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Development of Post-transcriptional Gene Silencing Constructs for Groundnut Bud Necrosis Virus Nucleocapsid Protein Gene

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Abstract: Groundnut bud necrosis caused by Groundnut bud necrosis virus (GBNV), an enveloped RNA virus is remerging as economically important viral disease on several crops in India. In the present study, an effort was made to develop various constructs for inducing post-transcriptional gene silencing (PTGS) of GBNV in host plants. Virus-infected groundnut leaf (*Arachis hypogae* L.) samples were collected and partial CP gene was amplified using gene specific primer, cloned and confirmed by sequencing. The clones with viral gene inserts were PCR amplified and restricted with endonucleases , *Bam* HI and *Kpn* I for antisense, *Nco* I and *Xho* I for sense cloning into generic intron hairpin and *Nco* I and *Xho* I for cloning into SHUTR (short heterologous 3'UTR) vector. Truncated CP gene sequence of GBNV was cloned in sense, antisense and both (sense/antisense) orientation in generic ihp vector to develop three PTGS constructs and in SHUTR vector to develop SHUTR construct against GBNV. The confirmed transformants of all four constructs pPK 14806, pPK 1806, pPK 4906 and pPK19906 were sub cloned into pCAMBIA 1305.1 promoter less plant transformation vector and the confirmed clones pPK 191106, pPK 291106, pPK 71206 and pPK 241206 were mobilized into *Agrobacterium tumifaciens* LBA 4404 with *E.coli* pPRK2013 as helper strain and then transformed to tobacco and transgenic confirmed by PCR and GUS assay.

Key words: GBNV, PCR, ihp, SHUTR, *Agrobacterium*, tobacco and transgenic

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is the thirteenth most important food crop of the world. It is the world's fourth most important source of edible oil and the third important source of vegetable protein. It is grown on 26.4 million ha worldwide with a total production of 36.1 million metric tons, and an average productivity of 1.4 metric tons ha^{-1}. India occupies the first position both with regard to area (5.7 million ha) and production (4.7 million metric ton) but productivity is at 0.8 metric ton/ ha [1]

Groundnut bud necrosis, which is a reemerging as one of the most important viral diseases on several crops was thought initially to be caused by a strain of tomato spotted field virus [23], but is now classified as a distinct virus in the genus Tospo and species groundnut bud necrosis [22]. Bud necrosis has been named variously in different parts of the world; as ring spot of peanut in Brazil [3] and South Africa [5 and 14] as chlorosis or spotted wilt in Australia and United States,[11 and 10] and as bud necrosis or bud blight disease in India[24]. The disease is reported to occur in all prominent groundnut-growing areas of India. In Karnataka, the disease was first reported from Dharwar [28]. The incidence of bud necrosis of groundnut ranges from 5 to 80 per cent in different parts of the Indian subcontinent. Of late the disease has been occurring on tomato in severe form and affected the cultivation of hybrid tomatoes in north India resulting in losses to the extent of 40-50 percent [1]. Worldwide it causes a loss of over one billion dollars annually [8,16,20].

GBNV is the type species of genus Tospovirus and family Bunyaviridae. The virus is vectored by thrips, *Frankliniella occidentalis, F. schultzei, F. fusca, Thrips tabaci* and *Scirtothrips dorsalis* which infest over 800 plant species, both dicots and monocots, in more than 80 plant families. Solanaceae and compositae families have the largest numbers of susceptible plant species [19]. The disease is characterized by bronze or purple coloured leaves, severe necrosis of buds and petiole and the affected plant has stunted growth and pale yellow concentric rings are observed on tomato and chilli fruits [21]. The virus has tripartite genome, composed of the large [L] segment [~9 kb] encoding RNA depended RNA polymerase in negative sense [9], the medium [M] segment [~4.8 kb] encoding the *NSm* and G1 and G2 precursor glycoprotein gene in

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ambisense orientation [26] and the small [S] segment [~3 kb] encodes *NSs* non-structural protein gene and the *N* or nucleocapsid gene protein in ambisense orientation [26].

The potential of genetic engineering to produce GBNV resistant plants has not yet been demonstrated however, several laboratories around the world have tried to introduce resistance to TSWV which is a related tospo virus by transforming N gene of the virus to plant. Gielsen *et al*. reported engineered resistance to TSWV in tobacco, although no correlation was observed between resistance and N protein level [7]. Sense and antisense orientation of TSWV *N* gene were used by Pang *et al.* and Kim *et al*. to produce transgenic tobacco and tomato[13,18]. In both the cases the orientation of the gene did not affect the level of protection. The expression of virus derived sense or antisense RNA in transgenic plants conferring RNA mediated virus resistance appear to induce a form of post transcriptional gene silencing [2].

Presently, the phenomenon of RNA silencing is used to describe all siRNA-mediated gene-silencing pathways that are evolutionarily conserved in most eukaryotic organisms. The phenomena has been referred to as, post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in nematode, flies and mammals and quelling in fungi [30,6 ,17] and is increasingly viewed as an adaptive immune system of plants against viruses [29]. The term RNAi indicates a gene silencing process induced by double stranded RNA (ds RNA) and is a widely used strategy for functional genomics, the primary advantage being its ability to knock down the specific target gene. The implication of the RNAi in science has lead to christening RNAi as "Technology of the year" by Science [4].

Initially, gene silencing was a puzzling phenomenon but great strides have been made in elucidating the basic biology of the complex mechanism [27]. The involvement of PTGS in viral resistance was first reported by Lindbo *et al* [15]. Plant viruses could be the inducers or the target of RNA silencing. Several lines of work around the world indicate that RNA silencing is a general antiviral defense in plants and can be induced and translocated in the system to trigger antiviral defense.

MATERIALS AND METHODS

GBNV infected groundnut leaf samples were collected from fields infected with GBNV in the U.A.S., campus Dharwad. Other samples were brought from infested fields in Raichur and Gokak districts of Karnataka, India.

RNA Isolation and Reverse Transcription: Total RNA was isolated from healthy and infected sunflower leaves using Eppendorf mini Eukaryotic RNA isolation kit and it were used as a template for reverse transcription that was done using Eppendorf RT- PCR kit. For the RT-PCR reaction about 500 ng of total RNA was added to 20µl RT-PCR mixture containing 1X RT-PCR buffer (Eppendorf, USA) 0.25mM each of dATP, dTTP, dCTP and dGTP, 65ng of oligo dT primer and 1.5 U of Reverse transcriptase [Eppendorf, USA]. The reaction mixture was cycled through $65C^0$ for 5 mins, $42C^0$ for 90 mins and finally cooled and kept at $4C^0$.

