RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2010.36.194

ScienceAsia 36 (2010): **194–198**

Detection of Tobacco streak virus by immunocapture-reverse transcriptase-polymerase chain reaction and molecular variability analysis of a part of RNA3 of sunflower, gherkin, and pumpkin from Andhra Pradesh, India

Bheemathati Sarovar*, **Y. Siva Prasad**, **D.V.R. Sai Gopal**

Department of Virology, S.V. University, Tirupati-517 502, Andhra Pradesh, India

*Corresponding author, e-mail: sarovarbhee@yahoo.co.in

Received 14 Oct 2009
Accepted 10 Aug 2010

ABSTRACT: The coat protein-coding and 3′UTR regions of the RNA3 of Tobacco streak virus infecting sunflower, gherkin, and pumpkin with the characteristic symptoms of necrosis were amplified by IC-RT-PCR. The amplicons were cloned and sequenced. The nucleotide sequences of TSV clones were determined as $\sim$ 1 kb. This length corresponds to 717 and 288 nucleotides of coat protein-coding and 3′UTR regions, respectively. Comparative sequence analysis of the coat protein-coding region of TSV isolates under study both at the nucleotide and the amino acid levels indicated $98 \pm 1\%$ and $97 \pm 1\%$ identity, respectively, with TSV reported from other hosts. The sequence analysis of the 3′UTR region at the nucleotide level showed $98 \pm 1\%$ and 88% identities with the Indian and US isolates, respectively. IC-RT-PCR was found to be more sensitive than RT-PCR, and hence could be used in quarantine programmes.

KEYWORDS: IC-RT-PCR, TSV isolates

INTRODUCTION

Tobacco streak virus (TSV) belongs to the genus *Ilarvirus* of the family Bromoviridae. It has a broad host range, worldwide distribution, and shows severe impact on agricultural as well as horticultural crops. These characteristics make it one of the economically most important plant viruses in commercially grown crops. It is known to infect more than 80 plant species belonging to the families Asteraceae, Brassicaceae, Cucurbitaceae, Fabaceae, Rosaceae, Solanaceae, and others [1] and is transmitted in nature vertically through seed and horizontally by pollen and thrips [2]. TSV contains a single-stranded, tripartite RNA genome; virions are quasi-isometric and 27–35 nm in diameter. RNA1 and RNA2 encode proteins involved in viral replication, whereas RNA3 encodes a protein required for cell-to-cell movement. The viral coat protein (CP) is expressed by a subgenomic RNA, designated RNA4, collinear with the 3′ end of RNA3 [3]. Being a destructive virus of economically important crops, disease diagnosis and reliable detection methods play a key role in disease management of ilarviruses. Various methods have been developed to detect TSV, including enzyme linked immunosorbent assay (ELISA), RT-PCR, and probe based techniques. A further refinement of PCR is immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) in which the virus particles are captured by immobilized antibodies for the detection of a specific virus [4]. Here, IC-RT-PCR was used for the characterization of coat protein and 3′ untranslated region (UTR) regions of the TSV infecting sunflower, pumpkin, and gherkin to define the relationships of TSV isolates present in different crops and to check the distribution and prevalence of strains or variants from different origins.

MATERIALS AND METHODS

Sample collection

Sunflower (*Helianthus annuus* var sunbreed), gherkin (*Cucumis anguira* var anguira), and pumpkin (*Cucurbita pepo* var coimbatore-1) plants with characteristic symptoms of necrosis were collected in and around Chittoor and Warangal districts of Andhra Pradesh, India. Leaf samples with the mosaic and systemic necrosis were taken for the molecular characterization of the virus.

