Plant Pathology (1989) **38**, 520–526

Isolation and characterization of a potyvirus associated with bushy dwarf symptom in chickpea, *Cicer arietinum*, in India

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A potyvirus that induced stunting and a characteristic bushy appearance at the apical region, due to proliferation of terminal branches with narrowed, reduced and deformed leaflets, was isolated from chickpea in India. The virus was sap-transmissible to 14 species of Chenopodiaceae, Leguminosae, Solanaceae and Malvaceae; *Chenopodium amaranticolor* was a good local lesion host. Virus particles, trapped by immunosorbent electron microscopy and stained with uranyl acetate, were 710 ± 10 nm long. Purified virus preparations contained a single polypeptide species of 32 500 Da and one nucleic acid species of $3{\cdot}1 \cdot 10^6$ Da. The virus was serologically related to soybean mosaic, azuki bean mosaic and peanut mottle viruses but not to clover yellow vein, pea seed-borne mosaic and bean yellow mosaic viruses.

On the basis of these properties, the virus was identified as a previously undescribed potyvirus in chickpea, for which the name chickpea bushy dwarf virus is proposed.

INTRODUCTION

Chickpea in India is known to be affected by four viruses, alfalfa mosaic (AMV), bean yellow mosaic (BYMV), cucumber mosaic (CMV), and chickpea stunt (CpSV) (Reddy *et al.*, 1986; Nene & Reddy, 1987). However, others may have escaped detection because not all macroscopic symptoms produced by chickpea viruses are distinctive (Kaiser & Danesh, 1971a).

During the 1987 rainy season at ICRISAT, chickpea plants were observed that showed stunting, bushy growth of the apical branches, and narrow and reduced leaflets. In leaf dip preparations stained with 2% uranyl acetate, potyvirus-like particles were seen, but the symptoms were different from those caused by two naturally occurring potyviruses in chickpea, BYMV in Iran and India (Kaiser & Danesh, 1971b; Chalam, 1982) and chickpea filiform virus (CFV) in the USA (Kaiser *et al.*, 1988).

The main aim of the study was to investigate the host range, symptomatology, electron microscopy, serological relationships and physicochemical properties of this virus.

MATERIALS AND METHODS

Virus culture and maintenance

The virus often occurred in mixed infections with CpSV. Leaf extracts prepared in 0·05 M phosphate buffer, pH 7·0, containing 0·75 g/l thioglycerol (PBT), were inoculated onto *Chenopodium amaranticolor*. The virus isolated from a lesion, after six successive single local-lesion transfers, was maintained in chickpea (cv. Annigeri). CpSV is not mechanically transmitted to *C. amaranticolor*.

Host range studies

Extracts from 1–2 g of systemically infected young chickpea leaflets, prepared in 10–15 ml of PBT, were inoculated onto six plants of each test species and maintained in a glasshouse for 40 days at 20–30°C. Inoculated and uninoculated leaflets of all species were checked for infection by sap inoculation to *C. amaranticolor* and by direct antigen coating (DAC) enzyme-linked immunosorbent assay (ELISA) using homologous antiserum.

Virus purification

The method followed was a modification of that reported by Rajeshwari *et al.* (1983). Infected chickpea leaflets, 4 weeks after inoculation, were homogenized (1 g per 3 ml) in a blender with chilled 0·1 M phosphate buffer, pH 6·0, containing 0·01 M sodium diethyl dithiocarbamate (DIECA) and 2 g/l thioglycerol. The sap was filtered through cheesecloth and shaken with 100 ml/l chloroform for 3–4 min. The emulsion was broken by centrifugation at 5000 revolutions/min (rpm) for 10 min in a Sorvall RC-5C centrifuge. The virus in the aqueous phase was precipitated by polyethylene glycol (PEG), collected by centrifugation, resuspended in 0·01 M borate-phosphate buffer, pH 8·3, containing 0·2 M urea (BPU) and clarified. Twenty-five ml of the suspension was layered on a 13-ml column of 300 g/l sucrose in BPU containing 40 g/l PEG and 0·2 M NaCl. Following centrifugation at 25000 rpm for 90 min in a Beckman SW-28 rotor, the pellets were resuspended in BPU and subjected to a rate-zonal density-gradient centrifugation in sucrose solution in BPU as described by Rajeshwari *et al.* (1983). The virus from the light-scattering zone (4·5–5·0 cm from the bottom of the tube) was removed and pelleted at 30000 rpm for 2 h in a Beckman R-50 rotor. The pellets were resuspended in BPU and subjected to centrifugation in a CsCl gradient. Gradients were prepared by layering, 1, 1·5 and 2 ml of 300, 400 and 500 g/l of CsCl in BPU, respectively, followed by storage for 2 h at 4°C before use; 0·5-ml of the virus preparation was layered on the gradient and centrifuged at 30000 rpm for 3 h in a Beckman SW-50 rotor. The virus from the single light-scattering zone was collected and dialysed overnight in BPU at 4°C. The virus was concentrated by pelleting at 30000 rpm for 2 h in a Beckman R-50 rotor.

