

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

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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 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 RNAagents 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 Knott's 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 ($\sim 600 \mu\text{l}$) 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 GCNCCNGTNGTNTGGAAAYTGG 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 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 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 -stock solution	3 μl	0.5-2.5 mM
PCR Dig labelling mix	5 μl	200 μM
Viral sense primer (PPVXv1)	1.25 μl	250 nM
Complementary sense primer (PPVXc2)	1.25 μl	250 nM
<i>Taq</i> DNA polymerase	0.25 μl	2.5 U
DNA	2.5 μl	1 fg to 500 ng

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.

(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 μ l of RT-PCR-DIG labelled amplified product with 20 μ l 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 μ l of hybridization solution containing 50 ng/ml of DNA capture probe.
3. Pipette 200 μ l 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 μ l of polyclonal anti-DIG Fab fragments, conjugated to peroxidase diluted 1:100 in Tris-HCl (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 μ l of substrate solution (100 μ g/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 CAAACAGAAATGATAGACACAGGCCCTATTCCAACGGCATCAGCAGAGCCAGACTGGCAGCA
 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 GCAATTAAGAGGGTGTGCACACTTAGGCAATTTTGCATGAAGTATGCCCCAGTGGTATGGAAC
 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 AAATTCGCAGCATTGACTTCTTCAATGGAGTCACCAACCCAGCTGCCATCATGCCCAAAGAG
 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

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