Aim: Genetic engineering of peanuts (*Arachis hypogaea* L.) via genes encoding for coat protein (*CP* gene) of Tobacco streak virus (TSV) and nucleocapsid protein (*NC* gene) of a Peanut bud necrosis virus (PBNV) were used to impart concurrent resistance against the stem and bud necrosis diseases. The main objective of this study was to determine whether the *CP/NC*-mediated resistance strategy could be applied for developing the transgenic peanut plants, by utilizing *CP* gene of TSV and *NC* gene of PBNV.

Methodology: The transgenic lines of peanut cv. K-6 were characterised for integration, inheritance and expression of transgenes through PCR, RT-PCR and quantitative PCR (qPCR) analysis. The transgenic plants were artificially challenged with viruses under confined glasshouse conditions and the load of virus particles were confirmed using DAC-ELISA, RT-PCR and histopathology in both transgenic and wild-type (WT) plants.

Results: The marker-free transgenic groundnut plants carrying TSV-CP+PBNV-NC genes witnessed delayed and less intense symptoms after viral inoculation, suggesting underlying resistance via a coat protein/nucleocapsid-mediated mechanism and indicated partial/non-durable resistance to TSV and PBNV.

Interpretation: The marker-free Agrobacterium-mediated transformation technique can be successfully used to generate transgenic peanuts having resistance to both Ilarvirus and Tospoviruses. This strategy may be applied to commercially important crops that are affected by Ilarvirus and Tospoviruses.

Key words: Allotetraploid, Biotic-stress resistance, necrosis virus, Tobacco streak, Transgenic plants virus

Evaluation of transgenic peanut plants encoding coat protein and nucleocapsid protein genes for resistance to tobacco streak virus and peanut bud necrosis virus
Introduction

Peanut (Arachis hypogaea L.) is a self-pollinated, allotetraploid legume of family Fabaceae, which can play a major role in bridging the edible oil gap in India. The current Indian peanut productivity of 1804 kg ha⁻¹ is deplorably low as compared to 3614 kg ha⁻¹ in China and 4496 kg ha⁻¹ in USA (FAOSTAT, 2014). About 80% of the peanut area in India is monsoon dependent and main reasons for low yield is not only the rainfed nature of the crop but also various biotic-stresses including viral diseases like peanut stem necrosis disease and peanut bud necrosis disease caused by Tobacco Streak Virus (TSV) and Peanut Bud Necrosis Virus (PBNV), respectively (Reddy et al., 2002; Mishra et al., 2015; Kandakoor et al., 2014, Singh et al., 2015a,b). Moreover, viral diseases are known to restrain the production in all peanut-growing areas of the world (Mehta et al., 2013).

TSV is distributed worldwide as it has been reported from North and South America, Europe, South Africa, India, Australia, New Zealand, Netherlands, Pakistan and Iran (Jain et al., 2008). In India, during the year Kharif 2000, TSV was epidemic in Anantapur district of Andhra Pradesh and caused inflicting loss to the tune of 47.0 million USD (Reddy et al., 2002; Prasad Rao et al., 2003). In peanut, TSV necrotic lesions appear first on young leaves and later spread to the petiole and stem, killing the top growing buds while, spots may appear on pegs and pods also (Reddy et al., 2002).

Furthermore, PBNV which is vectored by thrips is restricted to South and Southeast Asian countries, including India, Nepal, Sri-Lanka, Myanmar, Thailand and parts of China. In India, PBNV alone has been estimated to cause annual loss of 89 million USD (Reddy et al., 1995). Initial symptoms of PBNV appear on young quadrifoliolate leaves as mild chlorotic spots and later develop in to necrotic and chlorotic rings. In rainy and post rainy seasons, necrosis of terminal bud is the main characteristic symptom.

Strategies for management of viral diseases normally include control of vector population using insecticides, resistant cultivars and several cultural practices can reduce the incidence of TSV and PBNV. But, the above components of integrated diseases management so far did not prove competitively favourable. Moreover, for both TSV and PBNV the genetic resistance against virus has not been reported in the gene pool of cultivated peanut, hence no cultivar, resistant to these diseases could be developed till date. Under these circumstances, direct gene transfer using genetic transformation method remains the only choice to have peanut plants with genetic resistance to TSV and PBNV.

Coat protein mediated resistance is well-known as an effective means of protection against viral infection and avoidance of crop loss (Baulcombe, 1996; Beachy, 1997). CP genes has been reported to grant partial or complete resistance against TSV in tobacco (Pradeep et al., 2012) and potato virus Y in potato plants (Hefferon et al., 1997).

