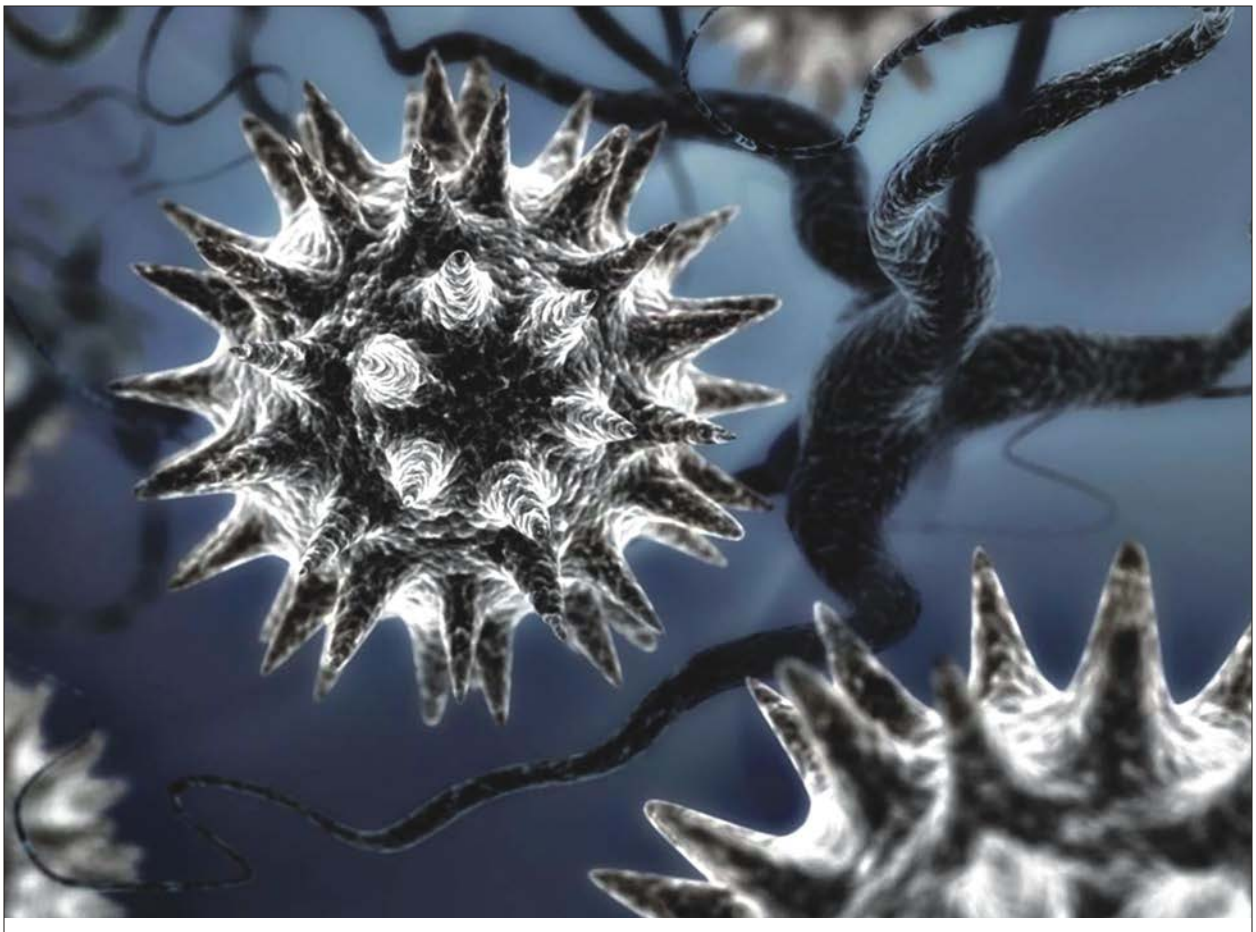

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Research Article
Serological and Molecular Identification of *Tobacco streak ilarvirus* Infecting Onion (*Allium cepa* L.) on Commercial Fields in Southern India

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Abstract

Tobacco streak virus (TSV), a member of the genus *Ilarvirus* (family: Bromoviridae) is an important viral pathogen infecting onion in Southern India. TSV isolates naturally infecting onion plants were collected from Andhra Pradesh, Tamil Nadu and Karnataka States in South India and characterized. The TSV infection was confirmed by direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA) using TSV antiserum. The CP gene was further amplified by using TSV coat protein primers. The amplicon (717 bp) was cloned, sequenced and sequence analysis revealed that the N gene shared 98.6-100 and 98.3-100% sequence identity with TSV at the nucleotide and amino acid levels, respectively. The phylogenetic relation based on the sequence of these isolates from different geographical regions was also analyzed in this study.

