the highest production of tomato is in the U.S.A. The percentages of total world production were 15%, and 0.62% for U.S.A. and the Middle East, respectively.

The most serious viral disease infecting tomatoes in the Mediterranean region is caused by the *Tomato Yellow Leaf Curl Virus* (TYLCV), which is the major factor limiting tomato production, during Summer, Fall and Winter cultivations. When necessary precautions are not taken (using nets or insecticides) infection may reach 100% depending on the age of plants and the time of infection. TYLCV is a monoparatite, circular, single-stranded DNA genome of approximately 2.8 kb (2). It belongs to the Geminiviridae family. Affected tomato plants are stunted, the shoots have short internodes (2). The leaves are small, curled, leathery and chlorotic (2). The most significant effect of TYLCV infection is flower abscission. Usually, less than one in ten flowers set fruit, thus severely reducing the yield.

This tomato virus attacks a great variety of hosts including lentil and tobacco. TYLCV is transmitted by cotton or tobacco whitefly (*Bemisia tabaci)* or silverleaf whitefly (*Bemisia argentifolii)*. A single whitefly is able to transmit the virus and the rate of transmission increases as the population density of the vector increases (3). No other means of transmission, such as mechanical were observed for this virus. The widespread occurrence of epidemics associated with TYLCV and its potential threat to tomato production, make it essential to develop procedures for TYLCV detection in both *B. tabaci* and plants for disease management. Serological methods have met limited success with the whitefly-transmitted Geminiviruses. But recently, nucleic acid hybridization techniques and polymerase chain reaction (PCR) provide sensitive methods for the detection and identification of TYLCV in infected plants, or the whitefly vector.

Materials and Methods

Tomato yellow leaf curl virus was detected by enhanced chemiluminescence system (ECL direct nucleic acid labeling and detection system-Amersham. RPN 3000). Infected tomato leaves and frozen whiteflies, squashed onto a dry nylon membrane. Cloned viral template labeled with enzyme horseradish peroxidase. Positively charged peroxidase attached loosely to completely denatured negatively charged template. Addition of glutaraldehyde strengthens the attachment through formation of chemical cross-links, so that the probe becomes covalently attached to the enzyme. In hybridization, the probe hybridized with target DNA immobilized on the membrane. Viral infection is detected by oxidation reduction reactions, using detection reagents, which produced blue light. The light output is increased and prolonged by the presence of an enhancer, so that it can be detected on a blue-light sensitive film (see kit leaflet for more details).

Preparation of TYLCV specific non-radioactive probe

Denature 100 ng of cloned TYLCV CP gene (available from the Hebrew University/Rehovot) at 100ºC for 10 min. Cool in ice for 5 min. Add 10 µl of DNA labeling reagent and 10 µl of glutaraldehyde solution to the cold DNA. Incubate the probe at 37ºC for 1 h. Hybridize the squash blots with the labeled probe for 13 h. Wash the blot twice with (1X SSC and 0.1% SDS) for 15 min.

Detection procedure

Cover the blots with equal volumes of both detection reagents (supplied with the kit). Place the blots in the film cassette. Expose top of the blots to autoradiography film for 1 min. Finally, remove and develop the film.

Results

Autoradiographic detection of TYLCV-DNA with TYLCV-specific non-radioactive probe, in squashes of tomato leaf tissues and single insect (Bemisia tabaci). Healthy: squashes of healthy tomato leaf. Infected: squashes of TYLCV infected tomato leaf. V.W.: squash of viruliferous whitefly.

References

(1) Nelson, P. 1996. Quality attributes of processed tomato products. Food Rev. Int. 12:375-401.
(2) http://hammock.ifas.ufl.edu/new/pg08400.htm
(3) Mansour, A., and Al-Musa, A. 1992. Tomato yellow leaf curl virus: host range and virus-vector relationship. Plant Pathol. 41:122 -125.

