Molecular characterisation of a strain of peanut stripe potyvirus from groundnut germplasm imported into South Africa

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Peanut stripe potyvirus (PStV) was identified in a groundnut plant grown from seed imported into South Africa. Host range, serology and electron microscopy studies indicated that the infective agent was an isolate of PStV. This was confirmed by sequencing the coat protein gene and a portion of the 3' untranslated region of the virus and comparing it with known strains of PStV. The local strain (PStV-95/399) was related to, but distinct from, strains of PStV reported from the USA. PStV-95/399 and the USA strains probably had a common ancestor, but the 95/399 sequence appeared to have diverged to a greater extent from this ancestral sequence. Phylogenetic analysis indicated that 95/399 probably originated in the USA.

Key words: germplasm, groundnut, peanut, peanut stripe potyvirus, South Africa, strain.

Peanut stripe potyvirus (PStV) is a member of the Potyvirus genus of plant viruses in the family *Potyviridae*. As such, it has a single-stranded positive-sense RNA genome of approximately 10 Kb (Demski et al. 1984). It contains a single open reading frame encoding a polyprotein which is post-translationally cleaved into at least 8 individual proteins required for virus replication and movement (Dougherty & Carrington 1988). Characterisation of viruses related to bean common mosaic potyvirus (BCMV) using serology and peptide profiling studies have led to PStV being taxonomically classified as a groundnut (*Arachis hypogaea* L.)-infecting strain of BCMV (McKern et al. 1992; Vetten et al. 1992; Saiz et al. 1994). Recent sequence-comparison studies (Berger et al. 1997; Higgins et al. 1998) have shown that, while PStV strains can be classified as members of the BCMV group, they are clearly distinct from the other viruses in this group.

PStV causes severe stunting of plants and great reductions in yield of cultivated groundnuts. Surveys of groundnut-producing areas have indicated that the virus is endemic in east and southeast Asia (Xu 1987). Xu et al. (1983) reported a potyvirus from China, describing it as 'virus producing mild mottle'. Demski et al. (1984) identified PStV in groundnuts in the USA and concluded that the virus had been imported from China. Further characterisation showed that the above two viruses were strains of the same virus. Choopanya (1973) reported a virus in Thailand that could have been PStV, while a virus collected in Thailand in 1972 was positively identified as PStV (Wongkaew & Dollet 1990). Considering that PStV was present in Thailand as early as 1972 and the large range of biological strains of PStV that have been identified from there, it has been suggested that Thailand is the centre of origin of PStV (Wongkaew & Dollet 1990). Phylogenetic analysis of PStV suggests a common ancestor for all PStV isolates studied thus far (Higgins et al. 1998). At present, the virus does not occur in Australia owing to rigorous quarantine procedures. There have, as yet, been no reports of PStV in African countries.

PStV is both aphid-transmitted in a non-persistent manner and seedborne in groundnut. The rate of seed transmission depends on the cultivar infected and the period of infection (Demski & Lovell 1985). Seed-transmission rates of up to 50 % have been observed (Xu et al. 1991), making it difficult to control the spread of PStV. Seed is imported and exported between various countries, both for processing and consumption and for incorporation of new germplasm into breeding programmes. Strict control of groundnut seed importation combined with sensitive diagnostic tests for PStV in groundnut material (Dietzgen et al. 1994) is needed to restrict further spread of the virus.

A diseased groundnut plant grown from seed imported into South Africa with symptoms resembling those reported for PStV was intercepted in 1995. In this paper, we report on the identification of the infective agent as PStV (designated

Table 1. Sequences and positions of the primers used for RT-PCR and sequencing of peanut stripe potyvirus strain 95/399. Nucleotide numbering follows that used by Flasinski et al. (1996).

Primer	Sequence	Position
PST1	5'- GCATGCCCTCGCCATTGCAA -3'	10003–9984
PST2	5'- GCACACACTTCTTGGCATGG -3'	9769–9788
PST4	5'- TACATAGCAGAATCAGCACT -3'	8810–8829
PST5	5'- GCCTTTCAGTATTCTCGCTG -3'	9740–9721
PST6	5'- CAATGAGAGACAAGGATG -3'	9081–9098

PStV-95/399), and describe its relationship with PStV strains reported elsewhere, as well as with the related viruses blackeye cowpea mosaic potyvirus (BlCMV), BCMV and bean common mosaic necrosis potyvirus (BCMNV).

