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Research article

PARTIAL CHARACTERIZATION OF REPLICASE GENE OF *TOBACCO STREAK ILARVIRUS* IN ONION (*ALLIUM CEPA*.L)

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ABSTRACT: *Tobacco streak* virus (TSV) is an important emerging virus belongs to the genus Ilarvirus and family *Bromovidae*. The Onion crop is infected by *Tobacco streak ilar* virus and it is major problem in different places of Andhra Pradesh in South India and also transmitted by thrips vector. TSV suspecting onion samples were identified by direct antigen coating enzyme linked immunosorbent assay using TSV polyclonal antiserum. The Replicase gene from each isolate was amplified using TSV replicase gene specific primers by using the RT-PCR. The ~530 bp product was amplified, cloned, sequenced and determined its length as 534 nucleotides and codes for 178 amino acids. The partial sequence of TSV-Rep shared identity of 87.6 -99.8% at nucleotide levels and 67.8-99.4% at amino acid levels respectively with other reported TSV isolates. The phylogenetic tree relationship based on the nucleotide sequence of present study isolate (AP-Onion-Chittoor) from different geographical regions was also analyzed in this study.

Key words: DAC-ELISA; Onion; RT-PCR; *Tobacco streak virus*

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important horticultural crop belongs to the family *Alliaceace* grown in India and other countries of the world. India occupies second position after China in the production of onion with the annual production of over 85 million tons. In India it is grown in an area of 12.04 lakhs ha with the productivity of 194.02 lakhs MT (NHRDF, 2015). It has a lot of medicinal properties by containing a chemical compound such as quercetin which shows anti-cancer, anti-cholesterol, anti-inflammatory, and anti-oxidant properties. Some reports showed that onion plays a significant role in preventing heart diseases (Augusti, 1990). Onion stocks are affected by more than 20 viruses in the world-wide and it belonging to the genera *Potyvirus*, *Carlavirus*, *Allexivirus*, *Ilarvirus* and *Tospovirus* which causes more qualitative and quantitative losses (Barg 1997; Pringle 1998; Van Dijk 1993a, 1993b; Walkey 1990; Maliogka *et al.* 2006; Sivaprasad *et al.* 2010; Sujitha *et al.* 2012 ; Hamed 2012). A disease characterized as *Tobacco streak virus* (TSV) with symptoms of straw colored, irregular, necrotic lesions of stalks in onion (*Allium cepa* L.) was reported in India based on the serological and molecular characterization (Sivaprasad *et al.* 2010). TSV was first described by Johnson (1936) which is a member of the genus *Ilarvirus* (family *Bromoviridae*) and consists of non-enveloped, linear, tripartite positive sense ssRNA with 50 terminal cap structures. The 30 terminus is not polyadenylated, sometimes forming strong tRNA-like structures. The RNA-1 (3.5 Kb) and RNA 2 (3.0 Kb) encode proteins involved in viral RNA replication. RNA-3 (2.3 Kb) encodes protein that is required for cell to cell movement. Only RNA 4 ‘‘encodes the coat protein of c.28 kDa (Xin *et al.* 1998; Scott 2001). It is emerging as an important virus infecting several economically important crops such as sunflower, peanut, cotton, tomato, chilli, black gram, green gram, okra, onion, guar, kenaf, lablab bean, castor bean etc. in India ((Bhat *et al.* 2002; Babu *et al.* 2003; Prasada Rao *et al.* 2003; Sivaprasad *et al.*, 2010; Sivaprasad *et al.* 2012; Bhaskara Reddy *et al.* 2013; Bhaskara Reddy *et al.* 2014).TSV has a wide host range, infecting more than 200 plant species belonging to 30 dicotyledonous and monocotyledonous plant families and its occurrence has been reported from more than 26 countries worldwide (Fulton, 1985). The virus is readily sap transmissible and is naturally transmitted through pollen from infected plants with the aid of thrips, such as *Scirtothrips dorsalis*, *Frankliniella schultzeii*, *Frankliniella fusca*, *Thrips palmi* and *Megalurothrips usitatus* (Reddy *et al.* 2002; Prasada Rao *et al.* 2003).

However, the molecular identity of TSV replicase gene of onion was not addressed. Thus, the present study was aimed to study the identity of replicase gene of *tobacco streak virus* infecting onion. The replicase protein gene from TSV isolates from onion samples were cloned, sequenced and compared with other reported *ilarvirus*. We here describe these aspects in detail.

