INHERITED ALTERATION UTILIZING THE COTYLEDONARY NODE OF PEANUTS (*ARACHIS HYPOGAEA* L.) AS A TRANSPLANT AND A PROMOTER-LESS *GUS::EPSPS* FUSION GENE-DERIVED VECTOR

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Abstract. This study focused on *Agrobacterium-mediated* transformation to develop potential transgenic lines of *Arachis hypogaea* L. using the promoterless *GUS::EPSPS* β-glucuronidase*::*5-Enolpyruvylshikimate 3-phosphate synthase gene and cotyledonary node (CN) explants. The most effective medium for inducing multiple shoot buds was Murashige and Skoog (MS) medium containing 0.4 mg/L BAP (6-benzylaminopurine) and 0.1 mg/L NAA (α-naphthaleneacetic acid). Investigations were conducted on parameters increasing genetic transformation, such as seedling age, co-cultivation periods, and *Agrobacterium* genetic background. Co-cultivating 6-day-old seedlings with *Agrobacterium* strain *GV3101* for three days resulted in a 45% induction of kanamycin-resistant shoots, and *GUS* assays confirmed a transformation frequency of approximately 31%. Using the promoterless *GUS::PxCSGm*-YFP::Yellow Fluorescence Protein *EPSPS* construct, 38% of transient *GUS* expression was observed. A total of 151 T0 seedlings were generated, of which 92 survived in the greenhouse, and five plants (4.54%) exhibited stable gene integration as confirmed by Southern blot, PCR, and *GUS* assays. *GUS-positive* expression was observed in 24 plants, with Mendelian segregation patterns identified in 15 T_0 progenies. The present study identified that the variable *GUS* expression patterns were discovered in the transgenic lines and demonstrates the successful introduction of the *GUS::EPSPS* gene without a promoter, offering a promising approach for peanut genetic engineering and herbicide resistance.
Keywords: *cotyledonary node, Agrobacterium tumefaciens, peanut, promoter-tagged lines, GUS::EPSPS bi-functional fusion gene*

Abbreviations: *GUS*: β-glucuronidase, EPSPS: 5-Enolpyruvylshikimate 3-phosphate synthase, CN: cotyledonary node, BAP," "6-benzyl amino-purine, NAA α-napthaleneacetic acid, *NPT-II*, neomycin phosphotransferase-II, *PxCSGm-YFP*, Yellow Fluorescence Protein, PCR, Polymerase Chain Reaction, .cv, cultivars, kinetin, KN, and 2,4-dichloro-phenoxy acetic acid (2,4-D), Murashige and Skoog, MS, Root induction medium, RIM, SEM, Stem Elongation Medium

Introduction

Besides cereals, legumes represent one of the most considerable classes of crop plants (Sharma et al., 2023). Among these, peanuts are a significant cash crop for growers, especially in the tropical regions that are semi-arid (Puli et al., 2021). However, due to their inherent confines in *in vitro* regeneration through conventional tissue culture methods, extensive research has focused on developing proficient and reproducible regeneration systems (Sehaole et al., 2022). The challenges associated with regenerating legumes via tissue culture have prompted the refinement of protocols, particularly in optimizing explant selection, hormonal balance, and

