Peanut Chlorotic Streak Virus, a New Caulimovirus Infecting Peanuts (*Arachis hypogaea*) in India

D. V. R. Reddy, R. D. Richins, R. Rajeshwari, N. Iizuka, S. K. Manohar, and R. J. Shepherd

First author, principal plant virologist, and third and fifth authors, research associates, Legumes Virology Unit and Electron Microscope Unit, respectively, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, India. Second and sixth authors, graduate student and professor, Department of Plant Pathology, University of Kentucky, Lexington 40546. Fourth author, visiting scientist from the Tropical Agricultural Research Center, Tsukuba-Science City, Yatabe, Ibaraki 305, Japan. Present address of R. Rajeshwari, School of Life Sciences, Hyderabad Central University, Gachibowli, Hyderabad.
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ABSTRACT

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Peanut (*Arachis hypogaea* [groundnut]) plants with reduced leaflets, chlorotic streaks, and stunting were observed during surveys for diseases caused by peanut viruses in India. These peanut plants were infected with a new caulimovirus designated peanut chlorotic streak virus (PClSV). PClSV was mechanically transmissible to several plants in Leguminosae and Solanaceae but was not transmitted by *Aphis craccivora* or *Myzus persicae*. Purified from *Nicotiana clevelandii* leaves, PClSV contained isometric particles 52 ± 3 nm in diameter. The virus was not related to cauliflower mosaic, figwort mosaic, or soybean chlorotic mottle viruses. Inclusion bodies similar to those produced by caulimoviruses were observed in the cytoplasm of infected *Nicotiana rustica* and *A. hypogaea* leaves. Purified PClSV contained two polypeptides with relative molecular masses of 58 and 51 kDa. The size of double-stranded DNA was estimated as approximately 8.1 kbp, which contained two single-stranded discontinuities. The physical map of the PClSV genome was distinctly different from those of other caulimoviruses.

Peanut (*Arachis hypogaea* L. [groundnut]) plants showing reduced leaflets, chlorotic streaks, and stunting were observed during surveys in Anantapur and Chittoor districts, Andhra Pradesh State, India. A similar disease was subsequently observed on peanut plants in the states of Karnataka, Maharashtra, and Tamil Nadu. The natural field incidence did not exceed 1%; infected plants occurred as scattered individuals. Spherical virus particles, 52 ± 3 nm in diameter, were consistently observed in leaflet extracts. In thin sections of leaflets, inclusion bodies similar to those produced by caulimoviruses were observed.

Peanut plants are susceptable to infection by many viruses (9,10); this, however, is the first report of a caulimovirus which infects peanut. We describe the host range, symptoms, purification, serological relationships, and some physico-chemical properties of the virus, designated peanut chlorotic streak virus (PClSV), isolated from peanut plants. Evidence shows that PClSV is a new member of the caulimovirus group.

MATERIALS AND METHODS

Virus culture and maintenance. Peanut plants showing chlorotic streaks on leaflets as well as stunting were collected during surveys, and the virus culture was established by graft-inoculating healthy peanut plants (cv. TMV 2). Extracts from leaflets on grafted plants showing initial symptoms were prepared in PBT (0.05 M phosphate buffer, pH 7.0, containing 0.075% thioglycerol) and were sap inoculated onto *Vigna unguiculata* L. *verde* (cowpea, cv. C 152). The virus, isolated from a single local lesion after five successive, single local-lesion transfers, was maintained in *Nicotiana clevelandii* A. Gray or in *Nicotiana rustica* L.

Assay host. Fully expanded primary leaves of *V. unguiculata* produced characteristic necrotic lesions and as a result, were used for assay.

Host-range studies. Extracts from systemically infected *N. clevelandii* or *N. rustica* leaves, prepared in PBT, were inoculated onto at least 10 plants of each of 23 test species belonging to five families and were maintained for 60 days at 25–30 C. Inoculated young leaves of all test species were examined for infection through sap inoculations into *V. unguiculata* and through direct antigen coating (DAC) enzyme-linked immunosorbent assay (ELISA), using homologous antiserum.

