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Identification and Molecular Characterization of Groundnut Bud Necrosis Virus (GBNV) Infecting Chilli (*Capsicum annuum* L.) in Andhra Pradesh, India

D. Bhagavathi Devi*, M. Krishna Reddy, B. Padmodaya, V.L.N. Reddy and V. Srilatha

Department of Plant Pathology, S.V Agriculture College, Tirupati, Andhra Pradesh, India

**Corresponding author*

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A B S T R A C T

Groundnut Bud Necrosis Virus (GBNV) (family Bunyaviridae, genus *Tospovirus*) is an emerging plant viral disease. The GBNV was a very broad host range infecting many economically important crops throughout in India. So, the aim of this study is identification of GBNV infecting chilli to know the genetic diversity. The plants showing chlorotic and nectrotic rings and apical bud necrosis in young leaves of chilli plants were collected from different locations in Andhra Pradesh. Total RNA was isolated from the positive ELISA samples and amplified with GBNV coat protein gene specific primers. GBNV chilli isolates shares a tripartite ssRNA genome structure and organization with other GBNV isolates, and more generally, other tospoviruses. The genome consists of L RNA (8911 nt), M RNA (4816nt) and S RNA (3059nt) segments.

Introduction

Chilli (*Capsicum annuum* L.). is an important spice crop in India and is grown for its pungency, color and flavor. Capsicum fruit are used in a wide range of fresh and processed food products and are an important source of vitamins viz., A and C and essential nutrients. Capsicum extracts are also used in pharmaceutical and cosmetic (Bosland and Votava, 2000) industries. Chilli belongs to the genus Capsicum and family Solanaceae. is herbaceous or semi-woody annual or perennial. *C. annuum* and *C. frutescens* are commercially cultivated species. Both the species are inter crossable (2n=24).

Chilli suffers from a large number of viral, bacterial, nematode and phytoplasma diseases causing considerable yield loss worldwide (Pemezny *et al.*, 2003). Among these diseases, viral infection reduces the yield and quality of Capsicum (Lee *et al.*, 2009; Anurag, 2012) drastically. Chilli is highly susceptible to a large number of viruses through natural infection and is known to be affected by 42

viruses worldwide which cause a great economic loss, in India a few viruses are detrimental affecting chilli crop, subsequently the economy of the country. The diseases caused by tospoviruses (familyBunyaviridae: genus Tospovirus) are emerging as a significant limiting factor for the sustainable production of chilli in India. Recently Groundnut bud necrosis virus (GBNV) (Satyanarayana *et al.,* 1996; Hemalatha *et al.,* 2008; Anjaneya Reddy *et al.,* 2008) and *Capsicum chlorosis virus* (CaCV) (Krishnareddy *et al.,* 2008) have been reported in India. The geographical expansion and 90 to 100 per cent incidences of tospovirus infections in chilli/ peppers has caused significant concern to fanners in Khammam and Warangal districts of the then Andhra Pradesh (Ravi *et al.,* 2007). Karnataka, Tamil Nadu. Tospoviruses form pleomorphic, spherical particles of approximately 80-120 nmin size within plant cells and are surrounded by a lipid envelope with two surface glycoproteins projections enclosing three nucleocapsids. The nucleocapsids contain three single stranded linear RNA segments which contain the genetic information essential for viral replication, movement and transmission. All tospoviruses are transmitted by thrips, sap-sucking insects within the order Thysanoptera. The virus genome consists of three linear single-stranded RNA molecules denoted as small (S) RNA, medium (M) RNA and large (L) RNA (Elliott. 1990). Both S and M RNA have an ambisense coding strategy. The S RNA encodes the nucleocapsid protein (N) and nonstructural proteins (NSs). The M RNA encodes the two envelope glycoproteins (Gn and Gc) and another nonstructural protein (NSm) (De Haan *et al.,* 1990).

Materials and Methods

Leaf samples of chilli plants showing typical symptoms of Tospovirus (GBNV) were collected from open fields Andhra Pradesh, India. Total RNA was isolated from diseased and healthy samples of chilli. Subsequently cDNA was synthesized from the RNA using oligo dT primer and reverse transcriptase enzyme.

Virus isolates and maintenance

The Groundnut Bud Necrosis Virus (GBNV) suspecting chilli samples were collected from different places in Andhra Pradesh, India. Naturally affected chilli samples showing concentric rings on leaves and apical bud necrosis were observed on the young leaves.

Maintenance of GBNV pure culture

The GBNV positive samples were maintained in *Vigna unguiculata* for further studies.

Isolation of total RNA and synthesis of C DNA

Total RNA from GBNV infected chilli leaf samples was isolated using RN easy plant Minikit according to the manufacturer's instructions. The RNA which is isolated from the plant samples was used for RT-PCR. First c-DNA was synthesized from total RNA in a 20µl reaction using BIOSCRIPT–RT enzyme (BIOLINE catalogue no. BIO-27036) as per manufacturer's instructions

Polymerase Chain Reaction (PCR) amplification

PCR reaction was carried out in 25µl volume reaction mixture. The cDNA samples prepared from infected leaf samples along with positive control (virus c-DNA) and negative control (distilled water) were used. Eppendorf PCR tubes of 0.2ml capacity were labelled and kept on thermo box which maintains temperature less than 40^0C.Three µl of cDNA was amplified in a 25 µl reaction volume

containing 2.5 units of Taq DNA Polymerase, 10 pmol of forward (SGF) and reverse primer (SGR), 2.5mM $MgCl_2$ and 0.2mM each dNTP's. PCR reaction mixture was prepared as given in the table and PCR amplification was carried out in a Eppendorf thermal cycler with the conditions; initial denaturation at 94°C for 3min followed by 35 cycles with 45 sec of denaturation at 94°C, 1 min of annealing at different temperature for each primer as mentioned above and extension for 1.30 min at 72^0C followed by a final extension for 20 min at 72^0C.