TECHNICAL SHEET No. 36

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV) and other begomoviruses

Method: Immunocapture PCR

General
Virus: Geminiviruses detection from whitefly and infected plants
General method: Immunocapture PCR (IC-PCR)

Developed by
Name of researcher: Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad @agri.huji.ac.il and czosnek@agri.huji.ac.il
Date: Oct. 8, 2002

Goals

To develop a sensitive and reliable method for the detection of begomoviruses, based on serology combined with PCR.

Introduction

The coat proteins of whitefly-transmitted geminiviruses (begomoviruses), whether from the New or the Old World, have a high degree of homology in their amino acid sequences. Hence capsids have common serological determinants and a polyclonal antibody raised against one begomovirus may detect the presence of others.

We have used a polyclonal antibody raised against the TYLCV (Israel) coat protein expressed in *E. coli*, a gift from Dr. R. Gilbertson (University of California-Davis). This antibody has a broad-spectrum and is able to detect several TYLCV isolates as well as other begomoviruses such as *African cassava mosaic virus* (ACMV); *Watermelon chlorotic stunt virus* (WmCSV) and *Abutilon mosaic virus* (AbMV).

Materials and Methods

Immunocapture (IC)

1. Add 200 µl of anti-TYLCV antibody (diluted 1:1000 in coating buffer) to each well of ELISA plate, or to PCR tube. The coating buffer is: for 1 liter, pH 9.6: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3.
2. Incubate at 37ºC for 3-4 h. Empty the ELISA wells/PCR tube and wash 3 times with TBST. TBST is 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20.
3. Homogenize infected leaves (1 g in 20 ml) with extraction buffer I. Alternatively, cut the infected leaf into small pieces and add 0.1 g to 100-200 µl extraction buffer II into the well or the tube. Alternatively, homogenize 1 to 5 whiteflies with 100-200 µl extraction buffer II. Extraction buffer I is for 1 liter, pH 9.0: 2.4 g Tris, 8 g NaCl, 0.5 ml Tween 20, 0.2 g KCl, 0.2 g NaN_3. Extraction buffer II is for 1 liter, pH 9.0: 2.4 g Tris, 20 g PVP, 8 g NaCl, 0.5 ml Tween 20, 0.2 g KCl, 0.2 g NaN_3.
4. Add 100-200 µl of the homogenate to the coated ELISA well/PCR tube and incubate for 18 h at 4ºC.

5. Empty the ELISA wells/PCR tubes and wash 3 times with TBST.
6. Dry the ELISA wells/PCR tubes, add 10 µl ddH_2O and boil at 100 ºC for 5 min.

PCR Primers

TYLCV: V61 (nt 61-80, viral strand, 5'ATACTTGGACACCTAATGG3') and C473 (nt 473-457, complementary strand, 5'AGTCACGGGCCCTTACAA3').

AbMV: AbAV356 (nt 356-379, virion strand, 5' CAAAATGCCTAAGCGCGATCTCCC 3') and AbAC1117 (nt 1117-1096, complementary strand, 5' TTTATTAATTCATGAGCGAATC 3').

PCR Reaction

1. The PCR reaction contains 5 µl from the IC reaction, 0.25 µl 25 mM dNTPs, 1 µl of each primers 1 and 2 (100 pmoles each), 2.5 µl Taq 10 x buffer and 1 unit Taq polymerase; add ddH_2O to a final volume of 25 µl.
2. Cycle: 1 cycle of 95ºC for 3 min 55ºC for 2 min, 72º C for 2 min; then 30 cycles of 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min; end with an additional cycle of 72 ºC for 10 min.
3. Subject the reaction products to 1% agarose gel electrophoresis.

Results

The method was applied to the detection of TYLCV in tomato plants and in whiteflies. The same procedure was used to detect another geminivirus present in Israel, AbMV, and ACMV and WmCSV from dry material. The following results are from:

Ghanim M, and Czosnek H (2000) Tomato yellow leaf curl geminivirus (TYLCV-Is) is transmitted among whiteflies (*Bemisia tabaci*) in a sex-related manner. Journal of Virology 74: 4738-4745.

