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Peanut bud necrosis tospovirus S RNA: complete nucleotide sequence, genome organization and homology to other tospoviruses

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Summary. The complete nucleotide sequence of the S RNA of peanut bud necrosis virus (PBNV) has been determined. The RNA is 3057 nucleotides in length, contains inverted repeats and two open reading frames (ORFs) with an ambisense coding strategy that are separated by an A + U-rich intergenic region. One ORF (1320 nucleotides in the viral sense strand) encodes a Mr 49.5 kDa protein, identified as the nonstructural (NSs) protein based on similarity to published tospovirus sequences. The second ORF (831 nucleotides in virus complementary strand) encodes a Mr 30.6 kDa protein. This protein was identified as the nucleocapsid (N) protein based on sequence similarities. Amino acid sequence comparison of N and NSs proteins revealed identities of 22–34% with the reported tospovirus isolates of serogroups I, II, and III, whereas it had 82–86% identity with viruses in serogroup IV, watermelon silver mottle virus (WSMV) and tomato isolate of peanut bud necrosis virus (PBNV-To). Two subgenomic RNA species detected in PBNV infected tissue corresponded to the predicted sizes (1.65 and 1.4 kb) of the NSs and N mRNAs. The data presented show conclusively that PBNV should be included in serogroup IV, along with WSMV and PBNV-To.

Introduction

Among the plant viruses, the *Tospovirus* genus of *Bunyaviridae* family is unique in that: (1) virus particles are quasi-spherical shape (80–110 nm in diameter); (2) the genome consists of three linear RNA species, small (S) RNA, medium (M) RNA and large (L) RNA, and; (3) the viruses are transmitted persistently by thrips [15, 25]. The genomic RNAs are tightly associated with the nucleocapsid

protein and form circular nucleocapsids [8, 24, 36]. The nucleotide sequence of the complete genome is known for only one tospovirus, a Brazilian isolate BR-01 of tomato spotted wilt virus (TSWV). The L RNA (8 897 nucleotides) is of negative polarity and encodes a putative RNA polymerase of 331.5 kDa [10]. The M and S RNAs encodes both structural and nonstructural proteins in the ambisense coding strategy. The M RNA (4 821 nucleotides) contains two ORFs, one in the viral sense that encodes a nonstructural (NSm) protein of 33.6 kDa and the other in the viral complementary sense that codes for a precursor to the two envelop glycoproteins G1 (78 kDa) and G2 (58 kDa) [20]. The S RNA of TSWV (2 916 nucleotides) has two ORFs, the one coding for the nucleocapsid (N) protein of 29 kDa is present in the viral complementary sense and a nonstructural protein (NSs) of 52.4 kDa is present in the viral sense [9]. Both the proteins are expressed from subgenomic mRNAs, transcribed from complementary strands via a process of cap-snatching [21].

The *Tospovirus* genus constitutes four highly diverged serogroups. The members within each serogroup were classified based on serological cross reaction and amino acid sequence identity among the members [5, 6]. Each species can be clearly differentiated by a weak or lack of serological cross-reaction between their N proteins [5, 7], in addition to the low amino acid sequence homology between their N proteins [6]. Serogroup I is comprised of TSWV type species [TSWV-BR 01, TSWV-L, TSWV-BL, TSWV-10W] [9, 23, 27] which react weakly with antibodies to serogroup II [TSWV-B (Brazil isolate), tomato chlorotic spot virus (TCSV) and groundnut ring spot virus (GRSV)] [6, 26] and do not react with antibodies raised against serogroup III [Impatiens necrotic spot virus (INSV-NL07, INSV-LI, INSV-Beg)] [11, 27] viruses. Recently serogroup IV [peanut bud necrosis virus (PBNV), watermelon silver mottle virus (WSMV) and tomato isolate of peanut bud necrosis virus (PBNV-To)] has been recognized on the basis of lack of serological cross reaction and nucleotide sequence homology of N gene with serogroups I, II, and III [1, 18, 30, 37].

PBNV was first reported from India in 1968 [32]. It is currently regarded as the most economically important virus in several crops in the Indian subcontinent [31]. In this paper we report the complete nucleotide sequence of the S RNA segment of PBNV and its coding strategy, and comparison of amino acid sequences of N and NSs proteins with those of other tospoviruses.

Materials and methods

Virus propagation, purification of nucleocapsids and extraction of viral RNA

Peanut (*Arachis hypogaea* L.) plants exhibiting typical symptoms of PBNV were collected from field plots at the ICRISAT Asia Center, India. The virus was isolated and maintained by serial mechanical transmission in the peanut cvs. TMV 2 or JL 24 as described by Reddy et al. [30]. PBNV nucleocapsids were purified from frozen (−70 °C) leaf tissue of systemically infected young quadrifoliates of peanut showing primary symptoms. Extracts were prepared in 0.1 M potassium phosphate buffer, pH 7.6, treated with 1.0% NP-40 (v/v) followed by sucrose gradient centrifugation. Nucleocapsids were lysed by heating at 65 °C for 15 min in

1% SDS. The lysate was then extracted with phenol followed by phenol:chloroform (1:1, v/v) and RNA was precipitated at −20 °C overnight after adding 2.5 volumes of ethanol in the presence of 300 mM sodium acetate, pH 5.2.

cDNA synthesis and cloning

To construct cDNA libraries specific to the PBNV S RNA, total viral RNA was fractionated on 1.0% LMP agarose gel under denaturing conditions [3]. The S RNA band was excised and eluted from the gel slice according to Sambrook et al. [33]. The cDNA synthesis was done by using random primers and AMV reverse transcriptase according to Gubler and Hoffman [17]. After second strand synthesis the resulting ds cDNA was cloned, blunt end, into Sma I cut dephosphorylated pUC 119. Prior to DNA sequencing, all cDNA clones, including those derived from RT-PCR and 5′ RACE (see below), were tested by dot blot and Northern blot hybridizations [33] to confirm the presence of S RNA fragment.

