

A New Mild Strain of Potato Spindle Tuber Viroid Isolated from Wild *Solanum* spp. in India

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ABSTRACT

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A disease syndrome whose symptoms include stunting, leaf crinkling, and severe veinal necrosis was observed among four wild *Solanum* spp. maintained at the Central Potato Research Institute, Simla, India. Consistent with a possible viroid etiology, transfer of the infectious agent to tomato (*Lycopersicon esculentum* 'Rutgers') produced symptoms that were indistinguishable from those induced by the mild strain of potato spindle tuber viroid (PSTVd), and dot blot hybridization analysis of infected plants using an RNA probe complementary to PSTVd yielded strongly positive reactions. Nucleotide sequence analysis of the isolates from *Solanum sucreuse*, *S. verrucosum*, and *S. chacoense* showed them to be identical, each containing two nucleotide substitutions within the central conserved region when compared with a mild strain of PSTVd isolated from potatoes grown in North America. The isolate from *S. multiinterruptum* was identical in sequence to the previously described mild strain of PSTVd.

Potato spindle tuber disease is known to occur in potato-growing regions of the United States, Canada, China, Argentina, and the former USSR (3,4). Although the infectious nature of this disease was recognized in 1923 by Schultz and Folsom (16), it was not until 1971 that Diener (2) demonstrated the agent responsible for the spindle tuber disease to be a viroid—a small, unencapsidated, single-stranded circular RNA molecule capable of replicating without a helper virus (see also Singh and Clark [19]).

Known strains of PSTVd contain 356–360 nucleotides (12,15), and the experimental host range includes a wide variety of *Solanaceous* spp. (17). The characteristic disease syndrome (epinasty, stunting, and rugosity) that appears after inoculation of certain tomato (*Lycopersicon esculentum* Mill.) cultivars with intermediate or severe strains of PSTVd is quite unlike that caused by other potato or tomato pathogens. Symptoms produced by mild strains of PSTVd in such bioassays, however, may be so mild or transient as to be easily overlooked (5).

Potato spindle tuber disease poses a potentially serious threat to the produc-

tion of seed potatoes, maintenance of potato germ plasm collections, and commercial cultivation. Crop losses are negligible when potatoes are grown under temperate conditions and disease incidence is kept low, i.e., approximately 1% (20). However, major efforts are now under way to adapt the potato for growth in subtropical and tropical climates, and under these conditions, spindle tuber may cause severe damage and total crop loss.

Some years ago, extensive veinal necrosis was noted among certain wild *Solanum* spp. in the germ plasm collection at the Central Potato Research Institute, Simla, India. Preliminary biological characterizations strongly suggested a viroid etiology (8). Here we report the results from a more complete series of biological characterization studies as well as nucleic acid hybridization and sequencing studies showing that three of the affected *Solanum* spp. contain a previously undescribed strain of PSTVd.

MATERIALS AND METHODS

Biological characterization. Foliage samples were collected from individual symptomatic *Solanum* spp. plants growing singly in clay pots under glasshouse conditions (27–35 C and 14 hr of natural daylight during the summer months). For sap (mechanical) inoculation, seedlings of tomato cv. Rutgers were inoculated either by the cut-leaf method (rubbing the fresh-cut edge of an infected leaf petiole on leaves pre-dusted with 600-mesh Carborundum) or

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, pH 7.2, containing a small quantity of Carborundum. Control plants were mock-inoculated with either leaf tissue collected from uninfected tomato/potato seedlings or sap extracts prepared from such tissue. Approximately 2–5 min after being inoculated, leaves were rinsed with distilled water. Test plants were kept under observation for 8 wk postinoculation, and symptom severity was scored on a scale of 0–3 (21). Samples collected from the uppermost leaves 3–4 wk postinoculation were assayed for the presence of PSTVd by molecular hybridization using a full-length ³²P-labeled RNA probe specific for PSTVd (11,14).

