Egyptian J. Virol. 1, 121-127 (2004)

Molecular Characterization of *Potato Spindle Tuber Viroid* Egyptian Isolate

E. K. Allam [1]; Kh. A. El-Dougdoug [1]; M. A. Amer[2] A. A. Abou-Zaid [2]; and S. M. Amin [2]

[1] *Microbiology Department (Virology lab.), Faculty of Agricultue,Ain Sha University,Shoubra El-Kheima, Cairo, Egypt.* [2] *Virus and Phytoplasma Research Departmen, Plant Pathology Research Institute, Agricultural Research Center , Giza . Egypt.*

Potato spindle tuber viroid (PSTVd) was detected in potato plants grown in Egypt using return –polyacrylamide gel electrophorsis (R-PAGE) and molecular techniques. The method involved the extraction of circular nucleic acids followed by electrophoretic analysis of the extracts on 5 % polyacrylamide gel. A reverse transcription-polymerase chain reaction (RT-PCR) assay was used for the isolation and identification of PSTVd cDNA from infected plant materials using specific primer to PSTVd. A major cDNA product of approximately 360 bp was successfully hybridized with digoxigenin labeled PSTVd cDNA probe.

INTRODUCTION

Viroids are unencapsidated single stranded, circular RNA molecules with a size ranging between 246 to 463 nucleotide residues (Diener, 1987 and Singh & Dhar, 1998). In their native state, viroids have been shown to exist as rod –like structures (Riesner, 1987). They replicate in plant cells via RNA –RNA pathway utilizing the RNA replicative machinery of the host cell (Sanger, 1987).

Potato spindle tuber viroid (PSTVd) is the first member of this peculiar group of plant pathogenic agents to have been characterized (Diener, 1987; Gross and Riesner, 1980; Riesner and Gross, 1985 and Semancik, 1987). It is also the first pathogenic agents to have been completely sequenced (Gross *et al.*, 1978) and for which infectious cDNA molecules have become available (Cress *et al.*, 1993). This single stranded, circular RNA molecules (356–360 nucleotides long) is an autonomous replicon which induces specific symptoms in its host while, at the same time completely lacking polypeptide coding capacity (Gross and Riesner, 1980; Riesner and Gross, 1985; Diener, 1987 and Semancik, 1987). PSTVd infection might cause as much as 65% reduction in yield of potato tubers (Singh *et al.*, 1971). PSTVd is readily transmissible by mechanical means and is highly contagious. It is also highly seed transmissible in several host plants (Benson and Singh., 1964; Hunter *et al.*, 1969; Singh and Finnie, 1973 and El-Doug-doug , 1988). Several methods have been described for the detection of PSTVd from infected tissues (Singh and Dhar, 1998). These include biological indexing on indicator plants, Return polyacrylamide gel electrophoresis (R-PAGE), reverse transcription-polymerase chain reaction (RT-PCR) and molecular hybridization using radioactive and non-radioactive labeled PSTVd cDNA probe.

Using of return polyacrylamide gel electrophoresis(R-PAGE) has facilitated detection of PSTVd from individual true potato seed (TPS) or TPS seedling grown *in vitro* (Singh *et al.*, 1988). Semancik and Weather (1972) first reported the isolation and detection of a similar viroid RNA

from citrus exocortis infected plants using polyacrylamide gel electrophoresis. Morris and Wright (1975) subsequently published a diagnostic procedure for PSTVd using nucleic acid extraction and polyacrylamide gel electrophoresis. In this paper the successful use of the molecular techniques for detection and identification of full length of PSTVd cDNA from infected potato plants were used.

MATERIAS AND METHODS

Source of viroid isolate

Foliage samples showing veinal necrosis were collected from individual symptomatic potato plants *Solaum tuberosum* cv. Sponta. For sap inoculation, seedlings of tomato cv. Rutegers were inoculated by rubbing with an extract prepared by grinding infected leaf tissue (1 g fresh weight) with 2-5 ml of 0.1 M Sodium phosphate buffer, pH 7.2 containing a small quantity of carborundum. Approximately 2-5 min after being inoculated, leaves were rinsed with distilled water. Test plants were kept under observation for 3-4 WK post inoculation, then assayed for the presence of PSTVd by different molecular techniques.