Gap filling and amplification of the S RNA termini

An internal portion of the S RNA not contained in the initial set of cDNA clones, and the 3′ terminus were cloned utilizing reverse transcriptase and the polymerase chain reaction (RT-PCR). Two oligonucleotides: 5′ GGACAAAGCTTTCAGTGCCAG, complementary to nucleotides 2233–2253 and 5′ TGGATCCCTTGAGTCCTG, corresponding to positions 1785–1802 (Fig. 1) were used to amplify the S RNA gap, essentially as described by Ausubel et al. [2]. The S RNA 3′ terminus was amplified using oligonucleotides: 5′ AGAGCAATCG, complementary to nucleotides 3527–3536 of PBNV-To S RNA [18] and 5′ TTGGTGCCAGACCTGTCAT, identical to positions 2489–2507 of PBNV S RNA, to prime cDNA synthesis and followed by PCR.

The PBNV S RNA 5′ terminus was cloned using the rapid amplification of cDNA ends (5′ RACE) system [16] from Gibco BRL (Gaithersburg, MD, USA). First strand cDNA was synthesized with a 23-mer oligonucleotide (5′ CTCCATTTGTAGCTATGTAAAAG) complementary to the 549–571 nucleotide positions of PBNV S RNA. The 3′ end of the first strand cDNA was then tailed with dCTP using terminal deoxynucleotidyl transferase. Tailed cDNA was amplified using an anchor primer that annealed to the homopolymeric tail, and another oligonucleotide (5′ CTTGATACCATCCAGAGGG) that was complementary to nucleotides 511–529 of PBNV S RNA. Purified PBNV S RNA was used for both RT-PCR and 5′ RACE. All the PCR products (RT-PCR and 5′ RACE) were eluted from agarose gels, as described earlier, and cloned into pGEM-T (Promega, Madison, WI) for sequence analysis. The PBNV S RNA primers were designed with the aid of the computer program OLIGO (Nat. Biosciences, USA).

Nucleotide sequencing and analysis

Plasmid subclones were sequenced directly by Taq cycle sequencing using fluorescence based chain termination chemistry (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) and an automated DNA sequencer (Perkin Elmer/Applied Biosystems, model 373A). The nucleotide sequences were determined from both DNA strands, first by the universal and reverse primers, and then by the internal primers specifically designed for sequencing the PBNV S RNA.

Electrophorograms were edited and cDNA contigs were assembled with the Sequencher 2.1 program (Gene Codes Corporation, Ann Arbor, MI, USA). Additional sequence analyses were done with the aid of programs developed by the University of Wisconsin Genetics Computer Group [12]. CLUSTAL V [19] was used for comparative analyses including

multiple alignments of tospovirus amino acid sequences and calculation of genetic distances. Distance was defined as (1-Identity)/100, sequence gaps were considered in the calculation, and no correction was made for multiple substitutions. Phenograms were constructed by cluster analysis employing UPGMA [34] using the program NTSY-pc (Exeter Software, Setauket, NY, USA).

In vitro transcription and detection of subgenomic viral mRNA species

Total RNA was extracted from healthy, and PBNV-infected peanut and tobacco plants according to the method of de Vries et al. [13], 6 μg RNA was fractionated on 1.0% agarose gel under denaturing conditions [3] and transferred onto nylon membranes. The blots were hybridized with the strand-specific RNA probes from N and NSs ORFs.

Strand-specific RNAs were generated using a riboprobe kit (Promega, Madison, WI, USA). Full-length N and NSs genes of PBNV were amplified by RT-PCR as described by Ausubel et al. [2]. The N gene was amplified using primers: 5′ ATGTCTAACGTCA AGCAACTC, complementary to nucleotides 2970–2990, and 5′ TTACAATTCCAG-CGAA, corresponding to nucleotides 2160–2175. The NSs gene was amplified using oligonucleotides: 5′ TTACTCTGGCTTCACAA and 5′ ATGTCAACCGCAAGGAGTG, complementary to nucleotides 1370–1386 and identical to 67–85 nucleotides, respectively. The RT-PCR amplified N and NSs genes were directly cloned into pGEM-T (Promega, WI,USA), which has the T7 and SP6 promoters flanking either side of the multiple cloning site. Plasmids were linearized with an appropriate restriction enzyme and ^{32}P-labeled transcripts were obtained in both orientations by performing synthesis using either the T7 or SP6 RNA polymerases.

Results

Cloning and DNA sequence analysis

The nucleotide sequence was initially determined from nine overlapping or independent cDNA clones representing nucleotides 17 to 1810, 1942 to 2169 and 2185 to 2720 of the PBNV S RNA. Later, two additional clones representing nucleotides 1785 to 2253 and 2489 to 3057 were amplified by RT-PCR using specific oligonucleotide primers and sequenced. Each clone hybridized specifically with the PBNV S RNA in Northern blots (data not shown). Comparison of the nucleotide sequence assembled from all cDNA and RT-PCR clones with the complete S RNA sequence of PBNV-To ([18], GenBank Accession No. Z 46419) showed that the assembled sequence of 3041 bp represented 99% of the S RNA genome, excluding 16 nucleotides at the extreme 5′ end of the S RNA (Fig. 1). The 5′ end of the S RNA was therefore, obtained using the 5′ RACE system. Five independent clones were sequenced with an oligonucleotide (5′ TTGTCTCTTGTGCCATAGC) that annealed close to the 5′ end of the PBNV S RNA (complementary to nucleotide positions 107 to 125 of PBNV S RNA). The nucleotide sequence of all five clones was identical and each contained the 5′ terminus of S RNA.

