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Sequence Diversity Analysis of *Tobacco Streak Virus* Infecting Okra (*Abelmoschus esculentus* L.) in India

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Tobacco streak virus **(TSV) is the most economically important virus infecting okra (*Abelmoschus esculentus* L.) and the samples collected from the field was detected by RT-PCR using coat protein and movement protein genes specific primers. 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 on cowpea plants cv. C 152 and resulted in the production of circular necrotic lesions and death of plants. The coat protein and movement protein genes were amplified with a size of 929 bp and 1.2 kb respectively including the UTR region as part of RNA3 of TSV. Sequence analysis of the coat protein gene had nucleotide similarity of 98.3 to 99.4 per cent with known strains of TSV. The multiple sequence alignment revealed that the sequence had two unique variations at the position 15 where cytosine was substituted with adenine and it produced unique variation at the position 526 where cytosine was substituted with thiamine. There was no deletion and addition between nucleotide sequences in the group, further confirms the placement of the okra strain of TSV in a single subgroup. The nucleotide sequence of movement protein okra strain had single unique variation at position 438, where thiamine was substituted with cytosine. Phylogenetic analysis of the amino acid confirms that the okra strain of TSV forms single subgroup with other crop of Indian isolates.**

Key words: Coat protein, Movement protein, *Tobacco streak virus* and Diversity analysis.

Okra (*Abelmoschus esculentus* L.) is an important vegetable crop in India and worldwide. Young fruits are consumed fresh or cooked. The okra stem provides fiber which is used in the paper industry. 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). Okra is susceptible to at least 19 plant viruses, *Tobacco streak virus* (TSV) being reported to be a emerging virus and major constrains in recent days infecting okra. Johnson (1936) reported infection of TSV in tobacco and it is a member of the genus *Ilarvirus* under the family *Bromoviridae*. In India, the TSV infects several other crops in addition to okra (Jain *et al*, 2005; Kumar *et al*., 2008; Sivaprasad *et al*., 2010; Bhaskara Reddy *et al*., 2012). 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 conditions (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

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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.

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 (Tamil Nadu) and used as inoculum of virus. The infected plants were identified by the presence of malformed fruits with extensive chlorotic streaks on the fruits and stunting of plants with chlorosis of leaves. 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. The cowpea C 152 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).

RNA extraction and Reverse transcription-PCR analysis

Coat protein gene

The inoculum of TSV okra isolate was maintained on cowpea cv. C152 and total RNA (approximately 65 µg/µl) was extracted from 100 mg leaves of cowpea infected with TSV inoculum from okra using RNeasy plant extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturers' protocol and resuspended in 50 µl nuclease free water. The isolated RNA was subjected to RT-PCR using OneStep RT-PCR kit (Bioline, USA Inc., USA) in 50µl reaction volume containing RNA and 2 units of enzyme mix and with coat protein gene specific primer pairs GKTSV CPF - 5' AGATAAGTCGCTTCTCGGAC 3' and GKTSV CPR - 5'TGCTCGCATGGGTCATAGAC 3' to amplify the complete coding region of CP gene with a part of UTR region of RNA3 genome of TSV. The RT-PCR was performed in Eppendorf Mastercycler Gradient ES with the following thermal programme: Reverse Transcription at 50°C for 60 min, initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min and final extension of 72°C for 10 min.

Movement protein gene

The movement protein gene specific primer pair GKTSV 1F - 5' GTATTCTCCGAGCTT AAGATAC 3' and GKTSV 1R - 5' ATGGTCTGGACCTTGGATCA 3' was used to amplify the coding region of movement protein (MP) gene. RT-PCR was carried out with the OneStep RT-PCR kit (Bioline, USA Inc., USA) in 50µl reaction volume containing RNA and 2 units of enzyme mix and with movement protein gene specific primers to amplify the complete coding region of MP gene of TSV isolate. The PCR settings comprised of 35 cycles of amplification including denaturation at 94°C for 2 min, annealing at 61°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR product was analyzed on a 1.2 % agarose gel, stained with ethidium bromide and viewed under transilluminator.

