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Satyanarayana Tatineni
USDA-ARS, Satyanarayana.Tatineni@ars.usda.gov

S. E. Mitchell
USDA-ARS

D. V. R. Reddy
International Crops Research Institute for the Semi-Arid Tropics

S. Brown
USDA-ARS

S. Kresovich
USDA-ARS

See next page for additional authors

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Authors
Satyanarayana Tatineni, S. E. Mitchell, D. V. R. Reddy, S. Brown, S. Kresovich, R. Jarret, S. Gowda, R. A. Naidu, and J. W. Demski

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Peanut Bud Necrosis Virus: Purification of Nucleocapsids and Sequence Homology of Nucleocapsid Protein and Glycoprotein Precursor with other Tospoviruses

T. Satyanarayana,[1] S.E. Mitchell,[2] D.V.R. Reddy,[1] S. Brown,[2] S. Kresovich,[2] R. Jarret,[2] S. Gowda,[3] R.A. Naidu,[1] and J.W. Demski[4]

[1]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India; [2]Plant Genetic Resources Conservation Unit, US Department of Agriculture, Agricultural Research Service, Griffin, GA 30223, USA; [3]Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA; and [4]Department of Plant Pathology, University of Georgia, Experiment Station, Griffin, GA 30233, USA

A procedure for the purification of peanut bud necrosis virus (PBNV) nucleocapsids was developed. Virus particles were treated with nonionic detergent to disrupt the envelope membrane and free nucleocapsids were separated into three light-scattering zones after sucrose gradient centrifugation. Nucleocapsids from the top zone contained S RNA and traces of M RNA; whereas, nucleoplasids from the middle zone contained M RNA with detectable levels of S RNA and those from the bottom zone contained L RNA with traces of M and S RNAs. Clones from a cDNA library made from the purified PBNV S and M RNAs were characterized and sequenced. Comparison of the amino-acid sequence of the nucleocapsid (N) protein (276 amino acids) encoded by the S RNA with corresponding sequences from other tospoviruses indicated that PBNV was closely related to members of serogroup IV, i.e., watermelon silver mottle virus (WSMV) and tomato isolate of PBNV (PBNV-To) (identity and similarity values were 86% and 94%, respectively). A more distant relationship was evident between PBNV and members of serogroups I, II, and III (the N protein showed 30–34% identity and 51–53% similarity). Sequence comparisons of the PBNV glycoprotein precursor (GP) protein (1121 amino acids, encoded by the M RNA), also showed distant relationship between PBNV and tomato spotted wilt virus (TSWV) (serogroup I) and impatiens necrotic spot virus (INSV) (serogroup III) (37% identity and 58–59% similarity). These findings suggest that PBNV is a distinct species in serogroup IV.

The genus *Tospovirus* consists of four highly diverged serogroups based on serological reactions of structural proteins (Adam et al. 1993; de Ávila et al. 1993a,b) and sequence homology of the N proteins (de Haan et al. 1990; Law et al. 1991; de Ávila et al. 1993a; Heinze et al. 1995; Satyanarayana et al. 1995; Yeh and Chang 1995). Serogroup I is comprised of TSWV isolates [TSWV-BR 01; TSWV-L; TSWV-BL; TSWV-10W] (de Haan et al. 1990; Maiss et al. 1991; Pang

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et al. 1994) that reacted weakly with antibodies to serogroup II [TSWV-B; tomato chlorotic spot virus (TCSV); groundnut ring spot virus (GRSV)] (de Ávila et al. 1993a; Pang et al. 1993) and did not react with antibodies to serogroup III (INSV) (de Haan et al. 1992). The serogroup IV isolates (PBNV, PBNV-To, WSMV) (Reddy et al. 1992; Heinze et al. 1995; Yeh and Chang 1995) did not react with antibodies to serogroup I, II, and III (Adam et al. 1993).

PBNV is by far the most important of all currently known virus diseases of peanuts in South Asia. It has also been reported on several crops in the Indian subcontinent (Reddy et al. 1992, 1995). PBNV was first reported from India by Reddy et al. (1968). We report here a procedure for the purification of nucleocapsids. We also compare the amino-acid sequences of both PBNV N protein and the GP with other tospoviruses, and derive relationships from these comparisons.

Materials and Methods

Virus isolate

PBNV-infected peanut (*Arachis hypogaea*) plants exhibiting typical symptoms were collected from field plots at ICRISAT. The virus was isolated and maintained by serial mechanical transmission in the peanut cultivars TMV-2 and JL-24 (Reddy et al. 1992).