Transgenic peanut progenies expressing nucleocapsid protein of TSWV, when subjected to natural virus infection under field conditions showed resistance to TSWV (Yang et al., 2004). In addition, transgenic plants harbouring sense and translationally defective or antisense NC protein gene have also been developed and tested for expression in progenies (Li et al., 1997 and Yang et al., 1998). Additionally, the transgenic progenies of peanut cultivar, MARC-1 expressing antisense NC gene, showed significantly lower occurrence of spotted wilt over wild-type (WT) plants, without fail over a 3-year period (Yang et al., 2004). In another report, transgenic peanut plants developed from coat protein gene for Tobacco streak virus showed resistance with traces or refusal systemic growth of virus up to T3 generation (Mehta et al., 2013).

Therefore, considering the economic importance of TSV and PBNV in peanut cultivation, we have resorted to the marker free Agrobacterium-mediated genetic transformation approach using CP and NC genes to develop virus resistant genotypes in peanut. The main objective of this study was to determine whether the CP/NC-mediated resistance strategy could be applied for developing the transgenic peanut plants, by utilizing the CP gene of the TSV and NC gene of PBNV.

Materials and Methods

Gene construct and plant materials: The 717 bp CP gene (GenBank Acc. No. AF400664.1) of TSV and 831 bp NC gene (GenBank Acc. No. FJ749261.1) of PBNV were introduced into the binary vector pCAMBIA 1305.1 by replacing HygR and GUS PLUSTM genes. The 2X CaMV 35S and CaMV 35S constitutive promoters from the cauliflower mosaic virus were used for constitutive expression controlling of TSV-CP and PBNV-NC genes, respectively. The dual construct transgenic peanut lines of Arachis hypogaea cv. Kadiri-6 (K-6), was transformed and available events were used as experimental material. The dual gene (TSV-CP and PBNV-NC) construct was obtained from Advanced Centre for Plant Virology, IARI, New Delhi, India.

Molecular analysis

PCR analysis: The PCR screening of putative transgenic plants were done to confirm the presence of transgenes. Genomic DNA was extracted from fresh terminal leaves (Cuc et al., 2008) and PCR was performed using gene specific primer pairs for TSV-CP and PBNV-NC genes (Table 1). The amplification of 717 bp for TSV-CP and 654 bp for PBNV-NC genes were obtained. For TSV-CP gene, PCR reaction was set in 25 µl volume containing 5 µl of 5x PCR buffer, 2 µl 25 mM MgCl₂, 2 µl of 2 mM dNTP mix, 25 pmol of each primers, 0.25 µl of 1U Taq DNA polymerase and 100 ng of...
Evaluation of transgenic peanut plants

Table 1: Details of primers used for PCR, RT-PCR and qPCR analysis of transgenic plants

<table>
<thead>
<tr>
<th>Gene and RT-PCR analysis</th>
<th>Primer sequence (5’-3’)</th>
<th>Tm (°C)</th>
<th>Amplicons size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSV-CP</td>
<td>Fwd-CCATGGATGAATCTTGATGCAAG</td>
<td>74.0</td>
<td>717</td>
</tr>
<tr>
<td></td>
<td>Rev-GGTNACCTCAGTCTGATGTCACCA</td>
<td>74.0</td>
<td></td>
</tr>
<tr>
<td>PBNV-NC</td>
<td>Fwd-CTTCGGTGGACTCTGCACTG</td>
<td>74.0</td>
<td>654</td>
</tr>
<tr>
<td></td>
<td>Rev-AGGTCGTTCGGAGTTGACTCTCG</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>qPCR analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSV-CP</td>
<td>Fwd-CCATGGATGAATCTTGATGCAAG</td>
<td>54.4</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>Rev-GGTNACCTCAGTCTGATGTCACCA</td>
<td>58.8</td>
<td></td>
</tr>
<tr>
<td>PBNV-NC</td>
<td>Fwd-CTTCGGTGGACTCTGCACTG</td>
<td>55.6</td>
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</tr>
<tr>
<td></td>
<td>Rev-GTACTGCTCTGATGTCAC</td>
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<td></td>
</tr>
<tr>
<td>18S rRNA*</td>
<td>Fwd-GGCTCAAGCAGTTGAAAGTG</td>
<td>60.0</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Rev-AGACGACAGCTTACGCA</td>
<td>64.0</td>
<td></td>
</tr>
<tr>
<td>RT-PCR analysis for detection of challenge inoculum load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSV-Putative viral replicase</td>
<td>Fwd-CTGGAAAGAACGCAAAACAC</td>
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<td>507</td>
</tr>
<tr>
<td></td>
<td>Rev-TATAGGCTACTTCTCGCATC</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>PBNV-Non-structural movement protein</td>
<td>Fwd-CTCCGTCCTTTACACAGC</td>
<td>60.0</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>Rev-ATCAGGAGATGTCACCA</td>
<td>62.0</td>
<td></td>
</tr>
</tbody>
</table>

