The Bioscan 10(4): 1787-1793, 2015 (Supplenent on Plant Pathology)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

COAT PROTEIN MEDIATED RESISTANCE AGAINST *TOBACCO STREAK VIRUS* IN *NICOTIANA TABACUM* L. THROUGH RNA SILENCING

S. RAJAMANICKAM*, G. CHANDRASEKAR AND G. KARTHIKEYAN
Department of Plant Pathology,
Tamil Nadu Agricultural University, Coimbatore - 641 003,INDIA
e-mail: rajamanickam.path@gmail.com

KEYWORDS
Tobacco streak virus
Coat protein
hpRNA
Viral resistance

Received on :
07.10.2015

Accepted on :
21.12.2015

***Corresponding author**

ABSTRACT
The transgenic tobacco (*Nicotiana tabacum* L.) cv Abirami plants expressing hairpin RNA transcript (hpRNA) targeting coat protein gene of *Tobacco streak virus* (TSV) were generated and analyzed at the molecular and phenotypic levels. The coat protein (CP) genes of five isolates of TSV were sequenced (designated as CPCBE1, CPCBE2, CPDHP, CPKAR and CPCBE3) with view to develop transgenic tobacco resistance to *Tobacco streak virus* (TSV). The sequence analysis showed that they had a sequence identity of around 99 per cent at nucleotide level. The hairpin (hpRNA) construct was generated using the conserved sequences of coat protein gene of above TSV isolates. The hpRNA constructs corresponding to nucleotide positions 560 and 898 in the coat protein gene of TSV were introduced into tobacco (*Nicotiana tabacum* L.) cv. Abirami through *Agrobacterium* mediated transformation. The transgenic plants were screened and confirmed through PCR and Southern blot analysis using the genomic DNA. The transgenic T_0 tobacco plants showed resistance against TSV upon artificial mechanical inoculation of TSV without producing any symptoms of TSV, which was also confirmed by DAC-ELISA.

INTRODUCTION

Tobacco necrosis disease was first described by Johnson (1936) and the casual agent of the disease is *Tobacco streak virus* (TSV). It is the type member of the genus *Ilarvirus* (Family: *Bromoviridae*). The virus has tripartite genome which consists of three ssRNA of positive polarity. The virus particles are quasi isometric in shape with diameters between 24-36 nm. The RNA1 consists of 3491 nucleotides (nt) and contains a single open reading frame (ORF), putative replicase, which encodes for a protein of 1094 amino acids (aa) (Scott et al. 1998). RNA3 carries two open reading frames, first represents movement protein and second separated by an intergenic region, encodes coat protein of 25 to 30 kD (Scott *et al.,* 1998; Guo *et al.,* 1999). TSV is causing necrosis in plants leads to significant loss of yield in wide range of crops (Kumar *et al.,* 2008; Sivaprasad *et al.,* 2010; Jagtap *et al.,* 2012). The virus causes asymptomatic infections in several common weed species, including *Parthenium hysterophorus, Ageratum conyzoides* and *Corchorus trilocularis,* whose pollen is a major source of TSV and these plants also harbour thrips. The transmission commonly occurs through different species of thrips *viz*., *Thrips tabaci*, *T. palmi*, *Megalurothrips usitatus*, *Frankliniella schultzei* and *Scirtothrips dorsalis* and infected pollen under field conditions (Prasada Rao *et al.,* 2003; Shukla *et al.,* 2005).

Management strategies based on cultural practices, such as seed treatment with imidacloprid to control the thrips vector, barrier crops with fast-growing tall cereals to prevent insect movement, removal of TSV susceptible weed hosts, and maintaining optimal plant density were shown to reduce disease incidence, but are seldom practiced under subsistence agriculture systems (Dasgupta *et al.,* 2003; Kalaria *et al.,* 2014; Ingle *et al.,* 2014). After the concept of pathogen-derived resistance (PDR) proposed by Sanford and Johnston (1985) and it was first demonstrated in transgenic tobacco plants expressing the coat protein gene of *Tobacco mosaic virus* (Powell-Abel *et al.,* 1986). The pathogen-derived resistance in plants is primarily due to transgene-induced post transcriptional gene silencing (PTGS) *via* formation of dsRNA, known as RNA silencing (Baulcombe, 2002). RNA silencing in plants is part of a natural defence mechanism against virus and triggered by the presence of specific double stranded RNA (dsRNA) molecule, which is manifested by specific degradation of cytoplasmic transgene RNAs in transgenic plants (Fagard and Vaucheret 2000). The RNAi can be engineered to effectively target RNA virus, for example Hu et al. (2011) demonstrated *Nicotiana tabacum* expressing hairpin RNA derived from TMV movement protein exhibited complete resistance to TMV. In the present study, we have investigated the use of coat protein gene of TSV to develop transgenic tobacco (*Nicotnana tabacum* L. cv Abirami) resistance to TSV through RNA silencing.

MATERIALS AND METHODS

Virus isolates

TSV isolates were collected from naturally infected field of

sunflower (*Helianthus annuus* L.), okra (*Abelmoschus esculentus* L.) and soybean (*Glycine max* L.) plants showing characteristic symptoms of TSV and used as inoculum. 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 ICRISAT, Hyderabad). The cowpea plants cv C152 was used for propagating the virus by the method suggested by Subramanian and Narayanasamy (1973).