Production of antiserum

Purified virus at 1 mg/ml was emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into a New Zealand White inbred rabbit at weekly intervals. Serum was collected 2 weeks after the fourth injection, and its titre determined by the precipitin ring test using purified virus (Reddy *et al.*, 1966).

Enzyme-linked immunosorbent assay (ELISA)

Direct antigen-coating (DAC) ELISA was used (Hobbs *et al.*, 1987). Healthy and infected leaf samples, ground in a mortar in 0·05 M sodium carbonate buffer, pH 9·6, containing 0·01 M Na

Fig. 1. Plants of chickpea cv. Annigeri, (a) infected with potyvirus showing typical bushiness with deformed leaves, (b) healthy.

DIECA, were used to coat wells of microtitre plates. Homologous and heterologous antisera (from potyviruses) were cross-absorbed with healthy chickpea leaf extracts. Antibodies for the Fc portion of rabbit r-globulin prepared in goats, conjugated with alkaline phosphatase, as described by Clark & Adams (1977), was used at a dilution of 1:1000; *p*-nitrophenyl phosphate was used at 0·25 mg/ml. Absorbance values were recorded at 405 nm in a Dynatech MR 590 ELISA reader.

Gel electrophoresis of virus protein and nucleic acid

Polyacrylamide gel electrophoresis (PAGE) for protein was done in 100 g/l slab gels, using the discontinuous buffer system of Laemmli (1970). Purified virus pellets were solubilized in 0·06 M Tris-HCl buffer, pH 6·8, containing 20 g/l sodium dodecyl sulphate (SDS), 10 g/l 2-mercaptoethanol and 6 M urea, and electrophoresed with markers at 25 V for 12–16 h. Gels were stained with Coomassie blue R-250, and the relative molecular weight of the viral coat protein was estimated. The following protein markers obtained from Sigma Chemical Co. were used: phosphorylase B (92 500), bovine serum albumin (66 200), ovalbumin (45 000) and carbonic anhydrase (31 000).

Nucleic acid was extracted from purified virus as described by Reddy *et al.* (1985). SDS at 5 g/l was added to purified virus in STE buffer (0·01 M Tris-HCl, 0·06 M NaCl, 0·003 M EDTA, pH 8·6). After 15 min at room temperature, the preparation was mixed for 3 min with an equal volume

Fig. 2. Local lesions induced by the potyvirus on *Chenopodium amaranticolor*.

of Kirby's mixture (STE-saturated phenol containing 100 g/l m-cresol and 1 g/l 8-hydroxyquinoline). The mixture was kept at 60°C for 2 min, and then centrifuged for 20 min at 5000 rpm. The aqueous phase was removed, extracted with chloroform + isoamyl alcohol, and the RNA precipitated with 70% ethanol at −20°C.

Viral RNA and marker RNA samples were denatured with formaldehyde and formamide (Maniatis *et al.*, 1982). Denatured RNA was electrophoresed in a horizontal slab gel of 10 g/l agarose in Tris-borate-EDTA buffer. Following electrophoresis for 4 h at 50 V, the gels were stained with ethidium bromide (1 μg/ml) for 30 min, destained in distilled water, and examined under a Spectroline (R) TR 302 UV transilluminator. The markers used were brome mosaic virus RNAs (molecular weights $1{\cdot}1 \times 10^6$, $1{\cdot}0 \times 10^6$, $0{\cdot}75 \times 10^6$ and $0{\cdot}28 \times 10^6$ Da) and Indian peanut clump virus RNAs (molecular weights $1{\cdot}83 \times 10^6$ and $1{\cdot}35 \times 10^6$ Da).

