Differentiation of Eastern and Western Strains of Peanut Stunt Cucumovirus Based on Satellite RNA Support and Nucleotide Sequence Homology

R. A. Naidu, C.-C. Hu, R. E. Pennington, and S. A. Ghabrial

Department of Plant Pathology, University of Kentucky, Lexington 40546-0091.
Address correspondence to S. A. Ghabrial.
Present address of first author: ICRISAT, Patancheru P. O. Andhra Pradesh 502 324, India.
Paper 94-11-157 of the Journal Series of the Kentucky Agricultural Experiment Station. This research was funded by a grant from the U.S. Department of Agriculture (Cooperative Agreement 58-6430-3-114).
We thank O. W. Barnett, J. Demski, J. M. Kaper, and S. A. Tolin for providing the various strains of peanut stunt virus, and M. J. Roossinck for supplying strain Fny of cucumber mosaic virus (CMV) and CMV satellite RNAs. The nucleotide sequence data of peanut stunt virus strains ER RNA 1, 2, and 3 have been deposited in the GenBank, EMBL, and DDJB under accession numbers U15728, U152729, and U15730.
Accepted for publication 25 January 1995.

ABSTRACT

Naidu, R. A., Hu, C.-C., Pennington, R. E., and Ghabrial, S. A. 1995. Differentiation of eastern and western strains of peanut stunt cucumovirus based on satellite RNA support and nucleotide sequence homology. Phytopathology 85:502-507.

Two western strains of peanut stunt cucumovirus (PSV), strains W and BV-15, were unable to support the replication of two distinct PSV satellite RNAs (satRNAs), or infectious transcripts from full-length satRNA cDNA clones, in either tobacco or several leguminous host species. Furthermore, these two strains did not support the replication of cucumber mosaic virus satRNAs in tobacco. All 10 eastern PSV strains tested, on the other hand, efficiently supported PSV satRNA replication. Strains PSV-W and BV-15 can be further differentiated from the eastern strain PSV-ER by Northern hybridization using cloned cDNA probes to PSV-ER RNAs 1, 2 and 3. Whereas the PSV-ER RNA-specific probes did not hybridize to any PSV-W RNAs, the PSV-ER RNA 3 probe hybridized strongly to PSV-BV-15 RNAs 3 and 4, thus supporting the finding that strain BV-15 is serologically closely related to the eastern strains. The PSV-ER RNA 2-specific probe, but not the PSV-ER RNA 1-specific probe, hybridized to the respective RNA of PSV-BV-15. The results of Northern hybridization with strain PSV-BV-15 support the contention that strain BV-15 represents a reassortant between western and eastern strains. The lack of cross-hybridization between PSV-ER and PSV-W RNAs in Northern hybridization at high stringency is consistent with finding that the percentages of nucleotide identity between the RNAs of strains PSV-ER and PSV-W were 75, 73, 74, and 74%, respectively, for RNAs 1, 2, 3, and 4. A procedure based on reverse transcription and the polymerase chain reaction (RT-PCR) was developed that utilized primers specific for PSV-ER RNA 2. Restriction enzyme digestion of the RT-PCR products generated distinct restriction patterns that clearly differentiated western from eastern strains.

Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* in the family *Bromoviridae*. Other members of the genus are cucumber mosaic virus (CMV), the type member, and tomato aspermy virus (TAV). Cucumoviruses have tripartite genomes of positive sense single-stranded RNAs, designated RNAs 1, 2, and 3 in order of decreasing size, that are packaged in isometric particles of about 28 nm in diameter. In addition to the genomic RNAs, the virions also encapsidate a fourth RNA (RNA 4), a subgenomic RNA that functions as mRNA for the viral coat protein (28). Naturally occurring virions of CMV and PSV, but not TAV, may also package a fifth RNA, designated satellite RNA (satRNA), along with their genomic and subgenomic RNAs (31). Depending on the helper virus strain and host species involved, satRNAs can modulate the symptoms incited by the helper virus (12,27,31). More than 25 satRNA variants, ranging in size from 330 to 405 nucleotides (nt), have been reported in association with CMV (3,8,31). The PSV satRNA variants, on the other hand, are of similar size, 391–393 nt (1,24). CMV and TAV, but not PSV, support the replication of CMV satRNAs. Only PSV supports the replication of PSV satRNAs (13).

PSV is an economically important pathogen of legumes worldwide (19,21,34). In Kentucky as well as in the southeastern United States, PSV is widespread in forage legumes and is considered a major constraint to productivity and stand longevity (11,20). We have recently reported the occurrence of attenuating PSV satRNAs that, when co-inoculated with PSV, elicit the suppression of virus replication and spread in infected tobacco plants (24). The symptom-attenuating properties of satRNAs have been successfully exploited in the development of satRNA-mediated transgenic protection against CMV and tobacco ringspot virus (4,7,10,35).

