Techniques

Electrophoretic Separation of a Severe From Mild Strains of Potato Spindle Tuber Viroid

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We thank Jatta Mueller-Derlich from the Abteilung Viroidforschung of the Max Planck Institute Fur Biochemie, Martinsreid near Munich, West Germany, for the initial assistance in the development of the method.

Accepted for publication 1 July 1987 (submitted for electronic processing).

ABSTRACT

Singh, R. P., and Boucher, A. 1987. Electrophoretic separation of a severe from mild strains of potato spindle tuber viroid. Phytopathology 77:1588-1591.

When nucleic acid extracts from leaves infected with various strains of potato spindle tuber viroid (PSTV) were electrophoresed by Return-polyacrylamide gel electrophoresis (R-PAGE), viroid RNA migrated more slowly than other nucleic acids in the extract. Electrophoretic mobility of viroid bands from samples containing a severe strain (S-PSTV) was 3–4 mm slower in the return direction than those containing mild strains (M-PSTV). Mixed preparations of severe and various mild strains separated into well-defined bands, which verified the differential migration of S-PSTV from M-PSTV strains. The origin of extracts from different organs of the potato (sprout, tuber, stolon, and leaf) or different plant species (tomato, potato, and *Scopolia sinensis*) did not affect the migration pattern of viroid strains. This method, if verified with more severe isolates, may enable separation and identification of mild and severe strains of PSTV within a few hours, as compared with several weeks required for the standard biological cross-protection test.

Additional key words: differential migration, secondary conformation, symptomatology.

Viroids are the smallest and structurally best-characterized infectious molecules. They are unencapsidated, single-stranded covalently closed RNA molecules (15) with a chain length of 240–380 nucleotides (4,14,23).

The potato spindle tuber viroid (PSTV) was the first plant viroid to be discovered (3,24). It consists of 359 nucleotides (6). Various PSTV strains produce disease in tomato (*Lycopersicon esculentum* Mill.) characterized by mild, intermediate, or severe symptoms (1,5,14,17). In New Brunswick potato fields, the mild strains occurred 10 times more frequently than severe strains (25), and monitoring of various germ plasm or field collections elsewhere yielded more mild strains than severe ones (1,2).

The determination of the existence of mild strains requires an initial inoculation of tomato plants with the suspected mild strain, followed by a challenge inoculation with a severe strain. Absence of symptom development in challenge inoculated (cross-protected) plants is considered evidence of the existence of mild strains (1,2,5,25). It is a cumbersome procedure that takes 5–7 wk for a diagnosis and requires growing tomato plants at high temperatures and using a severe PSTV strain. There is no laboratory test to differentiate mild and severe strains of PSTV for large-scale use.

Polyacrylamide gel electrophoresis has been used widely to identify and monitor the presence of viroid molecules in plant nucleic acid extracts (10,13,20). Recently, a sensitive method termed Return-polyacrylamide gel electrophoresis (R-PAGE) has been described for viroids and viruses with circular RNAs (18). In R-PAGE, the viroid molecules are subjected to denaturing conditions, thus achieving the separation by slower mobility of denatured viroid molecules. The method can detect PSTV in samples containing as little as 800 pg of viroid (18).

No information is available in the literature about the detectability of mild strains of viroids by R-PAGE. We report here on a modification to the method that enables separation and identification of mild and severe strains of PSTV on the basis of their differential migration.

MATERIALS AND METHODS

PSTV cultures and test procedures. One locally isolated severe strain of the potato spindle tuber viroid (S-PSTV) and three mild

strains (MA-PSTV and MB-PSTV, obtained from S. A. Slack, University of Wisconsin, Madison [22], and MF-PSTV, collected locally in 1984 [21]) were propagated in potato (*Solanum tuberosum* L. 'Tobique', 'Katahdin', or 'Russet Burbank'). Indicator plants were tomato cultivars Sheyenne, *Scopolia sinensis* Hemsl (19), or *Solanum berthaultii* Hawkes (21). Plants were manually inoculated with partially purified nucleic acid extracts or with excised gel bands (see identification of viroid band) and maintained under environmental conditions optimum for symptom development (21,23). Molecular hybridization tests were performed with samples prepared by the methods of Owens and Diener (12), applied to nitrocellulose membranes from Agdia Inc. (Mishawaka, IN), and further processed by Agdia.