Key words: ELISA, RT-PCR, sequence identity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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. Onion is the 17th most valuable food commodity and the second most valuable vegetable in the world followed by tomato. It is cultivated in an area of 42.90 million ha with a production of 85.37 million t in India (http://faostat3. fao.org/ browse/Q/QC/E/2012). The major onion producing countries in the world are China, India, USA, Turkey, Egypt, Pakistan, Russia, Iran, Brazil and Netherlands. Globally, India occupies second position after China in the production of onion about an area of 1.03 million ha with a production of 15.39 million Mt (http://www. indiastat.com/agriculture/2/stats.aspx/2012). Onion shows medicinal properties such as anti-cancer, anti-cholesterol, anti-inflammatory and anti-oxidant. Increased consumption of onions reduces the risk of head and neck cancer. Recent 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 *et al*., 1997; Pringle, 1998; Van Dijk, 1993a, b; Walkey, 1990; Maliogka *et al*., 2006; Sivaprasad *et al*., 2010; Sujitha *et al*., 2012; Hamed *et al*., 2012).

Tobacco streak virus (TSV) was first described by Johnson (1936) and is emerging as one of the most important viral diseases in several economically important crops. It 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. In India, TSV was first identified in sunflower in Karnataka in the year 1997 (Singh *et al*., 1997) and peanut in Andhra Pradesh during 1999-2000 (Prasada Rao *et al*., 2000; Reddy *et al*., 2002). Since then, TSV was found to be responsible for serious damage to peanut, sunflower, cotton, tomato, chilli, black gram, green gram, okra, onion, kenaf etc. Recently TSV has been observed in guar (Sivaprasad *et al*., 2012), kenaf (Bhaskara Reddy *et al*., 2012), lablab bean (Bhaskara Reddy *et al*., 2013) and castor (Bhaskara Reddy *et al*., 2014). 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*, *F. fusca*, *Thrips palmi* and *Megalurothrips usitatus* (Reddy *et al*., 2002; Prasada Rao *et al*., 2003).

Tobacco streak virus (TSV) is a member of the genus Ilarvirus (family Bromoviridae) and consists of non-enveloped, linear, tripartite positive sense ssRNA with 5' terminal cap structures. The 3' 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 'eencodes the coat protein of c.28 kDa (Xin *et al*., 1998; Scott, 2001). RNA-1-3 are genomic and encode proteins 1a (119 kDa), 2a (91 kDa) and 3a (32 kDa), respectively, whereas RNA-4a and RNA-4 are subgenomic expressed from RNA-2 and RNA-3 encodes 2b (22 kDa) and coat protein (28 kDa), respectively. It is infectious only in presence of its coat protein or RNA-4 (Fulton, 1985; Sanchez-Navarro and Pallas, 1994; Ansel-Mckinney *et al*., 1997).

The coat protein gene from TSV isolates from onion samples were cloned, sequenced and compared with other reported Ilarvirus.

MATERIALS AND METHODS

Virus isolates and maintenance: Onion samples were collected with typical virus symptoms such as straw colored, irregular, necrotic lesions according to the Sivaprasad *et al*. (2010). These onion samples were collected from the major producing states of Andhra Pradesh (Kadapa, Kurnool, Chittoor, Nellore, Medak), Tamil Nadu (Coimbatore, Perambadur, Dindigul, Thirichirapalli, Periyar) and Karnataka (Dharwad, Chitradurg, Gadag, Bagalkot) in South India during December, 2010 to January, 2013. A total number of 144 samples were obtained from three states Andhra Pradesh (59 samples), Tamil Nadu (42 samples) and Karnataka (43 samples) with zigzag type and randomly (Table 1). The onion plant samples were packed in plastic bags with proper labeled and keeps on ice coolers and transferred into the laboratory. The analyses were performed at the Department of Plant Pathology, Institute of Frontier Technology, Tirupati Andhra Pradesh, India. Association of an Ilarvirus with the samples was first established by direct antigen-coated enzyme-linked immunosorbent assay (Clark and Bar-Joseph, 1984) using polyclonal antiserum directed against the CP of TSV (Dr. P. Lava Kumar, ICRISAT). The virus isolates were then sap inoculated to cowpea (cv-C-152, a diagnostic assay host) plants using chilled 0.05 M phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol.