We are currently testing various PSV satRNAs, including those known to be attenuating in tobacco (24), for their symptom-attenuating activities in PSV-infected forage legumes so that a transgenic protection approach may be implemented for PSV control. The success of such a strategy depends on the ability of the incoming virus to amplify the transgenically expressed satRNA transcripts so that virus replication and spread is reduced and, as a consequence, symptom development is suppressed. The occurrence in nature of virus strains that do not support satRNA replication would therefore represent a significant drawback to this strategy.

The objectives of this study were to identify and characterize naturally occurring PSV strains that are unable to support satRNA replication/encapsidation/movement in infected plants. We show that, unlike several eastern PSV strains, two western strains are incapable of supporting satRNA replication in tobacco and sev-

© 1995 The American Phytopathological Society

eral leguminous host species, and that these two western strains can be distinguished from eastern strains by Northern hybridization and polymerase chain reaction (PCR).

MATERIALS AND METHODS

Virus strains and purification. Several PSV strains from diverse sources were obtained and maintained in cowpea (*Vigna unguiculata* (L.) Walp. 'Blackeye'). The strains used in this study and their sources are listed in Table 1. All strains were increased in cowpea or burley tobacco (*Nicotiana tabacum* L. 'Ky 14') and purified as described by Ghabrial et al (5). Viral RNAs were isolated from gradient purified virus using the sodium dodecyl sulfate (SDS)–phenol method of Peden and Symons (29).

Mechanical inoculations. Carborundum-dusted tobacco and cowpea leaves were inoculated with PSV RNA at 10 μg/ml in 20 mM Na_2HPO_4 with or without the addition of 5 μg/ml of gel purified G-satRNA, V-satRNA, or in vitro transcripts derived from cloned cDNA to G-satRNA or V-satRNA (clones pT7G4 and pT7V4, respectively) (26). In some experiments gel purified CMV WL1-satRNA or infectious transcripts of CMV B-satRNA (derived from clone pBsat5) were used. The CMV satRNAs as well as CMV strain Fny were supplied by M. A. Roossinck (Noble Foundation). Plants were maintained in a greenhouse at 25/22 C day/night and a 16 h photoperiod.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Viral coat proteins were analyzed in 12.5% polyacrylamide gels using the Laemmli system (16).

RNA analysis and detection of satRNA. Leaf samples were collected from inoculated and symptomatic upper leaves, 14 days after inoculation. Total nucleic acids were extracted and analyzed in 9% polyacrylamide gels containing 8 M urea as previously described (24). After staining with ethidium bromide, the samples were electrotransferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA) in 0.5 × TAE (TAE: 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8) at 80 V for 4 h using the Bio-Rad Trans-Blot apparatus. Membranes were baked at 80 C for 2 h, pre-hybridized, hybridized, and washed under high stringency conditions as previously described (24). For hybridization, a nick-translated ^{32}P-labeled probe was prepared from plasmid pT7G4 containing a full-length G-satRNA (26) using a commercial kit from Gibco-BRL (Gaithersburg, MD).

Northern hybridization analysis of viral RNAs. Viral RNAs were separated in 1.5% agarose-formaldehyde gels, transferred onto Zeta-Probe nylon membranes (Bio-Rad) and probed with ^{32}P-labeled probes specific for PSV-ER RNAs 1, 2, and 3. A cDNA library representing PSV-ER RNAs has been constructed and cDNA clones spanning the entire sequence of genomic RNAs 1, 2, and 3 have been identified and completely sequenced (GenBank accession numbers U15728, U152729, and U15730, respectively). Briefly, poly(A)$^+$ tails were added enzymatically to the viral RNAs by treatment with poly(A) polymerase, and first strand cDNA synthesis was primed with oligo $(dT)_{12-18}$ primers. Double-stranded cDNA (ds-cDNA) was synthesized by the procedures of Gubler and Hoffman (6) using the cDNA synthesis kit from Amersham (Arlington Heights, IL). The blunt-ended ds-cDNA was cloned in the *Sma*I site of the phagemid vector pUC119. Nucleotide sequences were obtained with both single- and double-stranded DNA templates using the dideoxynucleotide chain termination method (33).

The cDNA probes used in this study were prepared by nick translation (32) of the following plasmids containing cDNA to PSV-ER RNAs 1, 2, and 3, respectively (approximate size of insert in parenthesis): pER1-87 (1 kbp), pER2-60 (2.0 kbp), and pER3-19 (2.0 kbp).

