MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 3, pp. 268-275, 1991

Symptom-Modulating Properties of Peanut Stunt Virus Satellite RNA Sequence Variants

R. A. Naidu,[1] G. B. Collins,[2] and S. A. Ghabrial[1]

[1]Departments of Plant Pathology and [2]Agronomy, University of Kentucky, Lexington 40546 U.S.A.
Received 26 November 1990. Accepted 3 February 1991.

The symptom-modulating properties of three peanut stunt virus (PSV) satellite RNA (satRNA) sequence variants were studied. The (V)-satRNA did not affect symptom development in tobacco plants infected with PSV. The (G)- or (WC)-satRNA, on the other hand, attenuated the symptoms. In these plants, the symptoms of PSV were restricted primarily to the inoculated leaves, and in some cases, a few leaves above the inoculated leaf showed small chlorotic areas. Northern blot analysis of total nucleic acids from PSV-infected plants containing the (V)-satRNA revealed the presence of both satellite and viral RNAs in inoculated leaves as well as in systemically infected leaves. On the other hand, satellite and viral RNAs were detected in the inoculated but not in the noninoculated leaves from infected plants containing either (G)- or (WC)-satRNA. Although a decrease in the quantities of genomic RNAs 1, 2, and 3 was characteristic of all satRNA-containing plants, this effect was more evident in the case of (G)- and (WC)-satRNAs. The complete nucleotide sequences of the three satRNAs were determined and compared to the published sequence of PSV satRNA. The (V)-satRNA differed from the published sequence at two positions, whereas the (G)- and (WC)-satRNAs differed at six and eight positions, respectively. Comparison of the nucleotide sequence of the satRNA having no effect on PSV-induced symptoms with those reducing virus symptoms suggests that a single nucleotide change or as many as five nucleotide changes may distinguish between attenuating and nonattenuating satRNAs.

Additional keywords: cucumber mosaic virus group, cucumovirus.

Peanut stunt virus (PSV), a member of the cucumber mosaic virus (CMV, cucumovirus) group, is an economically important pathogen of legumes in many parts of the world (Mink 1972; Xu *et al.* 1986). Like CMV, PSV encapsidates a satellite RNA (satRNA) molecule along with its genomic RNAs (Kaper *et al.* 1978). PSV-associated satRNA is a 393-nucleotide, linear, single-stranded RNA that depends entirely on PSV for its replication and encapsidation. PSV satRNA has essentially no sequence homology with its helper virus genomic RNAs (Linthorst and Kaper 1984; Collmer *et al.* 1985). Likewise, it shares very little sequence homology with CMV satRNA. PSV, but not CMV, can support the replication of PSV satRNA (Kaper *et al.* 1978).

Disease symptom modulation that is mediated by satRNA has been reported for several plant viruses (Murant and Mayo 1982; Francki 1985; Simon 1988). In most cases, the presence of CMV satRNAs results in a significant reduction in virus yield and, in most host plants, an attenuation of the severity of symptoms induced by CMV (Kaper *et al.* 1976; Mossop and Francki 1979; Jacquemond and Leroux 1982). However, some CMV satRNAs are known to intensify the disease symptoms (Kaper *et al.* 1988; Masuta and Takanami 1989). The complete nucleotide sequences of a number of CMV satRNA variants that are associated with CMV strains from widely separated geographic locations have been reported (Garcia-Arenal *et al.* 1987; Kaper *et al.* 1988; Hidaka *et al.* 1988; Palukaitis 1988). The availability of sequencing data on biologically distinct CMV satRNA variants made it possible to identify specific sequences in the satRNA molecule that are responsible for differences in symptoms induced by these satRNA sequence variants (Kurath and Palukaitis 1989; Devic *et al.* 1989; Masuta and Takanami 1989; Devic *et al.* 1990). Moreover, the disease-attenuating properties of satRNA have recently been exploited in producing transgenic tobacco plants that express satRNA sequences, and as a result these plants exhibit resistance to infection with helper viruses (Harrison *et al.* 1987; Gerlach *et al.* 1987; Jacquemond *et al.* 1988).

Although PSV satRNA has been characterized biochemically and its complete nucleotide sequence has been determined, there is little or no information on its biological activity (Kaper and Collmer 1988). In this report, we describe the isolation and molecular characterization of PSV-associated satRNA sequence variants that show distinct symptom-modulating properties.

MATERIALS AND METHODS

Virus sources and purification. PSV isolates were obtained from several sources and maintained in cowpea (*Vigna unguiculata* (L.) Walp. 'Early Ramshorn'). Sources of PSV isolates are as follows: isolates 74-20, 74-23, and 76-69 were provided by S. A. Tolin, Virginia Polytechnic Institute and State University, Blacksburg; isolates G and ER were from J. W. Demski, University of Georgia, Athens; isolates V (a subculture of isolate PSV-74-23 that contains satRNA) and E were provided by O. W. Barnett, Clemson University, Clemson, SC; and isolates W and BV-15 were from J. M. Kaper, U.S. Department of Agriculture, Beltsville, MD. PSV isolate WC was obtained from a naturally

Address correspondence to S. A. Ghabrial.

Nucleotide and/or amino acid sequence data are to be submitted to GenBank, EMBL, and DDBJ as accession numbers JO3695, JO3696, and JO3697.

infected white clover plant in Kentucky. All isolates were increased in cowpea and purified as described by Ghabrial *et al.* (1977).

Mechanical inoculations. Carborundum-dusted leaves of tobacco plants, cultivar Burley 21, were inoculated at the four-leaf stage with PSV RNAs at a concentration of 10 μg/ml plus satRNA at 2.5 μg/ml in 0.1 M Na_2HPO_4. Inoculum of purified virus (50 μg/ml) was prepared in 0.01 M sodium phosphate buffer, pH 7.0. Plants were maintained in a greenhouse with day and night temperatures of 25° C and 20° C, respectively.