Reverse transcriptase -PCR and sequence analysis

The total RNA was extracted from TSV infected leaf to amplify the CP gene containing fragment with an amplicon size of 929 bp including UTR part of RNA3 genome of TSV (Table 1). RT-PCR was carried out in Eppendorf Master cycler Gradient ES with the OneStep RT-PCR kit (Bioline, USA Inc., USA) in 50μl reaction volume containing total RNA, 2 units of enzyme mix and fragment specific primers GKTSV CPF and GKTSV CPR used to amplify the complete coding region of CP gene of TSV. The amplified products were purified in 1% agarose gel, stained with ethidium bromide using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). The fragments were inserted into pGEM-T Easy vector (Promega, Madison, WI, USA) separately and transformed into *Escherichia coli* DH5α by following standard molecular biology procedures (Sambrook et al. 1989). Plasmid DNA was isolated and three independent clones were sequenced from both orientations for each fragment separately. The sequences were then edited using the BIOEDIT Software (Hall 1999). The nucleotide sequences of five isolates were then aligned to identify the consensus sequences using the MEGA 4.0 program.

Construction of vector

The vector pHANNIBAL obtained from CSIRO Plant Industry, Australia was used to clone the partial gene(s) encoding coat protein gene of TSV. The primers were designed based on the located regions of TSV to amplify 339 bp CP gene fragments. For effective cloning the suitable restriction enzymes were added to the primers to facilitate cloning into pHANNIBAL vector. The amplified PCR products were separated by electrophoresis on 1.2% agarose gels, recovered from the gel and purified. These products were cloned onto the pGEMT-easy vector (Promega) from where they were excised and cloned to pHANNIBAL vector into two steps. The recombinant plasmid pHANNIBAL containing hpRNA cassettes were identified by respective restriction endonuclease enzymes (Pradeep *et al.,* 2012). The hpRNA cassette from plasmid pHANNIBAL was sub cloned into pART 27, in order to transform tobacco plants.

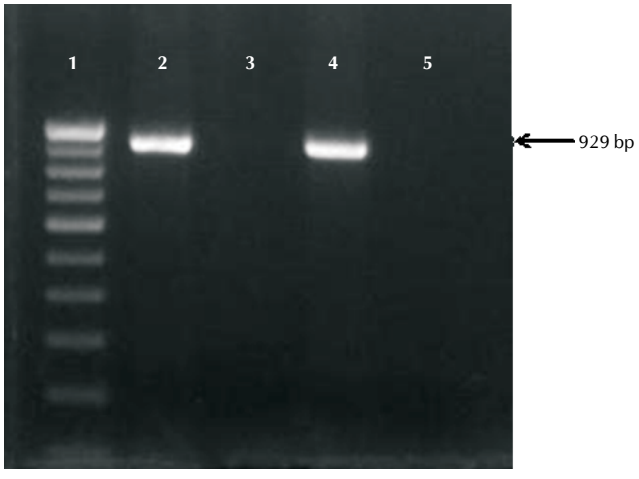

Figure 1: RT-PCR analysis of TSV using CP gene specific primers. Lane 1- 100 bp, Lane 2 &4- Positive Sample, Lane 3&5- Healthy Control

Tobacco transformation and molecular analysis

The recombinant binary vectors containing hpRNA cassettes were introduced into *Agrobacterium* strain LBA4404 (Jefferson 1987) *via* triparental mating using pRK2013 as a helper strain described by Ditta *et al.* (1980). The bacterium was grown in YEP for 16-24 hrs to obtain 1 O.D. (Optical density) culture. The *Agrobacterium* cells were pelleted by centrifugation at 4,000 rpm for 10 min and dissolved in equal volume of Schenk and Hildebrandt (SH) basal medium without hormone and the cells were grown for 3 h at 28°C with 175 rpm shaking. The protocol developed by Kutty *et al.* (2010) was followed for *Agrobacterium tumefaciens* mediated transformation of tobacco (*Nicotiana tabacum* L.) cv. Abirami. The transformed T_0 plants were generated under selection pressure of kanamycin and further screened by PCR using selectable marker *nptII* gene and coat protein gene fragment specific primers.

PCR detection of transformed plants

After planting in the greenhouse, plant tissue were collected from the regenerated tobacco plants and wrapped in aluminium foil together with the pestle and incubated at -80°C for at least 30 min upon removal from the freezer, the tissue was quickly ground with the chilled pestle and mortar and transferred to a chilled Eppendorf tube. The DNA extraction was carried out using CTAB method. The DNA was used as a template for the amplification of *nptII* gene (*nptII* F and *nptII*), partial fragments of CP gene (CPF1 and CPR1) gene fragments (Pradeep *et al.,* 2012). The PCR products were analyzed on a 1.2 % agarose gel, stained with ethidium bromide and viewed under transilluminator.