Direct double antibody sandwich (DAS-ELISA) demonstrated by Clark & Adams (1977) was used for detection of potato viruses: potato virus Y, potato virus X, potato virus A, potato virus M , potato virus S and potato leaf roll virus. ELISA kit's were supplied by SANOFI (Sante Animale, Paris, France).

Detection of PSTVd using (R-PAGE)

Extraction of the viroid- RNA procedure from leaf and tuber tissues was carried out using phenol according to Morris and Smith (1977).The extracted RNA was fractionated by LiCl. Analysis of low molecular weight RNA by R-PAGE was conducted essentially as described by Singh and Boucher (1987). After the second electrophoresis, RNA was visualized by silver staining.

Viroid cDNA synthesis and polymerase chain reaction

Specific oligonucleotide primers were used as published by Levy *et al.* (1994) and Shamloul *et al.* (1997). The primer A was synthesized complementary to nucleotides 69-88 (5`- ccc tga agc gct cct ccg ag -3` and primer B in sense orientation to nucleotides 89-113 (5`-atc ccc ggg gaa a cc t gga gcg aac -3`). The primers A and B define a target of 359 bp corresponding to the entire PSTVd sequence (Gross *et al.*, 1978).

For first stranded cDNA synthesis and PCR assay, purified circular RNA (1 µg) was mixed with 50 pmol of primer A, heated to 95 °C for 3 min and quickly cooled on ice. Reverse transcription of viroid RNA was carried out in a 20 µl reaction containing the template and primer A, 500 µM each DNTPs, 200 unit of MMLV reverse transcriptase (Promega). The mixture was incubated at 42 °C for 45 min.

PCR was carried out in a 50 µl reaction mixture containing 5 µl of cDNA reaction ,10 pmol each primer A and B, 200 µm of each DNTPs, 1.5 mM MgCl2, and 2.5 U of Taq DNA polymerase (Promega) in the reaction buffer provided by the same source. The mixture was incubated for 5 min at 85°C followed by thermal cycling for 40 cycles of 45 sec at 94°C for denaturation, 45 sec at 62°C for annealing and 1 min at 72°C for extention with final extention of 7 min at 72°C followed by cooling at 4°C.

Analysis of RT-PCR amplified product

The amplification products (5 µl) were analysed by electrophoresis in a 1 % agarose gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA pH 8.3) at 100 volt for 1 h. Gel was stained with ethedium bromide. 50 bp PCR marker (Promega) was used to determine the size of RT-PCR amplified products.

Preparation of cDNA PSTVd probe and southern blot hybridization.

The obtained inserts from RT-PCR products were labeled the PCR technique using the Genius TM System (Boehringer Mannheim Corp.) according to the manufacturer's instructions. DNA fragment (359 bp) was amplified by PCR from PSTVd infected potato plants using the same specific primer for PSTVd, which used as the template to synthesize a cDNA. The following components were added to a sterile microcentrifuge tube placed on ice during pipetting as follows: 5 µl 10 X PCR buffer without MgCl2; 1.5 mM MgCl2 stock solution; 5 µl 10 mM dNTP labeling mixture, 1 µl upstream and downstream primer (10 pmol) ;32.5 µl H2O; 0.5 µl Taq DNA polymerase; 5 µl template cDNA. The volume was completed to 50 µl with H2O. The reagents were mixed, and centrifuged briefly to collect the sample at the bottom of the tube and amplified in thermal cycler (Biometra Inc). Cycling parameters for the reaction were as follows: denatured at 95°C for 3 min before the first cycle. Denatured at 94°C for 45 sec, annealed at 62°C for 45 sec, extend at 72°C for 1 min for 40 cycles with final extension at 72°C for 7 min. The capillary transfer of plant DNA from the gel to nylon membrane support was achieved using the southern technique (Southern, 1975). Nucleic acid hybridization was done according to the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany).