*same 18S rRNA primers were used in RT-PCR and qPCR

genomic DNA as template. Thermal-cycler condition was set as initial de-naturation: 94°C for 3 min; then 30 cycles of 94°C-45 sec, 60-56°C-30 sec, 72°C-1 min and final extension at 72°C for 5 min and for PBNV-NC gene the PCR reaction was set in 25 µl reaction volume containing 2.5 µl of 5x PCR buffer, 2 µl of 25 mM MgCl₂, 1.60 µl of 2 mM dNTP mix, 25 pmol of each primers, 0.2 µl of 1U Taq DNA polymerase and 100 ng of genomic DNA as template. Thermal-cycler conditions were set as initial de-naturation: 94°C for 3 min; then 35 cycles of 94°C-30 sec, 65-60°C-40 sec, 72°C-1 min and final extension at 72°C for 10 min. The PCR products were analysed on 1.2% (w/v) agarose, stained with ethidium bromide, scanned and documented. The plants giving amplification using both gene-specific primers were considered positive.

Segregation analysis: To study the segregation pattern of transgene, T1 transgenic plants were grown in pots under controlled conditions in a confined glasshouse. The plantlets at 2-4 leaf stage were used for PCR analysis using PBNV-NC gene specific primers to score the amplicons.

\[ \chi^2 = \sum (O_i - E_i)^2 / E_i \]

Where, \( \chi^2 \) = Pearson’s cumulative test statistic; \( O_i \) = Number of observations of types \( i \); \( E_i \) = Expected (theoretical) frequency of type \( i \); \( n \) = Total number samples

Chi-square (\( \chi^2 \)) test was used to study segregation pattern of the transgene (Pearson, 1900). The \( \chi^2 \) analysis was carried out for the transgenic events (having at least five pods) for observance of Mendelian pattern of inheritance.

Reverse transcription PCR (RT-PCR): Total RNA was isolated from representative transgenic plants through Plant RNeasy Kit (Qiagen, GmbH). RNA was quantified through NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA). The cDNA was prepared from 1 µg of each RNA using First Strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer protocols. These cDNAs were used to set the PCR reaction for TSV-CP (717 bp fragment), PBNV-NC (654 bp fragment) and 18S rRNA (148 bp fragment) genes to confirm efficiency of cDNA synthesis reaction using gene-specific primer pairs (Table 1), respectively. To check stringency of the reaction conditions, 18S rRNA gene was also amplified from the same cDNA using 18S rRNA primer pair in same PCR conditions.

Quantitative PCR (qPCR): The qPCR was performed using an ABI StepOne real-time PCR machine (Applied Biosystem, California, USA) to quantify the transcripts for TSV-CP and PBNV-NC based on SYBR Green chemistry using Quantifast SYBR Green PCR Kit (Qiagen, GmbH). Primers used for the reaction were TSV-CP, PBNV-NC and 18S rRNA genes (Table 1). RTq PCR mixtures for expression of TSV-CP, PBNV-NC and 18S rRNA genes were prepared in MicroAmp Fast Optical 48 well reaction plate. Reactions were performed using Quantifast SYBR Green PCR mix (Qiagen, USA), 20 pmol of each primers and 1 µl of cDNAs following manufacturer protocols. After completion of 40 cycles, products were subjected to MelT-Curve analysis to check the specificity of amplification. The relative quantification of TSV-CP and PBNV-NC was normalized with respect to 18S rRNA as internal (housekeeping gene) control on Real-Time PCR system. Proportional fold expressions of transgenes were calculated in terms of 2^-ΔΔCT method (Livak and Schmittgen, 2001).

Table 1: Details of primers used for PCR, RT-PCR and qPCR analysis of transgenic plants

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<tr>
<td>PBNV-NC</td>
<td>Fwd-CTTCGGTGGACTCTGCACTG</td>
<td>74.0</td>
</tr>
<tr>
<td></td>
<td>Rev-AGGTCGTTCGGAGTTGACTCTCG</td>
<td>66.0</td>
</tr>
<tr>
<td>qPCR analysis</td>
<td>Fwd-CCATGGATGAATCTTGATGCAAG</td>
<td>54.4</td>
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<tr>
<td></td>
<td>Rev-GGTNACCTCAGTCTGATGTCACCA</td>
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</tr>
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<tr>
<td></td>
<td>Rev-GTACTGCTCTGATGTCAC</td>
<td>56.9</td>
</tr>
<tr>
<td>18S rRNA*</td>
<td>Fwd-GGCTCAAGCAGTTGAAAGTG</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>Rev-AGACGACAGCTTACGCA</td>
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<tr>
<td></td>
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<td>62.0</td>
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</table>