Properties in plant extracts. Extracts of *N. rustica* prepared in PBT were used to determine the dilution end point (DEP), the thermal-inactivation point (TIP), and the longevity in vitro (LIV). A 1:10 dilution of sap was used for the TIP and LIV tests.

Aphid transmission. Virus-free colonies of *Aphis craccivora* Koch. and *Myzus persicae* Sulz. were reared on peanut and cabbage, respectively. Aphids were starved for 2 h and were then provided access, for a period of 15 min to 1 day, to infected *A. hypogaea* and *N. clevelandii*. Fifteen to twenty aphids were transferred to each caged, healthy *A. hypogaea* and *N. clevelandii* plant. After an inoculation-access period of 3 days, the aphids were killed with 0.025% Metasystox spray. The test plants were kept in a glasshouse for 2 mo, at 25–30 C.

Seed transmission. Seeds (390) from mechanically inoculated peanut plants (cultivars TMV 2 and Robut 33-1) were planted in sterile soil, and the seedlings were observed for symptoms for 45 days. All seedlings were tested by DAC-ELISA.

Purification. All purification steps were conducted near 4 C. Systemically infected *N. clevelandii* leaves, harvested 2–3 wk after inoculation and stored at −80 C, were used in purification. The tissue was homogenized in a blender in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.5% thioglycerol (3 ml per gram of tissue). The extract was filtered through two layers of cheesecloth and was treated with chloroform (10%, v/v) for 15 min. The emulsion was centrifuged at 4,000 *g* for 15 min. The aqueous phase was centrifuged at 25,000 rpm for 2 h in a Beckman SW28 rotor. The pellets were suspended in 0.02 M phosphate buffer, pH 7.2, (PB) (0.15 ml per gram of tissue) and were clarified at 4,000 *g* for 10 min. The supernatant was layered onto a sucrose gradient composed of 8 ml each of 10, 20, 30, and 40% sucrose (w/v) in PB and was centrifuged for 1 h at 25,000 rpm in a Beckman SW28 rotor. A light-scattering zone, 4.5–5.0 cm, from the bottom was drawn off and diluted in PB (1:5). The virus was pelleted at 36,000 rpm for 2 h in a Beckman R40 rotor, suspended in PB (25 μl per gram of tissue) and centrifuged (0.5

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ml) in a CsCl gradient (prepared by layering 2.0, 1.5, and 1.0 ml of 30, 40, and 50% (w/v) CsCl in PB) for 4 h at 30,000 rpm in a Beckman SW39 rotor. A single sharp, light-scattering zone was removed, dialyzed overnight against PB, and centrifuged in a Beckman R40 rotor at 36,000 rpm for 2 h. Virus yield was assessed by assuming an extinction coefficient of 7.0 (1 mg per milliliter, at 260 nm) (15).

Electron microscopy. Leaf tissue of *A. hypogaea* and *N. rustica* showing distinct symptoms was cut into small pieces, fixed in 2% gluteraldehyde in 0.1 M PB, pH 7.0, and washed three times in the buffer. The leaf pieces were postfixed in 2% aqueous osmium tetroxide for 6 h and were washed three times in distilled water. Samples were dehydrated in a graded series of acetone, then embedded in Spurr's medium. Ultrathin sections were stained in uranyl acetate and lead citrate and examined with a Philips 201 C electron microscope (Philips Company, Holland).

Formvar-coated, carbon-stabilized 200 mesh-copper grids were floated on 10-μl drops of purified virus preparations for 1 h. Grids were washed with distilled water and stained with 2% aqueous uranyl acetate for 2 min.

Serology. A New Zealand white, inbred rabbit was immunized, at weekly intervals, with five intramuscular injections of purified virus (1 mg in 0.5 ml of PB) emulsified with an equal volume of Freund's incomplete adjuvant. Two weeks after the fifth injection, the rabbit was bled at weekly intervals. Titer was determined with the precipitin-ring interface test (11).

