J. Phytopathology 140, 326—334 (1994)

ISSN 0931-1785

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Identification of a Strain of Peanut Chlorotic Streak Virus Causing Chlorotic Vein Banding Disease of Groundnut in India

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With 4 figures

Received June 24, 1992; accepted February 17, 1993

Abstract

A virus disease characterized by chlorotic vein banding, chlorotic line pattern along the margins or midrib of mature leaflets and chlorotic spots/rings was observed on commercial groundnut crops in Rayalaseema area of Andhra Pradesh with an incidence from 1 % to nearly 60 %. The virus was transmitted by mechanical inoculation in extracts prepared with 0.01 M potassium phosphate buffer, pH 8.0 to 21 species from the Chenopodiaceae, Cruciferae, Leguminosae and Solanaceae. *Chenopodium quinoa* was found to be a good local lesion host. The virus was neither seed-transmitted through 1591 groundnut seeds nor aphid-transmitted by *Aphis craccivora, Myzus persicae* and *Rhopalosiphum maidis* either in non-persistent or semi-persistent manner. The virus remained infective in buffered tobacco leaf sap at a dilution of 10^{-3}; in a 10^{-1} dilution of buffered sap the virus was infective for 2–3 days at 22–29 °C or when heated to 65 °C for 10 min but not to 70 °C.

Clarification treatments with organic solvents with 10 % chloroform was least damaging. The virus was purified from *Nicotiana rustica* leaves. Purified virus contained isometric particles of 51 nm in diameter with an electron dense core of 22 nm and two major polypeptides of 76 kDa and 36 kDa. A polyclonal antiserum to this virus was produced. In agar gel double diffusion, enzyme-linked immunosorbent assay and in electro-blot immunoassay tests the virus was related to peanut chlorotic streak virus and not to cauliflower mosaic, figwort mosaic and soybean chlorotic mottle viruses.

Zusammenfassung

Die Identifizierung eines Erdnuß chlorotic-streak Virus-Stammes, der die chlorotic Vein-Banding-Krankheit der Erdnüsse in Indien verursacht

In kommerziell angebauten Erdnußfeldern im Rayalaseema-Gebiet des Andhra Pradesh wurde eine Viruskrankheit mit einer Häufigkeit von 1 % bis fast 60 % beobachtet, die durch chlorotische Bänder der Blattadern, chlorotischer Streifenbildung entlang den Blatträndern oder der Mittelrippe der vollentwickelten Blattfiedern sowie chlorotischen Flecken/Ringe charakterisiert wird. Das Virus konnte durch mechanische Inokulation mit Extrakten, die mit 0,01 M Kaliumphosphatpuffer (pH 8,0) hergestellt wurden, auf 21 Pflanzenarten der Chenopodiaceae, Cruciferae, Leguminosae und Solanaceae übertragen werden. *Chenopodium quinoa* zeigte sich als eine geeignete Wirtspflanze mit lokaler Läsionenbildung. Das Virus wurde weder samenbürtig in 1591 Erdnußsamen noch durch Blattlausarten wie *Aphis craccivora, Myzus persicae* oder *Rhopalosiphum maidis* weder in nicht persistenter noch in

semi persistenter Art übertragen. In gepuffertem Tabakblattsaft und bei einer Verdünnung von 10^{-5} behielt das Virus seine Infektivität. In einer 10^{-7} Verdünnung des gepufferten Saftes blieb das Virus 2–3 Tage bei einer Temperatur von 22–29 °C infektiv. Bei einer Erhitzung des Saftes auf 65 °C, aber nicht auf 70 °C, überlebt das Virus für 10 Min.