Genome organization of PBNV S RNA

The complete sequence of the PBNV S RNA is 3057 nucleotides (Fig. 1), which is similar to the predicted size based on relative electrophoretic migration (D.V.R.

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                                                  M  S  T  A  R  S  A  A  S  E  F  V  K  S  Y  Q  T  R
AGAGCAATCGGGGCATCTAATAATTCTAGAAAAACAACGATATACTCAAGAAATACTATTTCAGGCATGTCAACCGCAAGGAGTGCTGCTTCTGAATTCGTCAAAAGCTATGGCACAAGA 120

M  L  F  M  R  N  N  A  G  I  K  S  A  F  S  I  N  D  L  G  D  N  R  A  I  N  D  C  Y  S  V  F  S  G  E  G  V  N  F  L
GACAACAGAGCAATCAATGATTGCTATTCTGTCTTCTCTGGAGAAGGTGTCAACTTTCTCAATTTGTTCATCGCATAACAATGCAGGTATTAAATCTGCATTCAGCATCAACGATTTGGGC 240

R  N  E  D  I  K  I  N  E  A  E  V  I  D  S  C  N  D  T  H  Y  F  E  K  F  G  L  D  I  I  F  C  E  H  E  N  S  L  V  V
ATGAATGAGGATATCAAAATCCATGAAGCTGAAGTTATTGATTCATGCCATGATTATCATTACTTTGAGAAGTTTGGGTTAGACATAATATTCTGTGAGCATGAACTCAGTCTGGTTGTG 360

E  K  P  G  V  K  N  T  G  C  K  F  T  H  N  N  Q  I  F  N  P  N  S  D  T  L  A  L  T  P  G  T  V  S  E  N  E  F  Y  E
GGGAAACCAGGTGTGAAGAATACAGGCTGCAAATTCACAATGCACAACCAAATTTTCAATCCTAATTCTGATACACTTGCTTTAACTCCTGGAACAGTCTCAGAAAATGAGTTCTATGAA 480

K  I  K  I  R  S  N  G  L  L  P  S  G  V  Y  Q  D  E  C  C  K  N  F  Y  I  A  T  N  G  D  L  T  L  D  Y  G  F  S  V
AAAATCAAAATAAGATCAAATGGACTACTTCCCTCTGGATGGTATCAAGATGAATGCTGTAAAAACAACTTTTACATAGCTACAAATGGAGATTTGACTCTTGACTACGGGTTTCTGTT 600

M  G  K  T  T  S  Y  V  R  E  S  I  S  R  E  K  I  L  S  V  K  Q  K  C  L  P  D  N  T  V  P  T  N  R  L  L  S  T  S  T
ATGGGAAAGACAACATCCTATTGGAGAGAAAGATATCTCTAGGGAGAAGATTTTGTCTGTTAAGCAGAAGTGTTTACCTGATAACACTGTCCCTACCAACAGGCTCCTATCTACATCAACT 720

GTAAAAGGTATTCAGCTAGGATCTGAACTAGCCCCAGACACCACAGTGATACTATCACTTAAGCAAAATCTGAACGTTGACTTAAAATCTCAATACCGCGTTTCGTTTCATGGCATCCAA 840

E  E  G  A  F  A  R  T  F  C  V  P  F  E  N  K  S  R  N  I  C  L  Y  A  K  T  V  V  D  N  S  N  E  R  T  T  L  I  I  K
GAGGAAGGTGCTTTTCGCAAGGACTTTCTGTGTTCCTTTTGAAAACAAATCCAGGATGATATGCCTATACGCCAAAAACTGTTGTGGATAACAGTAATGAAAGGACTACTCTGATTATAAAG 960

V  V  T  K  T  I  E  S  N  L  V  I  P  I  R  N  H  I  N  C  V  K  K  I  Q  A  R  I  G  L  V  D  S  I  E  S  D  P  N  Y
GTAGTTACAAAAACTATTGAATCCCATCTAGTCATTCCTATCAGGAATCACATAAATTGTGTAAAGAAAATTGGAGCCAGGATAGGGCTTGTTGATTCTATTGAAAGTGATCCTAACTAT 1080

M  -  H  I  Y  K  E  L  L  Q  V  N  T  Q  F  A  I  N  L  S  L  V  I  K  K  P  I  V  F  K  M  Y  D  I  E  L  N  N  N
AATCAGATGATTGTTAAAGAGTTATTGGGTGTTCATACTCAATTTGCTATCAATTTATCCTTGGTCATAAAGAAGCCGATCATTGTGTTCAAAATGTATGATATTGAGTTGAACAACAAT 1200

M  I  D  V  T  G  R  I  L  N  Y  Q  T  D  A  E  G  N  A  Y  F  L  S  K  T  L  E  V  L  P  K  S  L  S  T  L  S  Y  L  G
CATATAGACGTTTATGGAAGGATCCTGAATTACCAGACAGATGCAGAGGGAAATGCCTATTTCCTTTCAAAAACCCTTGAAGTTCTTCCCAAATCACTTTCCACTCTGAGTTACTTAGGA 1320

S  I  A  P  I  Q  W  E  E  S  L  E  H  Q  N  F  I  V  K  P  E  *
AGCATTGCACCAATTCAATGGGAGGAATCTTTGGAGCACCAGCATTCATTGTGAAGCACGAGTAATTCAGGTTTATGTCCTTGTTCTACTTAAGTTTATTGTGTGCATGTTATTTAATA 1440

AGCAACTAATAAGTGTTTGCTTGTTTAAAGTATATAAGTATTTTAAGTATTACAGTATAATGTTATCAAATATAAGTTTATATAAATAAAATAAGCTAATACTAATAAGTGTAAGTTTAA 1560