MATERIALS AND METHODS

Virus isolates

Naturally affected Onion (*Allium cepa* L.) leaf samples exhibiting straw colored, irregular, necrotic lesions collected during 2011-2013 from Kadapa, Kurnool, Chittoor, Nellore, Guntur, Anantapur states in Andhra Pradesh. Onion leaf samples (n=38) were collected and initially screened by direct antigen coating-enzyme-linked immunoassay (DAC-ELISA) (Clark and Joseph 1984) using polyclonal antisera of TSV. The ELISA confirmed virus cultures were maintained on cowpea (cv.C-152, a diagnostic assay host) plant by sap inoculation in the net house. Healthy cowpea plants were also maintained as controls.

Analysis of replicase (Rep) gene of *Tobacco streak virus*

Isolation of RNA and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA from 100 mg of healthy and TSV confirmed onion leaf samples were isolated using RNase plant minikit according to the manufacture's instructions (Qiagen, Valencia, CA). The resulting total RNA was incubated with TSV-rep gene specific reverse primer at 65°C for 5 min and snap-chilled on ice for 2 min. cDNA was synthesised using M-MuLV reverse transcriptase (Fermentas, USA) at 42°C for 1 h. The genome sense primer TSV-Rep-F-5′ATTCCTTATCTTTACCCACCGTGA 3′; and antisense primer, TSV-Rep-R; 5′ CATGGGCTTCAGATAAGCTAAGG 3′ were used to amplify the replicase protein gene of TSV) Two micro litres of cDNA were amplified in a 25 ml reaction volume containing 2.5U of Taq DNA polymerase (Fermentas, Maryland, USA), 10 pmol of forward (TSV-Rep-F) and reverse primer (TSV-Rep-R), 2.5 mM MgCl2 and dNTPs of 10 mM each. PCR amplification conditions included an initial denaturation cycle of 2 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 52°C and extension for 1 min at 72°C with a final extension 60 min at 72°C. The amplified products were resolved following electrophoresis through a 1% agarose gel and visualised in a UV gel documentation system (Alpha Innotech, San Leandro, CA) after staining with ethidium bromide (10 mg/ml).

Cloning and sequencing of PCR products

The PCR product (~534 bp) was eluted by QIAquick gel extraction kit (Qiagen, Valencia, CA) and cloned into pTZ57R/T vector (Fermentas, Maryland, USA) according to the manufacture's instructions. The resulting recombinant plasmids were transformed into *Escherichia coli* strain DH5α cells. Recombinant clones were confirmed through colony PCR and restriction digestion using enzymes *BamH1* and *Xba1*. The resulting clones with expected size DNA inserts were sequenced using M13 universal primers at Eurofins Genomics India Pvt. Ltd, Bangalore.

Sequence analysis and phylogenetic relationship

The sequences of clones were assembled and a sequence identity matrix was generated using BioEdit sequence alignment editor (version 5.09) Hall (1999). Multiple alignment were performed, a phylogenetic tree was constructed, and a bootstrapped consensus dendrogram was generated with 1000 replications using Neighbour Joining algorithm of MEGA 4 (version 4.02) (Tamura *et al.* 2007). The replicate protein genes of present isolates were compared with other known TSV isolates from GenBank (Benson *et al.* 1999).

RESULTS AND DISCUSSION

The symptoms of TSV infected onion plants (n=38) showing straw colored, irregular, necrotic lesions were observed on young leaves. The symptoms are varied depending on the age of the plants and climatic conditions. The presence of TSV was detected in 20 of the 38 onion plants from Kadapa, Kurnool, Chittoor, Nellore, Guntur and Anantapur states in Andhra Pradesh by DAC-ELISA using the TSV polyclonal antiserum. After confirmation of ELISA, the positive onion samples were maintained on cowpea (Cv-c-152) by sap inoculation; both localized and systemic symptoms were observed on cowpea plants.

The total RNA was extracted from the infected onion samples and subjected to RT-PCR using the relicase gene specific primers and resulted in an amplicon of the expected size (~530 bp) (Fig. 1). The amplified products were purified, cloned into pTZ57R/T, transformed into *E.coli* DH5α cells and the recombinant clones were confirmed by restriction digestion and PCR methods. The positive clones were sequenced in both directions with M13 universal primers and BLAST analysis. The sequence (AP-Onion-Chittoor) was deposited at the NCBI GenBank under the accession number KC297129.