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environmental conditions, to overcome recalcitrance and improve regeneration success rates. These advancements are critical for both biotechnological applications and genetic improvement of legume crops. Due to their limited genetic variety and limited genetic foundation, Asian land species of peanuts are not resistant to numerous illnesses and pests. These may result in significant losses both in terms of quantity and quality. These issues can be resolved by genetic transformation, which makes it possible to transfer genes from alien sources to create transgenic plants that are resistant to both biotic and abiotic stressors. In plant transgene technology, increasing transformation frequency continues to be the most crucial component (Gheysen et al., 1998). Tissue-specific promoters are necessary for genetically modifying plants because, in many situations, targeted transgene expression is required to prevent undesired ectopic expression with constitutive promoters that are commonly utilized (Bailey, 1995); Fobert et al., 1994). Promoters provide a basic control over gene expression, and there is intense global a desire to recognize and define plant promoters (Datla et al., 1997). In the current investigation, a promoterless-*GUS*::*npt-II* bi-functional fusion gene is used as a promoter tagging strategy (Datla et al.,1991). T-DNA insertional mutagenesis has been used in the past to pinpoint plant promoters in a range of plants, including Arabidopsis (Koncz et al., 1989; Kertbundit et al., 1991; Topping et al., 1994), tobacco (Andre et al., 1986), and potatoes (Lindsey et al., 1993). For tagging, vector constructs with either a marker gene devoid of promoter elements or a reporter gene have been utilized (Teeri et al., 1986**)**. Plant regulatory sequences and promoters can be effectively isolated and characterized using T-DNA insertional mutagenesis (Walden, 2002). A comparable tagging strategy in tobacco that makes use of a promoter-less, the fusion gene study of *lacZ::nptII* was performed *(*Suntio and Teeri, 1994*)*. However, the intricacy of the *lacZ::nptII* fusion gene made expression analysis challenging. Promoter tagged lines were recently created using explants of hypocotyls and a promoter-free *GUS*::*nptII* fusion gene. Plasmid rescue was utilized to retrieve genomic regions preceding this construct, as reported by Bade et al. (2003). *Medicago truncatula*, a model legume used in T-DNA insertional mutagenesis experiments, showed that multiple transgenic lines with indicated genes and *GUS* fusions that occurred in vivo were also achieved in forage legumes (Scholte et al., 2002). The legume *Lotus japonicus* was used as a test subject for a promoter tagging technique to find genes found in plants that fix nitrogen (Webb et al., 2000). To produce T-DNA tagged lines, this calls for a high-frequency transformation procedure. Scientists have developed alternative transformation technologies that aim to affect the cotyledonary nodes' axillary meristems since many legumes are resistant to plant regeneration and tissue culture response (Somers et al., 2003). Here we report, employing the cotyledonary node (CN) as an explant, the first approach of genetic transformation in peanuts mediated by *Agrobacterium* tumefaciens. The primary goal of this work is to fuse transcriptional genes in the plant genome between the upstream promoter elements and the downstream fusion gene, which makes it possible to quantify the expression of the reporter gene. With a promoter-less *GUS*::*EPSPS* fusion vector with CN as an explant, we were able to create lines that were T-DNA tagged and showed different patterns of glucuronidase (*GUS*) expression. In several of these plants, using Southern analysis and polymerase chain reaction (PCR), the integration was verified. *GUS* and PCR tests verified the T_1 generations, inheriting the bi-functional fusion gene *GUS::EPSPS*, which is segregated according to the Mendelian pattern.

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Materials and methods

Plant material and explant preparation

The matured seeds of peanut (*Arachis hypogaea* L.) cv, Potohar and Bard-479 They were rinsed for 1 min in 70% (v/v) ethanol and 1 min in aqueous mercuric chloride at 0.1% (w/v) to surface sterilize them for 20 min. Several washes with sterile double-distilled water followed next on sterile filter paper wicks submerged in double-distilled sterile water. After that, the seeds were manually germinated and spent four to six weeks submerged in sterile water. In order to evaluate how explant age affects the frequency of transformations, explants were generated from seedlings that were 1-6 days old in the initial tests. The ensuing trials employed six-day-old seedlings. The longitudinal plane was used to bisect the embryo axis, remove the radical and seed coat, and excise the cotyledonary nodes by making incisions approximately 2 mm in both directions from the nodal region. A sterile surgical blade made six to eight diagonal shallow cuts in the meridional region of the nodal region, macerating the area. The shoot-induction medium (SIM) was used to in plant the nodes of the cotyledons, resulting in indirect contact between the medium and the cotyledon's adaxial surface as well as the wounded meridional nodal area. For every seed, two explants were produced. CN is the new designation for the explant. According to Townsend and Thomas (1993), the explanting procedure has been altered.

Regeneration

Testing different combinations of growth regulators, such as 6-benzyl amino purine (BAP), naphthalene acetic acid (NAA), kinetin (KN), and 2,4-dichloro-phenoxy acetic acid (2,4-D), in addition to basal salts (Murashige and Skoog, 1962), prepared with 3% sucrose as a carbon source and a pH of 5.6-7.8 before autoclaving, allowed for the standardization of regeneration using CN transplantation. Explants were grown on solidified SIM agar (0.8%) added BAP of 0.4 mg/l and NAA of 0.1 mg/l as supplements. to produce the greatest number of shoots possible for each explant. The cultures were kept at a consistent 28 ± 1°C and exposed to a light/dark cycle of 16/8 h. Approximately 1600 lux of cool white fluorescent lighting was used to provide the light. To induce adventitious shoot buds, 15-day intervals of SIM subcultures were performed, lasting two weeks apiece. SEM (shot elongation medium) containing 0.1 mg/l NAA and 2 mg/l BAP was introduced to explants that were yielding multiple shoots. The inter-nodal region of elongated shoots was clipped, MS basal salts and NAA at 0.8 mg/l were added to 5% agar gel (Sigma, USA) to solidify the root induction medium (RIM) as shown in *Figure 1*.