Polymerase chain reaction. cDNA was synthesized by reverse transcription (RT) with Superscript reverse transcriptase (Gibco-BRL) in 20-μl reaction mixtures prepared and incubated as suggested by the supplier. Reactions contained 100 ng viral RNA template and 50 pmol of an antisense primer (PSVER2r), 5′-GCGCTGACTTCTGTTTTTCC-3′, complementary to nt positions 2,436 to 2,455 in PSV-ER RNA 2. PCR amplification of cDNA templates was performed with AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT) in 50-μl reactions prepared as suggested by the supplier. PCR reaction mixtures contained 1 μl of cDNA reaction product and 50 pmol each of sense (PSVER2f) and antisense primer (PSVER2r). The sense primer PSVER2f, 5′-CTGCCTCTGGGCTCACTTAC-3′ was identical to nt positions 1,285 to 1,304 of PSV-ER RNA 2. Based on the nucleotide sequence of PSV-ER RNA 2, the pair of primers is predicted to direct the amplification of a PCR product of 1,171 bp. Amplification was carried out in a Perkin-Elmer Cetus DNA thermocycler model 480 programmed to perform 25 cycles of 1 min at 94 C, 1 min at 60 C, and 90 s at 72 C. The initial cycle was preceded by a 2-min denaturation step at 95 C and the final cycle was followed by an additional 3-min extension step at 72 C.

The RT-PCR products were concentrated by phenol/chloroform extraction and ethanol precipitation prior to digestion with *Nde*I, which cleaves the PSV-ER RNA 2 product into three fragments (623, 481, and 67 bp). Digested and undigested RT-PCR products were analyzed by agarose gel electrophoresis in 1.5% agarose followed by staining with ethidium bromide. The stained gels were visualized using a GDS 7500 gel documentation system (UVP, Inc., Upland, CA).

RESULTS

Satellite RNA support by various PSV strains. With two exceptions, PSV satRNA was detected in total nucleic acid extracts from all cowpea and tobacco plants previously co-inoculated with satRNA and one of several PSV strains as helper virus (Table 1). The PSV western strains W and BV-15, unlike the eastern strains tested, did not support satRNA replication. Furthermore, no satRNA support function could be demonstrated for the western strains in several other leguminous hosts including common bean (*Phaseolus vulgaris* L.), red clover (*Trifolium pratense* L.), and pea (*Pisum sativum* L.).

In a more detailed study, we used Northern hybridization to monitor satRNA in preparations of total nucleic acids from plants co-inoculated with satRNA and one of three PSV strains, BV-15, W, or ER, as helper virus. For each of the three strains, no satRNA was detected in any of the plants inoculated with the

TABLE 1. Sources of peanut stunt virus strains and their abilities to support satellite RNA (satRNA) replication

Virus strain	Source plant	Location	Collector/supplier (reference)	SatRNA support[a]
74-23 (PSV-V)	Peanut	Va.	S. A. Tolin (17)	(+)*[b]
76-69	Bean	Va.	S. A. Tolin (13)	(+)*
76-93	Tobacco	Va.	S. A. Tolin	(+)
H4D8	Peanut	Va.	S. A. Tolin	(+)
M-6	Bean	Md.	S. A. Tolin	(+)
M76-20	Lima bean	Md.	S. A. Tolin	(+)
WC	White clover	Ky.	S. A. Ghabrial (24)	(+)*
E	Bean	N.C.	O. W. Barnett	(+)
ER	Cowpea	Ga.	J. Demski (24)	(+)*
G	Cowpea	Ga.	J. Demski (24)	(+)*
W	Bean	Wash.	G. Mink/ J. M. Kaper	(–)
BV-15	Bean	Wash.	G. Mink/ J. M. Kaper	(–)

[a] (+) = satRNA was detected in total nucleic acids extracts as revealed by electrophoresis in 9% semidenaturing polyacrylamide gels (see Fig. 1A for an example); (–) = no satRNA was detected.

[b] (*) an asterisk denotes that virions from the designated strains have been previously reported to encapsidate satRNA; reference is indicated (in parenthesis) under collector/supplier column.

genomic RNAs (1+2+3) alone (Fig. 1, lanes 2, 4, and 7). Sat-RNA-specific bands including the slow migrating single-stranded form and the faster migrating double-stranded form were detected only in plants co-inoculated with PSV-ER and G-satRNA (Fig. 1, lanes 3A and 3B) or V-satRNA (data not shown). On the other hand, no satRNA specific bands were detected in plants co-inoculated with PSV-BV-15 or PSV-W plus G-satRNA (Fig. 1, lanes 5A and B, and 8A and B, respectively) or V-satRNA (data not shown). Similar results were obtained when in vitro transcripts from either the G-satRNA or the V-satRNA full-length cDNA clones were used instead of the naturally occurring sat-RNAs. Likewise, these results were reproducible regardless of the host plant used (tobacco or cowpea) or the tissue analyzed (inoculated leaves versus symptomatic upper leaves).

Virions were purified from the same plants used for total nucleic acids preparations and the encapsidated RNAs were analyzed by Northern blot hybridization, as described above. Whereas PSV BV-15 and W virions were satRNA-free, those of PSV-ER contained satRNA. Furthermore, Northern blot analysis of total nucleic acids preparations following 10 serial passages in either cowpea or tobacco did not reveal any satRNA replication in the presence of PSV strains BV-15 and W as helper viruses.