Amplified DNA fragments were electrophoresed in 1 per cent agarose gel according to the procedure outlined by Sambrook and Russel (2001).

Cloning of RT-PCR products and genome sequencing analysis

The amplified PCR product was eluted by FavorPrep™ Gel Extraction Kit and cloned into pTZ57R/T vector using InsT/A clone PCR product cloning kit (Cat# K 1214, MBI, Fermentas) by following the manufacturer's instruction. The resulting ligation products were transformed into *Escherichia coli* strain DH5α cells. Recombinant clones were identified by restriction endonuclease digestion and PCR.

The resulted positive clones sequenced at Medoxin, Sequencing, Bangalore. Multiple sequence alignments were generated using CLUSTAL X (Version 3.1). Sequence phylograms were constructed using TREEVIEW software (bootstrap analysis with 1000 replicates).

The coat protein genes of other known tospoviruses were collected from GenBank(NCBI). Both nucleotide and amino acid sequences of coat protein gene of different *Tospovirus* species were compared and the corresponding phylogenetic trees were generated.

Results and Discussion

Molecular characterization of Groundnut Bud Necrosis Virus (GBNV)

Sequencing and analysis of L RNA

Of the nine cDNA clones were amplified from GBNV L RNA by RT-PCR using the primers designed from the L RNAs of GBNV and WBNV. After assembling these seven sequences, the complete nucleotide sequence of the GBNV. Kurnool isolate, the L RNA is 8911 nt length [GenBank Accession no. KP827649] and encoded a single large ORF (RNA dependent RNA polymerase (RdRp), RdRp protein) that starts at nt 33 nt and terminates at nt 8,666 in the viral complementary strand. The deduced amino acid sequence of the ORF is 2877in length and is predicted to code for a 330.7 kDa protein. The 5' untranslatable region (UTR) 32 nt in length and 3' UTR of the L RNA was 245 nt in length. The 18-bp termini of the L RNA formed a panhandle structure with a mismatch at the 11th nucleotide. These UTRs shared the first 16 nt (agagcaatcaggtaac) that are conserved terminal sequences for Tospovirus genome segments (Table 1). The comparative sequence analysis of the L RNA of GBNV. Kurnool isolate shared highest nucleotide identity of 94.1 to 96.8% nucleotide identity with L RNA of GBNV isolates. The GBNV.Kurnool isolate shares high nucleotide identity of 68.8 to 81.7% with WSMoV serogroup tospovirus such as CaCV, CCSV, TNRSV, MYSV, MBV, TZSV, WBNV and WSMoV followed by the IYSV serogroup 64.6 to 75.9% nucleotide sequence identity, 51.1 to 53.5% nucleotide similarity with TSWV serogroup and 51.0 to 53.0% nucleotide sequence identity with INSV serogroup and others (Table 10). The L

protein amino acid sequence comparative analysis showed highest 98.5% with GBNV. Groundnut isolate (AF025538), with other GBNV isolates showed 95.3 to 98.0% amino acid homology. The Watermelon silver mottle virus (WSMoV) serogroup viruses showed 74.9 to 91.6%, IYSV serogroup viruses showed 63.2 to 67.1% identity, TSWV serogroup viruses showed 45.0 to 45.1% identity, INSV serogroup viruses showed 44.5 to 45.0% and other tospoviruses showed 35.9 to 42.0% identity (Table 1).

Sequencing and analysis of M RNA

Based on the M RNAs of GBNV and WBNV, primers were designed for amplifying the M RNA of GBNV. Fifteen cDNA fragments were amplified, with M RNA of GBNV as the template and assembled the sequences. The complete nucleotide sequence of the GBNV. Kurnool isolate M RNA is 4816nt in length and contains two ORFs encoding NSm and GP, in an ambisense coding strategy and separated by an IGR of 421 nts (GenBank accession number KM819699).

The 5' and 3' UTRs were 56 and 47 nt long, respectively and had the potential to form a stable hairpin structure. In the Virus sense strand, the first small ORF of 927 nt between nt 57 and 981 had AUG codon starting and terminated with an UAA stop codon, encoding the non-structural NSm protein of 307aa with a predicted molecular mass of 34.4 k Da.

The large ORF, which was 3366 nt long and located between nt 1404 and 4769 in viral-complementary strand. This ORF encode the viral glycoprotein precursor Gn/Gc of 1126 aa with predicted molecular masses of 127.6 k Da. The protein was identified as the glycoprotein precursor (GP) based on the sequence similarities to glycoproteins of other tospoviruses (Table 2).

The multiple sequence alignment comparison of the M RNA of GBNV. Kurnool isolate with that of other tospoviruses revealed that it showed highest nucleotide homology of 92.2 to 95.7% with GBNV isolates. Pair wise nucleotide comparison of GBNV. Kurnool isolate with the WSMoV serogroup tospoviruses showed nucleotide identity of 61.5 to 78.7%, the IYSV serogroup share 58.6 to 62.2% nucleotide identity, TSWV serogroup shared 42.8 to 44.3% nucleotide identity and INSV serogroup and others shared 35.6 to 45.2% nucleotide identity Table 2). Comparative sequence analysis of the NSm protein of GBNV. Kurnool isolate with other tospoviruses indicated that the highest identities of 91.2 to 100% nucleotide and 97.7 to 100% amino acid identity with GBNV isolates. The NSm protein of WSMoV serogroup viruses (CaCV, CCSV, MVBaV, MYSV, TNRSV, TZSV, WBNV, WSMoV) showed homology of 63.4 to 83.3% nucleotide and 58.8 to 85.9% amino acid identity. The IYSV sero group (HiCRSV, IYSV, TYRV and PolRSV), showed identity of 65.7 to 68.3% nucleotide and 62.9 to 65.4% amino acid, nucleotide identity of 49.3 to 52.3% and 37.0 to 39.2% amino acid identity with TSWV serogroup (ANSV, CSNV, GRSV, PNSV, MSMV, TCSV, TSWV, ZLCV) and nucleotide identity of 44.6 to 49.2% and 31.8 to 39.2% amino acid identity with INSV and others (INSV, BeNMV, SVNV, GCFSV) (Table 2).