Purification of nucleocapsids

All purification steps were carried out at 4°C. Systemically infected young quadrifoliates of peanut showing primary symptoms were frozen at -70°C. Leaflets were then homogenized (4 mL/g tissue) in chilled 0.1 M potassium phosphate, pH 7.6, containing 0.01 M sodium sulfite and 0.01 M EDTA, pH 8.0. Filtered extract was clarified at 2,500 rpm for 10 min and the supernatant centrifuged at 25,000 rpm in a Beckman 45 Ti rotor for 40 min. The pellets were suspended in a PSE buffer containing 10 mM each of potassium phosphate, pH 7.6, sodium sulfite and EDTA, pH 8.0, with 1.0% Nonidet P-40 added. After stirring for 30 min and clarification at 7,000 rpm for 10 min, the supernatant was centrifuged through a 30% sucrose cushion (15 mL) in PSE buffer for 1 h 15 min at 35,000 rpm in 45 Ti rotor. The pellet was suspended in PSE buffer and kept overnight at 4°C. The clarified supernatant (7,000 rpm for 10 min) was centrifuged in 20–40% sucrose gradients (3 mL of 40%, 2 mL each of 35%, 30%, 25%, and 20%) prepared in PSE buffer for 2.5 h at 33,000 rpm in a SW 40 rotor. The three light-scattering zones were collected separately, diluted in PSE buffer, and the nucleocapsids were pelleted at 35 000 rpm for 2 h in a 45 Ti rotor.

Extraction and analysis of viral RNA

Purified nucleocapsids from the top, middle, and bottom zones of the sucrose gradient were resuspended in 10 mM potassium phosphate buffer, pH 8.0. The

nucleocapsids were lysed with 1.0% SDS and 10 mM EDTA at 37°C for 15 min. The lysate was then extracted with Tris-saturated phenol, followed by phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) and chloroform:isoamyl alcohol (24:1, v:v). RNA was precipitated in 2.5 vol. of ethanol in the presence of 300 mM sodium acetate, pH 5.2, at -20°C overnight. The RNA was pelleted, suspended in DEPC water, and stored at -70°C. The RNA was analyzed on 1.0% agarose gel containing 10 mM methyl mercury (Bailey and Davidson 1976).

Synthesis and molecular cloning of cDNA

To construct cDNA libraries specific to the S and M RNAs of PBNV, RNA from the top and middle zones of the nucleocapsids, respectively, were fractionated on a 1.0% LMP agarose gel under denaturing conditions (Bailey and Davidson 1976). The S and M RNA bands were excised separately and eluted from the gel slices according to Sambrook et al. (1989). The cDNA was synthesized using random primers and AMV reverse transcriptase according to Gubler and Hoffman (1983). After second-strand synthesis, the resulting dsDNA was cloned into *Sma* I-cut, dephosphorylated pUC 119.

Nucleotide sequencing and analysis

Cloned cDNA inserts were sequenced on an automated DNA sequencer (Perkin-Elmer, Model 373A) using Taq cycle sequencing with fluorescence-based chain termination chemistry (Perkin-Elmer). The nucleotide (nt) sequences of cDNA clones were determined for both DNA strands, first by the universal and reversal primers, and then by internal primers specific to the cDNA insert. Electrophorograms were edited and cDNA contigs were assembled with the Sequencher 2.1 program (Gene Codes Co.). Nucleotide and amino-acid sequences were compiled and analyzed using programs developed by the Univ. of Wisconsin Genetics Computer Group (Devereux et al. 1984).

Results

Purification of nucleocapsids and spectral properties

The nucleocapsids were separated into three light-scattering zones (top, middle, and bottom) between 6.0–6.2, 5.5–5.8, and 4.5–5.0 cm, respectively, from the bottom of the tube after sucrose gradient centrifugation (Figure 1). The UV absorption spectrum of nucleocapsids from the three zones showed maximum and minimum absorption at 260 nm and 245 nm, respectively. The 260/280 ratio of nucleocapsids was between 1.38 and 1.40, which indicated that the nucleocapsids contained about 12% nucleic acid (Gibbs and Harrison 1976). The $A_{max/min}$ was between 1.08 and 1.12. The nucleocapsids from all the three zones contained a single polypeptide of 31 kDa, when analyzed by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

