Madras Agric. J., 103 (7-9): 254-257 September 2016
https://doi.org/10.29321/MAJ.10.001030

Complete RNA1 Genome Sequence of *Tobacco streak virus* Infecting Okra (*Abelmoschus esculentus* L.) and its Phylogenetic Relationship

S. Rajamanickam* and G. Karthikeyan
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 641 003

Tobacco streak virus **(TSV) was detected from infected okra (*Abelmoschus esculentus* L.) samples collected from the field. They were also serologically positive in direct antigen coating enzyme linked immunosorbant assay (DAC-ELISA). Sap inoculation of the okra strain induced local as well systemic infection with the production of circular necrotic lesions infection on cowpea cv. C 152 plants. TSV was characterized by RT-PCR using replicase gene specific primers with three different fragments. The replicase gene was amplified into three different fragments with a size of 1.11, 1.19 and 1.26 kb including the UTR region as part of RNA1 of TSV. The BLAST analysis of full length replicase gene had nucleotide similarity of 99.5 to 79.0 % and amino acid sequence shared sequence similarities of 99.4 to 86.8 % with known strains of TSV. Phylogenetic analysis of the nucleotide confirms that the okra strain of TSV forms single subgroup with other crop of Indian isolates.**

Key words: Replicase gene, *Tobacco streak virus*, Sequence analysis.

Okra (*Abelmoschus esculentus* L.) is an important vegetable crop in India and worldwide. Okra is a good source of vitamins A, B, C as well as protein, carbohydrates, fats, minerals, iron and iodine. Consumption of 100 g of fresh okra fruit provides 20%, 15% and 50% of the daily requirement of calcium, iron and ascorbic acid, respectively and older fruits are used in processed products (Fajinmi and Fajinmi, 2010). The crop has been infected by various viral diseases in India including *Yellow vein mosaic virus* and *etc*. Among the viral diseases *Tobacco streak virus* is a major problem in recent days infecting okra under field conditions. The infected plants were produced symptoms of malformed fruit with extensive chlorotic streaks on the fruits and stunting of plants with chlorotic lesion on leaves. Johnson (1936) reported infection of TSV in tobacco and it is a member of the genus *Ilarvirus* under the family *Bromoviridae*. TSV is a major limiting factor in okra production in India and in addition to okra, it infects various crops (Sivaprasad *et al*., 2010; Bhaskara Reddy *et al*., 2012) in India. TSV can be transmitted mechanically, but the transmission of TSV commonly occurs through different species of thrips *viz., Megalurothrips usitatus, Frankliniella schultzei* , *Scirtothrips dorsalis*, *Thrips palmi* and *Thrips* tabaci under field (Jagtap *et al*., 2012). Alternative host plants have been suspected to harbour TSV, which have contributed in its transmission. The virus causes asymptomatic infections in several common weed species, including *Parthenium hysterophorus, Ageratum conyzoides* and *Corchorus trilocularis,* whose pollen is a major source of TSV and these plants, also harbour thrips (Prasada Rao *et al*., 2003; Shukla *et al*., 2005). Though the occurrence of TSV has been reported from many hosts in India, only limited reports are available on the biological and molecular characterization of these isolates and their exact identification remains unaddressed in okra. In this study, we report the natural occurrence of TSV on okra and its molecular properties and phylogenetic relationship with other TSV isolates.

*Corresponding author's e-mail: rajamanickam.path@gmail.com

Materials and Methods

Virus isolates and propagation

Okra (*Abelmoschus esculentus* L.) plants showing characteristic symptoms of TSV were collected from naturally infected field at Coimbatore of Tamil Nadu state and used as inoculum of virus. The infected plants were identified by the presence of malformed fruit with extensive chlorotic streaks on the fruits and stunting of plants with chlorotic lesion on leaves (Fig. 1). The TSV infected samples collected from field were subjected to direct antigen coating-ELISA (DAC-ELISA) as per the procedure described by Hobbs *et al*. (1987) with the polyclonal antiserum specific to TSV (kindly provided by the ICRISAT, Hyderabad). The cowpea plants cv. C 152 was used for propagating the virus, since they produce typical local lesion symptoms within 3-4 days of inoculation. The cowpea plants were raised in the glasshouse under insect proof conditions. The virus extract was prepared by macerating TSV infected fruit samples with 0.1M sodium phosphate buffer pH 7.0 using ice tray and inoculated mechanically in cowpea cv. C125 cotyledonary leaves of six day old plants previously dusted with 600 mesh carborundum powder. The inoculated plants were kept under observation for 4-5

days for the expression of symptoms (Subramanian and Narayanasamy, 1973).