ELISA

Samples collected from the fields were subjected to direct antigen coating-ELISA (DAC-ELISA)[5] using specific polyclonal TSV and recombinant peanut bud necrosis virus (PBNV) antisera (courtesy of ICRISAT, Hyderabad). The polystyrene plates were coated with 200 µl of plant extract ground in 0.05 M carbonate buffer (pH 9.6, 1:10 dilution) incubated at 37 °C for 1.5 h. Antiserum and conjugate (goat anti rabbit immunoglobulins conjugated with alkaline phosphate, Genei, Bangalore) buffers were prepared in PBS-TPO (0.15 M NaCl in 0.1 M phosphate buffer pH 7.4, 0.05% Tween 20, 2% polyvinyl pyrrolidine, 0.2% ovalbumin). The plates were coated with antisera buffers (1:5000 dilution) and later with conjugate buffer (1:10 000 dilution) incubated at 37 °C for 1 h at both the steps. Between each coating of antigen, antibody, and conjugate, the plates were washed with PBS-T three times. The reaction was developed by adding 200 µl of the substrate solution (9.7% diethanolamine, 50 mg *p*-nitrophenyl phosphate pH 9.8) and kept for incubation at 37 °C in the dark for 20 min.

Maintenance of TSV pure culture

Local lesion assay host as *Vigna unguiculata* C 152 cultivar was used following sub-culturing for three generations on the same host and later maintained on their natural hosts for further studies.

Primer design

Upstream (ATG AAT AAT TTG ATC CAA RGT CCA) and downstream (GCA TCT GGT ATA AAG GAG GCA T) primers were designed for the coat protein and 3′UTR regions of TSV using OLIGO version 5 software based on reported sequence data of TSV RNA3[6].

IC-RT-PCR

The positive serological and uninfected samples were used for immunocapture (IC) and further used for RT-PCR amplification. TSV antiserum was diluted in coating buffer (0.05 M carbonate buffer pH 9.6) and the PCR tubes were coated with 50 µl of antiserum, incubated for 2 h at room temperature. Leaf tissue, 0.2 g, was homogenized in 1 ml of extraction buffer (500 mM Tris-HCl pH 8.3, 10 mM Na_2SO_3, 3 mM $NaNO_3$, 140 mM NaCl, 2% PVP, 0.05% Tween 20) and centrifuged at 5000*g* for 10 min. The tubes were washed three times with PBS-Tween (pH 7.4) with a 3 min interval between each wash. Then, 50 µl of sample supernatants were added to the PCR tubes and incubated for 2 h at room temperature. The tubes were washed three times with wash buffer and finally with sterile water. The tubes were left in air dry for 3 min before proceeding to the next step.

Comparative sensitivities of IC-RT-PCR and RT-PCR

To compare the sensitivity limit of IC-RT-PCR and RT-PCR, 10^{-1} to 10^{-6} dilutions of TSV infected sunflower leaf sap were prepared in grinding buffer and total RNA up to 10^{-6} dilutions. Ab coating and washing steps were performed as described earlier.

cDNA strand synthesis

Sterile water and 10 pmol of TSV specific reverse primer were added to the antigen and antibody coated PCR tubes, denatured at 70 °C for 5 min, and chilled on ice. For 20 µl of reaction, 4 µl of 5 × reaction buffer, 2 mM dNTPs, 20 units of ribonuclease inhibitor (Fermentas) were added to the above and incubated at 37 °C for 5 min. Then, 200 units of revert aid Moloney murine leukaemia virus (MuLV) reverse transcriptase (Fermentas) was added and the reaction was carried out according to the manufacturer's instructions.

PCR amplification

PCR was carried out using specific sense and antisense primers in a thermal cycler (Corbette Research, Australia). The PCR mixture for 50 µl of reaction contained 1 µl of cDNA, 10 pmol upstream and 10 pmol of downstream primers, 10 × *Taq* buffer, 2 mM dNTPs, 1.25 units *Taq* (Fermentas) and 1.75 mM, 1.5 mM, and 1.5 mM $MgCl_2$ for sunflower, gherkin, and pumpkin, respectively. The PCR programme comprised of 40 cycles of amplification including denaturation at 94 °C for 30 s, annealing at 58 °C, 57 °C, and 58 °C for sunflower, gherkin, and pumpkin, respectively, for 1 min, extension at 72 °C for 1.5 min and final extension at 72 °C for 30 min.