The Replicase gene of TSV revealed that sequenced region contained 534 nucleotides that could potentially code for a protein of 178 amino acids. The partial sequence of TSV-Rep shared identity of 87.6 -99.8% at nucleotide levels and 67.8-99.4% at amino acid levels respectively with other reported TSV isolates (Table. 1). The Phylogenetic tree based on the replicase protein gene sequences of present study isolate with other TSV isolates from different geographical locations in Indian subcontinent (Table 1). The present study isolate from AP-Onion-Chittoor grouped together along with Sunflower (GU371445) and Sunflower (EU649674) formed a separate clade (Clade I). Okra, Pumpkin, Sunflower (JN584481), Sunflower (JN584480) and Sunflower (JN584479) formed a separate clade (Clade II). Tobacco (JX073656), Soybean and Tobacco (U80934) formed a separate clade (Clade III) at nucleotide level (Fig. 2).

Tobacco streak virus has a wide host range, infecting different economically important crops like cotton, peanut, sunflower, tomato, chilli, black gram green gram, etc in India. TSV is easily transmitted by sap and thrips vectors in persistent manner. TSV suspected straw colour and necrotic onion samples were collected from major growing areas in Andhra Pradesh, India. These onion samples were initially screened by DAC-ELISA and further confirmed by RT-PCR method. After confirmation of ELISA, onion samples were maintained in cowpea plant for further studies. Among these onion samples (n=38), only 20 samples were confirmed by DAC-ELISA by using the TSV polyclonal antiserum. Sundaresha *et al.* (2012) developed the easy and high efficiency method of sap inoculation studies for TSV in sunflower crop, these samples were confirmed by ELISA and RT-PCR methods for detection of virus. Sharman *et al.*, 2015 developed strain-specific multiplex PCR for the three RNA segments of TSV-Parthenium, Crownbeard and also some strains of naturally infecting 41 plant hosts in Australia. Padmanabhan *et al.* (2014) reported the complete genome sequence of *tobacco streak virus* infecting zucchini squash in Florida by Sanger sequencing method. Dutta *et al.*, 2015 identified the soybean plants that exhibited symptoms of *tobacco streak virus* infection were sampled from different places of Oklahoma by RT-PCR method. Almeida *et al.*, (2005) characterized the soybean bud blight disease in Brazil caused by TSV (TSV-BR), is a distinct strain of TSV. Sharman *et al.* (2008) reported TSV naturally infecting sunflower, cotton, mungbean and chickpea in Australia. Sharman *et al.* (2009) studied the seed transmission of TSV on *Parthenium hysterophorus* in Central Queensland. Abtahi *et al.* (2009) studied the host range of TSV and characterization of different lettuce samples infected with TSV in Iran and also studied the seed transmission by TSV and confirmed by DAC-ELISA using polyclonal antibodies. Sarovar *et al.* (2010) studied the TSV variability studies of 3'UTR region in sunflower, gherkin and pumpkin, the sequence analysis at nucleotide level showed 98±1% and 88% identities with the Indian and US isolates. Sudeep *et al.* (2008) studied the genetic diversity of coat protein gene of TSV infecting groundnut, chilli and sunflower showed 96-100% at nucleotide and amino acid levels. The variability studies of TSV not only is useful in establishing differences among strains that infect different crops, but also aids in evolving transgenic plants with resistance to TSV.