RNA isolation. Viral RNAs were isolated from gradient-purified virus using the sodium dodecyl sulfate-phenol extraction method of Peden and Symons (1973). SatRNA was separated from viral RNAs by two cycles of centrifugation on 7.5–30% sucrose density gradients prepared in 10 mM Tris-HCl, pH 7.8, containing 100 mM NaCl, 1 mM EDTA, and 0.04 mg/ml bentonite (Kaper *et al.* 1976). SatRNA was further purified by polyacrylamide gel electrophoresis (PAGE) on 9% polyacrylamide gels containing 8 M urea as described by Avila-Rincon *et al.* (1986).

Electrophoretic analysis of nucleic acids on semidenaturing polyacrylamide gels. Total nucleic acid preparations from inoculated and noninoculated tobacco plants were made as follows: liquid nitrogen-powdered leaf tissue (corresponding to 1 g wet weight) was triturated with a mortar and pestle in a mixture containing 2 ml of STE buffer (0.05 M Tris-HCl, pH 7.2, 0.1 M NaCl, 1 mM EDTA) amended with a mixture of 2% sodium dodecyl sulfate and 0.2% 2-mercaptoethanol and an equal volume (2 ml) of phenol/chloroform (1:1, v/v). The aqueous phase was recovered by centrifugation at 10,000 × *g* for 10 min, and total nucleic acid was precipitated with 2.5 volumes of 95% ethanol. The pellets were washed with 70% ethanol, dried, and resuspended in 100 μl of 1× TAE (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) containing 8 M urea and 0.3% each of bromophenol blue and xylene cyanole. The samples were heated at 55° C for 5 min and centrifuged in a microcentrifuge for 2 min, and 15 μl from each sample was layered on 9% polyacrylamide gels containing 8 M urea (Kaper *et al.* 1981). The gels were run at 10 V/cm for 16 hr, stained in ethidium bromide, destained, and photographed using a UV transilluminator. To verify that the ethidium bromide-stained bands unique to the infected tissues were indeed the single-stranded (ss) and double-stranded (ds) forms of satRNA, their sensitivity to RNase A digestion in high salt was tested as described by White and Kaper (1989). Furthermore, authentic ss-satRNA samples were run on the same gel.

Purified viral RNAs were also analyzed by PAGE on semidenaturing polyacrylamide gels. The analysis, however, was performed on 4% gels containing 8 M urea.

Northern blot hybridization. After destaining, the gels were rinsed for 10 min in 0.05 M NaOH containing 0.5 M NaCl followed by two rinses in 5× TAE and two 10-min rinses in 1× TAE. The samples were electrotransferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA) in 0.5× TAE at 80 V for 4 hr using the Bio-Rad Trans-Blot apparatus. The membrane was baked at 80° C for 2 hr. Prehybridization, hybridization, and washing of the membrane were as described previously (M. Barinaga, R. Franco, J. Meinkoth, E. Ong, and G. M. Wahl. Methods for the transfer of DNA, RNA and protein to nitrocellulose and diazotized paper solid supports, Schleicher & Schuell, Keene, NH). The ^{32}P-labeled probes were prepared by either oligolabeling of cloned cDNA to satRNA (Feinberg and Vogelstein 1983) or by random-primed ss-cDNA synthesis using PSV genomic RNA as a template (Taylor *et al.* 1976). The blotted membranes were exposed to X-ray film using intensifying screens at −70° C.

For dot blotting, total nucleic acid samples (corresponding to the quantities used for PAGE analysis) were suspended in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) and applied to a wetted Zeta-Probe membrane using a Minifold microsample filtration manifold (Schleicher & Schuell). Samples were rinsed with 100 μl of 10× SSC per well. Filters were air-dried, baked for 2 hr at 80° C, and probed as described above with a probe prepared for PSV genomic RNAs. The detection limit of purified PSV RNA by this procedure was determined at 0.1–0.3 ng per spot.

Serology. An indirect enzyme-linked immunosorbent assay (ELISA) procedure similar to that described by Anderson *et al.* (1991) was used to compare the virus titer in infected tissue of tobacco plants in the presence or absence of satRNA. Four leaf disks were punched from each tobacco leaf using a No. 5 cork borer (8-mm-diameter). They were homogenized with a Teflon pestle in 250 μl of carbonate buffer, pH 9.6, in a 1.5-ml Eppendorf tube and centrifuged for 5 min in a microcentrifuge, and 100-μl samples were used per well. The IgG fraction, purified from an antiserum raised against the WC isolate of PSV, was used at a concentration of 0.5 μg per milliliter, and goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) was used at a dilution of 1:2,000. The detection threshold of purified PSV via indirect ELISA was determined to be in the range of 1.0–5.0 ng/ml.

cDNA synthesis and cloning. cDNAs representing satRNAs associated with PSV isolates V, G, and WC were synthesized essentially as described by Gubler and Hoffman (1983) using the Amersham cDNA synthesis system (Amersham International, Amersham, U.K.). A synthetic oligodeoxynucleotide primer 5′-GGGTCGTGTA (that is complementary to satRNA nucleotides 384–393, as deduced from the published sequence of Collmer *et al.* [1985]) was used to prime the synthesis of the first strand of cDNA. The ds-cDNA was blunt-ended with T4 DNA polymerse, ligated into the phagemid vector pUC118 or pUC119 at the *Sma*I site, and transformed into *Escherichia coli* JM101. The resultant white colonies were screened by isolating mini-preparation plasmid DNA (Sambrook *et al.* 1989) for the presence of satRNA-specific sequences by restriction enzyme analysis and Southern hybridization using ^{32}P-labeled randomly primed cDNA of satRNA as a probe.

DNA sequencing. Ss-cDNA inserts in both orientations were obtained with the aid of helper phage M13KO7 (Vieira and Messing 1987). cDNA was sequenced by the dideoxy chain termination method (Sanger *et al.* (1977) using the Sequenase 2.0 system (U.S. Biochemical Corporation, Cleveland, OH). At least three independent clones were

sequenced in each orientation for each of the satRNA variants.