Cloning and sequencing

The amplicon of coat protein and movement protein genes 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, 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 at Chromos

Biotech Pvt. Ltd., Bangalore 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 coat protein and movement protein genes were translated from the consensus nucleotide sequences 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. *Prunus ringspot necrotic virus* (PRNV) was used as a reference out group member of the genus *Ilarvirus* for rooting the phylogenetic tree.

RESULTS

Isolation of virus and serodiagnosis

The okra showing characteristic symptoms of TSV like presence of malformed fruits with extensive chlorotic streaks on the fruits and stunting of plants with chlorotic leaves were collected (Fig. 1) 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 the entire 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.

Cloning and sequencing

Total RNA extracted from cowpea samples infected with TSV was analyzed by RT-PCR with specific primers corresponding to coat protein and movement protein genes. The result revealed that, infected samples resulted in the amplification of products with amplicon sizes of 929 bp and 1.2 kb corresponding to CP and MP genes respectively (Fig. 2). The amplified DNA fragments were cloned into pGEM-T easy vector and sequence determined. The nucleotide sequence analysis confirmed the association TSV.

Table 1. Nucleotide (nt) and amino acid (aa) identities of the coat protein gene of *Tobacco streak virus* (TSV) okra strain (KF264467) with corresponding sequences of selected strains of TSV

Accession No	Strain /Host	Country	Percentage (%) identity	
			nt	aa
DQ864456	Okra	India	99.4	100.0
DQ864455	Okra	India	99.4	100.0
AY501481	Okra	India	99.4	100.0
AY510130	Okra	India	98.8	99.5
FJ561304	Okra	India	99.4	99.1
FJ655173	Okra	India	99.1	99.1
FJ655172	Gherkin	India	99.3	99.1
FJ655170	Squash	India	99.3	99.5
FJ655169	Marigold	India	99.3	99.1
GQ401240	Groundnut	India	99.0	99.1
DQ058079	Cowpea	India	99.1	99.5
DQ225172	Urdbean	India	99.0	99.1
AY590139	Chilli	India	98.3	98.3
AF515824	Cotton	India	99.3	99.5
AJ969095	Rose	India	40.6	18.2

Table 2. Nucleotide (nt) and amino acid (aa) identities of the movement protein gene of Tobacco streak virus (TSV) okra strain (KF264463) with corresponding sequences of selected strains of TSV

Accession No	Strain /Host	Country	Percentage (%) identity	
			nt	aa
FJ561304	Okra	India	99.1	99.3
FJ655173	Okra	India	99.5	99.6
FJ655172	Gherkin	India	99.1	99.3
FJ655171	Cowpea	India	98.8	98.9
FJ655170	Squash	India	99.6	100.0
FJ655169	Marigold	India	99.0	98.9
FJ608537	Watermelon	India	98.7	98.9
FJ403377	Soybean	India	89.6	93.1
FJ417083	Marigold	India	99.0	94.8
FJ417082	Amaranth	India	99.6	100.0
DQ141601	Sunflower	India	98.6	98.2
FJ561301	Pumpkin	India	99.5	99.6
AJ969095	Rose	India	42.0	22.5

Coat protein gene analysis

The amplified fragment corresponding to CP gene contains single ORF consist of 717 bases encoded the protein with 239 amino acids. The coat protein gene sequence of okra strain of TSV was submitted in NCBI Genbank database (Accession No. KF264467). The sequence (KF264467) was compared with corresponding genes from known TSV isolates at the nucleotide and amino acid sequence levels. The sequence had 98.4% nucleotide homology with other okra TSV strains (DQ864456; DQ864455 and AY501481), 99.8 and 99.4 % nucleotide homology with other TSV strains from okra (AY510130 and FJ561304) respectively (Table 1). Multiple nucleotide sequence alignment and phylogenetic analysis revealed very high homologies between the TSV strains and confirmed the formation of single subgroup (Fig 3 & 4). Multiple sequence alignment further revealed a near perfect homology between the nucleotide sequence of the okra strain and the nucleotide sequences of other strains except for a unique variation at position 15 where cytosine was substituted with adenine. Also strain produced unique variation at the position 526 where cytosine was substituted with thiamine (Fig. 5). The results revealed that no deletions and additions between nucleotide sequences in the subgroup, further confirms the placement of the okra strain of TSV as a single subgroup. Analysis of the 239 deduced amino acid sequence of the 3' end of the coat protein gene of RNA 3 revealed that the okra strain of our TSV had 100% homology except the okra strains (AY510130 and FJ655173), also had similarity of more than 98.3% with other strains of the same virus. Two of the five okra strain from other parts of India used for comparison, our strain had variation at the position 7th where the glycine was substituted with serine. Similarly, position 22 and 59 where threonine was substituted with aspargine respectively and aspartic acid was substituted with aspargine (Fig. 6).