RESULTS AND DISCUSSION

The biological characteristic of the agent responsible for veinal necrosis was consistent with a viroid etiology for this disease. Tests for a variety of common potato viruses (PVX, PVY, PLRV , PVM , PVS and PVA) were negative using DAS-ELISA. After mechanical inoculation S. tubersum cv Sponta plants became stunted and epinastic. Symptoms was visible in the dark green foliage of these plants and a few small tubers developed roughened skins without spindling (Fig.1). This viroid also readily infect tomato cv. Supermarmande and produced rugosity, epinasity and deformation of infected tomato plants. Fig (2).

In certain case, a viroid like RNA could be visualized in low molecular weight RNA preparation from infected plant tissues. Return polyacrylamide gel electrophoresis analysis of one such purified RNA revealed the presence of a viroid whose mobility was very similar or identical of that of the PSTVd. Fig (3) shows R-PAGE analysis of PSTVd isolated from potato tuber, leaves and tomato leaves and RNA extracted from uninfected potato tuber.

In preliminary experiments with return gel electrophoresis, a viroid like nucleic acid was detected in total nucleic acid extracted from potato plants. To determine the possible relation of this viroid like agent to PSTVd, DNA primers for PSTVd in an RT-PCR assay were used to amplify cDNA from purified nucleic acid extracts of infected potato plants. For control we used nucleic acid extracts of uninfected potato

plants. The amplification of PSTVd cDNA infected potato tuber and tomato leaves by RT-PCR was specific. Fig(4) shows agarose gel electrophoresis analysis of RT-PCR amplified products of PSTVd that was reverse transcribed from RNA of PSTVd infected potato tuber and tomato leaves .A major DNA product of approximately~360 bp (full length), A (lane 1 and 2) , that hybridized with digoxigenin labeled PSTVd cDNA probe , B (lanes 1 and 2). No amplified PSTVd cDNA product or hybridization was detected with extracts from uninfected potato tuber A and B (lane 3).

PSTVd is a single stranded circular RNA molecules of commonly 359 nucleotides (Gross *et al.*, 1978). Since it contains no capsid proteins in contrast to viruses (Diener, 1979), serological methods like enzyme – linked immunosorbent assay (ELISA) are not be used for viroid detection.

PSTVd is commonly detected by bioassay with tomato cv. Rustgers (Raymer and O'Brin, 1962) or Scopolia sinensis (Singh *et al.*, 1971). In RT-PCR as in other assays, a critical step in the procedure is sample preparation. The extreme sensitivety of the PCR assay necessitates carful consedration of potential pathway for samples to become contaminated. The very sensitivity of the PCR can render it useless if precautions are not be taken to prevent contamination by nucleic acid spot hybridization using radioactive labeled cDNA probes (Owens and Diener , 1981), non-radioactive cDNA and cRNA probes (Roy *et al.*, 1989; Welnicki and Hiruki, 1992 and Romero-Duriban *et al.*, 1995) , and R-PAGE (Schumacher *et al.*, 1986 and Schroeder & Weidemann, 1989), but the technique involves extensive sample preparation and requires considerable technical expertise.

The non-radioactive labeling systems are safer and more convenient to use than P^{32} radioactive labelling system. The digoxigenine probes are more stable than their radioactive counterparts. The non-radioactive probes are stable for at least a year when stored at -20 C. Bioassay are unreliable because symptoms may be absent particularly with mild PSTVd strains. Nucleic acid hybridization and R-PAGE methods are laborious time consuming. RT-PCR has proved to be rapid and sensitive techniques for detection of RNA viruses (Henson and French, 1993) and viroids (Hadidi and Yang, 1990; Yang *et al.*, 1992; Rezaian *et al.*, 1992; Levy *et al.*, 1994 and Shamloul *et al.*, 1997).

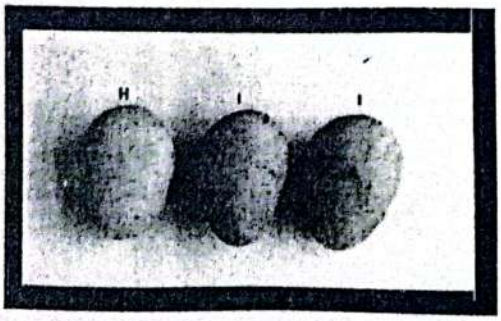

Fig (1) Left: Healthy and, Right: PSTVd infected potato tubers cv. Sponta showing roughened skins with spindling.