RESULTS

Characterization of PSV satRNA. Electrophoretic analysis of RNA isolated from purified preparations of various PSV isolates revealed that isolates 74-23, 74-20, ER, W, BV-15, and E were satellite-free, whereas isolates 76-69, V, G, and WC encapsidated satRNA. The purified satRNAs were further characterized by electrophoretic analysis on a semidenaturing 9% polyacrylamide gel system (Kaper *et al.* 1981). All PSV satRNAs that were examined had identical electrophoretic mobilities (data not shown) and comigrated with the PSV satRNA (obtained from J. M. Kaper) whose complete nucleotide sequence has been previously reported by Collmer *et al.* (1985). Thus, it appears that all PSV satRNAs, so far examined, are of similar size. The satRNAs that are associated with PSV isolates V, G, and WC were selected for further studies. These satRNAs will hereafter be referred to as (V)-satRNA, (G)-satRNA, and (WC)-satRNA, respectively. The satRNA sequenced by Collmer *et al.* (1985) was derived from a satRNA-containing culture of PSV isolate 74-23 and presumed to be identical to the (V)-satRNA used in this study.

Symptom-modulating properties of PSV satRNAs. We have examined several PSV isolates, which were collected from diverse sources, for the presence of satRNA as well as for symptom induction in a number of host species. We found that the ER isolate of PSV (PSV-ER), a satellite-free isolate, produced more severe symptoms in tobacco than any of the other satellite-free isolates. Therefore, the ER isolate was selected as a helper virus to study the disease-modulating properties of (V)-, (G)-, and (WC)-satRNAs. Ten to 15 plants were used per treatment, and the experiments were repeated four times. Symptoms on the inoculated leaves appeared at the same time in all plants whether or not satRNA was included in the inoculum. In all cases, the inoculated leaves produced chlorotic spots 4–5 days postinoculation. These spots enlarged with time and appeared as concentric rings. Systemic symptoms in satellite-free plants appeared about 10 days postinoculation and could be visualized on the third and fourth leaf above the inoculated leaf. These leaves showed severe mottling, general chlorosis, chlorotic spots, and oak-leaf line patterns. Leaves that developed subsequently showed similar severe symptoms, but chlorosis was less extensive (Fig. 1). These symptoms persisted in all leaves, and the plants were severely stunted. Plants that were inoculated with PSV-ER containing (V)-satRNA had symptoms similar to those observed with the satellite-free plants. On the other hand, those inoculated with PSV-ER plus (G)- or (WC)-satRNA showed greatly reduced symptoms. The majority of these plants developed small chlorotic areas and spots on a few of the newly developed leaves above the inoculated leaf. However, in about 30–40% of the plants, disease symptoms were primarily restricted to the inoculated leaves (Fig. 1). Leaves that developed subsequently were symptomless, and the plants grew as vigorously as the noninoculated plants.

Viral and satRNA content in relation to symptom development. To relate disease symptom modulation to accumulation of viral and satRNAs, total nucleic acids were extracted from the fourth and eighth leaves above the inoculated leaf (2 and 4 wk postinoculation, respectively) of plants inoculated with either PSV-ER alone or PSV-ER in the presence of various satRNAs. Total nucleic acid preparations were analyzed by PAGE on semidenaturing polyacrylamide gels and by northern blotting. Preliminary analyses of noninoculated leaves from plants receiving PSV-ER plus (G)- or (WC)-satRNA treatments indicated that viral and satRNAs can be detected in symptomatic, but not in symptomless areas in such leaves. Therefore, symptomatic regions of the leaves representing these treatments were used for analyses that were made with the fourth leaf above the inoculated leaf. The eighth leaf was symptomless in all plants receiving inocula containing (G)- or (WC)-satRNA. No satRNA was detected in the fourth leaf above the inoculated leaf from plants infected with PSV-ER alone (Fig. 2, A and B, lane 2), indicating that the ER isolate has maintained its satellite-free status. In plants inoculated with PSV-ER plus any of the three satRNAs, satRNA-specific bands were readily detected (Fig. 2, lanes 3, 4, and 5). In these plants, the faster of the two unique bands (Fig. 2, arrow) migrated to a position

Fig. 1. Effect of peanut stunt virus (PSV) satellite RNA (satRNA) isolates on the severity of symptoms induced by PSV isolate ER (PSV-ER) infection of tobacco cultivar Burley 21. Systemic mottling, chlorotic spots, and oak-leaf line patterns are produced in plants infected with PSV-ER alone (**A** and **B**, left) or in the presence of (V)-satRNA (**A**, right). Tobacco plants infected with PSV-ER plus (G)-satRNA showed no systemic symptoms (**B**, right). The effects of (WC)-satRNA on virus symptoms are similar to those observed with (G)-satRNA.

predicted for ds-satRNA. The double-stranded nature of these bands was further confirmed by their resistance to RNase A treatment in high salt. The unique slower bands migrated to the position of authentic ss-satRNA, which was purified from virions, and were sensitive to the RNase treatment (data not shown). In northern blots, the nucleic acids in these bands hybridized with a satRNA-specific probe, confirming that they are satellite-related RNAs (Fig. 2B, lanes 3, 4, and 5). The relative proportions of ss- and ds-satRNAs that accumulated varied with the satRNA isolate involved as well as with the method of detection, whether it was by ethidium bromide staining or by northern blotting (Fig. 2). We have followed the procedures of White and Kaper (1989) who detected no discrepancy between ethidium bromide staining and northern blotting in estimating the relative proportions of CMV ss- and ds-satRNAs in plant extracts. If band intensity in the ethidium bromide-stained gel accurately reflects the distribution of the two forms of PSV satRNA, the ss-satRNA should have proportionately much stronger hybridization signals than the ds-satRNA. The ds-satRNA may be less efficiently transferred to the membrane or less accessible to the hybridization probe due to the semidenaturing nature of the gel. It was, therefore, necessary in some cases to overexpose the X-ray film so that the dsRNA bands would be clearly visible in the autoradiogram.