Isolation of total RNA and RT-PCR: Total RNA (100 mg) was isolated by using RNase plant minikit according to the manufacturer's instructions (Qiagen USA) from the healthy and TSV infected leaf samples which were confirmed

Table 1: Survey and collection of TSV infected onion samples from different parts of the South India

Crops	Locations	Samples	States	Total samples (%)
Onion	Kadapa	12	Andhra Pradesh	59 (67.79)
Onion	Kurnool	15		
Onion	Nellore	10		
Onion	Chittoor	13		
Onion	Medak	9		
Onion	Coimbatore	12	Tamil Nadu	42 (59.52)
Onion	Perambadur	8		
Onion	Dindigul	9		
Onion	Thirichirapalli	6		
Onion	Periyar	7		
Onion	Dharwad	14	Karnataka	43 (41.86)
Onion	Chitradurg	9		
Onion	Gadag	12		
Onion	Bagalkot	8		
Total	144			

TSV: *Tobacco streak ilavirus*

through DAC ELISA. The resulting total RNA was incubated with TSV-CP gene specific reverse primer at 65°C for 5 min and snap-chilled on ice for 2 min. cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas Canada) at 42°C for 1 h. The genome forward primer- 5′-ATGAATACTTTGATCCAAGG-3′ and reverse primer- 5′-TCAGTCTTGATTCACCAG-3′ were used to amplify the complete CP gene of TSV (Bhat *et al*., 2002). Two µL of cDNA was amplified in a 25 µL reaction volume containing 2.5 U of *Taq* DNA polymerase (Fermentas Canada), 10 pmol of forward (TSV-CP-F) and reverse primer (TSV-CP-R), 2.0 mM $MgCl_2$ and 10 mM each dNTP's. The PCR amplification conditions included an initial denaturation cycle of 5 min at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 48°C and extension for 1 min at 72°C with final extension for 15 min at 72°C. Amplified products were resolved following electrophoresis through a 1% agarose gel containing ethidium bromide (10 mg mL^{-1}).

Cloning and genome sequencing analysis: The PCR product was eluted by QIAquick gel extraction kit (Qiagen, USA) and cloned into pTZ57R/T vector (Fermentas Canada) according to the manufacturer's instructions. The resulting recombinant plasmids were transformed into *Escherichia coli* strain DH5α cells. Recombinant clones were identified by restriction endonuclease digestion and colony PCR. The resulted clones with expected size DNA inserts were sequenced at Eurofins Genomics India Pvt. Ltd, Bangalore. Multiple sequence alignments were generated using CLUSTAL W (Thompson *et al*., 1994). Sequence phylograms were constructed using TREEVIEW software (bootstrap analysis with 1000 replicates) (Page, 1996). The coat protein genes of other known ilarviruses were collected from GenBank (Benson *et al*., 1996). Both nucleotide and amino acid sequences of CP gene of different Ilarvirus species were compared and the corresponding phylogenetic trees were generated.

RESULTS AND DISCUSSION

The *Tobacco streak virus* (TSV) infections on onion were characterized by straw coloured; irregular, necrotic lesions in onion were observed (Fig. 1). These symptoms are very similar to Groundnut Bud Necrosis Disease (GBND) caused by *Groundnut bud necrosis virus* (GBNV) and they can't be differentiated in field level. The symptoms varied depending on the age of the plants and were collected from different regions of Andhra Pradesh, Karnataka and Tamil Nadu States in India during 2010-2013. The presence of the virus in symptomatic leaves was confirmed by DAC-ELISA using TSV polyclonal antibodies. In DAC-ELISA of the onion samples (n = 144) were tested, 40 (67.79%) in Andhra Pradesh, 25 (59.52%) in Tamil Nadu and 18 (41.86%) in Karnataka were found to be infected with TSV. The ELISA confirmed TSV isolates were found to be mechanically transmissible as the sap inoculated cowpea (Cv-c-152) plants showed the development of chlorotic rings and interveinal necrotic spots and streaks after a fortnight of inoculation. The symptoms gradually increased in size and adjacent spots coalesced resulting in plant death. The virus-affected plants reacted with the polyclonal antiserum directed against nucleocapsid protein of TSV (A405 = 1.95).

