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

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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^{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. 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:

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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
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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.

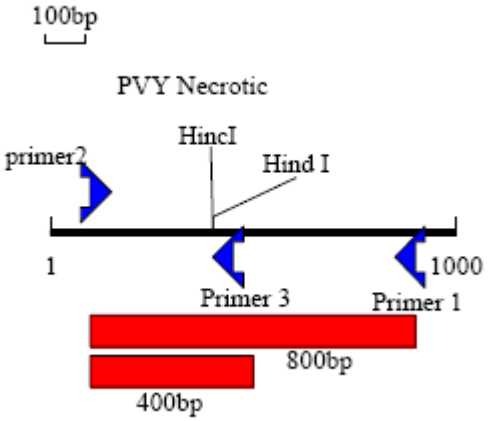
One step RT-PCR

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

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