Electrophoretic analysis of total nucleic acid preparations that were made 4 wk after inoculation (from the eighth leaf above the inoculated leaf) showed no satRNA in plants which were inoculated with PSV-ER alone (Fig. 3, A and B, lane 2) or with PSV-ER plus (G)- or (WC)-satRNA (Fig. 3, lanes 4 and 5, respectively). The smear observed in lane 5 of Figure 3B is due to overexposure of the film so that satRNA and related molecules (if present) would be clearly revealed, as was discussed earlier. SatRNA, however, was readily detected in plants inoculated with PSV-ER plus (V)-satRNA (Fig. 3, A and B, lane 3). We cannot readily explain the origin of the hybridization signal that was detected above the ss-satRNA band in lane 3 of Figure 3B, but it may represent a multimeric form (probably a dimer) of satRNA that we have detected in a few preparations. The occurrence of multimeric forms of PSV satRNA that are encapsidated with the viral RNAs has been reported previously (Linthorst and Kaper 1984). Electrophoretic analyses of total nucleic acid preparations from satellite-free and satRNA-containing plants were performed four times with reproducible results.

The level of viral RNA that accumulated in infected plants was also evaluated by dot blot hybridization using labeled cDNA representing PSV-ER genomic RNA as a probe. No differences were observed in the amounts of PSV genomic RNA that accumulated in leaves inoculated with either PSV-ER alone or mixed with (V)-satRNA. However, leaves that were rubbed with a virus inoculum containing either (G)- or (WC)-satRNA accumulated lower amounts of viral RNA (Fig. 4, row A). At 2 and 4 wk postinoculation, viral RNA was only detected in satellite-free plants and in those containing (V)-satRNA. The latter, however, had relatively lower levels of virus RNA. No virus

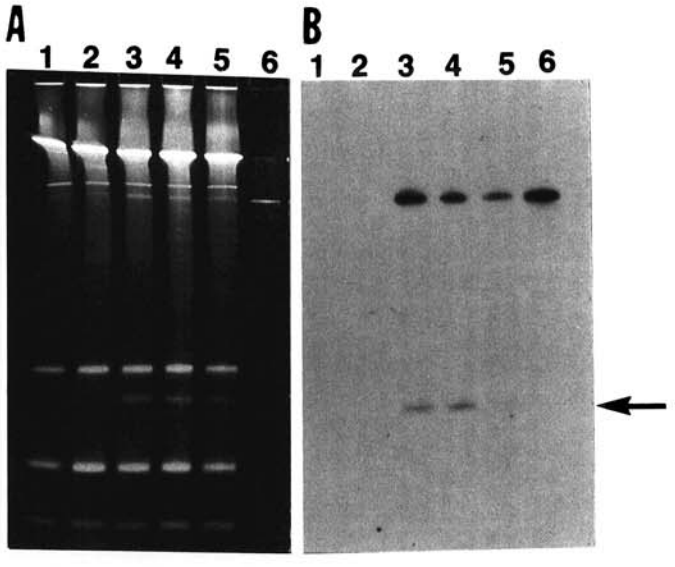

Fig. 2. Northern blot analysis of total nucleic acid extracts from tobacco (fourth leaf above inoculated leaf) prepared 2 wk after inoculation with peanut stunt virus (PSV) isolate ER (PSV-ER) alone or mixed with (V)-, (G)-, or (WC)-satellite RNA (satRNA). Total nucleic acid extracts were subjected to electrophoresis on semidenaturing 9% polyacrylamide gels and photographed after ethidium bromide staining (**A**) or electrotransferred to Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, CA) and hybridized to ^{32}P-labeled nick-translated cloned satRNA cDNA (**B**). Total nucleic acid preparations were from noninoculated plants (lanes 1), plants inoculated with PSV-ER alone (lanes 2), PSV-ER plus (V)-satRNA (lanes 3), PSV-ER plus (G)-satRNA (lanes 4), and PSV-ER plus (WC)-satRNA (lanes 5). Purified satRNA was applied to lanes 6. The arrow indicates the position of double-stranded satRNA.

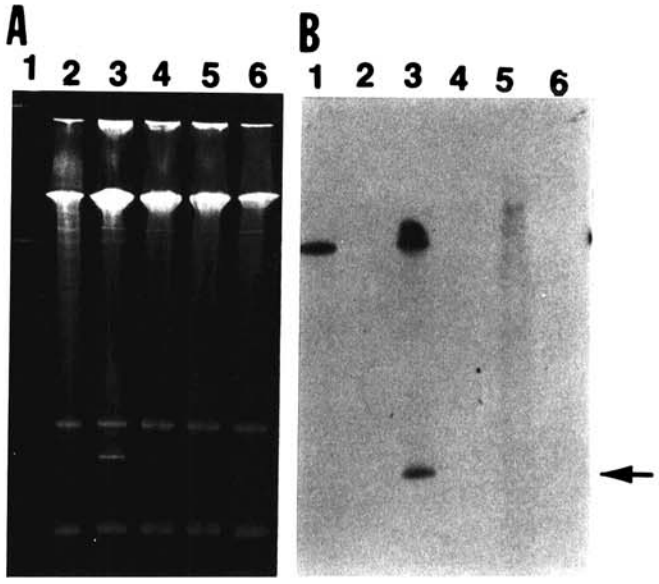

Fig. 3. Northern blot analysis of total nucleic acid extracts obtained from tobacco 4 wk after inoculation (eighth leaf above inoculated leaf) with peanut stunt virus (PSV) isolate ER (PSV-ER) alone and in the presence of (V)-, (G)-, or (WC)-satellite RNA (satRNA). **A**, Gel stained with ethidium bromide. **B**, Northern blot. (For conditions of electrophoresis and northern blot analysis, see the legend to Fig. 2.) Lanes 1 contain purified satRNA; lanes 2–5 contain total nucleic acid preparations from plants inoculated with PSV-ER alone, PSV-ER plus (V)-satRNA, PSV-ER plus (G)-satRNA, and PSV-ER plus (WC)-satRNA, respectively. Lanes 6 have total nucleic acid from noninoculated control plants.