Amplification and Cloning of GBNV Partial Coat Protein Gene Sequence: For the PCR experiment, about 100 ng of cDNA was added to 20µl PCR mixture containing 1.5U of Taq polymerase (Bangalore genei, India), 0.2mM each of dATP, dTTP, dGTP and dCTP, 10mM Tris-HCl pH 8.5, 50mM KCl, 1.5mM $MgCl_2$ and 5pM of the genome forward primer with *BamH1* site 5'ggATCCgCgTCTTTCgATACATgTgCTT 3' and reverse primer with *Kpn1* site 5'ggTACCCATTgAAAgACTggCACTgAAAg3' for antisense cloning and with *Nco* I site 5'CCATgggCgTCTTTCgATACATgTgCTT 3' as reverse and with *Xho* I site 5'CTCgAgCATTgAAAgACTggCACTgAAAg 3' as forward for sense orientation cloning. The reaction mixture was cycled through the following temperature profile: 94 C^0 for 5 mins, $94C^0$ for 1 mins, 57.5 C^0 for 1 mins, 72 C^0 for 2 mins the PCR was run for 35 cycles and finally terminated at $72C^0$ for 30 mins and therefore cooled at $4C^0$. PCR fragment was analyzed by applying 5µl of sample to 1.5 per cent agarose gel and run at 50V for one hour in TAE 1X buffer (0.04 M Tris-acetate and 1mM EDTA) and subsequently the gel was documented with gel doc (Uvi tec Cambridge, England).

The single sharp amplicons of both sense and antisense partial Cp gene (approximately 355bp) was eluted from the agarose block by using the Eppendorf gel clean up kit as described in user's manual. The purified PCR amplicons was ligated to pTZ57 R/T cloning vector (2886bp) as described in ins T/A Clone ™ PCR product cloning kit MBI, Fermentas, USA and transformed to *E.coli* DH5α following standard molecular biology procedures[25]. The recombinant clones were identified by PCR amplification and digestion with restriction endonucleases *Kpn*1 and *Bam*H1 for sense and *Nco* I and *Xho* I (Bangalore Genei Pvt.Ltd, Bangalore) and the confirmed clones were sequenced using M13F/R primers employing primer-walking technique at Bangalore Genei Private Ltd., Bangalore.

Sequence Analysis: The sequences obtained were subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. and the related sequences. The nucleotide sequence of coat protein gene of Peanut Bud Necrosis virus was downloaded from NCBI database. The gene sequence from different host was taken and multialigned using CLUSTAL-X and the PCR primer was designed to amplify 355 bp of the coat protein gene sequence using FASTA PCR. The multiple sequence alignments of both the nucleotide sequence and amino acids were made using CLC Free Workbench version 3.1

Preparation of Generic Ihp Vector for Sense, Antisense and Both [Sense/ Antisense] Cloning to Get Ihp Construct: The generic ihp vector is derived from pRT100, a cloning vector carrying plant expression T-DNA cassette. The generic ihp vector has a functional catalase [CAT] intron from castor, downstream to CaMV 35S promoter. The presence of multiple cloning sites on either side of the intron allows directional cloning. For cloning of CP into generic ihp vector [provided by Dr. Dinesh Kumar, Directorate of Oil seeds Research, Hyderabad, INDIA], the vector DNA was isolated using the alkaline lysis protocol of Brimbion and Dolly (1979) with certain modifications and quantified using nano drop.

Sequential digestion of generic ihp vector (Fig. 1a) was done separately with *BamHI* and *KpnI* for cloning in antisense orientation and with *Nco* I and *Xho* I for cloning in sense orientation and similarly the 355bp Cp gene PCR amplicon was restricted with the same combination of enzyme in separate tubes. The linearized vector and the insert was eluted and extracted using eppendorf gel cleanup kit as per the protocol given in user's manual and quantified using Nanodrop.

The recombinant Generic ihp plasmid clones having antisense insert of CP was digested separately with two restriction enzymes *NcoI* and *XhoI* for cloning in sense orientation. PCR amplification of Cp gene was done with respective gene specific primers having *NcoI* and *XhoI* restriction sites in forward and reverse primer respectively, to help sense cloning. The specific amplicons from above PCR reactions were digested with *NcoI* and *XhoI* and purified using eppendorf PCR purification kit as per user's manual and quantified using nanodrop.

Cloning into Generic Shutr Vectors: The generic SHUTR vector [provided by Dr. Dinesh Kumar, Directorate of Oil seeds Research, Hydrabad, INDIA], is derived from pRT100, a cloning vector carrying plant expression T-DNA cassette. For constructing generic SHUTR vector, functional catalase (CAT) intron from castor was cloned downstream to CaMV 35S promoter. On both sides of the introns, poly (A) was inserted in both sense and antisense orientation to give hairpin structure of 3' UTR (untranslated region) (Fig: 1b).

Sequential digestion of generic SHUTR vector was done separately with *Nco*I and *XhoI* for cloning. The linearized vector was eluted and extracted using eppendorf gel cleanup kit as per the protocol given in user's manual.

For cloning CP gene in SHUTR, PCR amplification was done with respective gene specific primers having *Nco*I and *Xho*I restriction sites in forward and reverse primer respectively. The specific amplicons from above PCR reactions were digested with *Nco*I and *Xho*I and purified using eppendorf PCR purification kit as per user's manual and quantified.

Ligation and Transformation: The ligation reaction, preparation of competent cells, transformation of *E. coli* DH5a and confirmation of clones were done as described earlier. The ligation mixture composition is shown in (Table I).

Subcloning into Plant Expression Vectors PCAMBIA 1305.1: Plant expression vector pCAMBIA1305.1 (Fig.2) is a promoter less vector used for cloning inserts along with regulatory elements for expression studies. The vector was digested with *Pst* I restriction enzymes for cloning the entire expression cassette with insert. The linearized vector was eluted and extracted using eppendorf gel cleanup kit as per the protocol given in user's manual.

For cloning entire expression cassette of sense, antisense, ihp and SHUTR of CP, respective recombinant plasmids were used. Plasmid clones were digested with *PstI* and the released expression cassettes were gel eluted separately and purified using Eppendorf gel cleanup kit, as per user's manual and quantified using Nanodrop.

Ligation and Transformation: The ligation reaction, preparation of competent cells, transformation of *E. coli* DH5a and confirmation of clones were done as described earlier. Initially recombinants were selected based on blue/white assay. The ligation mixture is shown in (Table II).

