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Genome characterization of a new strain of peanut chlorotic streak virus causing chlorotic vein banding disease of groundnut (*Arachis hypogaea* L.) in India

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The double-stranded DNA of the chlorotic vein banding isolate of peanut chlorotic streak virus (PClSV-CVB), isolated from purified virus, resolved into circular and linear molecules similar to those of other caulimoviruses. A physical map of viral DNA was constructed, which showed the PClSV-CVB DNA to be circular and composed of approximately 8·2 kbp. A number of restriction sites were found to be shared with a similar caulimovirus, PClSV. Nevertheless, several differences between physical maps of the two viruses suggest that PClSV-CVB should be considered as a distinct strain of PClSV. *Bam* HI-cleaved PClSV-CVB DNA was cloned into pUC 118 and was infectious when cleaved from the cloning vector and inoculated onto *Vigna unguiculata*.

INTRODUCTION

Peanut chlorotic streak virus (PClSV), a caulimovirus, has been reported to infect naturally groundnut (peanut) in India (Reddy *et al.*, 1993). A virus serologically related to PClSV but causing different symptoms on groundnut was collected from groundnut and named chlorotic vein banding isolate of PClSV, i.e. PClSV-CVB (Satyanarayana *et al.*, 1994). The virus had isometric particles of diameter 51 nm and was sap transmissible to 21 species in the families Chenopodiaceae, Cruciferae, Leguminosae and Solanaceae. It differed from PClSV in host range and reaction and was therefore regarded as a distinct strain of PClSV (Satyanarayana *et al.*, 1994). The present paper reports on restriction mapping and cloning of the PClSV-CVB genome and points to further differences between PClSV and PClSV-CVB.

MATERIALS AND METHODS

Virus culture maintenance, purification of virus and extraction of viral nucleic acid

The virus from field-collected infected groundnut plants was transmitted initially by grafting, and subsequently by mechanical inoculation to cowpea (*Vigna unguiculata* cv. Local) in a screen house. After three successive passages of virus from single lesions in cowpea, the virus was maintained in groundnut or in *Nicotiana rustica* by mechanical inoculation.

PClSV-CVB was purified from *N. rustica* as described by Satyanarayana *et al.* (1994), and the nucleic acid was extracted according to the procedure described for CaMV DNA (Shepherd *et al.*, 1970; Gardner & Shepherd, 1980), except that pronase was replaced with proteinase K at 1 mg/ml in the presence of 1% SDS and 20 mM EDTA. This suspension was extracted three times with phenol-chloroform (1:1, v/v) and once with chloroform, and the nucleic acid was precipitated by the addition of 2·5 volumes of ethanol in the presence of 300 mM sodium acetate, pH 5·2.

Native viral DNA and restriction mapping of viral nucleic acid

Electrophoresis of native viral DNA in 0·7% non-denaturing agarose gels using Tris-acetate-EDTA buffer (TAE: 0·04 M Tris-acetate, pH 7·6 and 0·001 M EDTA, pH 8·0) was as described by Sambrook *et al.* (1989). Cauliflower mosaic virus (CaMV) DNA was used as a reference.

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Restriction enzymes *Bam* HI, *Pst* I, *Eco* RI, *Eco* RV, *Bgl* II, *Hind* III, *Sal* I and *Pvu* II were used according to the manufacturers' recommendations. The activity of enzymes that did not cut the viral DNA was tested using pUC 118 as substrate. Restriction fragments were separated in 1% or 1·2% agarose gels in TAE buffer under submarine conditions (Sambrook *et al.*, 1989). The sizes of the PClSV-CVB DNA fragments generated by restriction endonuclease digestion were determined by comparison with lambda DNA digested with *Eco* RI and *Hind* III (New England Biolabs, Beverly, MA, USA). The location of different restriction sites and the sizes of restriction fragments were determined using both native viral DNA and an infectious clone of PClSV-CVB. The infectious clone, when inoculated onto groundnut, produced symptoms similar to those that of DNA extracted from purified PClSV-CVB.