Total RNA isolation and RT-PCR analysis

Total RNA was extracted from putative transgenic and non transgnic tobacco plants using total RNA isolation kit (Qiagen Inc., Chatsworth, CA, USA). The RNA was converted into cDNA using Revert-AidTMH minus first strand cDNA Synthesis Kit (MBI Fermentas, USA). The first strand (cDNA) was used as a template for the amplification of CP gene fragment with the primers namely CPF1-5′ TTATTAGGTACCATACCT ACCGCAGCCGAG 3′ and CPR1-5′ TTATTACTCG AGTGCGCGGCA GCTATGCATG 3′. The presence of amplicon was checked through 1.2 % agarose gel electrophoresis and stained with ethidium bromide and viewed under transilluminator (Elayabalan *et al.,* 2013).

Southern blot analysis

To confirm the stable integration of transgene into the tobacco genome and to evaluate transgene copy number, Southern blot analysis was performed as described by Sambrook et al. (1989). Total genomic DNA was isolated from young leaves

of control and the putative transgenic plants expressing RNAi constructs using a modified CTAB protocol (Rogers and Bendich 1988). Thirty micrograms of genomic DNA was digested with the restriction enzyme *HindIII* for 12 hrs and separated on a 1% (W/V) TBE agarose gel overnight at 37 V and transferred onto Biodine B 0.45 μm positively charged Nylon 6.6 transfer membrane (Life Science Products Inc., Boston, MA, USA). Membranes were hybridized overnight with ^{32}P-labelled probe of 339 bp fragment of coat protein gene. The probe was generated by amplifying the specific fragments that included the coat protein gene used to generate the hairpin RNAi constructs. After hybridization the membranes were washed with 0.1X SSC, 0.5% SDS (W/V) at 58^{o}C for 15 min and then blots were exposed to X-ray films.

Viral resistance assay of transformed plants

The sap from cowpea cv C152 infected with TSV was used as an inoculum. Approximately 1.0 g of infected leaf tissue was ground with 1 ml of inoculation buffer [0.1 M sodium phosphate buffer (pH 7.0)] and mechanically inoculated to putative transgenic tobacco plants at the 3-4 four leaf stage as described earlier. A control was also maintained by inoculating TSV sap on nontransformed/wild tobacco plants. The inoculated plants were incubated under greenhouse conditions at 22 $\pm$ 2^{o}C for the development of symptoms (Elayabalan et al. 2013). The detection of TSV infection was also done by direct antigen coating-ELISA (DAC-ELISA) using specific polyclonal TSV antibody at 4 weeks of post-inoculation.

RESULTS

Isolation of virus and serodiagnosis

The crop plants *viz*., sunflower, okra and soybean showing characteristic symptoms of TSV like severe stunting, distortion of leaves, malformation of heads, necrosis of leaves, petioles, stem and floral calyx in sunflower and chlorotic streaks on the fruits in case of okra were collected and inoculated separately on cowpea cv C152 plants through mechanical sap inoculation. The assay host plant 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 led to the collapse of inoculated plants. The results of DAC-ELISA revealed that, the samples exhibiting characteristic symptoms of TSV showed strong positive reaction with approximately five fold increases in absorbance values than the apparently healthy samples.

Location of conserved sequences

The fragment containing coat protein gene was amplified with a size of 929 bp by RT-PCR (Fig.1) and inserted into pGEM-T easy vector for sequence determination. The sequence of TSV CP consists of 717 bases encoded the protein with 238 amino acids. The GenBank accession numbers of these coat protein genes are KF264467; KF264468; KF264469; KF264470 and KJ825822. The comparative analysis of the sequence of CP gene of TSV isolates showed nucleotide identities of above 99.4% between themselves. The region of conserved sequence sharing the highest nucleotide sequence identity was located in the gene sequences corresponding to nucleotide positions 560 and 898 of the coat protein gene of TSV. The hpRNA targeting the coat protein gene was successfully generated using the conserved nucleotide sequences of coat protein gene of TSV.

Construction of target vector and tobacco transformation

The target sequences of 339 bp of in length were cloned in to pHANNIBAL vector with respective cloning sites for making hairpin construct under the control of CaMV35S promoter.

Table 1: Primers used in this study

Primer name	Primer sequence	Restriction sites
GKTSV CPF	AGATAAGTCGCTTCTCGGAC	-
GKTSV CPR	TGCTCGCATGGGTCATAGAC	-
CPF1	ttattaggtaccATACCTACCGCAGCCGAG	*KpnI*
CPR1	ttattactcgagTGCGCGGCAGCTATGCATG	*XhoI*
CPF2	ttattaatcgatATACCTACCGCAGCCGAG	*ClaI*
CPR2	ttattaggatccTGCGCGGCAGCTATGCATG	*BamHI*
nptII F	TTGAGGCGCTAAATGAAACC	-
nptII F	ATTTG CCGACTACCTTGGTG	-

Table 2: Bioassay of transgenic tobacco plants (T_0)