Mobilizing Recombinant Clones into *Agrobacterium Lba4404:* The confirmed recombinant clones were mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating. The vector, pCAMBIA1305.1 is capable of replicating in both *E.coli* as well as *Agrobacterium* and T-DNA has gene for hygromycin resistance, which is used as selectable marker in plants.

Table I: Ligation Mixture Composition for cloning in GENERIC ihp and SHUTR vector

Genric Vector Volume in µl	insert volume in µl Volume in µl	T_4 DNA Ligase in µl	Sterile Water Volume in µl	Total Volume in µl
Sense 2	3	2	11	20
Antisense 2	3	2	11	20
Sen/ant 2	3	2	11	20
Shurt 2	2	2	14	20

Table II: Ligation mixture composition for cloning in pCAMBIA 1305.1

Components	Generic Ihp Vector		Ihp Sense+antisense (µl)	GENERIC SHURT VECTOR (µl)
	Antisense (µl)	Sense (µl)		
p Cambia 1305.1	0.5	0.5	0.5	0.5
Insert	3	3	6	4
T_4 DNA ligase	2	2	2	2
Sterile nano pure water	14.5	14.5	11.5	13.5
Total	20	20	20	20

The strain has chromosomal selection rifampicin (25 mg/ml) and disarmed Ti- plasmid pAL4404, having gene for streptomycin resistance (100mg/ml) as selectable marker. *E.coli* strain containing pRK2013 vector with gene for kanamycin resistance was used as a helper for mobilizing recombinant vector into *A. tumefaciens* LBA4404.

E. coli DH5α cells having recombinant plant transformation vector pCAMBIA1305.1 were grown overnight in Luria both containing 50 mg/ml of kanamycin overnight at 37°C. The *A. tumefaciens* LBA4404 was grown for 16-22 hours at 28°C in Yeast Extract Mannitol Agar containing rifampicin (25 mg/ml) and streptomycin (100 mg/ml). The *E. coli* DH5α pRK2013 strain was grown overnight in LB containing kanamycin (50 mg/ml).

The overnight grown cultures were centrifuged at 15,700 rcf for 1 min. The supernatant was discarded and the pellet was washed with 0.01 $MgSO_4$ for 2-3 times to remove traces of antibiotics. It was again centrifuged at 13,000 rpm for 1 min and pellet was dispensed in 50 ml of 0.01 M $MgSO_4$. *A. tumefaciens* LBA4404, *E. coli* DH5α (pRK2013) and *E. coli* containing recombinant plasmids were mixed in 1:1:1 ratio separately. The mixture was spotted on plain LA medium and incubated overnight at 28°C. The spotted culture was scraped and dissolved in 200 ml of 0.01 M $MgSO_4$ and spotted on YEMA medium containing streptomycin (100 mg/ml), rifampicin (25 mg/ml) and kanamycin (50 mg/ml) along with *A. tumefaciens* LBA4404, *E. coli* (pRK2013) helper strain and *E. coli* with recombinant vectors as negative controls.

The presence of recombinant plasmid in the *Agrobacterium* was confirmed by PCR amplification.

Transgenic Tobacco Development: The *Agrobacterium* containing recombinant plasmid was used for tobacco transformation by using protocol mentioned by Hooykaas *et al.* (1992) with some modification.

Agrobacterium tumefaciens LBA4404 transformed with recombinant construct was grown in YEMA (Yeast Extract Mannitol Agar) with streptomycin (100 mg/L), rifampicin (25 mg/L) and kanamycin 950 mg/L) as selection at 28°C for 24 hours. Leaves of tobacco (5-6 week old plants) were surface sterilized by rinsing them for 1 min in 0.1 per cent mercuric chloride. The sterilized leaves were given 3-4 times water wash, to remove the traces of the mercuric chloride. The leaves were cut into 1 to 2 cm² disks and incubated in acetosyringone (200 mM) treated *Agrobacterium* culture and left for 20 minutes for agroinfection.

These explants were dried by blotting onto the sterile filter paper and placed on solid Murashige and Skoog (MS) medium without any hormones and kept in dark for 2 days for co-cultivation. Leaf disks were later transferred to test tubes containing MS medium with Napthalene acetic acid (NAA) (0.5 mg/L), benzylaminopurine (BAP) (1 mg/L) for callus induction and cephotaxime (200 mg/L) to avoid *Agrobacterium* growth. The cultures were incubated at 25°C and 16 hrs photoperiod for 3-4 weeks. The shoots and excised calli were subcultured and incubated for another 4 weeks at same culture conditions. Young shoots were then transferred to hormone free MS medium with 200 mg/L cefotaxime and 200 mg/L kanamycin as selection for rooting. Cultures were incubated for 3-4 weeks and survived plants were transferred to pots containing peat and acclimatized in green house.

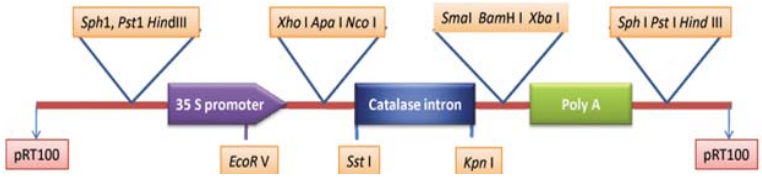

Fig. Ia: Generic ihp vector

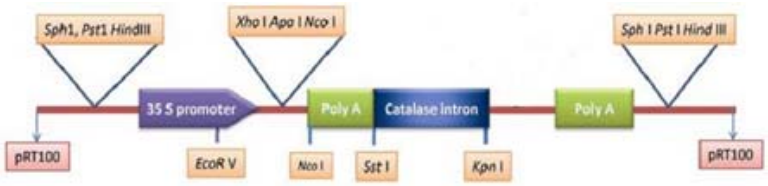

Fig. Ib: Generic SHUTR vector

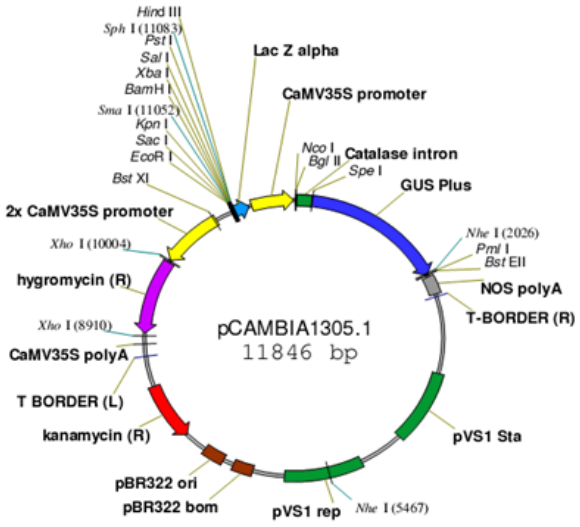

Fig. 2: Vector backbone map of pCAMBIA1305.13.

