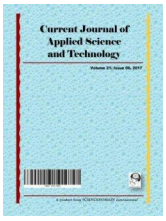

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Characterisation of the Nucleocapsid Protein Gene of *Groundnut bud necrosis virus* (GBNV) in Tamil Nadu and its Phylogenetic Relationships

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The nucleocapsid protein (*N*) gene of *Groundnut bud necrosis virus* (GBNV) associated with bud blight of tomato plants from different locations of Tamil Nadu *viz*., Coimbatore, Krishnagiri, Dharmapuri and Madurai was cloned and sequenced. The *N* gene comprised of 831 nucleotides and 277 aminoacids. The nucleotide sequence identities of the present isolates of the study ranged from 97.1% to 100%. Coimbatore isolate (MK032858) shared maximum sequence identity of 100% with Dharmapuri (MK032860) and Coimbatore isolates (AY618561) of tomato. Krishnagiri isolate (MK032859) shared maximum nucleotide sequence identity of 100% with Andhra Pradesh tomato isolate (AY510133). The aminoacid sequence identities of our isolate ranged from 97.8% to 100%. Krishnagiri isolate (MK032859) shared maximum aminoacid sequence identity of 100% with Andhra Pradesh isolate (AY510133). On this basis, it is concluded that the GBNV isolates from different crops and different agro-ecological zones have not much variation in nucleotide and aminoacid levels.

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Keywords: Tospovirus; GBNV; Nucleocapsid protein gene.

1. INTRODUCTION

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop, standing next to potato, and belongs to the family *Solanaceae*. It is highly sensitive to viral diseases *viz., Tomato spotted wilt virus* (TSWV) [1], *Tomato leaf curl New Delhi virus* (ToLCNDV) [2], *Tomato torrado virus* [3], *Tomato marchitez virus* [4], *Tomato infectious chlorosis virus* [5], *Tomato chlorosis virus* [6], *Tomato necrotic spot virus* [7], *Tomato pelargonium zonate spot virus* [8], *Tomato yellow ring spot virus* [9], *Capsicum chlorosis virus* [10], *Tomato necrotic spot virus* [11], *Pepino mosaic virus* [12] and *Tomato yellow leaf curl virus* and related species [13]. In the Indian subcontinent, *Groundnut bud necrosis virus* (GBNV) is one of the most destructive viruses in tomato. GBNV belongs to the family *Bunyaviridae,* composed of 19 species [14]. Among them, 14 have been identified in Asia [15]. Six tospoviruses have been reported from India viz., *Groundnut bud necrosis virus* [16,17], *Groundnut yellow ringspot virus* [18], *Watermelon bud necrosis virus* [19], *Iris yellow spot virus* [20], *Capsicum chlorosis virus* [21,22] and *Tomato spotted wilt virus* [23]. Tospoviruses belong to the family *Thysanoptera* and are transmitted through thrips by circulative and propagative manner [24]. Among various tospoviruses, GBNV particles are enveloped, quasi-spherical in shape and 80-110 nm in diameter with a tripartite single-stranded RNA genome. The large (L) RNA has a single open reading frame (ORF) and encodes for RNA dependent RNA polymerase (RdRp); the M- RNA has two ORFs encoded in ambisense strategy for glycoprotein precursors in a viral complementary sense and nonstructural movement (NSm) protein in a viral sense. S-RNA also has an ambisense strategy, two ORFs encoded for Nucleocapsid protein (N) in a viral complementary sense and nonstructural small (NSs) in a viral sense.

Phylogenetic analysis of nucleocapsid protein gene sequences revealed a classification of tospovirus based on serogroups. Isolates of serogroup I (TSWV) and serogroup III (INSV) were closely related with each other. Serogroup II has been distinct from *Tomato chlorotic spot virus* and *Groundnut ring spot virus* [25]. *Watermelon bud necrosis virus* (WBNV), *Groundnut bud necrosis virus* (GBNV) and *Watermelon silver mottle tospovirus* were related to serogroup IV. Serological classification in Tospoviruses was based on the nucleocapsid protein sequence. Bhat et al. [26] explained that sequence identity in the nucleocapsid protein gene sequences from GBNV causing bud blight of soybean is 98% identical to that of other isolates of GBNV.