Plants	PCR /Southern blot results	Absorbance value at 405nm in DAC-ELISA	Symptom development
IR-CP-1	+	0.129	-
IR-CP-2	+	0.162	-
IR-CP-3	+	0.168	-
IR-CP-4	+	0.153	-
IR-CP-5	+	0.173	-
IR-CP-6	+	0.108	-
IR-CP-7	+	0.171	-
IR-CP-8	+	0.152	-
IR-CP-9	+	0.144	-
IR-CP-10	+	0.174	-
Wild type (Nontransformed)	-	1.078	+

(+) – Positive ; (-) - Negative

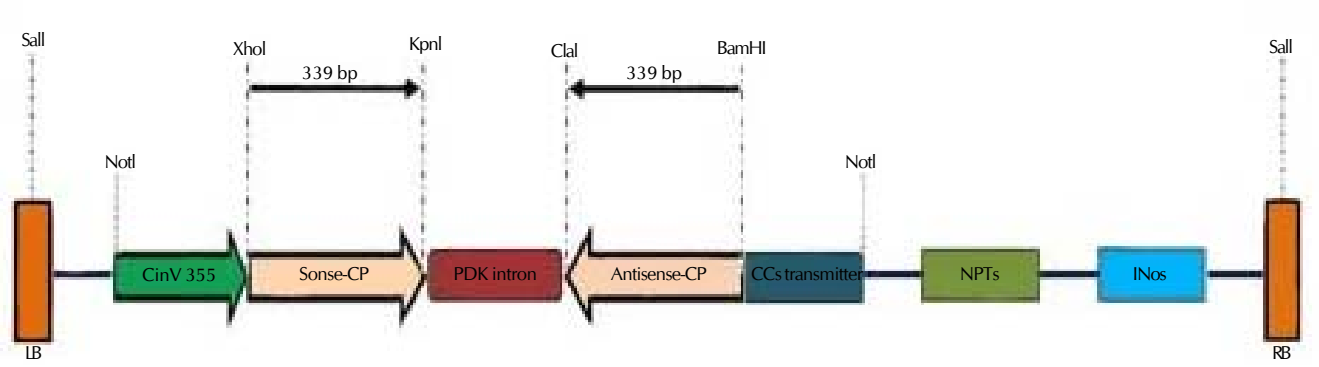

Figure 2: Schematic representations of hpRNA construct. TSV-CP gene fragments of 339 bp were amplified and used to form inverted repeats under the control of CaMV35S promoter

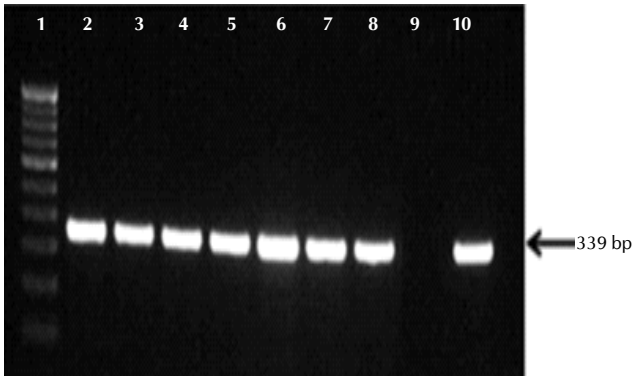

Figure 3: PCR analysis of total genomic DNA from transgenic tobacco (T_0) plants. A DNA fragment of 339 base pairs specific to the TSV-CP gene fragments was amplified in positive transformants (Lane 2 to 8). The plasmid served as positive control (Lane 10). Total DNA from non-transformants did not show any amplification (Land 9). 100 bp DNA molecular weight marker is shown on left side (Lane 1)

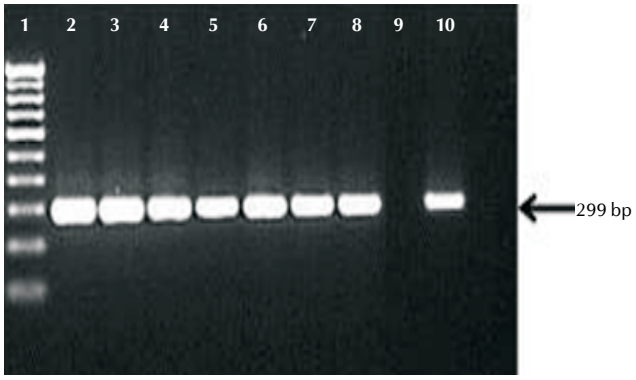

Figure 4: PCR analysis of total genomic DNA from transgenic tobacco (T_0) plants. A DNA fragment of ~299 base pairs specific to the *nptII* gene was amplified in positive transformants (Lane 2 to 8). The plasmid served as positive control (Lane 10). Total DNA from non-transformants did not show any amplification (Land 9). 100 bp DNA molecular weight marker is shown on left side (Lane 1)

The resultant pHANNIBAL (CP) was digested with restriction enzymes, *KpnI & XhoI + ClaI & BamHI*, about 339 bp fragment were obtained. The binary vectors were successfully constructed by cloning hpRNA in to plant expression vector pART27. The tobacco leaf discs were transformed with *Agrobacterium tumefaciens* LBA 4404 containing binary plasmids harbouring hpRNA cassette of coat protein gene (Fig. 2). The transgenic lines were regenerated on Murashige and Skoog (MS) medium containing vitamins, 0.8% (w/v) agar and 3% (w/v) sucrose supplemented with 1mg/l benzylaminopurine, 100 mg/l kanamycin and 250 mg/l cephotaxime. Putative transgenic plants were screened on MS medium containing kanamycin 100 mg/l. Fifteen days after regeneration, 5 cm long plantlets along with meristem and few leaves were transferred to rooting medium for subsequent rooting. After substantial growth the shoots were transferred to greenhouse for hardening.