Materials and methods

Virus identification

The virus characterised in this investigation originated from a single cultivated groundnut plant showing dark green blotches that was grown in an experimental plot at the ARC-Grain Crops Institute at Brits, North-West Province, South Africa.

The virus was isolated by two local lesion transfers on *Chenopodium quinoa* Willd. and was maintained and propagated on *Nicotiana benthamiana* Domin. Isolated virus, designated strain 95/399, was also back-inoculated to two local cultivars of *A. hypogaea,* Sellie and Natal Common. A host-range study was carried out using leguminous and non-leguminous plants. All mechanical inoculations were performed by grinding tissue in a pre-chilled pestle and mortar with ice-cold 0.01 M phosphate buffer, pH 7.1, containing 0.01 M sodium sulphite and a small amount of celite. All plants used in the study were inoculated when young and growing vigorously, and included *A. hypogaea* cvs Sellie and Natal Common, *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa, Glycine max* Merr. cvs B66S10 and Forrest, *Lupinus albus* L., *N. benthamiana, N. clevelandii* Gray, *Phaseolus vulgaris* L. cv. Top Crop and *Vigna unguiculata* (L.)Walp. cv. Blackeye. Plants were maintained in a greenhouse at the ARC-Plant Protection Institute in Pretoria (PPRI) and observed daily. Symptom development was recorded over a three-week period. All plants were tested in a $F(ab')_2$-ELISA (Barbara & Clark 1982) for the presence of PStV three weeks after inoculation. The antiserum used was produced at ARC-PPRI against a PStV strain received from J. Demski (University of Georgia, USA). Immunosorbent electron microscopy (ISEM) studies were also carried out using this antiserum. ISEM and antibody coating were performed as described by Roberts (1986).

Immunocapture RT-PCR

The coat protein sequence of strain 95/399 was isolated by immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR). Immunocapture was done as previously described (Cook et al. 1998). A single vial, two-stage protocol was followed with RT and PCR reagents combined in a single mix. With the captured virus as template, the components of the reaction were as described by Cook et al. (1998), except that the primers were used at 0.5 µM each. The following combinations of primers (Table 1) were included: PST1 & PST2, PST1 & PST4, PST1 & PST6, PST4 & PST5 and PST5 & PST6. Synthetic primers were purchased from Bresatec Ltd (Adelaide, Australia), Centre for Cellular and Molecular Biology, University of Queensland or Life Technologies (Gaithersburg, USA). All primers were designed from PStV sequences that had previously been published (Cassidy et al. 1993; Gunasinghe et al. 1994; Teycheney & Dietzgen 1994; Flasinski et al. 1996). First strand cDNA synthesis was at 42 °C for 45 min. The amplification conditions were: 94 °C for 2 min (1 cycle) and 35 cycles of 94 °C for 45 sec; 55 °C for 45 sec; 72 °C for 90 sec followed by one cycle of elongation at 72 °C for 10 min.

The PCR products were electrophoresed in 1 % agarose/TBE gels (Sambrook et al. 1989) and purified using Geneclean (BIO 101, Vista, USA)

according to the manufacturer's instructions. Purified fragments were cloned into the T-tailed cloning vector pCR2.1 according to the instructions from Invitrogen. Ligation reactions were transformed into *Escherichia coli* DH5α using standard chemical transformation techniques (Sambrook et al. 1989) and clones were identified either by digestion with *Eco*RI or by PCR of bacterial colonies using universal forward and reverse primers.

DNA sequence determination

Plasmid DNA was prepared and sequenced using an Applied Biosystems model ABI 373 automated sequencer according to the recommendations of the manufacturer. Sequencing was done by the dideoxy chain termination method of Sanger et al. (1977) using the recommended PRISM dye terminator premix and reaction conditions. Sequence was obtained from three clones of each PCR product in both directions to rule out possible mis-incorporated bases during PCR.