During the field survey conducted in different districts of Tamil Nadu *viz.,* Dharmapuri, Krishnagiri, Coimbatore and Madurai, various symptoms were observed on tomato plants. Symptoms of GBNV on tomato were chlorotic and necrotic spots on young leaves, necrotic streaks on the stem, bud blight on young buds, bronzing, stunting and wilting of entire plants. The GBNV disease incidence in tomato ranged from 18% to 35% in different fields of Tamil Nadu [27]. Understanding the genetic diversity of GBNV will be helpful to develop strategies for management of bud necrosis disease of tomato. Nucleocapsid sequences are highly conserved for identification of Tospoviruses. The present study was carried out to investigate the variability of the nucleocapsid protein gene GBNV in Tamil Nadu. Nucleocapsid protein gene sequences were used for sequence analysis to study genetic diversity and phylogenetic analysis. The information on the variability of nucleocapsid protein gene may help to generate GBNV resistant transgenic plants through pathogen-derived resistance.

2. MATERIALS AND METHODS

2.1 RNA Extraction and RT- PCR Analysis

Leaf samples from tomato plants showing characteristic symptoms of GBNV *viz.,* chlorotic and necrotic spots on infected leaves, stem necrosis, bronzing, bud blight, wilting and stunting of the infected plant were collected from different parts of Tamil Nadu *viz.*, Coimbatore, Dharmapuri, Krishnagiri, Salem and Madurai. Collected GBNV infected tomato leaves were inoculated on cowpea plant cv. CO5 under insect-proof glass house condition. Extraction of the virus was done by macerating GBNV infected fresh leaves with 0.1 M of sodium phosphate buffer pH 7.0 at the ratio of 1:2 (infected leaf tissue: buffer volume) using ice tray. The cowpea plants were used for propagating the virus since they produce characteristic local lesion symptoms within 3-4 days after inoculation. Inoculation was carried out by gentle rubbing with inoculum using the broad end of the pestle

on the cotyledonary leaves of six-day-old cowpea plants previously incubated in the dark for 12 hrs and dusted with 600 mesh carborundum powder. The excess inoculum was washed with a jet of sterile distilled water using a wash bottle. The inoculated plants were kept under observation for 4-5 days for the expression of symptoms [28].

The total RNAs were extracted from cowpea leaf samples (100 mg) showing local lesions of GBNV using the Trizol method by TRI reagent developed by Chomczynski and Sacchi [29]. The plant samples showing symptoms along with healthy samples were homogenised separately using liquid nitrogen. One ml of Trizol reagent (Sigma- Aldrich now Merck) was added, transferred to a 1.5 ml centrifuge tube and incubated at under room temperature for 2 min and centrifuged @ 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a new 1.5 ml centrifuge tube and 250 µl of chloroform was added, mixed well and the centrifuged at 13,000 rpm for 15 min. The aqueous layer was transferred to another new tube and 250 µl of isopropanol and 250 µl NaCl was added. Then it was incubated over the ice for 10 min and then centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the pellets retained in the tube were washed with 75% ethanol. The pellets were air dried and dissolved in 30 µl of RNase free water under the sterile conditions and stored at -80°C. First strand cDNA synthesis was carried out using the first strand cDNA synthesis kit (Thermo scientific RevertAid first strand cDNA synthesis kit, USA) as per manufacturer's instruction. The reaction was performed at 42°C for 60 min followed by incubation at 70°C for 5 min.