Preparation of nucleic acid extracts. One gram of tissue (leaf or tuber) was prepared as described previously (13,21) by homogenization in 3 ml of extraction buffer (0.53 M NH_4OH, 0.013 M disodium ethylenediaminetetraacetate [EDTA] adjusted to pH 7.0 with Tris, 4 M LiCl, 1% purified bentonite) and 4 ml of Tris-saturated phenol (containing 0.1 g of 8-hydroxyquinoline per 100 ml). The samples and extraction buffer were maintained at 4 or 5 C throughout the extraction procedure. A Polytron PT-35, equipped with a PT 10-ST microgenerator was used for homogenization, and the generator was rinsed twice with water between each sample to prevent contamination. The suspension was centrifuged (15 min, 7,710 *g*) at 4 C, and the nucleic acids were ethanol precipitated (−20 C, 30 min) from the aqueous top layer. The precipitate was collected by centrifugation, dried with a current of N_2, and dissolved in 100 μl of "high salt" buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) (18) containing 40% glycerol.

Return-polyacrylamide gel electrophoresis. The electrophoretic procedure of Schumacher et al (18) was used with some modifications. Nucleic acids were first separated on 5% nondenaturing gels (5% acrylamide, 0.125% bisacrylamide) in "high salt" buffer, in a slab gel (14 × 16 × 0.15 cm) using 6 μl of sample mixed with the dyes xylene cyanol FF and bromophenol blue. For the first electrophoresis, a constant current of 46 mA at 20 C took about 2.5 hr to move xylene cyanol to the bottom of the gel. After the first electrophoresis, the buffer was exchanged for a "low salt" buffer (10 mM Tris, 10 mM boric acid, 0.3 mM EDTA, about 1:8 dilution of the "high salt" buffer). About 2 L of buffer heated to 87–90 C was poured into the lower electrophoresis chamber to denature the "viroid," and the remaining chambers were filled with 3 L of buffer heated to 70 or 71 C. The polarity was reversed and a second electrophoresis was performed at 70 or 71 C (46 mA constant current for 2 hr).

For the silver staining (18), gels were shaken 2 × 5 min in 10% ethanol and 0.5% acetic acid, then 15 min in 0.2% silver nitrate, followed by 4 × 15-sec wash in distilled water. Subsequent treatment was 7–15-min wash in a fresh solution of 375 mM NaOH, 2.3 mM $NaBH_4$, 0.4% HCHO (37% w/v) with a final 5 min in 70 mM Na_2CO_3 for the preservation of the gels.

Identification of viroid bands. The following procedures were used to determine the relative location of circular (slow migrating) and linear (fast migrating) forms of PSTV. After the first electrophoresis, a 1-cm-wide strip of the viroid region across all the lanes was excised from the lower part of the gel. The strip was placed onto the bottom of a gel chamber, a 5% gel polymerized on it, and a second electrophoresis carried out in the return direction under denaturing conditions. Nucleic acid bands from the entire R-PAGE or circular and linear regions were excised and ground with a glass rod in microtubes containing either electrophoretic or glycine-phosphate buffer (21). After centrifugation (5 min) in a microcentrifuge (Eppendorf 5414, Brinkmann Instrument Division, Sybron, Canada), a 50-μl sample of supernatant was removed for infectivity or molecular hybridization.