Electroblot immunoassay

Electroblotting and immunological detection of viral polypeptides was done as described by Burgermeister & Koenig (1984). Purified virus was subjected to SDS-polyacrylamide gel electrophoresis, as described above, and transferred to nitrocellulose membranes, using a Biorad Trans-Blot cell at 50 V for 4 h at 4 C. Membranes were blocked with 10 g/l bovine serum albumin prepared in Tris-buffered saline (TBS), and then incubated in a 1/1000 dilution of antiserum in TBS for 1 h. After washing in TBS containing 0·5 ml/l Tween-20, the membrane was incubated in 1/

Fig. 3. Veinal necrosis induced by the potyvirus on *Phaseolus vulgaris* cv. Local.

500 dilution of alkaline-phosphatase-labelled goat anti-rabbit Fc-specific antibodies. The substrate used was naphthyl phosphate (5 mg in 20 ml 0·2 M Tris-HCl) and the dye used to visualize the reaction was Fast Blue RR salt (Sigma 25 mg in 5 ml water).

Electron microscopy

Measurements were made on particles in crude chickpea sap, using carbon-filmed grids. The grids were coated with protein A (5 μg protein A per ml) for 15 min, washed with a few drops of 0·1 M phosphate buffer, pH 7·0 (PB), and then floated on a 10-μl drop of homologous antiserum (1/500) for 30 min. The grids were washed in PB, floated on a drop of infected chickpea leaf extract for 45 min, washed with several drops of distilled water, then stained with 20 g/l aqueous uranyl acetate. The grids were viewed in a Philips 201 C electron microscope at an instrument magnification of 13 500. Magnification was calibrated using a carbon grating replica with 2160 lines per mm.

RESULTS

Symptoms of disease

Symptoms appeared on chickpea plants 10 days after sap inoculation, as chlorosis and reduction in leaflet size. The plants later became bushy due to proliferation of the axillary buds and stunting (Fig. 1). Leaflets were brittle, deformed and reduced in size. Plants field-infected early were severely stunted. Terminal branches of late-infected plants were bushy. No pods were produced on early-infected plants, and only few pods were observed on late-infected plants. Seeds were small and deformed. In field infections, the virus often occurred in association with CpSV.

Host range

Of the 27 plant species tested, the virus infected 14 species in four families. Numerous chlorotic local lesions were produced, 8–10 days after inoculation, on leaves of *C. amaranticolor* (Fig. 2) and *C. murale* without systemic infection.

Systemic necrosis was produced on *Canavalia ensiformis*, *Chenopodium quinoa*, *Gossypium herbaceum*, *Nicotiana benthamiana*, and *P. vulgaris* cultivars Local (Fig. 3), Red Kidney, Bountiful, Pinto and Kintoki. The virus induced chlorotic lesions on *Cyamopsis tetragonoloba*, and necrotic lesions on *Cassia obtusifolia* and *Macrotyloma uniflorum*.

The following plant species were not infected: *Arachis hypogaea* cv. TMV-2, *Cucumis sativus* cv. National Pickling, *Glycine max* cv. Bragg, *N.*

Table 1. Serological relationships of chickpea potyvirus in DAC ELISA

	Dilution of chickpea leaf extracts[b]		
		Infected with chickpea potyvirus	
Antiserum to[a]	Healthy 1/100	1/100	1/1000
Pea seed-borne mosaic	0·13[c]	0·16	0·17
Bean yellow mosaic	0·12	0·12	0·12
Bean yellow mosaic	0·11	0·13	0·12
Blackeye cowpea mosaic	0·13	0·40	0·18
Soybean mosaic	0·12	1·56	0·92
Clover yellow mosaic	0·12	0·11	0·11
Azuki bean mosaic	0·48	1·19	1·02
Peanut mottle virus	0·36	1·10	0·96
Peanut stripe virus	0·14	0·48	0·44
Chickpea potyvirus (homologous)	0·18	<2·00	1·60

[a] Antisera to 1 and 3 supplied by Dr R. O. Hampton, 4 and 5 by Dr D. E. Purcifull, 6 by Dr A. J. Cockbain, 7 by Dr N. Iizuka, 2 and 9 by Dr J. W. Demski; antisera of 8 and 10 were produced at ICRISAT.