The sequence of the Gn/Gc precursor protein of GBNV. Kurnool isolate shared 90.6 to 97.6 % nucleotide homology and 93.8 to 99.1% amino acid homology with the GBNV isolates. The GBNV. Kurnool isolate shared 65.6 to 81.5% nucleotide identity and 64.8 to 90.1% amino acid identity with WSMoV serogroup viruses, 63.5 to 65.3% nucleotide and 60.3 to 63.1% amino acid identity with IYSV serogroup viruses, 44.3 to 46.5% nucleotide identity and 33.3 to 46.7% amino

acid identity with TSWV serogroup viruses and share 42.0 to 46.0% nucleotide and 28.2 to 33.0% amino acid identity with INSV serogroup and others. The intergenic region (IGR) between Nsm and Gp genes, which is highly variable, the chilliGBNV. Kurnool isolate showed highest nucleotide similarity of 81.7 to 88.1% with GBNV isolates. The GBNV. Kurnool isolate shared 20.6 to 56.7% nucleotide identity with WSMoV serogroup viruses, 27.9 to 36.3% nucleotide identity with IYSV serogroup viruses, 14.5 to 30.7% nucleotide identity with TSWV serogroup viruses and share 20.2 to 35.5% nucleotide identity with INSV serogroup and others (Table 2).

Sequencing and analysis of S RNA

For the complete genome sequencing of S RNA of GBNV. Kurnool isolate, three cDNA fragments, located in the NSs gene, the N gene, the intergenic region (IGR) and the 5' and 3' terminal regions of S RNA were amplified by RT-PCR using primers designed based on S RNA of GBNV. The results showed that the complete nucleotide sequence of the GBNV. Kurnool isolate S RNA is 3059 nucleotides (nt) in length. The S RNA contains two open reading frames (ORFs) in an ambisense arrangement separated by an A-U-rich intergenic region (IGR). The GBNV. Kurnool isolate had 33.10% A, 18.06% C, 16.72% G, 32.12% T, with GC content of 34.58% and AP content of 65.42%. The 5' had 66 nt length and 3' had 67 nt untranslated regions of the S RNA can form a panhandle structure with 13 base pairs and two mismatches. An ORF of 1320 nt between nt 67 and 1386 of the viral RNA (vRNA) strand encoded the non-structural NSs protein of 439 amino acids (aa) with a predicted molecular mass of 49.6 k Da. The other ORF, located between nt 2162 and 2992 of the viral RNA, was 831 nt long with predicted molecular masses (Mr) of 30.5 kDa and encoded the N protein of 276 aa in the viral complementary RNA (vcRNA) (Table 3). The IGR between the NSs and N genes (IGR-S) was 775 nt long, with an A-U content of 76.6%. The multiple sequence alignment comparison of the S RNA, NSs, N and IGR regions with that of other tospoviruses revealed that the complete nucleotide sequence of GBNV. Kurnool isolate had highest identity of 93.3 to 96.2% with the reported GBNV isolates. Whereas with other WSMoV group isolates share nucleotide homology of 58.9 to 69.5% with members of Watermelon silver mottle virus serogroup such as Watermelon silver mottle virus (WSMoV), Watermelon bud necrosis virus (WBNV), Capsicum chlorosis virus (CaCV), Calla lily chlorotic spot virus (CCSV), Mulberry vein banding virus (MVBV), Melon yellow spot virus (MYSV), Tomato necrotic ring spot virus (TNRSV) and Tomato zonate spot virus (TZSV). The IYSV group viruses such as Iris yellow spot virus (IYSV), Hippeastrum chlorotic ring spot virus (HCRSV), Polygonium ring spot virus (PoRSV), and Tomato yellow ring virus (TYRV) have 43.7 to 55.3% nucleotide identity, whereas American tospoviruses TSWV group such as Groundnut yellow spot virus (GYSV), Tomato chlorotic spot virus (TCSV), Groundnut ring spot virus (GRSV), Chrysanthemum stem necrosis virus (CSNV), Zucchini lethal chlorosis virus (ZLCV), Pepper necrotic spot virus (PNSV), melon sever mosaic virus (MSMV), Impatiens necrotic spot virus (INSV) and Tomato spotted wilt virus (TSWV) share 32.1% to 35.0% nucleotide homology. The BeNMV group isolates such as Bean necrotic mosaic virus (BeNMV), and Soybean vein necrosis virus (SVNV) viruses showed 29.7% to 31.1% nucleotide homology and GYSV group viruses such as share 35.6% nucleotide homology (Table 2). The two ORF were similar in length to nucleotide sequences of the known isolates of GBNV.

Table.1 Comparison of L RNA complete sequence of GBNV.Kurnool isolate with RdRp of chilli tospovirus isolates and other tospoviruses

