Genetic transformation of peanut (*Arachis hypogaea* L.) using cotyledonary node as explant and a promoterless *gus::npt*II fusion gene based vector

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We have generated putative promoter tagged transgenic lines in *Arachis hypogaea* cv JL-24 using cotyledonary node (CN) as an explant and a promoterless *gus::npt*II bifunctional fusion gene mediated by *Agrobacterium* transformation. MS medium fortified with 6-benzylaminopurine (BAP) at 4 mg/l in combination with 0.1 mg/l α-napthaleneacetic acid (NAA) was the most effective out of the various BAP and NAA combinations tested in multiple shoot bud formation. Parameters enhancing genetic transformation viz. seedling age, *Agrobacterium* genetic background and co-cultivation periods were studied by using the binary vector p35SGUSINT. Genetic transformation with CN explants from 6-day-old seedlings co-cultivated with *Agrobacterium* GV2260 strain for 3 days resulted in high kanamycin resistant shoot induction percentage (45%); approximately 31% transformation frequency was achieved with p35S GUSINT in β-glucuronidase (GUS) assays. Among the *in vivo* GUS fusions studied with promoterless *gus*::*npt*II construct, GUS-positive sectors occupied 38% of the total transient GUS percentage. We have generated over 141 putative T_0 plants by using the promoterless construct and transferred them to the field**.** Among these, 82 plants survived well in the green house and 5 plants corresponding to 3.54% showed stable integration of the fusion gene as evidenced by GUS, polymerase chain reaction (PCR) and Southern blot analyses. Twenty-four plants were positive for GUS showing either tissue-specific expression or blue spots in at least one plant part. The progeny of 15 T_0 plants indicated Mendelian inheritance pattern of segregation for single-copy integration. The tissue-specific GUS expression patterns were more or less similar in both T_0 and corresponding T_1 progeny plants. We present the differential patterns of GUS expression identified in the putative promoter-tagged transgenic lines in the present communication.

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1. Introduction

Legumes are the most important group of crop plants next to cereals and much effort has been devoted to develop efficient *in vitro* regeneration systems because of their recalcitrance to tissue culture regeneration. Among them, peanut is an important cash crop for farmers particularly in the semi-arid tropics. Asian land races of peanut have a narrow genetic base and low genetic diversity because of which they lack resistance to many pests and diseases. These can cause major losses in terms of quality and quantity. Genetic transformation offers a solution to these problems by making the transfer of genes from alien sources feasible for generating transgenic plants possessing resistance to biotic and abiotic

Keywords. *Agrobacterium tumefaciens*; cotyledonary node; *gus::npt*II bifunctional fusion gene; peanut; promoter tagged lines

Abbreviations used: AMV, Alfalfa mosaic virus; BAP, 6-benzylaminophrine; CN, cotyledonary node; GUS, β-glucuronidase; NAA, α-naphthaleneacetic acid ; PCR, polymerase chain reaction; RIM, root induction medium.

stresses. Improving transformation frequency remains the most important factor in plant transgene technology (Gheysen *et al* 1998). Genetic modification of plants is dependent on tissue-specific promoters, as the targeted expression of transgenes is needed in many cases to avoid undesirable ectopic expression with widely used constitutive promoters. Promoters offer a fundamental control in gene expression and there is a great deal of interest in isolating and characterizing plant promoters worldwide (Datla *et al* 1997). The present study involves a promoter tagging approach with a promoterless *gus::npt*II bifunctional fusion gene (Datla *et al* 1991). This strategy based on T-DNA insertional mutagenesis was exploited earlier to identify plant promoters in different plants like tobacco (Andre *et al* 1986; Fobert *et al* 1994), Arabidopsis (Koncz *et al* 1989; Kertbundit *et al* 1991; Topping *et al* 1994) and potato (Lindsey *et al* 1993). Vector constructs containing either a reporter gene or a marker gene lacking promoter elements have been used for tagging (Teeri *et al* 1986**)**. T-DNA insertional mutagenesis acts as a powerful tool in the isolation and characterization of plant regulatory sequences / promoters (Walden 2002). A similar tagging approach in tobacco using a promoterless *lacZ::npt*II fusion gene was carried out (Suntio and Teeri 1994). However, expression analysis was difficult because of the complexity of *lacZ:: npt*II fusion gene. Recently, by employing hypocotyls as explants and a promoterless *gus::npt*II fusion gene, promoter tagged lines were produced and genomic sequences upstream of this construct were isolated via plasmid rescue (Bade *et al* 2003). Among forage legumes, T-DNA insertional mutagenesis programme in the model legume *Medicago truncatula* revealed that several genes were tagged in the transgenic lines and *in vivo* GUS fusions were also obtained (Scholte *et al* 2002). A promoter tagging approach in the legume, *Lotus japonicus* was initiated to identify plant genes involved in the nitrogen fixation (Webb *et al* 2000). A high frequency transformation protocol is required for generating T-DNA tagged lines. The recalcitrance of many legumes to tissue culture response and plant regeneration has driven researchers to develop alternate transformation systems that target axillary meristem in the cotyledonary nodes (Somers *et al* 2003). We report here for the first time the mode of genetic transformation using cotyledonary node (CN) as an explant in peanut mediated through *Agrobacterium tumefaciens*. The main objective of this work is to create transcriptional gene fusions between the upstream promoter elements in the plant genome and the downstream fusion gene and thus the reporter gene expression can be assayed. By using CN as an explant and a promoterless *gus::npt*II fusion vector, we have generated T-DNA tagged lines with a wide range of β-glucuronidase (GUS) expression patterns. The integration was confirmed by polymerase chain reaction (PCR) and Southern analyses in some of these plants. The inheritance of the *gus::npt*II bifunctional fusion gene in the T_1 generation was confirmed by GUS and PCR analyses and followed Mendelian pattern of segregation. To the best of our knowledge this is the first report on promoter tagging in grain legumes.