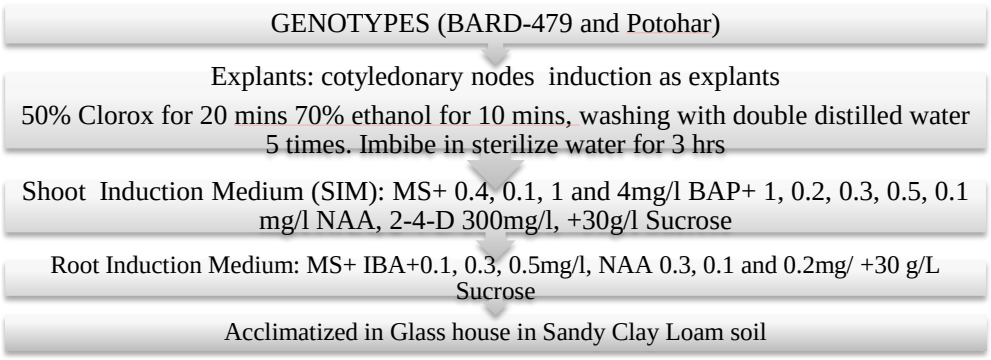

Figure 1. *Tissue culture procedure*

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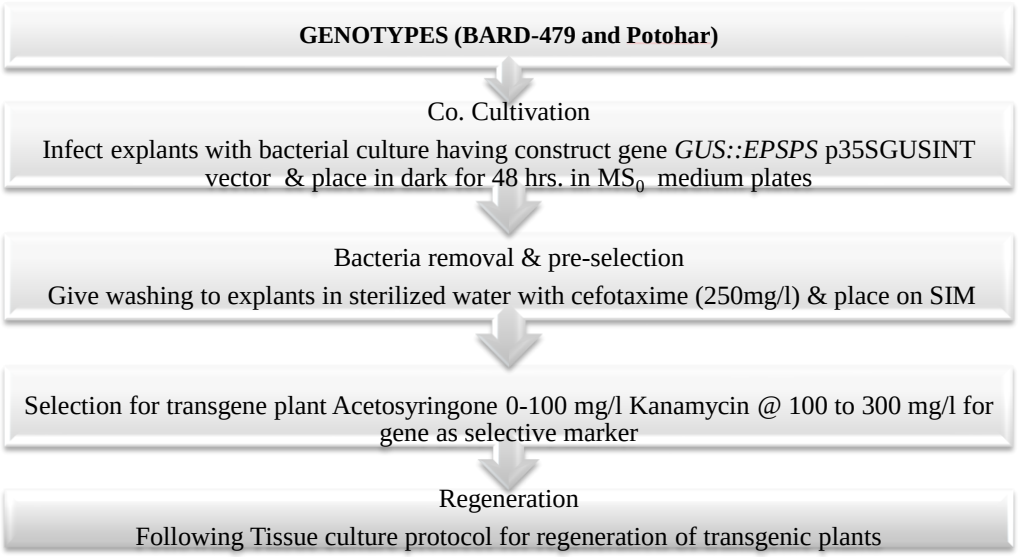

Figure 2. *Transformation procedure*

Agrobacterium strain and plasmid construct

A synthetic gene called *EPSPS*, or *GUS::EPSPS* bi-functional fusion gene, is included in a binary p35SGUSINT vector variant containing a translational enhancer of the cauliflower mosaic virus (CMV) (Datla et al., 1993). *Agrobacterium tumefaciens* strain *GV3101*, which is resistant to carbenicillin and rifampicin, was utilized to transform peanuts using *NOSTER*, or the 5′end has distinct restriction sites, the right border (RB), the left border (LB), and the terminator of Nopaline synthase. On frozen Luria agar plates using 100 mg/l of carbenicillin added as a supplement, 50 mg/l of kanamycin mono sulphate and 100 mg/l of rifampicin, the strain was kept alive. Following the inoculation of one *Agrobacterium* colony with the appropriate antibiotics in Luria broth, a fresh overnight culture was generated. Before infection, the culture was centrifuged at 8000 rpm for 10 min, reconstituted in double-distilled sterile water, and chilled for 2 h at 4°C. Initially, to maximize the transformation conditions, a binary vector called p35SGUSINT (Vancanneyt et al., 1990) was employed.