RESULTS

Detection of mild strains of PSTV. The standard R-PAGE method (18) did not separate viroid bands from other nucleic acids in extracts containing the mild strains, MF-PSTV, although the severe strain (S-PSTV) band was well separated (Fig. 1A). However, raising the temperature of the second electrophoretic buffer to 87–90 C from the recommended 60 C and carrying out the electrophoresis at 70 or 71 C, gave separation of both MF-PSTV and S-PSTV (Fig. 1B). The presence of PSTV in the lowest band of both sets (Fig. 1) of nucleic acids except healthy was also confirmed

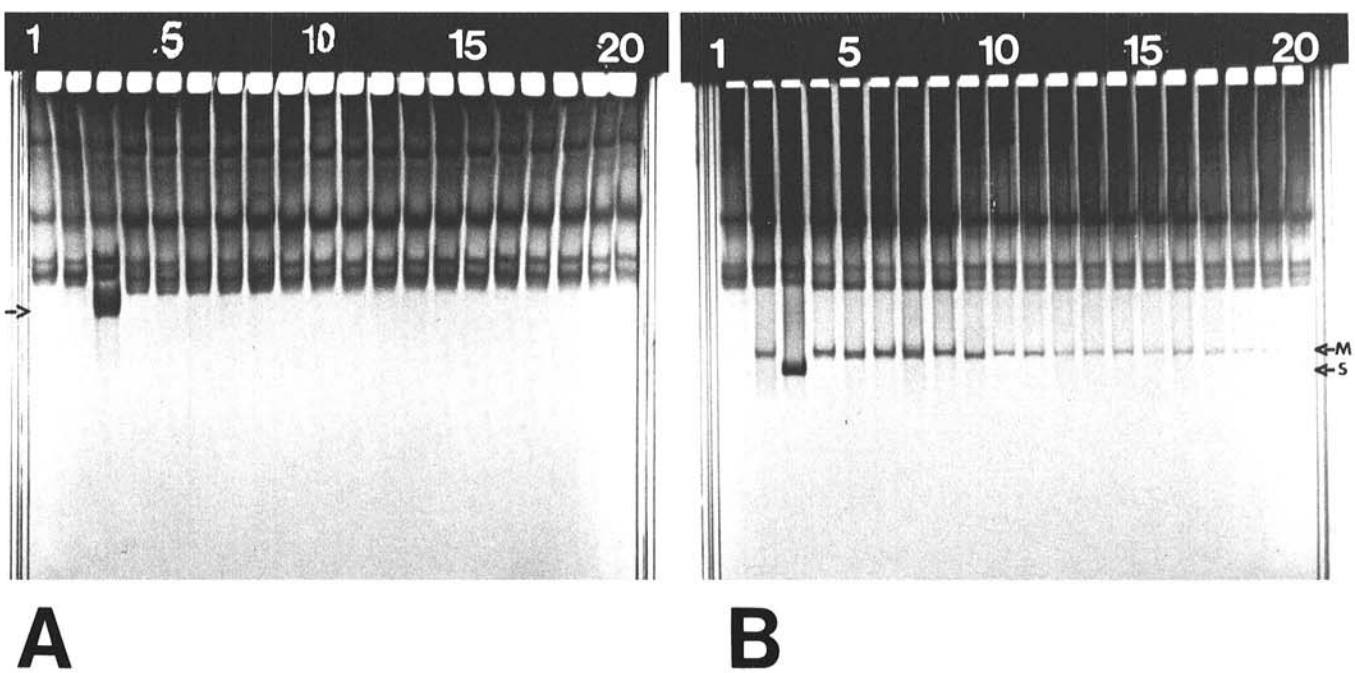

Fig. 1A and B. Return-polyacrylamide gel electrophoresis of mild strains of potato spindle tuber viroid. **A,** Second electrophoresis was carried out at 60 C and the "low salt" buffer for the lower chamber was heated to 60 C, before electrophoresis. Lanes 1 and 2 contain nucleic acid from healthy plants; lane 3 severe PSTV; lanes 4–8, mild PSTV (MF) from Tobique potato tuber; and lanes 9–20, mild PSTV (MF) from Katahdin potato tubers. **B,** Second electrophoresis was carried out at 70 or 71 C and the "low salt" buffer for the lower chamber was heated to 87–90 C, before electrophoresis. Except for lane 2, which contained mild PSTV from Russet Burbank tubers, other lanes contained samples as in A. First electrophoresis was on 5% gels under nondenaturing conditions at 46 mA for 2.5 hr; second electrophoresis under denaturing conditions for 2 hr at 46 mA. M = mild; S = severe strain.

by molecular hybridization. Repeated R-PAGE tests showed that the high temperature of the buffer in the lower chamber near the viroid band was essential to cause the denaturation of the viroid molecule (Fig. 2). Once denaturation had occurred, the migration remained slow even if the subsequent electrophoresis was carried out at 70 or 71 C.