Morin S, Ghanim M, Sobol I, and Czosnek H (2000) The GroEL protein of the whitefly *Bemisia tabaci* interacts with the coat protein of transmissible and non-transmissible begomoviruses in the yeast two-hybrid system. Virology 276:404-416.

1) Detection of TYLCV in whiteflies and tomato plants

Fig 1. WF* and WF: viruliferous and non-viruliferous whitefly. P*: infected plant. 0A: no antibody; 0E: no plant or whitefly extract.
1 to 9: samples tested.

2) Detection of AbMV in whiteflies and infected Abutilon plants

Fig 2.
M: molecular weight markers.
W* and W viruliferous and non-viruliferous whiteflies (20 individuals).
P*: infected tomato plant.
C: Cloned AbMV DNA-A.
0A: no antibody; 0: no plant or whitefly extract.
1,2 or a,b: samples tested.

Technical Sheet No. 37

Virus Detection: *Tomato yellow leaf curl virus* Isolates

Method: Multiplex Polymerase Chain Reaction

General
Virus Detected: TYLCV isolates from tomato plants.
Method: Multiplex Polymerase Chain Reaction

Developed By
Name of researchers: Ghandi ANFOKA, Al-Balqa Applied University; Mohammad ABHAARY, Al-Balqa Applied University; Mark NAKHLA, University of Wisconsin-Madison
Addresses (e-mail): anfoka@cyberia.jo, ghanfoka@yahoo.com, mkabhary@yahoo.com, mknakhlaakhla@plantpath.wisc.edu

Goal

The development of a rapid multiplex PCR protocol to detect and differentiate between isolates of TYLCV from tomato tissues.

Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most damaging diseases of tomatoes worldwide. Several virus species belonging to the genus Begomovirus of the family Geminiviridae have been reported to cause TYLCD (Rybicki et al., 2000). Two TYLC virus species are known to cause TYLCD in the Western Hemisphere: *Tomato yellow curl virus* (TYLCV, formerly TYLCV-Israel) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV, formerly TYLCV-Sardinia). Both species occur in Spain and Italy (Kheyr-Pour et al., 1991; Moriones et al., 1993; Navas-Castillo et al., 1999; Accotto et al., 2000; Sánchez-Campos et al., 2002) and recently TYLCSV was reported in Morocco (K. El Mehrach and D. P. Maxwell, personal communication). Identification of the virus isolates at the molecular level is a prerequisite to developing effective control measures against the most severe isolates of this virus. Therefore, the aim of this study was to develop a fast, simple, reliable and one-step PCR technique to detect two isolates of TYLCV and one isolate of TYLCSV.

Materials and Methods

Nucleic Acid Extraction

Total nucleic acid was extracted from infected tomato leaves according to the Dellaporta heat extraction method as described previously by Potter *et al.* (2003). In brief, 50-100 mg of tomato leaves were ground in liquid nitrogen using sterilized Kontes micropestle (Kontes Glass, Vineland, NJ, USA). To each tube, 500µl of Dellaporta extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM 2-β-mercaptoethanol) was added. The tubes were incubated at 65°C for 10 min. After centrifugation at 14,000 rpm for 10 min, the supernatants were transferred into new tubes and used for PCR.

Design of isolate-specific PCR primer pairs

To develop isolate-specific primer pairs, the sequence of three TYLCV isolates (TYLCV, X15656, TYLCSV-ES[2], L27708 and TYLCV-Mld, X76319) were analyzed using the DNAMAN software program (Lynnon BioSoft, Que., Canada). The consensus sequence was analyzed as a total genome and for all ORF of the virus. Different dissimilarity regions of the genome were used to generate isolate-specific primer pairs to anneal to the different virus isolates. The sequences of the generated primer pairs are shown in table 1 and the location of each primer on the genome of TYLCV is shown in Fig. 1. Before use, the concentration of all primers was adjusted to 10 µM with MilliQ water.

