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Molecular characterization of the *Tobacco streak virus* infecting peanut

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Abstract
Tobacco streak virus (TSV) is belongs to the family Bromoviridae and genus Ilarvirus; fast increasing plant pathogen infecting more than 200 plant species belonging to 30 dicotyledonous and monocotyledonous plant species and transmitted by the Arthropod vector thrips. Peanut is very prone to *Tobacco streak virus*; causing peanut stem necrosis leads to huge economic losses. In present study we have collected the peanut plant showing characteristics symptom of the TSV; diagnosed through Direct antigen coating- Enzyme linked imminosorbent assay (DAC-ELISA) as well as Dot immunobinding assay. Coat protein gene of the TSV was amplified, cloned and sequenced. The identified coat protein gene sequenced was compared with the sequence available in NCBI data base. The identified peanut strain of TSV showed 99.78% homology to the Kurnool peanut strain of TSV (Accession no AY50508).

Keywords: Ilarvirus, Polymerase chain reaction, cloning, coat protein

Introduction
Peanut is a very important leguminous oilseed crop belongs to the family of "Fabaceae" and genus of "Arachis"; growing in tropical and subtropical region of the world. It is top oilseed crop growing in Asian countries. India stands second in production next to the China in Asian countries. There are several uses of the peanut seed apart from the oil. It is used in culinary preparation, peanut flour and protein. Peanuts are rich in essential nutrients, so it is used in variety of foods, such as desserts, cakes, confectionery, snacks, and sauces. Peanuts are rich in protein, fat, and various healthy nutrients. Peanut production is limited due to various abiotic and biotic stresses. Among the Biotic stresses, *Tobacco streak virus* and *Peanut bud necrosis virus* are major constraints (Reddy *et al*. 2002) [2]. In the present study, we mainly focused on the Tobacco streak virus (TSV). To brief about the TSV;
First the natural incidence of the TSV was reported by the on tobacco (Nicotiana tabacum) in 1936 by Johnson (Johnson, 1936) [1]. Later, it has been reported from more than 26 countries worldwide. Host of the TSV is very wide ranges. Its host ranges are increasing day by day after breaching the host pathogen barriers. The total size of TSV genome varies from 8.5 to 9.0 kb. The whole genome is segmented, tripartite in nature (each segment is encapsulated individually) and consist of linear, positive-sense, single stranded RNA. RNA1 contains a single ORF that encodes a polypeptide (1a protein) of 123 kDa. The polypeptide contains two domains with characteristics of methyltransferase and NTP-binding activities. RNA2 contains 2 ORFs. The product of the larger ORF (2a protein) is 94 kDa and contains a domain with sequence characteristics of an RNA dependent RNA polymerase. It is required to perform replication, transcription, cap- snatching and genomic strand selection. The smaller 2b ORF begins within the larger ORF and extends towards the 3' terminus of the molecule. The product (2b protein) is 22 kDa and appears to be expressed via a subgenomic RNA4a. RNA3 is bi-cistronic and codes for a movement (3a) protein of 31 kDa and the coat (3b) protein of 28 kDa. The coat protein gene is expressed via subgenomic RNA4. Cap structure present at the 5' end of each RNA helps in the translation and protect from its degradation. In the present study we have collected the virus infected peanut samples from the farmer's field of the Patancheru, Medak district, Telangana and characterized at molecular level.

Materials and methods
Virus culture
Peanut plants showing typical symptoms of TSV were collected from Patancheru, Medak district of Telangana. Collected sample were mechanically inoculated to cowpea, *Vigna unguiculata* cv. C-152; three consecutive single lesion transfers carried out by mechanical sap inoculations for getting the pure virus and finally maintained in cowpea.

Isolation of Total RNA

Total RNAs were extracted from 100 mg from a single lesion of TSV maintained in cowpea; ground in the 600 µl lysis buffer (0.1 M glycine pH 9.2, 40 mM EDTA, 100 mMNaCl, 2% SDS and 0.05% bentonite). Tube was incubated in a water bath at 42ºC for 15 min and equal volume of phenol and chloroform in 1:1 ratio was added and mixed vigorously. The supernatant was collected after centrifugation at 15,000 rpm for 15 min. To precipitate the RNA, equal volume of isopropanol was added to the supernatant and it was freeze-thawed 3 times using liquid nitrogen. It was again centrifuged at 15,000 rpm for 15 min at 4ºC. The pellet was washed with 1 ml of 70% ethanol by centrifuging at 15,000 rpm for 10 min and then air dried. The dried pellet was dissolved in 50 µl of milliQ water. 2 µl of total RNA was mixed with 1 µl of 6 M urea, 4 µlMilliQ and 1 µl of 6X DNA loading dye and heated at 65ºC for 5 min. Agarose gel electrophoresis was performed.