Statistical analysis

TSV infected leaf samples of sunflower, gherkin, and pumpkin were collected in and around areas of Andhra Pradesh and detected by IC-RT-PCR. The infection rates were analysed by using the Kruskal Wallis test (Table 1).

Cloning of IC-RT-PCR products and sequencing

The PCR amplicons were ligated with T_4 DNA ligase, incubated at 37 °C for 1 h, and cloned into pGEM-T easy vector (Promega). The cloned products were transformed into DH5α $CaCl_2$ competent cells and

Table 1 Performance of IC-RT-PCR in detecting TSV from sunflower, gherkin, and pumpkin leaf samples.

Host	Tested	Positive	Negative
Sunflower	15	14	1
Gherkin	13	11	2
Pumpkin	16	15	1

plated onto Luria agar with ampicillin and IPTG/X-gal plates. Recombinant plasmids were isolated by alkaline lysis (Qiagen) and the positive clones were subjected to restriction enzyme digestion with *Eco*RI (New England Biolabs). The positive clones identified by restriction enzyme analysis were sequenced in both orientations by using T7 and SP6 forward and reverse primers (MWG, Sequencing Department, Bangalore). Comparison of the nucleotide sequences of the positive clones was performed by using BLAST V.2.1.2[7,8] with those available in GenBank. Sequence analysis and the calculation of identity and similarity were carried out using BIOEDIT V.5.0.9[9] for all the nucleotide and deduced amino acid sequences. The phylogenetic relationships were established using MEGA 4.1 beta software by bootstrap test with 100 replicates and 11 random odd seed numbers, in which *Parieteria mottle virus* (PMV) served as the outgroup.

RESULTS AND DISCUSSION

Detection of TSV by IC-RT-PCR

In the field survey of Tirupathi and Warangal regions of Andhra Pradesh, the sunflower, gherkin, and pumpkin plants showing chlorosis and necrotic symptoms on leaves, stem, and inflorescence were serologically detected by TSV antiserum. Many scientists have reported the association of TSV with *Tospovirus* on sunflower[10] in Karnataka and PSND and PBNV on groundnut[11] in Anantapur. In Iran, it has been observed that many of the TSV infected samples had mixed infections with tomato spotted wilt virus, cucumber mosaic virus, potato virus y, and tobacco mosaic virus[12]. TSV and PBNV antisera were used for the serological detection because, in late stages of infection, it is often difficult to distinguish TSV and Tospovirus symptoms from those of mixed infections. The TSV-positive tested samples in DAC-ELISA were inoculated onto the cowpea C152 leaves. After 3 days, necrotic rings or pinpoint necrotic lesions on primary leaves were observed on cowpea leaves. The resulted amplicons from IC-RT-PCR of TSV on the above three hosts yielded fragments of $\sim$ 1 kb (Fig. 1). No amplification was observed from uninfected leaf tissues used in the experiment. The primer pair used in the amplification of CP and 3′UTR regions proved to have a significantly broad spectrum to amplify the TSV on different crops.

Comparison of sensitivity between IC-RT-PCR and RT-PCR

The relative sensitivities of IC-RT-PCR and RT-PCR were compared by using serial dilutions of TSV infected sunflower leaf sap. The RNA of the TSV was amplified in RT-PCR to 10^{-5} dilution, while IC-RT-PCR amplified to a sap dilution of 10^{-6}. No virus was detected with the healthy samples. High intensity and no bands were observed with the crude samples of RT-PCR and IC-RT-PCR, respectively (Fig. 2). In IC-RT-PCR there is a protein-dependent step where the virus particles are bound to the surface of the PCR tube, but from then onwards the reaction is entirely nucleic acid-based for the amplification of the viral RNA. Amplification of TSV was observed at the least dilution used in IC-RT-PCR and so the sensitivity limit in detecting the virus was higher than with the RT-PCR. The intensity of the observed bands on the gel is reduced with the increase in the dilutions of leaf sap and total RNA and this correlates with the virus concentration. Hence, IC-RT-PCR is more specific and sensitive than RT-PCR.