2. Materials and methods

2.1 *Plant material and explant preparation*

Mature *Arachis hypogaea* cv. JL-24 seeds were surface sterilized by rinsing with 70% (v/v) ethanol for 1 min and 7 min with 0.1% (w/v) aqueous mercuric chloride followed by several washes with sterile double distilled water. They were then imbibed in sterile double distilled water for 4-6 h and germinated on autoclaved filter paper wicks soaked in sterile double distilled water. Explants were prepared from 1-6-day-old seedlings in the initial experiments to evaluate the influence of explant age on transformation frequency. In the subsequent experiments, 6-day-old seedlings were used. The seed coat and the radicle were removed and the cotyledonary nodes were excised by cutting both epicotyls and hypocotyls approximately 2 mm above and below the nodal region and the embryo axis was bisected along the longitudinal plane. The meristematic region present in the nodal region was macerated by 6–8 diagonal shallow cuts by a sterile surgical blade and the cotyledons along with their nodes were embedded into the shoot induction medium (SIM) in such a way that the wounded meristematic nodal region and the adaxial surface of the cotyledon were in direct contact with the medium. From each seed, two explants were obtained. The explant is now designated as CN. The method of explanting has been adapted from Townsend and Thomas (1993).

2.2 *Regeneration*

Regeneration with CN explant was standardized by testing various combinations of growth regulators like 6-benzylaminopurine (BAP), kinetin (KN), 2,4-dichloro-phenoxyacetic acid (2,4-D) and α-napthaleneacetic acid (NAA) with MS basal salts (Murashige and Skoog 1962), pH 5.6–5.8 before autoclaving and 3% sucrose as a carbon source. Explants were cultured on agar (0.8%) solidified SIM containing 4 mg/l BAP and 0.1 mg/l NAA that induced maximum number of shoots per explant. The cultures were maintained at 28 ± 1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux. Subcultures were done at 15-day interval with 2-week duration each on SIM for the development of adventitious shoot buds. Explants producing multiple shoots were transferred

to shoot elongation medium (SEM) comprising 2 mg/l BAP and 0.1 mg/l NAA. Elongated shoots were cut at the internodal region and transferred to root induction medium (RIM) solidified with 5% agargel (Sigma, USA) comprising of 0.8 mg/l NAA with MS basal salts.

2.3 *Plasmid construct and* Agrobacterium *strain*

A derivative of binary vector pRD400, harbouring a promoterless synthetic *gus::npt*II bifunctional fusion gene (Datla *et al* 1991) with an alfala mosaic virus (AMV) translational enhancer (Datla *et al* 1993) at the 5′ end and nos terminator was used to transform peanut through a disarmed rifampicin and carbenicillin resistant *Agrobacterium tumefaciens* strain, GV2260. The strain was maintained on solidified Luria agar plates with 100 mg/l rifampicin, 100 mg/l carbenicillin and 50 mg/l kanamycin monosulphate. A fresh overnight culture of the *Agrobacterium*, obtained by inoculating single colony in Luria broth containing appropriate antibiotics with an OD_{600nm} 0.8, was pelleted by centrifugation at 5000 rpm for 5 min, resuspended in sterile double distilled water and stored at 4°C for 2 h before infection. A binary vector p35SGUSINT (Vancanneyt *et al* 1990) was used initially to optimize the transformation conditions.

2.4 *Transformation*

Freshly cut CN explants were infected by dipping in the bacterial suspension by their proximal cut ends and incubated for 5–10 min in 90 × 15 mm sterile petridishes. They were co-cultivated on SIM for 72 h in 16/8 h photoperiod at 25 ± 2°C. After a 4-day recovery period on SIM containing 250 mg/l cefotaxime, explants were transferred to a fresh SIM supplemented with 175 mg/l kanamycin monosulphate (Sigma, USA) and 250 mg/l cefotaxime. Cultures with completely green shoots were maintained on SEM with 175 mg/l for two selections of two-week duration each. Cultures with green and white shoots intermingled were transferred to medium without kanamycin and there after maintained on MS medium without any selection agent. Shoots with two internodes were cut and transferred to RIM for root induction. Plants with well-developed roots were transplanted to autoclaved soil and vermiculite mixture (1:1 ratio) in plastic pots and were hardened under culture conditions for 2 weeks prior to their transfer to greenhouse. Later they were transferred to bigger pots and allowed to flower and set seed in the greenhouse.

2.5 *Selection*

An effective kanamycin concentration for the selection of transformed shoots was initially standardized by culturing control untransformed CN explants on SIM containing different concentrations of kanamycin (100, 125, 150, 175, 200, 250 mg/l). Two subcultures were carried out on fresh SIM having the same level of antibiotic and then scored for the regeneration percentage and number of shoots per explant. Similarly, kanamycin concentration that inhibited root formation was determined by transferring control shoots (2–3 cm in length) regenerated from non-transformed explants to RIM supplemented with different kanamycin levels (20, 30, 40, 50, 60, 70 mg/l etc.).

2.6 *GUS analysis*

Phenotypic GUS expression was determined by staining unfixed leaflets, roots, flower and shoot parts of over 141 putative T_0 plants analysed so far. GUS assay was carried out by incubating the above plant tissues from the putative transformants in a buffer comprising 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, Biosynth, Switzerland), 100 mM sodium phosphate (pH 7.2), 0.1% Triton X-100, 0.5 mM potassium ferricyanide and 10% methanol (Jefferson *et al* 1987) overnight at 37°C and stained tissues were cleared of chlorophyll by soaking in 70% ethanol. GUS assays were also performed with the progeny of 15 T_0 plants. Transient GUS assays were done in triplicate to study *in vivo* GUS fusions, by staining young leaflets and shoot parts, which were picked up randomly from the cultures.