RNA was detected in the symptomless noninoculated leaves of plants known to contain either (G)- or (WC)-satRNA in their inoculated leaves (Fig. 4, rows B and C). These results were reproducible in two experiments.

To find out whether satRNA had influenced the relative proportions of genomic RNAs encapsidated in virus particles, RNA was extracted from purified virions that were isolated from inoculated tobacco leaves and analyzed by PAGE on semidenaturing 4% polyacrylamide gels. As shown in Figure 5, a significant reduction in the levels of genomic RNAs 1, 2, and 3 was detected in the presence of satRNA. The reduction in viral RNA was more evident in the presence of (G)- or (WC)-satRNA than in the presence of (V)-satRNA. These results were reproducible in two experiments.

Influence of satRNA on movement and systemic spread of PSV as determined by ELISA. Results of ELISA tests to assess the accumulation of virus coat protein in infected plants were consistent with those obtained for monitoring viral RNA by dot blot analysis. Little or no virus was detected in the noninoculated leaves of plants that had been inoculated with PSV-ER plus either (G)- or (WC)-satRNA, whereas PSV was readily detected in the leaves of systemically infected plants previously inoculated with PSV-ER alone or in the presence of (V)-satRNA (data not shown).

To determine whether virus spread may be affected in infections containing satRNA, small areas (about 4 cm^2) of tobacco leaves were gently rubbed with viral RNAs alone or mixed with one or the other of the satRNA variants. Virus spread was monitored by punching leaf disks that were 1–2 cm away from the inoculated area and using ELISA to determine PSV content. In general, the virus titer in the noninoculated regions was significantly lower than that in the inoculated areas of the same leaf (Table 1). Nevertheless, PSV was readily detected in noninoculated areas away from the treated area (14 days postinoculation) in leaves inoculated with PSV-ER alone or PSV-ER plus (V)-satRNA. On the other hand, in the presence of either (G)- or (WC)-satRNA, the virus titers in the inoculated and noninoculated regions of the treated leaves at 14 days postinoculation were, respectively, about 24 and 61% lower than those of the satellite-free treatment. As expected, plants that were inoculated with either PSV-ER alone or mixed with (V)-satRNA showed systemic symptoms, whereas those inoculated with PSV-ER plus either (G)- or (WC)-satRNA did not show systemic symptoms and no virus was detected by ELISA (Table 1).

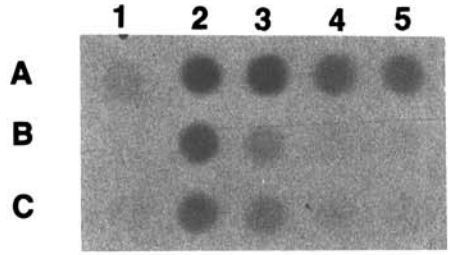

Fig. 4. Dot blot hybridization assay of viral RNA in total nucleic acid samples from tobacco inoculated with peanut stunt virus (PSV) isolate ER (PSV-ER) in the presence or absence of satellite RNA (satRNA) variants. Total nucleic acid preparations were made at different intervals after inoculation, spotted onto Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, CA), and probed with ^{32}P-labeled PSV cDNA. Samples were from inoculated leaves (row A) and fourth and eighth leaves above the inoculated leaf (rows B and C, respectively). Lanes 1–5 represent the sources of leaves: lane 1, noninoculated control plants, and lanes 2–5, plants inoculated with PSV-ER alone or mixed with (V)-, (G)-, and (WC)-satRNAs, respectively.

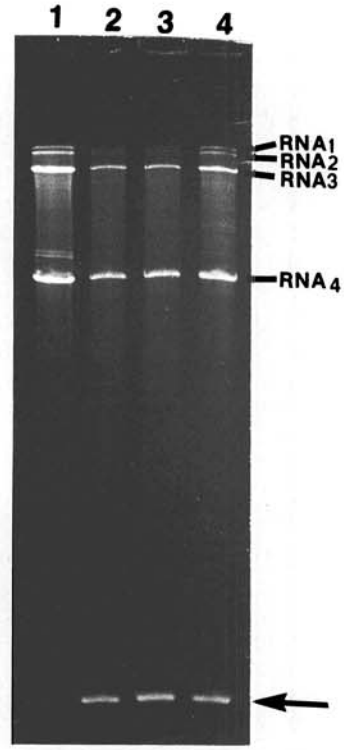

Fig. 5. PAGE on semidenaturing 4% polyacrylamide gels of peanut stunt virus (PSV) genomic RNA from purified virus. The virus was purified from tobacco plants inoculated with PSV isolate ER alone (lane 1) or mixed with (G)-satellite RNA (satRNA) (lane 2), (WC)-satRNA (lane 3), and (V)-satRNA (lane 4). Positions of viral RNAs 1, 2, 3, and 4 are indicated to the right of the gel. The arrow indicates the position of satRNA.