TAATCGAATATAAATTAAATAAATAAAACCCATAAAAATCAAAAAACGGAAAACCAAAAAGACCTCGAAAGAGGCAATCTTGGCTTAACTGCTTTAATTAAACTCATTTATCAAAAGTT 1680

TTTTACACGGAATTCATGTTTCATGGAATTTGAGATCTTTCGAGTATTTTAATCTCATTTATCAACTTACATGATTTAATCAACATGAGGAACTATACTGAATTTGGATCCCCTGAGTCC 1800

TGTAAATTAATCCGAGCTAGATAATCAATTCCAGAAATAAAGCAAATCTATGTTCAAGACCGGCCAATCGGTTGCCTCCGAAGAGGTCTTTTTTGGTTTTTGATTTTTTGATTTTTTTG 1920

GGTTTTTTGTTATTTATTTATTTTACTTATTAATTATTATTTGGTTTATTTATTATCTTACTCTTATATTTATTTTAAACACAAACACAATAACACAATAAGTAAAGTAACATTTAAATT 2040

TAATACACAATGTAAACACATAAAGAACTTATAAACACACAAAAGAGCTTATATTACTTAACAACCACACATAAACTTAGATACATATTAACATGCATAAACACCTTATATGAAACAATT 2160

TACAATTCCAGCGAAGGACCAGCCTTTGAACTCTTCTTCTTAGAATGTTCACCATAATCATCCATTGAGAGACTGGCACTGAAAGCTTTGTCCATGTATTTGACTTGTTCATCATATTTC 2280
*  L  E  L  S  P  G  A  K  S  S  K  K  K  S  H  E  G  Y  D  D  N  S  L  S  A  S  F  A  K  D  M  Y  K  V  Q  E  D  Y  K

TTGCGTGAGATAGAGCTGGCTGTTCCAGGGTTGCTCTCAATCAATAGCTTTACAGCTGATTTAAACATTTCCTCTAGTTCATTTTTGAATTCAATCTGTGAAGCAGAAAGTACTTTGGCT 2400
K  L  S  I  S  S  A  T  G  P  N  S  E  S  L  L  K  V  A  S  K  F  M  E  E  L  E  N  K  F  E  I  Q  S  A  S  L  V  K  A

ACTTTGCAAACCTGTTCATAAGTAGAAAAGTTCTTAATTCCTCAATTTCTCTTTCTTCACATTTTGATAATAAGCCAGAGGGAATATAATTGGTGCCAGACCTGTCATGCTAGACAAGAGA 2520

GGTAGAGGACCTCGAATGCAGAGCATCATCCTTAAAGCACATGTATCGAAAGATGCAGGGACATTTAATCCATAAGCTGCCACTAATGGTAATTCCATTATTTTATTGTACATCTCCTGC 2640
P  L  P  G  G  I  C  L  M  N  R  L  A  C  T  D  F  S  A  P  V  N  L  G  Y  A  A  V  L  P  L  E  M  I  K  N  Y  M  E  Q

TTAGCAGCATCATTCTTGCTCTTCTCAACCATACTAGCCATTTTGGTCCTGATGAAAGCTTCTGTCCTTTTGAAGGTCCAGTCATCTGGCCCTACATCAGTATTTGTAGCAACAATAGTT 2760
K  A  A  D  N  K  S  K  E  V  M  S  A  N  K  T  R  I  F  A  E  T  R  K  F  T  W  D  D  P  G  V  D  T  N  T  A  V  I  T

TTTTCACAAAAACATACTTACCACTTTACAAGCAGCAAAGATCTGCTTCCTGCACTTCAAAATATTCAAACAGTTTGATAAAAGTTATTTCAAGATTTTTGTGGTGTCATAGAAAGCT 2880
K  -  C  F  V  Y  K  G  S  K  C  A  A  F  I  Q  K  R  C  K  L  I  M  L  C  N  T  F  T  I  E  L  N  K  N  T  D  Y  F  A

TTAAAGCTAAATCCGGGAGTGGAATCTTCTGTTTCAATTTCAACATCTGCAGAGCCACCAGCCAAAAGTTCTTTGATTTTCTTCTCGGTGAGTTGCTTGACGTTAGACATGGTGTTTACT 3000
K  F  S  F  G  P  T  S  D  E  T  E  I  E  V  D  A  S  G  G  A  L  L  E  K  I  K  K  E  T  L  Q  K  V  N  S  M

CTGGAAACGTCTAGTATATTGACTTTGAATGAATTTTATTAGCGCCTCGATTGCTCT 3057
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Fig. 1. The complete nucleotide sequence of PBNV S RNA. The PBNV S RNA sequence (3057 nucleotides) is presented as DNA sequence in the 5′ to 3′ viral sense polarity and is numbered on the right. The deduced amino acid sequence of the protein encoded by the viral sense RNA is shown above the RNA sequence. The sequence of the protein encoded by the viral complementary sense RNA strand is shown below the RNA sequence. Potential N-glycosylation sites are underlined. Asterisks indicate the stop codons

Reddy and S. Gowda, unpubl.). The sequence is composed of 32.9% A, 32.4% U, 17.8% C and 16.9% G. The 3′ and 5′ terminal sequences are complementary over a stretch of 48–51 nucleotides with exact matching of up to 10 nucleotides.