Molecular analysis of transgenic plants

The PCR amplifications were demonstrated for the presence of CP and *nptII* gene fragments with the genomic DNA isolated from putative transformants. All the transgenic plants were confirmed the presence of CP gene fragments, by producing amplicon size of 339 bp (Fig 3). The *nptII* gene also targeted for PCR analysis of transformed tobacco plants. The results revealed that transformants produced ~299 bp amplicon which was integrated previously in binary vector (Fig 4). Further these putative transgenic tobacco plants were subjected to RT-PCR analysis using RNA from leaf tissue in order to verify CP gene fragments for transgene expression. The CP gene transcript amplification of expected fragment size 339 bp was obtained from RNA samples of all the tested transgenic plants. The putative transgenic plants were transferred to pots under greenhouse conditions and used for further studies.

Southern blot analysis of transgenic plants

The Southern blot analyses were performed on selected putative transgenic plants to analyse the transgene integration of the introduced hpRNA-CP gene cassettes and estimating the transgene copy numbers. The results of Southern blot analysis revealed the stable integration of transgene and multiple-copy transgene integration into the tobacco genome of the transformants. Of the five transformants analysed for RNAi-CP, two showed multiple copy integration of transgene

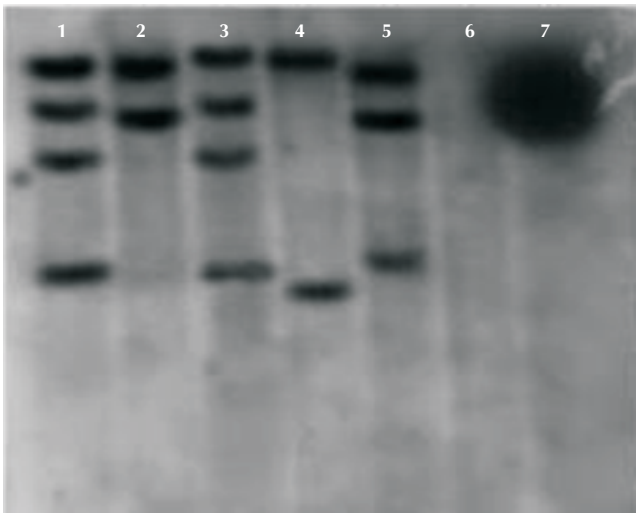

Fig. 5. Southern blot analysis of PCR positive tobacco transformants carrying RNAi-CP constructs. The *HindIII*-digested total genomic DNA was probed with a 339 bp probe of CP gene fragment. The samples in lanes 1-5 are RNAi-CP tobacco transformants and lane 6 is non-transformed tobacco sample with plasmid control (Lane 7)

(lanes 1 and 3). However, three transformants showed double copy transgene integration respectively (lanes 2, 4 and 5-). The pHANNIBAL vector with CP gene served as positive control which hybridized with the CP probe respectively (Fig 5). The non-transgenic tobacco plant did not show any signal.

Response of transgenic tobacco lines with TSV

The transgenic tobacco plants were inoculated with TSV at the four leaf stage in order to investigate the level of resistance against TSV. Ten days after inoculation, characteristic symptoms of TSV were observed on the leaves of the inoculated untransformed control plants. The transformed plants were completely symptomless even after four weeks of inoculation (Fig 6). After 4 weeks of inoculation, the leaf samples were also analysed by DAC-ELISA for the presence of TSV. The TSV could not be detected in transgenic tobacco plants expressing hairpin construct containing coat protein gene, while the TSV was detected in non-transformed (Table 2).

DISCUSSION

TSV infected samples exhibited the severe characteristic symptoms of TSV on cowpea cv C152 were produced by upon mechanical inoculation with typical necrotic lesions. Similar type of symptoms were noticed on cowpea by mechanical inoculation of TSV collected from various host crops (Ramiah *et al.,* 2001; Reddy *et al.,* 2002; Kumar *et al.,* 2008; Vemana and Jain 2010). It was also confirmed by DAC-ELISA and RT-PCR for the presence of TSV. The amplified coat protein genes with a product size of 929 bp were successfully cloned and sequence determined. The sequence analysis showed high homologies between the target nucleotide sequences of five TSV isolates. Sivaprasad et al. (2013) amplified the coat protein gene of TSV from different host crop and studied the genetic diversity. The sequence analysis revealed that the CP gene shared 91-100% and 91-99% sequence identity with TSV at nucleotide and amino acid level. Bhat *et al*. (2002) conducted serology and characterization of coat protein studies, reported that strain of TSV belonging to subgroup I, designated as TSV-SF. 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. The expression of coat protein gene of plant viruses usually resulted in different types of resistance could occurred in plants (Golemboski et al. 1990; Krubphachaya et al. 2007). In the present study, transgenic tobacco plants were developed using hairpin interference sequence targeting the conserved sequence of coat protein gene of TSV. In PDR, a part or a complete viral gene is introduced into a plant, which, subsequently interferes with essential step in the life or infection cycle of the virus and results in resistance to the pathogen. In this study, we used highly conserved region of coat protein gene of TSV for making hairpin RNA construct for tobacco transformation. RNA mediated virus resistance is a homology dependent gene silencing. It is possible to obtain transgenic plants resistance to multiple virus using template DNA fragments with high identity selected from different viruses (Xu et al. 2009). The hpRNA constructs containing sense/anti-sense arms ranging from 98 to 853 nt gave efficient silencing in a wide range of plant species (Wesley et al. 2001).