Table 1. Influence of satellite RNA (satRNA) on peanut stunt virus (PSV) movement and systemic spread in inoculated tobacco plants as determined by ELISA

	ELISA absorbance values ($A_{405\ nm}$) for extracts from[b]				
	Inoculated area		Noninoculated area		Systemic leaf[c]
Treatment[a]	7	14	7	14	14
	(days after inoculation)				
PSV	1.644[d]	1.560	0.019	0.180	0.943
PSV + (V)-satRNA	1.617	1.473	0.013	0.192	0.660
PSV + (G)-satRNA	1.525	1.176	0.017	0.070	0.031
PSV + (WC)-satRNA	1.502	1.200	0.016	0.069	0.034

[a] Treatment was with PSV isolate ER (PSV-ER) and PSV-ER plus one of the three satRNAs associated with PSV isolates V, G, and WC.
[b] Leaf extracts from comparable mock-inoculated plants served as controls and gave ELISA absorbance values of 0.01–0.03.
[c] Noninoculated upper leaf tested for systemic infection with PSV-ER.
[d] Absorbance values are the means of three replications. ELISA absorbance values higher than twice the readings for comparable mock-inoculated control plants were considered to be positive.

Comparison of satRNA sequences. The first-generation satRNA cDNA clones that were produced in this study were not full-length; nucleotide sequencing revealed that they were 375–380 nucleotides long. When compared to the published sequence of PSV satRNA of 393 nucleotides, we discovered that our clones were missing a fragment consisting of nucleotides 1 through 10–15. The missing sequence at the 5′ end of the satRNA variants was determined by primer extension (Sambrook *et al.* 1989) using a synthetic primer complementary to nucleotides 36 to 52 of PSV satRNA. Figure 6 shows an alignment of the complete nucleotide sequences of four satRNA variants that includes the sequences of the three satRNAs selected for this study along with the published sequence. The most striking feature of the sequences of the satRNAs is the extremely high degree of homology; these four satRNA variants are identical at 97–99% of the nucleotide positions (Fig. 6). (V)-satRNA differs in two positions from the published sequence with nucleotide substitutions being at positions 166 (G→T) and 247 (T→C). (G)- and (WC)-satRNAs showed more variation than (V)-satRNA; they varied in six to eight positions from the published sequence. The (G)-satRNA differs at six positions; a nucleotide deletion at position 366 and nucleotide substitutions at positions 56 (G→C), 57 (C→G), 226 (C→T), 247 (T→C), and 362 (A→C). The (WC)-satRNA is almost identical to (G)-satRNA, except for a nucleotide deletion at position 263 and a nucleotide substitution at position 377 (C→T). Thus, the length of (G)-satRNA is 392 nucleotides, whereas the (WC)-satRNA is 391 nucleotides.

DISCUSSION

This study represents the first report demonstrating the symptom-modulating properties of PSV satRNA. Of the three satRNA variants studied, two, namely (G)- and (WC)-satRNAs, exhibited disease-attenuating properties. Analysis of plants showing symptom attenuation revealed that both satellite and viral RNAs were restricted to the inoculated leaves. In some cases, the satellite and viral RNAs were detected at a very low level in a few leaves above the inoculated leaves. In such leaves, the satellite and viral

```
Sat:  GTTTTGTTTTGTCGGGAGTCACCGCGTAAAAACCCACTGTAATGGTGATGCGACAGCAGG      60
V  :  ------------------------------------------------------------
G  :  ------------------------------------------------------CG---
WC :  ------------------------------------------------------CG---

Sat:  TGAAGAAATGCCTGTATGTGCGTGGCGCTACGCCTTTGCCCGGGGGGGATATACTGGGGGG     120
V  :  ------------------------------------------------------------
G  :  ------------------------------------------------------------
WC :  ------------------------------------------------------------

Sat:  GGGCGTATGCACGTAGGTGACCGATGAGAGAGGACGCCTCCGGGGGCGGAGGTAAGTCGG      180
V  :  ---------------------------------------------T--------------
G  :  ------------------------------------------------------------
WC :  ------------------------------------------------------------

Sat:  GGAGGGCGGCGTTCGTAGTGAAACGACCTCCTATCAGGACTGCTGCACCGTCGGGGGGAA      240
V  :  ------------------------------------------------------------
G  :  ---------------------------------------------T--------------
WC :  ---------------------------------------------T--------------

Sat:  ACCCCCTTGGGTCGAGCCCGATCCCTCTTCTTGTAAAACCGGTGGAACTTAACCTCATTT      300
V  :  ------C-------------------------------------------------------
G  :  ------C-------------------------------------------------------
WC :  ------C---------------Δ---------------------------------------

Sat:  CTGGAGAAGTCCGGACAACTGTGATGGAAGAAAGCAGTGTGGTGCCCCAGCGGCGACCAC      360
V  :  ------------------------------------------------------------
G  :  ------------------------------------------------------------
WC :  ------------------------------------------------------------

Sat:  GAAAGGCCACGGCCTTCAGCTCCTACACGACCC   393
V  :  ---------------------------
G  :  -C---Δ---------------------
WC :  -C---Δ----------T----------
```

Fig. 6. Nucleotide sequences of cDNA clones of three peanut stunt virus (PSV) satellite RNAs (satRNAs): (V)-satRNA, (G)-satRNA, and (WC)-satRNA. The top line shows the complete sequence of PSV satRNA as published by Collmer *et al.* (1985). For the three satRNA clones, only those nucleotides that differ from the published sequence are shown. A dashed horizontal line indicates identity and Δ indicates deletion. The numbering system for these sequences corresponds to the system used by Collmer *et al.* (1985).

RNAs were detected in the symptomatic, but not in the symptomless regions of the leaf. In the CMV-satRNA system, a large accumulation of ds-satRNA was observed in symptom-attenuated tissue, accompanied by significant reduction in virus titer (Mossop and Francki 1979; Habili and Kaper 1981; Jacquemond and Leroux 1982; Kaper *et al.* 1981; Piazolla *et al.* 1982). In the PSV-satRNA system, on the other hand, we did not observe any correlations between ds-satRNA accumulation and symptom attenuation. This conclusion was made regardless of the method of ds-satRNA detection, whether it was by ethidium bromide staining or northern blotting (Fig. 2). These observations suggest that, in addition to differences in replication specificity and to lack of sequence homology between the satRNAs of PSV and CMV, a different mechanism(s) of disease attenuation may be involved in the PSV-satRNA system. When transgenic tobacco plants that express the tobacco ringspot virus (TRSV) satRNA sequence were challenge-inoculated with the helper virus, TRSV was mostly restricted to the inoculated leaves in these plants (Gerlach *et al.* 1987). This similarity between the PSV-satRNA system and TRSV-satRNA system is of interest because their respective helper viruses belong to distinct virus groups with different genome expression strategies.

