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# Comparison of *Groundnut bud necrosis virus* isolates based on movement protein (NSm) gene sequences

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## Summary

The nucleotide and amino acid sequences of the movement protein (NSm) genes of five isolates of *Groundnut bud necrosis virus* (GBNV) originating from different hosts and parts of India such as cowpea and tomato from Kerala, groundnut from Tamil Nadu, and potato from Madhya Pradesh and Rajasthan were determined and compared to the known NSm sequences. Sequence analysis revealed that the NSm genes of GBNV isolates were identical in length (924 bp encoding 307 amino acids). GBNV isolates shared maximum identity (98-100%) at amino acid levels with GBNV-Type isolate, while 82-83% and 34-65% amino acid sequence identities were observed with *Watermelon silver mottle virus* and other Tospoviruses respectively. The NSm genes among GBNV isolates originating from different hosts and locations appeared highly conserved (93-100%), suggesting their common origin.

**Key words:** *Groundnut bud necrosis virus*, NSm gene, tospovirus

## Introduction

Tospoviruses contain three RNA segments, small (S), medium (M) and large (L), in quasispherical (80-110 nm in diameter) enveloped particles and are exclusively vectored by several thrips species in a circulative and propagative manner (Mumford *et al*., 1996; Moyer, 1999). They have emerged as serious pathogens affecting a wide range of crop plants in the Indian sub-continent (Varma *et al*., 2002). Three distinct Tospoviruses, Groundnut bud necrosis (GBNV) and Groundnut yellow spot (GYSV) from groundnut and Watermelon bud necrosis (WBNV) from watermelon have been recognised from parts of India on the basis of nucleocapsid protein (NP) gene properties (Reddy *et al.*, 1992; Jain *et al.*, 1998; Satyanarayana *et al.*, 1998). Recently, analysis of the NP gene sequence (located on the S RNA) was used to determine the extent of GBNV infection in various leguminous (cowpea, mungbean and soybean) and solanaceous (potato and tomato) hosts (Bhat *et al*., 2002; Jain *et al*., 2002; Thien *et al*., 2003; Umamaheswaran *et al*., 2003). In order to further characterise the viral genome and confirm earlier identifications based on the NP gene, the movement protein (NSm) genes (located on M RNA) from five GBNV isolates originating from cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea*), potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) were cloned, sequenced and compared for nucleotide and amino acid sequence identity in this study. This study extends the data presented in Akram *et al*. (2003).

## Materials and Methods

### *Sources and maintenance of virus isolates*

Naturally affected samples showing chlorotic and brown necrotic spots on leaves in cowpea and tomato (Kerala), severe necrosis on stem and leaves in potato (Madhya Pradesh and Rajasthan) and chlorotic and necrotic ring spots on leaves and necrosis on stem and buds in groundnut (Tamil Nadu) were collected (Table 1). Association of a tospovirus with the samples was first established by direct antigen-coated enzyme-linked immunosorbant assay (Clark & Joseph, 1984) using polyclonal antiserum directed against the NP of *Watermelon silver mottle virus* (WSMoV) (Yeh *et al.,* 1996). The virus was subsequently identified as *Groundnut bud necrosis virus* (GBNV) on the basis of NP gene sequences (Jain *et al*., 2002; Umamaheswaran *et al*., 2003). The virus isolates were then sap inoculated to cowpea (cv. Pusa Komal, a diagnostic assay host) plants using chilled 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol.

### *Nucleic acid extractions and reverse transcription -polymerase chain reaction*

Total RNA from freshly desiccated infected tissues

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Table 1. *Sources of virus isolates used in this study and their reaction against polyclonal antiserum directed against nucleocapsid protein of* Watermelon silver mottle virus *in direct antigen-coated enzyme-linked immunosorbant assay*

| Isolates | Host | Origin | Absorbance at 405 nm[a] |
|---|---|---|---|
| GBNV-CP | Cowpea | Kerala | 0.542 (0.01) |
| GBNV-POMP | Potato | Madhya Pradesh | 0.587 (0.12) |
| GBNV-PORJ | Potato | Rajasthan | 0.534 (0.12) |
| GBNV-TO | Tomato | Kerala | 0.423 (0.10) |
| GBNV-GNTN | Groundnut | Tamil Nadu | 0.425 (0.13) |

[a] Average absorbance of three replicates 1 h after substrate addition. Values in the parentheses are absorbance of healthy plant extracts

of cowpea, tomato, potato and groundnut (100 mg) was extracted using the RNeasy kit according to the manufacturer's instructions (Qiagen Inc., Chatsworth, CA, USA). RT-PCR was based on the method of Pappu *et al*. (1993). The primer pair used for amplification of NSm genes was derived from the previously reported NSm gene sequence of GBNV (type isolate; U42555) (Satyanarayana *et al.,* 1996). The upstream primer 5' ATGTCTCGCTTDT CTAAHGTB 3' and downstream primer 5' TTATATTTCAAGAAGATTATC 3' represented the first and the last 21 bases of the coding region of the NSm gene, respectively. Prior to amplification, the template was incubated at 72°C for 5 min and snap-cooled on wet ice for 2 min. Reverse transcription-PCR was performed in an automated thermal cycler (Biometra) using the following parameters: one cycle at 42°C for 45 min for cDNA synthesis, then 40 cycles at 94°C for 30 s, 48°C for 1 min, and 72°C for 1 min followed by one cycle at 72°C for 60 min. Products were resolved following electrophoresis through a 1% agarose gel containing ethidium bromide.