Southern hybridization of PClSV-CVB DNA

The entire DNA sequence of an infectious clone of PClSV has been determined and a map showing the genomic organization of the virus has been constructed (Richins, unpublished data). Using probes derived from PClSV, Southern hybridizations were performed and the PClSV-CVB map was oriented in relation to that of PClSV.

DNA fragments electrophoresed for Southern hybridizations included the gel-isolated fragment used for probe construction (homologous control DNA), PClSV DNA digested with *Eco* RI, and cloned PClSV-CVB DNA digested with *Bam* HI and one of the following enzymes: *Bgl* II, *Eco* RI or *Eco* RV. Biotinylated *Hind* III-digested lambda DNA (Sigma Chemical Company, St Louis, MO, USA) was used to determine the size of each of the hybridizing fragments. The nucleic acids were transferred from agarose gels to nitrocellulose membranes using with a vacuum transfer apparatus.

Biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany)-labelled probes were prepared by random priming (Sambrook *et al.*, 1989) of PClSV restriction fragments isolated from agarose gels using DEAE-cellulose membranes (Whatman DE 81).

The probes used in these experiments were a 0·7-kbp *Hind* III-*Pst* I fragment, a 0·9-kbp *Pst* I fragment, and a 1·1-kbp *Bgl* II fragment of PClSV (Fig. 3). After hybridization, the biotin-labelled DNA was detected using streptavidin and alkaline phosphatase conjugated biotin (Bethesda Research Laboratories, Bethesda, MD, USA). The substrate was a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/BNT).

Molecular cloning

Recombinant plasmids were prepared by inserting *Bam* HI-digested DNA into a similarly digested pUC 118 plasmid vector (Sambrook *et al.*, 1989). *Escherichia coli* strain DH5α was used as the recipient host for $CaCl_2$-mediated transformations. Immediately before plating the cells onto a selective medium, 50 μl of 2% X-gal (in N-N'dimethylformamide) and 20 μl of 2% isopropylthiogalactoside (in water) were added to the cells. The cells were then plated onto LB agar medium containing 500 μg/ml penicillin G and incubated overnight at 37°C. White colonies were inoculated to culture tubes containing 4 ml of LB medium and penicillin G (500 μg/ml) and grown for 8–10 h at 37°C. Plasmid DNA was extracted from these cultures by the alkaline lysis DNA miniprep method (Sambrook *et al.*, 1989). The isopropanol precipitated DNA was washed with 70% ethanol and suspended in TE buffer. Plasmid DNA was re-extracted with an equal volume of phenol, followed by a phenol-chloroform mixture, and was then precipitated with 2 volumes of ethanol in the presence of 300 mM sodium acetate, pH 5·2.

The isolated plasmid DNAs were screened for the presence of full-length viral DNA insert by digesting with *Bam* HI followed by electrophoresis in 1·0% agarose gel in TAE buffer. Plasmid DNAs of the appropriate size were subjected to further restriction endonuclease digestion and compared with restriction digests of viral DNA in order to ensure that no rearrangements had occurred during the cloning of viral genome.

To test the infectivity of the full-length viral genome, the cloned viral genome (5 μg in 50 μl TE buffer) was excised from the plasmid and inoculated onto the fully expanded primary leaves of *Vigna unguiculata*.