Transgene Expression Analysis:

PCR Analysis: DNA isolated from putative transgenic lines from each construct was tested for the presence of insert. PCR was performed using *hpt*II specific primers in a 20 ml PCR reaction containing 1U of Taq DNA polymerase, 2mM dNTP mix, 5 pmoles of each primer, 1x Taq assay buffer. For PCR standard protocol was followed except the annealing temperature (55^0 C for 1 min). Vector DNA and non-transgenic plant DNA were used as negative and positive control, respectively. The following primer combination was used for *hpt*II amplification; Forwad-5' CGACCTGATGCAGCTCTCGGAGGGC3'; reverse 5' CGATTGCGTCGCATCGACCCTGCGC3'. Ampicons were visualized by running 1% agarose gel.

Gus Histochemical Assay: To confirm expression of transgene in transgenic plants, GUS histochemical assay was performed as described by Jefferson [12] with some modifications. The leaves from PCR positive plants were cut radially and was then washed for 30-60 min with several changes of ice-cold distilled water. The tissue was incubated with incubation medium in dark at 37^o C until distinct staining appeared. The tissue was held in the distilled water and incubated with absolute alcohol for several hours until all the chlorophyll content was removed. The ethanol was then removed and the tissue was mounted in 100% glycerol on a slide and observed under the microscope.

RESULT AND DISCUSSION

The partial coat protein gene of GBNV was successfully amplified by reverse transcription followed by PCR, giving a product of expected size 355bp (Fig 3: Plate 3A-B) which were eluted from the preparative

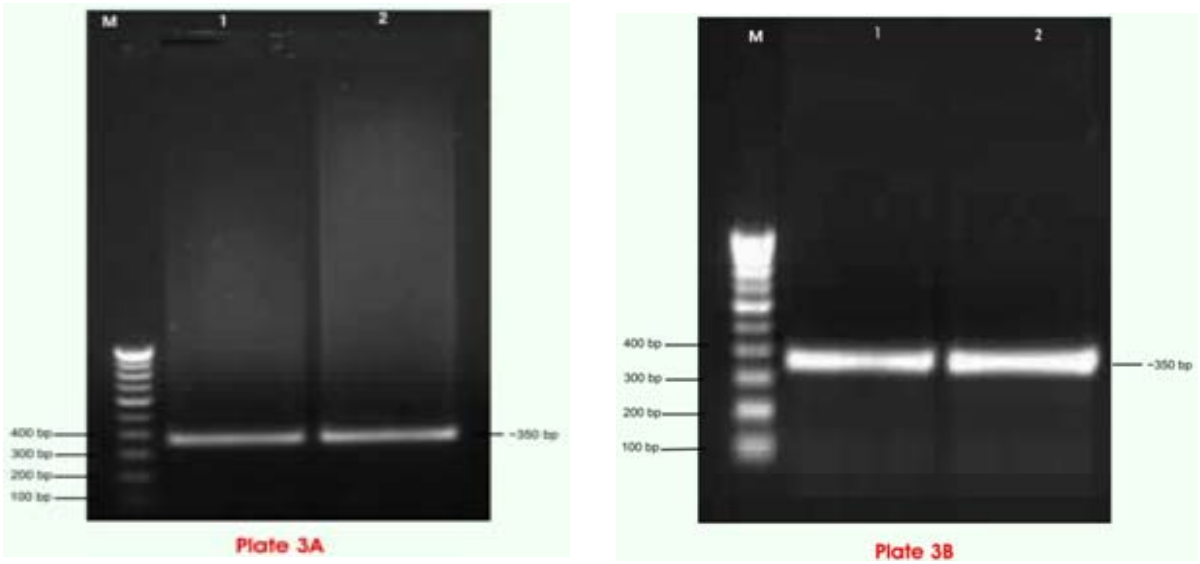

Fig. 3: Plate 3A-3B - Large-scale amplification of GBNV partial CP gene with primer having *BamH1* and *Kpn1* and *Nco1* and *Xho1*.

gel and ligated to pTZ57R/T cloning vector. The recombinant product of vector pTZ57R/T+ 350bp CP (sense) and 350 bp CP (antisense) gene were transferred individually to *E.coli* DH5α.

The recombinant cells were selected based on blue/white assay on LA+Amp_{50}+X-gal+IPTG. In both the cases fifteen transformants were obtained out of which nine and five clones were white for sense and antisense CP, respectively. Further, the PCR (Fig:4 Plate 4A-B) and restriction analysis of both the clones confirmed the presence of 350 bp fragment (Fig:4 Plate 5A-B) and the constructs were named as pPK 8706 and pPK 15706 (Fig. 5 A and 5B).

Clonal Confirmation of Generic Ihp Vector and SHUTR Vector: Truncated CP gene in antisense orientation defined by specific primers with *Bam* HI and *KpnI* sites and sense primer with *Nco* I and *Xho* I sites was obtained by digestion of the pTZ clones pPK 80706 and pPK 15706 with the respective enzymes and purified using eppendorf PCR purification kit as per user's manual and quantified. The linerized vector was ligated with insert at 1:3 molar concentrations and then transformed to *E.coli* DH5a.

The transformants were picked and streaked on Luria agar containing ampicillin (50mg/ml). Plasmid DNA was isolated from the respective clones and confirmed through PCR (Fig: 6 Plate 6) and restriction analysis using *PstI* to release the cassette (Fig: 6 Plate 7). The clone was named as pPK 14806 and pPK 1806 (Fig. 7 A and Fig. 7 B)

For cloning in both sense/antisense [ihp] fragment into the vector, the recombinant ihp vector with cloned antisense fragment was restricted with *Nco* I and *Xho*I to facilitate cloning. The linerised recombinant vector was then ligated with Cp gene amplicon with *Nco*I and *Xho*I cohesive ends at 1:3 molar concentration and transformed to *E.coli* DH5a and the recombinant clone were picked up on Ampicillin (50 mg/ml) Luria agar. Plasmid DNA isolated from these clones was confirmed by PCR (Fig 6: Plate 6) and restriction (Fig: 8 Plate 8). The clones were named pPK 4906 (Fig. 7 C).