*Cloning, sequencing and sequence analyses*

The product amplified from each sample was purified after electrophoresis using Qiax II gel purification kit (Qiagen Inc., Chatsworth, CA, USA). Purified DNA fragments were ligated into a pGEM-T Easy vector (Promega, Madison, WI, USA) and competent *Escherichia coli* cells (DH 5α) were transformed by following standard molecular biology procedures (Sambrook & Russell, 2001). Two clones of each isolate were sequenced in both directions (by the Department of Biochemistry, University of Delhi, India). Sequences were compared with published NSm gene sequences of GBNV and other known Tospoviruses (Table 2) using BIOEDIT Version 5.0.9. Sequence phylograms were constructed using TREECON Version 1.3b (bootstrap analysis with 500 replicates) and unrooted trees were generated.

## Results

GBNV isolates originating from cowpea (GBNV-CP), groundnut (GBNV-GNTN), potato (GBNV-POMP, GBNV-PORJ), and tomato (GBNV-TO) reacted with polyclonal antiserum directed against the NP of WSMoV (Table 1) and were easily sap-transmitted to cowpea. Similar symptoms (chlorotic/necrotic spots or lesions, followed by veinal and systemic necrosis) were induced by all isolates.

Table 2. *Sources of movement protein (NSm) gene sequences of* Groundnut bud necrosis virus *isolates and other* tospoviruses

| Virus Isolates[a] | GenBank Accession no. | No. of amino acid | Reference |
|---|---|---|---|
| GBNV-CP | AY221023 | 307 | This study |
| GBNV-POMP | AY221024 | 307 | This study |
| GBNV-PORJ | AY259522 | 307 | This study |
| GBNV-TO | AY259523 | 307 | This study |
| GBNV-GNTN | AY259524 | 307 | This study |
| GBNV-GNAP[b] | U42555 | 307 | Satyanarayana *et al*., 1996 |
| WSMV | U75379 | 312 | Chu & Yeh, 1998 |
| IYSV | AF213677 | 311 | Silva *et al.,* 2001 |
| INSV | M74904 | 303 | Silva *et al.,* 2001 |
| ZLCV | AF213676 | 303 | Silva *et al.,* 2001 |
| TSWV | S48091 | 302 | Silva *et al.,* 2001 |
| CSNV | AF213675 | 303 | Silva *et al.,* 2001 |
| TCSV | AF213674 | 303 | Silva *et al.,* 2001 |
| GRSV | AF213673 | 303 | Silva *et al.,*2001 |

[a] CP = cowpea, POMP = potato Madhya Pradesh, PORJ = potato Rajasthan, TO = tomato, GNTN = groundnut Tamil Nadu, GNAP = groundnut Andhra Pradesh, GBNV = *Groundnut bud necrosis virus*, WSMV = *Watermelon silver mottle virus*, IYSV = *Iris yellow spot virus*, INSV = *Impatiens necrotic spot virus*, ZLCV = *Zucchini lethal chlorosis virus*, TSWV = *Tomato spotted wilt virus*, CSNV = *Chrysanthemum stem necrosis virus*, TCSV = *Tomato chlorotic spot virus*, GRSV = *Groundnut ringspot virus*.
[b] Type isolate

*Cloning and sequence determination*

The NSm genes were cloned following RT-PCR amplification of viral RNA from cowpea, groundnut, potato and tomato, growing at four locations, as indicated in Table 1. The complete nucleotide sequences of five NSm genes were determined; no sequence differences were seen between the two clones of each isolate. Sequences have been deposited in the Gen Bank database as detailed in Table 2. The sequenced region in all five isolates had an open reading frame (ORF) of 924 bases and could potentially code for a protein of 307 amino acids. Although the conserved 'D-motif' of the '30K superfamily' of virus movement proteins (Melcher, 2000) was present in all GBNV isolates, the conserved glycine (G-residue; Melcher, 2000) was absent in all but GBNV-GNTN (Fig. 1).

