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

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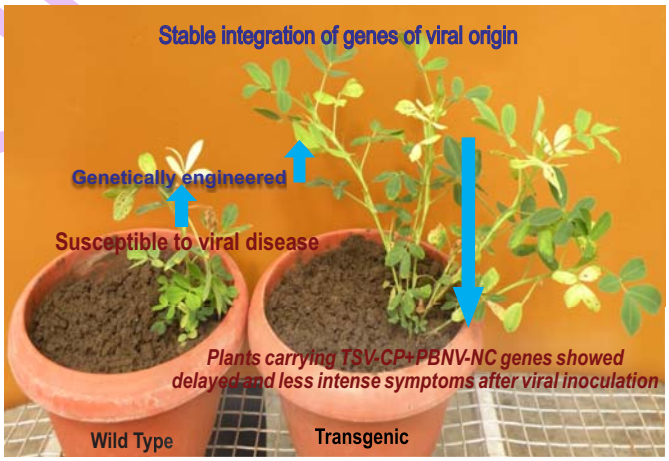
Abstract

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 PNBV.

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*.



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, PBND alone has been estimated to cause annual loss of 89 million USD (Reddy *et al.,* 1995). Initial symptoms of PBNV appear on young quadrifoliate 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 competently 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 PNBV.

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 1305.1by replacing HygR and GUS PLUS™, 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 $MgCl_2$, 2 µl of 2 mM dNTP mix, 25 pmol of each primers, 0.25 µl of 1U *Taq* DNA polymerase and 100 ng of

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

Gene	Primer sequence (5'-3')	Tm (°C)	Amplicons size (bp)
PCR and RT-PCR analysis			
TSV-CP	Fwd-CCATGGATGAATACTTTGATCCAAGG	74.0	717
	Rev-GGTNACCTCAGTCTTGATTCACCAG	74.0	
PBNV-NC	Fwd-ACT TCT GGC TGG TGG CTC TGC	74.0	654
	Rev-ACT GGC TGT TCC AGG GTT GCT	66.0	
qPCR analysis			
TSV-CP	Fwd-GAATGACCG CAC CAATTC CT	54.4	149
	Rev-TGG GTA GCT TCAACGATG TCT TC	58.8	
PBNV-NC	Fwd-CTG CAT CTT TTG ATA CAT GTG CTT T	55.6	436
	Rev-GCC AGA CCT GTC ATG CTA GAC A	56.9	
18S rRNA*	Fwd-GGC TCAAGC CGATGGAAGT	60.0	148
	Rev-AGC ACG ACA GGG TTT AAC AAG A	64.0	
RT-PCR analysis for detection of challenge inoculum load			
TSV-Putative	Fwd-GTT GAA GAAACG GGAAAAACAC	62.0	507
viral replicase	Rev-TATAGG GCT ACC ACT TCG CATC	66.0	
PBNV- Non-structural	Fwd-CTT TCC GTC CTT CAAACA GC	60.0	406
movement protein	Rev-ATC AGG GAT AGT TGG CAC A	62.0	

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

genomic DNA as template. Thermal-cycler condition was set as initial de-naturation: 94ºC for 3 min; then 30 cycles of 94ºC-45 sec, 60-56ºC-30 sec, 72ºC-1 min and final extension at 72ºC 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 $MgCl_2$, 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: 94ºC for 3 min; then 35 cycles of 94ºC-30 sec, 65-60ºC-40 sec, 72ºC-1 min and final extension at 72ºC 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.

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where, X^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 (χ^2) test was used to study segregation pattern of the transgene (Pearson, 1900). The χ^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 18 *S 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 (Table1). 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* were 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 $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The ΔC_T values were determined by

subtracting 18S rRNA C_T from *TSV-CP* and *PBNV-NC* C_T in a given sample. The $\Delta\Delta C_T$ values were determined by subtracting the ΔC_T of calibrator from ΔC_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® 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).Thereafter, 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-inoculated 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 antiserum (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^{-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® 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 bpf or *PBNV*-non-structural movement protein gene. For *TSV*- putative viral replicase gene, RT-PCR reaction was set in 25 µl reaction volume containing 2.5 µl of 5x PCR buffer, 2 µl 25 mM $MgCl_2$, 1.6 µl of 2 mM dNTP mix, 25 pmol of each primers, 0.20 µl 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 µl reaction volume containing 5 µl of 5x PCR buffer, 2 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTP mix, 25 pmol of each primers, 0.25 µl 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 lacto-phenol-trypan blue (Takemoto *et al.,* 2003). First, infected leaves were boiled for 2 min(s) in lacto-phenol-tryphan blue stain (10 ml of H_2O, 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 1hr and the stain was replaced with distaining 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.