Fig. 1. Symptoms of TSV on Okra *(Abelmoschus esculentus L.)* with chlorotic streaks on fruits

Reverse transcription-PCR

Total RNA approximately, 65 µg/µl were extracted from 100 mg leaves of cowpea infected with different isolates of TSV separately using RNeasy plant extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturers protocol and resuspended in 50 µl nuclease free water. To obtain full length of replicase gene of TSV, three different pair of primers viz., GKTSV 4F - 5' ATCCGAAACAGAACCTCCAG 3' and GKTSV 4R - 5' TCCATCAACGCGTACCTAAC 3'**;** GKTSV5F - 5' ACCTGTAAGTGCCGAATGG 3' and GKTSV 5R - 5' TAGCTCTTTCGTACTGACCG 3' and GKTSV6F - 5' AGTGGTACCCCT CTGCACC 3' and GKTSV 6R- 5' TCGCAGGTTCCCTGCGGC 3' were designed. RT-PCR was carried out in Eppendorf Mastercycler Gradient ES with the OneStep RT-PCR kit (Bioline, USA Inc., USA) in 50µl reaction volume containing total RNA, 2 units of enzyme mix and replicase gene fragments specific primers to amplify the complete coding region of replicase gene of TSV. The amplified products were analyzed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager).

Cloning and sequencing

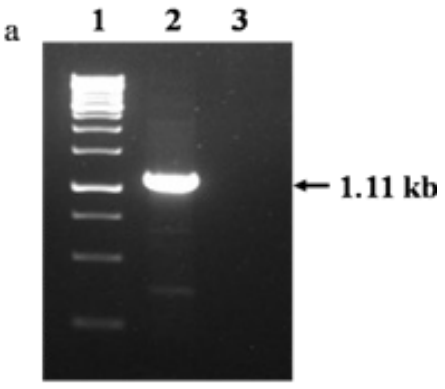
The amplicon of replicase gene fragments were purified using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions and transformed into *Escherichia coli* DH5α by following standard molecular biology procedures (Sambrook *et al*., 1989). Plasmid DNA was isolated from the potential recombinant clones using Wizard plus DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol and the positive clones were identified by restriction digestion analysis using *EcoRI* enzyme. The three independent clones were sequenced from both orientations for each fragment separately. The sequences were then edited using the BIOEDIT Software (Hall, 1999). Sequence similarity search of the GenBank database was done using the Basic Alignment Search Tool (BLAST) program.

Sequence diversity analysis

The amino acid sequences of the TSV replicase gene was translated from the consensus nucleotide sequence using the EMBOSS Transeq program (Rice *et al*., 2000). Both the nucleotide and amino acid sequences were then aligned with selected sequences of TSV strains using the CLUSTAL W program (Larkin *et al*., 2007). Phylogenetic analysis was done on MEGA 5.1 (Tamura *et al*., 2011) and trees were created using the neighbour-joining method (Saitou and Nei, 1987). The robustness of the trees was determined by bootstrap using 1,000 replicates. PRNV was used as a reference out-group member of the genus *Ilarvirus* for rooting the phylogenetic tree.

Results and Discussion

The okra plants showing characteristic symptoms of TSV like presence of malformed fruit with extensive chlorotic streaks on the fruits and stunting of plants with chlorotic lesion on leaves were collected and inoculated separately on cowpea cv. C152 plants through mechanical sap inoculation. The assay host cowpea cv. C152 expressed distinct local lesions on 3 to 4 days after inoculation. The inoculated cowpea cotyledonary leaves developed necrotic lesions and then the systemic veinal necrosis occurred. The veinal necrosis resulted in severe stem necrosis and lead to the collapse of inoculated plants. The results of DAC-ELISA revealed that, the samples exhibited characteristic symptoms of TSV showed strong positive reaction with approximately five fold increase in absorbance values than the apparently healthy samples. The TSV infected okra shows symptoms viz., stunting, chlorosis and chlorotic streaks on the fruits under field conditions. The okra plants that are infected fruit exhibiting typical symptoms of TSV were collected and inoculated on cowpea cv. C 152 plants, which produced typical necrotic lesions on inoculated primary leaves, systemic veinal necrosis and death of plants under glasshouse condition.



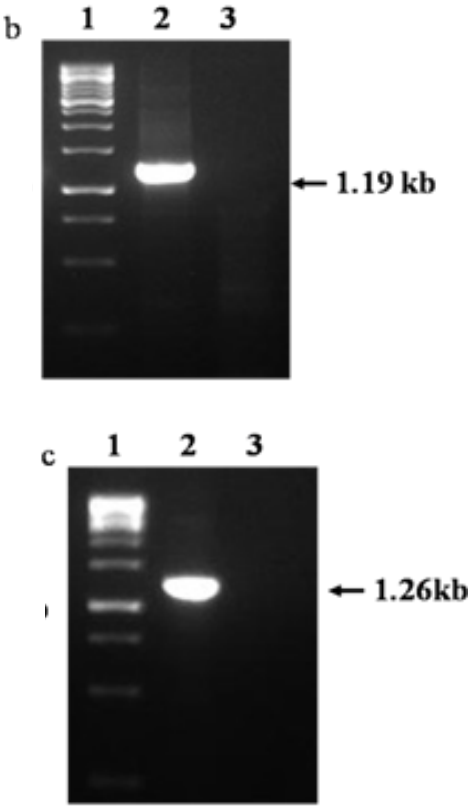


Fig. 2. RT-PCR amplification of TSV replicase gene from okra infected sample. Lane 1: 1 kb ladder; Lane 2: amplified DNA fragment from infected samples; Lane 3: healthy samples (Control)