The sequence analysis of PBNV S RNA revealed two nonoverlapping ORFs with an ambisense arrangement similar to other S RNAs of tospoviruses (Fig. 1). The large ORF (1 320 nucleotides) is located on the viral strand, initiating with an AUG codon at nucleotide 67 and terminating at an UAA stop codon at nucleotide 1 386 (numbered from the 5′ end of the viral strand) (Fig. 1). This ORF encoded a protein of 439 amino acids with a predicted molar mass of 49.5 kDa. Since a protein of 49.5 kDa is not found in SDS-PAGE analysis of purified virus particles [30], it is considered to be a non-structural protein and referred to as NSs. The deduced amino acid sequence of this ORF contains two potential N-glycosylation sites (Fig. 1). The second ORF, on the viral complementary RNA strand, starts with an AUG codon at position 2 990 and terminates at an UAA stop codon at position 2 160 (numbered from the 5′ end of the viral strand) (Fig. 1). This ORF encodes a protein of 276 amino acids with a predicted molar mass of 30.6 kDa, and when expressed in *E. coli*, it reacted with an antiserum raised against PBNV nucleocapsid (data not shown). Based on similarity to other tospovirus sequences, it is identified as the N protein. The deduced amino acid sequence of N protein contains one potential N-glycosylation site at amino acid residue 187 (Fig. 1). The N gene of PBNV is 828 nucleotides long. It is 3 to 54 nucleotides longer (depending on the isolate) than the homologous genes of other tospoviruses [6, 9, 11, 18, 23, 26, 37].

The two ORFs are separated by a noncoding, A + U-rich intergenic region of 773 nucleotides. Size differences between the S RNA molecules of different tospovirus isolates have been attributed to variation in the lengths of these intergenic regions. The PBNV S RNA intergenic region is 145, 272, 190 and 132 nucleotides longer than that of TSWV-B, TSWV-BR 01, TSWV-L3 and INSV, respectively [9, 11, 23, 26] and 489 nucleotides shorter than PBNV-To [18].

Comparison of the PBNV N and NSs proteins to those of other tospoviruses

The amino acid sequence of the N and NSs proteins of PBNV are aligned with presumed homologous sequences from other tospoviruses (Fig. 2). The multiple alignments of the NSs protein sequences from 6 tospoviruses representing all four serogroups revealed only a small domain of 7 conserved amino acid residues at positions 423–430 (Fig. 2a). Alignments of N protein sequence from 13 different tospoviruses revealed three small conserved domains with the most notable conservation occurring between residues 94 and 167. Interestingly, the methionine residues conserved in other tospovirus N proteins [6] are not present in the PBNV N protein (Fig. 2b).

The identity and similarity values based on the amino acid sequences of the tospovirus N and NSs proteins and the associated phenograms (Fig. 3) are completely consistent with the known serological groupings. The amino acid sequence of PBNV N protein showed 30–34% identity and 51–53% similarity

a

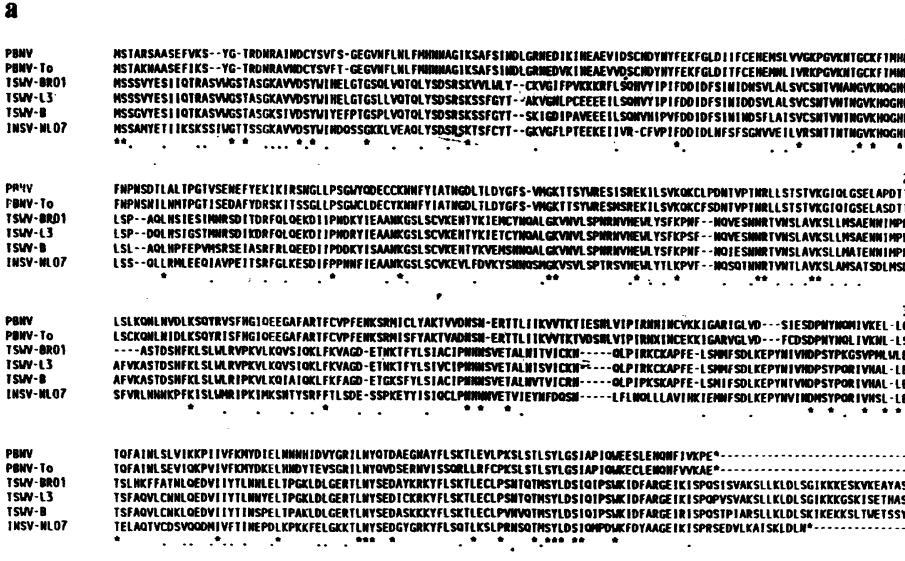

Fig. 2 (continued)