RNA extraction and return-polyacrylamide gel electrophoresis (R-PAGE). Procedures for the extraction and purification of low molecular weight RNA from frozen tomato leaf tissue by phenol-chloroform extraction and LiCl fractionation have been described by Owens and Diener (11). Although removal of DNA (by digestion with DNase) and polysaccharides (by extraction with ethylene glycol monomethyl ether) is not required for analysis by R-PAGE, these steps were included to ensure reliable and efficient synthesis and amplification of PSTVd cDNAs.

Analysis of low molecular weight RNA by R-PAGE was conducted essentially as described by Singh and Boucher (18) except that: 1) the polyacrylamide concentration was increased to 7.5% (39 acrylamide/1 N,N'-methylenebisacrylamide [w/w]) and 2) the "low-salt" buffer used for the second electrophoresis was heated to 100 C before being added to the upper and lower reservoirs. After the second electrophoresis, RNAs were visualized by silver staining.

Synthesis, amplification, and sequence analysis of viroid cDNAs. Owens et al (10) have described a strategy for the amplification and sequence analysis of randomly primed viroid cDNAs synthesized from low molecular weight RNA templates. Three different primer pairs were used to determine the complete nucleotide sequence of our four PSTVd

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isolates via the polymerase chain reaction (PCR). Specific cDNAs representing the left and right sides of PSTVd were amplified in PCR reactions containing equimolar mixtures of primer pairs HAD3 (5'-CTCCAGGTTTCCCGGG-3') plus RAO2 (5'-GCGGATCCG-GTGGAAACAACACTGAAGC-3') or HAD4 (5'-AGGGCTAACACCCTCGCCC-3') plus RAO14 (5'-AGGGATCCCCG-GGAAACC-3'), respectively; a third cDNA containing the upper portion of the central conserved region was generated using primers RAO2 and T2 (5'-CTGTTTCGGCGGGAATTA-3'). The single-stranded DNA templates required for nucleotide sequence analysis were then synthesized in asymmetric PCR reactions containing a 20:1 molar ratio of the original primer pair, and nucleotide sequence analysis was accomplished using the TaqTrack protocol (Promega Biotec, Madison, WI) and 5'-³²P-labeled primers. Annealing sites for all five PCR primers are shown in Figure 1.

RESULTS AND DISCUSSION

The biological characteristics of the agent(s) responsible for veinal necrosis in four wild *Solanum* spp. (*S. sucrense*, *S. chacoense*, *S. verrucosum*, and *S. multiinterruptum*) were consistent with a viroid etiology for this disease. Tests for a variety of common potato viruses (PVA, PVS, PVM, PVX, PVY, and PLRV) were negative, and the disease was readily transmissible via true seed, tubers, and contact. Nearly all the seedlings derived from seeds collected from diseased plants were stunted, and their foliage showed both crinkling and extensive, severe veinal necrosis. After mechanical inoculation, *S. tuberosum* L. 'Kufri Jyoti' and 'Kufri Chandramukhi' plants became stunted and epinastic.

Etching was visible in the dark green foliage of these plants, berry set was much reduced or absent, and the few, small tubers developed roughened skins without spindling. The disease agent also readily infected *Gomphrena globosa* L. and *Capsicum pendulum* Willd. plants, producing an ash-gray mottle and systemic necrosis, respectively.

Transfer of the agent to tomato cv. Rutgers produced neither stunting nor epinasty, but the leaf petioles and midveins of infected plants showed a slight twisting and the lamina tended to fold upward. Ultrastructural studies of leaf primordia revealed several types of aberrations previously reported to be associated with PSTVd infection, including unusual undulations and thickening of the cell walls and abnormal paramural bodies (9,22). Symptom development was accompanied by the appearance of small circular RNAs with electrophoretic properties similar to those of the mild strain of PSTVd (Fig. 2). Strong positive reactions were observed when samples of chloroformclarified sap prepared from infected plants were analyzed by dot blot hybridization using an RNA probe complementary to the intermediate strain of PSTVd (results not shown).