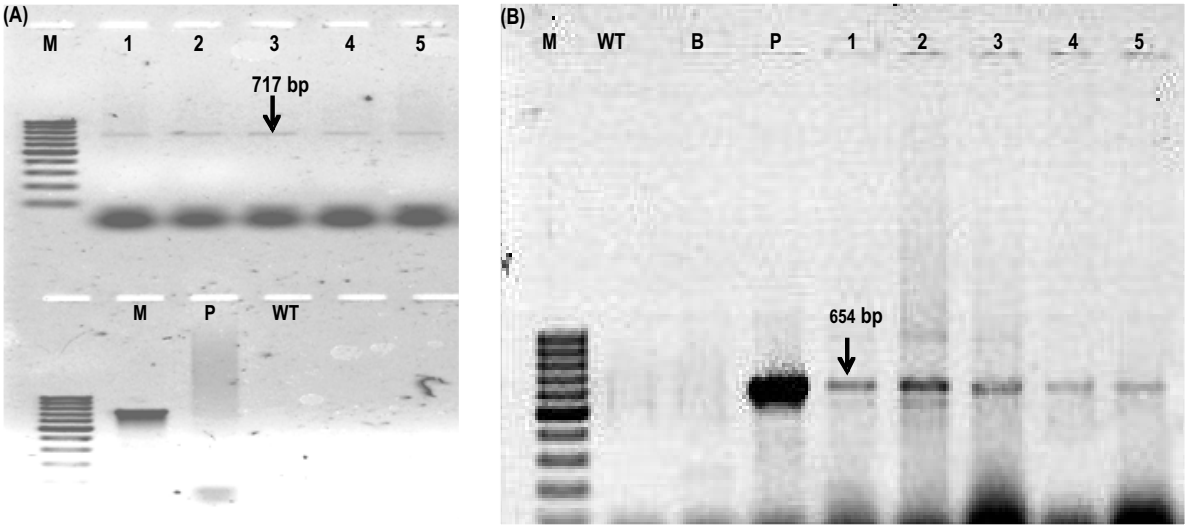


Fig. 1: PCR analysis. (A) PCR screening of *TSV-CP* gene in transgenic peanut plants. *TSV-CP* gene-specific primers (RKJ-1) were used for PCR amplification and the size of expected product was 717 bp as that of the full-length *TSV-CP* coding sequences (cds). (B) PCR screening of *PBNV-NC* gene in transgenic peanut plants. *PBNV-NC* gene-specific primers (RK4) were used for PCR amplification and the size of expected product was 654 bp as that of the partial-length *PBNV-NC* cds. Lanes, M: 100 bp ladder, WT: wild type plant (cv.K-6), B: blank, P: positive control (pCAMBIA1305.1 *TSV-CP+PBNV-NC* plasmid), WT: wild type peanut plant (cv.K-6) and 1–5: transgenic peanut lines represented by their identity number (E1 to E5)

Table 2: Segregation analysis transgenic peanut lines in T1 generation

Events	Total no. plants	No. PCR positive plants	No. PCR negative plants	Expected ratio	Observed ratio	X^2	*P*
E1	10	7	3	3:1	2.33:1*	0.13	0.40
E3	13	10	3	3:1	3.33:1*	0.03	0.35
E4	6	5	1	3:1	5.00:1*	0.22	0.42
E5	7	2	5	3:1	0.40:1	8.05	0.95

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

The χ^2 analysis was carried out to check goodness of fit. On the basis of expected and observed frequencies of gene specific amplicons, X^2 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 3:1was 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 X^2 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