Klarifikationsbehandlungen mit organischen Lösungsmitteln zeigten, daß eine 10 prozentige Chloroformlösung die wenigsten Schäden hervorrief. Das Virus wurde aus *Nicotiana rustica*-Blättern gereinigt. Gereinigtes Virus enthielt isometrische Partikel mit 51 nm Durchmesser mit einem elektronendichten Kern von 22 nm sowie zwei Hauptpolypeptiden von 76 kDa und 36 kDa. Ein für dieses Virus polyklonales Antiserum wurde hergestellt. Es konnte in Agargel double diffusion-, ELISA- sowie electro-blot immunoassay-Tests gezeigt werden, daß das Virus mit dem Erdnuß Chlorotic-Streak-Virus, aber nicht mit den Blumenkohlmosaik-, Figwortmosaik- und Sojabohne-chlorotic-Mottle-Viren verwandt war.

Many virus diseases occur on groundnut worldwide (SREENIVASULU *et al.* 1991) and they can cause considerable yield losses. Although many virus diseases of groundnut have been described in India (REDDY 1988, 1991), only those caused by peanut mottle (PMV), bud necrosis virus (BNV), peanut green mosaic (PGMV), Indian peanut clump (IPCV), cowpea mild mottle (CMMV), peanut yellow spot (PYSV), groundnut veinal chlorosis (GVCV) and groundnut carlaviruses are fully characterized (REDDY 1991, SREENIVASULU *et al.* 1993). Peanut chlorotic streak virus (PCISV) was earlier reported from India and found to be a caulimovirus (IIZUKA *et al.* 1981, IIZUKA and REDDY 1986, REDDY *et al.* 1993). A disease characterized by veinal chlorosis and chlorotic vein banding symptoms on young groundnut leaves was observed in 1986 on commercial groundnut fields in Rayalaseema area (Anantapur, Chittoor, Cuddapah and Kurnool districts) of Andhra Pradesh with incidence upto nearly 60 % (SATYANARAYANA 1991). In preliminary experiments, utilizing direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) infected leaf extracts reacted positively with an antiserum of PCISV (SATYANARAYANA *et al.* 1990). This paper reports on detailed studies on the PCISV isolate that cause chlorotic vein banding symptoms in groundnut.

Materials and Methods

Virus maintenance

A culture of the virus was maintained in a screen house from field-collected groundnut plants by grafting on to healthy groundnut plants (cv. TMV 2). Subsequently the virus was inoculated onto cowpea (*Vigna unguiculata* cv. Local). The virus isolated from a single lesion produced in cowpea, following five serial transfers was maintained either in groundnut (cv TMV 2) or in *Nicotiana rustica*. All mechanical sap inoculations were made on carborundum dusted leaves with sap prepared in 0.01 M potassium phosphate buffer, pH 8.0 containing 0.2 % 2-mercaptoethanol (PBM).

Host range

At least six plants of each of 37 species belonging to 9 dicotyledonous families were mechanically inoculated with sap from groundnut plants. Extracts from inoculated and subsequently developed leaves were tested by DAC-ELISA and by back inoculation onto groundnut to confirm virus presence.

Properties in buffered sap

For dilution end point (DEP) determination, 10-fold dilution of the leaf sap from groundnut prepared with PBM was used. To determine the thermal inactivation point (TIP), 1 ml of sap of 1/10 dilution was heated for 10 min at various temperatures. Longevity *in vitro* was determined at room temperature (22–29 °C) at 1 day intervals. *Chenopodium quinoa* was used as the local lesion assay host.

Seed transmission

Seeds collected from infected groundnut plants (cv TMV 2) with conspicuous symptoms were sown in sterile soil and kept in a wire mesh house. Seedlings (5–6 weeks old) were tested for virus presence by ELISA.

Aphid transmission

Non-viruliferous *Aphis craccivora*, *Myzus persicae* and *Rhopalosiphum maidis* were reared on cowpea, tobacco and sorghum, respectively. In non-persistent transmission studies, aphids were starved for 6–8 h and then given 5 min acquisition access period on detached young infected groundnut leaflets. Five aphids were transferred to each groundnut seedling and they allowed 2–3 h inoculation access periods. Subsequently aphids were killed by spraying with 0.02 % rogor. In semi-persistent transmission studies aphids were given 2–3 h starvation period, 3–4 h acquisition access period and 2 days inoculation access period. Five aphids were transferred to each groundnut seedling. All the test plants were assayed by DAC-ELISA for virus presence.