DAC-ELISA was employed (4), utilizing healthy and infected *A. hypogaea* and *N. clevelandii* leaf extracts to test serological relationships. Heterologous antisera were cross-absorbed either with *A. hypogaea* or *N. clevelandii* leaf extracts, depending on the source of the antigen. Rabbit Fc-specific immunoglobulins from antisera produced in goats were conjugated to alkaline phosphatase and used at 0.5 μg per milliliter dilutions (2). *P*-Nitrophenyl phosphate was used as the substrate. Absorbance values were recorded at 405 nm for three replications of each sample.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of viral-coat proteins. SDS-PAGE analysis of virus proteins and molecular-weight standards was done according to the procedure of Laemmli (6), as described by Naidu et al (8). Only freshly purified virus was used for each analysis. Gels were stained with 0.2% Coomassie Brilliant Blue R-250.

Nucleic acid characterization. Restriction endonucleases and modification enzymes used for routine molecular manipulations were purchased from several manufacturers—New England Biolabs (Beverly, MA), American Allied Biochemicals (Denver, CO), and United States Biochemicals (Cleveland, OH)—and were used in accordance with the manufacturers' recommendations. Viral DNA was isolated from PClSV-infected *N. clevelandii* plants following the procedure of Shepherd et al (18), was treated with one of several restriction endonucleases, and was analyzed by agarose-gel electrophoresis (7). Treatment of viral DNA with *Bam*HI or *Kpn*I yielded, on agarose gels, a single band of approximately 8 kbp. Recombinant plasmids were prepared by inserting *Bam*HI- or *Kpn*I-digested DNA into similarly digested pUC119. *Escherichia coli* K12, strain JM101 (19), was used as the recipient bacterial host for plasmid transformations (3). Immediately before plating the cells on selective medium 50 μl of 2% X-Gal (in dimethylformamide) and 20 μl of 2% isopropylthiogalactoside (in water) were added to the transformed cells. The cells were plated on YTA medium (0.5% [w/v] yeast extract; 0.8% [w/v] bacto-tryptone; 0.5% [w/v] NaCl; and 1.5% [w/v] bacto-agar) containing per milliliter 500 μg of penicillin (Sigma Chemical Company, St. Louis, MO) and were incubated at 37 C, overnight.

White colonies were inoculated into 12-ml culture tubes containing 3 ml of YT medium (0.5% [w/v] yeast extract; 0.8% [w/v] bacto-tryptone; and 0.5% [w/v] NaCl) and were grown in an orbital shaker/incubator for 8–24 h at 37 C. Plasmid DNA was isolated from these cultures using the alkaline-lysis DNA-miniprep method of Birnboim (1).