Separation of the mild and the severe strains. To determine whether the method was applicable to other mild strains, nucleic acid extracts from tomato and potato infected with S-PSTV were combined in equal volumes (10 μl each) with nucleic acid extracts containing mild strains MA, MB, and MF before electrophoresis. Nucleic acids from tissues uninfected or infected with S-PSTV or M-PSTV strains were used for comparison. The migration on the electropherogram of M-PSTV isolates individually or in mixtures differed from the one of S-PSTV (Fig. 3A). All isolates of the three mild strains had an identical migration distance of about 30–32 mm, while the severe strain had a migration distance of 26–28 mm when measured from the bottom of the gel. There was no difference in the migration distance of S-PSTV from tomato or potato tissues (Fig. 3A).

In additional tests, R-PAGE of MF- and S-PSTV was repeated at four different temperatures. The mean difference in the migration distance of both strains was 0.5, 2.0, 2.5, and 1.5 mm at 60, 65, 70, and 75 C, respectively (data not shown).

The bands of mixed samples after R-PAGE did not contain the two forms of PSTV, but only the circular forms. Under denaturing conditions, contrary to nondenaturing conditions, both forms of viroid separate out, the linear migrating faster than the circular ones (Fig. 2). With S-PSTV and M-PSTV, corresponding circular and linear molecules maintained their differential migration, characteristic of each strain (Fig. 2). The linear bands migrated close to a component from the host-plant and were difficult to visualize as separate bands on the electropherograms.

Identification of the strains was attempted using R-PAGE and cross-protection tests on eight PSTV-infected samples obtained as tubers from a potato breeding program. The PSTV content of the tubers was predetermined by molecular hybridization, but their reaction on tomato plants was unknown. Nucleic acid extracts from each tuber tested by R-PAGE revealed a definite mild strain electrophoretic pattern. The cross-protection test also confirmed their mild strain nature (data not shown).

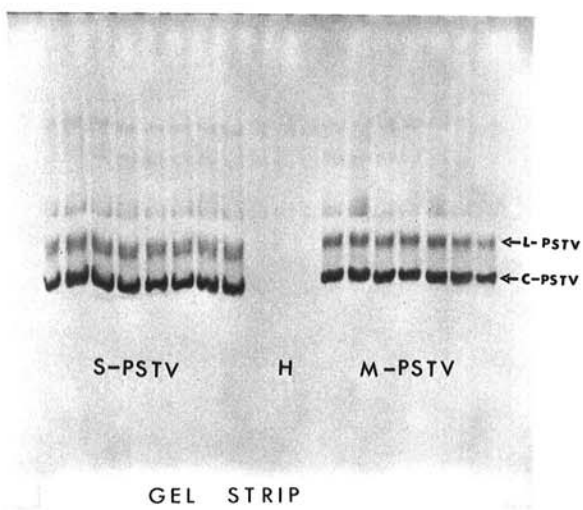

Fig. 2. Separation of the circular (C-PSTV) and the linear (L-PSTV) forms of mild (MA-PSTV) and severe (S-PSTV) strains of potato spindle tuber viroid by Return-polyacrylamide gel electrophoresis. After first electrophoresis under nondenaturing conditions, a strip containing viroid band was excised and placed in the bottom of the gel chamber. A 5% gel was polymerized on top of it, and second electrophoresis was carried out as described in Figure 1B. Left, the nucleic acid extracts containing S-PSTV; center, healthy; and right, M-PSTV; from *Scopolia sinensis* plants.