Transformation

After being dipped by their bacterial suspension proximal cut ends, cut-fresh CN explants were placed in 15 mm sterile media in petri dishes at 90°C and incubated for 5–10 min. For 72 h, they were co-cultivated on SIM at 25 ± 2°C with a 16/8 photoperiod. Following four days of recuperation on SIM supplemented explants were given 250 mg/l of cefotaxime, moved to a fresh SIM that has 175 mg/l of kanamycin and 250 mg/l of cefotaxime mono-sulphate (Sigma, USA). Two selections, each lasting two weeks, of cultures on SEM (Stem Elongation Medium) that had fully green branches at a dose of 175 mg/l. After being transferred to a kanamycin-free medium, cultures containing mixed green and white shoots were kept on MS media without the use of any selection agent. After being cut, to stimulate roots, two internoded shoots were transferred to RIM. Fully developed roots on the plants were transferred to plastic containers filled with a 1:1 mixture of autoclaved soil and vermiculite, and they were hardened for two weeks in these conditions before being moved to a greenhouse. They

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were afterwards moved to larger pots and given time in the greenhouse to blossom and set seed as shown in *Figure 2.*

Selection

In order to select transformed shoots, an effective kanamycin concentration was first established by cultivating kanamycin was applied in several concentrations (100, 150, 200, 250 and 300 mg/l) along with acetosyringone with different concentrations (0, 40, 60, 80 and 100 mg/l) to untransformed CN explants on SIM. The same amount of antibiotic was added to fresh SIM for two subcultures, which were then assessed for how many shoots there are per explant and the regeneration percentage. After recovering from non-transformed explants, control shoots, measuring 2-4 centimeters were added to RIM enriched with different amounts of kanamycin (100, 150, 200, 250 and 300 mg/l) to ascertain the kanamycin concentration that prevented root formation.

GUS analysis

By staining the variable like leaflets, roots, flowers, and shoots, more than 151 potential T0 plants that have been examined thus far, phenotypic *GUS* expression was ascertained. To perform the *GUS* assay. The plant tissues that were extracted from the potential transformants were kept within a buffer containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl α-D-glucuronic acid, Biosyntheses, Switzerland). Ten percent methanol, Triton X-100 at 0.1%, potassium ferricyanide at 0.5 mM, and sodium phosphate at 100 mM (pH 7.2) (Jefferson et al., 1987), after soaking in 70% ethanol for the entire night staining tissues showed no chlorophyll at 37°C. The offspring of 15 T_0 plants were also used in *GUS* experiments and staining juvenile leaflets and shoot portions that were randomly selected from the cultures, to study *GUS* fusions in vivo transient *GUS* studies were carried out in triplicate.

PCR analysis and DNA isolation

Using the conventional CTAB approach, from immature leaves of putative transformants and control plants, complete genomic DNA was extracted (Begum et al., 2014). The plants were shade-grown for two days before their leaves were harvested. Using particular primers for the *EPSPS* and *GUS* genes, a PCR was run to check for the fusion gene's existence in T1 generation plants and presumed T_0 transformants.2.5 units of Taq DNA polymerase that is recombinant (Invitrogen Corporation, USA) with a single genomic DNA ng of 100 were used as templates in the PCR experiments. *EPSPS* F5′-GAGGCTATTCGGCTATGACTG-3′ and *EPSPS*R 5′-ATCGGGAGCGGCGATACGTA-3′, 21-mer oligonucleotide primers, were employed to amplify the *EPSPS* fragment's 1371 bp. The cycling parameters included an 8-minute initial denaturation at 94°C, then 37 cycles: 94°C for 1 min, 60°C for forty seconds, and 72°C for 1 min. The last extension at 72°C lasted for 10 min. The primers for the 22-mer oligonucleotide *GUS*R 5′CTTCTCTGCCGTTTCCAAATCG-3′ and *GUS*F 5′-TACCTCGCATTACCATTACGCG-3′ were used the 285 bp *GUS* fragment was amplified. Similar cycling conditions were used, with 63°C for 55 s. Ethidium bromide was used to visualize the amplified products after they had been electrophoresed on 1.8% agarose gels. Following their transfer by Southern blotting to Hydrogen bond N+, the agarose gel resolution of the fragments revealed that were combined with *GUS* fragments obtained from PCR amplified plasmids tagged using α–32P.