[b] Dilutions are based on original weight of tissue

[c] Absorbance at 405 nm (see text).

Fig. 4. Chickpea potyvirus particles stained with 20 g/l uranyl acetate in immunosorbent electron microscopy. Bar represents 115 nm.

edwardsoni, *N. glutinosa*, *N. rustica*, *Petunia hybrida*, *Phaseolus vulgaris* cv. Topcrop, *Pisum sativum* cv. Bonneville, *Sesbania grandiflora*, *Psophocarpus tetragonolobus*, *Vigna mungo* and *V. unguiculata* cv. C-152.

Virus purification

The purification method adopted yielded 10–20 mg virus (assuming an extinction coefficient of 3·0) from 1 kg chickpea tissue. Electron microscopy of purified preparations detected no host contamination. The UV absorption spectrum of the purified virus had a shoulder at 290 nm, and the $A_{260/280}$ and $A_{260/245}$ ratios were 1·31 and 1·11, respectively.

Serology

The titre of the antiserum as determined by the precipitin ring test was 1/640. In DAC ELISA, the virus reacted strongly with antisera to azuki bean mosaic virus (ABMV), peanut mottle virus (PMV) and soybean mosaic virus (SMV), and weakly with peanut stripe virus (PStV) and blackeye cowpea mosaic virus (BICMV) antisera. Several other potyvirus antisera, including BYMV from two sources, did not react with the virus (Table 1).

In electro-immunoblot assay, the virus reacted strongly with homologous and SMV antisera, and weakly with PMV, PStV and BICMV antisera. It did not react with BYMV antiserum.

Analysis of protein and nucleic acid

In PAGE, the virus coat protein formed one band with an estimated molecular weight of 32 500 Da. However, with some preparations, another polypeptide of 29 000 Da was observed. The nucleic acid migrated as a single band, with an estimated molecular weight of $3{\cdot}1 \times 10^6$ Da.

Electron microscopy

Negatively stained leaf-dip preparations revealed the presence of flexuous rods. The majority of the particles (42 of 64) measured between 700 and 720 nm (Fig. 4) while the width was between 13 and 15 nm.

DISCUSSION

The size and morphology of virus particles,

molecular weights of coat protein sub-units and nucleic acid, and serological tests show that the virus isolated from chickpea belongs to the potyvirus group. Virus symptoms observed in inoculated chickpea were similar to those observed in the field.

On the ICRISAT Centre farm, the incidence of the potyvirus alone in chickpeas did not exceed 1%. The virus was also observed in 1988 in several chickpea-growing areas in north India, often in association with CpSV. We are currently investigating its economic importance. Since seed transmission is a significant means of spread of some chickpea viruses (Kaiser *et al.*, 1988), we are currently investigating seed transmission of the potyvirus.

BYMV and CFV appear to be the only potyviruses previously reported as occurring naturally in chickpeas, the latter only in the USA (Kaiser & Danesh, 1971b; Nene & Reddy, 1987; Kaiser *et al.*, 1988). In indirect ELISA tests and electroblot immunoassays, the new potyvirus did not react with BYMV antisera from two sources. The BYMV isolate from chickpea in India produced different symptoms on chickpea and had a narrower host range. It did not induce local lesions on *C. amaranticolor* (Chalam, 1982). Although the potyvirus isolated showed serological relationships with ABMV, PMV and SMV, host-range studies indicated that it differed from these viruses. PMV produces local lesions on *P. vulgaris* cv. Topcrop, unlike the chickpea potyvirus. Additionally PMV infects *A. hypogaea*, *P. sativum*, *G. max* and *V. unguiculata* (Kuhn, 1965). The potyvirus failed to infect *G. max* and *V. unguiculata*, unlike SMV (Galvez, 1963) and ABMV (Hampton *et al.*, 1978).

On the basis of the information presented, the potyvirus is considered to be a distinct and previously undescribed virus and we suggest it be named chickpea bushy dwarf virus.

ACKNOWLEDGEMENTS

The authors thank Dr Wendy Sacks for help in conducting gel electrophoresis for nucleic acids, and Drs D. McDonald, J. H. Williams and M. V. Reddy for their valuable comments. We thank Drs R. O. Hampton, D. E. Purcifull, A. J. Cockbain, N. Iizuka and J. W. Demski for supplying antisera.

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