Total RNA from Elisa positive samples (83) were used for cDNA synthesis by using RT-PCR which results in a single band of expected size (~717 bp) corresponding to the CP gene (Fig. 2). Among these, the selected samples (Onion-Andhra Pradesh, Onion-Tamil Nadu and Onion-Karnataka) were confirmed by cloning and sequencing. These samples weresequenced and deposited in NCBI GenBank with the

Fig. 1: TSV infecting onion leaves showing straw colored, irregular, necrotic lesions

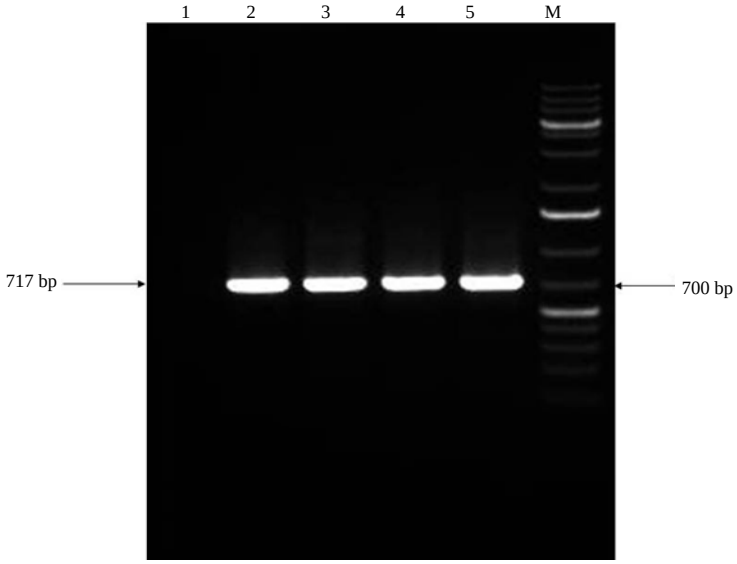

Fig. 2: RT-PCR amplicons resolved by 1% agarose gel electrophoresis. M: 1 Kb DNA Ladder, 1: Healthy onion, 2, 3: Onion-Andhra Pradesh, 4: Onion-Tamil Nadu, 5: Onion-Karnataka

Accession No. JQ269830, JX294487 and JX294488, respectively. The CP gene of TSV was 717 nucleotides long and coded for a protein of 239 amino acids. The coat protein gene sequence of our TSV isolates was compared with other reported Ilarvirus at nucleotide and amino acid levels (Table 2). The complete sequence of TSV-CP shared identity of 98.6 -100% at nucleotide and 98.3-100% at amino acid levels (Table 3), respectively with other reported TSV isolates.

The coat protein gene sequence of our TSV isolates was compared with other reported ilarviruses at nucleotide and amino acid levels. Our isolates Onion-Andhra Pradesh (JQ269830) and Onion-Karnataka (JX294488) clustered along with Onion (HM131490) and Helianthus (AY061929) as a separate clade (Clade 1). Onion-Tamil Nadu (JX294487) clustered along with Sun-hemp (AF515825), Okra (DQ864456) and Marigold (AY510129) as a separate clade (Clade 2) Sunflower (AF400664), Mungbean (AF515823), HM131488 (Groundnut), Cyamopsis (JQ269831) and Green gram (HM131487) as a separate clade (Clade 3). Soybean (DQ518916) isolate formed a separate clade (Clade 4), Cotton (AF515824) isolate formed a separate clade (Clade 5), Calotropis (HQ199846) isolate formed a separate clade (Clade 6) and Chilli (AY590139) isolate formed a separate clade (Clade 7) (Fig. 3).

The TSV is easily sap transmissible and transmitted by thrips (Prasada Rao *et al*., 2003). The generally TSV infecting