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

Lane 1- 100 bp/1 kb ladder
Lane 2- Positive Sample
Lane 3- positive Sample
Lane 4- Healthy Control

Fig. 2. RT-PCR analysis of TSV using CP (Fig a) and MP (Fig b) gene specific primers

Movement protein gene analysis

The movement protein ORF consist of 873 bases encoded the protein with 291 amino acids. The okra strain of TSV movement protein gene sequence was submitted in NCBI GenBank database (Accession No. KF264463). The okra strain (KF264463) was compared with corresponding genes from known TSV isolates at the nucleotide and amino acid sequence levels. The strain of okra had 99.1 and 99.5% nucleotide homology with members of TSV isolates from okra (FJ561304 and FJ655173) respectively. The strain

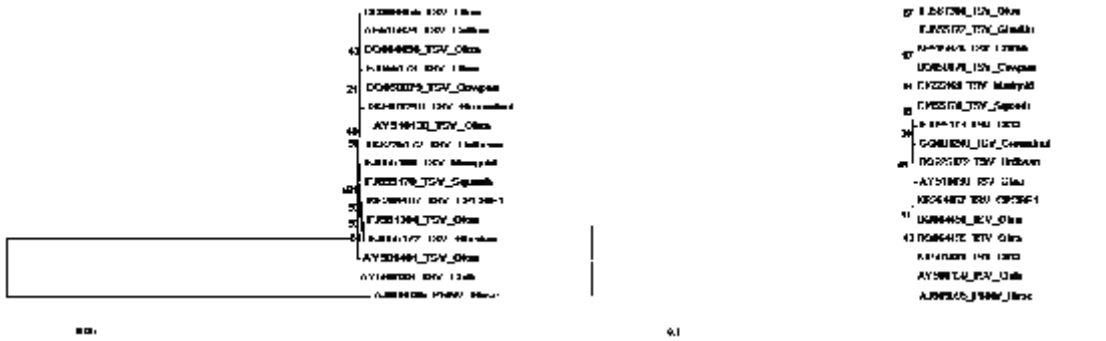

Fig. 3. Neighbour-joining phylogenetic tree based on (a) the nucleotide sequences of the coat protein gene and (b) the amino acid sequences of the okra strain of *Tobacco streak virus* (TSV) (KF264467) and *Prunus ringspot necrosis virus* is defined as an out-group.

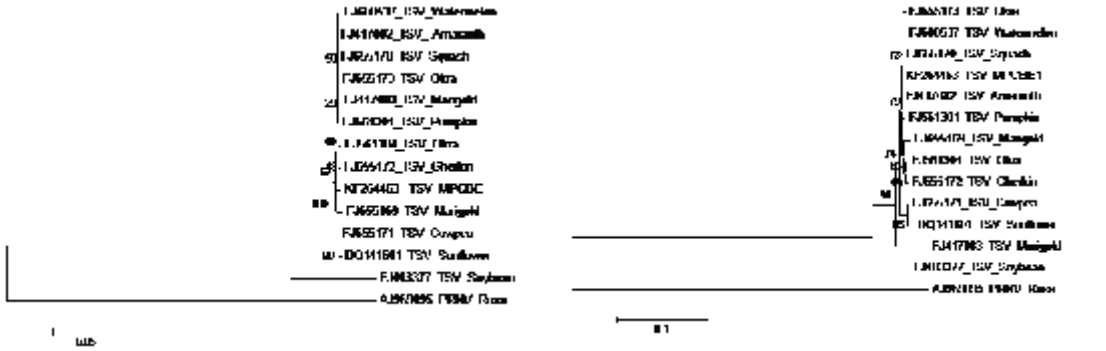

Fig. 4. Neighbour-joining phylogenetic tree based on (a) the nucleotide sequences of the Movement protein gene and (b) the amino acid sequences of the okra strain of *Tobacco streak virus* (TSV) (KF264463) and *Prunus ringspot necrosis virus* is defined as an out-group.