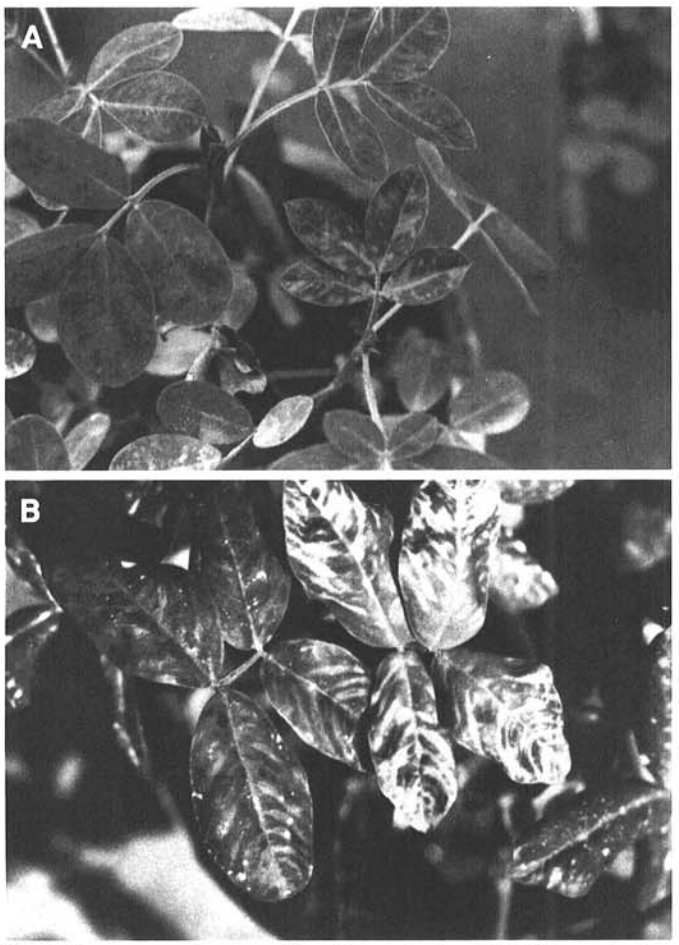

Fig. 1. Symptoms of peanut chlorotic streak virus (PClSV) on *Arachis hypogaea*. **A,** Initial symptoms (chlorotic spots). **B,** Typical symptoms (chlorotic streaks).

TABLE 1. Host range of peanut chlorotic streak virus

Host species	Symptoms[a]	
	Local	Systemic
Arachis hypogaea	CST[b]	M
Canavalia ensiformis	CL	...[c]
Cyamopsis tetragonoloba	CNL	...
Datura stramonium	CL	VC, C, P
Glycine max (cv. Bragg)	VN	CS, VN
Nicandra physaloides	NL	NL, TN
Nicotiana benthamiana	CL	M
N. clevelandii	CL	M
N. edwardsonii	CL	M, P
N. glutinosa	CL	M, P
N. rustica	CL	M, C, P
N. tabacum (cv. White Burley)	CL	M
Petunia hybrida	CL	M, C
Phaseolus vulgaris		
(cv. Dwarf)	NL	...
(cv. Topcrop)	CL	...
Spinacia oleracea (cv. Local)	CL	SL
Vigna radiata	NL	VN
V. unguiculata		
(cv. C 152)	CLN	...
(cv. California Blackeye)	CL	...

[a] Virus infection was confirmed in ELISA tests.

[b] C = curling, CL = chlorotic lesions, CLN = chlorotic lesions with necrotic center, CNL = concentric necrotic lesions, CS = chlorotic spots, CST = chlorotic streaks, M = mosaic, NL = necrotic lesions, P = puckering, SL = symptomless, TN = total systemic necrosis, VC = vein clearing, and VN = veinal necrosis.

[c] No symptoms were observed, and no virus was recovered in ELISA tests.

The isolated plasmid DNA were screened for the presence of full-length viral-DNA inserts by digesting the DNA with *Kpn*I or *Bam*HI, followed by electrophoresis through 1% (w/v) agarose gels. *Sac*I-digested pFMV-Sc3 DNA (18) was used as a molecular-weight marker during electrophoresis. Agarose gels were stained for 10–20 min in 0.0001% (w/v) ethidium bromide, then viewed under an ultraviolet transilluminator. Appropriately sized plasmid DNAs were subjected to further restriction-endonuclease analysis (and compared to restriction digests of viral DNA) to insure that no rearrangements had occurred during the cloning of the viral genome.

PClSV/pUC119 recombinant plasmids were also screened for infection on *Datura stramonium*, as previously described (18), except that plants were incubated in a growth chamber, at 34 C, for 7–10 days beginning 2 days after inoculation. Several infectious, full-length clones were obtained. One, pPClSV K-1, was selected for further characterization.

Restriction endonuclease sites within the PClSV genome were identified by analyzing the sizes of DNA fragments resulting from treatment of pPClSV K-1 with combinations of restriction endonucleases. *Hind*III-treated pFMV-Sc3 (13,18) provided molecular-weight markers for this analysis. The presence and location of the characteristic single-stranded discontinuities in PClSV viral DNA were determined according to the method of Schoelz et al (14).