Viruses/isolates	FG	5UTR	3NTR	RdRp	
	nt	nt	nt	nt	aa
MKMD2324.Kurnool	100.0	100.0	100.0	100.0	100.0
AF025538.GBNV.Groundnut	96.8	100.0	93.4	96.8	98.5
KX950791.GBNV.PB197	95.7	100.0	90.2	95.7	98.0
MK204380.GBNV.CBE.Tomato	95.6	100.0	93.0	95.6	97.3
MK204382.GBNV.DMP.Tomato	95.0	ID	93.4	95.0	96.2
MK204383.GBNV.MDU.Tomato	94.9	100.0	93.4	94.9	96.1
KX965704.GBNV.Capsicum	94.4	100.0	90.6	94.4	95.3
KX965705.GBNV.Guntur	94.1	100.0	91.8	94.1	95.5
MH779497.ChYRSV.14YV855	81.7	90.6	74.5	81.8	91.6
JX177647.WSMoV.GZ	81.3	100.0	71.2	77.8	87.5
AF133128.WSMoV.	80.7	100.0	71.3	80.9	90.8
GU735408.WBNV.JT	80.4	100.0	72.4	80.6	90.3
KJ874251.WBNV.TN	78.4	100.0	13.0	80.2	89.5
KM589493.CaCV.Qld-3432	80.1	93.7	69.2	80.3	89.6
KT876916.CaCV-TN-CBE.Chilli	79.7	44.1	69.2	80.1	89.1
GU199334.CaCV.Ch-Har	79.7	96.8	68.4	80.0	88.6
DQ256124.CaCV.AIT	79.3	96.8	67.6	79.5	89.5
KM819698.MVBV.XCSY-3	75.9	96.8	55.0	76.4	84.5
MG252780.CCSV-Cel	70.7	55.5	39.2	71.4	75.9
AB061774.MYSV.melo.Japan	70.5	58.3	36.4	71.1	75.5
KT984754.TNSAV	70.4	52.7	38.0	71.1	76.3
KX213531.Tospovirus	70.4	52.7	40.0	71.2	76.5
EF552435.TZSV	70.0	52.7	39.6	70.6	74.9
KY315809.PCSV.TwPep3	69.0	51.4	32.5	69.8	71.9
JN560178.TYRV.T	65.8	50.0	39.3	62.5	63.2
MF469033.AYSV.Als-2000	65.7	58.3	39.2	66.7	66.7
KY484838.HCRV-CM	65.7	56.7	41.4	66.5	67.0
FJ623474.IYSV	65.6	55.5	42.0	66.5	66.8
KJ541746.PoRSV.Plg13	64.6	56.7	39.0	65.5	67.1
MK348942.TSWV.Tarquinia	53.5	54.2	33.8	54.3	45.1
KT717691.TSWV-QLD1.Capsicum	53.5	52.7	36.1	54.2	44.9
MF159048.TSWV.MJ	53.4	54.2	37.3	54.2	45.0
HM581934.TSWV.Tomato.NJ-JN	53.4	52.7	36.5	54.1	44.9
KJ575620.TSWV.p105.Italy	53.1	52.7	35.0	53.8	45.0
KF493773.CSNV.TcCh07A	53.4	52.7	27.0	54.3	45.0
DQ425094.INSV.Tomato.Italy	53.1	44.8	25.8	54.2	44.5
HM581937.TSWV.Pepper1.CY-CN	53.0	52.7	36.5	53.8	45.0
MG696851.AlNSV.San_Vicente3	53.0	52.7	15.7	54.0	45.0
KX698424.MeSMV.VE440-A	52.9	75.0	37.3	53.9	45.5
KU681010.ZLCV-DF	52.8	50.0	31.1	53.9	45.4
MH742956.GRSV.SA-05	52.6	53.8	29.6	53.4	45.0
KX463272.TCSV.DR	35.6	51.3	31.2	54.0	45.1
JF417980.BNMV	51.0	24.2	28.4	51.4	41.9
HQ728385.SVNV.TN	50.6	27.7	25.6	51.1	42.0
KP146140.GCFSV	48.2	75.7	16.0	48.8	35.9

Table.2 Comparison of M RNA complete sequence of GBNV.Kurnool isolate with chilli tospovirus isolates and other tospoviruses

Tospoviruses	FG	5UTR	3NTR	IR	NSmnt	NSmaa	GP	GPaa
	Nt	Nt	Nt	Nt	Nt	aa	Nt	aa
MKMD2327.Kurnool	100	100	100	100	100	100	100	100
KY006470.GBNV.Bangalore	95.7	96.4	97.8	85.9	91.3	98.0	97.6	99.1
MK617532.GBNV.Tumkur-ch	95.7	96.4	97.8	85.9	91.2	98.0	97.6	99.1
AY871097.GBNV.Mungbean	95.3	98.2	97.8	88.1	92.3	97.7	96.5	97.8
U42555.GBNV.Groundnut	93.9	94.7	93.6	81.7	92.2	98.3	95.4	99.0
KX347892.GBNV.brinjal.Bagalkot	93.8	100.0	95.7	84.6	92.0	97.0	94.8	95.8
KX244326.GBNV.brinjal	92.5	100.0	93.6	86.3	100.0	100.0	90.6	93.8
MK548887.GBNV.Guntur	92.2	96.4	87.2	87.9	92.0	97.7	92.4	94.7
MH779496.ChYRSV.14YV855	79.5	85.9	89.3	56.7	83.3	85.9	81.1	90.1
GU474545.WBNV.wDel	78.7	92.9	82.9	42.3	80.3	83.7	81.0	88.2
GU584185.WBNV.JT	78.4	92.9	87.2	42.7	80.4	84.0	80.5	88.2
JX177646.WSMoV.GZ	76.5	91.2	89.3	37.3	76.3	80.4	81.5	88.6
U75379.WsMoV.Taiwan	76.2	91.2	87.2	37.2	76.8	80.7	81.1	88.2
KM589494.CaCV.Qld-3432	76.5	82.7	85.1	33.6	81.0	87.3	79.9	87.7
KX499515.CaCV.TN-CBE	76.5	82.7	87.2	34.2	81.1	87.6	79.8	87.9
DQ256125.CaCV.AIT	76.4	85.9	89.3	46.9	81.7	87.9	79.0	86.9
FJ011450.CaCV.Ch-Pan	75.5	81.0	71.1	30.8	81.1	87.3	78.7	86.2
KM819699.MVBV	73.6	74.1	76.5	38.1	75.2	81.2	76.8	83.6
KT984753.TNSaV.2009-GZT	66.3	25.7	72.3	72.4	70.2	72.8	70.3	73.5
KX213532.Tospovirus.kiwifruit.	66.5	72.4	70.2	20.6	70.8	73.4	70.5	74.1
MG252781.CLCSV.Cel	66.5	65.5	72.3	33.3	71.8	71.8	69.5	73.2
EF552434.TZSV	65.3	67.2	70.8	31.1	71.5	74.4	69.9	73.1
KY315810.PCSV.TwPep3	64.5	67.2	57.1	29.3	67.6	65.7	66.3	66.9
FJ947152.TNRSV.TT1	63.4	62.2	57.1	36.3	68.6	68.4	65.6	65.0
AB061773.MYSV.melo.Japan	63.1	60.6	67.3	33.6	63.4	58.8	66.1	64.8
JN560177.TYRV.T	62.6	48.4	52.0	33.3	66.1	65.4	65.3	63.1
FJ361359.IYSV.USA	61.2	48.4	10.6	33.0	67.0	64.8	63.9	61.3
MF469034.AYSV.Als-2000	61.2	45.3	48.0	27.9	65.7	63.6	64.2	61.9
KJ541745.PoRSV.Plg13	60.9	46.8	50.0	28.0	66.8	63.8	64.0	60.3
KY484837.HCRSV.CM	60.7	21.8	48.0	36.3	68.3	62.9	63.5	61.0
DQ425095.INSV.Tomato.Italy	45.6	30.6	19.0	35.5	49.2	35.1	46.0	33.0
MF159060.TSWV.MJ	45.3	31.6	29.7	25.2	50.5	38.9	46.5	33.5
HM581935.TSWV.NJ-JN.Tomato	45.1	30.6	29.7	25.1	50.1	38.6	46.3	33.3
MG983522.TSWV.Tarquinia	45.1	29.7	29.7	23.5	50.6	38.3	46.3	33.3
HM581938.TSWV.Pepper1	45.0	29.7	29.7	24.4	49.5	38.0	46.3	33.4
KJ575621.TSWV.p105	45.0	30.6	29.7	23.7	50.1	38.3	45.9	33.1
KT717692.TSWV-QLD1	44.8	30.6	29.7	21.4	50.4	37.3	45.6	33.5
KF493772.ChSNV.TcCh07A.Japan	45.1	31.6	31.3	14.5	51.2	39.2	45.9	33.7
MH742957.GRSV.SA-05	44.9	30.6	30.9	29.8	52.3	38.3	44.8	33.4
KX463273.TCSV.DR	44.8	30.6	30.9	30.7	51.7	38.0	44.3	33.5