The Southern blot analysis confirmed the integration of transgenes into the tobacco genome and detected multiple-copy integration of the transgenes in transformants expressing coat protein genes. The majority of the transformants showed multiple copy transgene integration, indicating that the intact T-DNA was integrated into the tobacco genome. Pandolfini et al. (2003) demonstrated that, transgenic *N. benthamiana* lines expressing hairpin construct under the control of *rolC* promoter produced single and multiple copy of integration of transgenes which showed resistance to *Plum pox virus* systemic infection. The transformed tobacco plants conferred resistance to TSV through mechanical inoculation without producing any necrotic lesions on the inoculated leaves, systemic veinal necrosis and death of plants. Whereas non-transformed plants produced characteristic symptom of TSV after 10 days of inoculation under greenhouse conditions and it was also confirmed by DAC-ELISA using polyclonal antiserum specific to TSV. RNA mediated virus resistance seems to be effective only against viruses with closely related sequences (Balcombe 1996; Bau et al. 2003). The small interfering RNAs (siRNAs) were detected in the resistant plants, indicating that the resistance is attributed due to RNA silencing (Nomura et al. 2004). In siRNA mediated silencing, it was observed that siRNA sequences have non-random distribution along the length of viral genome (Molnar et al. 2005). Jinlong Guo et al. (2015) developed the virus resistance transgenic sugarcane expressing hairpin interference sequence targeting the conserved region of coat protein of *Sugarcane mosaic virus* (SCMV). Vimal Kumar *et al*. (2015) reported coat protein mediated resistance in transgenic lines of *Nicotiana tabacum* cv. Petit Havana against *Cucumber mosaic virus* (CMV) subgroup IA using *Agrobacterium tumefaciens*-mediated transformation. Similarly, Bag et al. (2007) reported that the transgenic groundnut transformed with hairpin construct of TSV-CP through *Agrobacterium* mediated transformation

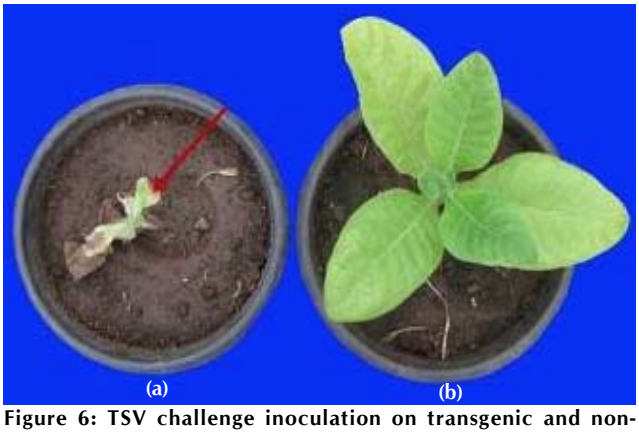

Figure 6: TSV challenge inoculation on transgenic and non-transformed control tobacco plants. Transgenic tobacco plant expressing TSV-CP hairpin construct (b) and nontransformed control (a) plant showing characteristic symptoms on leaves at 10 days post inoculation

confers transgene integration into groundnut showed resistance against TSV. In conclusion, RNA mediated virus resistance is homology dependent gene silencing. It is possible to obtain transgenic plants with viral coat protein gene showing homology to selected isolates of the same virus or different strain infecting various crops. Our results confirmed that tobacco plants with inverted repeat of TSV-CP gene under the control of the constitutive CaMV35S promoter through *Agrobacterium*-mediated transformation enhanced the resistance against TSV.

ACKNOWLEDGEMENT

The authors acknowledge the Department of Science and Technology (SERB), Government of India, New Delhi for the financial support of the project and also CSIRO Plant Industry, Australia for providing pHANNIBAL vector.

REFERENCES

Almeida, A. M. R., Sakai, J., Hanada, K., Oliveira, T. G., Belintani, P., Kitajima, E. W., Souto, E. R., Novaes, T. G. and Nora, P. S. 2005. Biological and molecular characterization of an isolate of *Tobacco streak virus* obtained from soybeans in Brazil. *Fitopatol Bras.* **30:** 366-373.