Similarly for cloning into SHUTR vector its plasmid was isolated and restricted with *Nco*I and *Xho*I. The insert was obtained by digestion of pPK 15706 with *Nco*I and *Xho*I and ligated by taking each vector and amplicon at 1:3 molar concentration and then transformed to *E.coli* DH5a. The transformants were picked up and streaked on Luria agar containing ampicillin (50mg/ml). Plasmid DNA was isolated and confirmed by PCR (Plate 7) and restriction by using *PstI* enzyme to release the cassette (Fig:8 Plate 8).The clones were named (pPK 19906) (Fig. 7 D).

Clonal Confirmation in Plant Expression Vector PCAMBIA1305.1: The maxi prep isolation of plasmid DNA of the recombinant clone and the pCAMBIA 1305.1 vector was done and then restricted with *PstI* to release the entire expression cassette. The linerised vector was ligated separately with each expression cassette in 1:3 molar concentrations and then transformed into *E.coli* DH5a cells. The transformants cells were picked and streaked on Luria agar containing Kanamycin [50 ppm], Xgal 932 ppm) and iso propyl β-D- thiogalactoside [IPTG]. The

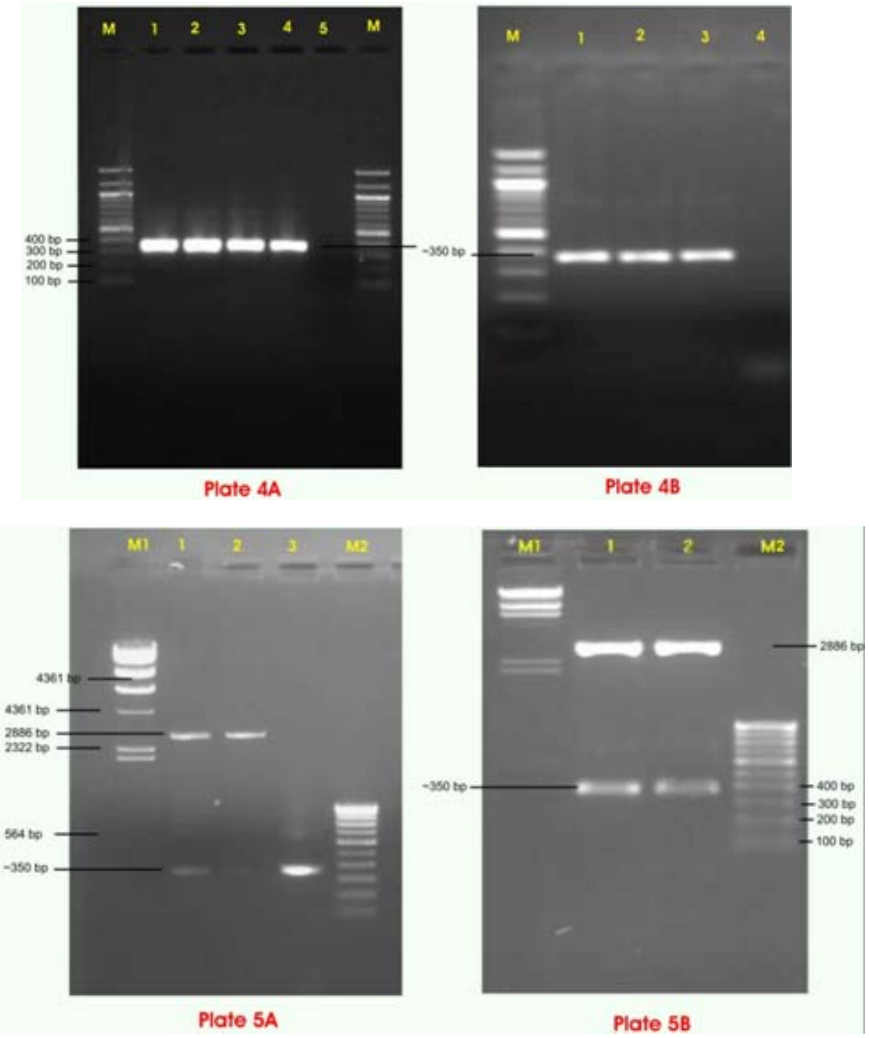

Fig. 4: Plate 4A-B - PCR confirmation of pTZR/57 clones containing GBNV partial CP antisense insert(A) and sense insert(B)

Fig. 4: Plate 5A-B - Restriction analysis of pTZ57R/T clones containing GBNV CP antisense insert(5A) and GBNV CP sense insert (5B)

recombinant clones were selected by blue white assay. Plasmid DNA was isolated from the recombinant clones and was confirmed by PCR [Fig 8: plate 9] followed by restriction with PstI enzyme (Fig:9 Plate 10, Plate 11). The clones were named pPK 191106 for antisense, pPK 291106 for sense, pPK 71206 for ihp and pPK 241206 for SHUTR (Fig. 10 A, B, C and D) in pCAMBIA1305.1.

Mobilizing Recombinant Clones into *Agrobacterium*: The confirmed pCAMBIA clones with sense construct(pPPK 291106), antisense construct (pPPK 191106), ihp construct (pPPK 71206) and SHUTR construct (pPK 241206) were mobilized into Agrobacterium tumefaciens LBA4404 via triparental mating using E.coli carrying pRK2013 as helper plasmid. Patch mating was done in the concentration of 1:2:2 (recipient: helper: donor) was done and except SHUTR all other were found to be successful. The transcongugants were picked up on yeast extract mannitol agar (YEMA) medium having kanamycin (50?g/ml), streptomycin (100?g/ml) and rifampicilin (25?g/ml). Plasmid DNA was isolated from the Agrobacterium clone and was confirmed by PCR (Fig: 11 Plate 12) and the transconjugants were named pPK 14107, pPK 15107, pPK 21107and pPK 23107.