Statistical analysis was performed by taking three crops to validate the IC-RT-PCR method. The Kruskal Wallis test showed that there is no significant difference between the crops ($\chi^2 = 1.0466$, $p > 0.05$).

Molecular variability analysis of TSV isolates

The fragment included the genes of 717 nucleotides (nts) CP (Open Reading Frame II) and 288 nts of 3′UTR and the translation product is of 238 amino acids. The determined sequences were deposited at GenBank under the accession number EF159704 (sunflower), EF159702 (gherkin), EF159703 (pumpkin). Comparative analysis of coat protein gene of TSV-sunflower, gherkin, and pumpkin showed amino acid sequence identity of $94 \pm 1\%$ with all other TSV Indian isolates (AY940152, EU085385, DQ864449, DQ864460, DQ864448, AY940153, DQ864454, EU085386, AF515825, DQ518916, DQ864457, AF515823, DQ225172, AY590139, AY940158, DQ864456, AY501481, DQ864455, AY940157, AY505081, AY940155, AY510129, DQ864458, EF159704, EF159702, EF159703). Phylogenetic analysis of the CP region of the three crops revealed that at the protein level sunflower and pumpkin forms one clade which are closely related to sunn hemp, sunflower, mungbean, chilli (India), soybean, cowpea, urdbean (Tamil Nadu), and groundnut (Andhra

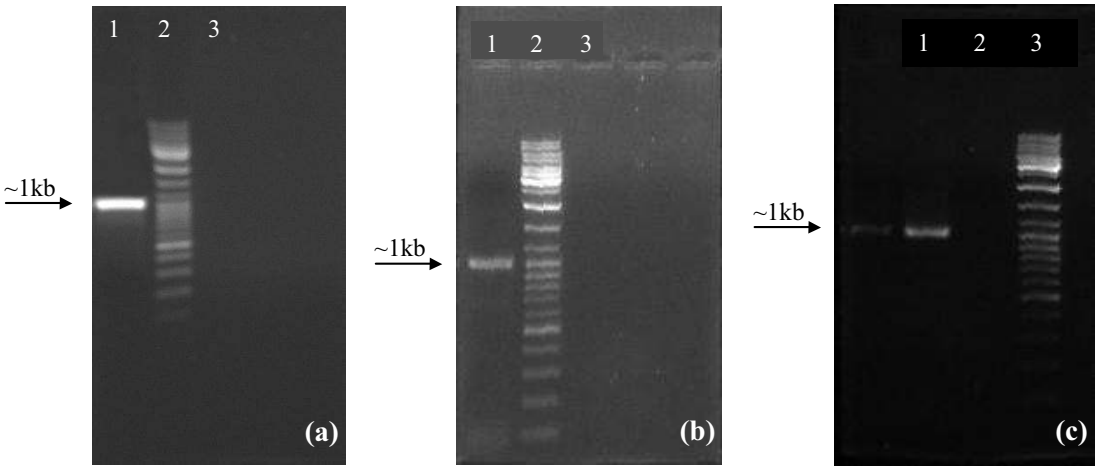

Fig. 1 Gel electrophoretic analysis of IC-RT-PCR product of TSV isolate from (a) sunflower, (b) gherkin, and (c) pumpkin. (a) Lane 1: infected; Lane 2: 1 kb DNA ladder; Lane 3: healthy (b) Lane 1: infected; Lane 2: 1 kb DNA ladder; Lane 3: healthy (c) Lane 1: infected; Lane 2: healthy; Lane 3: 1 kb DNA ladder.