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GBNV-GNAP       MSRFSNVLESFRPSNSSNKELVPAVKKENNRSILARNVSKKDVDSAIMNKAKTLNGKQYV
GBNV-GNTN       ***L************************************************************
GBNV-CP         ***L************************************************************
GBNV-PORJ       ***L************************************************************
GBNV-POMP       ***L************************************************************
GBNV-TO         ***L************************************************************

GBNV-GNAP       SSGDSSVLGTYSSESAVEATSDDILSRLVVEQSTHLSNWKNDSLVGNGNDKVSFTISIMP
GBNV-GNTN       ************************************************************
GBNV-CP         ************************************************************
GBNV-PORJ       ************************************************************M**
GBNV-POMP       ************************************************************M**
GBNV-TO         ************************************N***********************

GBNV-GNAP       TWNSNRRYMHISRLIIWVVPTIPDSKNNVKASLIDPNKMTKEEKIIISRQASLKDPMCFI
GBNV-GNTN       ************************************************************
GBNV-CP         ************************************************************
GBNV-PORJ       ******K*****************************************************
GBNV-POMP       ******K*****************************************************
GBNV-TO         ************************************************************

GBNV-GNAP       FHLNWSFPKERNTPKQCMQLNLTSDEKYAKGVSFASVMYSWVKNFCDTPIAAENNTCDVV
GBNV-GNTN       *********************************G**************************
GBNV-CP         ************************************************************
GBNV-PORJ       ************************************************************
GBNV-POMP       ************************************************************
GBNV-TO         ************************************************************

GBNV-GNAP       PINRAKVIQSAALIEACKLMIPKGTGGKQISNQIKSLQKAAERLALEAENDDESLDVDIE
GBNV-GNTN       *********************************************D**N*********
GBNV-CP         ************************************************************
GBNV-PORJ       *****************************************T******************
GBNV-POMP       *****************************************T******************
GBNV-TO         ************************************************************

GBNV-GNAP       MDNLLEI
GBNV-GNTN       *******
GBNV-CP         *******
GBNV-PORJ       *******
GBNV-POMP       *******
GBNV-TO         *******
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Fig. 1. CLUSTAL W generated multiple alignment of movement protein (NSm) gene sequences of *Groundnut bud necrosis virus* isolates. Asterisks indicate amino acids identical to GBNV-GNAP at a given position; differences are shown in bold. The 'D-motif' and the 'G-residue' conserved within the '30K superfamily' (Melcher, 2000) are in bold and underlined.

*Sequence comparisons*

The sequence similarities between tospovirus NSm genes and the alignment of GBNV NSm amino acid sequences are shown in Table 3 and Fig. 1, respectively. In all, NSm genes of 14 isolates representing nine Tospoviruses were used for analysis. The GBNV isolates showed maximum levels of sequence identity with the corresponding gene sequence of GBNV-Type isolate (GBNV-GNAP). Percent identity ranged from 92-95% and 98-100% at the nucleotide and amino acid levels respectively (Table 3). Further, GBNV isolates shared a considerable identity with WSMoV both at the nucleotide (77-79%) and amino acid sequence levels (82-83%). In contrast, only 50-68% (nucleotide) and 34-65% (amino acid) sequence identities were observed with other Tospoviruses (Table 3).

The NSm proteins of the GBNV isolates from this study differed from the Type isolate at eight amino acid positions (Fig. 1). Phenylalanine (amino acid 4) in the Type isolate was replaced by leucine in all of the GBNV isolates. In the tomato isolate (GBNV-TO), lysine (amino acid 100) was replaced by asparagine. In potato isolates (GBNV-POMP and GBNV-PORJ), isoleucine, arginine and alanine (amino acids 118, 127 and 281) were substituted by methionine, lysine and threonine respectively and in the groundnut isolate (GBNV-GNTN), serine, alanine and aspartic acid (amino acids 213, 288 and 291) were substituted by glycine, aspartic acid and asparagine respectively (Fig. 1).

Phylogenetic analysis of the amino acid sequences of the NSm genes showed that GBNV isolates formed one cluster along with WSMoV and IYSV. The remaining six tospoviruses, CSNV, GRSV, INSV, TCSV, TSWV and ZLCV formed a second cluster (Fig. 2).