A mechanism that has often been proposed to explain the satRNA-mediated disease symptom attenuation entails that satRNA competes with viral RNAs for binding to the RNA replicase and cellular factors essential for replication, and as a result, both viral replication and disease symptoms are reduced (Piazolla *et al.* 1982; Kaper 1982, 1984). In this study, analysis of progeny viral RNA in tobacco plants infected with PSV showed that the amount of viral RNA accumulated was markedly lower in the presence of (G)- or (WC)-satRNA than in infections with satellite-free PSV. The viral genomic RNAs appear to have been markedly reduced (Fig. 5). These results are consistent with the competition mechanism and further indicate that some PSV satRNAs (e.g. G- and WC-satRNAs) are more efficient competitors of viral RNAs than others (e.g. (V)-satRNA).

The (G)- and (WC)-satRNAs appear to interfere with movement and systemic spread of PSV. Our results (Table 1) cannot distinguish among interference with cell-to-cell movement, reduction in virus replication, and interference with entry and movement in the vascular tissues as factors contributing to virus restriction to the inoculated leaf. Although the mechanism(s) of PSV restriction via satRNA remains obscure, the sequence data of biologically distinct PSV satRNAs being made available in this study should be valuable in helping to understand the molecular basis for interference with the helper virus.

The satRNAs associated with CMV have been extensively studied, and at present, more than 25 satellite variants originating from widely separated geographic areas have been characterized and sequenced (Garcia-Arenal *et al.* 1987; Kaper *et al.* 1988; Hidaka *et al.* 1988; Palukaitis 1988). Although our study included a limited number of PSV isolates, we have shown that sequence variants do occur among PSV satRNAs. The three variants that we have included in our study showed extensive sequence homology with each other (Fig. 6) as well as with the PSV satRNA sequence reported by Collmer *et al.* (1985). Whereas the nucleotide sequence of (V)-satRNA differs from the published sequence at two positions, those of (G)- and (WC)-RNAs differ at six and eight positions, respectively. For (G)- and (WC)-satRNAs, most of the variations occurred in the 3′ terminal half of the RNA molecule, and they are one and two nucleotides shorter, respectively, than the published sequence. A comparison of the nucleotide sequences of PSV satRNAs that attenuate symptoms (satRNAs associated with isolates G and WC) with those that have no effect on symptom development (e.g., the satRNA associated with isolate V) suggests that a single nucleotide change or as many as five nucleotide changes may be responsible for the differences in properties between attenuating and nonattenuating satRNAs. It is of interest in this regard to note that a limited number of substitutions and/or deletions within a particular sequence background in certain CMV satRNA variants may result in a modification of the tripartite interaction involving satRNA, host, and helper virus (Palukaitis 1988; Masuta and Takanami 1989; Sleat and Palukaitis 1990; Devic *et al.* 1990). For example, Sleat and Palukaitis (1990) have recently shown that a single nucleotide change in certain CMV satRNA variants can convert the satRNA phenotype in tomato from ameliorative to necrogenic.

We have preliminary data, which are based on symptom development and ELISA monitoring, that suggest that the effects of the three satRNA variants on PSV accumulation and pathogenicity are apparently PSV strain-specific. Whereas results similar to those reported in this study were obtained when the satellite-free PSV isolate 74-20 was used as the helper virus, none of the three PSV satRNAs appeared to have any effect on symptom development and virus accumulation when the satellite-free PSV isolate 74-23 was the helper virus. Studies that involve production of infectious transcripts from cloned PSV satRNA cDNAs and construction of chimeric satRNA molecules combined with site-directed *in vitro* mutagenesis are underway in our laboratory to determine the role of specific nucleotide changes in the satRNA molecule in the observed symptom amelioration in tobacco.

ACKNOWLEDGMENTS

We thank O. W. Barnett, J. W. Demski, J. M. Kaper, and S. A. Tolin for supplying PSV isolates. This work is supported in part by a grant from the R. J. Reynolds Tobacco Co., Winston-Salem, NC, and is published as journal series paper 90-3-11-126 of the Kentucky Agricultural Experiment Station.

LITERATURE CITED

Anderson, J. A., Ghabrial, S. A., and Taylor, N. L. 1991. Natural incidence of peanut stunt virus infection in hybrid populations of *Trifolium ambiguum* × *T. repens*. Plant Dis. 75:156-159.

Avila-Rincon, M. J., Collmer, C. W., and Kaper, J. M. 1986. *In vitro* translation of cucumoviral satellites. I. Purification and nucleotide sequence of cucumber mosaic virus-associated RNA5 from cucumber mosaic virus strain S. Virology 152:446-454.

Collmer, C. W., Hadidi, A., and Kaper, J. M. 1985. Nucleotide sequence of the satellite of peanut stunt virus reveals structural homologies with viroids and certain nuclear and mitochondrial introns. Proc. Natl. Acad. Sci. USA 82:3110-3114.

Devic, M., Jaegle, M., and Baulcombe, D. 1989. Symptom production on tobacco and tomato is determined by two distinct domains of the satellite RNA of cucumber mosaic virus (strain Y). J. Gen. Virol.