2.7 *DNA isolation and PCR analysis*

Total genomic DNA was extracted from young leaves of putative transformants and control plants using standard CTAB method (Doyle and Doyle 1987). Plants were kept under shade for 2 days prior to harvesting leaves from them. The PCR was performed to screen putative T_0 transformants and T_1 generation plants for the presence of the fusion gene by using specific primers for *npt*II and *uid*A genes. The PCR reactions were carried out using 100 ng of purified genomic DNA as template and 2.5 U of recombinant *Taq* DNA Polymerase (Invitrogen Corporation, Sao Paulo, Brazil). The 700 bp of the *npt*II fragment was amplified by using 21-mer oligonucleotide primers (*npt*IIF 5′-GAGGCTATTCGGCTATGACTG-3′ and *npt*IIR 5′-ATCGGGAGCGGCGATACGTA-3′). The cycling conditions comprised an initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 61°C for 45 s, 72°C for 1 min and a final extension of 10 min at 72°C. The 469 bp GUS fragment was amplified by using 22-mer oligonucleotide primers (GUSF 5′-TACCTCGCATTACCATTACGCG-3′ and GUSR 5′-CTTCTCTGCCGTTTCCAAATCG-3′). Cycling conditions were similar as in PCR

using *npt*II primers except for the annealing temperature at 63°C for 55 s. The amplified products were electrophoresed on 1.2% agarose gels and visualized with ethidium bromide. The fragments resolved on agarose gels were transferred to Hybond N+ by Southern blotting and the blots were hybridized with GUS fragment, PCR amplified from the respective plasmid labelled with α–^{32}P.

2.8 *Southern hybridization*

Genomic DNA (15 μg) from T_1 plants was separately digested to completion with *Hin*dIII that does not cut within the T-DNA region and the restriction fragments were resolved by electrophoresis on 0.8% agarose gels and blotted by capillary method onto Hybond N+ membrane (Amersham Pharmacia, UK) using 20X SSC as a transfer buffer. Membranes were probed with α-^{32}P dATP labelled 700 bp *npt*II fragment amplified from the plasmid having *gus::npt*II gene. Following 16 h of hybridization at 65°C, membranes were washed for 20 min each at 65°C in 2X SSC, 0.1% SDS, 1X SSC, 0.1% SDS and finally with 0.1X SSC, 0.1% SDS for 10 min. The washed membranes were wrapped in saran wrap and subjected to autoradiography (Sambrook *et al* 1989).

2.9 *Statistical methods*

Standard statistical methods were used. Since the number of seeds obtained in transgenic peanut plants would be insufficient to conduct Chi-square test on individual progenies except three, Brandt and Snedecor's rows and columns method was used in Chi-square analysis (Bailey 1965). For the three progenies with a minimum population of 12, Chi-square test was conducted individually also.

3. Results

3.1 *Regeneration*

Cotyledonary node explants excised from 1-8-day-old seedlings were cultured on different media with various growth regulator combinations for multiple shoot induction (data not presented). Six-day old seedlings responded well in terms of regeneration and transformation (figure 1). The percentage regeneration and average number of shoots were highest on MS media fortified with BAP and NAA. Among the various BAP and NAA combinations tested, high frequency regeneration was obtained following culture of explants on MS medium supplemented with 3, 4 and 5 mg/l BAP along with 0.1 and 0.2 mg/l NAA (table 1). BAP at 4 mg/l and 0.1 mg/l NAA was most effective for multiple shoot bud formation. Both the percent regeneration as well as the average number of shoot buds per explant was found to be higher (82% regeneration, 28 ± 1.5), when the adaxial side of the cotyledon was in direct contact with the medium compared to the abaxial side (58% regeneration, 8.75 ± 2.4). Elongated shoots when cultured on RIM developed adventitious roots within 15 days of culture. An average of 6 plants were recovered from each explant.

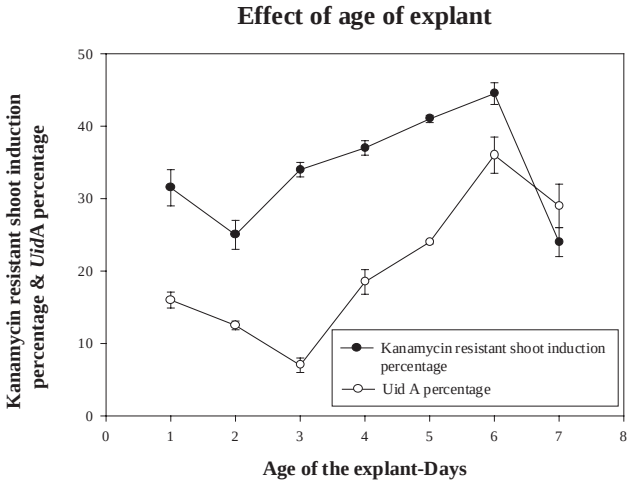

Figure 1. Effect of explant age in days on regeneration and *uid*A percentage.

Table 1. Effect of different concentrations of BAP and NAA on regeneration from 6-day-old CN explants of *A. hypogaea* cv JL-24.

MS medium supplemented with BAP and NAA (mg/l)		Regeneration (%)	Average number of shoot buds / explant ± SE
3	0.1	69	19 ± 2.1
4	**0.1**	**82**	**28 ± 1.5**
5	0.1	76	21 ± 2
3	0.2	64	16 ± 2.5
4	0.2	73	23 ± 1.75
5	0.2	71	14 ± 3.4

Each mean value was an average calculated from three experiments ± SE.
Optimum growth regulator combination has been shown in bold face.