Table.3 Comparison of S RNA complete sequence of GBNV.Kurnool isolate with with chilli tospovirus isolates and other tospoviruses

Tospoviruses	FG	5UTR	3NTR	IR	NSs		GP	
	Nt	Nt	Nt	Nt	Nt	aa	Nt	aa
MKMD2325.Kurnool	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
U27809.GBNV.Groundnut	96.2	96.9	100.0	96.3	94.6	96.1	98.0	99.2
MH940242.GBNV.BL-C	95.9	95.4	100.0	96.1	94.3	96.1	98.0	100.0
AY871098.GBNV.Mungbean	95.6	96.9	100.0	95.2	94.4	95.4	97.3	99.6
MK875278.Tumkur	95.5	96.9	98.5	96.2	94.2	96.7	98.1	96.5
KX244321.GBNV.Brinjal.GKVK	95.5	98.4	100.0	94.8	94.5	95.6	97.1	99.2
KX244320.GBNV.Brinjal.Bagalkot	95.2	98.4	100.0	94.9	94.0	94.7	96.9	98.9
MK617533.GBNV.Guntur-ch	93.3	96.9	98.5	94.7	92.1	91.1	93.3	93.4
MH779495.ChYRSV.14YV855	69.5	95.4	91.0	20.5	82.3	81.5	86.7	86.2
EU249351.WBNV.	67.9	92.4	89.5	15.5	80.6	85.6	79.6	85.1
GU584184.WBNV.JT	67.5	89.3	85.0	15.6	80.6	85.8	79.6	84.7
U78734.WSMoV.Taiwan	65.4	89.3	79.1	18.6	81.3	85.1	80.0	85.5
JX177645.WSMoV.GZ	64.3	89.5	80.5	19.7	80.2	85.4	80.6	85.8
FJ011449.CaCV-Ch-Pan	63.3	89.3	86.5	76.1	77.8	81.0	78.0	84.4
DQ256123.CaCV.AIT	62.0	90.9	88.0	19.1	78.0	81.3	80.2	84.4
KM589495.CaCV.Qld-3432	59.4	90.9	85.0	18.3	76.9	80.8	77.8	84.0
KY308185.CaCV-TN-CBE	59.3	81.8	85.0	18.3	76.2	78.8	79.1	84.0
KM819701.MVBV.XCSY-3	58.9	58.2	79.1	25.8	71.7	69.9	70.0	71.4
KX213533.Tospovirus.kiwifruit.	55.3	31.0	65.2	47.7	60.9	60.1	64.9	65.3
KM355773.TNSaV.2009-GZT	55.2	31.0	66.1	45.6	60.9	59.9	64.6	65.3
MG252782.CLCSV. Cel	53.9	64.7	52.8	33.0	59.9	60.4	64.8	64.2
EF552433.TZSV. China	52.9	50.0	60.2	30.3	61.3	59.4	64.6	62.2
AB038343.MYSV. melo.Japan	52.5	60.0	64.1	30.2	53.2	45.8	63.5	59.1
FJ489600.TNRSV.TT1	48.3	55.8	56.9	49.7	53.5	47.5	59.5	56.9
KF383956.PCSV. TwPep3	48.0	57.3	56.9	20.5	54.7	45.4	60.6	57.6
AF001387.IYSV.USA	47.9	53.4	62.8	34.8	53.8	48.5	51.7	43.2
MF469035.AYSV. Als-2000	47.8	52.7	61.9	20.4	57.3	50.5	54.4	42.1
AY686718.TYRV. T	47.7	45.9	66.1	25.5	56.1	48.5	52.8	42.1
KY484836.HCRSV.CM	45.6	47.9	60.5	20.4	54.7	47.4	52.3	41.7
KJ541744.PoRSV.Plg13	43.7	48.6	61.1	9.8	54.9	46.9	53.0	41.4
HM581939.TSWV. Pepper1	35.0	34.0	13.1	25.4	36.8	17.1	40.7	27.4