Transgenic Development: Agrobacterium tumefaciens LBA4404 pCAMBIA1305.1 constructs carrying CP-sense (pPK 14107), CP-antisence (pPK 15107),

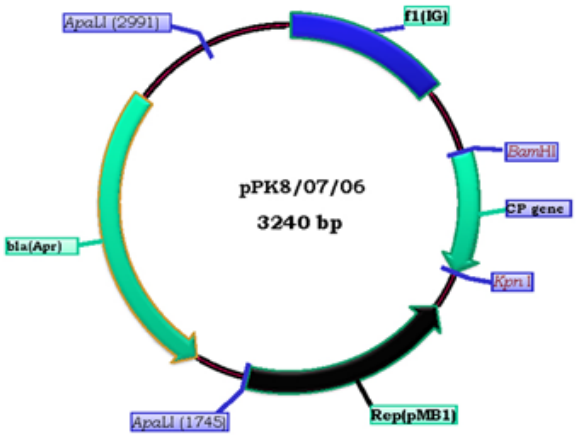

Fig. 5A: Partial CP gene with *BamHI* and *KpnI* restriction sites to facilitate antisense cloning

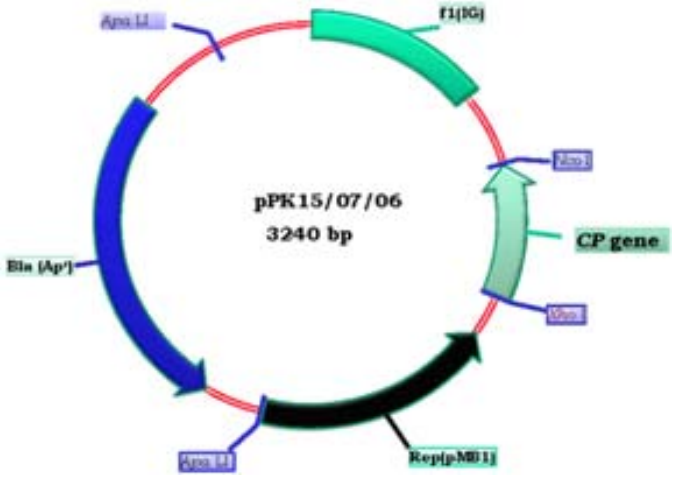

Fig. 5B: Partial CP gene with *NcoI* and *XhoI* restriction sites to facilitate sense cloning

Table 3: Percent success of transformation shown by various analyses

Construct	Pcr Analysis	Gus Assay
Cp (s)	6/8 (75.0%)	4/6 (66.6%)
Cp (as)	8/9 (88.8%)	5/8 (62.5%)
Cp (Shurt)	6/8 (75.0%)	4/6 (66.6%)

CP-ihp (pPK 21107) and CP- SHUTR (pPK 23107). The leaf discs co-cultivated (Fig 12 a) for 48 hours on MS medium were further transferred on to the MS medium with napthalene acetic acid (NAA) (0.5 mg/L), benzylaminopurine (BAP) (1 mg/L) and cephotaxime (200 mg/L). Explants produced calli and direct shoot within three weeks (Fig: 12 b). The shoots and calli were excised and transferred to hormone free MS medium with 200mg/L cefotaxime and 200mg/L kanamycin. Approximately 75 per cent of the total cultured leaf disc turned albino on both callus induction and shoot regeneration medium. Only surviving green shoots were transferred to rooting medium with kanamycin 200mg/L after about 4 weeks (Fig 12 c).

PCR Analysis of Transformed Plants: Screening of transgenic plants (T_0) by PCR using *hpt*II specific primers resulted in 75.0% (6/8) of the transformed plants from CP (sense), 88.8% (8/9) of the transformed plants from CP- (antisense), 87.5% (7/8) of the transformed plants from CP-ihp (sas/ihp), 75.0% (6/8) of the transformed plants from CP- (SHUTR) (Table III) but no such amplicon was observed in untransformed (negative control) plants (Fig 13).

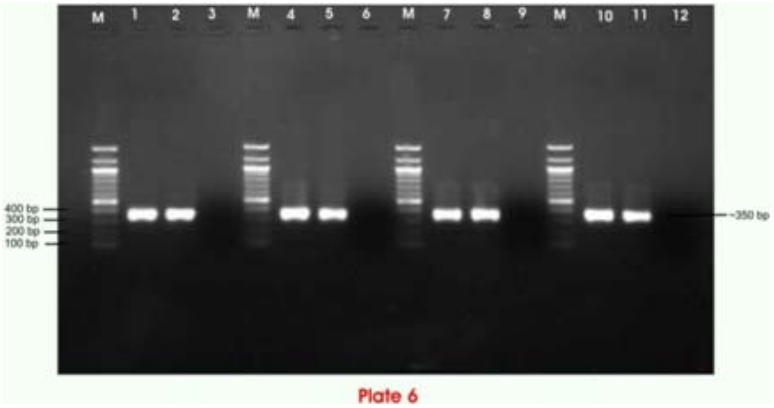

Plate 6

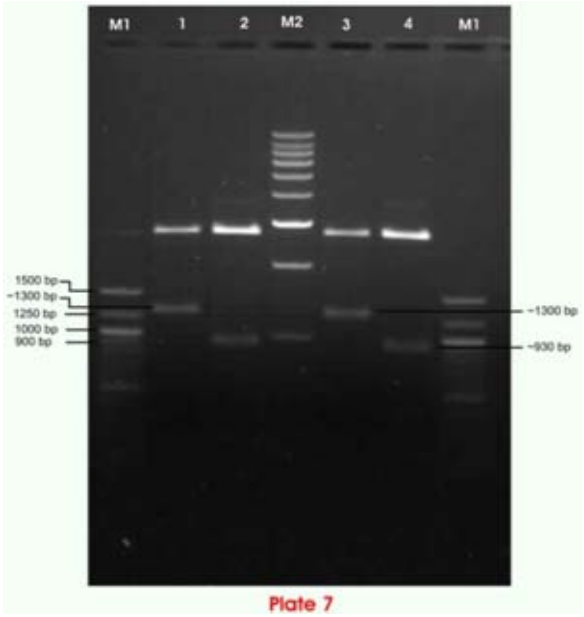

Plate 7

Plate. 6: PCR confirmations of generic ihp antisense, sense and IHP insert and SHUTR carrying partial Cp insert.
Plate. 7: Restriction analysis of IHP vector carrying antisense and sense CP insert.

Gus Histochemical Assay: The pCAMBIA construct used in this study has a GUS reporter along with the selectable marker *hpt*II. To confirm expression of inserted gene in the transformed plants, the putative transgenic plants of T_0-generation were subjected to GUS histochemical assay. Among the putative transgenic plants, 66.6% (4/6) of CP-(sense), 62.5% (5/8) of CP (antisense), 57.1 (4/7) of CP-ihp (sas/ihp) and 66.6% (4/6) of CP-(SHUTR) were GUS positive, but no GUS activity was observed in untransformed (negative control) plants (Fig 14). The percent success of transformation for GUS histochemical assay is summarized in (Table III).