3.2 *Genetic transformation*

To evaluate the transient GUS frequency as well as the stable transformation efficiency, a number of parameters enhancing genetic transformation were studied by using binary vector p35SGUSINT. Optimized conditions determined were followed in the subsequent experiments. Parameters that

were tested included the seedling age, *Agrobacterium* strains and co-cultivation periods. The total number of GUS spots and GUS positive sectors on different leaf and shoot parts were scored. The GUS positive sectors are the deeply stained blue regions on different plant parts such as leaves, roots, stem etc. Kanamycin resistant shoot induction percentage was calculated at the end of first subculture as the percentage of shoots growing on medium containing kanamycin to the total number of explants cultured. Transient transformation frequency was determined 72 h after co-cultivation in order to assess the efficiency of transformation and is the percentage of explants showing at least one discrete dark blue GUS positive sector or GUS spot, whereas stable transformation efficiency is the percentage of transformed shoots showing positive for GUS staining, PCR and Southern analysis (Egnin *et al* 1998). For each factor tested, at least three experiments were performed and a minimum of 20 explants was used in each experiment. Kanamycin resistant shoot induction percentage as well as transient transformation frequencies were studied in these experiments.

3.2a *Effect of age of explant:* Cotyledonary nodes obtained from 1-8-day-old seedlings were used as explants and two transformation experiments were carried out in order to determine the transient GUS expression as well as kanamycin resistant shoot regeneration percentage. Transient transformation frequency or GUS staining percentage in 1, 2 and 3-day-old CN explants after co-cultivation was low when compared with that of 6-day-old CN explants**.** The possible fact could be that the surface area of the existing meristematic cells might be more in the 6-day-old CN explants facilitating efficient T-DNA transfer.

The transient GUS percentage in both 5- and 6-day-old explants after co-cultivation was more or less similar but they differed in shoot regeneration response; hence the latter was employed for further experiments (figure 1). The possible reason can be attributed to the actively dividing state of explant cells during 5–6 days of development whereas, the low *uid*A and regeneration response of 8-day-old CN explants could be due to the further differentiation of primary meristem and the availability of less number of actively dividing cells.

3.2b *Effect of* Agrobacterium *genetic background:* The transient GUS percentage was studied with two disarmed *Agrobacterium* strains, GV2260 and LBA4404. Of the two strains, GV2260 was found to enhance transient transformation efficiency of 6-day-old nodes by at least 3 times compared to LBA4404. Also, more dark blue GUS positive sectors were observed in cultures with strain GV2260. Hence, GV2260 was used in subsequent experiments (data not shown).

3.2c *Effect of co-cultivation period:* When 6-day-old explants were co-cultivated with GV2260 for 1, 2 and 3 days, kanamycin resistant shoot regeneration percentage

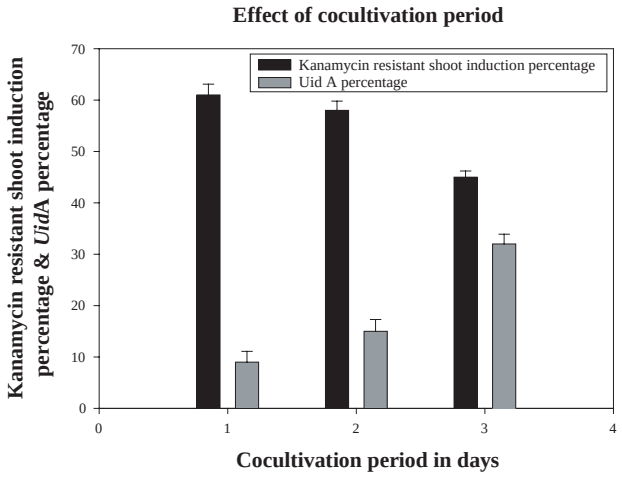

Figure 2. Effect of co-cultivation period on regeneration and *uid*A percentage.

was high with 1-day-old co-cultivated explants and decreased gradually within 2 and 3 days (figure 2). Transient GUS staining percentage increased considerably when explants were co-cultivated for 1–3 days. When the co-cultivation period exceeded 3 days, it resulted in necrosis of the explants. The high shoot induction percentage of 1- and 2-day-old co-cultivated explants is likely due to the reduced damage caused by *Agrobacterium* during co-cultivation. Approximately, 31% of transformation frequency was observed with p35SGUSINT based on transient GUS staining. The data obtained so far was collected based on transient GUS analyses.

3.3 *Selection*

Further, we determined the optimal concentration of kanamycin for the selection of transformed shoots by culturing the uninfected control CN explants on SIM containing kanamycin in dose-dependent concentrations (100, 125, 150, 175, 200 and 250 mg/l). Kanamycin resistant shoot induction percentage decreased with increasing kanamycin concentration (data not shown). At 175 mg/l kanamycin, regeneration percentage decreased drastically and the shoots produced were chlorotic. Hence, this concentration was chosen for the selection of putative transformants. Explants showed necrosis and shoot induction diminished completely at kanamycin concentrations 200 and 250 mg/l. Similarly, a kanamycin concentration of 60 mg/l caused complete inhibition of root induction from control shoots (data not shown). It is well known that root induction is more sensitive to kanamycin than shoot organogenesis and further explains the fact that the utility of any antibiotic depends on both plant species as well as the explant involved (Saini *et al* 2003).

3.4 *Regeneration of transformants*

Genetic transformation experiments with 6-day-old CN explants co-cultivated with GV2260 carrying p35SGUSINT for 3 days resulted in more number of kanamycin resistant shoots and higher frequency of GUS positive sectors in transient GUS analysis. Further, transformation experiments were conducted using a promoterless *gus::npt*II bifunctional fusion gene construct following the parameters investigated thus far. After co-cultivation and a short recovery period, explants were cultured on SIM supplemented with 175 mg/l kanamycin until two selections and later some of the cultures were maintained on medium free of any selection agent. To study the transient GUS expression, different tissues were collected randomly from the cultures and stained in the X-Gluc solution. Among *in vivo* GUS fusions, the range of GUS expression patterns include GUS positive sectors on leaves, shoot parts, brown calli, deep blue staining along the midrib of leaves, cut regions of leaves and also GUS spots on various plant parts. The GUS positive sectors on the leaves were found to occupy maximum (38%) of the total transient GUS percentage. The total number of GUS positive sectors on shoot parts and brown calli were found to be less than the number of GUS spots on leaves and petiole (figure 3). No staining was observed in any tissue of the untransformed shoots studied. These results suggest that the GUS expression observed was only because of the productive transcriptional fusions between the upstream regulatory elements in the plant genome and the downstream *gus::npt*II fusion gene devoid of promoter in the vector. However, the appearance of GUS spots on leaves clearly infers that some of the shoots were chimeric and does not necessarily indicate tissue-specific expression.