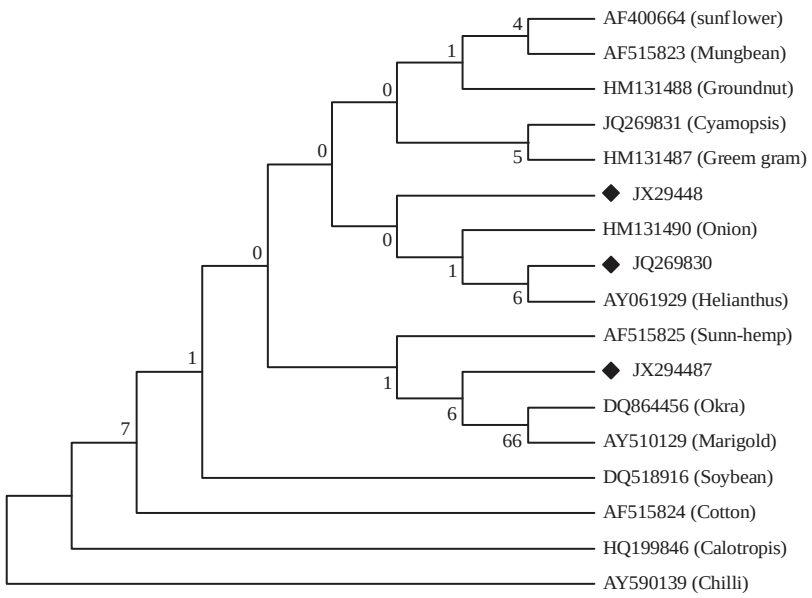

Fig. 3: Phylogenetic tree constructed from the alignment of amino acid sequences of our TSV isolates with those of other reported TSV isolates. The phylogenetic tree was constructed using MEGA version 4.0

Table 2: Members of the genus Ilarvirus used for sequence analysis in the present study

TSV-CP Isolates	GenBank Accession numbers
Onion-Tamil Nadu	JX294487
Onion-Karnataka	JX294488
Onion-Andhra Pradesh	JQ269830
Cotton-New Delhi	AF515824
Sunflower-Coimbatore	AF400664
Helianthus-Bangalore	AY061929
Cyamopsis-Tirupati	JQ269831
Groundnut-Ananthapur	HM131488
Okra-Coimbatore	DQ864456
Soybean-Coimbatore	DQ518916
Sun-hemp-New Delhi	AF515825
Onion-Kurnool	HM131490
Mungbean-New Delhi	AF515823
Calotropis-Nellore	HQ199846
Green gram-Kurnool	HM131487
Marigold-Anantapur	AY510129
Chilli-New Delhi	AY590139

Source: Present study and reported isolates

plants showed the mosaic and necrotic lesions were observed in plants. Later this leads to the total necrosis of the leaflets and spreads to the petiole, stems and growing buds. In severe infection the entire plant becomes necrotic and dies prematurely.

Earlier reports indicated that TSV infects several economically important crops in India such as sunflower, groundnut, cotton, sunn-hemp, mungbean, okra, cucumber, safflower, chilli, urdbean, nizer, soybean, onion and guar. Sivaprasad *et al*. (2010, 2012). Recently, straw coloured, irregular, necrotic lesions on leaves were observed in onions in Kurnool district of Andhra Pradesh, India (Sivaprasad *et al*., 2010). Sivaprasad *et al*. (2012) identified the foliar mosaic and necrotic spotting as well as necrotic streaks on buds and stems were observed in guar fields of Chittoor district of Andhra Pradesh, India. Bhaskara Reddy *et al*. (2013) identified leaf veinal necrosis and necrotic spotting and necrotic streaks on stems and petioles were observed on Lablab purpureus in farmer's fields in the Chittoor district Andhra Pradesh, India. Bhaskara Reddy *et al*. (2014) identified the necrotic spots and vein mosaics were observed in castor bean fields of Tirupati Andhra Pradesh, India. Hence, the present TSV isolates

Table 3: Sequence identity of present study isolates at amino acid (above the diagonal) and nucleotide (below the diagonal) levels, respectively, with other reported TSV isolates

Isolate	JX294487	JX294488	JQ269830	AF515824	AF400664	AY061929	JQ269831	HM131488	DQ864456	DQ518916	AF515825	HM131490	AF515823	HQ199846	HM131487	AY510129	AY590139
JX294487	100.0	100.0	98.7	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
JX294488	100.0	100.0	98.7	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
JQ269830	99.4	99.4	100.0	98.7	98.7	98.7	98.7	98.7	98.3	98.7	98.7	98.7	98.3	98.7	98.3	98.3	97.4
AF515824	99.8	99.8	99.3	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
AF400664	99.8	99.8	99.3	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
AY061929	99.8	99.8	99.3	100.0	100.0	100.0	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
JQ269831	99.7	99.7	99.1	99.8	99.8	99.8	100.0	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
HM131488	99.7	99.7	99.1	99.8	99.8	99.8	99.7	100.0	99.5	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
DQ864456	99.7	99.7	99.1	99.8	99.8	99.8	99.7	99.7	100.0	99.5	99.5	99.5	99.1	99.5	99.1	100.0	98.3
DQ518916	99.7	99.7	99.1	99.8	99.8	99.8	99.7	99.7	99.7	100.0	100.0	100.0	99.5	100.0	99.5	99.5	98.7
AF515825	99.7	99.7	99.1	99.8	99.8	99.8	99.7	99.7	99.7	99.7	100.0	100.0	99.5	100.0	99.5	99.5	98.7
HM131490	99.5	99.5	99.0	99.7	99.7	99.7	99.5	99.5	99.5	99.5	99.5	100.0	99.5	100.0	99.5	99.5	98.7
AF515823	99.5	99.5	99.0	99.7	99.7	99.7	99.5	99.5	99.5	99.5	99.5	99.4	100.0	99.5	99.1	99.1	98.3
HQ199846	99.4	99.4	98.8	99.5	99.5	99.5	99.4	99.4	99.4	99.4	99.4	99.5	99.3	100.0	99.5	99.5	98.7
HM131487	99.4	99.4	98.8	99.5	99.5	99.5	99.4	99.4	99.4	99.4	99.4	99.5	99.3	99.4	100.0	99.1	98.3
AY510129	99.5	99.5	99.0	99.7	99.7	99.7	99.5	99.5	99.8	99.5	99.5	99.4	99.4	99.3	99.3	100.0	98.3
AY590139	98.8	98.8	98.3	99.0	99.0	99.0	98.8	98.8	98.8	98.8	98.8	98.7	98.7	98.6	98.6	98.7	100.0

