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
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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'GCGTTAATTAATTTTTTTTTTTTTTTTT-TTTTTTTTTTGTCTCCTGATTGAAGTTTACAGTC3' 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

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