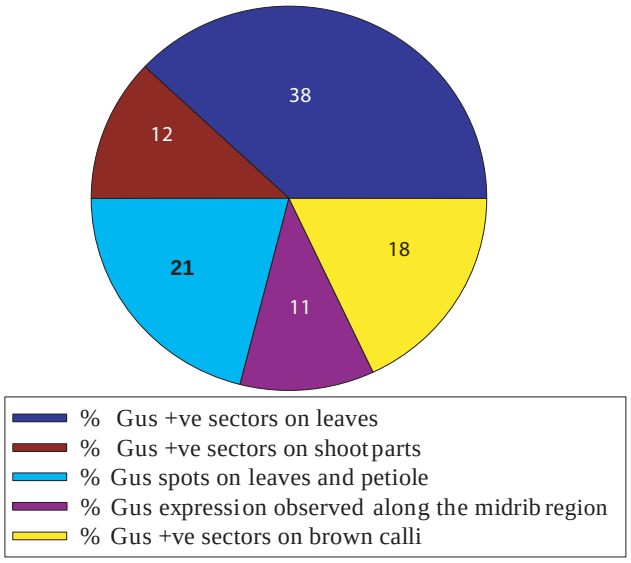

Figure 3. Distribution of *in vivo* GUS fusions among cultures.

We have generated over 141 putative T_0 plants by using the promoterless construct (figure 4A) and transferred them to green house. Among these, 82 plants survived well in the greenhouse and 5 plants corresponding to 3.54% showed stable integration of the fusion gene as evidenced by GUS, PCR and Southern analyses. Three out of thirty three shoots rooted well on RIM with kanamycin. These plants were phenotypically similar to normal control plants. Figure 4B represents different regeneration stages of peanut from explant stage to plant development.

3.5 *GUS expression in individual transgenic plants*

In order to investigate the *uid*A gene expression patterns in various plant tissues, GUS assays were carried out with leaves, roots, shoot parts and floral organs of over 141 T_0 putative transformants. Twenty-four plants were found to be positive for GUS showing either tissue-specific expression or blue spots in at least one plant part. GUS studies in flowers revealed that 3 putative transformants out of 24 were GUS positive exhibiting differential *uid*A expression patterns. These plants did not reveal any detectable GUS staining in any other plant parts studied. Relatively high frequency of GUS staining was observed in leaves of putative transformants (75%; 18/24), while in roots it was low (41.66%; 10/24). Among these, only 16.66% of the transformants (4/24) exhibited GUS expression in shoot regions. Plants that have been rooted on RIM supplemented with kanamycin were found to be positive for GUS, PCR and Southern hybridization. Faint blue colouration was observed in many of the plant parts that were analysed for GUS. Deep blue sectors were found on the leaves of plant C-53 (figure 4C.). Plants C-23, C-27, C-28, C-31, C-33, C-44 and C-52 did not show any GUS staining in the subsequent assays and the blue spots observed in these plants can be considered as artifacts. The cultures, from which these plants were obtained, were initially maintained on SIM containing 175 mg/l kanamycin for up to two selections and later transferred on to a medium free of any selective agent. Some of these GUS positive plants can be considered as putative promoter tagged lines. These results indicate that, within a population of transformed plants, expression of a promoterless *gus::nptII* gene occurs at high frequency in a wide range of plant parts.

3.6 *Segregation analysis of the* uid*A expression in the progeny*

It is necessary to characterize the inheritance pattern of fusion gene in the T_1 generation in order to understand the