In further studies, we investigated whether the western strains can support CMV satRNA. The results indicated that whereas the CMV strain Fny efficiently supported the replication of CMV B- or WL1-satRNA, neither western nor eastern strains of PSV could do so (data not shown).

Comparative biochemical properties of the three viral strains. The coat protein and genomic RNAs of the three strains were partially characterized. When analyzed by SDS-PAGE in 12.5% gels using the discontinuous buffer system of Laemmli (16), the coat proteins of PSV strains ER, BV-15, and W strains migrated as a single polypeptide with an M_r of 30,000–31,000 (Fig. 2). These values are higher than those predicted from the deduced amino acid sequences, M_r of 24,000–25,000 (25). The coat proteins of cucumoviruses have recently been reported to migrate anomalously in SDS–polyacrylamide gels (9). Interestingly, the relative migration of the coat protein from strain BV-15, originally isolated in Washington State, was similar to that of the PSV eastern strain ER, and the coat protein from both strains BV-15 and ER migrated faster than that from the western strain W.

Analysis of purified viral RNAs by PAGE in 2.4% semidenaturing polyacrylamide gels revealed the typical pattern of genomic and subgenomic RNAs for each of the three PSV strains (Fig. 3A). All three strains showed similar profiles of four distinct RNA bands with the relative migration distances of the individual RNA species differing slightly among strains. Furthermore, RNA 1 and 2 from PSV strains ER and BV-15 appeared to occur in approximately equimolar ratios (Fig. 3A), whereas RNA 1 from strain W consistently occurred in disproportionately higher amounts relative to RNA 2. This was particularly evident when RNA analysis was carried out in 3% gels (Fig. 3B).

Nucleotide sequence homology. The results of Northern blot hybridization of PSV-ER, -W, and -BV-15 RNAs with cloned probes specific for PSV-ER RNAs 1, 2, and 3 are shown in Figure 4. Whereas the PSV-ER RNA 1-specific probe hybridized only to RNA 1 from strain ER (Fig. 4A), the PSV-ER RNA 2-specific probe hybridized to RNA 2 from both PSV-ER and PSV-BV-15. A somewhat weaker hybridization signal, however, was obtained with BV-15 RNA 2 (Fig. 4B). The PSV-ER RNA 3-specific probe hybridized strongly to RNA 3 and 4 from both PSV-ER and PSV-BV-15. None of the PSV-ER probes hybridized with PSV-W RNAs.

Differentiation of PSV strains by RT-PCR. RT-PCR amplification with primers PSVER2f and PSVER2r of RNA from PSV strains W, BV-15, and ER yielded the expected size product of approximately 1.2 kbp (Fig. 5, lanes 2, 3, and 4, respectively). For strain BV-15, two additional bands (1.0 and 0.6 kbp in size) of similar intensity to the 1.2-kbp band, were produced (Fig. 5, lane 3). Digestion with *Nde*I of the RT-PCR product of PSV-ER is predicted, based on the nucleotide sequence of PSV-ER RNA 2 (Genbank accession number U152729), to yield three fragments, two of which, 0.6 and 0.5 kbp, were observed in the stained gel (Fig. 5, lane 7). The third predicted product of 67 bp could not be detected by our gel electrophoresis system. In contrast, the RT-

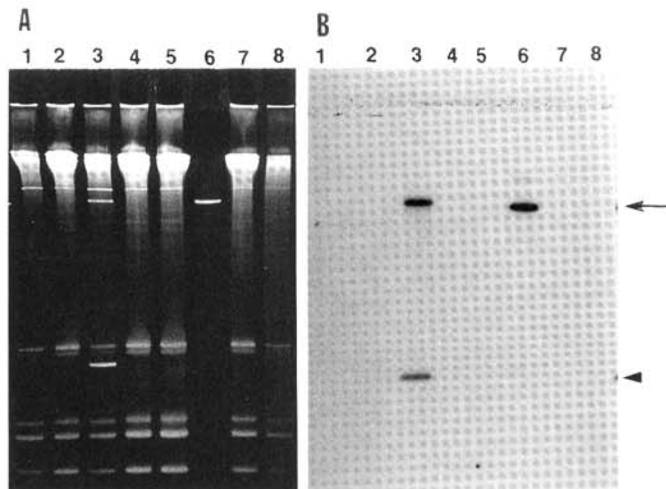

Fig. 1. Northern blot analysis of total nucleic acid extracts from tobacco plants 2 wk after inoculation with each of three strains of peanut stunt virus (PSV) alone or mixed with G-satellite RNA (satRNA). Total nucleic acid extracts were subjected to electrophoresis on semidenaturing 9% polyacrylamide gels and **A,** photographed after ethidium bromide staining or **B,** electrotransferred to Zeta-Probe membranes and hybridized to ^{32}P-labeled nick-translated cloned satRNA cDNA. Total nucleic acid preparations were from mock-inoculated plants (lanes 1), plants inoculated with PSV-ER alone (lanes 2), PSV-ER plus G-satRNA (lanes 3), PSV-BV-15 alone (lanes 4), PSV-BV-15 plus G-satRNA (lanes 5), PSV-W alone (lanes 7) and PSV-W plus G-satRNA (lanes 8). Purified G-satRNA was applied to lanes 6. The arrow indicates the position of single-stranded satRNA, and the arrowhead denotes the position of double-stranded satRNA.