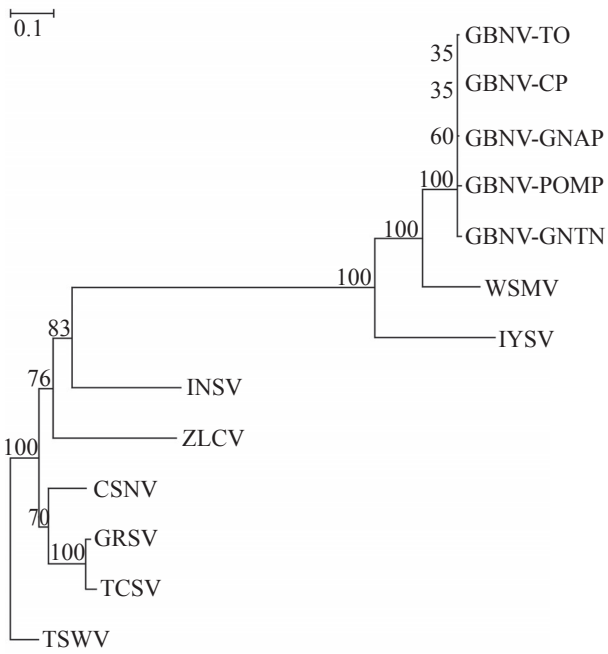


Fig. 2. Cluster dendrogram showing the relationships between the deduced amino acid sequences of the movement protein (NSm) gene of *Groundnut bud necrosis virus* isolates with those of known Tospoviruses. The dendrogram was constructed using the neighbour-joining method with bootstrapping (500 replicates) in TREECON for Windows version 1.3b on sequences aligned using CLUSTAL W 1.7 version. Vertical distances are arbitrary. Horizontal distances are proportional to genetic distances (bar represents 0.1). The number at nodes refer to number of times (in percentages) in which branching was supported. The tree was rooted on the TSWV sequence.

Table 3. *Per cent nucleotide (above diagonal line) and amino acid (below the diagonal line) sequence identity of movement protein (NSm) genes between* Groundnut bud necrosis virus *isolates and other Tospoviruses*

| Tospovirus isolates[a] | GBNV-CP | GBNV-POMP | GBNV-TO | GBNV-GNTN | GBNV-GNAP | WSMV | IYSV | INSV | ZLCV | TSWV | CSNV | TCSV | GRSV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GBNV-CP | | 93 | 99 | 97 | 92 | 78 | 67 | 53 | 51 | 51 | 53 | 53 | 53 |
| GBNV-POMP[b] | 99 | | 93 | 93 | 95 | 77 | 67 | 52 | 51 | 50 | 52 | 53 | 53 |
| GBNV-TO | 99 | 98 | | 97 | 92 | 78 | 67 | 53 | 51 | 51 | 53 | 53 | 53 |
| GBNV-GNTN | 99 | 98 | 98 | | 92 | 79 | 68 | 53 | 52 | 51 | 52 | 53 | 53 |
| GBNV-GNAP[c] | 99 | 98 | 99 | 98 | | 77 | 67 | 52 | 51 | 51 | 52 | 53 | 53 |
| WSMV | 83 | 82 | 83 | 82 | 83 | | 65 | 52 | 53 | 51 | 52 | 52 | 53 |
| IYSV | 65 | 64 | 64 | 65 | 64 | 60 | | 51 | 51 | 53 | 52 | 52 | 52 |
| INSV | 39 | 39 | 39 | 39 | 39 | 37 | 34 | | 66 | 66 | 68 | 67 | 66 |
| ZLCV | 34 | 34 | 34 | 34 | 34 | 33 | 32 | 59 | | 70 | 75 | 72 | 73 |
| TSWV | 38 | 37 | 37 | 38 | 38 | 36 | 36 | 64 | 64 | | 76 | 75 | 73 |
| CSNV | 39 | 38 | 38 | 38 | 39 | 36 | 37 | 66 | 70 | 80 | | 76 | 77 |
| TCSV | 38 | 38 | 38 | 38 | 38 | 37 | 36 | 68 | 70 | 79 | 84 | | 93 |
| GRSV | 39 | 38 | 38 | 36 | 39 | 37 | 37 | 67 | 70 | 80 | 85 | 96 | |

[a] Abbreviations are as in the footnote to Table 2.
[b] GBNV-POMP and GBNV-PORJ are 100% identical at the amino acid level
[c] GBNV-GNAP is the type isolate

## Discussion

The NSm gene from five GBNV isolates originating from different hosts and locations in India were sequenced and compared to known NSm sequences. The NSm coding region of all the isolates was the same; 924 bases encoding 307 amino acids. This is in contrast to the considerable heterogeneity (303-312 amino acids) observed between the NSm proteins of other Tospovirus species (Silva *et al*., 2001). Sequence analysis of the NP genes of the Indian GBNV isolates (Bhat *et al*., 2002; Jain *et al*., 2002; Thien *et al*., 2003; Umamaheswaran *et al*., 2003) showed them to be highly conserved and similar conservation was seen in the NSm genes analysed in this study.

Our data show that GBNV isolates originating from different hosts and locations in India are highly similar and are indistinguishable on the basis of their NP and NSm gene sequences. However, comparison of other genes on the three viral RNA species could allow the isolates to be distinguished. The sequence conservation within the NP and NSm genes may facilitate the use of pathogen derived resistance strategies to generate virus resistant transgenic plants (Prins & Goldbach, 1998; Rudolph *et al*., 2003).

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