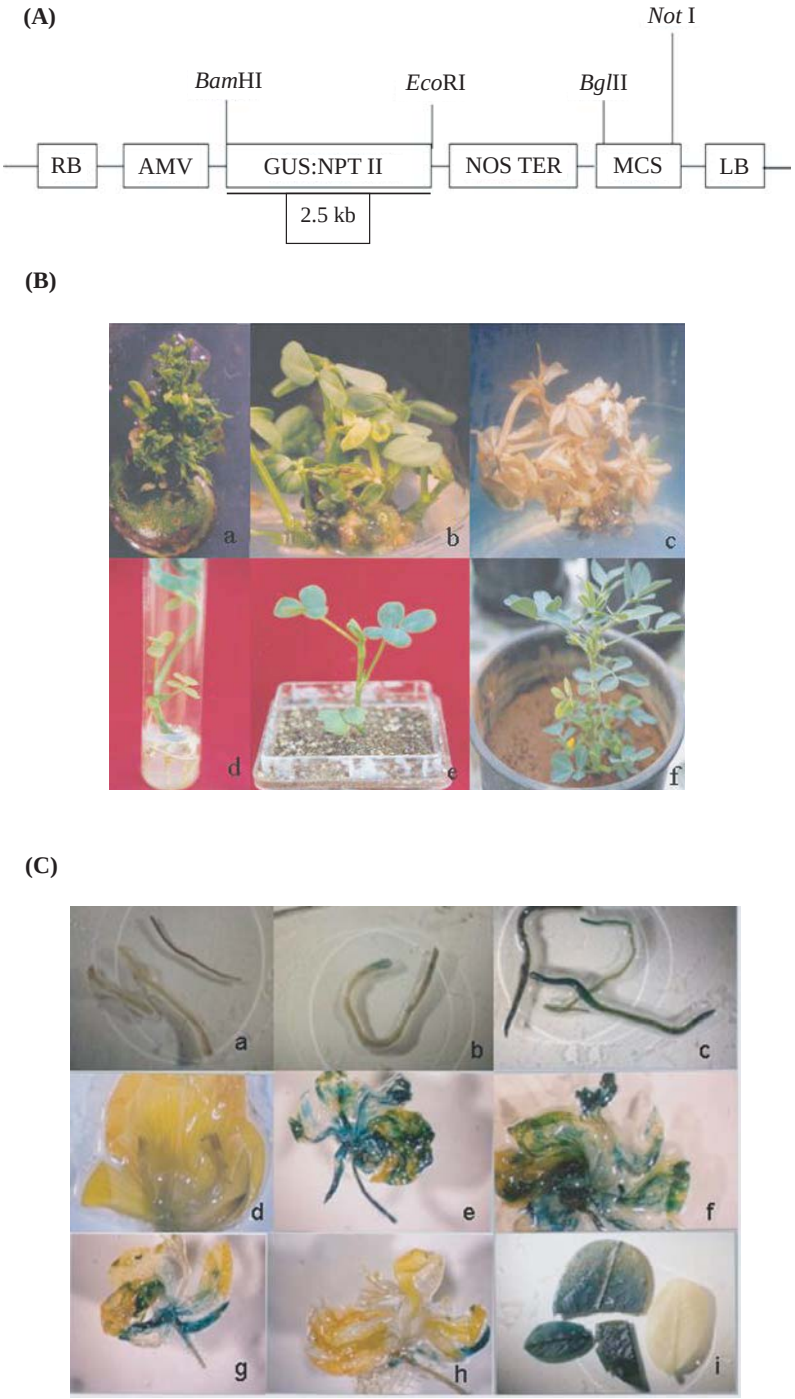

Figure 4. **(A**) Synthetic T-DNA region showing the AMV enhancer sequence, promoterless *gus:: npt*II bifunctional fusion gene, *NOS-TER*, Nopaline synthase terminator; *RB*, right border; *LB*, left border and unique restriction sites. (**B**) Regeneration and *Agrobacterium*-mediated transformation with CN in *A. hypogaea* CV JL-24. (**a**) CN explants showing multiple bud induction after three weeks on SIM containing 175 mg/l kanamycin. (**b**) Cultures with complete green multiple shoots after six weeks on SIM containing 175 mg/l kanamycin. (**c**) Completely bleached control cultures on SIM containing 175 mg/l kanamycin. (**d**) Profusely rooted shoot on RIM. (**e**) Hardening of plant in soil: vermiculite mixture. (**f**) Acclimatization of plant in greenhouse conditions. **(C)** GUS expression observed in various plant parts of different T_0 plants. (**a**) Control JL-24 roots. (**b**) GUS expression in root tip. (**c**) GUS expression in roots. (**d**) Control JL-24 flower. (**e** and **f**) GUS expression in complete flower of C-1 plant showing stained floral parts. (**g**) GUS stained keel petal and pedicel of plant C-2. (**h**) GUS expression only in keel petal of plant C-13. (**i**) GUS expression in leaves in contrast to bleached control leaf.

stability of foreign gene integration. The progeny of 15 GUS positive plants were analysed to study the segregation pattern of the fusion gene. For undertaking the Chi-square test for segregation, the minimum population size is 12. However, we have the minimum population size only in three samples and they showed good fit for monogenic segregation suggesting single copy integration. However, for none of these three lines, Southern data were available. Chi-square test was also conducted on pooled data from all the lines following the rows and columns method, which indicated a fit for monohybrid ratio indicating single copy integration. However, plant No. C-43 has been observed to have two copies of the T-DNA in Southern analysis. Root and leaf-specific expression was found to be predominant among the progeny of plants C-12 and C-17 (table 2). The tissue-specific GUS expression patterns were more or less similar in both parent and progeny plants. Segregation of *gus* and *npt*II genes in the progenies was confirmed through PCR.

3.7 *PCR analysis and Southern hybridization*

The presence of the fusion gene was confirmed in about 10 T_0 putative transformants by PCR using specific primers for *uid*A and *npt*II genes. To check the fidelity of the amplicons in PCR reaction, the PCR products were transferred to nylon membranes for Southern hybridization and probed with radiolabelled GUS fragment. This experiment confirmed the transgenic nature of the T_0 plants. Figure 5A shows the Southern of PCR products from 12 T_0 plants. The PCR screening was carried out in the T_1 progenies in order to ascertain the inheritance pattern of the integrated fusion gene. Figure 5B shows the amplification of *npt*II and *uid*A genes in the progeny of 5 PCR confirmed T_0 plants.

Southern hybridization analysis revealed the integration and copy number of *npt*II gene among the progeny of 5 individual T_0 plants analysed. Digestion of genomic DNA of T_1 plants with *Hin*dIII that does not cut internally within the T-DNA region of pRD400 *gus::npt*II fusion gene should yield unique restriction patterns when probed with a *GUS* or *npt*II fragment and the number of bands should correspond to the copy number.

Southern analysis of the progeny of the 5 GUS or PCR confirmed T_0 plants showed differential banding patterns upon probing with *npt*II fragment. Plants C-1a, C-17a, C-4a and C-53a were shown to contain independent single copy insertions whereas; C-43a plant showed two copy insertions respectively (figure 6). The hybridized bands were greater than the size of T-DNA and this confirms the integration of T-DNA into plant genome suggesting the origin of plants as a result of independent transformation events.

Table 2. Segregation pattern of the GUS expression in T_1 generation.