Computer analysis of DNA sequences

The sequence of 95/399 was compared at the nucleotide and amino acid levels with the corresponding sequences from PStV isolates from Thailand (T1 no. Y11776, T3 no. Y11771, T5 no. Y11772, T6 no. Y11773, T7 no. Y11774, Higgins et al. 1998), USA (USA1 no. U34972, Flasinski et al. 1996, USA2 no. U05771, Gunasinghe et al. 1994, USA3 no. X63559, Cassidy et al. 1993) and Indonesia (Ib no. X21700, Teycheney & Dietzgen 1994). These sequences were compared with the W strain of BICMV (no. S66253, Khan et al. 1993), the NL4 strain of BCMV (no. L21766, Berger et al. 1997) and the NL3 strain of BCMNV (no. U20818, Fang et al. 1995).

Programs networked by the Australian National Genome Information Service (ANGIS) were used to compile and analyse the nucleotide and predicted amino acid sequences. Sequence alignments were done using CLUSTALW (Thompson et al. 1994) and phylogenies were inferred from these alignments using version 3.5 of the Phylip package (Felsenstein 1989).

Results

The virus characterised in this investigation (strain 95/399) was originally noticed as producing dark-green blotches on a groundnut plant grown from seed imported into South Africa (Fig. 1a). Only a single plant of unknown origin appeared to have these symptoms.

Strain 95/399 was identified as PStV by host-range, symptoms and serology. Mechanical inoculation of two groundnut cultivars grown in South Africa, Natal Common (Fig. 1b) and Sellie (data not presented), produced systemic dark-green stripe symptoms in both cultivars. The isolate also caused chlorotic lesions on *Chenopodium* species and systemic infection in *L. albus, N. benthamiana*, and *V. unguiculata* (Table 2), typical for isolates of PStV (Edwardson & Christie 1991). The virus causing symptoms in the indicator plants reacted specifically with the PStV antiserum, identifying it as a strain of PStV (Table 2). Furthermore, immunosorbent electron microscopy studies revealed the presence of long flexuous rod-shaped potyvirus-like particles in the infected material, which were both trapped and decorated by the PStV antiserum (data not presented).

Since the origin of the 95/399 strain was unclear, and since the CP gene sequences of PStV strains group according to their geographical origin (Higgins et al. 1998), it was of interest to sequence the coat protein gene of 95/399 to determine its relationship with other PStV strains. Initial analysis of the isolate using immunocapture RT-PCR with primers PST1 and PST2 gave the expected 234 bp PCR product (Fig. 2). Sequencing of the PCR product derived from amplification of the 3′-end of the coat protein gene confirmed its identity as PStV. However, primers PST1 and 4, which had been used previously to efficiently amplify the full-length coat protein gene from other strains (Higgins et al. 1998), resulted in preferential amplification of a 370 bp product (Fig. 2), with only a very faint product of the expected size of 1194 bp. While this smaller product has been observed for other strains, it was not amplified preferentially. Sequence analysis of the 370 bp product showed that it was derived from the 5′-end of the coat protein gene and resulted from mispriming of PST1 at position 9161–9182, according to the numbering used by Flasinski et al. (1996).

RT-PCR incorporating other primer combinations that had been used previously for other strains resulted in the expected PCR products (Fig. 2). The combination of PST 1 and 6 gave a product of 922 bp as well as one of about 450 bp (Fig. 2). The smaller product produced when primers PST1 and 6 were used, was probably also derived from the mispriming of one of these primers. In this case, the mispriming is probably not