The present study was done only with the aim of development of Post Transcriptional Gene Silencing Constructs against Groundnut Bud necrosis virus so that the most efficient silencing methodology could be found out which may assist later in the development of transgenic groundnut resistant to Bud necrosis virus. However, the further works are still need to be done by subjecting these transgenic plants with varying loads of viruses and study their resistance level. Further the level of siRNA produced in these transgenic could also be quantified by Real time PCR using siRNA specific primers.

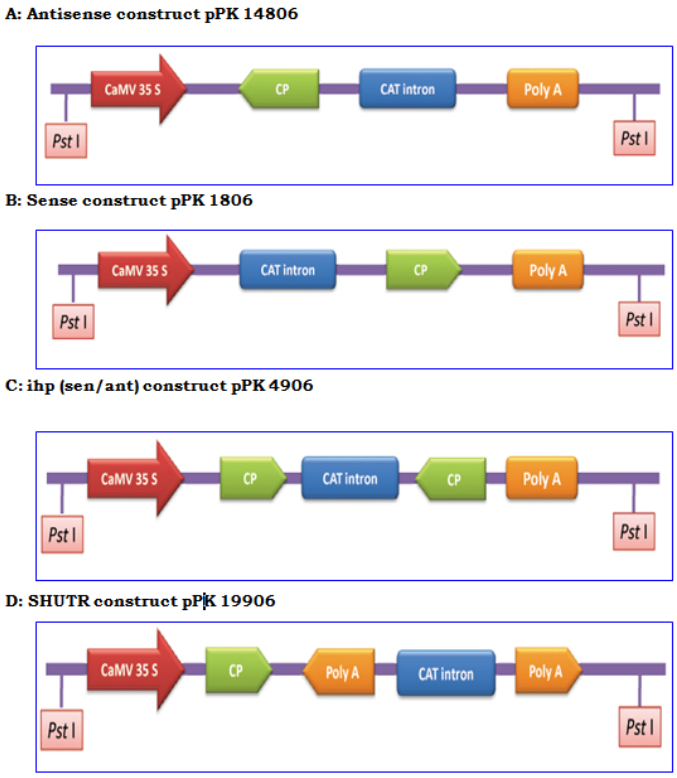

Fig. 7: Construct maps of generic ihp vector and SHUTR vector carrying partial GBNV CP gene

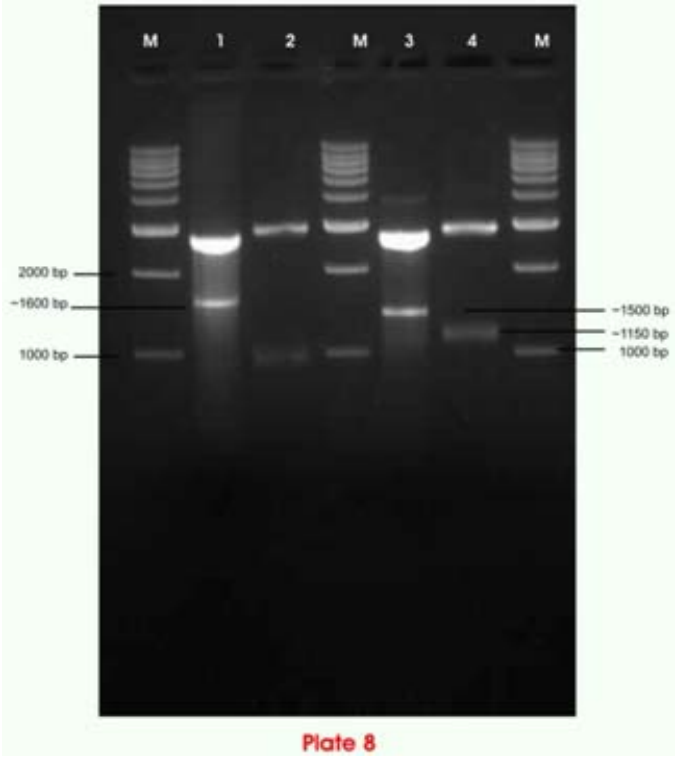

Plate. 8: Restriction analysis of ihp (sen/ant) and SHUTR clone.

Plate. 9: PCR confirmation of pCAMBIA vector carrying antisense, sense, Ihp and SHUTR constructs.

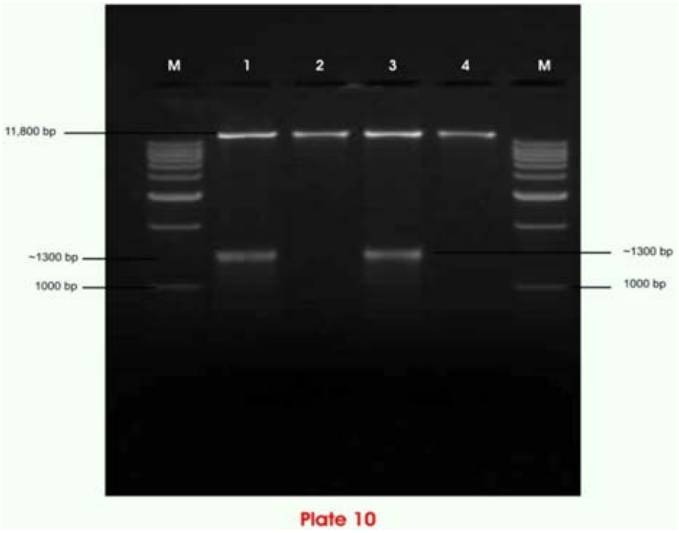

Plate. 10: Restriction analysis of pCAMBIA vector carrying antisense and sense ihp constructs

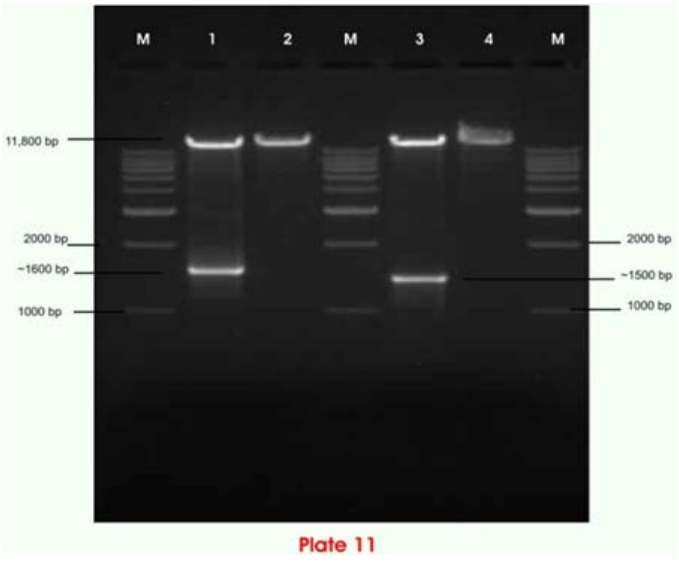

Plate. 11: Restriction analysis of pCAMBIA carrying ihp (sen/ant) constructs and SHUTR construct