Complete nucleotide sequence of PBNV S RNA

b

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                                                                                                                          120
WSMV       MSNVKQLTEKKIKCLLAGGIADVEIETEDSTPGFSFKAFFYNNKNI--EITFTNCLNILKCRKQIFAACKSGKYNFCG---KNIVATSVDVGPEDWTFKRTEAFIRTKIVSMVEKSKNEA
PBNV-To    MSNVKQLTEKKIKELLAGGIADVEIETEDSTPGFSFKAFFDNNKNI--EITFTNCLNILKCRKQIFAACKSGKYNFCG---KNIVATSVDVGPEDWTFKRTEAFIRTKIVSMVEKSKNEA
PBNV       MSNVKQLTEKKIKELLAGGSADVEIETEDSTPGFSFKAFYDTNKNL--EITFTNCLNILKCRKQIFAACKSGKYVFCG---KTIVATNTDVGPDDWTFKRTEAFIRTKMASMVEKSKNDA
INSV-L1    MDKAK-ITKENIVKLLTQS-DSLEFEETQNEGSFNFTDFFTNNREKIQNMTTASCLSFLKNRQSIMRVIKSADFTFGSVTIKKTRNNSERVGVNDMTFRRLDAMVRVHLVGM--IKDNGS
INSV-NL07  MNKAK-ITKENIVKLLTQS-DSLEFEETQNEGSFNFTDFFTNNREKIQNMTTASCLSFLKNRQSIMRVIKSADFTFGSVTIKKTRNNSERVGVNDMTFRRLDAMVRVHLVGM--IKDNGS
INSV-Beg   MDKAK-ITKENIVKLLTQS-DSLEFEETQNEGSFNFTDFFTNNREKIQNMTTASCLSFLKNRQSIMRVIKSADFTFGSVTIKKL-EHSERVGVNDMTFRRLDAMVRVHLVGI--DKDNGS
TSWV-BR01  MSKVK-LTKESIVALLTQG-KDLEFEEDQNLVAFNFKTFCLENIDQIKKMSVISCLTFLKNRQSIMKVIKQSDFTFGKITIKK---TSDRIGGTDMTFRRLDSLIRVRLVE---ETGNSE
TSWV-L3    MSKVK-LTKESIVALLTQG-KDLEFEEDQNLVAFNFKTFCLENLDQIKKMSVISCLTFLKNRQSIMKVIKQSDFTFGKITIKK---TSDRIGATDMTFRRLDSLIRVRLVE---ETGNSE
TSWV-BL    M--VK-LTKESIVALLTQG-KDLEFEEDQNLVAFNFKTFCLENLDQIKKMSIISCLTFLKNRQSIMKVIKQSDFTFGKITIKK---TSDRIGATDMTFRRLDSLIRVRLVE---EIGNSE
TSWV-10W   M--VK-LTKESIVALLTQG-KDLEFEEDQNLVAFNFKTFCLENLDQIKKMSIISCLTFLKNHQSIMKVIKQSDFTFGKITIKK---TSDRIGATDMTFRRIDSLIRVRLVE---ETGNSE
TSWV-B     MSKVK-LTKENIVSLLTQS-ADVEFEEDQNQVAFNFKTFCQENLDLIKKMSITSCLTFLKNRQGIMKVVNQSDFTFGKVTIKK---NSERVGAKDMTFRRLDSMIRVKLIE---ETANNE
GRSV       MSKVK-LTKENIVSLLTQS-EDVEFEEDQNQVAFNFKTFCQENLDLIKKMSITSCLTFLKNRQSIMKVVKQSDFTFGKVTIKK---NSERVEAKDMTFRRLDSMIRVKLIE---ETANNE
TCSV       MSKVK-LTRENIISLLTQA-GEIEFEEDQIKATFNFEDFCGENLDSIKKMSITSCLTFLKNRQSIMKVVNLCDFTFGKITIKK---NSGRVGANDMTFRRLDSMIRVKLIE---ETGKAE

                                                                                                                          240
WSMV       ARQEMYGKIMELPLVAAYGLNVPASYDSCALRMMLCIGGPLPLLSSIRGLATIIFPLAYYQNIKKEKLGI--KNFSTYEQVCKVAKVLSASQVEFKDDLDVMFKQAVKILSFSHTGTASS
PBNV-To    ARQEMYGKIMELPLVAAYGINVPASYDSCALRMMLCIGGPLPLLSSIRGLAPIIFPLAYYQNIKKEKLGI--KNFSTYEQVCKVAKVLSASQVEFKDDLDVMFKQAVKILSESHPGTASS
PBNV       AKQEMYNKIMELPLVAAYGLNVPASFDTCALRMMLCIGGPLPLLSSMTGLAPIIFPLAYYQNVKKEKLGI--KNFSTYEQVCKVAKVLSASQIEFKNELEEMFKSAVKLLSESNPGTASS
INSV-L1    ALTEAINSLPSHPLIASYGLATT---DLKSCVLGVLLGGSLPLIASVLNFEIAALVLAIYQDAKHVELGIDMSKFSTKEAVGKVCTVLKSKGYSMNSVEIGKAKQYADILKACSPKAKGI
INSV-NL07  ALTEAINSLPSHPLIASYGLATT---DLKSCVLGVLLGGSLPLIASVLNFEIAALVPAIYQDAKHVELGIDMSKFSTKEAVGKVCTVLKSKGYSMNSVEIGKAKQYADILKACSPKAKGL
INSV-Beg   ALIEAINSLPSHPLIASYGIATT---DLKSCVLGVLLGGSLPLIASVLNFEIAALVLAIYQDAKHVELGIDMSKFSTKEAVGKVCTVLKSKGYSMNSVEIGKAKQYADILKACSPKAKGL
TSWV-BR01  NLNTIKSKIASHPLIQAYGLPLD---DAKSVRLAIMLGGSLPLIASVDSFEMISVVLAIYQDAKYKDLGIDPKKYDTKEALGKVCTVLKSKAFEMNEDQVKKGKEYAAILSSSNPNAKGS
TSWV-L3    NLNTIKSKIASHPLIQAYGLPLD---DAKSVRLAIMLGGSLPLIASVDSFEMISVVLAIYQDAKYKDLGIDPKKYDTKEALGKVCTVLKSKAFEMNEDQVKKGKEYAAILSSSNPNAKGS
TSWV-BL    NLNTIKSKIASHPLSQAYGLPLD---DAKSVRLAIMLGGSLPLIASVDSFEMISVVLAIYQDAKYKDLGIDPKKYDTREALGKVCTVLKRKAFEMNEDQVKKGKEYAAILSSSNPNAKGS
TSWV-10W   NLNTIKSKIASHPLIQAYGLPLD---DAKSVRLAIMLGGSLPLIASVDSFEMISVVLAIYQDAKYKDLGIDPKKYDTREALGKVCTVLQRKAFEMNEDQVKKGKEYAAILSSSNPNAKGS
TSWV-B     NLAIIKAKIASHPLVQAYGLPLA---DAKSVRLAIMLGGSIPLIASVDSFEMISVVLAIYQDAKYKELGIEPTKYNTKEALGKVCTVLKSKGFTMDDAQINKGKEYAKILSSCNPNAKGS
GRSV       NLAIIKAKIASHPLVQAYGLPLD---DAKSVRLAIMLGGSIPLIASVDSLEMISVVLAIYQDSQVQELGIEPTKYNTKEALGKVCTVLKSKGFTMDDAQDNKGKEYAKILSSCNPNAKGS
TCSV       NLAIIKSKIASHPLVQAYGLPLT---DAKSVRLAIMLGGSIPLIASVDSFEMISIILAIYQDAKYKDLGIEPSKYNTKEALGKVCTVLKSKGFTMDEEQVQKGKEYATILSSCNPNAKGS