*same 18S rRNA primers were used in RT-PCR and qPCR

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subtracting 18S rRNA C\textsubscript{T} from TSV-CP and PBNV-NC C\textsubscript{T} in a given sample. The \(\Delta C\textsubscript{T}\) values were determined by subtracting the \(\Delta C\textsubscript{T}\) of calibrator from \(\Delta C\textsubscript{T}\) of different. The outcomes were evaluated statistically by relative fold expression of TSV-CP and PBNV-NC transcripts. The primers for TSV-CP, PBNV-NC and 18S rRNA genes used for quantitative PCR expression analysis were designed using Primer Express\textsuperscript{\textregistered} version 3.0 from Applied Biosystems and synthesized from IDT Inc., USA.

Virus resistance assays: Transgenic plants and WT were grown in confined glasshouse for 2 wk(s) before virus inoculation. Plants were mock-inoculated with phosphate buffer or inoculated with leaves sap extracts (diluted in 0.1 M phosphate buffer (pH 7.2)) from cowpea plants infected with TSV and PBNV. Leaves from infected cowpea plants with outstanding symptoms were selected for inoculums. The leaves were macerated using mortar and pestle in chilled inoculation buffer (for 100 mg leaves 0.1 M phosphate buffer, (pH 7.0) in 1:10 w/v ratio). Leaves of transgenic plants were dusted with carborundum (320 grit) powder and then inoculums of TSV and PBNV were applied using a cotton swab (Mehta et al., 2013). Then after, leaves were washed with distilled water and the inoculated plants were observed for viral symptoms.

Direct antigen coated enzyme linked immuno sorbent assay (DAC-ELISA): DAC-ELISA was performed for the detection of TSV and PBNV with 500 mg leaf samples of infected transgenic, WT and un-infected healthy plants (Hobbs et al., 1987). 96-well polystyrene micro titer plates were coated with leaf extracts prepared in 50 mM sodium carbonate buffer, (pH 9.6). The Polyclonal antisera (obtained from ICRISAT, Hyderabad) was raised against CP of TSV and NC of PBNV and used in dilutions of 1:10,000. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis) were used at 1:2,000 dilution of commercial stock. P-nitrophenyl phosphate at 0.5 mg ml\textsuperscript{-1} was used as substrate. Absorbance was recorded at 405 nm after incubation for 1 hr at room temperature following the totalling of the substrate using a BioTek Make ELISA Plate Reader (Model Epoch). The samples showing at least three times more value than their respective healthy controls were considered positive.

RT-PCR base detection of challenge inoculum load of TSV and PBNV: After challenge inoculum of TSV and PBNV to transgenic and WT peanut, RT-PCR analysis was performed to determine the existence of virus inoculums load. Total RNA from the leaves of transgenic and WT was extracted, using RNeasy plant mini kit (Qiagen, GmbH). Following first strand cDNA synthesis (Thermo Scientific, USA), the RT-PCR was performed with TSV (Putative viral replicase; GenBank Acc. No.: NC_003844.1) and PBNV (Nonstructural movement protein; GenBank Acc. No.: HQ259244.1) gene specific primers (Table 1) designed using Primer Express\textsuperscript{\textregistered} software version 3.0 from Applied Biosystems and synthesised from IDT Inc., USA. The primer pairs gave amplification of 507 bp for TSV-putative viral replicase gene and 406 bp or PBNV-non-structural movement protein gene. For TSV-putative viral replicase gene, RT-PCR reaction was set in 25 \(\mu\)l reaction volume containing 2.5 \(\mu\)l of 5x PCR buffer, 2 \(\mu\)l 25 mM MgCl\textsubscript{2}, 1.6 \(\mu\)l of 2 mM dNTP mix, 25 pmol of each primers, 0.20 \(\mu\)l of 1U Taq polymerase and 100 ng of cDNA as template. Thermo-cycler conditions were set as initial de-naturation: 94ºC for 5 min; then 35 cycles of 94ºC-30 sec, 58ºC-30 sec, 72ºC-1 min and final extension at 72ºC for 7 min and for PBNV-non-structural movement protein gene RT-PCR reaction was set in 25 \(\mu\)l reaction volume containing 5 \(\mu\)l of 5x PCR buffer, 2 \(\mu\)l of 25 mM MgCl\textsubscript{2}, 2.5 \(\mu\)l of 2 mM dNTP mix, 25 pmol of each primers, 0.25 \(\mu\)l of 1U Taq polymerase and 100 ng of cDNA as template. Thermo-cycler condition was set as initial de-naturation: 94ºC for 5 min; then 35 cycles of 94ºC-15 sec, 52ºC-15 sec, 72ºC-1.30 min and final extension at 72ºC for 7 min. The PCR products were analysed on 1.2% (w/v) agarose stained with ethidium bromide, scanned and documented using a Fuji FLA5200 imaging system. The plants whose sample gave amplification with respective primer pairs were confirmed for presence of virus. A control devoid of template cDNA was also used as reaction control.