Although the viroid isolated from *S. multiinterruptum* was similar to the mild strain of PSTVd with respect to both biological properties and electrophoretic behavior (Fig. 2, lanes 3 and 4), those isolated from the other three wild *Solanum* spp. appeared to be somewhat different. Comparison of lanes 5-7 with lane 3 (PSTVd mild strain) and lane 4 (viroid isolated from *S. multiinterruptum*) suggested a slight difference between the electrophoretic mobilities of these viroids and that of the mild strain

of PSTVd. Previous attempts to resolve three mild North American PSTVd isolates by R-PAGE were unsuccessful (18), but whether or not these isolates actually differed in their nucleotide sequences is not clear.

To complete our characterization of the PSTVd-like viroids isolated from these four wild *Solanum* spp., we determined the complete nucleotide sequence of each isolate. The first step in our sequencing strategy involved the amplification (via the PCR) of randomly primed viroid cDNAs that had been synthesized from low molecular weight RNA templates. Three different primer pairs (RAO2 + HAD3, HAD4 + RAO14, and RAO2 + T2) were used to generate the overlapping double-stranded cDNAs necessary to determine the complete nucleotide sequence of each PSTVd isolate. The results from our sequencing studies with uncloned viroid cDNAs (Fig. 1) show that the four *Solanum* spp. examined contain two different PSTVd variants.

Consistent with its behavior during R-PAGE, the PSTVd variant isolated from *S. multiinterruptum* proved to be identical in sequence to the PSTVd mild strain commonly isolated from potatoes grown in North America and Europe. Compared with the intermediate or "type" strain of PSTVd, this viroid contains two changes in the pathogenicity domain, i.e., an A->U substitution at position 309 and a U insertion between positions 310 and 311, and a single change in the variable domain, i.e., an AA->U substitution at positions 120 and 121 (Fig. 1). The variant recovered from *S. sucrense*, *S. chacoense*, and *S. verrucosum* contained additional substitutions at positions 255 and 258 within the central conserved domain. None of

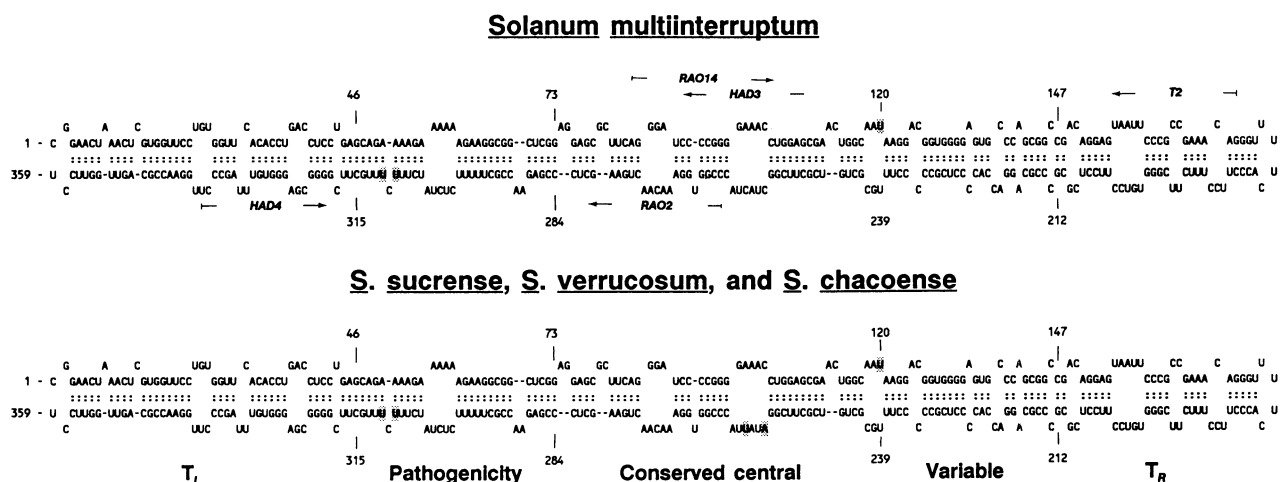


Fig. 1. Nucleotide sequence and possible secondary structures for two PSTVd-mild strains isolated from wild *Solanum* spp. Sequence differences from PSTVd intermediate strain (6) are shaded, and the boundaries of the five structural domains (i.e., left and right terminal loops, pathogenicity domain, central conserved region, and variable domain [7]) are marked. Secondary structures are patterned after those of Schnölzer et al (15). (Top) PSTVd mild strain isolated from *S. multiinterruptum*. The sequence of this variant is identical to that previously reported by Schnölzer et al (15). Horizontal arrows indicate annealing sites for the five primers used for PCR amplification and sequence analysis. (Bottom) PSTVd mild strain isolated from *S. sucrense*, *S. verrucosum*, and *S. chacoense*. Two additional nucleotide substitutions are present at positions 255 and 258 in the lower portion of the central conserved region.