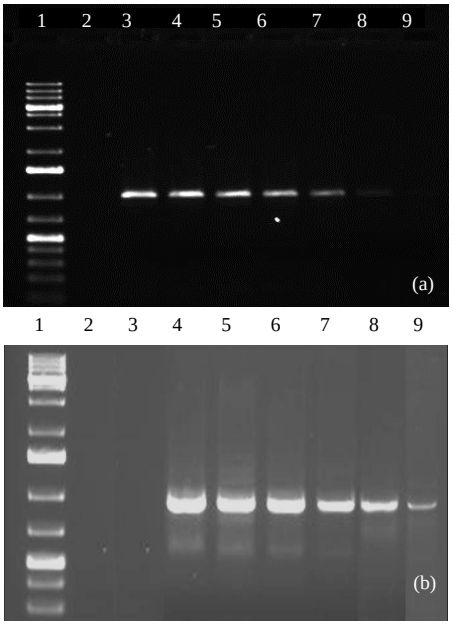

Fig. 2 Sensitivity of TSV detection using (a) RT-PCR, (b) IC-RT-PCR Lane 1: 100 bp DNA ladder; Lane 2: healthy; Lane 3: crude; Lanes 4–9: 10^{-1} to 10^{-6} – tenfold serial dilution of TSV infected sunflower.

Pradesh). Gherkin forms another clade closely related to globe amaranth, marigold (Andhra Pradesh), sunflower, niger (Karnataka), cotton, and soybean (Maharashtra isolates, Fig. 3). The 3′UTR region of TSV isolates, as in sunflower, gherkin, and pumpkin, consists of 288 nts. Comparative sequence analysis of 3′UTR region showed 3% variability with the Indian and 89% of identity with that of US isolates. This region contains 5 repeats of AUGC motifs in the proximal position of the 3′ terminal end. The 3′UTR contains a single septet GATGCCT (2195–2201 nts) in APct, APKu, APWa, and the same was noticed in slightly differed regions, i.e., 2192–2198 nts (Indian isolate) and 2183–2189 nts (US isolate). Phylogenetic analysis of 3′UTR of the above isolates showed that pumpkin forms a separate clade. Sunflower and gherkin form another clade (data not shown).

In TSV isolates the N-terminal region of the CP is highly conserved and is necessary for binding the CP to the 3′ end of Ilarviruses and AMV RNAs[13]. The N-terminal region of the coat protein amino acid sequences of the Bromoviridae family is predominantly basic due to the presence of a high proportion of Arg and Lys residues. In accordance with the above data, the N-terminal region is highly conserved and highly basic in nature. For phylogenetic studies, CP sequences or their structures are taken because they are more conserved than 3a proteins during evolution[14]. The 97% identity of the CP region shows that it is mostly conserved. It has usually been found that 3′UTR of TSV RNAs have more divergence than the 3′UTRs of other ilarviruses[15]. However, here the least divergence was observed by sequence analysis. Phylogenetic analysis of a part of viral genome segment including coding and non-coding regions did not reveal any divergence.

The high sensitivity and reliability of the IC-RT-PCR described was used for indexing local field samples and in quarantine practices. It is considered to be a rapid and simple method because crude sap extracts

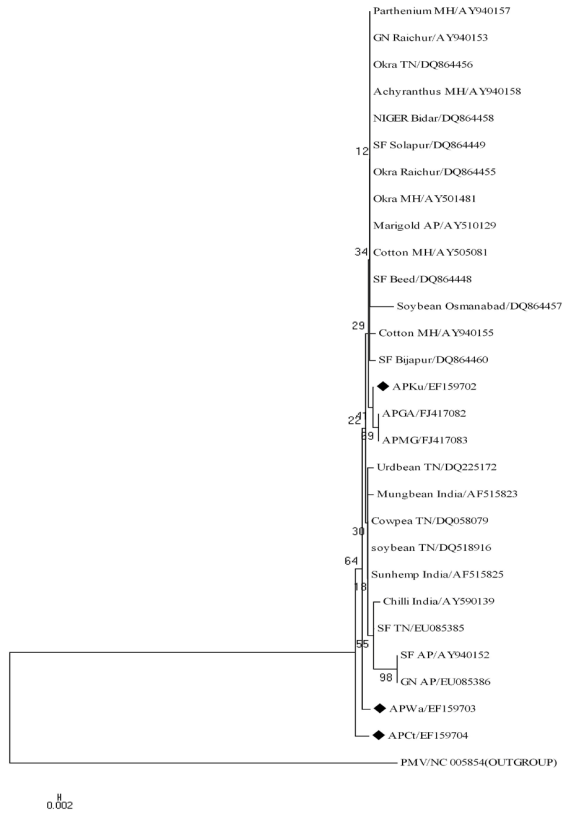

Fig. 3 Dendrogram of the encoded amino acid sequences of coat protein of TSV isolates under study (APct, APku, APwa). TSV-Indian isolates are for comparison. PMV is the outgroup.