Histopathology of wild type and transgenic leaves for micro-necrosis symptoms of TSV and PBNV: To visualize plant cell death (micro-necrosis), leaves of TSV and PBNV infected transgenic and wild type peanut plants were stained with lactic-pheno1-typ Re blue (Takemoto et al., 2003). First, infected leaves were boiled for 2 min(s) in lactic-pheno1-typ Re blue stain (10 ml of H\textsubscript{2}O, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol and 10 mg of trypan blue). Afterwards, the leaves were allowed to cool at room temperature for 1 hr and the stain was replaced with disaining solution (75 ml of glycerol, 75 ml of acetic acid and 225 ml ethanol). Blue colour stained leaves were monitored and photographed.

Results and Discussion

In PCR, gene-specific primer pairs, RKJ-1 and RK-4 gave full-length amplification for TSV-CP (717 bp) and partial amplification for PBNV-NC genes (654 bp), respectively, in transgenic lines and positive control (pCAMBIA1305.1 TSV-CP-PBNV-NC plasmid) (Fig. 1). No amplification was observed in non-transgenic peanut (cv. K-6) lines. Mehta et al. (2013) also reported ~750 bp amplified fragment of TSV-CP through gene-specific primers in PCR analysis of single gene transgenic peanut lines, respectively.

A total of 354 plants (T3) from five events (E1-E5) were PCR screened with PBNV-NC gene specific primer from which 192 plants were found PCR positive. Those above T3 five events (E1-E5) plants were used for further characterization. In T3 progeny highest numbers of PCR positive transgenic plants were observed for event E3.
The χ² analysis was carried out to check goodness of fit. On the basis of expected and observed frequencies of gene specific amplicons, χ² test was found non-significant (p= 0.05), indicating close agreement between the observed and expected frequencies (Table 2). The T1 progeny segregation ratio of 3:1 was confirmed in three events (E1, E3 and E4); whereas other one event (E5) yielded non-segregating progenies. However, in the event E2, no line was found having minimum sample size of five plants, so they were not included χ² analysis. Transgene was found behaving as monogenic dominant gene due to their hemizygous state in recipient genome, and thus segregated as dominant loci in a classic 3:1 Mendelian ratio (Cambell et al., 2000). Two major characteristics of transgenic populations are that the transgene may be present in many copies and molecular rearrangements can create an unstable transgenic locus, which leads to segregate independently and evolve continuously. Also, deletion, duplication, repeated sequence recombination, as well as gene interaction, nature of recipient genome, nature of transgene and interaction between them seems to contribute to non-Mendelian segregation of transgenes as causal factor (Zhimin et al., 2004). Mehta et al. (2013) and Chander Rao et al. (2013) also reported segregation of TSV-CP and PBNV-NC genes in some of T1 peanut progeny lines in 3:1 Mendelian fashion. Similarly, Gao et al. (2015) also reported that the majority of transgenic soybean lines tested for Soybean mosaic virus resistance through inverted repeat-SMV-HC-Pro genes showed Mendelian pattern of inheritance with the expected ratio of 3:1.

Normal expression of transgenes in transgenic lines at transcript level was confirmed by reverse transcriptase PCR (RT-PCR) and quantitative (real-time) PCR (qPCR). RT-PCR analysis of TSV-CP and PBNV-NC transcripts gave an amplification of 717 bp and 654 bp in transgenic lines, respectively. However, no amplification was recorded in wild type plants. To check the

Table 2: Segregation analysis transgenic peanut lines in T1 generation

<table>
<thead>
<tr>
<th>Events</th>
<th>Total no. plants</th>
<th>No. PCR positive plants</th>
<th>No. PCR negative plants</th>
<th>Expected ratio</th>
<th>Observed ratio</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>3:1</td>
<td>2.33:1*</td>
<td>0.13</td>
<td>0.40</td>
</tr>
<tr>
<td>E3</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>3:1</td>
<td>3.33:1*</td>
<td>0.03</td>
<td>0.35</td>
</tr>
<tr>
<td>E4</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>3:1</td>
<td>5.00:1*</td>
<td>0.22</td>
<td>0.42</td>
</tr>
<tr>
<td>E5</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>3:1</td>
<td>0.40:1</td>
<td>8.05</td>
<td>0.95</td>
</tr>
</tbody>
</table>

df=1; * Observed ratio is not significantly different from expected ratio at p=0.05