RT-PCR was carried out with the GBNV nucleocapsid protein gene primer pair, GK-GBNV-F (5'-ATGTCTAACGTYAAGCAGCTC-3') and GK-GBNV-R (5'-TTACAATTCCAG CGAAGGAC-3') [30] corresponding to complete nucleocapsid protein gene of GBNV. The PCR was carried out with the master mix (Smart prime, India) in 50 µl reaction volume containing master mix- 25 µl; GK-GBNV-F- 5 µl; GK-GBNV-R- 5µl; distilled water- 10 µl; cDNA- 5 µl. The PCR setting comprised an initial denaturation for 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, the extension at 72°C for 1 min and a final extension of 72°C for 10 min. The RT-PCR product was then analysed on 1.2% agarose gel, stained with ethidium bromide and viewed under transilluminator.

2.2 Cloning, Sequencing and Sequence Analysis

The amplicon of complete nucleocapsid protein gene was purified using GenJET PCR purification kit (Thermo Fischer Scientific Inc.,) and ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) at 4°C following the manufacturer's instructions. The ligated product was transformed into *Escherichia coli* DH5α competent cells by following standard molecular biology procedures [31]. Plasmid DNA was isolated from the potential recombinant clones using GenJET plasmid Midiprep kit (Thermo Fischer Scientific Inc.,) according to the manufacturer's protocol. Potential recombinant clones were identified by restriction digestion analysis using *EcoRI* enzyme. Two independent clones were sequenced in both the orientations by using universal sequencing primers M13 (forward and reverse) and sequencing was done with M/s Chromous Biotech Pvt. Ltd., Bangalore. The PCR products were sequenced and the sequence results were analysed in the BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To investigate the phylogenetic relationship among GBNV isolates of Tamil Nadu were compared with other isolates of India. Closely related nucleocapsid protein gene sequences were obtained from NCBI as mentioned in Table 1. The consensus sequences of the nucleocapsid protein gene of GBNV isolates were analysed and compared by using CLUSTAL W. The phylogenetic trees at nucleotide and aminoacid levels were constructed by using MEGA version 4.0. and Bioedit 5 software.

3. RESULTS AND DISCUSSION

3.1 Sequence Determination and Data Analysis

The GBNV infected tomato leaf samples were identified by the presence of chlorotic and necrotic spots on leaves (Fig. 1) from different parts of Tamil Nadu. Symptomatic tomato leaf samples were inoculated on cow pea cv. CO5 leaves on cotyledonary leaves under insect-proof condition. Cowpea leaves exhibited chlorotic to necrotic lesions of inoculated cotyledonary leaves (Fig. 2). Though samples were collected from both Dharmapuri (DMP) and Salem (SLM) districts, they were considered one isolate as Dharmapuri (DMP) for sequencing, since these districts are very closer and have similar agro-climatic conditions. RNA extracted from symptomatic cowpea and non-symptomatic

leaves were subjected for RT-PCR assay with GBNV nucleocapsid protein gene-specific primers, GK-PBNV-CP-F (5'-ATGTCTAACGTYAAGCAGCTC-3') and GK-PBNV-CP-R (5'-TTACAATTCCAGCGAAG GAC-3'). The symptomatic leaf samples resulted in the amplification of an approximately 831 bp amplicon and there was no amplification from non-symptomatic leaves (Fig. 3). The nucleocapsid protein gene sequences of four isolates were determined, analysed and deposited in the GenBank database (accession numbers MK032858 (CBE); MK032859 (KGI); MK032860 (DMP); MK032861 (MDU)).