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Southern hybridization

With the help of *Hind-III*, T_1 plant was complete digested of 15 μg of genomic DNA independently, avoiding cutting within the T-DNA region. Utilizing a capillary method and a transfer buffer of 20X SSC, the restriction fragments were placed on a Hydrogen bond N + membrane via blotting (Amersham Pharmacia, UK) following their resolution using 0.8% agarose gel electrophoresis. To probe the membranes, a 1371-bp *EPSPS* fragment produced from the *GUS::EPSPS* gene plasmid, tagged with α-32P dATP. The membranes were washed at 65°C for 20 min in 1X, 2X, SSC, 0.1% SDS and finally for 10 min in 0.1% SDS with 0.1X SSC after being hybridized for 16 h at 65°C. After washing, the membranes underwent autoradiography after being wrapped in saran wrap (Sambrook et al., 1989).

Statistical analysis

Box-plot was performed in R (4.3.2) using "ggbiplot" package. While, in box-plots T test were employed to understand the significance for all studied traits between the treated and non-treated plants. Analysis of variance (ANOVA) was performed in SPSS (https://www.ibm.com/products/spss-statistics) software to estimate the variable response of genotypes against the selection treatments. ANOVA also observed the impact of different concentration of selection treatments. Bar charts were made in excel with standard error, whereas, lettering on bar plots showed the individual statistical difference based on Tukey test. Tukey test was performed in SPSS to understand the impact of individual treatments on selection efficiency.

Results

Regeneration

Explants of cotyledonary nodes were acquired from seedlings ranging in age from one to eight days were cultivated on diverse conditions using varying combinations of growth regulators to induce numerous shoots (information not provided) (Dhuha et al., 2023). Seedlings that were six days old demonstrated good transformation and regeneration responses. The BAP and NAA-supplemented MS medium exhibited the maximum regeneration percentage and average shoot count. High frequency regeneration was achieved across the several BAP and NAA combinations examined after explants were cultivated in MS medium enriched with NAA at 0.1, 0.2 and 0.5 mg/l and BAP at 2, 4, and 5 mg/l as shown in *Tables 1* and *2*.

Table 1. *Cotyledonary node in Bard 479*

Replication	400 mg/L BAP & 200 mg/ l NAA		400 ml/l BAP & 100 mg/L NAA		400 mg/L BAP & 500 mg/L NAA		200 mg/L BAP &100 mg/ l NAA		200 mg/L BAP& NAA 200 mg/L		200 mg/L BAP& NAA 500 mg/L	
	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq
1	22	88	20	80	7	28	4	16	2	8	0	0
2	23	92	21	84	7	28	5	20	1	4	1	4
3	20	80	21	84	5	20	1	4	1	4	0	0
4	22	88	20	80	4	16	4	16	2	8	1	4
Total	87		82		23		14		6		2	

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Table 2. *Cotyledonary node in Potohar*

Replication	400 mg/L BAP & 100 mg/LNAA		400 mg/L BAP & 200 mg/ l NAA		400 mg/L BAP & 500 mg/LNAA		200 mg/L BAP & 100 mg/ l NAA		200 mg/L BAP & NAA 200 mg/L		200 mg/L BAP & NAA 500 mg/L	
	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq	Number of plants	Freq
1	16	64	16	64	6	24	3	12	2	8	0	0
2	19	76	15	60	6	24	2	8	0	0	1	4
3	17	68	16	64	5	20	3	12	3	12	2	8
4	23	92	17	68	2	8	3	12	3	12	1	4
Total	75		64		19		11		8		4	

Morphological parameters including Plant height, leaf length, Leaf width, Plants tiller, Flowers plant, Chlorophyll content, 100 seed weight grams, Fruit size cm (*Figure 3*).