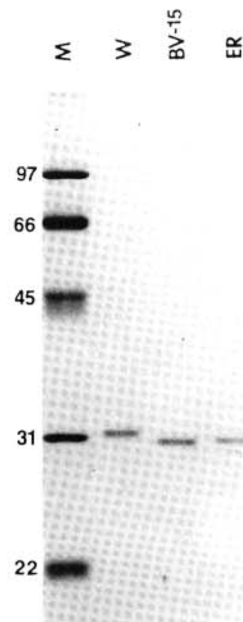

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the coat proteins from three strains of peanut stunt virus (PSV). Electrophoresis was performed in 12.5% polyacrylamide gels using the Laemmli system. Lanes W, BV-15, and ER represent protein samples from purified virions of PSV-W, PSV-BV-15, and PSV-ER, respectively. Molecular weight protein standards were applied to lane M.

PCR products of PSV-BV-15 were not digested by *Nde*I (Fig. 5, lane 6) whereas that of PSV-W was cleaved at least once resulting in a single visible fragment of approximately 1 kbp but no smaller fragments were resolved in our gel system (a fragment of 200 bp would be predicted if the RT-PCR product was cleaved only once) (Fig. 5, lane 5). The yields of RT-PCR products of PSV strains BV-15 and W were consistently lower than that of strain ER. To obtain stained bands of RT-PCR products of comparable intensity (Fig. 5), the RT-PCR products of strains W and BV-15 were concentrated 10- to 25-fold compared with that of strain ER. Furthermore, *Nde*I digestion patterns of three other eastern strains, PSV-V, PSV-E, and PSV-76-69, were identical to that of PSV-ER (data not shown).

DISCUSSION

The availability in our laboratory of full-length cDNA clones of PSV satRNAs from which infectious transcripts can be generated (26) has allowed us to test a set of PSV strains from diverse geographical sources for their abilities to support the replication of satRNA. Two western strains of PSV, unlike 10 eastern strains tested, were incapable of supporting the replication of two different naturally occurring PSV satRNAs or full-length transcripts of cDNA clones derived from them. Furthermore, neither the western nor the eastern PSV strains could support the replication of two distinct CMV satRNAs. The finding that total nucleic acids extracts from infected plants were devoid of satRNA (Fig. 1) does not by itself rule out the possibility that the western strains may support the replication but not the encapsidation of satRNAs. SatRNA may be replicated in the initially infected cells but its cell-to-cell and long distance movement may require its encapsidation in particles (28). This possibility, however, appears unlikely because progeny virions from plants co-inoculated with satRNA and pseudorecombinants composed of RNA 1 and 2 from the western strain W and RNA 3, which contains the coat protein gene, from the eastern strain ER were devoid of satRNA (C.-C. Hu, *unpublished*).

It is well recognized that the efficiency with which a given strain of CMV will support the replication of satRNA may vary with the host species and satRNA isolate involved (12,23,30). To our knowledge, there are no known CMV strains that, like the western strains of PSV, are generally incapable of supporting satRNA replication. This distinction between the two cucumoviruses may not be too surprising considering that PSV satRNAs, unlike CMV satRNAs, show little variability in size and structure (1,24). Furthermore, PSV has a relatively limited host range compared with CMV, and is mainly considered as a pathogen of legumes (21,34). It cannot be ruled out, however, that alternative host species may still be found in which the western strains of PSV can support satRNA replication.

It is noteworthy that all eastern PSV strains tested supported satRNA replication. The strategy of satRNA-mediated transgenic protection of forage legumes against PSV may therefore be implemented in the eastern United States provided that satRNAs that attenuate PSV symptoms in forage legumes are identified and measures are taken to prevent the introduction of western strains. The potential risks of implementing a transgenic satRNA strategy for CMV control have been described to include the mutation of the transgenic attenuating satRNA to a symptom-intensifying satRNA and the encapsidation and mobilization of transgenic satRNA to nontarget plants where it may exacerbate symptoms (31). These risks should be minimal or nonexistent with the PSV-satRNA system because, despite an extensive search, none of the PSV satRNAs so far isolated have been found to intensify symp-