Fig. 1. Appearance of chlorotic and necrotic spots on tomato leaves

3.2 Comparison of Sequence and Phylogenetic Analysis of GBNV Isolates

Pair wise sequences identity of the complete nucleocapsid protein gene among four isolates ranged from 97.1% to 100% at the nucleotide level (Table 2). Coimbatore, Dharmapuri and Krishnagiri isolates were very closely related with each other showing sequence identities from 99.1% to 100%. The Madurai isolate had similar sequence identities of 97.1% to 97.4% with other isolates. The nucleocapsid protein gene of GBNV isolates of Tamil Nadu was compared with the corresponding gene from known GBNV isolates from other states of India at the nucleotide sequence levels. Sequences were obtained from the National Center for Biotechnology Information (AY618561, EU373768, AY510132, AY512648, AY173043, AY882000, AY510133, GQ844886, EU373786, EF179100, EU373791, AY727923, AY618563, KX244337) and were aligned with multiple sequence alignment (BioEdit 5). The nucleotide sequence identities of the present isolates ranged from 97.1% to 100%. Coimbatore isolate (MK032858) shared maximum sequence identity of 100% with Dharmapuri (MK032860) and Coimbatore isolate (AY618561) of tomato. The same isolate shared a minimum identity with Karnataka isolate (AY173043) of tomato as 99.6%. Krishnagiri isolate (MK032859) shared maximum nucleotide sequence identity of 99.6% with Tamil Nadu isolate (AY618561) of tomato, Maharastra isolate (EU373768) of tomato, Karnataka isolate (AY512648) of brinjal and Karnataka isolate (AY173043) of tomato. Madurai isolate (MK032861) shared maximum nucleotide sequence identity of 98.5% with Maharastra isolate (AY510132) of tomato. The aminoacid sequence identities of our isolate ranged from 97.8% to 100%. Coimbatore isolate (MK032858) shared maximum aminoacid sequence identity of 100% with Dharmapuri (MK032860) and Coimbatore isolate (AY618561) of tomato. Krishnagiri isolate (MK032859) shared maximum aminoacid sequence identity of 100% with Andhra Pradesh isolate (AY510133) of tomato. Madurai isolate (MK032861) shared maximum aminoacid sequence identity of 98.3% with Maharastra isolate (AY510132) of tomato.

The GBNV nucleocapsid protein gene sequences were subjected to phylogenetic analysis, with those of closely related GBNV isolates from India and outgroup as *Tobacco streak virus* (KR017710). The GBNV isolates *viz.,* MK032858 (CBE), MK032860 (DMP), AY618561 (tomato, Coimbatore), EU373768 (tomato, Maharastra), AY512648 (Brinjal, Karnataka), AY510132 (tomato, Andhra Pradesh), MK032861 (MDU), AY173043 (tomato, Banglore, Karnataka), EF179100 (Peanut, Andhra Pradesh), AY882000 (tomato, Maharastra), GQ844886 (Brinjal, Maharastra), EU373786 (tomato, Maharastra), AY510133 (tomato, Andhra Pradesh) and MK032859 (KGI) formed a single cluster.

Tomato is one of the most important vegetable crops in India. Tospovirus is one of the most destructive diseases of tomato and has been recorded in almost all of the major tomato producing countries. According to our study, the disease incidence ranges from 18% to 35% in the major tomato growing areas of Tamil Nadu [27]. GBNV isolates of Tamilnadu along with

other isolates of India were grouped into a single clade. The cause of Tomato spotted wilt disease in India was reported in 1985 [32]. However, the presence of TSWV in India has been reported in Chrysanthemum recently by Renukadevi et al. [23]. Isolates in the *Tospovirus* genus with greater than 90% nucleocapsid protein (N) gene sequence identity are delineated as strains of the same virus (Moyer et al., 1999). Aminoacid level variability in 76 GBNV isolates infecting different vegetable crops has been studied by Kunkalikar et al. (2011) and they reported 91.3% to 100% similarities. Our present isolates found 97.8% to 100% identity at the aminoacid level and 97.1% to 100% identity at the nucleotide level. Nucleotide sequences of the NP gene of GBNV isolates including four tomato isolates (present study) infecting different hosts in different agro-ecological zones across the India were recorded with 97% - 100%. There were no variations in sequences of GBNV isolates either based on geographical locations or with hosts.