KT717693.TSWV-QLD1	34.8	32.9	24.0	24.0	37.3	17.4	40.4	27.0
MF159072.TSWV.MJ	34.8	32.9	15.5	24.8	36.8	17.1	40.3	27.0
MG983521.TSWV.Tarquinia	34.6	32.9	15.1	23.5	36.7	17.1	40.7	27.0
HM581936.TSWV.Tomato	34.5	31.8	14.4	22.6	37.0	17.1	40.2	27.0
DQ376178.TSWV.P105	34.0	31.2	13.8	5.2	37.0	16.9	40.7	27.4
KX463274.TCSV.DR	34.0	34.0	12.4	29.5	36.1	17.9	40.3	26.3
KF493771.ChSNV.TcCh07A.Japan	33.9	33.7	19.0	21.5	35.3	17.4	40.6	26.2
MG696853.ANSV. San_Vicente3	33.6	28.7	21.3	26.2	34.9	16.4	39.4	26.6
MH742958.GRSV.SA-05	33.5	34.0	13.7	23.8	34.4	17.1	42.4	27.7
EU275149.MSeMV.VE440	33.1	38.7	16.3	29.5	35.2	17.1	43.4	29.4
KU681011.ZLCV-DF	32.2	38.6	9.9	18.5	35.4	16.1	41.5	26.9
DQ425096.INSV.Tomato.Italy	32.1	44.3	32.3	24.1	36.9	16.7	38.5	26.0
AF013994.GYSV.Groundnut	35.6	38.3	53.9	26.2	34.3	11.0	36.1	16.3
HQ728387.SVNV.TN	31.1	35.1	40.0	12.4	36.5	16.0	39.5	30.9
JN587268.BNMV.TF-SP	29.7	36.9	38.7	12.3	37.2	15.5	39.4	28.3

Fig.1

Necrotic rings symptoms of GBNV

Local lesions in cowpea

Apical stem necrosis of GBNV

Fig.2 Phylogenetic tree of GBNV.Kurnool isolate L RNA with the L RNA sequences of GBNV and other tospoviruses. The dendrogram produced using the Neighbour - Joining algorithm with 1000 bootstrap replicates

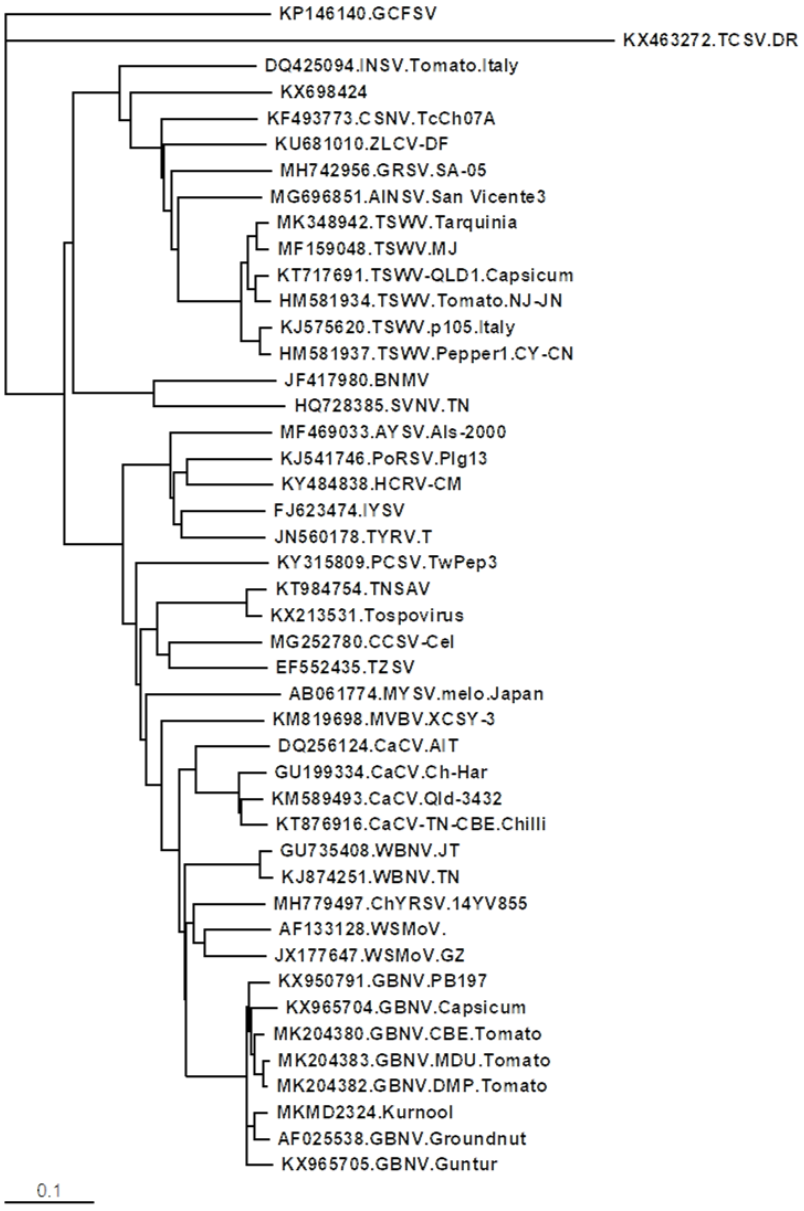


Fig.3 Phylogenetic tree of GBNV.Kurnool isolate M RNA with the M RNA sequences of GBNV and other tospoviruses. The dendrogram produced using the Neighbour- Joining algorithm with 1000 bootstrap replicates