The χ² analysis was carried out to check goodness of fit. On the basis of expected and observed frequencies of gene specific amplicons, χ² test was found non-significant (p= 0.05), indicating close agreement between the observed and expected frequencies (Table 2). The T1 progeny segregation ratio of 3:1 was confirmed in three events (E1, E3 and E4); whereas other one event (E5) yielded non-segregating progenies. However, in the event E2, no line was found having minimum sample size of five plants, so they were not included χ² analysis. Transgene was found behaving as monogenic dominant gene due to their hemizygous state in recipient genome, and thus segregated as dominant loci in a classic 3:1 Mendelian ratio (Cambell et al., 2000). Two major characteristics of transgenic populations are that the transgene may be present in many copies and molecular rearrangements can create an unstable transgenic locus, which leads to segregate independently and evolve continuously. Also, deletion, duplication, repeated sequence recombination, as well as gene interaction, nature of recipient genome, nature of transgene and interaction between them seems to contribute to non-Mendelian segregation of transgenes as causal factor (Zhimin et al., 2004). Mehta et al. (2013) and Chander Rao et al. (2013) also reported segregation of TSV-CP and PBNV-NC genes in some of T1 peanut progeny lines in 3:1 Mendelian fashion. Similarly, Gao et al. (2015) also reported that the majority of transgenic soybean lines tested for Soybean mosaic virus resistance through inverted repeat-SMV-HC-Pro genes showed Mendelian pattern of inheritance with the expected ratio of 3:1.
stringency of reaction conditions, 18S rRNA gene was amplified from the same cDNA, using gene specific primer pairs and under similar PCR conditions which resulted in 148 bp amplicon. RT-PCR results showed that five transgenic events expressed TSV-CP and PBNV-NC gene(s) at transcript level. Mehta et al. (2013) and Chander Rao et al. (2013) also reported similar kind of RT-PCR analysis for TSV-CP and PBNV-NC transcripts in transgenic peanuts, respectively.

The TSV-CP and PBNV-NC gene expressions were analyzed at transcript level through qPCR. The C_\text{t} values obtained were used for calculation of relative fold expression of transgene through ΔΔC_t method (Table 3). The transgenic event E1 showed highly significant expression levels (1.40 times), compared to the calibrator (Table 3). Similar reports of multiple folds increase in the transcript expression was recorded by Mehta et al. (2013), compared to the lowest expressing line for TSV-CP transcript in transgenic peanut plants. The TSV-CP transcripts showed similar trends of expression among different event plants. Similarly, Valkonen and Savenkov (2001) also reported steady-state level of CP gene mRNA were greatly increased in the plants infected with PVY. Also, the PBNV-NC transcripts showed more or less even trends of expression among different events. The transgenic event E1 showed highly significant expression levels (1.85 folds), compared to the calibrator (Table 3). Overall, qPCR results indicated that five events showed differential folds mRNA expressions in dual construct transgenic peanut plants.

Transgenic and wild type peanut plants were grown in glasshouse conditions for 2 weeks before virus inoculation and plants were mock-inoculated with TSV and PBNV and observed for virus symptoms. The TSV symptoms in transgenics were delayed and showed chlorotic spots on young leaves and necrotic lesions on young quadrifoliates leaflets. Transgenics showed declining TSV expressions, this might be due to high expression of TSV-CP protein which in turn retarded and delayed the TSV disease development. While wild type inoculated plants showed sudden wilting of leaflet and petiole, head drooping, upward killing of bud, and within a month plant died of necrosis.

The CP genes have been successful in averting the viral infection or reducing the diseases caused by homologous and narrowly related viruses (Gonsalves et al., 1993). Mehta et al. (2013) also reported resistance in different events to TSV in T2 and T3 progenies with no early symptoms of disease development. The studies by other workers have also reported delayed symptoms (Srivastava and Raj, 2008; Nakajima et al., 1993). Srivastava and Raj (2008) reported resistance in transgenic plants that remained symptomless throughout their life cycle, although virus accumulated at high level in their upper
Evaluation of transgenic peanut plants

Table 3: Level of TSV-CP and PBNV-NC transcripts in five transgenic events as determined by qPCR