Virus purification

Various buffers and organic solvents were initially tested on the infectivity of the virus by local lesion bio-assay and phosphate buffer and chloroform were chosen for virus purification. The procedure for purification of virus from *N. rustica* leaves was essentially that reported by REDDY *et al.* (1993). Infected *N. rustica* leaves were homogenized in 0.1 M potassium phosphate buffer, pH 8.0 and treated with chloroform (100 ml/l). After clarification at 5000 g for 10 min, the aqueous phase was collected and centrifuged (27,000 rpm for 2 h in Sorvall AH 629 rotor). The pellets were suspended in 0.02 M potassium phosphate buffer, pH 7.5 (PPB). The virus was further purified by centrifugation through 10–40 % sucrose gradients (7 ml each of 40, 30 and 20 % and 5 ml of 10 % sucrose (w/v)) in PPB (in Sorvall AH 629 rotor at 20,000 rpm for 1 h at 4 °C). A single light scattering zone between 5.2–6.0 cm from the bottom of the tube was collected and centrifuged at 35,000 rpm for 2 h. At certain times this material was also centrifuged through cesium chloride gradient (20–45 % in PPB) in SW-50 rotor for 2.5 h at 35,000 rpm at 4 °C. Purified virus suspended in 0.01 M potassium phosphate buffer, pH 7.0 was scanned in Hitachi UV-visible recording spectrophotometer from 200–300 nm.

Serology

An antiserum was produced in New Zealand white inbred rabbits following four intramuscular injections (at weekly intervals) with 1 mg of purified virus emulsified with an equal volume of Freund's incomplete adjuvant. Starting 1 week after the last injection, the rabbit was bled at weekly intervals for 6 weeks. Serum from each bleed was tested by DAC-ELISA utilizing healthy and infected groundnut leaf extracts. The titre of antiserum was determined by agar gel double diffusion test (AGDDT) (PURCIFULL and BATCHELOR 1977). The antiserum, cross-absorbed with healthy leaf antigens, was used in serological tests.

Heterologous antisera used in this study were generous gifts from ICRISAT [peanut chlorotic streak virus (PClSV)]; Dr R. J. SHEPHERD, USA (cauliflower mosaic virus [CaMV] and figwort mosaic virus [FMV]) and Dr M. IWAKI, Japan (soybean chlorotic mottle virus [SoCMV]). Serological

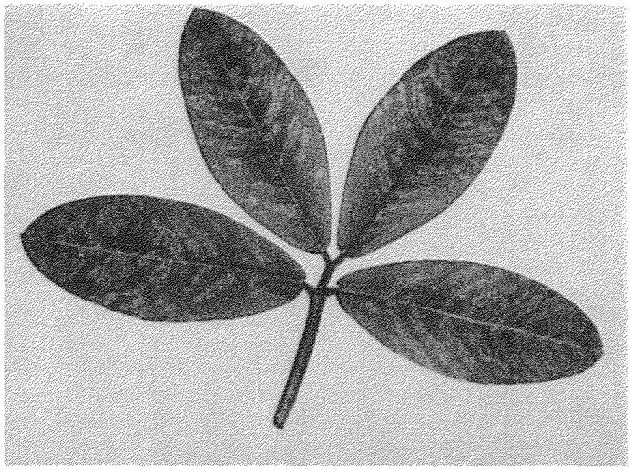

Fig. 1. Chlorotic vein banding symptoms induced by PCISV-CVB on groundnut (cv. TMV 2)

relationships of the virus with other caulimoviruses was determined by AGDDT and DAC-ELISA (HOBBS *et al.* 1987).

Electron microscopy

Purified virus processed through cesium chloride gradients was stained with 1 % uranyl acetate and observed in Philips 201 C transmission electron microscope.