used in IC-RT-PCR replaces more time-consuming total nucleic acid extraction protocols.

In conclusion, serological relatedness, high sequence similarities at nucleotide and amino acid levels, and closely related clusters in dendrogram analysis establishes the TSV on three different crops as TSV isolates and does not reveal any strains of TSV. In the present study, the gene encoding coat protein and 3′UTR regions were cloned and analysed. Exploitation of this clone could result in transgenic studies to produce plants resistant against sunflower necrosis disease.

Acknowledgements: The first author is greatly thankful to CSIR-New Delhi for financial support during the period of the research work.

REFERENCES

1. Scott SW (2001) Tobacco streak virus. In: *Descriptions of Plant Viruses*, CMI/AAB, Surrey, UK, No. 381.
2. Sdoodee R, Teakle DS (1993) Studies on the mechanism of transmission of pollen associated *Tobacco streak ilarvirus* by *Thrips tabaci*. *Plant Pathol* **42**, 88–92.
3. Bol JF (1999) *Alfalfa mosaic virus* and *ilarviruses*: involvement of coat protein in multiple steps of the replication cycle. *J Gen Virol* **80**, 1089–102.
4. Hull R (2001) Methods involving properties of the viral nucleic acid. In: Harrison BD, Murant AF (eds) *Matthews Plant Virology*, 4th edn, Academic Press, New York, pp 671–5.
5. Hobbs HA, Reddy DVR, Rajeswari R, Reddy AS (1987) Use of antigen coating method and protein-A coating ELISA procedures for detection of three peanut viruses. *Plant Dis* **71**, 747–9.
6. Cornelissen BJC, Janssen H, Zuidema D, Bol JF (1984) Complete nucleotide sequence of *Tobacco streak virus* RNA3. *Nucleic Acids Res* **12**, 2427–37.
7. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215, 403–10.
8. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al(1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–402.
9. Hall T (1999) Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–8.
10. Jain RK, Bhat AI, Byadgi AS, Nagaraju Harvir singh Halker AV, Anahosur KH, Varma A (2000) Association of a *Tospovirus* with sunflower necrosis disease. *Curr Sci* **79**, 1703–5.
11. Reddy AS, Prasada Rao RDVJ, Thirumala-Devi K, Reddy SV, Mayo MA, Roberts I, Satyanarayana T, Subramaniam K, Reddy DVR (2002) Occurrence of *Tobacco streak virus* on peanut (*Arachis hypogaea* L.) in India. *Plant Dis* **86**, 173–8.
12. Khateri H, Moarrefzadeh N, Koohi-Habibi M, Mosahebi G, Hosseini A, Hamez N (2006) High incidence of *Tobacco streak virus* in the tobacco fields of Iran. *Communications in Agricultural and Applied Biological Sciences* **71**, (3 Pt B), 1213–6.
13. Baer ML, Houser F, Loesch-Freis LS, Gehrke L (1994) Specific RNA binding by amino-terminal peptides of *Alfalfa Mosaic Virus* Coat Protein. *EMBO J* **13**, 727–35.
14. Koonin EV, Dolja VV (1993) Evolution and taxonomy of Positive – strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Crit Rev Biochem Mol Biol* **28**, 375–430.
15. Scott SW, Zimmerman MT, Ge X (1998) The sequence of RNA1 and RNA2 of Tobacco *streak virus*: additional evidence for the inclusion of alfalfa mosaic virus in the genus *Ilarvirus*. *Arch Virol* **143**, 1187–98.