Effect of plant organs. Aerial and underground organs have been shown to affect the electrophoretic pattern of a plant virus (11); therefore, experiments were made to check this possibility with M-PSTV and S-PSTV isolates, using leaf, tuber, stolons, and sprouts. There was no difference in the electrophoretic mobility of the S-PSTV isolate or of the various M-PSTV isolates from leaves, tubers, stolons, or sprouts (Fig. 3B). The three M-PSTV isolates had identical migration distances. Similarly, S-PSTV from *S. sinensis* had identical migration distances to S-PSTV from potato (Fig. 3B).

Infectivity and symptomatology of various electrophoretic isolates. The isolated electrophoretic bands were excised and used to inoculate tomato plants to compare symptomatology and *S. sinensis* or *S. berthaultii* to test infectivity. The lowest band was always infectious; the band corresponding to linear molecules was

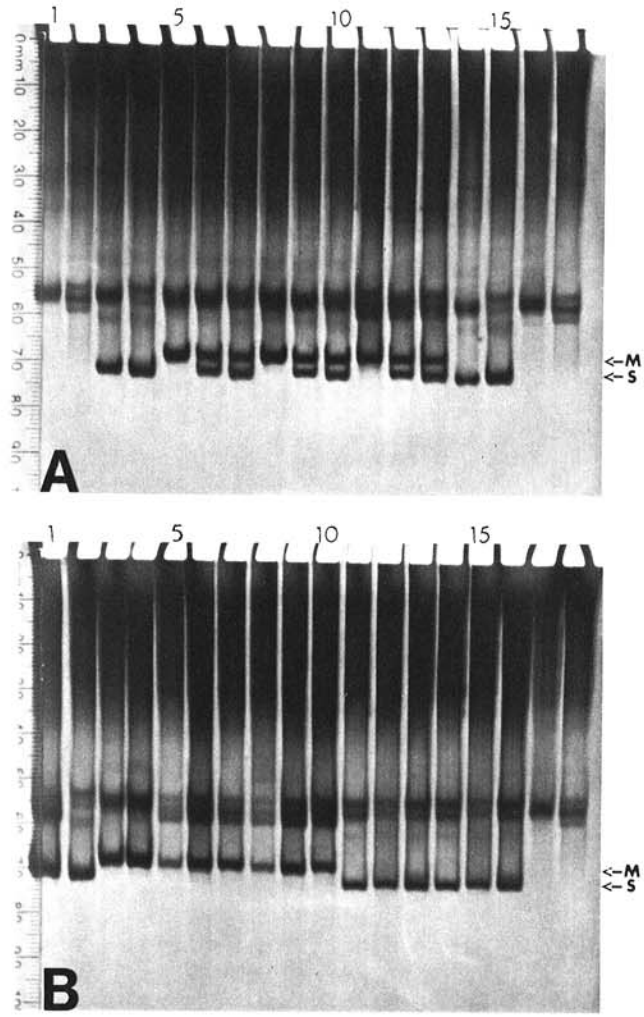

Fig. 3A and B. Separation of mild (M-PSTV) and severe (S-PSTV) strains of potato spindle tuber viroid by Return-polyacrylamide gel electrophoresis. The conditions were similar to those described in Fig. 1B. **A,** Lane 1 nucleic acid extracts from healthy tomato; lane 2 healthy potato; lane 3 S-PSTV from tomato; lane 4 S-PSTV from potato; lane 5, MA-PSTV from tomato; lane 6 MA + S-PSTV from tomato; lane 7, MA + S-PSTV from potato; lane 8, MB-PSTV from tomato; lanes 9 and 10, MB + S-PSTV from tomato and potato, respectively; lane 11, MF-PSTV from potato; lanes 12 and 13, MF + S-PSTV from tomato and potato, respectively; lanes 14 and 15, repeat of lanes 3 and 4; and lanes 16 and 17, repeat of lanes 1 and 2. **B,** Lane 1, S-PSTV from *Scopolia sinensis* leaves; lane 2, S-PSTV from potato; lane 3 MA-PSTV from tomato; lane 4, MB-PSTV from tomato; lanes 5–7, MF-PSTV from tuber, sprout, and leaf, respectively; lanes 8–10, repeat of lanes 5–7; lanes 11–13, S-PSTV from potato tuber, stolon, and leaves, respectively; lanes 14–16, repeat of lanes 11–13; and lanes 17 and 18, nucleic acids from healthy tomato and potato, respectively. M = mild; S = severe strain.