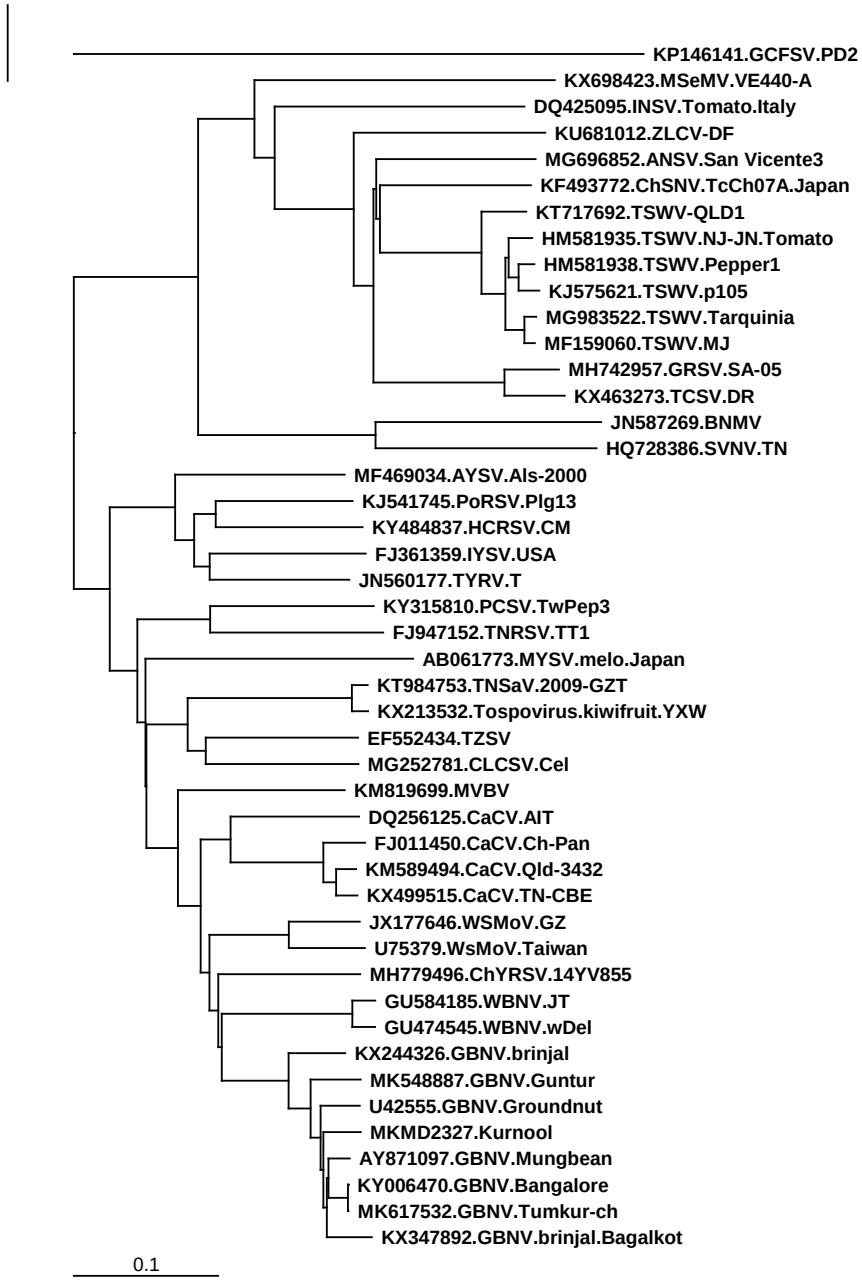
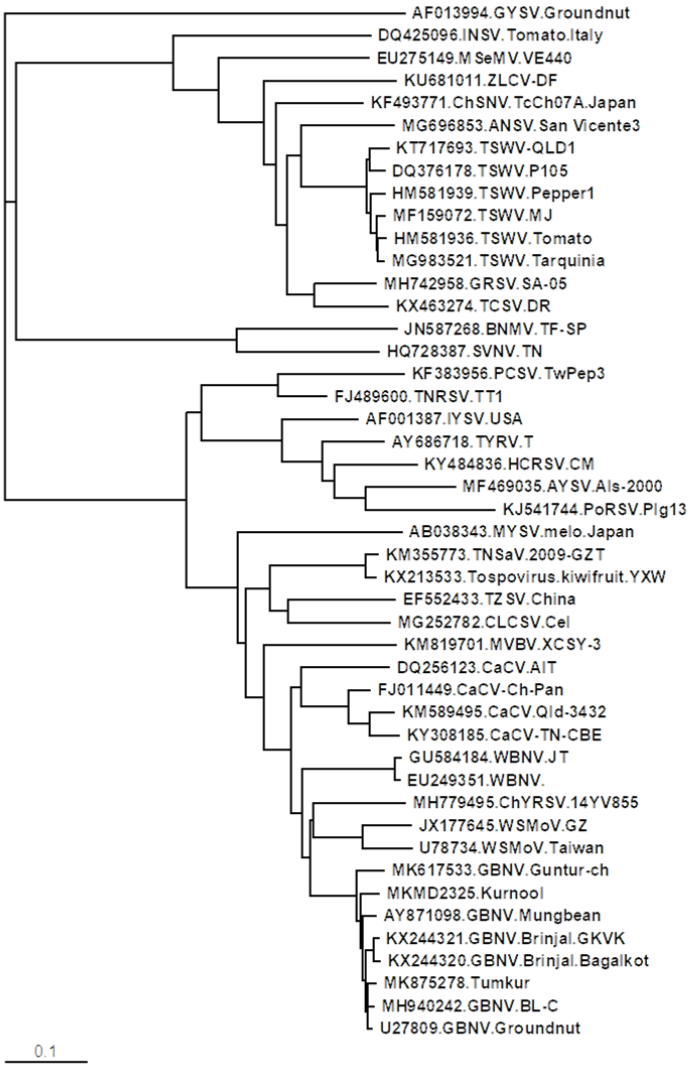