Plant	PCR amplification	Copies of the fusion gene integrated (Southern analysis)	Total No. of seeds collected	GUS and PCR +ve	GUS and PCR -ve	Chi-square value	Probability (*P*)	Segregation ratio
C-53	+	+/1	9	8	1			
C-4	+	+/1	6	5	1			
C-17	+	+/1	12	9	3	0	0.001	3:1
C-12	+	_	5	4	1			
C-25	+	_	21	17	4	0.209	0.05	3:1
C-43	+	+/2	7	3	4			
C-47	+	_	2	2	0			
C-11	+	_	7	6	1			
C-1	+	+/1	7	7	0			
C-13	+	_	11	7	4			
C-51	+	_	18	15	3	0.15	0.05	3:1
C-2	+	_	4	1	3			
C-50	+	_	11	5	6			
C-48	+	_	6	3	3			
C-49	+	_	6	2	4			

Minimum sample size for Chi-square test is 12. Only three samples have the minimum size of twelve and Chi-square test was conducted on these samples.

Brandt and Snedecor's rows and columns method was also used in Chi-square analysis (Bailey 1965) on the pooled data of GUS positive and negative segregation with a Chi-square value =24.9887, degrees of freedom 14, and probability $P \leq 0.05$ indicating a fit for monohybrid ratio for all lines. Plant C-43 has been observed to have two copies of the transgene.

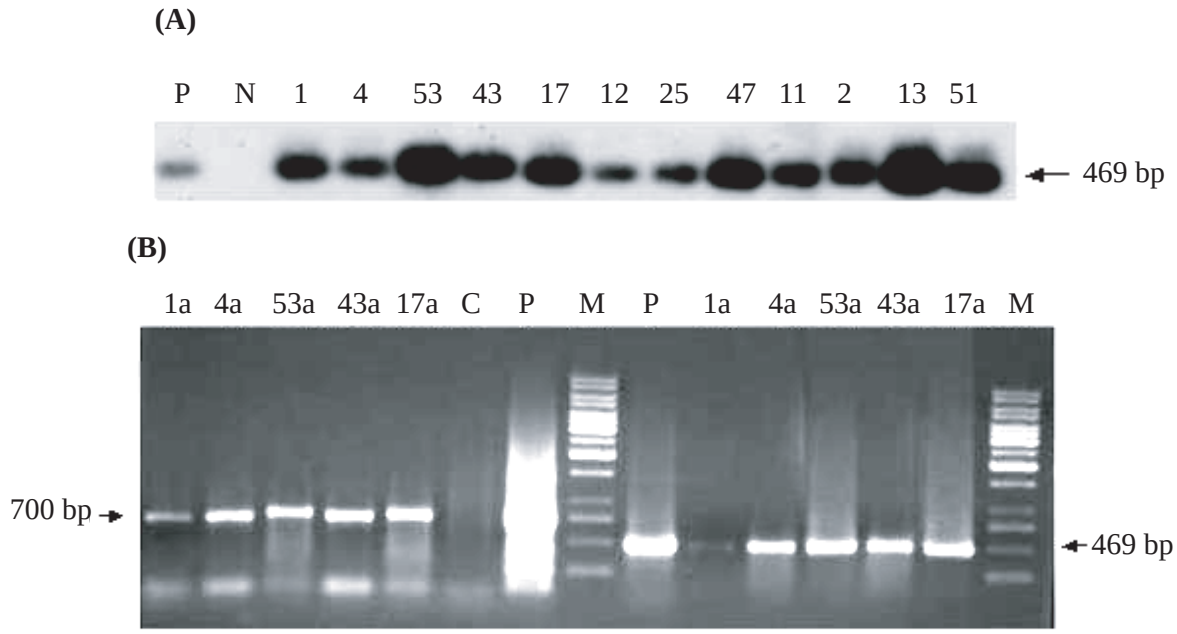

Figure 5. **(A)** PCR Southern of T_0 transformants using GUS as a probe. P, *gus::nptII* bifunctional fusion gene as a positive control; N, control plant. Lanes 3–14 represents T_0 putative transformants. **(B)** Analysis of T_1 transformants using the *uid*A (right) and *npt*II (left) primers. Lane M, 1 kb marker DNA. Lane C, DNA from non-transformed control plant. Lanes 1–5 and 10–14, T_1 transformed plants, +ve plasmid DNA.

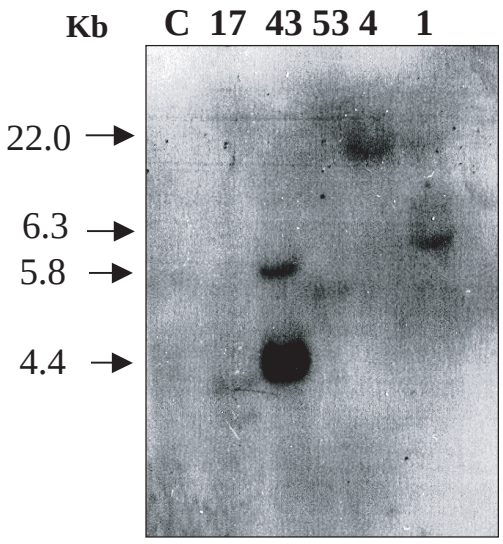

Figure 6. Southern blot analysis of T_1 transgenic peanut plants for the presence of *npt*II gene. Genomic DNA's were digested with enzyme *Hin*dIII and probed with PCR amplified *npt*II fragment from plasmid DNA. Lanes 1, Control plant. Lanes 2 to 6, T_1 transgenic plants.

When the same blot was stripped and re-probed with GUS fragment, similar banding pattern was noticed confirming the integration of the fusion gene into the genomes of transgenic plants (data not shown). These results show the co-integration and co-segregation of both *npt*II and gus genes respectively in the T_1 generation.