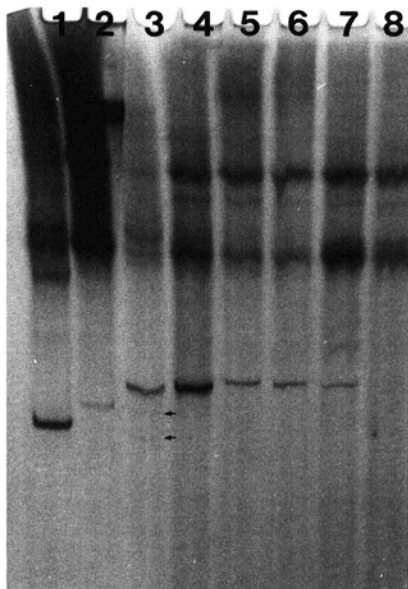


Fig. 2. Return-polyacrylamide gel electrophoresis of potato spindle tuber viroid (PSTVd) mild strains isolated from wild *Solanum* spp. All samples except that in lane 2 contain 10–20 μ g of 2 M LiCl-soluble RNA extracted from inoculated tomatoes. Lanes 1–3 and 8 are mobility standards: lane 1, tomato apical stunt viroid (TASVd); lane 2, PSTVd intermediate strain; lane 3, PSTVd mild strain; and lane 8, mock-inoculated tomato. Lanes 4–7 contain PSTVd strains isolated from four wild *Solanum* spp.: lane 4, *S. multiinterruptum*; lane 5, *S. sucrense*; lane 6, *S. verrucosum*; and lane 7, *S. chacoense*. The PSTVd mild strain preparation analyzed in lane 3 appears to contain trace amounts of both PSTVd intermediate strain (top arrow) and a second, slowly migrating sequence variant (bottom arrow).

these four independent isolates appeared to contain more than a single sequence variant, either by R-PAGE or by nucleotide sequence analysis.

Our results have several implications for future studies of both PSTVd epidemiology and molecular biology. With respect to the origin of both the PSTVd mild strain isolated from *S. multiinterruptum* and the novel variant present in the other three wild *Solanum* spp., it is likely that these germ plasm accessions were inadvertently infected with PSTVd during repeated propagation in the absence of rigorous screening by dot blot hybridization. Although Singh and Slack (21) found three of these wild *Solanum* spp. to be susceptible when mechanically

inoculated, PSTVd has never been detected in wild *Solanum* spp. growing in the Andes of South America (L. F. Salazar, *personal communication*). Furthermore, if these variants had arisen in the wild *Solanum* spp. rather than in the cultivated *S. tuberosum*, one would expect to observe latent infections rather than severe veinal necrosis, stunting, and epinasty.

Singh and Boucher (18) suggested that R-PAGE is able to resolve mild and severe strains of PSTVd because of conformational differences within the pathogenicity domain, or, more specifically, because of changes within a comparatively unstable portion of this domain known as “premelting region 1.” PSTVd and related viroids actually contain three premelting regions (13), but none of these regions includes positions 255 and 258. Additional studies will be required to identify all the structural features that influence the electrophoretic mobility of these stiff, basically rodlike molecules.

Finally, it is interesting to note that PSTVd positions 255 and 258 are also part of an ultraviolet light-sensitive structural element that is present in such disparate RNA molecules as hepatitis delta virus RNA, host 5S ribosomal and 7SL signal recognition particle RNAs, and PSTVd (1). Although the precise role of this conserved structural element in viroid replication and/or pathogenicity is unclear, the element seems able to tolerate a certain amount of sequence variability without loss of biological function.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. M88681.

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