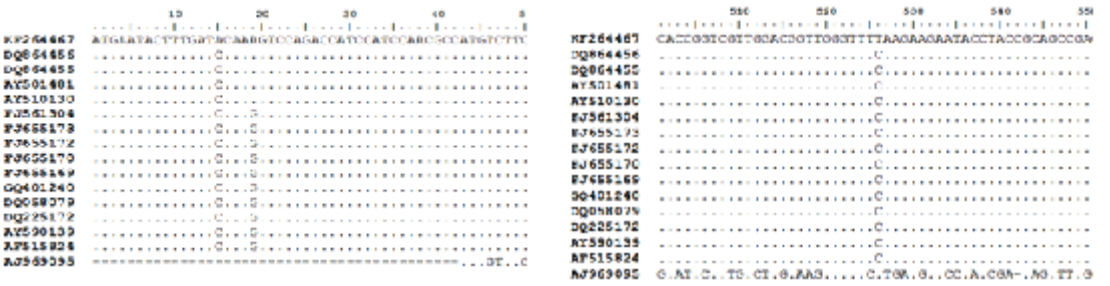

Fig. 5. Multiple sequence alignment of the 717 nucleotide sequences of the coat protein gene of the okra strain of *Tobacco streak virus* (TSV) and corresponding sequences of 14 selected strains of TSV. *Prunus ringspot necrosis virus* is defined as an out-group.

also showed 89.6 to and 99.0 % nucleotide homology with members of known TSV isolates available in NCBI genbank (Table 2). Multiple sequence alignment revealed the nucleotide sequence MP gene of okra strain had single unique variation at position 438, where thiamine was substituted with cytosine. It also had deletions in the positions 478, 479 and 525 against marigold strain (FJ417083) respectively where guanine was present. Similarly strain produced deletion at the position 305 to 310 against watermelon strain (FJ608537), where adenine was present (Figure 7). Analysis of the 291 deduced amino acid sequence of the 5' end of the movement protein gene of RNA 3 revealed that the okra strain of our TSV had 100% homology with the squash strain (FJ655170) and amaranth (FJ417082) and also had similarity of more than 99.1% with other strains of the same virus. Multiple sequence alignment revealed the amino acid had deletion at the position 101 and 102 against

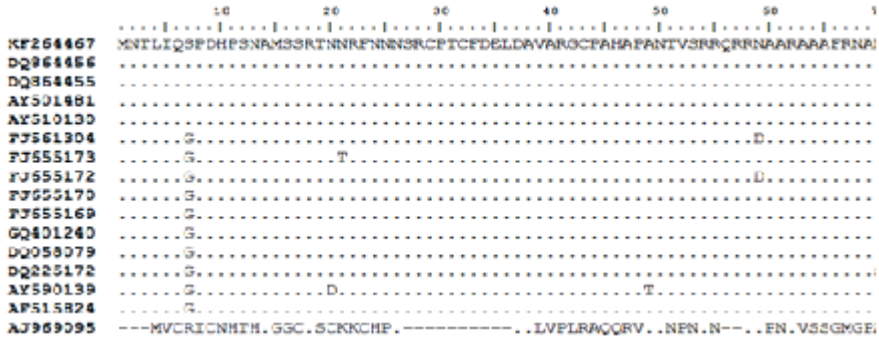

Fig. 6. Multiple sequence alignment of the 239 amino acid sequences of the 3' end of the coat protein gene of the okra strain of *Tobacco streak virus* (TSV) and corresponding sequences of 14 selected strains of TSV. *Prunus ringspot necrosis virus* is defined as an out-group.

