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
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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).

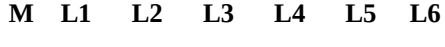

Fig. 2. Agarose gel electrophoresis analysis of DIG-labelled RT-PCR products of PLRV. M: 100 bp ladder; L1: 250 ng total of Dig-labelled RT-PCR product; L2: 25 ng total of Dig-labelled RT-PCR product; L3: 250 pg total of DIG-labelled RT-PCR product; L4: 25 pg total of DIG-labelled RT-PCR product L5: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