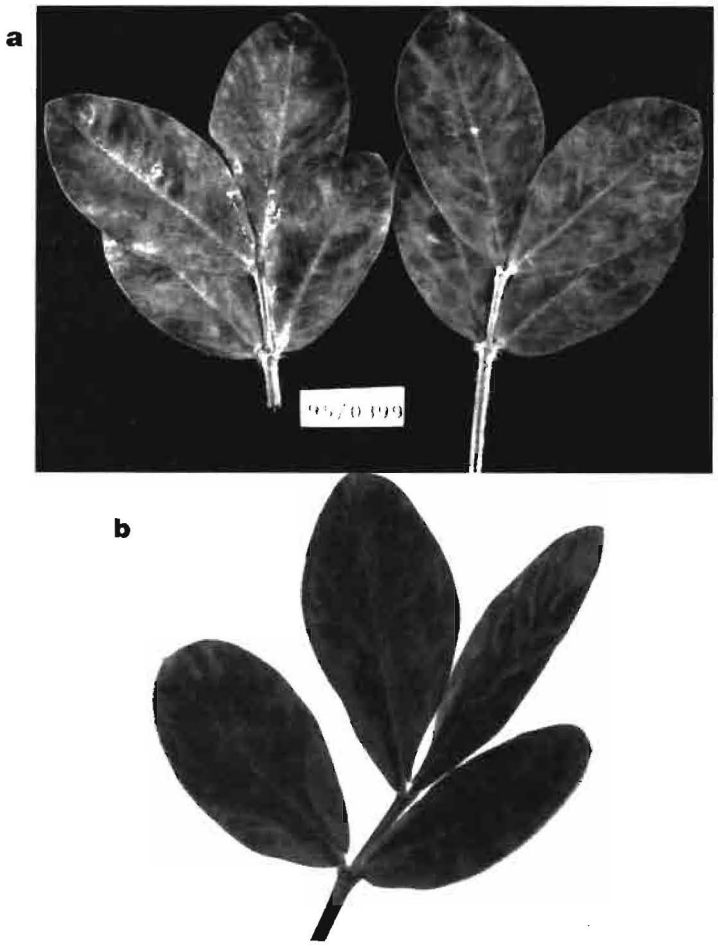

Fig. 1. Symptoms caused by peanut stripe potyvirus strain 95/399 on groundnut; (**a**) symptoms on the infected groundnut plant observed originally; (**b**) symptoms produced by mechanical infection of cv. Natal Common with the local lesion passaged strain.

Table 2. Symptoms in indicator plants mechanically inoculated with peanut stripe potyvirus strain 95/399.

Species		Symptoms	
		Local	Systemic
Arachis hypogaea	cv. Natal Common	No symptom	Dark green striping (+)[a]
	cv. Sellie	No symptom	Dark green striping (+)
Chenopodium amaranticolor		Chlorotic local lesions(+)	No systemic infection (–)
C. quinoa		Chlorotic local lesions (+)	No systemic infection (–)
Glycine max	cv. B66S10	No infection (–)	
	cv Forrest	No infection (–)	
Lupinis albus		No symptom	Mottle and leaf curl (+)
Nicotiana benthamiana		No symptom	Mild mottle (+)
N. clevelandii		No infection (–)	
Phaseolus vulgaris	cv. Top Crop	No infection (–)	
Vigna unguiculata	cv. Blackeye	No symptom	Slight stunting (+)

[a]ELISA results using anti-PStV antiserum are given in brackets.

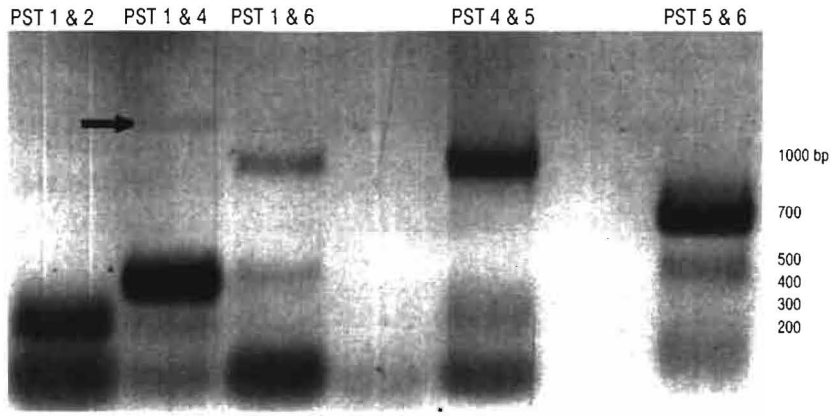

Fig. 2. Agarose gel of the RT-PCR products generated from peanut stripe potyvirus strain 95/399 using PStV primers. Primer combinations are shown at the top of the gel and the sizes of the molecular weight markers are on the right. The product corresponding to the full-length coat protein is indicated with an arrow.

efficient since most of the product produced is of the expected size. The primer combinations of PST4/PST5 and PST5/6 amplified the expected products of 930 bp and 659 bp, respectively (Fig. 2).