<table>
<thead>
<tr>
<th>Plant No.</th>
<th>TSV-CP C</th>
<th>18 S C</th>
<th>ΔC</th>
<th>ΔΔC</th>
<th>Normalized quantity of TSV-CP transcript relative to calibrator 2^ΔΔC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>10.47</td>
<td>26.31</td>
<td>-15.84</td>
<td>-0.47</td>
<td>1.40</td>
</tr>
<tr>
<td>E2*</td>
<td>10.62</td>
<td>25.99</td>
<td>-15.37</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>E3</td>
<td>10.54</td>
<td>26.27</td>
<td>-15.74</td>
<td>-0.37</td>
<td>1.29</td>
</tr>
<tr>
<td>E4</td>
<td>10.59</td>
<td>26.35</td>
<td>-15.76</td>
<td>-0.39</td>
<td>1.31</td>
</tr>
<tr>
<td>E5</td>
<td>10.42</td>
<td>26.05</td>
<td>-15.63</td>
<td>-0.26</td>
<td>1.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plant No.</th>
<th>PBNV-NC C</th>
<th>18 S C</th>
<th>ΔC</th>
<th>ΔΔC</th>
<th>Normalized quantity of PBNV-NC transcript relative to calibrator 2^ΔΔC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>29.23</td>
<td>28.61</td>
<td>0.62</td>
<td>-0.89</td>
<td>1.85</td>
</tr>
<tr>
<td>E2</td>
<td>29.34</td>
<td>28.66</td>
<td>0.68</td>
<td>-0.83</td>
<td>1.78</td>
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<tr>
<td>E3</td>
<td>29.29</td>
<td>28.52</td>
<td>0.77</td>
<td>-0.74</td>
<td>1.66</td>
</tr>
<tr>
<td>E4*</td>
<td>29.64</td>
<td>28.13</td>
<td>1.51</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>E5</td>
<td>29.63</td>
<td>28.51</td>
<td>1.12</td>
<td>-0.39</td>
<td>1.31</td>
</tr>
</tbody>
</table>

*Calibrator

Table 4: Concentration of TSV and PBNV through DAC-ELISA after challenge inoculation with TSV and PBNV on transgenic peanut seedlings (T5) and wild type after 3 weeks of sowing

<table>
<thead>
<tr>
<th>Details of samples</th>
<th>DAC-ELISA reading for TSV titer (A_{max}) (1hr)</th>
<th>Details of samples</th>
<th>DAC-ELISA reading for PBNV titer (A_{max}) (1hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating Buffer</td>
<td>0.00</td>
<td>Coating Buffer</td>
<td>0.00</td>
</tr>
<tr>
<td>Wild type (WT)</td>
<td>0.17</td>
<td>Wild type (WT)</td>
<td>0.44</td>
</tr>
<tr>
<td>Healthy peanut (K-6)</td>
<td>0.00</td>
<td>Healthy peanut (K-6)</td>
<td>0.00</td>
</tr>
<tr>
<td>Cowpea infected</td>
<td>0.20</td>
<td>Cowpea infected</td>
<td>0.15</td>
</tr>
<tr>
<td>E1</td>
<td>0.06</td>
<td>E1</td>
<td>0.15</td>
</tr>
<tr>
<td>E2</td>
<td>0.06</td>
<td>E2</td>
<td>0.08</td>
</tr>
<tr>
<td>E3</td>
<td>0.09</td>
<td>E3</td>
<td>0.09</td>
</tr>
<tr>
<td>E4</td>
<td>0.06</td>
<td>E4</td>
<td>0.07</td>
</tr>
<tr>
<td>E5</td>
<td>0.13</td>
<td>E5</td>
<td>0.07</td>
</tr>
</tbody>
</table>

leaves (but less than control). Higher CP level in inoculated transgenic plants have most likely affected the uncoating of the virus particle at later stages. Reimann-Philipp (1998) reported a reduced rate of virus growth in inoculated leaves and slower spread were frequently observed in transgenic CP-accumulated plants owing to slower replication rate or interference with local and systemic virus transport. Higher accumulation of TSV in inoculated leaves but no systemic spread may be due to interference either with entry into the phloem and vascular long-distance transport (Taliiansky and Gracia-Arenel, 1995). Sharma and Anjaiah (2000) created transgenic peanuts carrying coat protein gene (IPCVcp) for resistance to Indian peanut clump virus (IPCV). The resistance shown by transgenic peanut plants to TSV is likely to be protein-mediated as CP was detected by ELISA in the leaves of transgenics. It may be due to the interactions of TSV-CP in the transgenic plant and viruses in the challenge inoculums (Bendahmane et al., 2007).

Moreover, Asurmendi et al. (2007) postulated that the state of aggregation of CPs is correlated with the level of CP-mediated resistance (CPMR). As changed levels of resistance are indicated, there could be a connection of several mechanisms. A transgene can confer both protein and RNA-mediated protection (Prins et al., 2008). However, Chapman et al. (2004) suggested that resistance may also be conferred by inhibiting cell to cell movement of viral particles since coat protein is often a necessary cofactor in that reaction.