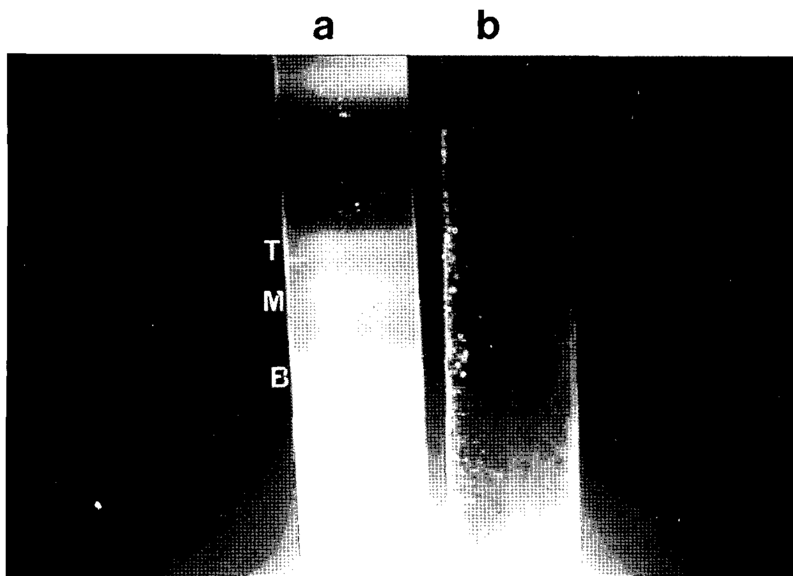

Figure 1. Sucrose gradient (20–40%) centrifugation of PBNV nucleocapsids: (A) infected; and (B) healthy. The light scattering zones top (T), middle (M), and bottom (B) are marked on the left side of the tube.

Analysis of viral RNA

RNA extracted from the three nucleocapsid zones was analyzed by electrophoresis on agarose gels containing 10 mM methyl mercury. The top zone contained S RNA and traces of M RNA, the middle zone contained M RNA and detectable levels of S RNA, and the bottom zone mainly contained L RNA and traces of M and S RNAs (Figure 2). When the virus was maintained continuously by mechanical inoculations, two additional low molecular weight RNA species (2,700 and 2,100 nt) were also observed. The size of L, M, and S RNA species was estimated to be 8,900, 4,800, and 3,050 nucleotides, respectively (Figure 2).

Homology of PBNV N protein with other tospoviruses

The S RNA of PBNV contained 3,057 nucleotides with inverted repeats and two open-reading frames (ORFs) in ambisense orientation that were separated by an A+U-rich intergenic region (Satyanarayana et al. 1995). The ORF coding for the N protein is 830 nucleotides in length, located on the virus complementary strand, and encodes a protein of 30.6 kDa. Comparison of the amino-acid sequences of the N proteins of tospoviruses representing all four serogroups were made and the values for identities and similarities are summarized in Table 1. The amino-acid sequence of PBNV N protein showed 30–34% identity and 51–53% similarity with the members of serogroups I, II, and III. The N protein sequence of PBNV showed

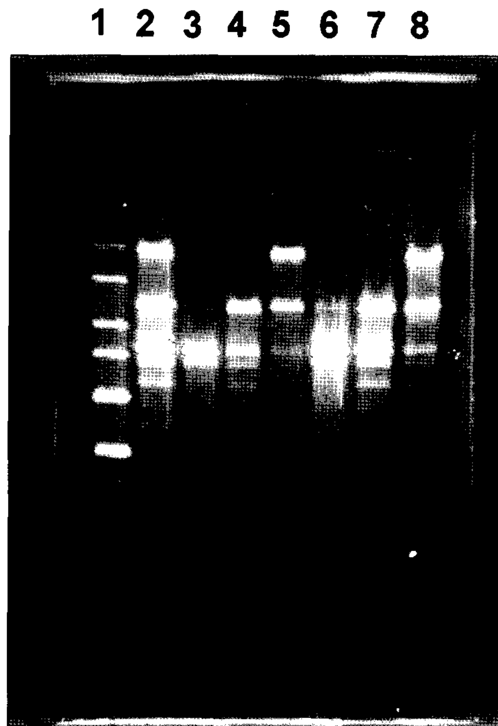

Figure 2. Size analysis of PBNV RNAs electrophoresed in 1.0% agarose gel. RNA markers: 9.5, 6.2, 3.9, 2.8, 1.9, 0.9, and 0.6 kb (lane 1); total PBNV RNA, 1.5 µg (lane 2); 0.5 µg RNA from top (lane 3), middle (lane 4), and bottom (lane 5) zones; lanes 6, 7, and 8 are double the concentrations of lanes 3, 4, and 5.

86% identity and 94% similarity with WSMV and PBNV-To of serogroup IV, which confirmed the serological relationships reported by Adam et al. (1993).

Homology of glycoprotein precursor with TSWV and INSV

The M RNA of PBNV contained 4,801 nt with inverted repeats and ambisense coding strategy (T. Satyanarayana et al., unpubl. results). The ORF coding for the GP is 3,366 nucleotides in length and is also on the virus complementary strand. This ORF codes for 1121 amino acids and showed 37% identity and 58–59% similarity with that of TSWV and INSV. Furthermore, a search of the EMBL protein database revealed that PBNV GP showed 21–23% identity and 44–47% similarity with the GP of snowshoe hare virus, La Crosse virus, Germiston virus, and Bunyamwera virus of the Bunyaviridae family.