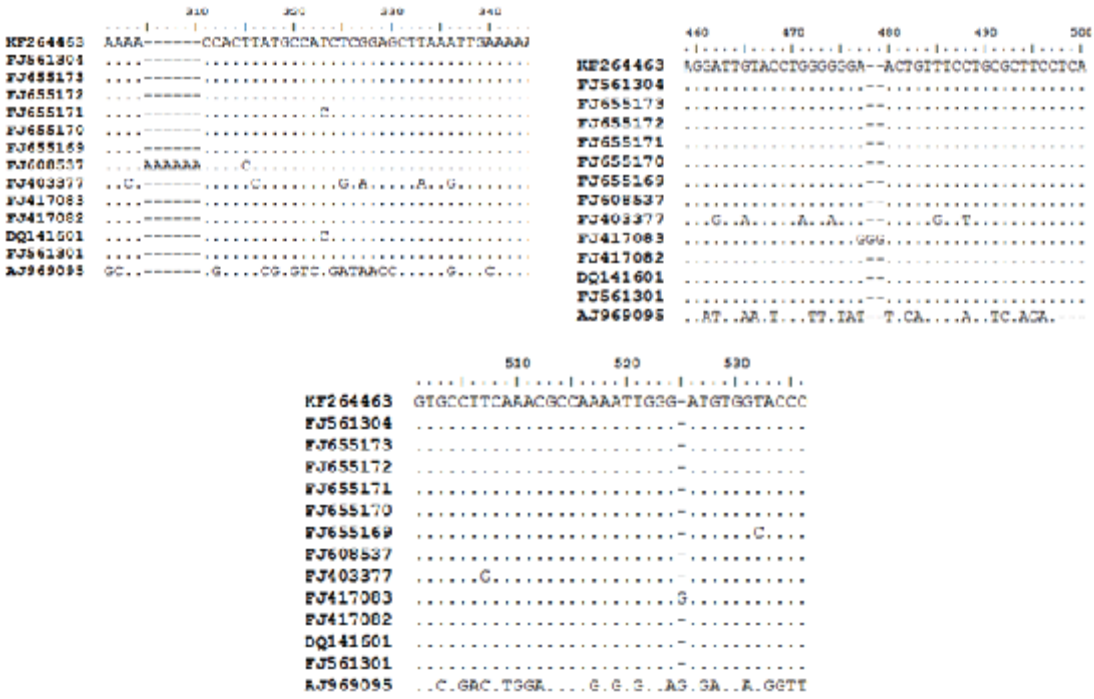

Fig. 7. Multiple sequence alignment of the 873 nucleotide sequences of the movement protein gene of the okra strain of *Tobacco streak virus* (TSV) and corresponding sequences of 12 selected strains of TSV. *Prunus ringspot necrosis virus* is defined as an out-group.

the strain marigold (FJ417083), where glycine was present. Similarly, it produced deletion at 159th the position against the watermelon strain (FJ608537), where lysine was present (Fig.8).

DISCUSSION

Molecular characterization of *Tobacco streak virus* infecting okra (*Abelmoschus esculentus* L.) in Tamil Nadu provides knowledge of better understanding the genetic composition, variation caused by mutation and recombination and correct taxonomic position. TSV had wider host range and extending its host range day by day. Due to different environmental constraints on the evolution of new strains, it is important to study the phylogenetic relationship of the viruses locally. TSV on okra showed symptoms *viz*., stunting, chlorosis and chlorotic streaks on the fruits under field conditions. The okra plant samples exhibiting typical symptoms of TSV were collected and inoculated on cowpea cv. C 152 plants, resulted in production of typical necrotic lesions on inoculated primary leaves, systemic veinal necrosis and death of plants under glasshouse conditions.

The production of brown necrotic lesions is the characteristic symptoms on cowpea by mechanical inoculation of TSV (Ramiah *et al*., 2001; Reddy *et al*., 2002; Arun Kumar *et al*., 2008; Ladhalakshmi *et al*., 2006). Serological or immunological assays have been developed and successfully used for a number of years for the detection of plant viruses. TSV infected okra collected from field were found to be positive for TSV specific polyclonal antibody. This type of results was supported by Bhaskara Reddy *et al*. (2012) who raised the polyclonal antibody against the TSV and showed positive reaction for sample collected from natural infection of *Hibiscus cannabinus* in DAC-ELISA. Prasad Rao *et al.* (2000) also proved the detection of a new strain SB-10 of TSV from potato through DAC-ELISA.