Table 1. Specific Polymerase Chain Reaction (PCR) primer pairs designed to differentiate among isolates of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV).

Isolates	Primers	nt. Position	Sequence (5'-3')	Size (bp)
TYLCSV-ES[2]	Almv2516	2516	TTTTATTTGTTGGTGTTTGTAGTTGAAG	433
	Almc115	115	ATATTGATGGTTTTTTCAAAACTTAGAAG	
TYLCV	TYv2337	2337	ACGTAGGTCTTGACATCTGTTGAGCTC	634
	TYc138	138	AAGTGGGTCCCACATATTGCAAGAC	
TYLCV-Mld	TYm2664	2664	ATTGACCAAGATTTTTACACTTATCCC	316
	TYc138	138	AAGTGGGTCCCACATATTGCAAGAC	

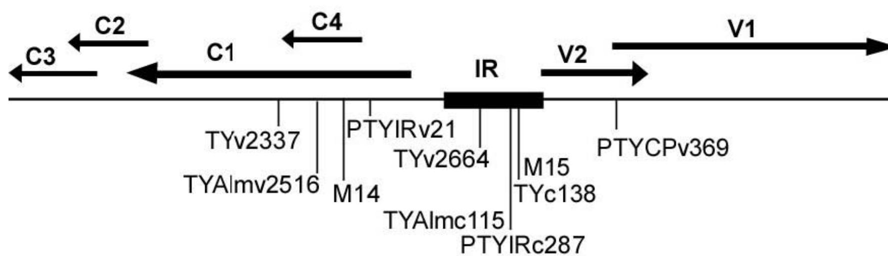


Figure 1. Genome organization of *Tomato yellow leaf curl virus* (TYLCV). Open reading frames (ORFs) are shown as black arrows. The ORFs of plus-strand (virion strand) polarity are designated *V1* and *V2*, ORFs of minus-strand (complementary strand) are designated *C1* through *C4*. IR indicates the intergenic region, the position of the developed primers is indicated.

Polymerase Chain Reaction

All components of the PCR reaction were from Promega, Co., Madison, WI, USA, unless otherwise stated. The parameters for the PCR reaction were optimized for 25 µl and the final concentrations of reaction components were: 25 µM deoxynucleotide triphosphate (dNTPs), 2.5 µl of 10X PCR buffer, 2.5 mM $MgCl_2$, 5 units *Taq* DNA polymerase, 0.5µl of 10 µM each complementary and viral-sense primers (1µl of primer TYc138) and 3 µl of six dilutions of DNA

(520 µg/ml, 52 µg/ml, 5.2 µg/ml, 520 ng/ml, 52 ng/ml, 26 ng/ml 13 ng/ml and 6.5 ng/ml) were used as target templates. PCR cycle parameters were as follows: one cycle at 94°C for 2 min; 30 cycles at 94°C for 1 min, 62°C for 90 sec, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 62°C for 1 min, and 72°C for 5 min. All PCR reactions were performed in a programmable thermal controller (model PTC-200, MJ Research Inc., Watertown, MA, USA).

Results and Discussion

Different PCR primer pairs were developed to differentiate between isolates of TYLCV and TYLCSV. To test the specificity of the generated primer pairs DNA from clones of different TYLCV and TYLCSV-ES[2] isolates were used as target template for the PCR reaction. As shown in Fig. 2, the developed primer pairs were able to specifically amplify the expected sizes from each virus isolate.