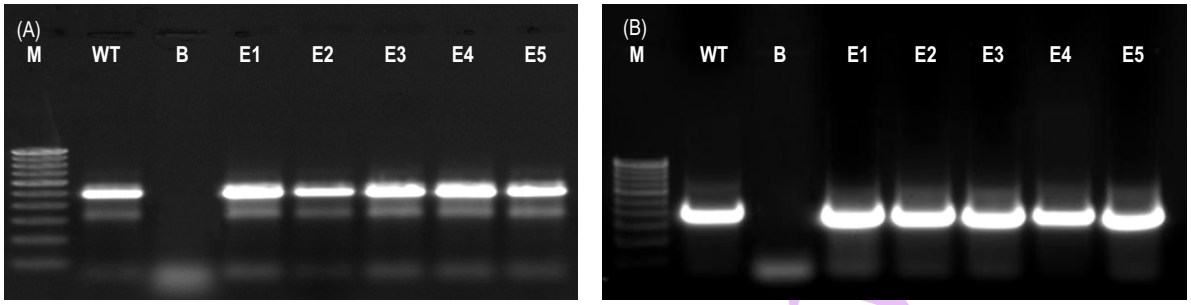


Fig. 2: RT-PCR base detection of challenge inoculum load of TSV and PBNV on transgenic (T5) and WT peanut plants to confirm the presence of challenge virus inoculums. RT-PCR analysis of (A) *TSV*-Putative viral replicase gene; the total mRNA from the TSV challenge inoculated transgenic and WT peanut plants were extracted and the first strand cDNA was prepared. Gene-specific primers were used to amplify 507 bp fragment of the *TSV*-Putative viral replicase gene. Lanes, M: 100 bp ladder, WT: wild type peanut plant, B: Blank, E1–E5: transgenic events. (B) *PBNV*-Nonstructural movement protein gene; the total mRNA from the PBNV challenge inoculated transgenic and WT peanut plants were extracted and the first strand cDNA was prepared. Gene-specific primers were used to amplify 406 bp fragment of the *PBNV*-Nonstructural movement protein gene. Lanes, M: 100 bp ladder, WT: wild-type peanut plant, B: Blank, E1–E5: transgenic events

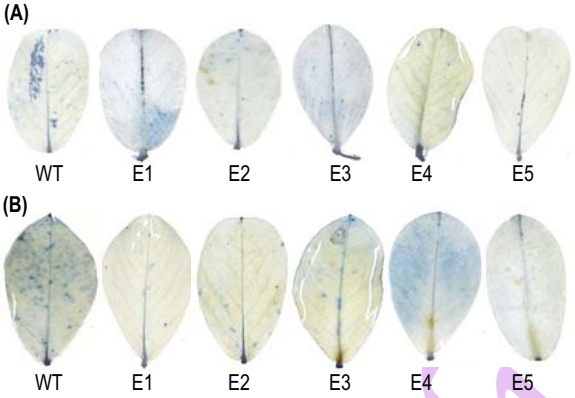


Fig. 3: Histopathology of WT and transgenic (T5) leaves for micro-necrosis symptom challenged inoculated with TSV and PBNV and stained with lacto-phenol-trypan blue (A) TSV infection and (B) PBNV infection

stringency of reaction conditions, 18S rRNAgene 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_T values obtained were used for calculation of relative fold expression of transgene through $\Delta\Delta C_T$ 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 (Table3). 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

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

Plant No.	*TSV-CP* C_T	18 S C_T	ΔC_T	$\Delta\Delta C_T$	Normalized quantity of *TSV-CP* transcript relative to calibrator $2^{-\Delta\Delta C_T}$
E1	10.47	26.31	-15.84	-0.47	1.40
E2*	10.62	25.99	-15.37	0.00	1.00
E3	10.54	26.27	-15.74	-0.37	1.29
E4	10.59	26.35	-15.76	-0.39	1.31
E5	10.42	26.05	-15.63	-0.26	1.20
Plant No.	***PBNV-NC*** C_T	**18 S** C_T	ΔC_T	$\Delta\Delta C_T$	**Normalized quantity of *PBNV-NC* transcript relative to calibrator** $2^{-\Delta\Delta C_T}$
E1	29.23	28.61	0.62	-0.89	1.85
E3	29.34	28.66	0.68	-0.83	1.78
E3	29.29	28.52	0.77	-0.74	1.66
E4*	29.64	28.13	1.51	0.00	1.00
E5	29.63	28.51	1.12	-0.39	1.31

*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

Details of samples	DAC-ELISA reading for TSV titer (A_{405nm}) (1hr)	Details of samples	DAC-ELISA reading for PBNV titer (A_{405nm}) (1hr)
Coating Buffer	0.00	Coating Buffer	0.00
Wild type (WT)	0.17	Wild type (WT)	0.44
Healthy peanut (K-6)	0.00	Healthy peanut (K-6)	0.00
Cowpea infected	0.20	Cowpea infected	0.15
E1	0.06	E1	0.15
E2	0.06	E2	0.06
E3	0.09	E3	0.09
E4	0.06	E4	0.07
E5	0.13	E5	0.07

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 (Taliansky 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 signiûcantly 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 titer values 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 inoculums 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 al*.(2014) and Prasada Rao *et al.* (2003).

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 Permar *et al.* (2014) in *Vigna unguiculata* for *Tospo* viral infection.

In the present inv estigation, the evaluation of the transgenic peanutlines 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 *Ilarvirus* and *Tospoviruses*. 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.

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