occasionally infectious. No infectivity was observed in any other bands. The S-PSTV containing bands caused stunting, bunchy-top, and necrosis of tomato leaf veins; the MB- and MA-PSTV containing bands caused varying degrees of stunting and bunchy-top symptoms but no veinal necrosis of tomato leaves; and MF-PSTV containing bands did not cause any visible symptoms. However, symptoms of local lesions and systemic necrosis of leaves in *S. sinensis* and necrosis of petiole, leaves, and stems in *S. berthaultii* were caused by all strains. Isolation of nucleic acids from tomato plants infected with S-PSTV or M-PSTV produced the expected migration pattern when electrophoresed.

DISCUSSION

Because M-PSTV and S-PSTV differ only in three nucleotide exchanges (7), their clear separation by modified R-PAGE may seem surprising. It could have been an artifact of the extraction procedure. However, extraction by another procedure (10) did not change the migration pattern (unpublished data). Therefore, the separation of mild and severe strains must be attributed to the resolving power of R-PAGE under the modified denaturing conditions of this study. We attribute the separation to the use of high temperature buffer (87–90 C). It is possible that S-PSTV molecules are fully denatured at relatively low temperature and, because of their fully opened secondary structure, migrate more slowly than M-PSTV strains. The M-PSTV may be only "incompletely denatured" at this low temperature. Because of their partially compact secondary structure the M-PSTV may migrate faster than the S-PSTV. This is supported by our knowledge that "pre-melting loop 1" within the virulence modulating region of the mild isolates requires a high melting temperature of 80 C, whereas those isolates with increasing virulence have a melting temperature around 50 C (14,17). That the separation of M-PSTV and S-PSTV forms could be caused by conformational changes of their secondary structure rather than to differences in chain length is further substantiated by the observation that the nine PSTV isolates ranging from mild to lethal in their response to tomato plants each have 359 nucleotides in their molecule (7,17).

If, in fact, the separation of mild from severe isolates occurs on the basis of secondary structure, as presumably is the case in this study, this method could have great potential with other viroids where chain length variants are known, which cause different symptoms. Although no PSTV isolates with different chain length have been reported, they are common in other viroids. Of the 18 isolates of citrus exocortis viroid studied so far, 12 have a chain length of 371, five over 371, and one under 371 nucleotides (26,27). Two isolates of chrysanthemum stunt viroid have 354 and 356 nucleotides (8,9). Three viroids naturally infecting cucumber, hops, and grapevine are considered to be strains of one viroid but have different chain lengths (16). With the variation in chain length, the secondary structure is expected to be different and R-PAGE may yield differential migration. In preliminary tests, the citrus exocortis and tomato apical stunt viroids, when compared by R-PAGE, displayed differential migration (unpublished data).

Recently, it has been reported that mixtures of several sequence variants could exist together (27). The mild strains in our study appear to be homogeneous on the basis of their migration, although MA and MB came from the United States and MF has been in New Brunswick potatoes for several years. The mild strains of our study have not been sequenced, but if we accept that all mild strains are similar to those sequenced so far (7), all three isolates may be identical in their sequences. However, this remains to be tested.

The modified R-PAGE can separate mild and severe strains of PSTV (Fig. 3), thus providing a rapid method for identifying strains, and is suitable for large-scale identification and separation. Each PAGE test could accommodate up to 40 samples (Fig. 1). Further, it eliminates potential cross-contamination from S-PSTV needed for confirmation in the usual cross-protection scheme.

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