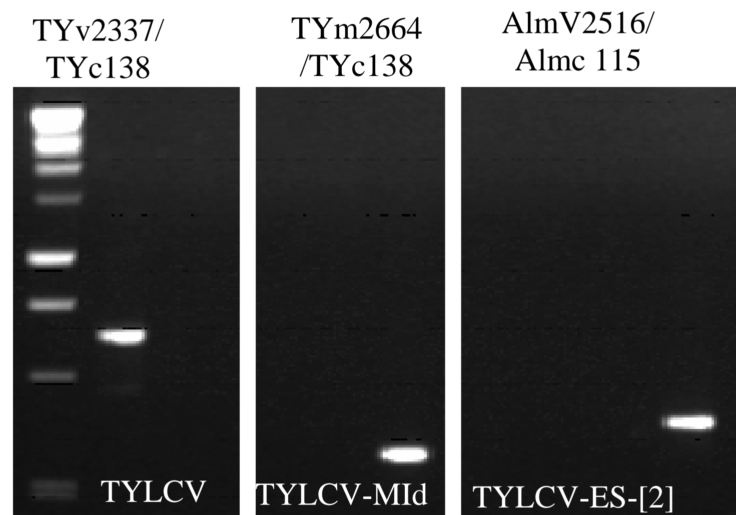


Figure 2. Specific amplification of two isolates of TYLCV and one isolate of TYLCSV using specific primer pairs by one-step multiplex polymerase chain reaction (PCR). Lane 1, 1 Kb DNA marker; lanes 2, 5 and 8, TYLCV; lanes 3, 6 and 9 TYLCV-Mld; lanes 4, 7 and 10 TYLCSV-ES[2].

To determine the detection level (sensitivity) of the multiplex PCR protocol developed using the specific primer pairs, different dilutions (52 µg/ml, 5.2 µg/ml, 520 ng/ml, 52 ng/ml, 26 ng/ml and 13 ng/ml) of the template DNA were used in the PCR reaction mixture. As shown in Fig. 3, multiplex PCR is very sensitive and could detect TYLCV and TYLCV-Mld at a dilution level of the template DNA of 6.5 ng/ml.

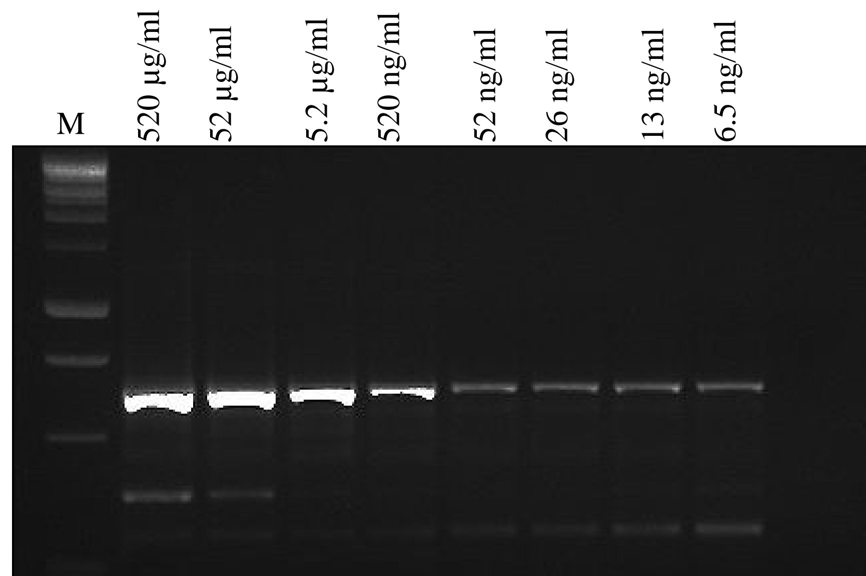


Figure 3. Detection of TYLCV, TYLCV-Mld and TYLCSV-ES[2] by multiplex PCR at different concentrations of the template DNA. M: DNA marker.

Since the main aim of developing this protocol is to be able to detect different isolates of TYLCV in infected tomato field samples in one PCR reaction, DNA from tomato plants known to be infected with two isolates of TYLCV and one isolate of TYLCSV was extracted as mentioned above and used as template in the PCR reaction. Fig. 4 shows that the developed protocol is efficient in detecting TYLCV, TYLCV-Mld and TYLCSV-ES[2] in infected tomato tissues.