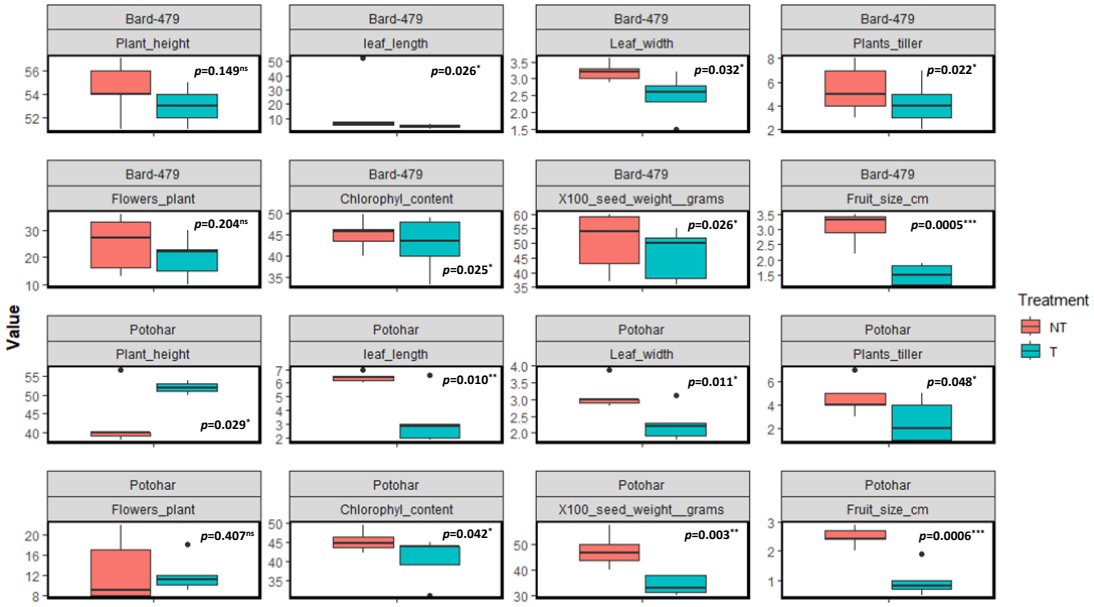

Figure 3. *Box plots showing morphological variable patterns between treated and non-treated plant of Bard-479 and Potohar. The box contains the middle 50% of the data, and the median is indicated by a line. The whiskers reach the minimum and maximum values. Dots outside the whisker showed > 90 coefficient of interval. T test results indicate the significance level between the treatments. Red color highlights NT (non treated), whereas blue color shows the T (treated)*

The best results for multiple shoot bud development were obtained with BAP at 4 mg/l and NAA at 0.1 mg/l. The majority of the shoot buds per explant (82% regeneration, 28 ± 1.5) and it was discovered that there were often more shoot buds while the cotyledon's adaxial side was in close touch 5.8% regeneration, 8.75 ± 2.4, with the medium compared to the abaxial side. After 15 days of culture, elongated shoots grown on RIM formed adventitious roots. From each explant, averages of six plants were retrieved.

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Agrobacterium genetic transformation

Using the binary vector p35SGUSINT, the evaluation of both the consistent transformation effectiveness and the transient *GUS* frequency involved examining a variety of characteristics that increase genetic transformation. The ensuing experiments were conducted under the optimized circumstances that were identified. The age of the seedlings, strains of *Agrobacterium* and co-cultivation times were among the parameters that were examined. Each leaf and shoot section's *GUS* positive sector and overall *GUS* spot count were given a score. On many plant parts, including leaves, roots, and stems, the highly stained blue areas are known as *GUS* positive sectors. By the time the first subculture ended, the proportion of kanamycin-resistant shoots was calculated using the ratio of shoots growing on kanamycin-containing medium to all explants grown. The transitory transformation frequency, which measures the success of the transformation, the percentage of explants with at least one distinguishable dark blue *GUS* positive sector, or *GUS* spot, was calculated 72 h after co-cultivation. In contrast, the proportion of altered shoots that test positive for Southern analysis, PCR, and *GUS* staining is known as the stable transformation efficiency (Egnin et al., 1998). At least three trials were conducted for each element examined, and each experiment included a minimum of twenty explants. These trials examined transitory transformation frequencies and the proportion of shoots induction resistant to kanamycin.

Impact of the explant age

Two transformation experiments were conducted to ascertain the proportion of shoot regeneration resistant to kanamycin and temporary *GUS* expression in cotyledonary nodes derived from seedlings ranging in age from one to eight days. In comparison to 6-day-old CN explants, compared to CN explants that were 1, 2, and 3 days old with reduced *GUS* staining or transient transformation frequency percentage during co-cultivation. The meristematic cell surface area may be greater in CN explants that were 6 days old; it promotes the efficient transfer of T-DNA. After co-cultivation, the 5- and 6-day-old explants had nearly the same transitory *GUS*%, but the latter was chosen for further testing since the reaction of shoot regeneration differed. Possibly, because of the explant cells were actively dividing during the first five to six days of development, while the eight-day-old CN explants' low *EPSPS* and lack of regeneration response may have resulted from the primary meristems' continued differentiation and the scarcity of cells that are actively dividing.

Impact of the genetic background of agrobacterium

Using disarmed *Agrobacterium* strains, *GV3101*, the transient *GUS* percentage was examined. The strain *GV3101* cultures exhibited a greater number of dark blue *GUS* positive sectors (information not given).