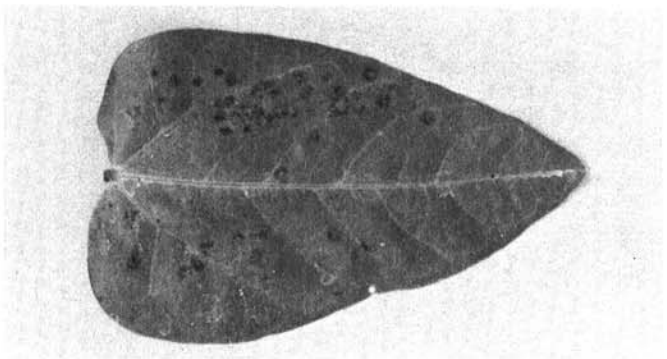

Fig. 2. Necrotic lesions on *Vigna unguiculata*.

Fig. 3. Vein clearing, curling, and puckering on leaves of *Datura stramonium*.

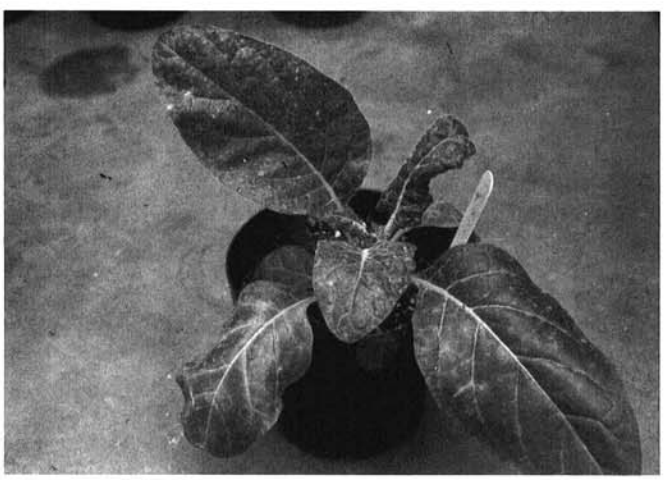

Fig. 4. Curling and puckering on leaves of *Nicotiana rustica*.

RESULTS

Symptomatology and host range. Initial symptoms in peanut plants appeared 3–4 wk after inoculation. Young leaflets showed oval chlorotic streaks along the veins (Fig. 1A and B). Streaks were not distinct in older leaflets, and they could be seen only when viewed against light. In field infections, diseased plants appeared conspicuous because of stunting and chlorotic streaks on younger leaflets.

Hosts infected by PClSV are listed in Table 1. Symptoms on *V. unguiculata, D. stramonium,* and *N. rustica* are presented in Figures 2, 3, and 4, respectively.

The following plants were not infected by the virus: *Chenopodium amaranticolor, C. quinoa, Gomphrena globosa,*

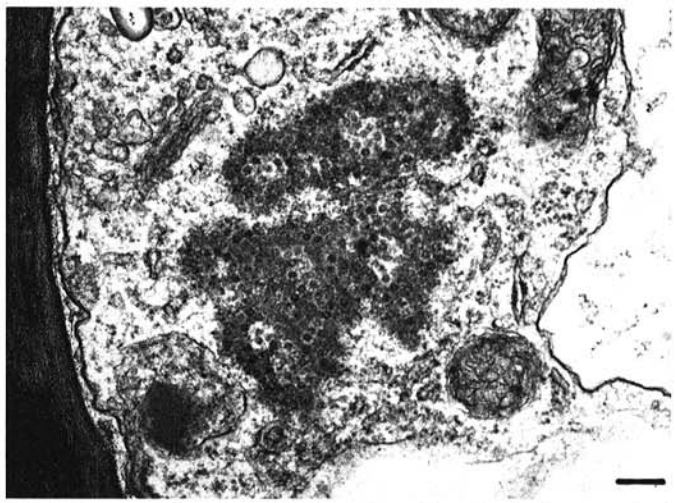

Fig. 5. Electron micrograph of thin section of an *Arachis hypogaea* leaf infected with peanut chlorotic streak virus (PClSV), showing inclusion bodies (bar represents 200 nm).