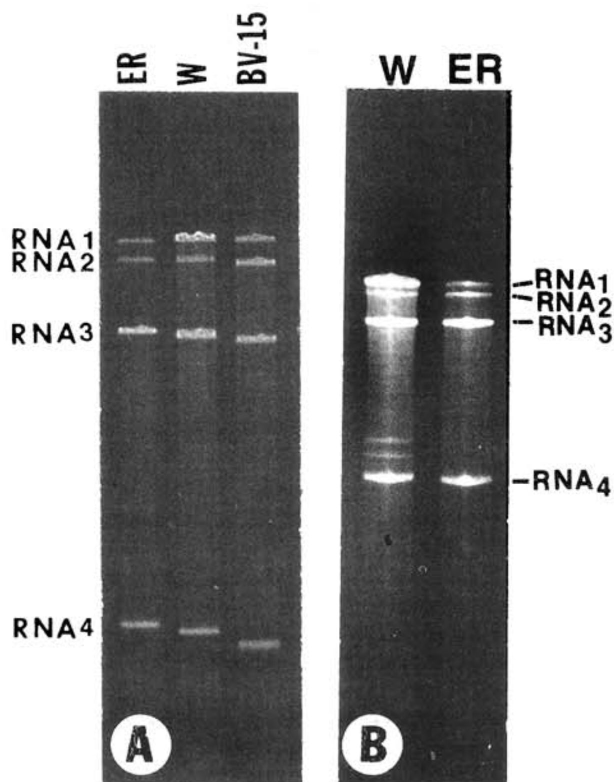

Fig. 3. Polyacrylamide gel electrophoresis of RNA from purified virions of three strains of peanut stunt virus (PSV). Electrophoresis was performed in either **A,** 2.4% or **B,** 3% semidenaturing polyacrylamide gels. Lanes ER, W, and BV-15 represent RNA samples from strains PSV-ER, PSV-W, and PSV-BV-15, respectively.

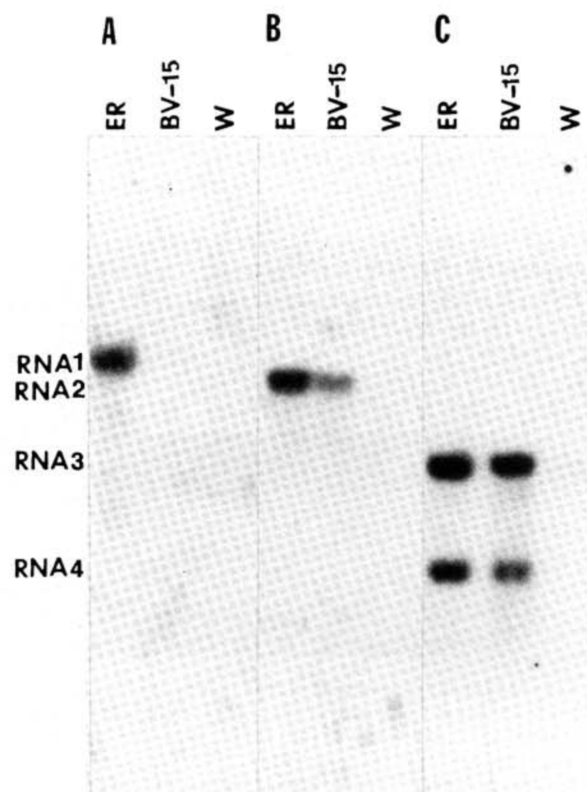

Fig. 4. Northern hybridization analysis of RNA from three strains of peanut stunt virus (PSV). Purified viral RNAs from strains PSV-ER (lanes ER), PSV-BV-15 (lanes BV-15), and PSV-W (lanes W) were separated in 1.5% agarose-formaldehyde gels and transferred by capillary action onto Zeta-probe nylon membranes. The blots were probed with ^{32}P-labeled nick-translated cDNA probes specific for **A,** PSV-ER RNA 1, **B,** RNA 2, and **C,** RNA 3. The positions of RNA 1, 2, 3, and 4 are indicated to the left.

toms and because PSV, compared with CMV, has a limited host range (21,24,31). Furthermore, the introduction of new PSV strains distinct from the eastern strains in genomic sequences may not pose any risks for mobilizing satRNAs to nontarget hosts because such strains may not replicate and encapsidate satRNAs, as was shown for the western strains (this study).

On the basis of serology and nucleic acid competition hybridization, strains of PSV have been differentiated into eastern and western subgroups (2,21,22). The BV-15 strain of PSV is of special interest because it appears to share properties with both groups. Although the original isolate of BV-15 supplied to researchers in the eastern United States was believed to be a subculture of the W strain (G. Mink, *personal communication*), published reports indicated that strain BV-15 has partial nonoverlapping sequence homology with both eastern and western subgroups in competition hybridization experiments (2). The finding that the PSV-ER RNA 3-specific probe hybridized strongly to BV-15 RNAs 3 and 4 (Fig. 4C) is in agreement with the finding that BV-15 is serologically closely related to the eastern strains but distinct from the western strain W in both gel double diffusion assays (S. A. Tolin, *personal communication*) and enzyme-linked immunosorbent assay/western blotting (C.-C. Hu, *unpublished*).