Similar symptoms of TSV have been reported in different crops *viz.,* blackgram (Ladhalakshmi *et al*., 2006), soybean (Arun Kumar *et al*., 2008) and knaf (Bhaskara Reddy *et al*., 2012). Bhaskara Reddy *et al*. (2012) found that, the polyclonal antibody raised against the TSV showed positive reaction for sample collected from natural infection of *Hibiscus cannabinus* in DAC-ELISA.

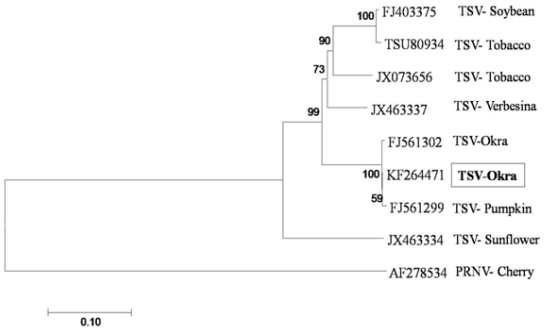


Fig. 3. Neighbour-joining phylogenetic tree based on the nucleotide sequences of the replicase gene sequences of the okra strain of Tobacco streak virus (TSV) (KF264471) and Prunus ringspot necrosis virus is defined as an out-group.

The complete genome (RNA1) encoding replicase of TSV was amplified by RT-PCR using three different pair of primers. The amplicon sizes of 1.11 kb, 1.19 kb and 1.26 kb were obtained corresponding to replicase gene fragments, respectively (Fig. 2). The amplified DNA fragments of replicase were cloned into pGEM-T easy vector and transformed into *E. coli* strain DH5α. Three independent clones were sequenced in both the orientations using universal M13 primers for above three different fragments separately and consensus sequences were determined. The replicase gene sequence of TSV okra isolate was submitted in NCBI genbank database (Accession No. KF264471). The replicase ORF of each of TSV isolates consists of 3279 bases encoded the protein with 1093 amino acids. The sequence analysis of nucleotide sequence of replicase gene revealed that they shared nucleotide sequence similarities of 99.5 to 79.0% and amino acid sequence similarities of 99.4 to 86.8 % (Table 1). The replicase gene of TSV isolates were compared with corresponding gene from known TSV isolates at the nucleotide and amino acid sequence levels. Cluster dendrograms revealed that, TSV were most closely related and forming one cluster (Fig. 3). The nucleotide and deduced amino acid analysis of replicase gene of TSV isolates revealed that, they shared nucleotide

Table 1. Nucleotide (nt) and amino acid (aa) identities of replicase gene of TSV okra strain (KF264471) with corresponding sequences of selected strains of TSV and PRNV.

Accession No.	Strain	Country	Per cent similarity	
			Nucleotide (nt)	Amino acid (aa)
FJ561302	TSV-Okra	India	99.5	99.4
FJ561299	TSV- Pumpkin	India	99.3	99.0
JX073656	TSV- Tobacco	USA	87.5	93.2
FJ403375	TSV- Soybean	USA	86.4	93.1
TSU80934	TSV- Tobacco	USA	86.1	89.3
JX463334	TSV- Sunflower	Australia	79.0	86.8
JX463337	TSV- Verbesina	Australia	88.3	93.6
AF278534	PRNV- Cherry	USA	47.0	36.1

identities of 99.5% and amino acid sequence identities of 99.4% to the TSV isolates from okra (FJ561302), shared nucleotide identities of 99.3% and amino acid sequence identities of 99.0% to the TSV isolates from pumpkin (FJ561299) available in NCBI genbank (Table 1). Bhat *et al*. (2002) conducted serology and characterization of coat protein studies for the sunflower *ilarvirus* from India and they reported that it should be regarded as a strain of TSV belonging to subgroup I, designated as TSV-SF, which shared 90 % amino acid sequence identity with TSV (strain WC). Almeida *et al*. (2005) amplified coat protein gene of TSV with a size of 717 nucleotides along with 287 nucleotides at 3' untranslated region using RT-PCR and the results revealed that nucleotides and amino acids showed 96 to 98 % similarity to other TSV isolates. They also reported TSV isolate causing soybean bud blight disease in Brazil was reported to be a distinct strain of TSV (TSV-BR), which shared 81.3 and 80.7% nucleotide sequence homology with the CP gene of TSV-WC and TSV-MB (mungbean isolate from India). Ravi *et al*. (2001) characterized the coat protein gene of TSV infecting sunflower and based on sequence analysis of the TSV, they reported as a strain of TSV distinct from the type strain TSV-WC.

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Received after revision: September 09, 2016 ; Accepted: September 30, 2016