Fig. 2. GBNV inoculated cowpea Cv. CO5 with chlorotic spots at 4 days after post inoculation

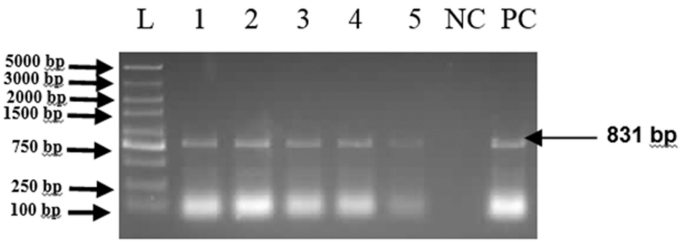

Fig. 3. RT-PCR analysis for nucleocapsid protein gene of GBNV isolates with the primer pair, GK PBNV CP F (5'-ATGTCTAACGTYAAGCAGCTC-3') and GK PBNV CP R (5'-TTACAATTCCAGCGAAG GAC-3') corresponding to the nucleocapsid protein gene of GBNV. DNA fragments of approximately 831 base pairs specific to the *N* gene of GBNV were amplified from the RNAs extracted from GBNV infected cowpea (Lane, 1-5), while no amplification was observed in RNA extracted from cowpea sample inoculated with healthy tomato (Lane, NC). L-100bp Ladder; 1. Dharmapuri sample; 2. Coimbatore sample; 3. Krishnagiri sample; 4. Salem sample 5. Madurai sample; PC- Positive control (GBNV infected groundnut leaf)

Table 1. Sources and Genbank accession numbers of Nucleocapsid protein gene isolates obtained from NCBI

Accession numbers	*Virus*	*Isolate*	*Source*
MK032858	GBNV	Coimbatore, Tamil Nadu	Tomato
MK032859	GBNV	Krishnagiri, Tamil Nadu	Tomato
MK032860	GBNV	Dharmapuri, Tamil Nadu	Tomato
MK032861	GBNV	Madurai, Tamil Nadu	Tomato
AY618561	PBNV	Coimbatore, Tamil Nadu	Tomato
EU373768	GBNV	Jalna, Maharastra	Tomato
AY510132	PBNV	Jalna, Maharastra	Tomato
AY512648	PBNV	Rainbennur, Karnataka	Brinjal
AY173043	PBNV	Banglore, Karnataka	Tomato
AY882000	PBNV	Arvikendra, Pune	Tomato
AY510133	PBNV	Kurnool, Andhrapradesh	Tomato
AQ844886	PBNV	Jalna, Maharastra	Brinjal
EU373768	GBNV	Jalna, Maharastra	Tomato
EF179100	PBNV	Jalna, Maharastra	Peanut
EU373791	GBNV	Jalna, Maharastra	Tomato
AY727923	GBNV	Pune, Maharastra	Tomato
AY618563	PBNV	Gulburga, Karnataka	Tomato
KX244337	GBNV	Banglore, Karnataka	*Solanum nigrum*
KR017710	TSV	USA	*Dahlia pinnata*

Table 2. Per cent homology of aminoacid (below diagonally) and nucleotide sequences (above diagonally) identities of the GBNV nucleocapsid protein gene of different isolates