The complete nucleotide sequences of PSV-ER RNA 1, 2, and 3 (GenBank accession numbers U15728, U152729, and U15730, respectively) and partial sequences (1–2 kb) of the corresponding RNAs of PSV-W (C.-C, Hu, *unpublished*) have been determined. Computer-assisted analysis of the available PSV-W and ER sequences contained in the three PSV-ER probes indicated that the percentages of nucleotide identity between the RNAs of strains ER and W were 75, 73, 74, and 74%, respectively, for RNAs 1, 2, 3, and 4. This may explain the lack of cross-hybridization between the corresponding genomic RNAs of PSV-ER and PSV-W because only sequences with at least 90% homology to the probe can be detected under the high stringency conditions used for hybridization (50% formamide at 42 C) (18). It is of interest to note that the percentages of nucleotide identity between the RNAs of strain ER and the Japanese strain, PSV-J (14,15), are 91, 94, 91, and 89%, respectively, for RNAs 1, 2, 3, and 4. Therefore, the J strain is more closely related to the eastern than to the western subgroup.

Although PSV-BV-15 RNA 1 did not hybridize to the PSV-ER RNA 1-specific probe, BV-15 RNAs 3 and 4 hybridized strongly to the PSV-ER RNA 3 probe. Furthermore, PSV-ER RNA 2 probe hybridized with the BV-15 RNA 2 but not as strongly as with its own RNA 2. Diaz-Ruiz and Kaper (2) proposed that strain BV-15 may represent a reassortant between eastern and western strains. Combining their data with those presented here, it can be inferred that BV-15 RNA 1 is derived from a western strain and BV-15 RNA 3 is of eastern origin, whereas BV-15 RNA 2 is at least partly similar to eastern strains but may be of more complex origin. This conclusion is supported by the results of the RT-PCR experiments using primers specific for PSV-ER RNA 2. Whereas an RT-PCR product of the expected size (1.2 kbp) was obtained with strains ER and W, three RT-PCR products, including the 1.2-kbp product and two smaller fragments, were produced with strain BV-15 (Fig. 5). The origin of the smaller products, however, is unknown and sequencing of the three fragments will be required in order to differentiate between at least two possibilities: the primer pair used may anneal to other sites in PSV-BV-15 RNA 2; or, alternatively, strain BV-15 may contain a heterogeneous population of RNA 2 molecules. The finding that the smaller two fragments were generated by PCR amplification of the larger RT-PCR product (data not shown) favors the contention that the primer pair anneals to alternative sites in RNA 2 within the sequence corresponding to the large RT-PCR product. The properties of RNA 2 of strain BV-15 are thus typical of neither eastern nor western strains and it may represent the product of intermolecular recombination involving RNA 2 sequences from both eastern and western strains. Alternatively, BV-15 RNA 2 may be derived from a still uncharacterized PSV strain.

In conclusion, we have identified two strains of PSV, a western strain and a reassortant (or possibly a recombinant) between western and eastern strains, that are incapable of supporting satRNA replication in tobacco and several leguminous host species. In addition to differences in the satRNA support function, these two strains could be readily distinguished from eastern strains by Northern hybridization and RT-PCR assays.

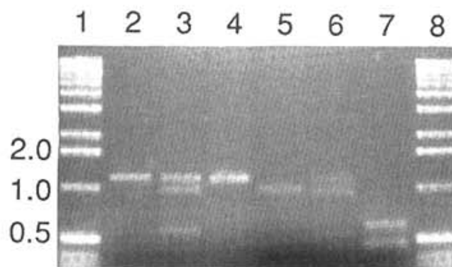

Fig. 5. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA from three strains of peanut stunt virus (PSV) using a PSV-ER RNA 2-specific primer pair. Lanes 1 and 8: 1 kb ladder; lanes 2, 3, and 4: RT-PCR amplification products of RNAs from PSV-W, PSV-BV-15, and PSV-ER, respectively; lanes 5, 6, and 7: *Nde*I digestion patterns of RT-PCR products of PSV-W, -BV-15, and -ER, respectively. The primers used in RT-PCR (PSVER2r and PSVER2f) are specific for PSV-ER RNA 2 and are predicted to prime the amplification of a product of 1,171 bp in length. The RT-PCR products were concentrated by phenol/chloroform extraction and ethanol precipitation prior to digestion with *Nde*I and visualization by 1.5% agarose gel electrophoresis and ethidium bromide staining. RT-PCR products of the predicted size and *Nde*I digestion patterns identical to that of PSV-ER (lanes 4 and 7) were obtained with three other eastern strains, PSV-V, -E, and -76-69.