Agarose gel electrophoresis

1% (W/V) of agarose was melted in 0.5X TAE buffer (20 mM Tris-acetate and 0.5 mM of EDTA of pH 8.0). Ethidium bromide is added to a final concentration of 0.5 µg/ml of gel. After cooling to 50ºC, the mixture is poured onto a preset template with an appropriate comb. The comb is removed after solidification and the gel with template was placed in an electrophoresis chamber containing the running buffer (0.5X TAE). DNA to be analysed is mixed with the gel loading buffer (6X buffer contains 0.25% bromophenol Blue, 30% glycerol in 0.5 X TAE buffer) at 5:1 ratio and loaded into the well. Electrophoresis was carried out at 120V (Sambrook *et al.*, 1989) [5].

Designing of primers

Coat protein sequence of TSV were retrieved from the NCBI data bank and aligned by the Bioedit software. Conserved regions were selected for designing of primers. The forward primer and reverse primer were designed manually. Both primers were checked for melting temperature using IDT oligocal online tool.

First strand cDNA synthesis

Isolated total RNAs was used as a template for 1st strand cDNA synthesis by using EPICENTRE Biotechnologies Kit. The cDNA was synthesized; according to manufacturer's protocol. The 2 µl (100 ng) of RNA was mixed with 8.5 µl nuclease free water and 10.0 µM gene specific reverse primer and it was incubated at 65ºC for 2 min in a water bath and it was cooled down at RT. To this mixture, 7.5 µl cocktail (2.0 µl 10X reaction buffer, 2.0 µl of 100 mM DTT, 2.0 µl of 5 mM dNTP, 0.5 µl of ScriptGuard RNAse inhibitor and 1.0 µl MMLV Reverse Transcriptase) was added and incubated at 37ºC for 90 min. After incubation, the reaction was terminated by heating at 85ºC for 5 min. This mixture was kept on ice for 1 min and used directly to amplify the gene by PCR with the specific sets of primers.

Polymerase Chain Reaction (PCR) and cloning

PCR is performed as described by Sambrook *et al*. (1989) [5]. Template was added to the cocktail of 25 µl reaction containing 2.5 µl of 10X buffer (50 mMTris-HCL pH 8.8 and 50 mM KCL, 1.5 mM $MgCl_2$)50 µM dNTP's, 0.5 µM of each specific forward and reverse primers and 1unit of *taq* DNA polymerase. Initial denaturation step was carried out at 94ºC for 4 min, then 30 cycles of denaturation at 94ºC for 50 sec, different annealing temp (depending on GC% of oligonucleotides) and extension time (depending on the amplicon size) generally 1 min+1 min/ 1kb of template @ 68ºC followed by final extension at 68ºC for 10 min. The amplified PCR product is subjected to electrophoresis on 1% agarose gel and was documented by UV-platinum gel documentation instrument. PCR product was cut from the gel, purified and used for cloning in pTZ57R/T cloning vector. Purified PCR product was cloned in pTZ57R/T as per the manufactures instructions.

Plasmid isolation

Recombinant plasmids harbouring coat protein gene were isolated from colonies using alkaline lysis method. Single colonies were picked and grown in 3 ml of LB broth containing ampicilin (100 mg/l) for 16 h at 37ºC and centrifuged at 10000 rpm for 2 min at 4ºC. The supernatant was discarded and the cells were re-suspended in 100 µl TES (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.9% Sucrose) and kept on ice for 5 min. To the re-suspended cells, 200 µl of lysis solution (0.2 N NaOH + 1.0% SDS) was added, mixed well and kept on ice for 5-10 min. After lysis, 150 µl of neutralization solution (1.32 M sodium acetate pH 4.8-5.2) was added, mixed well by gentle inversion and kept on ice for 5 min. The supernatant was taken by centrifugation at 12000 rpm for 10 min and 300 µl of cold isopropanol was added to precipitate the plasmid DNA. Centrifugation was done for 5 min at 12000 rpm and the supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol and air-dried. It was then dissolved in a 40µl of 10 mM Tris-HCl and was stored at -20ºC for further use.

Sequencing of plasmids

For the sequencing of the recombinant plasmid harbouring CP gene was isolated and confirmed through the restriction digestion. Restriction digestion is enzymatic technique that can be used for cleaving DNA molecules at specific sites using specific restriction endonucleases. The restriction digestion of plasmids and PCR product was performed according to standard procedures (sambrook *et al*., 1989) [5] using restriction endonuclease in appropriate buffer at their respective optimum temperature. The digested product was analysed in the 1% agarose gel electrophoresis. The confirmed positive plasmids through restriction digestion; were purified through column and send for sequencing to the Scigenom Pvt Ltd and Ocimum Biosolltuion Pvt Ltd. Plasmids were sequenced either through universal primer (T7, SP6 and M13) or gene specific primer. Nucleotide sequence and dendogram obtained after sequencing used as raw material for further analysis of sequencing result.

Analysis of sequencing result

The FASTA format nucleotides sequence obtained from the Scigenom and Ocimum;used for BLASTn analysis. FASTA of nucleotide sequence was pasted in the dialog box indicating "enter accession number (s), or FASTA sequences" and non reductant data base selected for the analysis. Results were predicted based on the following parameter; maximum score, total score, query coverage, E value and indent. Overlapping clones were analysed with BioEdit (version 5.0.9) and restriction sites within the overlaps were clones were determined with nebcutter.