associated with chlorotic/mosaic and necrosis disease of onion in Andhra Pradesh, Tamil Nadu and Karnataka were characterized at genome level to confirm their exact identity.

The CP gene of TSV isolates was initially targeted for the genetic characterization of TSV associated with mosaic and necrosis disease of onion in India. Sequence analysis of CP gene helps to exploit it for production of recombinant TSV-CP and thereby facilitates the production of rTSV-CP based polyclonal antibodies for the sensitive detection of TSV. Hence primers were designed for the amplification of TSV-CP based on the RNA4 sequence of onion infecting TSV isolates. As an initially BLAST analysis of the obtained sequence clearly related that the coat protein gene of TSV.

The CP gene of TSV revealed that the sequenced region contained a single Open Reading Frame (ORF) of 717 nucleotides that could potentially code for a protein of 238 amino acids. The sequences (AP-Onion (JQ269830), TN-Onion (JX294487) and KA-Onion (JX294488)) were deposited at the NCBI GenBank. The identity values of indian TSV-CP isolate in comparison to other isolates at nucleotide and amino acid levels shows some discrepancy with the data that has already been published as disease reports (Sivaprasad *et al*., 2010).

The TSV was identified from several crops and weeds in the peninsular India (Prasada Rao *et al*., 2003; Kumar *et al*., 2008). The TSV isolate from groundnut positively reacted with TSV-WC antiserum. The nucleotide sequence of RNA3 shared 88.4% similarity with TSV-WC sequences available in the databases and thus it was considered as a strain of TSV. Subsequently, several isolates from naturally infected crops in India were isolated, their genomes were partially sequenced and identified as strains of TSV (Kumar *et al*., 2006). Further, the CP gene sequence analysis of the TSV isolates characterized from various crops and locations of India was reported to share 99-100% nucleotide sequence homology among each other and shared 88-89% sequence homology with the type strain (Ravi *et al*., 2001; Bhat *et al*., 2002). The TSV isolate causing soybean bud blight disease in Brazil (TSV-BR) was reported to be a distinct strain of TSV, which shared 81.3-80.7% nucleotide sequence homology with the CP gene of TSV-WC and TSV-MB, respectively.

Sivaprasad *et al*. (2012) characterized the TSV isolates naturally infecting groundnut, onion, black gram, green gram, jute, tagetes, sunflower, calotropis, watermelon, pumpkin and kenaf plants were collected from fields in different regions of Andhra Pradesh, Tamil Nadu and Karnataka. The above sequence isolates analysis revealed that the TSV-CP gene shared 91-100% and 91-99% sequence identity with TSV at nucleotide and amino acid levels, respectively.

The variability studies of TSV not only useful in establishing differences among the strains that infect different crops but also aid in evolving transgenic plants against TSV. The genetic relationships of various TSV isolates infecting onion crop in South India is especially molecular characteristic based on a gene, may not represent the picture of a genome and so, multiple genes as well as whole genome analysis should be used.

CONCLUSION

The variability studies of TSV not only useful in establishing differences among the strains that infect different crops but also aid in evolving transgenic plants against to TSV. The genetic relationships of various TSV isolates infecting Onion crop in South India is especially lacking and this study contributes in understanding the sequence diversity among TSV isolates infecting onion crop in South India.

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