Electrophoresis of virus coat protein

Purified virus, following centrifugation in cesium chloride gradients and high speed pelleting (30,000 rpm for 2 h) was suspended in 0.0625 M Tris-HCl, pH 6.8 containing 2 % SDS, 1 % 2-mercaptoethanol and 20 % glycerol and analysed by SDS- polyacrylamide slab gel electrophoresis (LAEMMLI 1970). The resolving gel was 12 % and the stacking gel was 4 %, electrophoresis was at 60 V for 12 h at room temperature. The gels were stained with Commassie brilliant blue R-250. Bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), lactaglobulin (18,000) and lysozyme (14,300) (from Sigma Chemicals) were used as molecular weight markers.

Electro-blot immunoassay (EBIA)

Electro-blot immunoassay of viral proteins was as described by REDDY *et al.* (1990). Homologous antiserum was used at 1 : 500 dilution and heterologous antisera at 1 : 250 dilution.

Results

Symptoms in groundnut

The virus produced veinal chlorosis, chlorosis along the lateral veins (chlorotic vein banding, CVB) (Fig. 1) and downward rolling of leaf margins 20–45 days (about 20 days

when temperature was between 30–40 °C and nearly 40 days when temperature was between 25–35 °C) after sap inoculation. These initial symptoms were followed by a chlorotic line pattern along the leaf margins and midrib of leaflets, chlorotic rings/spots and crinkling of the leaves.

Host range

The virus infected 22 plant species belonging to Chenopodiaceae, Cruciferae, Leguminosae and Solanaceae. The virus induced local chlorotic or necrotic lesions on *Cassia occidentalis, Chenopodium amaranticolor, C. quinoa, Clitorea ternatea, Dolichos lablab, Macrotyloma uniflorum, Phaseolus vulgaris* cvs. Bountiful, Contender and Local, *Raphanus sativus, Vigna unguiculata* cvs. C-152, Early Ramshorn and Local. The virus induced local infections followed by systemic infection in *Canavalia ensiformis, Cyamopsis tetragonoloba, Datura metel, Glycine max, Nicotiana glutinosa, N. rustica, N. tabacum* var. White Burley, *Petunia hybrida, Physalis floridana, P. minima, Solanum tuberosum, V. radiata* and only systemic symptoms on *V. mungo* and *N. tabacum* var. Turkish and Xanthi NC. The virus failed to infect *Amaranthus viridis, Beta vulgaris, Cajanus cajan, Capsicum annuum, Cassia tora, Celosia viridis, Crotalaria verrucosa, Cucumis sativus, Gomphrena globosa, Hibiscus sabdariffa, Luffa acutangula, Lycopersicon esculentum, Nicotiana tabacum* var. Horrisson Special, *Phaseolus vulgaris* cvs. Kentucky and Top crop, *Pisum sativum, Sesbania grandiflora, Tagetus indicus, Tridax procumbens* and *Vinca rosea*.

Properties of the virus in buffered sap

The TIP was between 65 °C and 70 °C, the DEP was 10^{-5}, LIV (at 25–29 °C) was 3 days.

Aphid and seed transmission

The virus was not transmitted non-persistently by *A. craccivora* (0/115, number of plants infected/number of plants exposed), *M. persicae* (0/91) and *R. maidis* (0/61) and semi-persistently by *A. craccivora* (0/120), *M. persicae* (0/98) and *R. maidis* (0/36). None of 1591 groundnut seedlings were found virus infected.