RESULTS AND DISCUSSION

The native PClSV-CVB DNA resolved into circular and linear molecules in agarose gels (Fig. 1) in a manner similar to CaMV DNA (Volovitch *et al.*, 1976; Meagher *et al.*, 1977; Lebeurier *et al.*, 1980). The slowest migrating bands (Fig. 1) were composed of circular

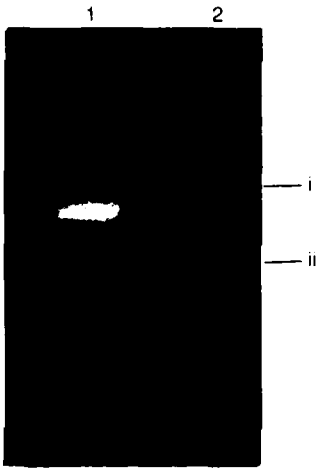

Fig. 1 Electrophoresis of native DNAs of (1) CaMV and (2) PClSV-CVB in 0·7% agarose gels (i) circular molecules, (ii) linear molecules

molecules (Howell & Hull, 1978). Multiple bands of circular form were caused by different degrees of twistedness (Lawson & Civerolo, 1976; Hull & Shepherd, 1977; Meagher *et al.*, 1977; Hull & Donson, 1982; Hibi *et al.*, 1986). The viral DNA appeared to contain more circular than linear molecules. In contrast, carnation etched ring virus (CERV) DNA consisted mainly of linear molecules (Lawson & Civerolo, 1978).

In Southern hybridization experiments, the 0·7-kbp *Hin*d III-*Pst* I fragment of PClSV hybridized to the following PClSV-CVB fragments: the *Bgl* II fragment from map positions 6·9 to 0·4, the *Eco* RI fragment from map positions 7·0 to 0·5, and the *Eco* RV fragment from map positions 7·1 to 2·2 (Fig. 2a). The 1·1-kbp *Bgl* II fragment of PClSV hybridized with the following PClSV-CVB fragments: the *Bam* HI to *Eco* RI fragment from map positions 4·0 to 7·0 and the *Eco* RV fragment from map positions 4·4 to 7·1. This probe also hybridized and co-migrated with the 1·2-kbp *Bgl* II fragment of PClSV-CVB DNA (Fig. 2b). The 0·9 kbp *Pst* I fragment of PClSV hybridized with the following PClSV-CVB fragments: the *Eco* RI fragment from map positions 0·5 to 1·6, the *Eco*RV fragment from map positions 7·1 to 2·2 and the *Bgl* II to *Bam*HI fragment from map positions 0·4 to 4·0 (Fig. 2c).

The results of the Southern hybridization experiments allowed the construction of a physical map of the PClSV-CVB genome and its orientation with respect to that of PClSV (Reddy *et al.*, 1993). Several sites were found to be shared with those of PClSV, but they differed in the number of restriction sites with respect to *Bgl* II, *Eco* RI, *Eco* RV, *Hind* III and *Pst* I. There is a single *Bam* HI and *Pst* I site in the viral DNA shown by *Bam* HI- and *Pst* I- digested DNAs co-migrating with the fastest migrating band (linear molecules) of uncut DNA. By using further digestions of *Bam* HI- or *Pst* I- linearized molecules with *Bgl* II, *Eco* RI and *Eco* RV, a restriction endonuclease map of PClSV-CVB

