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

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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 (Scott et al., 2008; Sivaprasad et al., 2010; Jagtap et al., 2012). The virus causes asymptomatic infections in several common weed species, including Parthenium hysterophorus, Agaratum 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 (Nicotiana 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 GKT3V 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 and viewed under transilluminator. The characteristic symptoms of TSV and used as inoculum. The recombinant plasmid pHANNIBAL containing hpRNA cassettes were identified by respective restriction endonuclease enzym (Pradeep et al., 2012). The hpRNA cassette from plasmid pHANNIBAL was sub cloned into pART 27, in order to transform tobacco plants.

Tobacco transformation and molecular analysis

The recombinant binary vectors containing hpRNA cassettes were introduced into Agrobacterium strain LBA4404 (Jefferson 1987) via trip parental 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 nptII1), 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 transgenic 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’ TTATTAGGTACTACCT ACCGCCCGCAT 3’ and CPR1-5’ TTATTACTGCCAGTGGCGGCGCA GTATGCGCAT 3’. The presence of amplicon was checked through 1.2 % agarose gel electrophoresis and stained with ethidium bromide and viewed under transilluminator (Elayabalani 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 32P-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°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±2°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>
<thead>
<tr>
<th>Plants</th>
<th>PCR /Southern blot results</th>
<th>Absorbance value at 405nm in DAC-ELISA</th>
<th>Symptom development</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-CP-1</td>
<td>+</td>
<td>0.129</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-2</td>
<td>+</td>
<td>0.162</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-3</td>
<td>+</td>
<td>0.168</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-4</td>
<td>+</td>
<td>0.153</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-5</td>
<td>+</td>
<td>0.173</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-6</td>
<td>+</td>
<td>0.108</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-7</td>
<td>+</td>
<td>0.171</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-8</td>
<td>+</td>
<td>0.152</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-9</td>
<td>+</td>
<td>0.144</td>
<td>-</td>
</tr>
<tr>
<td>IR-CP-10</td>
<td>+</td>
<td>0.174</td>
<td>-</td>
</tr>
<tr>
<td>Wild type (Nontransformed)</td>
<td>-</td>
<td>1.078</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) – Positive ; (-) – Negative
The resultant pHANNIBAL (CP) was digested with restriction enzymes, KpnI & XhoI + Clal & 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 cephotoxime. 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
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 vein 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 et al. 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.
serological and coat protein sequence studies suggest that necrosis


ringspot virus

interference.

RNA as target and an initiator of post-

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Tobacco streak

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