The 1194 bp full-length PCR fragment from PStV-95/399 obtained with primer pair PST1 and 4, contained a single open reading frame, which terminated in a UAG stop codon at nucleotide 991. For the other PStV strains, a second in-frame stop codon was located 45 nucleotides further downstream and there were two putative Q/S polyprotein cleavage sites. It is believed that the upstream site is the one most likely to be used in vivo since cleavage at this site would result in a coat protein of the expected size of about 32.5 kDa (Shukla et al. 1994). PStV-95/399 had the DAG motif required for aphid transmission (Atreya et al. 1991) at amino acid position 10–12 (data not presented). This nucleotide sequence has been deposited in the EMBL nucleic acid database under accession number Y11775.

Sequence comparisons and phylogenetic analysis confirmed that 95/399 should be classified as a strain of PStV (Fig. 3). The neighbour-joining tree in Fig. 3a shows that all the PStV strains, including 95/399, formed a distinct monophyletic group from the other viruses in this study. This grouping is significant with a bootstrap value of 100. Strain 95/399 showed only 87.8 % and 88.7 % nucleotide sequence identity with BlCMV and BCMN-NL4, respectively, and 76.5 % identity with BCMNV-NL3.

Since PStV-95/399 is not identical to any other PStV CP-sequence at the nucleotide or amino acid level, it is a distinct strain of PStV. The level of sequence identity observed between 95/399 and the other PStV strains ranged from 94.3 % (T6) to 98.8 % (USA1) for the nucleotide sequences and 95.5 % (T1) to 99.0 % (USA1 and 3) for the amino sequences. The latter comparison corresponded to 3–13 amino acid changes over the length of the coat protein. Strain 95/399 is, therefore, most closely related to the USA strains, particularly USA1. This was confirmed by phylogenetic analysis (Fig. 3b), which indicated that these strains formed a separate clade from the other strains of PStV. This grouping was significant with a bootstrap value of 98.

The 95/399 strain of PStV was closer to PStV-Ib than to the Thai strains. PStV-Ib showed 96.6 % nucleotide sequence identity with 95/399 compared to 94.3–94.8 % for the Thai strains. Amino acid sequence comparisons revealed that 95/399 had seven changes from the PStV-Ib sequence or 97.6 % identity while the Thai strains had 10–13 amino acid changes or 95.5–96.5 % sequence identity. The most distantly related strain to 95/399 was T6 at the nucleotide level and T1 at the amino acid level.

A study by Revers et al. (1996) suggested that the Indonesian blotch isolate PStV-Ib may be a recombinant between PStV and BlCMV and that this recombination occurred in the 3'-end of the coat protein coding sequence. Comparison of the final 38 bp of the coat protein coding sequence and 234 bp of the 3' untranslated sequence showed that the 95/399 sequence remained in the same