Discussion

The enveloped tospoviruses are difficult to isolate without some contamination of host cellular components. Different purification methods (de Ávila et al. 1990; Law

Table 1. Amino-acid sequence comparison of the N protein from different tospovirus isolates of serogroups I, II, III, and IV.[a,b,c]

		I		II			III	IV		
		A	B	C	D	E	F	G	H	I
Serogroup I	A	**100**	98	79	78	77	55	35	35	33
	B	99	**100**	80	79	78	55	36	36	34
Serogroup II	C	91	91	**100**	95	84	55	33	33	34
	D	90	90	97	**100**	81	54	33	33	34
	E	89	89	91	89	**100**	55	33	33	33
Serogroup III	F	69	68	69	69	71	**100**	32	32	30
Serogroup IV	G	51	53	52	52	54	49	**100**	99	86
	H	51	53	52	52	54	49	99	**100**	86
	I	51	51	53	53	53	53	94	94	**100**

[a]A = TSWV BR 0; B = TSWV-L3; C = TSWV-B; D = GRSV; E = TCSV; F = INSV; G = PBNV-To; H = WSMV; I = PBNV.

[b]The percent identities (above diagonal) and percent similarities (below diagonal) of amino acids of N protein as deduced from the nt sequence data using the GAP program of the GCG sequence analysis software package.

[c]The sequence data were taken from the following publications: TSWV (de Haan et al. 1990); TSWV-L3 (Maiss et al. 1991); TSWV-B (Pang et al. 1993); GRSV and TCSV (de Ávila et al. 1993a); INSV (de Haan et al. 1992); WSMV (Yeh and Chang 1995); PBNV-To (Heinze et al. 1995).

and Moyer 1990; Reddy et al. 1992) were tried to purify PBNV to extract genomic RNAs for construction of cDNA libraries and to produce high-quality antiserum. None of these methods yielded purified virus preparations free of contaminating host material. A purification protocol that separated PBNV nucleocapsids in sucrose gradients based on the molecular weight of encapsidated RNAs was developed. Our method yielded relatively pure preparations of S, M, and L RNAs that were free of host RNA. Therefore, we were able to construct cDNA libraries for the S and M RNA species. The size of the three PBNV RNAs, 8,900 (L RNA), 4,800 (M RNA), and 3,050 (S RNA) nt is comparable with that of TSWV and INSV RNAs (de Haan et al. 1989; Law and Moyer 1990). The two RNA species of 2,700 and 2,100 nt length were observed only in samples prepared from plants in which the virus was passed by many serial mechanical inoculations in a greenhouse. In Northern blots, these RNA species reacted with M RNA cDNA clones, and we presume that they are defective interfering RNAs of the M RNA (data not shown).

The N protein of PBNV contained 18 amino acids more than that reported for the members in serogroups I and II (TSWV-BR 01; TSWV-B; GRSV; TCSV), 14 amino acids more than that of members in serogroup III (INSV), and one amino

acid more than that of members in serogroup IV (WSMV and PBNV-To) (de Haan et al. 1990, 1992; de Ávila et al. 1993a; Pang et al. 1993; Heinze et al. 1995; Yeh and Chang 1995). PBNV N protein showed 30–34% identity and 51–53% similarity with the members of serogroups I, II, and III of the *Tospovirus* genus. This value is in contrast to 77–80% identity and 89–91% similarity reported between serogroups I and III, and II and III. These data suggest that members included in serogroup IV are distantly related to other members in serogroups I, II, and III.

The GP of PBNV exhibited 37% identity and 58–59% similarity with that of serogroup I (TSWV) and serogroup III (INSV). In contrast, identity and similarity values between TSWV and INSV were 65% and 79%, respectively. The M RNA sequence is not available for other members of the *Tospovirus* genus for comparison.

The PBNV N protein showed 86% identity and 94% similarity with WSMV and PBNV-To, which indicates that PBNV, WSMV, and PBNV-To are closely related and should be considered as members of a distinct group, serogroup IV. Among the serogroup IV members, the N protein of WSMV and PBNV-To are 99% identical and it is concluded that WSMV and PBNV-To are strains of the same virus. The GP of PBNV showed only 37% identity with TSWV and INSV, which indicates that PBNV is a distinct tospovirus. This finding also supports the conclusion that the glycoprotein was indeed coded by virus RNA and not host derived (Peters et al. 1995). Although we presume very close relationships among GPs of tospoviruses in serogroup IV, this assumption will only be confirmed when the M RNA sequence of other members becomes available.

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