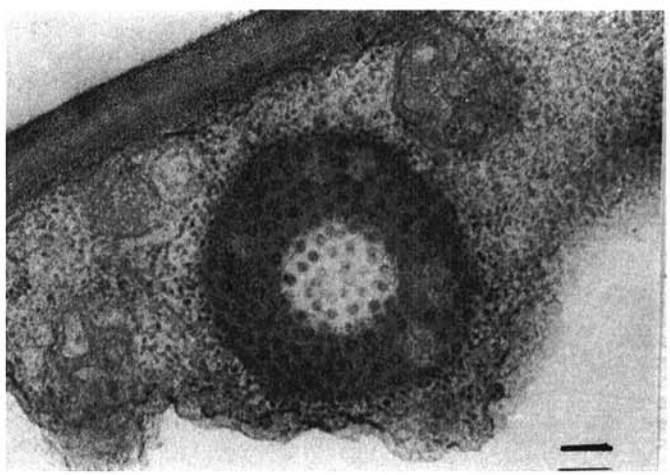

Fig. 6. Electron micrograph of thin section of a *Nicotiana rustica* leaf infected with peanut chlorotic streak virus (PClSV), showing inclusion bodies (bar represents 400 nm).

Phaseolus vulgaris (cv. Kintoki), *Pisum sativum* (cv. Bonneville), and *Vicia faba*.

Properties in plant extracts. Virus in crude plant extracts retained infectivity after dilution of 10^{-3}, but not of 10^{-4}, and after exposure to 25 C for 1 day, but not for 2 days. The TIP was between 85 and 90 C.

Aphid and seed transmission. After various access periods, *A. craccivora* exposed to 180 *A. hypogaea* and 45 *N. clevelandii* plants, as well as *M. persicae* exposed to 171 *A. hypogaea* plants, failed to transmit the virus. Every plant was tested by ELISA to confirm visual observations. Of 390 seeds collected from infected peanut plants, 291 germinated. None of the plants showed symptoms, and all were negative in ELISA tests.

Purification. The purification method yielded intact virus particles (Fig. 5). Centrifugation in CsCl gradients was essential for yielding particles devoid of host-plant material, judged using electron microscopy. One kilogram of systemically infected *N. clevelandii* leaves yielded 0.35–1 mg of virus. Purified PClSV preparations contained spherical virus particles 52 ± 3 nm in diameter (Fig. 5).

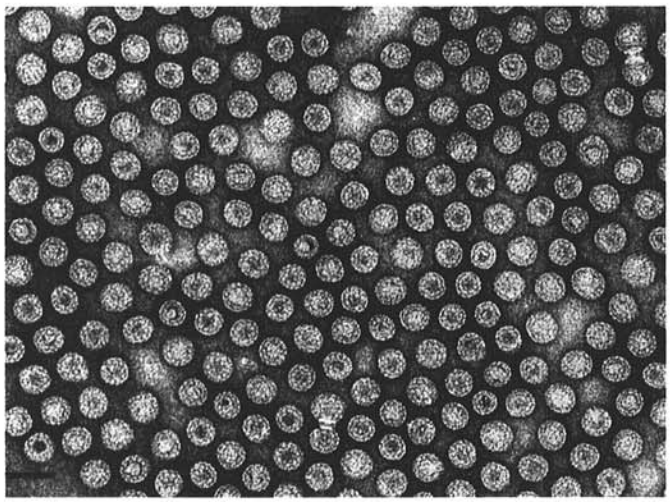

Fig. 7. Electron micrograph of purified peanut chlorotic streak virus (PClSV) particles stained with uranyl acetate (bar represents 100 nm).