Figure 4. Agarose gel electrophoresis of multiplex polymerase chain reaction (PCR) for the detection of TYLCV isolates from symptomatic field samples. Lane 1, 1Kb DNA marker; lane 2, 3 and 4, represent DNA from TYLCV, TYLCSV-ES[2] and TYLCV-Mld clones, respectively. Lane 5; DNA extracted from healthy tomato plant, lanes 6-10; DNA obtained from symptomatic tomato plant.

References

Accotto, G. P., Navas-Castillo, J., Noris, E., Moriones, E., and Louro, D. 2000. Typing of tomato yellow leaf curl viruses in Europe. European Journal of Pathololgy 106: 179 – 186.

Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, GP., Crespi, S., and Gronenborn, B. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Research 19: 6763 - 6769.

Moriones, E., Arnó, J., Accotto, G. P., Noris, E., and Cavallarin, L. 1993. Firs report of tomato yellow leaf curl virus in Spain. Plant Disease 77: 953.

Navas-Castillo, J., Sánchez-Campos, S., Díaz, J. A., Sáez-Alonso, E., and Moriones, E. 1999. Tomato yellow leaf curl virus-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. Plant Disease 83: 29 - 32.

Potter, J. L., Nakhla, M. K., Mejía, L., and Maxwell, D.P. 2003. PCR and DNA hybridization methods for specific detection of bean-infecting begomoviruses in the Americas and Caribbean. Plant Disease 87: 1205-1212.

Rybicki, E. P. 1994. A phylogenetic and evolutionary justification for three genera of Geminiviridae. Achieves of Virology 139: 49 - 77.

Sánchez-Campos, S., Díaz, J. A., Monci, F., Bejarano, E. R., Reina, J., Navas-Castillo, J., Aranda, M. A., and Moriones, E. 2002. High genetic stability of the begomovirus *Tomato yellow leaf curl Sardinia virus* in southern Spain over an 8-year period. Phytopathology 92: 842 - 849.

TECHNICAL SHEET No. 38

Virus Detection: *Tomato yellow leaf curl virus* (TYLCV)

Method: Squash Blot PCR

General
Virus detected: TYLCV from tomato plants and whitefly
Method: Squash blot PCR

Developed by
Name of researcher: Gil Atzmon, Fouad Akad and Hanokh Czosnek, The Hebrew University
Address (Email): akad @agri.huji.ac.il; czosnek @agri.huji.ac.il
Date: Oct. 8, 2002

Goals

To develop a rapid and very sensitive method for the detection of TYLCV in the tissues of whiteflies and plants. This method allows detection of TYLCV in a large number of samples (leaves and insects) from the field. The nucleic acid is stable up to several weeks at ambient temperature.

Introduction

Tomato yellow leaf curl virus, TYLCV, is the name given to a large number of genetically diverse whitefly-transmitted viruses infecting tomato. TYLCV infection reduces yields considerably; losses may reach 100% of the crop. Information about geminivirus can be found on the Web at Gemininet (http://www.danforthcenter.org/iltab/geminiviridae).

Based on sequence comparison, the various TYLCV isolates or different species can be grouped according to a geographically based scheme (Zeidan et al., 1998). 1. From the Middle East (Israel, Egypt, Jordan, Lebanon, Northern Saudi Arabia) (TYLCV) (Navot et al., 1991). 2. From Southwest Europe (Italy) (*Tomato yellow leaf Sardinia virus*, TYLCSV) (Kheyr-Pour et al., 1991). 3. From tropical Africa (Senegal, Tanzania), and 4. From Southeast and East Asia (Thailand, China). TYLCV from the Caribbean Islands and from the Southeast USA originated from the Middle East. All these isolates, except TYLCV from Thailand, have a monopartite genome. This virus is also called *Tomato yellow leaf curl Thailand virus*. Some related whitefly-transmitted viruses infecting tomato are also called *Tomato leaf curl virus*, ToLCV, and have been found in India and Australia. The ToLCV isolates have monopartite genome, except of ToLCV from Northern India. For some of the tomato-infecting begomoviruses there is evidence of recombination (Chatchawankanphanich and Maxwell, 2002).