Virus purification

Virus extracts were most infective in 0.01 M potassium phosphate buffer, pH 8.0 when compared to potassium phosphate (0.5, 0.1, 0.01 M; pH 7.0 and 0.5, 0.1M; pH 8.0), Tris-Cl (0.1, 0.01M; pH 8.0), borate (0.2, 0.1M; pH 8.0) and sodium citrate (0.5, 0.1, 0.05M; pH 6.5) buffers. In general, the extracts prepared with high molarity (0.05–0.5 M) buffers were less infective. Little or no infectivity was lost following treatment with 10 % chloroform. n-Butanol and n-butanol: chloroform (1:1, v/v), used to clarify other caulimoviruses, abolished the infectivity. The amount of infective virus recovered in various steps of virus purification was estimated by local lesion assay on *C. quinoa*. Purified virus retained about 60 % of the infectivity as compared to initial crude plant extracts. Assuming an extinction coefficient of 7 (uncorrected for light scattering), the virus yield was 2–3 mg per 100 g fresh leaves. Purified virus had A_{max} and A_{min} between 259–260 nm and 242–243 nm, respectively. The $A_{max/min}$ and $A_{260/280}$ ratios were 1.25 and 1.42, respectively.

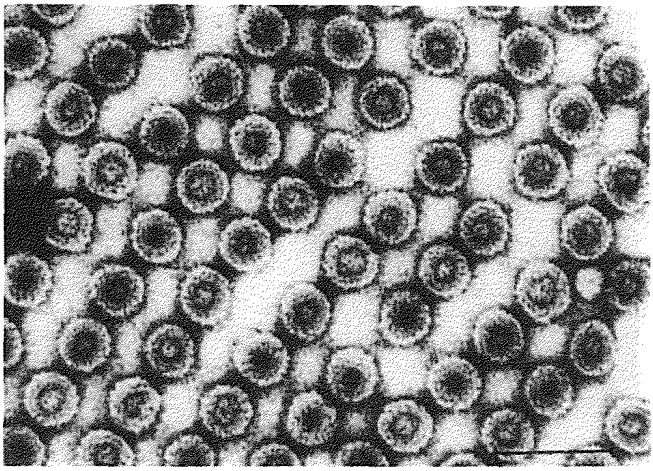

Fig. 2. Electron micrograph of purified preparation of PClSV-CVB negatively stained in uranyl acetate. Bar represents 100 nm

Particle morphology

Purified virus particles stained with uranyl acetate were 51 ± 2 nm diameter with an electron dense core of 22 ± 1 nm (Fig. 2).

Serology

The titre of cross-absorbed antiserum was 1/64 with purified virus in AGDDT. The viral antigen strongly reacted with antiserum to PClSV in DAC-ELISA and AGDDT. The virus did not react with antisera to CaMV, FMV or SoCMV (Fig. 3, Table 1).

Virus coat protein and electro-blot immunoassay

In SDS-PAGE, the virus coat protein resolved into two major polypeptides of 76 kDa and 36 kDa (Fig. 4A). These two polypeptide species reacted with homologous and PClSV antisera, but not with CaMV and FMV antisera (Fig. 4B).

Discussion

Based on particle morphology and serological tests the virus has been identified as a member of the caulimovirus group. The virus was found to contain dsDNA genome (SATYANARAYANA 1991) characteristic of caulimovirus group. However, like many caulimoviruses, it was not vectored by aphids.

The virus reacted both in ELISA and EBIA tests with PClSV reported to occur naturally on groundnut (REDDY *et al.* 1993). Although CaMV can infect groundnut in laboratory tests (HULL and DAVIES 1983) it was serologically not related to present virus and also different in host range. It also differed from soybean chlorotic mottle virus reported from Japan in not reacting with its antiserum and in host range (IWAKI *et al.* 1984). The

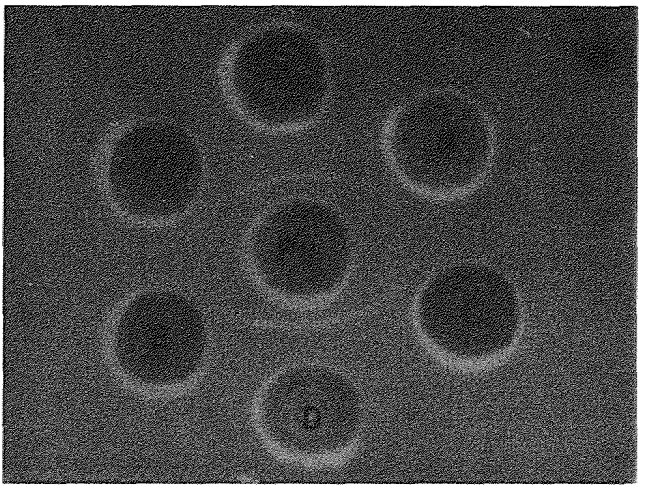

Fig. 3. Agarose gel double diffusion test: central well, PClSV-CVB purified virus (PV); peripheral well, antisera to (A) PClSV (B) FMV (C) CaMV (D) PClSV-CVB (E) SoCMV (F) Buffer (PBS)