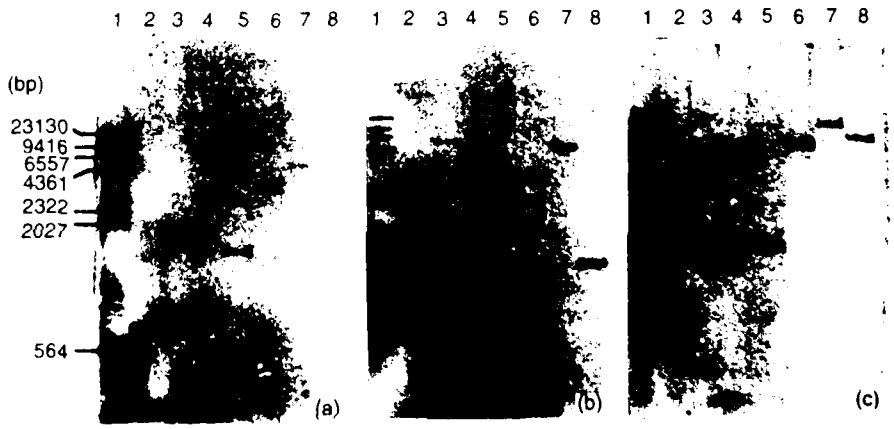

Fig. 2 Southern hybridization of restricted PClSV-CVB DNA probed with biotin-labelled 0·7-kbp *Hin*d III-*Pst* I fragment (a), 1·1-kbp *Bgl* II fragment (b) and 0·9 kbp *Pst* I fragment (c) of PClSV. The samples were biotin-labelled *Hin*d III digested lambda DNA (1), gel eluted respective DNA fragment (homologous control) (2), PClSV DNA digested with *Hin*d III and *Pst* I (3) and *Eco* RI (4). PClSV-CVB DNA digested with *Bam* HI and *Eco* RI (5), *Bam* HI and *Eco* RV (6), *Bam* HI and *Pst* I (7) and *Bam* HI and *Bgl* II (8).

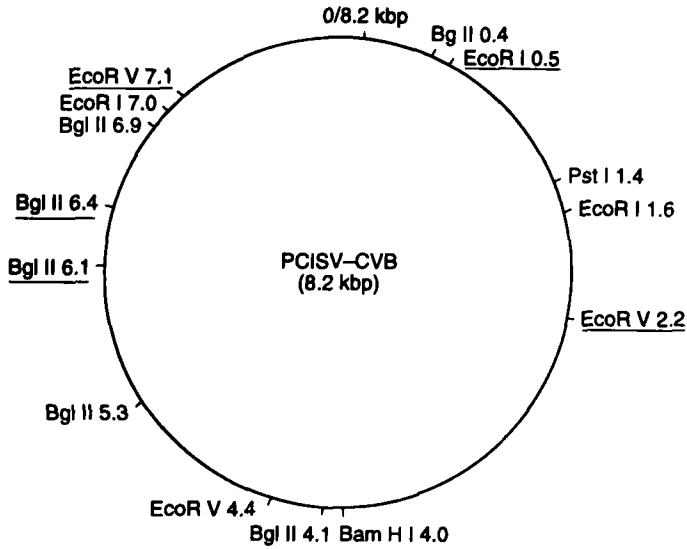

Fig. 3 Physical map of PCISV-CVB DNA. Sites not present in PCISV are underlined.

DNA was constructed (Fig. 3). The size of PCISV-CVB DNA was 8·2 kbp, as estimated from restriction enzyme digests. PCISV-CVB DNA is very similar in size to soybean chlorotic mottle virus (SoCMV) (8·2 kbp) (Hibi *et al.*, 1989) and PCISV (8·2 kbp) DNAs (Reddy *et al.*, 1993; Richins, unpublished data). As observed in the case of PCISV, the restriction map of PCISV-CVB DNA revealed no similarity to other caulimoviruses (Meagher *et al.*, 1977; Volovitch *et al.*, 1979; Hull, 1980; Hull & Donson, 1982; Donson & Hull, 1983; Richins & Shepherd, 1983; Hibi *et al.*, 1986). The differences that exist between the physical maps of PCISV (Reddy *et al.* 1993) and that constructed for PCISV-CVB and the differences in host range and reaction lend support to our conclusion that PCISV-CVB is a distinct strain of PCISV.

PCISV-CVB DNA was cloned into pUC 118. A full-length clone pPCISV-CVB was obtained that produced 10–12 local chlorotic lesions per cowpea plant when excised from the cloning vector. Fragment size and locations were confirmed by restriction digests of native viral DNA and suggested that no rearrangement had occurred during cloning.

The molecular characteristics of the PCISV-CVB genome described here reinforce its earlier identification as a distinct strain of PCISV based on its biological properties (Satyanarayana *et al.*, 1994). The PCISV genome has been completely sequenced (Richins, unpublished data). It would be interesting to sequence the PCISV-CVB genome in order to determine the critical differences between the two viruses.

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