After challenge inoculum of PBNV, the wild type peanut plant was visualized with symptoms of chlorotic lesions on terminal leaflets, necrosis of terminal bud (exposed with arrow) and axillary shoot proliferation with small and deformed leaflets. Infected plants remain stunted. Whereas, transgenic peanut plants with NC protein showed deferred (of 2-3 weeks) in symptom expression and attenuation with PBNV concluded that high expression of PBNV-NC protein retarded and delayed the disease development by PBNV. Interestingly, in transgenics plants no axillary shoot proliferation was observed. Introduction of viral NC gene by genetic transformation is a speedy and effective means to generate novel resistance against viral diseases in crop.
plants. Chander Rao et al. (2013), engineered popular Spanish
groundnut cv. JL-24, using two methods of gene transfer with
PBNV-NC gene, and T1 generation transgenic peanut plants
revealed that 16 of the 35 tested transgenic plant lines (45.7 %)
did not acquire virus under greenhouse condition.

RNA expression and production of nucleocapsid protein
from sense NC gene among the progenies of transgenic peanut
plants witnessed a delay in symptom development by 10–15 days
after mechanical inoculation with donor isolate of TSWV and it
attributes to hybridization of sense NC gene, RNA with the
genomic RNA of incoming virus causing inhibition of viral RNA
replication after introduction into plant cells (Yang et al., 2004).
In another report, transgenic plants developed against the same
virus using antisense NC gene had also been evaluated in field for
expression and noted significantly effective up to 76.0% symptomless plants for TSWV infection after 10 and 14 wk(s) of
sampling points (Magbanua et al., 2000). Similarly, Peart et al.
(2002) investigated the role of tobacco NC orthologue in
transgenic N. benthamiana showing resistance to a tobacco
mosaic virus.

The DAC-ELISA conducted on the upper leaves of
transgenic plants showed reduced virus accumulation as
compared to wild type. All the transgenic lines tested showed
resistance to TSV and PBNV infection in ELISA experiments.
The results of DAC-ELISA indicated more virus titer values in wild
type than transgenic peanut lines for TSV and PBNV (Table 4). Similar
observations of DAC-ELISA were also recorded for more virus
number in wild type than transgenic peanut plants for TSV
(Mehta et al., 2013). In addition, transgenic peanut plants
harbouring NC gene were also tested for expression through
ELISA in the progenies (Yang et al., 1998).

After challenge inoculum of TSV and PBNV to
transgenic and wild type peanut plants, RT-PCR analysis were carried out to
determine the presence of virus inoculum load. The RT-PCR
with primer pair of TSV-Putative viral replicase gene amplified a
507 bp fragment and PBNV-Nonstructural movement protein
gene, a 406 bp fragment, respectively. RT-PCR results confirmed
the presence of virus on both, transgenic events and wild type
challenge inoculated plants (Fig. 2).

Quick and proficient detection of any pathogen is decisive
for the progress and deployment of disease management
strategies. In the study, two viral agents that cause peanut stem
necrosis diseases and peanut bud necrosis disease were
developed and tested. These were the most devastating peanut
viral diseases whose diagnosis becomes very difficult when there
is co-infection of TSV and PBNV, because both the viruses
produce terminal bud necrosis. This RT-PCR based detection
contributed to the development of diagnostic tools necessary for
developing host plant resistance. The RT-PCR based detection of
different viruses in peanut was also reported by Anitha et

Staining of infected peanut leaves (A) TSV and (B) PBNV
with trypan blue to wild type and transgenic were visualized as a
dark blue and brown coloration, respectively, for cell death due to
micro-necrosis. Wild type peanut leaves showed larger
phenotypic symptoms of micro-necrosis spots at infection site
than the transgenic peanut leaves (Fig. 3). Similar type of micro-
necrosis symptom development with trypan blue was reported by
Pemmar et al. (2014) in Vigna unguiculata for Tospo viral infection.

In the present investigation, the evaluation of the
transgenic peanut lines showed that the resistance could be
obtained for both the viruses. The novelty of the work is that the
marker free Agrobacterium-mediated transformation was used to
generate transgenic plants for the first time for concurrent
resistance to TSV and PBNV. Therefore, this strategy can be
applied to commercially important crops that are affected by
Harvirus and Tosposviruses. Information on molecular
characterization of dual construct transgenic peanut plants has
provided a better understanding of the viral diseases and
contributed to the development of diagnostic tools necessary for
developing host plant resistance. Further investigations would be
taken up in future for determining the efficacy and performance of
identified transgenic lines at field level under natural infection
pressures.

Acknowledgment
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gratefully acknowledged for financial support.

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