Table 1
Reaction of PClSV-CVB with antisera to other caulimoviruses in DAC-ELISA[a]

	Antigens			
	Infected		Healthy	
Antisera[b] to	10^{-1}	10^{-2}	10^{-1}	10^{-2}
PClSV-CVB	1.49	1.15	0.27	0.12
PClSV	1.05	1.02	0.21	0.12
SoCMV	0.20	0.12	0.12	0.10
CaMV	0.36	0.34	0.26	0.30
FMV	0.26	0.29	0.21	0.20

[a]Readings taken at 405 nm over antigen buffer control. Each value is an average of three replicates. Experiment conducted twice.
[b]Antisera at 1 : 500 dilution.

virus was serologically related to PClSV and therefore named as an isolate of PClSV (i.e. PClSV-CVB). It differed from PClSV in symptomatology and host range (Table 2). Additionally the restriction map for PClSV-CVB differ from PClSV (SATYANARAYANA 1991).

The first author is grateful to Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing financial assistance (in the form of Senior Research Fellow). We thank Mr A. K. MURTHY, Engineer, EM unit of ICRISAT for electron microscopy and Dr M. A. MAYO, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland for his suggestions and for critical evaluation of the manuscript.

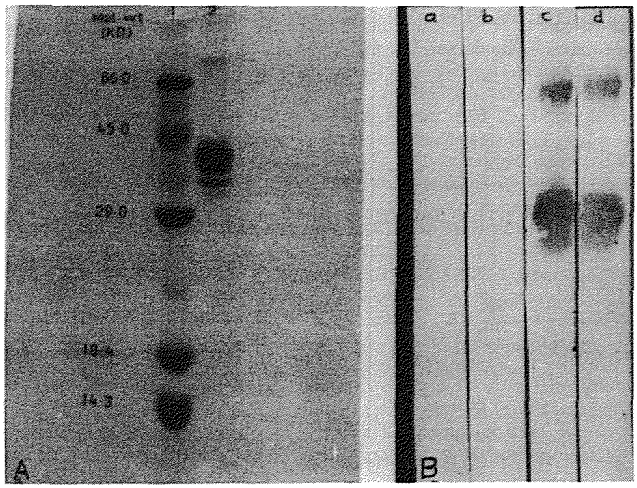

Fig. 4. (A) SDS-polyacrylamide slab gel showing virus coat protein of PClSV-CVB (lane 2) with molecular weight markers (lane 1). (B) Immunoblot of PClSV-CVB coat protein from 'A' after probing with (a) CaMV (b) FMV (c) PClSV and (d) homologous antisera

Table 2
Comparative host range of PClSV-CVB and PClSV

Host	PClSV-CVB	PClSV
Canavalia ensiformis	CLL,SCP,M	CLL
Chenopodium amaranticolor	CLL	—
Cyamopsis tetragonoloba	NLL,CS	NLL
Gomphrena globosa	—	PLL
Petunia hybrida	CLL,CS,M,Mo,LC	M,Mo,LC
Phaseolus vulgaris cv. Top crop	—	CLL
Vigna mungo	SN	NLL,SN

—Not infected; CLL: chlorotic local lesions; CS: systemic chlorotic spotting; LC: leaf curling; M: systemic mosaic; Mo: systemic mottling; NLL: necrotic local lesions; PLL: purple local lesions; SCP: systemic chlorotic patches; SN: systemic necrosis.

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