PCR has been a common method for the detection of geminiviruses using degenerative primers (Rojas et al., 1993; Wyatt and Brown, 1996). Discrimination between the different geminiviruses can be done using specific PCR primers.

Materials and Methods

Tomato squashes were done using symptomatic leaves from the field, inoculated in the lab with viruliferous whiteflies or by agroinoculation. The method is based on (but not identical to) the one reported in Atzmon et al. (1998):

Sampling

1. Squash tomato leaves (young leaf from the shoot apex, or individual whitefly) on Whatman 3MM paper using a glass rod, an Ependorf tube or a pen cover.
2. Cut out a ~ 1 x 2 mm piece and put it into a PCR tube.
3. Add 0.1 ml 1% Tween-20, and incubate at 65°C for 15 min.
4. Discard the solution and add 0.5 ml 70% ethanol and incubate at room temperature for 10 min.
5. Discard the ethanol and dry the squash at 65°C.

PCR primers

TYLCV (X15656) DNA fragments were amplified using the following pair of primers (0.2 mM each) deduced from the nucleotide (nt) sequence of the genome of TYLCV from Israel (Navot et al., 1991):

Specific primer pairs:

1) V61 (nt 61-80, viral strand, 5'ATACTTGGACACCTAATGG3') and C473 (nt 473-457, complementary strand, 5'AGTCACGGGCCCTTACAA3').

2) V781 (nt 781-800, viral strand, 5'CTCACAGAGTGGGTAAGAGG3') and C1256 (nt C1256-1229, complementary strand, 5'TTAATTTGATATTGAATCATAGAAATAG3').

3) V1769 (nt 1769-1790, viral strand, 5'GCGAACAGTGGCTCGTAGAGGG3') and C2120 (nt 2120-2097, complementary strand, 5'CAGGCAAAACAATGTGGGCCAGG3').

PCR

1. Add the PCR reaction mix (with TYLCV-specific primers, 0.2 mM each) to the microfuge tube with the squash blot.
2. Cycle: 1 cycle of 95°C for 3 min, 55°C for 2 min, 72°C for 2 min; then 30 cycles of 95°C for 1 min, 60°C for 1min, 72°C for 1 min; end with an additional cycle of 72°C for 10 min.
3. Subject PCR products to electrophoresis in 1% agarose gel in TAE buffer.

Results

Amplified TYLCV DNA: ~ 400-bp PCR product, primer pair V61 and C473.

M I 0 2 5 7 C

M: molecular weight markers
I: infected plant,
0: non-infected plant,
C: no template.
2, 5, 7: days after squash sampling

References:

Atzmon G, van Hoss, H., and Czosnek, H. 1998. PCR-amplification of tomato yellow leaf curl virus (TYLCV) from squashes of plants and insect vectors: application to the study of TYLCV acquisition and transmission. European Journal of Plant Pathology 104:189-194

Chatchawankanphanich, O. and D.P. Maxwell. 2002. *Tomato leaf curl Karnataka virus* from Bangalore, India appears to be a recombinant *Begomovirus*. Phytopathology 92:637-645.

Navas-Castillo, J., Sánchez-Campos, Noris, E., Louro, D., Accotto, G. P., and Moriones, E. 2000. Natural recombination between Tomato yellow leaf curl virus-Is and Tomato leaf curl virus. J. Gen. Virol. 81:2797-2801.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. Tomato yellow leaf curl virus: A whitefly-transmitted geminivirus with a single genomic component. Virology 185:151-161. (GenBank no. X15656)

Rojas, M. R., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis. 77:340-347.

Wyatt, S. D., and Brown, J. K. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. Phytopathology 86:1288-1293.

Zeidan, M., Green, S. K., Maxwell, D. P., Nakhla, M. K., and Czosnek, H. 1998. Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia. Trop. Agr. Res. Exten. 1:107-115.