Results and Discussions:

First strand c DNA synthesis and PCR

The RNA isolated from single lesion maintained cowpea plant was used as a template for cDNA synthesis. PCR was

performed with coat protein specific primer (FP and RP), 10X buffer, dNTP's, and *Taq* DNA polymerase as mentioned in material and method section. Thermo profile was optimized with the help of gradient PCR. The best annealing temperature for amplification of the coat protein of TSV found 57ºC for 30 sec. Amplified PCR was loaded in 1% agarose gel along with 1.0 kb ladder. The PCR product showed sharp band near the 717 bp peanut strain of TSV (Fig 1). PCR is a very specific and sensitive method for virus identification that is based on the presence of unique nucleic acid sequence in the genome of a virus (Bartlett, 2003). Here, PCR amplified product was showed band near the 717 bp and concurrent the reported by Reddy *et al*., 2002; Krishna reddy *et al*., 2003 and Sharman *et al*., 2008) [2, 4, 3]

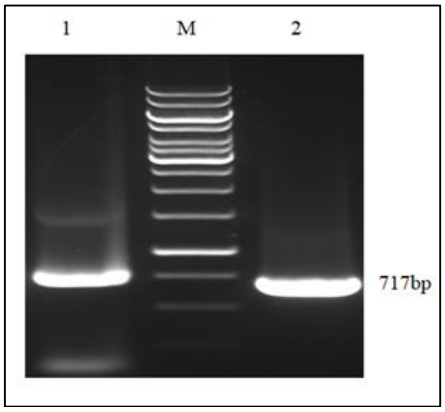

Fig 1: Reverse transcription –polymerase chain reaction (RT-PCR) for coat protein gene of TSV in 1% agarose gel. Lane 1 and 2; PCR amplified product for coat protein gene of TSV- Peanut. M; 1 kb ladder, right side of the gel indicate 717 bp for PCR amplified product

Confirmation of the plasmid by restriction digestion

Column purified plasmid was taken for restriction digestion. The total 10 µl of reaction contains; 4.0 µl plasmid, 1.0 µl 10X 'R buffer', 0.3 µl *Eco*RI, 0.3µl *Hin*dIII restriction enzyme and 4.4 µl of nuclease free milli Q water. After 2 h of incubation at 37ºC, reaction was heat inactivated at 65ºC for 20 min. Restriction digested product loaded into 1% agarose gel. The restriction digested products were showed 792 bp (717 bp of CP + 75 bp of multiple cloning sites of TA vector) of insert size (Fig 2). This indicates the presence of coat protein gene sequence in the plasmid. Further the presence of insert was confirmed by both direction of plasmid.

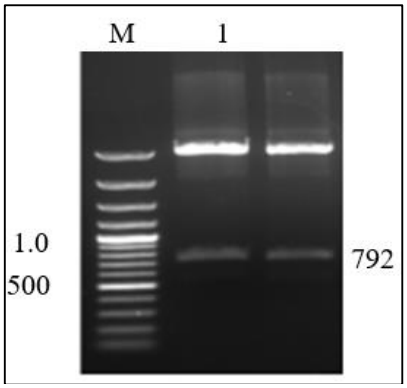

Fig 2: Restriction digestion pattern of recombinant pTZ57R/T plasmid having TSV coat protein gene of peanut strain in 1% agarose gel. In Lane 1 and 2; restriction digestion pattern for coat protein gene of TSV-Peanut present in pTZ57R/T vector, right side of the gel indicate 792 bp released CP gene as an insert and left side of the gel showed molecular weight of 1.0 kb DNA ladder.

Analysis of the sequencing results

Column purified plasmid was checked by Nano drop as well as in gel before sending for sequencing. 10 µl of restriction digestion confirmed plasmid having 200 ng /µl along with M13 forward and reverse primer was send for sequencing. The plasmid was sequenced from both direction to avoid the mismatch and gap. The quality of result was checked through the dendogram that showed very sharp and non-overlapping peak and very less noise in dendogram.

The sequence obtained in FASTA format of the coat protein gene used as the template for analysis of the result. The identified peanut strain of TSV showed 99.78% homology to the Kurnool peanut strain of TSV (Accession no AY50508), 99.72% homology with the cotton strain of TSV (Accession no AY505082), 99.58 homology to sunflower strain (Accession no AY501479) with coverage of 100% sequence. The identification of the viruses based on the symptom in the field condition is very difficult and undistinguishable. While collection of the field sample assuming a particular virus infection was in most cases gave contradictory results in laboratory. In comparative analysis of coat protein gene at nucleotide and amino acid level were analysed. The maximum mismatches were observed at the C-terminal region of the coat protein i.e. 684 nt to 711 nt. However, we have found that 100% conserved amino acid between peanut and other strains of the TSV at protein level.

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