RT-PCR has been shown effective in rapid and sensitive detection of many plant viruses (Candresse, 1998). The two set of primers used in this study were self-designed and amplified the CP and MP gene of TSV okra strain. Approximately 929 bp including UTR corresponding to CP gene were amplified using specific primer, when total RNA extracted from infected tissues, while no such band was observed when total RNA extracted from healthy tissue. Similarly, the movement protein gene also amplified with an amplicon size of approximately 1.2 kb including UTR using RT-PCR. The products were cloned and its nucleotide sequences were determined. The coat protein gene of TSV isolates were compared with corresponding gene from known TSV isolates at the nucleotide and amino acid sequence levels. Phylogenetic analysis revealed very high homologies between the TSV strains and confirmed the formation of single subgroup (Figure 3). Multiple sequence alignment revealed a near perfect homology between the nucleotide sequence of the okra strain and the nucleotide sequences of other strains. The results showed no deletions and additions between nucleotide sequences okra strain, further confirms the placement of the okra strain of TSV as a single subgroup. Similarly movement protein gene showed single unique variation and some of deletion in nucleotide sequences as well some of the deletion in amino acid sequence against the

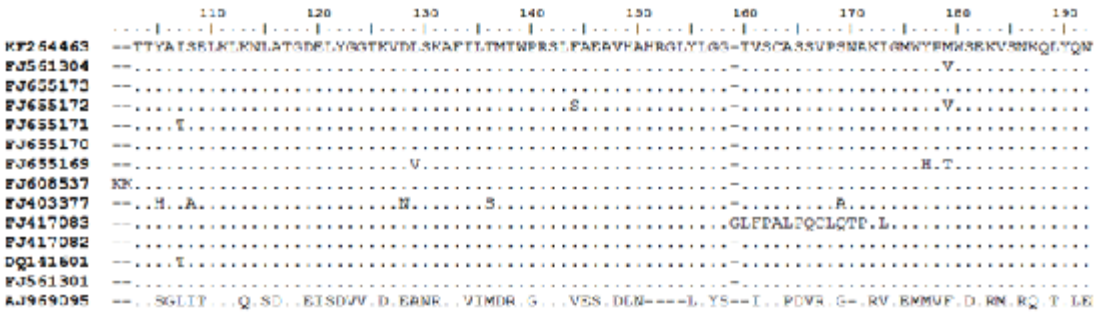

Fig. 8. Multiple sequence alignment of the 291 amino acid sequences of the movement protein gene of the okra strain of *Tobacco streak virus* (TSV) and corresponding sequences of 12 selected strains of TSV. *Prunus ringspot necrosis virus* is defined as an out-group.

strains of marigold (FJ417083) and watermelon (FJ608537). Cornelissen *et al*. (1984) cloned and sequenced TSV RNA3 genome reveals that, complete sequence of 2,205 nucleotides of TSV RNA 3, confirming 140 bp 3'-terminal residues. Two long open reading frames starting with a methionine codon are revealed by this sequence. One begins at the first AUG triplet from the 5'-end at nucleotides 211-213 and terminates with an UGA triplet at residues 1,078-1,080. 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 per cent 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 per cent 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. Reddy *et al*. (2002) used RT-PCR to amplify the RNA 3 sequence correspond to the 3' terminal part of the movement protein and the 5' terminal part of the coat protein gene of TSV and it revealed that, over the entire sequenced portion (868 nucleotide), the sequence identity was 88.4 per cent to RNA 3 of TSV (WC strain). The study of CP gene of okra strain of TSV revealed there was no amino acid changes except, three positions compared to other Indian isolates used in the study. Same study revealed MP gene showed some of the deletion in between the nucleotide and amino acid sequence. This could help in the studying genetic diversity of virus to understand the evolutionary mechanisms that generate and/or maintain variation in viral populations and their evolution. Such studies may help in the development of strategies for the control of viral diseases.

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