Fig.4 Phylogenetic tree of GBNV.Kurnool isolate S RNA with the S RNA sequences of GBNV and other tospoviruses. The dendrogram produced using the NeighbourJoining algorithm with 1000 bootstrap replicates



Secondary structure prediction of the S RNA using the Gene Bee-Molecular Biology Server showed that the IGR-S formed a hairpin structure with loops and three major branches (data not shown). The first and last 14 nucleotides at the 5' and 3' terminal regions of S RNA are inverted with one nucleotide mismatch. The terminal 9 nucleotides (5' AGAGCAAUC 3') are completely conserved in S, M and L RNAs of GBNV.

Phylogenetic analysis of GBNV

Molecular phylogeny of the complete SRNA sequences of known tospovirus were analyzed to study its molecular phylogeny for SRNA nucleotide sequence, NSS nucleotide and amino acid, NP nucleotide and amino acid and IGR sequences. The evolutionary history was infrared by using the maximum likely wood method based on the Tamura–Nei model. The phylogenetic tree shows that all known S RNA sequences of tospovirus are formed 4 sub clades (Fig. 1, 2 and 3). The S RNA sequence of GBNV. Kurnool isolate clustered with GBNV isolates of Chilli, brinjal, Cowpea, Groundnut, mungbean and tomato (Fig. 3). This is a major clade consisting of WSMoV serogroup which are of Asiatic origin such as groundnut bud necrosis virus (GBNV), watermelon silver motel virus (WSMoV), watermelon bud necrosis virus (WBNV), Capsicum chlorosis virus (CaCV), mulberry vein banding virus (MVBV), Calla lily Chloratic spot virus (CCSV), Tomato zonate spot virus (TZSV), Melon Yellow Spot virus (MYSV) and Tomato necrotic ring spot virus (TNRSV). Along with these clades a subgroup of IYSV serogroup was formed with iris yellow spot virus (IYSV), Tomato yellow ring spot virus (TYRSV), Hippeastrum chlorotic ring spot virus (HCRSV) and polygoneum ring spot virus (PolRSV). Another major clade consists of America, Euro and Asiatic isolates which have 3 subgroups. The group 1 consists of TSWV serogroup of tospovirus such as groundnut ring spot virus (GRSV), Chrysanthemum stem necrosis virus (CSNV), Pepper necrotic spot virus (PNSV), Melon sever mosaic virus (MSMV), Tomato Chloratic spot virus (TCSV) and Tomato spot wilt virus (TSWV).

The second subgroup consists of Impatiens necrotic spot virus (INSV) and Third group consists of bean necrotic mosaic virus (BeNMV) and Soybean vein necrosis virus (SVNV). The Phylogenetic analysis of NSS gene of both nucleotide and amino acid sequence analysis showed similar grouping of tospo viruses. The GBNV isolates clustered as a subgroup along with WSMoV serogroup clade. The phylogenetic tree generated based on nucleoprotein of both nucleotide and amino acid sequences showed grouping of chilli tospo isolates along with GBNV as subgroup within the major WSMoV serogroup clade. However, the IGR sequence-based tree generated showed grouping of all WSMoV serogroup as a major clade and subgrouping of different viruses into different subgroup one isolate of CaCV from India grouped along with GBNV indicating recombination with GBNV (Fig. 4).

The molecular phylogenetic relationship of M RNA complete nucleotide sequences showed similar grouping of subgroup and clades to that of S RNA. The Chillitospo isolates grouped along with GBNV isolates as a subgroup in the major clade of WSMoV (Fig. 2).

The phylogenetic analysis of NSm both nucleotide and amino acid sequences are showed similar grouping of chilli tospo isolates in the GBNV subgroups within major clade of WSMoV. Similarly, the glycoprotein precursors of both nucleotide and amino acid sequences grouped along with the GBNV subgroup within the WSMoV serogroup major clade. The IGR sequence-based tree generated

indicated chilli isolates along with other GBNV isolates clustered with tomato necrotic ring spot virus (TNRSV). Phylogenetic trees on the basis of NSm and Gn/Gc proteins alignments revealed a clustering similar to S RNA. The complete L RNA sequences of known tospoviruses were analyzed to study its molecular phylogeny the phylogenetic tree showed that the L RNA sequences formed 3 groups. The Chilli tospo isolates clustered with GBNV isolates along with WSMoV serogroup clade (Fig. 1). The molecular phylogenetic RdRp gene both nucleotide and amino acid sequences showed similar grouping of Chilli tospoviruses (Fig. 1).

GBNV chilli isolates shares a tripartite ssRNA genome structure and organization with other GBNV isolates, and more generally, other tospoviruses. The genome consists of L RNA (8911 nt). M RNA (4816nt) and S RNA (3059nt) segments (Table 1, 2, 3) with sizes of ORFs and untranslated regions (UTR) similar to those of other GBNV isolates reported form groundnut (Satyanarayana *et al.,* 1996) and mungbean (Saritha and Jain, 2007). The nine terminal nucleotides (50 AGAGCAAUC 30) in all three genomic RNA segments of GBNV chilli are completely conserved. In addition, the 50- and 30-terminal sequences of the RNA segments are reverse complements with perfect or nearly perfect base paring in the terminal 18 (L). 9 (M) and 14 (S) nucleotides.

N protein aa sequence analysis confirmed that GBNV chilli belongs to the Watermelon silver mottle virus (WSMoV) group and clusters in clade I of GBNV isolates (Saritha and Jain, 2007) with isolates from groundnut and mungbean.

The variability studies of GBNV not only useful in establishing differences among the different isolate infecting different crops but also aid in evolving transgenic plants against to GBNV.

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