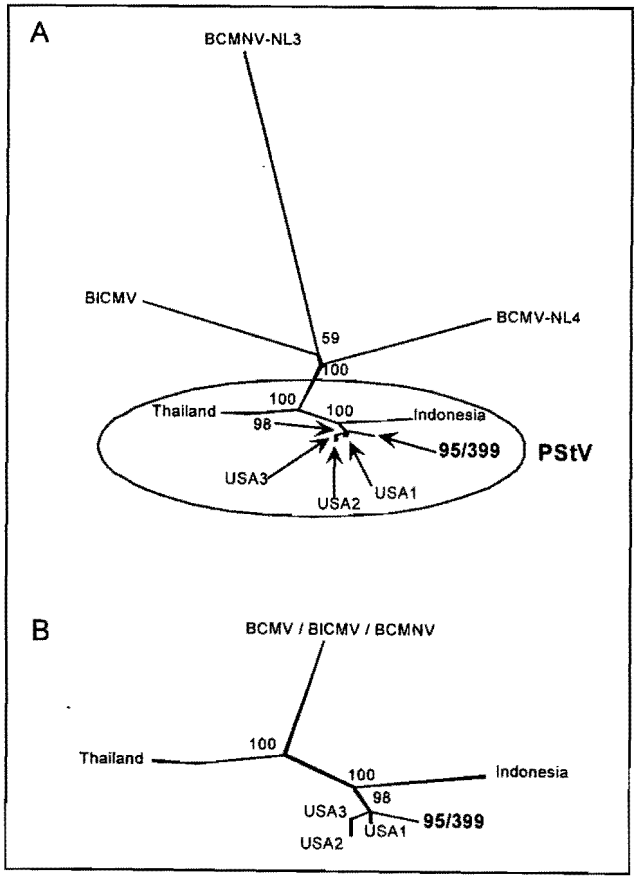

Fig. 3. A: neighbour joining tree showing the phylogenetic relationship of peanut stripe potyvirus strain 95/399 coat protein nucleotide sequence to that of other PStV strains and the related viruses, BICMV, BCMV-NL4 and BCMNV-NL3; **B**: enlargement of the PStV portion of the tree shown in (A), illustrating more clearly the relationship of 95/399 with other PStV strains. The relevant bootstrap values are indicated at the nodes.

position on the phylogenetic tree, irrespective of the region of coat protein and 3'UT region compared (data not presented). This suggests that the 95/399 strain of PStV is not a recombinant between PStV and BICMV in this region of the genome, nor a recombinant of different PStV strains.

Discussion

A single groundnut plant showing symptoms indicative of PStV infection was intercepted in South Africa among plants grown from imported seed. Host-range analysis, serological studies and electron microscopy indicated that the infective agent was PStV. Cloning and sequencing of the coat protein region confirmed that this plant was indeed infected with PStV.

RT-PCR amplification of the full-length CP fragment (1194bp) of PStV-95/399 with the primers PST1 and PST4 was not efficient, even though the expected product of the diagnostic primers for PStV, PST1 and PST2 amplified very well. It is likely that the inefficient annealing of PST1 to its predicted binding site would not become apparent until attempts are made to amplify a larger product such as the full-length CP sequence. PST1 is currently used in combination with PST2 as primer in a diagnostic test for PStV infection in groundnut leaves (RGD, unpubl.). Since the PST1/PST2 primer combination did not result in erroneous PCR products with the 95/399 template through the mispriming of PST1, this primer can still be used with confidence in the diagnostic RT-PCR test for PStV.

Sequence comparisons with other PStV isolates

showed that PStV-95/399, while being a strain of this virus, was distinct from all other PStV sequences characterised so far. It was most closely related to isolates from the USA indicating that it probably originated in that country. If viral evolution is heading towards increased sequence diversity, it is possible that PStV-95/399 and PStV strains in the USA may have had a common ancestor. If so, PStV-95/399 seems to have diverged from this original sequence to a greater extent than the USA sequences. PStV entered the USA through infected groundnut germplasm from China (Demski et al. 1984). It is unknown whether PStV-95/399 evolved from this original virus population since its introduction into the USA, or emanated from a subsequent introduction or, indeed, originated from China and transported to the USA. More sequences of PStV from both of these countries need to be studied to determine the evolutionary relationship between strains.

The interception of PStV in South Africa shows that this seedborne virus can emerge in groundnut planting and breeding stock through importation of infected germplasm. The African continent must still be considered free from this virus and vigilance exercised to prevent its introduction. However, the possibility that groundnut-infecting strains of BCMV may evolve in situ from a progenitor virus cannot be eliminated. As demonstrated here, reliable and sensitive diagnostic assays, international collaboration and stringent quarantine procedures are essential to prevent the introduction of exotic diseases and their spread in planting stock.

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