TABLE 2. Serological relationships of peanut chlorotic streak virus (PClSV), determined by the direct antigen coating form of enzyme-linked immunosorbent assay

Dilution of leaf extracts[a]	Antisera			
	PClSV[b]	Cauliflower mosaic[c]	Figwort[c]	Soybean chlorotic mottle[c,d]
Arachis hypogaea				
Healthy				
10^{-1}	0.15[e]	0.21	0.13	0.28
10^{-2}	0.14	0.18	0.11	0.26
10^{-3}	0.12	0.19	0.12	0.24
PClSV-infected				
10^{-1}	0.84	0.23	0.15	0.29
10^{-2}	0.37	0.16	0.12	0.22
10^{-3}	0.14	0.18	0.12	0.22
Nicotiana rustica				
Healthy				
10^{-1}	0.19	0.20	0.14	0.25
10^{-2}	0.16	0.18	0.12	0.24
10^{-3}	0.16	0.19	0.16	0.18
PClSV-infected				
10^{-1}	>2.0	0.19	0.18	0.29
10^{-2}	1.32	0.18	0.15	0.20
10^{-3}	0.72	0.18	0.16	0.18
10^{-4}	0.36	0.17	0.14	0.18
10^{-5}	0.11	0.13	0.12	0.17

[a] Dilutions are based on the original tissue weight.
[b] Used at 1:1,000 dilution.
[c] Used at 1:500 dilution.
[d] Gift from Dr. Kameya-Iwaki, Japan.
[e] The mean A_{405nm} value for three replicates.

Electron microscopy. Naturally infected *A. hypogaea* leaflets and mechanically inoculated *N. rustica* leaves contained intracellular inclusions typical of caulimoviruses (Figs. 6 and 7). The majority of inclusion bodies were circular, 4–11 μm in diameter. They contained particles 40–45 nm in diameter, either embedded in an electron-dense granular matrix surrounded by vacuoles or in an amorphous matrix. Inclusion bodies were present only in the cytoplasm.

Serology. The antiserum had a titer of 1/600 in the precipitin-ring interphase test. In ELISA tests, homologous antiserum reacted with infected *A. hypogaea* leaf extracts up to a dilution of 10^{-4} and with *N. rustica* leaf extracts up to a dilution of 10^{-4}. Cauliflower mosaic, figwort, and soybean chlorotic mottle virus antisera failed to show any reaction with either of the plant extracts tested (Table 2).

Capsid protein analysis. Purified PClSV contained two polypeptides with approximate relative molecular masses of 58 and 51 kDa (average of six determinations) analyzed in 10% acrylamide gels (Fig. 8).

Nucleic acid. Agarose-gel electrophoresis of uncut PClSV viral DNA yielded an array of bands strikingly similar to the pattern observed for other uncut, similarly analyzed caulimovirus (CaMV) DNA. In comparison gels, linear (form I) and open circular (form II) molecules migrated at a rate identical to corresponding forms of CaMV DNA, and faint bands of knotted molecules made up a series of more rapidly migrating components (16). The results of mapping restriction endonuclease sites and single-stranded discontinuities are shown in Figure 9. PClSV DNA consists of circular, double-stranded DNA approximately 8.1 kbp in length

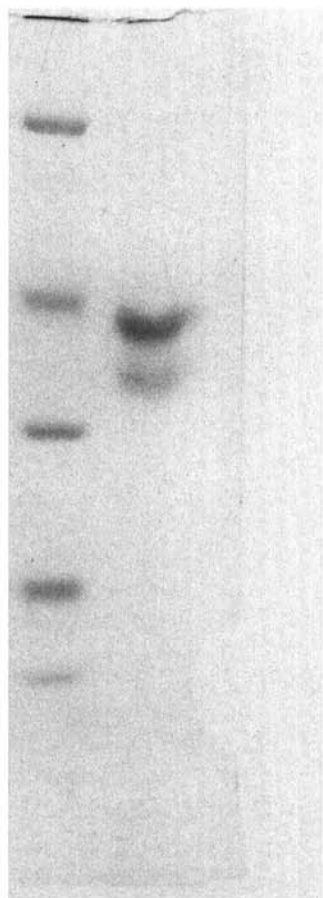

Fig. 8. Polyacrylamide gel of the coat proteins of peanut chlorotic streak virus (PClSV). The gel to the left shows molecular-weight markers. Relative molecular masses are provided to the right.