                                                      284
WSMV       ISLKKYEDQVKYMDRVFSANLSVDDYGDHS-KKSSKPSTSLEV*
PBNV-To    ISLKKYEDQVKYMDRVFSANLSVDDYGDHS-KKSSKPSTSLEV*
PBNV       ISLKKYDEQVKYMDKAFSASLSMDDYGEHSKKKSSKAGPSLEL*
INSV-L1    AAMDHYKEGLTSIYSMFNATIDFGKNDSI*--------------
INSV-NL07  AAMDHYKEGLTSIYSMFNATIDFGKNDSI*--------------
INSV-Beg   AAMDHYKEGLTSIYSMFNATIDFGKNDSI*--------------
TSWV-BR01  VAMEHYSETLNKFYEMFGVKKQ-AKLAELA*-------------
TSWV-L3    IAMEHYSETLNKFYEMFGVKKQ-AKLTELA*-------------
TSWV-BL    IAMEHYSETINKFYEMFGVKKQ-AKLTELA*-------------
TSWV-10W   TAMEHYSETLNKFYEMFGVKKQ-AKLIELA*-------------
TSWV-B     IAMDYYSDNLDKFYEMFGVKKE-AKIAGVA*-------------
GRSV       IAMDYYSDNLEKFYEMFGVKKE-AKIAGVA*-------------
TCSV       IAMEHYSEHLDKFYAMFGVRKE-AKISGVA*-------------
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Fig. 2. Multiple alignment of the NSs protein sequence of six tospoviruses (**a**) and N protein sequence of thirteen tospovirouses isolates (**b**) of four serogroups. The alignment was done with the CLUSTAL V program. Dashes represent gaps introduced to reach optimal alignment. Perfectly conserved residues are indicated by an asterisk and highly conserved residues by a dot. The sequence data were taken from the following publications: WSMV [37]; PBNV-To [18]; INSV-L1 [27]; INSV-NL07 [11]; INSV-Beg [27]; TSWV-BRO1 [9]; TSWV-L3 [23]; TSWV-BL [27]; TSWV-10W [27]; TSWV-B [26]; GRSV and TCSV [6]

with the isolates of serogroups I, II and III. The N protein sequence of PBNV showed 86% identity and 94% similarity with WSMV and PBNV-To, and confirmed the serological relationships proposed by Adam et al. [1]. The PBNV NSs protein sequence has 22–28% identity and 45–54% similarity with serogroups I, II and III members, whereas it showed 83% identity and 91% similarity with PBNV-To.

Analysis of subgenomic RNA species of S RNA in PBNV infected tissue

In order to confirm the ambisense coding strategy, total RNA from healthy and PBNV-infected peanut and tobacco was hybridized to strand-specific RNA probes derived from both the N and NSs ORFs. Subgenomic RNA species of approximately 1.4 kb and 1.65 kb were detected only in PBNV-infected tissues by probes transcribed from the viral sense strand of the N and viral complementary sense strand of NSs genes, respectively (Fig. 4). They were not detected in RNA from healthy tissue and from purified virus preparations.

Discussion

Overlapping clones which covered the complete S RNA of PBNV were generated by synthesizing cDNA, and by utilizing RT-PCR and 5′ RACE techniques. The S RNA is 3 057 nucleotides in length, as in the case of other tospoviruses. The ambisense coding strategy is similar to that of other tospoviruses (NSs gene coded by the viral strand and the N gene coded by the viral complementary strand). The S RNA contained 3′ and 5′ nontranslated regions and inverted repeats of 10 highly conserved nucleotides, the first eight of which are identical to TSWV and INSV isolates. The two ORFs are separated by a 773 nucleotide A + U-rich intergenic region, which is also present in the S and M RNAs of INSV and TSWV [9, 11, 20, 22]. The predicted structure and stability of the PBNV S RNA intergenic region between 1590 and 1924 nucleotides (free energy −96.6 kcal/mol) is comparable to the intergenic regions of TSWV S RNA and INSV M RNAs which have a calculated free energy of −108.2 and −81.2 kcal/mole, respectively [9, 22].

The 5′ and 3′ termini are partially complementary (48–51 nucleotides) which could result in the formation of a stable panhandle. These structures are believed to be important in the formation of circular RNA molecules in other Bunyaviridae members and thought to be important for encapsidation of full length viral sense and viral complementary sense RNA by the N protein [15].

Northern blot analysis using strand-specific riboprobes demonstrated that the N and NSs ORFs on the S RNA are expressed by two subgenomic mRNAs transcribed from opposite strands confirming the ambisense coding strategy as in the case of S RNA segment of TSWV [9], and the M RNAs of TSWV and INSV [20, 22]. The estimated sizes of the subgenomic RNA species of PBNV S RNA (1.4 and 1.65 kb) are similar to those of TSWV [9].