Seq->	*MK032858 (CBE)*	*MK032859 (KGI)*	*MK032860 (DMP)*	*MK032861 (MDU)*	*AY618561*	*EU373768*	*AY510132*	*AY512648*	*AY173043*	*AY882000*	*AY510133*	*GQ844886*	*KX244337*
MK032858 (CBE)	100	99.10	100	97.40	100	100	99.60	99.60	99.60	100	99.60	99.60	100
MK032859 (KGI)	99.60	100	99.1	97.10	99.6	99.60	99.20	99.20	99.20	99.60	100	99.20	99.60
MK032860 (DMP)	100	99.6	100	97.4	100	100	99.6	99.60	99.60	100	99.60	99.60	100
MK032861 (MDU)	98.10	97.80	98.10	100	98.10	98.10	98.50	97.80	97.80	98.10	97.80	97.80	98.10
AY618561	100	99.10	100	97.40	100	99.30	99.30	99.20	99.20	99.20	99.10	99.00	98.70
EU373768	99.30	99	99.30	97.30	100	100	99.20	99.10	99.10	99.30	99.00	99.10	98.60
AY510132	99.30	99.00	99.30	98.00	99.60	99.60	100	99.30	99.10	99.10	99.00	98.90	98.40
AY512648	99.20	99.30	99.20	97.40	99.60	99.60	99.20	100	99.00	99.00	99.30	98.70	98.30
AY173043	99.20	98.90	99.20	97.20	99.60	99.60	99.20	99.20	100	99.00	98.90	98.70	98.30
AY8820	99.20	98.90	99.20	97.20	100.00	100.00	99.60	99.60	99.60	100	98.90	99.20	98.50
AY510133	99.10	100.00	99.10	97.10	99.60	99.60	99.20	99.20	99.20	99.60	100	98.60	98.40
GQ844886	99.00	98.60	99.00	97.10	99.60	99.60	99.20	99.20	99.20	99.60	99.20	100	98.50
KX244337	98.70	98.40	98.70	96.50	100.00	100.00	99.60	99.60	99.60	100.00	99.60	99.60	100

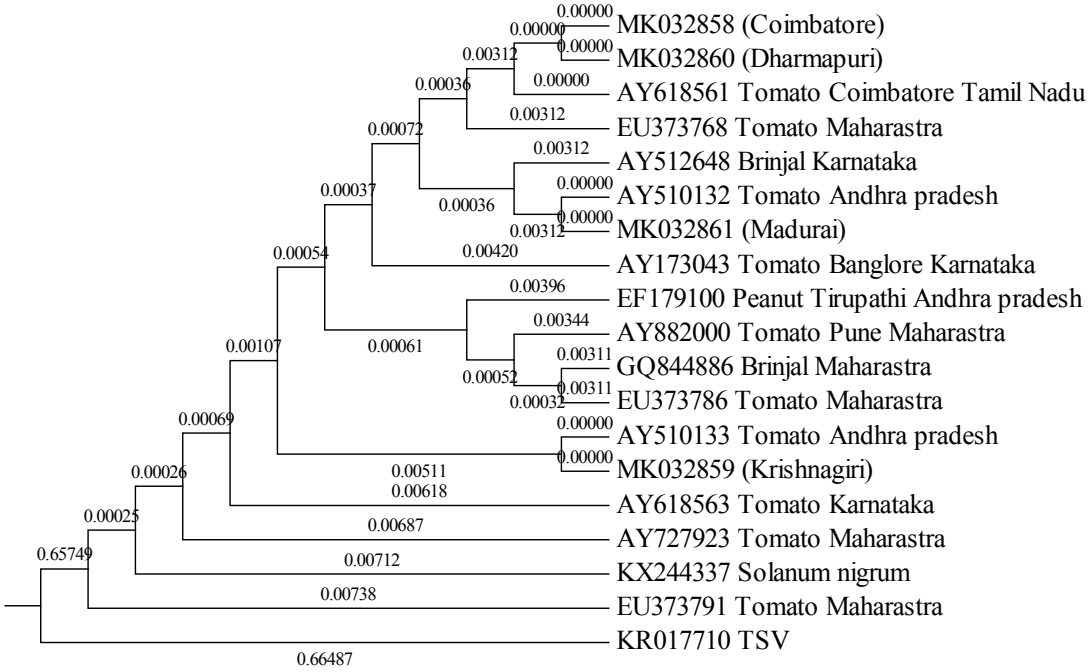

Fig. 4. Phylogenetic tree constructed using MEGA version 4.0 illustrating phylogenetic relationships based on the multiple alignments of the replicase gene sequences of different isolates of GBNV

4. CONCLUSION

Nucleocapsid protein gene of GBNV isolates of Tamil Nadu compared with different crops and different agro-ecological zones in India have no much variation in nucleotide and aminoacid levels.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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