Impact of the co-cultivation time

The percentage of kanamycin-resistant shoot regeneration was higher when *GV3101* was co-cultivated with 6-day-old explants for 1, 2, and 3 days, and the results showed a gradual reduction in two to three days. The percentage of *GUS* staining in transit rose significantly after explants were co-cultivated for one to three days Explants necroses when the co-cultivation time was longer than three days. Due to less *Agrobacterium*

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damage during co-cultivation, the 1- and 2-day-olds' high shot induction percentage explants grown in co-culture is probably the result. Based on transitory *GUS* staining, using p35SGUSINT, a transformation frequency of about 31% was noted. The information gathered thus far was done so using transient *GUS* analysis (Begum et al., 2022).

Selection

To determine the ideal concentration of kanamycin with acetosyringone to select transformed shoots, we also cultured the control, SIM-based uninfected CN explants that were dose-dependently exposed to kanamycin at concentrations of 100, 150, 200, 250 and 300 mg/l and acetosyringone at concentrations 0, 40, 60, 80 and 100 mg/l as shown in (*Figure 4)*. The optimal dose for kanamycin-resistant shoot induction declined as kanamycin concentration rose, with 100 mg/l being the lowest (data not shown). The regeneration sharply dropped and chlorotic shoots occurred at 100 mg/l kanamycin concentrations. Therefore, this concentration was used to identify potential transformants. Explants at aceto100 and 300 mg/l kanamycin demonstrated full reductions in necrosis and shoot induction. The induction of roots from control shoots was completely inhibited by a 100 mg/l of kanamycin (*Table 3*). It is commonly recognized that Kanamycin is more likely to affect root induction than shoot organogenesis, which helps to explain why the effectiveness of any antibiotic relies on the type of plant being treated in addition to the particular explant (Saini et al., 2003).

Table 3. *Impact of different plant selection treatments on different genotypes*

SOV	DF	Aceto		Kanamycine	
		MS	F	MS	F
Genotype	1	353.63^{ns}	3.88	97.20^{ns}	2.00
Treatment	4	4644.0^{***}	49.78	8489.37^{***}	172.84
Geno. × Treatment	4	93.133^{***}	31.75	50.53^{***}	23.15

Regeneration of transformants

Transient *GUS* analysis revealed a greater frequency of *GUS* positive sectors and a greater number of kanamycin-resistant shoots in 6-day-old CN explants grown in conjunction with *GV3101* harboring p35SGUSINT following a three-day genetic transformation. Using a promoter-less *GUS::EPSPS* bi-functional fusion gene construct, further transformation studies were carried out according to the previously studied parameters. After two selections, explants were cultivated on SIM in addition to 300 mg/l kanamycin and Acetosyringone 100 mg/l; thereafter, some of the cultures were kept on a medium with no selection agent. This was done after co-cultivation and a brief recuperation interval. To investigate the transitory *GUS* expression a variety of randomly chosen tissues from the cultures those were dyed with X-Gluc. The GUS expression patterns displayed by in vivo GUS fusions are diverse and include brown calli, and GUS positive areas on shoot segments cut leaf regions, leaf midribs with a strong blue staining and GUS patches on different plant parts. The highest percentage (38%) of the overall transitory GUS percentage was found to be occupied by the leaf segments that are GUS positive. It was discovered that there were fewer GUS-positive sectors overall on brown calli and shoot portions than there

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were on leaves and petioles. None of the studies' untransformed shoots' tissues showed any staining. These findings imply that the only reason for the GUS expression seen was because downstream of the vector's *GUS::EPSPS* fusion gene, which lacks a promoter, successfully underwent between transcription and the plant genome's upstream regulatory regions. Though it does not always imply tissue-specific expression, the presence of GUS spots on leaves suggests that a portion of the shoots were chimera. Using the promoterless design, we produced over 151 putative T_0 seedlings and moved them into a greenhouse. Out of these, 92 plants fared well in the greenhouse, and five plants, or 4.54% of the total, had stable fusion gene integration, as shown by Southern, PCR, and GUS tests (*Figure 5*). 33 shoots total rooted nicely on RIM when given kanamycin. From a phenotypic standpoint, these plants resembled ordinary control plants.