The NSs protein of PBNV is 25, 28 and 10 amino acids shorter than the NSs proteins of TSWV (serogroup I), TSWV-B (serogroup II) and INSV (serogroup

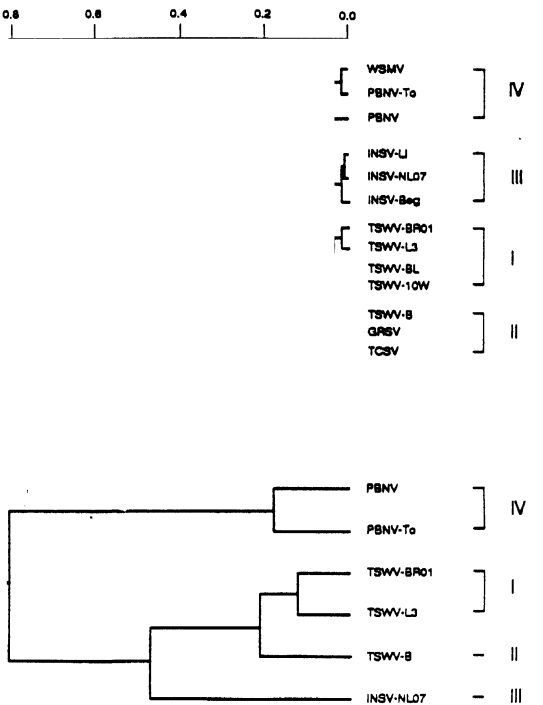

Fig. 3. Phenograms showing the genetic relationships among various tospovirus isolates based on the amino acid sequences of the N (**a**) and NSs (**b**) proteins. The scale at the top of the figure indicates genetic distance. Roman numerals on the right denote the various serogroups

III), respectively [9, 11, 26]. The PBNV NSs protein had only 22–28% identity and 45–54% similarity with tospoviruses belonging to serogroups I, II and III, and 83% identity and 91% similarity with PBNV-To (serogroup IV).

Data on serological differences in the structural proteins and sequence homologies of N protein have been used for the classification of tospoviruses into serogroups [1, 5, 6]. The PBNV N protein possesses 18 amino acids more than that reported for the members in serogroups I and II (TSWV-BR 01, TSWV-B,

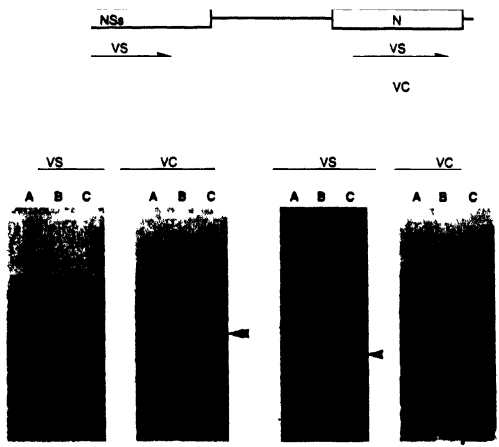

Fig. 4. Detection of S RNA-specific subgenomic RNAs Purified PBNV viral RNA (20 ng) (*A*), and total RNA from PBNV infected *N tabacum* (*B*) and peanut (*C*), were prepared and analyzed according to procedures given in Materials and methods Duplicate Northern blots were probed with strand-specific ^{32}P labeled RNA transcripts, viral sense (*VS*) or viral complementary sense (*VC*), prepared from NSs and N open reading frames The relative position and orientation of the ^{32}P labeled RNA transcripts are denoted by arrows The position of the subgenomic RNAs are marked with arrowheads

GRSV and TCSV), 14 amino acids more than that of members in serogroup III (INSV) and 1 amino acid more than that of members in serogroup IV (WSMV and PBNV-To) [6, 9, 11, 18, 26, 37] The N protein of PBNV exhibited 30–34% identity with the members of serogroups I, II and III of tospovirus genus This value is in contrast to 77–80% identity reported between serogroups I and II and 55% identity observed between serogroups I and III, and serogroups II and III The alignment data and cluster analysis also indicate that serogroup I, which includes various TSWV isolates, is fairly closely related to serogroup II (TSWV-B, GRSV, TCSV), while serogroup III (the INSV isolates) is distantly related (Fig 3)

The N protein of PBNV showed 86% sequence identity to WSMV and PBNV-To, indicating that PBNV, WSMV and PBNV-To are closely related and should be considered as members of a distinct group, serogroup IV Within serogroup IV, the N protein of WSMV and PBNV-To are 99 0% identical, with the exception of three amino acids exchange at 41, 172 and 235 residues (Fig 2b) Comparison of available nucleic acid sequences from the intergenic regions (276

nucleotides) of WSMV [37] with PBNV-To [18] indicate that they are 99.3% identical. Based on the identities of N protein and available intergenic region, it is concluded that WSMV and PBNV-To are strains of the same virus. Alignment of the nucleotide sequences of the NSs gene, intergenic region and N gene from PBNV and PBNV-To with GAP program resulted in 82, 52 and 82% identity, respectively. Indeed, the intergenic region of PBNV (773 nucleotides) is 489 nucleotides shorter than that of PBNV-To (1262 nucleotides). The observed 82% identity in N and NSs ORFs, and 83–86% identity in N and NSs proteins of PBNV and PBNV-To, together with the length variation of the intergenic region, not only supports the separation of these two viruses into distinct species, but may also provide a framework by which other serologically related serogroup IV isolates may be classified. The status of WSMV will only be apparent when the complete S RNA sequence becomes available.

PBNV infects many different crop plants and is currently regarded as one of the most economically important viruses in South Asia [29, 31]. Although resistance to PBNV or its thrips vector, *Thrips palmi* has been observed [4, 14], under high disease pressure nearly 30% of the resistant plants can become infected. It has already been demonstrated with other tospoviruses that expression of the N gene can be used to induce resistance in previously susceptible cultivars [27, 28, 35]. The determination of the complete nucleotide sequence of the PBNV S RNA should, therefore, provide a stepping off point for the development of transgenic peanut plants resistant to PBNV.

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