LITERATURE CITED

1. 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. Nat. Acad. Sci. USA 82:3110-3114.
2. Diaz-Ruiz, J. R., and Kaper, J. M. 1983. Nucleotide sequence relationships among thirty peanut stunt virus isolates determined by competition hybridization. Arch. Virol. 75:277-281.
3. Fraile, A., and Garcia-Arenal, F. 1991. Secondary structure as a constraint on the evolution of a plant viral satellite RNA. J. Mol. Biol. 221:1065-1069.
4. Gerlach, W. L., Llewllyn, D., and Haseloff, J. 1987. Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature 238:802-805.
5. 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.
6. Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.
7. Harrison, B. D., Mayo, M. A., and Baulcombe, D. C. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature 238:799-802.
8. Hidaka, S., and Hanada, K. 1994. Structural features unique to a new 405-nucleotide satellite RNA of cucumber mosaic virus inducing tomato necrosis. Virology 200:806-808.
9. Hu, C.-C., and Ghabrial, S. A. 1993. Factors contributing to the anomalous electrophoretic mobility of cucumoviruses coat proteins in SDS/polyacrylamide gels. (Abstr.) Phytopathology 83:1374.
10. 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.
11. Jones, R. T., and Diachun, S. 1976. Identification and prevalence of viruses in red clover in central Kentucky. Plant Dis. Rep. 60:690-694.
12. Kaper, J. M., and Tousignant, E. M. 1977. Cucumber mosaic virus associated RNA5. 1. Role of host plant and helper virus in determining the amount of associated RNA5 with virions. Virology 80:186-195.
13. Kaper, J. M., Tousignant, M. E., Diaz-Ruiz, J. R., and Tolin, S. A. 1978. Peanut stunt virus-associated RNA5: Second tripartite genome virus with

an associated satellite-like replicating RNA. Virology 88:166-170.
14. Karasawa, A., Kazuhiro, N., Kakutani, T., Minobe, Y., and Ehara, Y. 1991. Nucleotide sequence of RNA 3 of peanut stunt cucumovirus. Virology 185:464-467.
15. Karasawa, A., Kazuhiro, N., Kakutani, T., Minobe, Y., and Ehara, Y. 1992. Nucleotide sequence analyses of peanut stunt cucumovirus RNAs 1 and 2. J. Gen. Virol. 73:701-707.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 227:680-685.
17. Lot, H., and Kaper, J. M. 1976. Physical and chemical differentiation of three strains of cucumber mosaic virus and peanut stunt virus. Virology 74:209-222.
18. Memelink, J, Swords, K. M. M., Staehelin, L. A., and Hoge, J. H. C. 1994. Southern, Northern and Western blot analysis. Pages F1:1-23 in: Plant Molecular Biology Manual. 2nd ed. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer Academic Publishers, London.
19. McLaughlin, M. R., and Boykin, D. L. 1988. Virus diseases of seven species of forage legumes in the southeastern United States. Plant Dis. 72:539-542.
20. McLaughlin, M. R., Pederson, G. A., Evans, R. R., and Ivy R. L. 1992. Virus diseases and stand decline in a white clover pasture. Plant Dis. 76:158-162.
21. Mink, G. I. 1972. Peanut stunt virus. In: CMI/AAB Descriptions of Plant Viruses, No. 92.
22. Mink, G. I., Silbernagel, M. J., and Saksena, K. N. 1969. Host range, purification, and properties of the western strain of peanut stunt virus. Phytopathology 59:1625-1631.
23. Moriones, E., Diaz, I., Rodriguez-Cerezo, E., Fraile, A., and Garcia-Arenal, F. 1992. Differential interactions among strains of tomato aspermy virus and satellite RNAs of cucumber mosaic virus. Virology 186:475-480.
24. Naidu, R. A., Collins, G. B., and Ghabrial, S. A. 1991. Symptom-modulating properties of peanut stunt virus satellite RNA sequence variants. Mol. Plant-Microbe Interact. 4:268-275.
25. Naidu, R. A., Collins, G. B., and Ghabrial, S. A. 1991. Nucleotide sequence analysis of a cDNA clone encoding the coat protein gene of peanut stunt virus. Plant Mol. Biol. 17:175-177.
26. Naidu, R. A., Collins, G. B., and Ghabrial, S. A. 1992. Peanut stunt virus satellite RNA: analysis of sequences that affect symptom attenuation in tobacco. Virology 189:668-677.
27. 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.
28. Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., and Francki, R. I. B. 1992. Cucumber mosaic virus. Adv. Virus Res. 41:281-348.
29. Peden, K. W. C., and Symons, R. H. 1973. Cucumber mosaic virus contains a functionally divided genome. Virology 53:487-492.
30. Roossinck, M. J., and Palukaitis, P. 1991. Differential replication in zucchini squash of a cucumber mosaic virus satellite RNA maps to RNA 1 of the helper virus. Virology 181:371-373.
31. Roossinck, M. J., Sleat, D., and Palukaitis, P. 1992. Satellite RNAs of plant viruses: structures and biological effects. Microbiol. Rev. 56:265-279.
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY.
33. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
34. 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.
35. Yie, Y., Zhao, F., Zhao, S. Z., Liu, Y. Z., Liu, Y. L., and Tien, P. 1992. High resistance to cucumber mosaic virus conferred by satellite RNA and coat protein in transgenic commercial tobacco cultivar G-140. Mol. Plant-Microbe Interact. 5:460-465.