Bag, S., Singh, R. S. and Jain, R. K. 2007. *Agrobacterium* mediated transformation of groundnut with coat protein gene of *Tobacco streak virus*. *Ind. J. Virol.* **18:** 65-69

Bau, H. J., Cheng, Y. H., Yu, T. A., Yang, J. S. and Yeh, S. D. 2003. Broad spectrum resistance to different geographic strains of *Papaya ringspot virus* in coat protein gene transgenic papaya. Phytopatholoy, **93:** 112-120.

Baulcombe, D. C. 1996. RNA as target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol.* **32:** 79-88.

Baulcombe, D. C. 2002. Viral suppression of systemic silencing. *Trends in Microbiology*. **10:** 306-308.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. H. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. **409:** 363-366

Bhat, A. I., Jain, R. K., Kumar, A., Ramiah, M. and Varma, A. 2002. Serological and coat protein sequence studies suggest that necrosis disease on sunflower in India is caused by a strain of *Tobacco streak ilarvirus*. *Arch Virol.* **147:** 651-658.

Dasgupta, I., Malathi, V. G. and Mukherjee, S. K. 2003. Genetic engineering for virus resistance. *Curr Sci.* **84:** 341-354

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci* USA. **77:** 7347-7351.

Elayabalan, S., Kalaiponmani, K., Subramaniam, S., Selvarajan, R., Panchanathan, R., Muthuvelayoutham, R., Kumar, K. K. and Balasubramanian, P. 2013. Development of *Agrobacterium*-mediated transformation of highly valued hill banana cultivar Virupakshi (AAB) for resistance to BBTV disease. *World J. Microbiol and Biotechnol.*, **29:** 589-596.

Fagard, M. and Vaucheret, H. 2000. Gene silencing in plants: How many mechanism?. *Annu. Rev. Plant Mol. Biol.* **51:** 167-194.

Golemboski, D. B., Lomonossoff, G. P. and Zaitlin, M. 1990. Plants transformed with a *Tobacco mosaic virus* non structural gene sequence are resistant to the virus. *Proc Natl Acad Sci USA.* **87:** 6311-6315.

Guo, D., Edgar, M. and Gunter, A. 1999. Ilarvirus isolation and RNA extraction. In: Methods in Molecular Biology, Plant Virology Protocol. Foster, G.D. and Taylor, S.C. (Eds.) Humana Press Inc Totowa: pp.171-181.

Hall, T. A. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symposium Series*. **41:** 95-98.

Hobbs, H. A., Reddy, D. V. R., Rajeshwari, R. and Reddy, A. S. 1987. Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. *Plant. Di.* **71:** 747-749.

Hu, Q., Niu, Y., Zhang, K., Liu, Y. and Zhou, X. 2011. Virus-derived transgenes expressing hairpin RNA give immunity to *Tobacco mosaic virus* and *Cucumber mosaic virus*. *Virology J.* **8(41):**1-11.

Ingle, Y. V., Patil, C. U., Thakur, K. D. and Ingle, K. 2014. Effect of fungicides and plant resistance activator on *Colletotrichum* leaf spot of soybean. *The Bioscan.* **9(3):** 1187-1190

Jagtap, G. P., Jadhav, T. H. and Utpal, D. 2012. Occurrence, distribution and survey of *Tobacco streak virus* (TSV) of cotton. *Sci J Crop Sci.* **1:** 16-19.

Jain, R. K., Bag, S. and Awasthi, L. P. 2005. First report of natural infection of *Capsicum annuum* by *Tobacco streak virus* in India. *Plant Pathol.* **54:** 257.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Bio. Rep.* **5:** 387-405.

Jinlong Guo, Shiwu Gao, Qinliang Lin, Hengbo Wang, and Youxiong Que and Liping Xu. 2015. Transgenic sugarcane resistant to *Sorghum mosaic virus* based on coat protein gene silencing by RNA interference. *BioMed Research International*. ***1:*** *1-9.*

Johnson, J. 1936. *Tobacco streak*, a virus disease. *Phytopathology*, **26:** 285-292.

Kalaria, R. K., Digvijay, C., Mahesh Kumar, M. and Lalit, M. 2014. Identification of RAPD and ISSR makers for resistance against *Mungbean yellow mosaic virus* in mungbean (*Vigna radiata* L.) under South Gujarat agro climatic condition of India. *The Bioscan.* **9(3):** 1177-1182.

Krubphachaya, P., Jurick, M. and Kertbundi, S. 2007. Induction of RNA-mediated resistance to *Papaya Ringspot Virus* Type *W. J. Biochem Mol. Bio.* **40:** 404-411.

Kumar, A. N., Lakshminarasu, M., Zehr, U. B. and Ravi, K. S. 2008. Molecular characterization of *Tobacco streak virus* causing soybean necrosis in India. *Indian J. Biotechnol.* **7:** 214-217

Kutty, P. C., Parveez, G. K. A. and Huyop, F. 2010. An easy method

for *Agrobacterium tumefaciens* mediated gene transfer to *Nicotiana tobacum* cv. TAPM26. *J. Biol. Sci.* **10:** 480-489.

Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C. and Burgyan, J. 2005. Plant virus derived small interfering RNAs originate predominantly from highly structured single stranded viral RNAs. *J Virol.* **79:** 7812-7818.