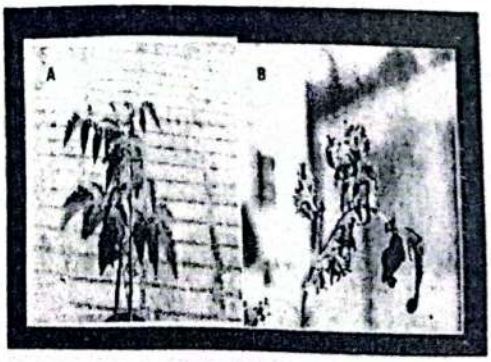

Fig (2) Left: (A) Healthy tomato plant, Right (B)Tomato plants cv. Supermarmande i nfected with PSTVd showing rugosity, epinasty and leaflets deformation.

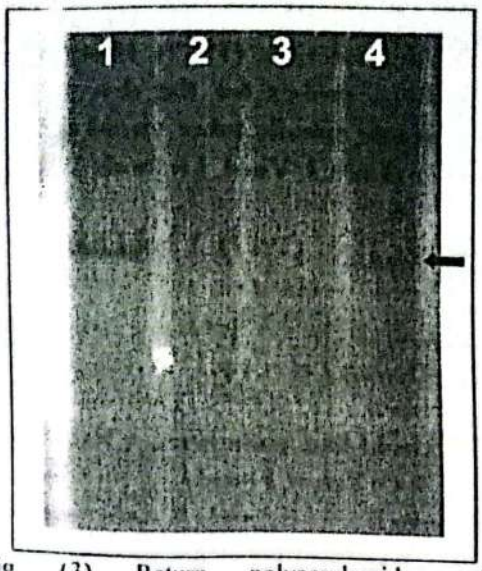

Fig (3) Return polyacrylamide gel electrophoresis silver nitrate stained showing fractionated RNA extracted from 5 g samples of potato and tomato plants. The gel contain nucleic acid extracted from potato tuber and leaves (lane 1 and 2) tomato leaves (lane 4) and uninfected potato tuber (lane 3). The arrow locates the position of the potato spindle tuber viroid RNA.

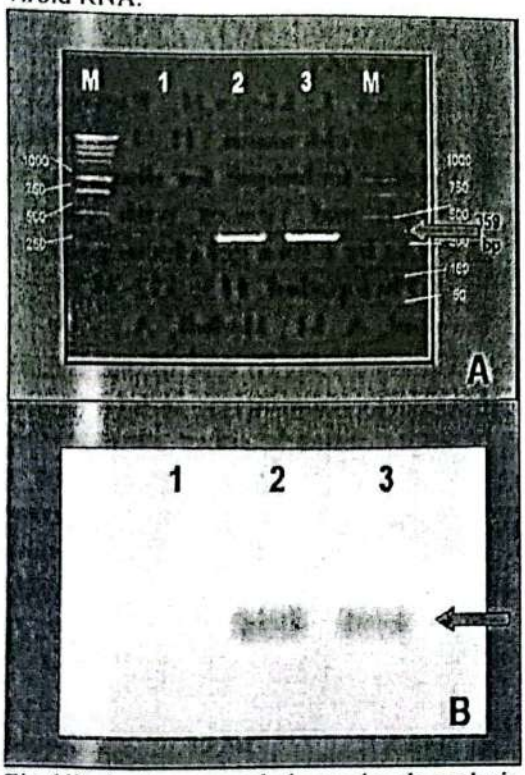

Fig (4): A: Agarose gel electrophersis analysis of RT-PCR amplified PSTVd cDNA products and B: Southern blot hybridization analysis of PSTVd cDNA with a DIG labeled PSTVd cDNA probe. Molecular PCR marker (50, 150 , 300, 500, 750 and 1000 bp) (lane M). The arrow indicates 360 bp, Total nucleic acid from PSTVd nfected potato tuber and tomato leaves (Lanes 2 and 3) and uninfected potato tuber (lane 1).

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