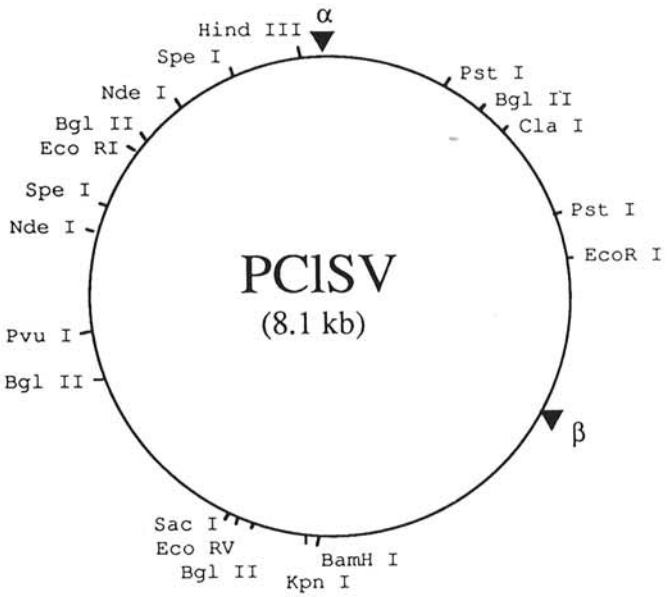

Fig. 9. Restriction endonuclease map of the peanut chlorotic streak virus (PClSV) genome. The circular, double-stranded DNA consisting of approximately 8.1 kbp is represented by the circle. The single-stranded discontinuities that occur in the viral DNA are indicated by arrows. The map is oriented so the single-stranded discontinuity, which occurs at the $tRNA_i^{Met}$ primer binding site, is positioned at the top.

and contains two single-stranded discontinuities. The physical map has been oriented so the single-stranded discontinuity occurring at the $tRNA_i^{Met}$ primer binding site is positioned at the top of the diagram (12).

PClSV symptoms (prominent vein clearing, blistering, distortion, and mottling) were observed in a small percentage of *D. stramonium* inoculated with *Kpn*I-digested pPClSV K-1. Plants exhibiting these symptoms tested positive (serologically) for the presence of PClSV.

DISCUSSION

From the results, it is apparent that PClSV is a member of the caulimovirus group but distinct from the known members. PClSV has a host range wider than any reported caulimovirus. The only other caulimovirus that can naturally infect legumes is soybean chlorotic mottle virus (SoyCMV) (5). PClSV differs markedly from SoyCMV in host range. While PClSV produced veinal necrosis on *Glycine max*, SoyCMV produced a chlorotic mottle (5). Both viruses produced local lesions on inoculated *P. vulgaris* leaves, but SoyCMV produced a systemic infection unlike PClSV. On *V. unguiculata*, PClSV produced characteristic local lesions; SoyCMV did not produce visible symptoms. Unlike SoyCMV, PClSV infects *A. hypogaea, D. stramonium, N. clevelandii, Nicotiana glutinosa, Nicotiana tabacum, Petunia hybrida, Spinacia oleracea,* and *Vigna radiata.*

The biological properties of PClSV are similar to those of other caulimoviruses with regard to stability in crude plant extracts, particle morphology, and characteristic inclusion bodies. Initial attempts to transmit PClSV by two aphid species were unsuccessful, similar to results with other caulimoviruses that could not be transmitted by aphids (17).

The physical nature of double-stranded DNA of PClSV is similar to that of other caulimoviruses. In agarose-gel electrophoresis, native PClSV DNA showed several bands, which are presumed to be the result of different forms of DNA (i.e., linear, circular, and supercoiled). The PClSV genome contains two single-stranded discontinuities. The polarity of the genome (as presented) is based on DNA sequencing (12). The physical map of PClSV DNA is different from any other reported caulimovirus.

According to recent surveys conducted in India, PClSV is widely distributed, and in some locations, the incidence exceeds 10% (P. Sreenivasulu, *personal communication*). This indicates that caulimoviruses could become economically important in tropical countries.

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