70:2765-2774.
Devic, M., Jaegle, M., and Baulcombe, D. 1990. Cucumber mosaic virus satellite RNA (strain Y): Analysis of sequences which affect systemic necrosis on tomato. J. Gen. Virol. 71:1443-1449.
Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
Francki, R. I. B. 1985. Plant virus satellites. Annu. Rev. Microbiol. 39:151-174.
Garcia-Arenal, F., Zaitlin, M., and Palukaitis, P. 1987. Nucleotide sequence analysis of six satellite RNAs of cucumber mosaic virus: Primary sequence and secondary structure alterations do not correlate with differences in pathogenicity. Virology 158:339-347.
Gerlach, W. L., Llewellyn, D., and Haseloff, J. 1987. Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature (London) 328:802-805.
Ghabrial, S. A., Pickard, C. M., and Stucky, R. E. 1977. Identification and distribution of virus diseases of soybean in Kentucky. Plant Dis. Rep. 61:690-694.
Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.
Habili, N., and Kaper, J. M. 1981. Cucumber mosaic virus-associated RNA 5. VII. Double-stranded form accumulation and disease attenuation in tobacco. Virology 112:250-261.
Harrison, B. D., Mayo, M. A., and Baulcombe, D. C. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature (London) 328:799-802.
Hidaka, S., Hanada, K., Ishikawa, K., and Miura, K. 1988. Complete nucleotide sequence of two new satellite RNAs associated with cucumber mosaic virus. Virology 164:326-333.
Jacquemond, M., and Leroux, J.-P. 1982. L'ARN satellite du virus de la mosaique du concombre. II. Étude de la relation virus-ARN satellite chez divers hôtes. Agronomie (Paris) 2:55-62.
Jacquemond, M., Amselem, J., and Tepfer, M. 1988. A gene coding for a monomeric form of cucumber mosaic virus satellite RNA confers tolerance to CMV. Mol. Plant-Microbe Interact. 1:311-316.
Kaper, J. M. 1982. Rapid synthesis of double-stranded cucumber mosaic virus-associated RNA 5: Mechanism controlling viral pathogenesis? Biochem. Biophys. Res. Commun. 105:1014-1022.
Kaper, J. M. 1984. Plant disease regulation by virus-dependent satellite-like replicating RNAs. Page 317 in: Control of Virus Diseases. E. Kurstak, ed. Marcel Dekker, New York.
Kaper, J. M., and Collmer, C. W. 1988. Modulation of viral plant diseases by secondary RNA agents. Pages 171-194 in: RNA Genetics, Variability of RNA Genomes, Vol. III. E. Domingo, J. J. Holland, and P. Ahlquist, eds. CRC Press, Boca Raton, FL.
Kaper, J. M., Tousignant, M. E., and Lot, H. 1976. A low molecular weight replicating RNA associated with a divided genome plant virus: Defective or satellite RNA? Biochem. Biophys. Res. Commun. 72:1237-1243.
Kaper, J. M., Tousignant, M. E., Diaz-Ruiz, J. R., and Tolin, S. A. 1978. Peanut stunt virus-associated RNA 5: Second tripartite genome virus with an associated satellite-like replicating RNA. Virology 88:166-170.
Kaper, J. M., Tousignant, M. E., and Thompson, S. M. 1981. Cucumber mosaic virus-associated RNA5. VIII. Identification and partial characterization of a CARNA5 incapable of inducing tomato necrosis. Virology 114:526-533.
Kaper, J. M., Tousignant, M. E., and Steen, M. T. 1988. Cucumber mosaic virus-associated RNA 5. XI. Comparison of 14 CARNA5 variants relates ability to induce tomato necrosis to a conserved nucleotide sequence. Virology 163:284-292.
Kurath, G., and Palukaitis, P. 1989. Satellite RNAs of cucumber mosaic virus: Recombinants constructed *in vitro* reveal independent functional domains for chlorosis and necrosis in tomato. Mol. Plant-Microbe Interact. 2:91-96.
Linthorst, H. J. M., and Kaper, J. M. 1984. Replication of peanut stunt virus and its associated RNA 5 in cowpea protoplasts. Virology 139:317-329.
Masuta, C., and Takanami, Y. 1989. Determination of sequence and structural requirements for pathogenicity of a cucumber mosaic virus satellite RNA (Y sat. RNA). Plant Cell 1:1165-1173.
Mink, G. I. 1972. Peanut stunt virus. No. 92 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.
Mossop, D. W., and Francki, R. I. B. 1979. Comparative studies on two satellite RNAs of cucumber mosaic virus. Virology 95:395-404.
Murant, A. F., and Mayo, M. A. 1982. Satellites of plant viruses. Annu. Rev. Phytopathol. 20:49-70.
Palukaitis, P. 1988. Pathogenicity regulation by satellite RNAs of cucumber mosaic virus: Minor nucleotide sequence changes alter host responses. Mol. Plant-Microbe Interact. 1:175-181.
Peden, K. W. C., and Symons, R. H. 1973. Cucumber mosaic virus contains a functionally divided genome. Virology 53:487-492.
Piazolla, P., Tousignant, M. E., and Kaper, J. M. 1982. Cucumber mosaic virus-associated RNA 5. IX. The overtaking of viral RNA synthesis by CARNA5 and ds CARNA5 in tobacco. Virology 122:147-157.
Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
Simon, A. E. 1988. Satellite RNAs of plant viruses. Plant Mol. Biol. Rep. 6:240-252.
Sleat, D. E., and Palukaitis, P. 1990. Site-directed mutagenesis of a plant viral satellite RNA changes its phenotype from ameliorative to necrogenic. Proc. Natl. Acad. Sci. USA 87:2946-2950.
Taylor, J. M., Illmensee, R., and Summers, J. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta 442:324-330.
Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
White, J. L., and Kaper, J. M. 1989. A simple method for detection of viral satellite RNAs in small plant tissue samples. J. Virol. Methods 23:83-94.
Xu, Z., Barnett, O. W., and Gibson, P. B. 1986. Characterization of peanut stunt virus strains by host reactions, serology, and RNA patterns. Phytopathology 76:390-395.