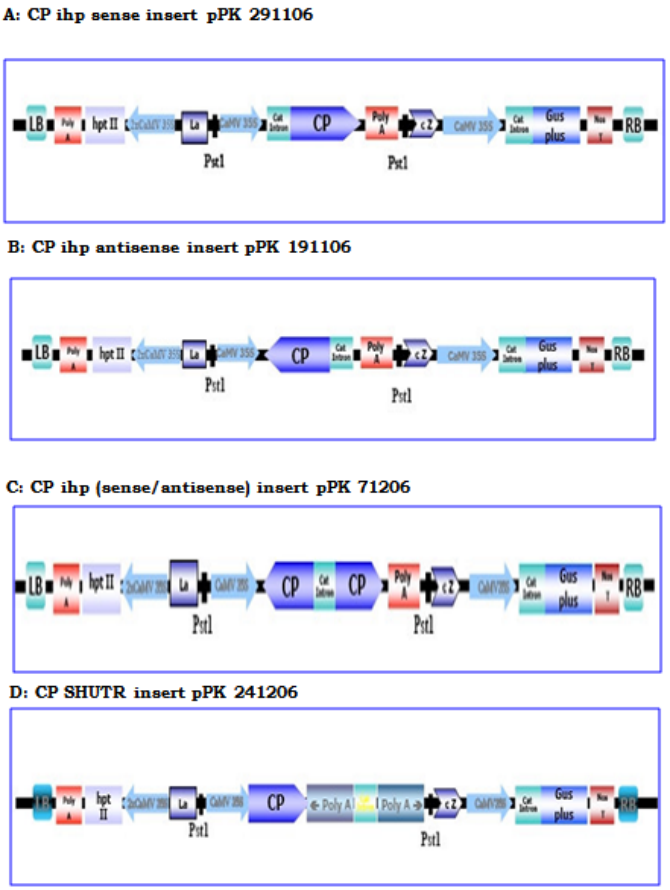

Fig. 10: Construct maps of pCAMBIA 1305.1 carrying various PTGS constructs

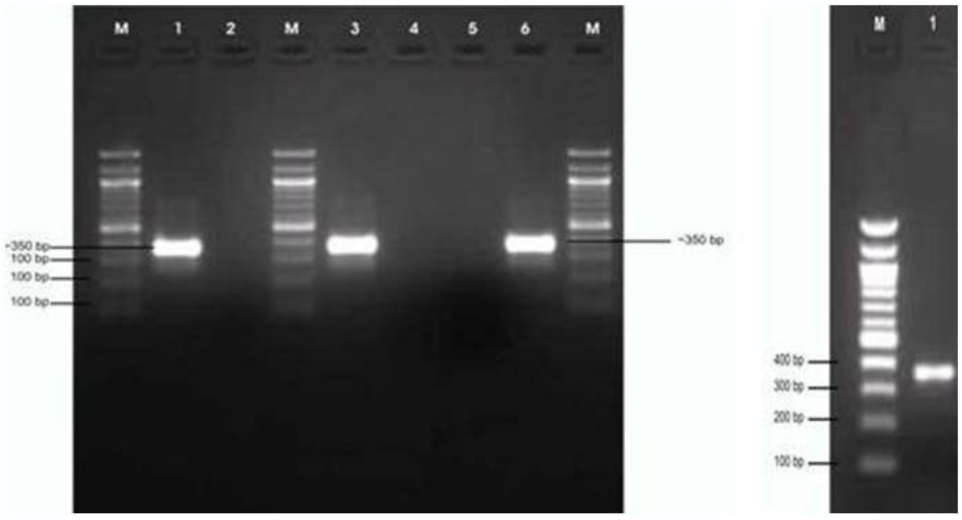

Plate. 12: PCR confirmation of Agrobacterium clones carrying PTGS constructs

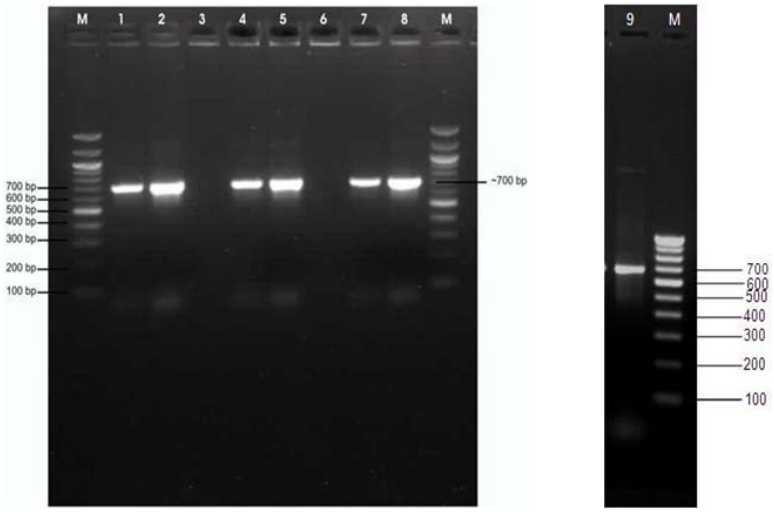

Plate. 13: PCR confirmation of transgenic tobacco carrying PTGS constructs with hptII primer

A B

C

Fig. 12: A- The leaf disc co-cultivated in MS medium
B - Callus induction in MS medium with kanamycin (100mg/L)
C - Plants in rooting medium with kanamycin (200mg/L)

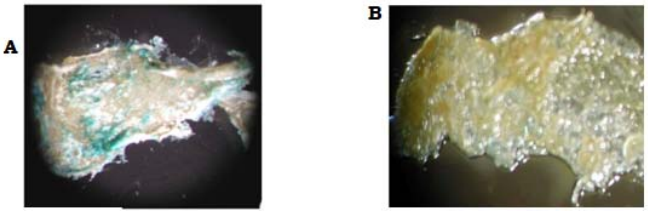

Fig. 14: A-GUS positive B-Control GUS negative.

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