Nomura, K., Ohshima, K., Anai, T., Uekusa, H. and Kita, N. 2004. RNA silencing of the introduced coat protein gene of *Turnip mosaic virus* confers broad-spectrum resistance in transgenic Arabidopsis. *Phytopathology*. **94:** 730-736.

Pandolfini, T., Molesini, B., Avesani, L., Spena, A. and Polverari, A. 2003. Expression of self complementary hairpin RNA under the control of the *rolC* promoter confers systemic disease resistance to *Plum pox virus* without preventing local infection. *BMC Biotechnol.* **3:** 7-22

Powell Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T. and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the *Tobacco mosaic virus* coat protein gene. *Science.* **232:** 738-743.

Pradeep, K., Satya, V. K., Selvapriya, M., Vijayasamundeeswari, A., Ladhalakshmi, D., Paranidharan, V., Rabindran, R., Samiyappan, R., Balasubramanian, P. and Velazhahan, R. 2012. Engineering resistance against *Tobacco streak virus* (TSV) in sunflower and tobacco using RNA interference. *Biologia Plantarum.* **56:** 735-741

Prasada Rao, R. D. V. J., Reddy, A. S., Reddy, S. V., Thirumaladevi, K., Chander Rao, S., Manoj Kumar, V., Subramanyam, K., Yellamanda Reddy, T., Nigam, S. N. and Reddy, D. V. R. 2003. The host range of *Tobacco streak virus* in India and transmission by thrips. *Ann Appl Biol.* **142:** 365-368.

Ramiah, M., Bhat, A. I., Jain, R. K., Pant, R. P., Ahlawat, Y. S., Prabhakar, K. and Verma, A. 2001. Partial characterization of an isometric virus causing sunflower necrosis disease. *Indian Phytopath.*, **54:** 246-250.

Reddy, A. S., Prasada Rao, R. D. V. J., Thirumaladevi, K., Reddy, S. V., Mayo, M. A., Roberts, I., Satyanarayana, T., Subramaniam, K. and Reddy, D. V. R. 2002. Occurrence of *Tobacco streak virus* on Peanut (*Arachis hypogaea*) in India. *Plant Dis.* **86:** 173-178.

Rogers, S. O. and Bendich, A. J. 1988. Extraction of DNA from plant tissues. In: Gelvin, S.B. and Schilperoort, R.A. (EDS), Plant molecular biology manual, Kluwer, Dordrecht: pp. l-10

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*

Sanford, J. C. and Johnson, S. A. 1985. The concept of pathogen derived resistance. *J. Theor Biol.* **113:** 395-405.

Scott, S. W., Zimmerman, M. T. and Ge, X. 1998. The sequence of RNA 1 and RNA 2 of *Tobacco streak virus*: additional evidence for the inclusion of *Alfalfa mosaic virus* in the genus *Ilarvirus*. *Arch Virol.* **143:** 1187-1198.

Shukla, S., Kalyani, G., Kulkarni, N., Waliyar, F. and Nigam, S. N. 2005. Mechanism of transmission of *Tobacco streak virus* by *Scirtothrips dorsalis, Frankliniella schultzei* and *Megalurothrips usitatus* in groundnut, *Arachis hypogaea L. Oilseeds Res.* **22:** 215-217.

Sivaprasad, Y., Bhaskara Reddy, B.V., Rekha Rani, K., Raja Reddy, K. and Sai Gopal, D. V. R. 2010. First report of *Tobacco streak ilarvirus* infecting onion (*Allium cepa*). *New Disease Reports.* **22:** 17.

Sivaprasad, Y., Bhaskara Reddy, B. V., Sujitha, A. and Sai Gopal, D. V. R. 2013. Genetic variability of tobacco streak virus in South India based on the analysis of coat protein gene. *Arch Phytopathol Plant Protect.* **46(2):** 172-179

Subramanian, K. S. and Narayanasamy, P. 1973. Mechanical transmission of *Whitefly borne yellow mosaic virus* of *Lablab niger* Medikus. *Curr Sci.* **47:** 92-93.

Vemana, K. and Jain, R. K. 2010. New experimental hosts of *Tobacco streak virus* and absence of true seed transmission in leguminous hosts. *Indian J. Virol.* **21:** 117-127.

Vimal Kumar, D., Kottakota, C., Alok, S., Aminuddin, Vijai Pal, S., Kartik, D. and Pankaj Kumar, A. 2015. Expression of coat protein gene of *Cucumber mosaic virus* (CMV-subgroup IA) Gladiolus isolate in *Nicotiana tabacum*. *J. Plant Interactions.* **10(1):** 296-304.

Wesley, V. S., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., Stoutjesdijk, P. A., Robinson, S. P., Gleave, A. P., Green, A. G. and Waterhouse, P. M. 2001. Construct design for efficient, effective and high throughput gene silencing in plants. *Plant J.* **27:** 581-590.

Xu, L., Song, Y., Zhu, J. and Guo, X. 2009. Conserved sequence of replicase gene mediated resistance to *Potyvirus* through RNA silencing. *J. Plant Biol.* **52:** 550-559.