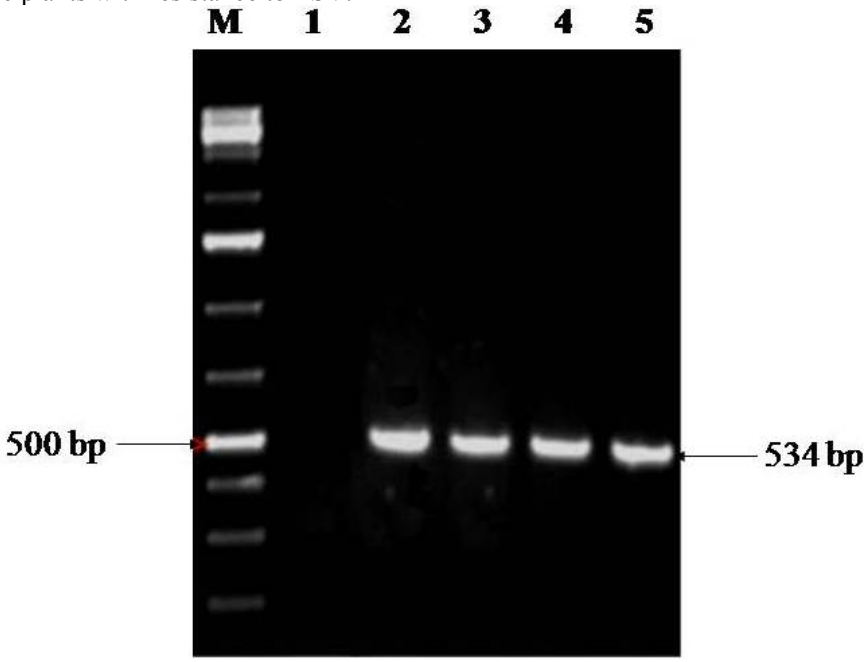

Fig.1: RT-PCR amplicons of TSV-Rep analyzed by 1% agarose gel electrophoresis. Lane M: 1 Kb DNA Ladder, 1: Onion healthy plant; 2, 3, 4 and 5: TSV infected onion plant samples.

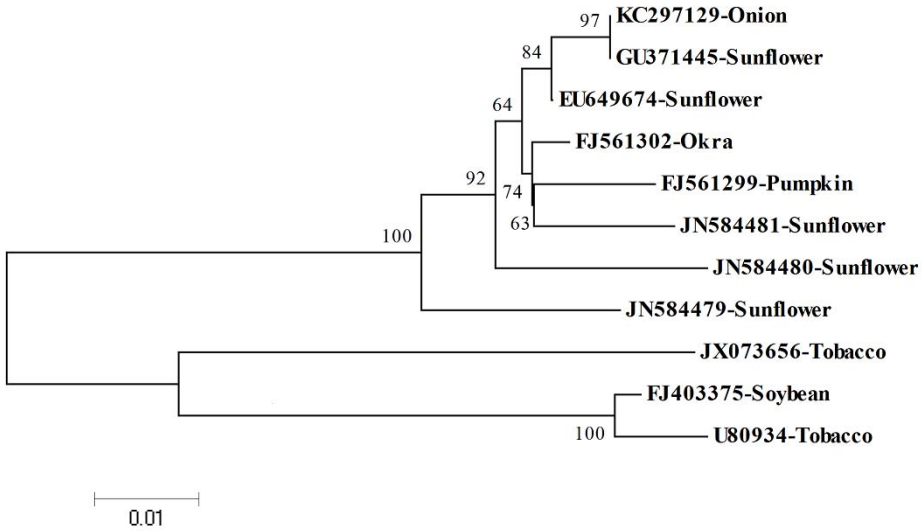

Fig.2: Phylogenetic trees constructed using neighbour joining method of MEGA version 4.1. The values at the forks indicate the number of trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base. Phylogenetic trees constructed based on the replicase protein sequences of TSV nucleotides.

Table.1: Percent identities of TSV-Replicase gene (Rep) isolates under studies with other reported TSV isolates at nucleotide (below the diagonal) level and amino acid (above the diagonal) level respectively

Isolates	KC297129	GU371445	EU649674	FJ561302	FJ561299	JN584481	JN584480	JN584479	FJ403375	JX073656	U80934
KC297129	100	99.4	98.8	97.6	97	95.2	92.2	90.5	69.5	71.5	69
GU371445	99.8	100	98.2	97	96.4	94.6	91.6	90	69	71	68.4
EU649674	99.4	99.2	100	98.8	97.6	96.4	93.4	91.7	70.7	71.5	70.1
FJ561302	98.6	98.5	99.2	100	97.6	96.4	93.4	91.7	70.7	71.5	70.1
FJ561299	97.9	97.7	98.5	98.5	100	95.2	92.2	90.5	69	71	68.4
JN584481	97.7	97.5	98.3	98.3	97.5	100	91.1	90	69	70.5	68.4
JN584480	96.8	96.6	97.3	97.3	96.6	96.4	100	89.4	68.4	69.8	67.8
JN584479	96.4	96.2	96.8	96.8	96	96	95.6	100	71.3	69	70.7
FJ403375	89.3	89.1	89.8	89.5	88.3	88.3	88.2	89.8	100	76	97.6
JX073656	89.3	89.1	89.5	89.1	88.3	88.2	88.2	88	91.1	100	76
U80934	88.7	88.5	89.3	88.9	87.8	87.8	87.6	89.3	98.8	90.8	100

CONCLUSION

The results indicate that standardization and amplification of *Tobacco streak virus* replicase gene in onion crop by using the TSV-Rep primers by RT-PCR method.

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