4. Discussion

Plant regeneration is usually a bottleneck for the development of highly reproducible transformation protocols. Adventitious shoot formation via organogenesis becomes more efficient, once a suitable explant has been identified. Previously, leaf discs and leaf-section explants were used to generate transgenic peanut plants but with low frequencies (Eapen and George 1994; Cheng *et al* 1996). In the recent studies, cotyledons were used as a source of explants for achieving high frequency of transformation in peanut (Venkatachalam *et al* 2000), more particularly in the cultivar JL-24 (Sharma and Anjaiah 2000). However, the regeneration response from the cotyledonary nodes may be comparatively higher than the cotyledons and other explants reported so far. The junction of the cotyledon and the embryo axes contain axillary meristematic cells that are highly regenerable and hence could be useful as potential targets for gene delivery. Cotyledonary nodes from mature seeds have been proved to be most responsive for the induction of multiple shoots and to generate transgenic plants in other grain legumes studied; viz. soybean (Olhoft *et al* 2003), pea (Bean *et al* 1997), pigeon pea (Geetha *et al*

1999), mungbean (Jaiwal *et al* 2001), black gram (Saini *et al* 2003). *Agrobacterium*-based genetic transformation leads to the production of large number of stable transgenic plants and more preferred to other gene transfer mechanisms.

Promoter tagging system with *gus::npt*II bifunctional fusion gene imparts both kanamycin resistance and GUS activity (Datla *et al* 1991). As proven earlier, AMV translational enhancer sequence elevates the expression levels of the reporter gene (Datla *et al* 1993) and thereby facilitating the recovery of weak promoters also. Insertion of the promoterless reporter gene downstream to a promoter by chance not only disrupts normal gene function but also activates the expression of the reporter gene i.e., it sequentially comes into the transcriptional control of the upstream plant promoter. The inserted element acts as a tag for gene identification. This approach is based on the assumption that even highly regulated genes are expressed to some extent in the undifferentiated cells of the explant. Productive fusion events render plant cells resistant to kanamycin and are thus selected (Datla *et al* 1997). Selection agent kanamycin played a significant role in recovering transformed shoots. Some of the control explants did not bleach completely, but shoot bud formation was suppressed to a maximum possible extent. However, shoots in which promoterless gene inserted downstream to weak promoters showing minimal activity can also be recovered by culturing them on medium without any selective agent. Apparent chlorosis of some of these shoots enabled us to separate these shoots from normal green shoots and thereafter maintained on media free of kanamycin. Some of the chlorotic shoots later cultured on media free of kanamycin were GUS-positive showing expression in the floral parts that subsequently confirmed Southern positive.

GUS analysis revealed a wide variety of expression patterns. The GUS data of independent transformants is in accordance with transient GUS expression profile. The total number of GUS hits was more in the randomly stained leaf parts than in the other shoot parts. Deeply stained GUS positive sectors on explants among the cultures were more in number, which indicates that the shoots arising from those areas could be transformed. Our studies revealed that among the 141 T_0 plants analysed so far, expression of GUS in leaves was more frequent compared to that in root, stem, and flower ($P \leq 0.05$), the hypothesis tested being that all plants expressing in leaf do express in root or stem also. Lindsey *et al* (1993) reported high frequency *uid*A expression in roots of tobacco plants when compared to other plant parts. The possible reason for which integration is more in leaves might be due to the occurrence of more number of recombinational hot spots forming loci close to genes that are active within leaf tissues. Foreign gene integration into the transcriptionally active chromatin of dividing meristematic leaf cells may enhance potential reporter gene expression.

The different patterns of GUS expression indicate that different regulatory sequences were tagged in each plant. The results indicate that the type of promoter that gets hooked onto the fusion gene influenced transient expression of the GUS gene. Intense blue staining observed was more in young compared to older leaves. Chi-square analysis and segregation ratio's for the introduced genes indicates that GUS characters are following 3:1 ratio as $P \leq 0.05$. Hence, the hypothesis tested being that this segregation pattern due to chance is low and the distribution is significant. The plants, in which T_1 progeny followed Mendelian inheritance pattern, were found to have single copy of exogenous gene. The GUS expression segregated in non-Mendelian fashion in the progeny of the plant C-43 accounting for the 2 copies of the integrated transgene.

In plants transformed with constructs having constitutive promoters, a negative correlation can be observed between copy number and the quantum of expression due to homology-dependent transcriptional silencing. In case of plants transformed with promoterless construct, no correlation between low expression and elevated copy number could be observed and the range of GUS expression patterns and the T-DNA copy number cannot be interlinked (Datla *et al* 1993; Lindsey *et al* 1993). The level of expression and specificity also varied among different plants having similar copy number and this might depend on the type of the upstream plant regulatory sequence that gets hooked on to promoterless fusion gene (Datla *et al* 1991). For example, in plant C-43a GUS expression was confined more to leaves, but it was shown to have 2 copies of fusion gene. The thick hybridization signal observed at size 4.4 kb in plant C-43a might correspond to two copies, and this plant needs to be studied further. In contrast, GUS staining in leaf lamina and root tip was noticed in plant C-53 showing single copy insertion. Plant C-1, which had single T-DNA copy showed more restricted GUS expression pattern than did plants C-4, C-53, C-17, which also had single copy of integrated GUS gene indicating that the tagged regulatory sequences may have varied activities. The genes identified by techniques such as cDNA library screening represent those that are more transcriptionally active, producing more abundant or stable mRNAs (Lindsey *et al* 1993). Some of the promoter tagged plants in which fusion gene inserted downstream to a tissue-specific promoters were selected for further promoter analysis.

To the best of our knowledge, this is the first report of efforts aimed at tagging regulatory elements in peanut, which is a crop of worldwide importance. Selected promoter tagged plants are being analysed at present to clone the upstream unknown sequences flanking the T-DNA region. This has implications for the spectrum of promoters that will be discovered by this method. Our future plan of work focuses on the reintroduction of the isolated regulatory

elements into peanut and other heterologous systems to test the specificity of the tagged promoter sequences. Thus, this approach offers new opportunities to generate a bank of tissue-specific promoters for potential applications in the field of plant genetic engineering for improvement of agronomic traits.

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