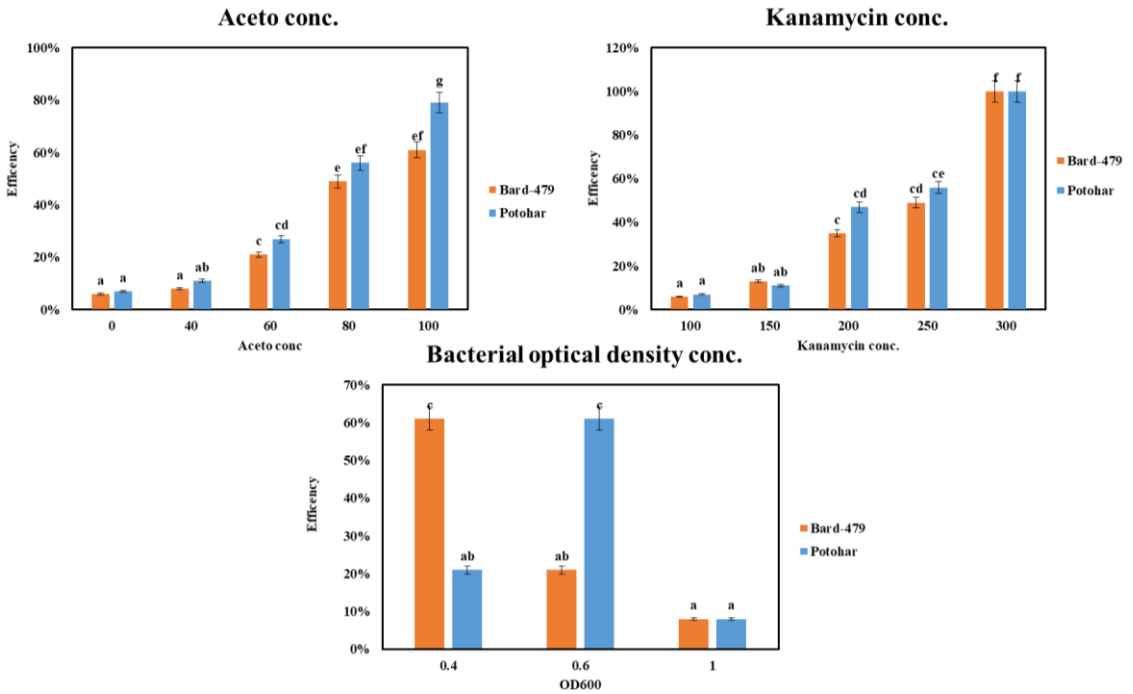

Figure 4. *Impact of concentrations of bacterial optical density, kanamycin, and aceto (acetosyringone) on both cultivars Bard-479 and potohar transformation efficiency. Bar represent mean SE. Different letters indicate significant differences ($p < 0.05$). Different letters indicate significant differences between treatments. Same letters indicate no significant difference. Red color represents Bard-479 and blue color shows Potohar genotype*

GUS expression in individual transgenic plants

More than 151 T_0 putative transformants' parts of shoots, roots, leaves, and floral organs were used in *GUS* experiments to examine the patterns of *EPSPS* gene expression in diverse plant tissues. As a consequence of showing blue dot expression localized to a tissue in at least one part of the plant, 24 plants tested positive for the *GUS* test. Out of 24, three possible transformants were determined to be *GUS*-positive and to have varied patterns of *EPSPS* expression in flowers, according to *GUS* research. None of the other plant parts that were examined showed any discernible *GUS* staining in these plants. Compared to the roots (41.66%; 10/24) of the putative transformants,

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the leaves showed a relatively high frequency of *GUS* staining (75%; 18/24). Out of these, only 4 out of 26 transformants (16.66%) showed *GUS* expression in the shoot areas. The Southern hybridization, PCR, and *GUS* results of plants based on RIM that had been supplemented with kanamycin were shown to be positive. Many plant components that were examined for *GUS* had faint blue coloration. The C-53 plant's leaves have deep blue sections. The blue dots seen in plants B-23, B-27, B-28, P-31, P-33, and P-52 can be regarded as artifacts because these plants the subsequent assays showed no signs of *GUS* staining. The cultures used to create these plants were initially maintained on SIM enhanced with a maximum of two choices of 175 mg/l kanamycin shown in (*Figure 4)*. Following this, they were transferred to a medium that was devoid of any form of selective agent. A few of these plants that test positive for *GUS* may be presumptive lines marked with promoters. These findings suggest that a wide variety of plant sections exhibit high frequency production of a promoter-less *GUS::EPSPS* gene inside a group of transformed plants (Rajyaguru et al., 2024).

Segregation study of the progeny's EPSPS expression

The T_1 generation's fusion gene's inheritance pattern must be described to understand how long-lasting foreign gene integrated.

Examining the fusion gene's potential segregation pattern, the offspring of fifteen *GUS*-positive plants were examined. A minimum population size of 12 is required in order to conduct the Chi-square segregation test. Only three of the samples met the minimal population size requirement, and they demonstrated an excellent suitable for monogenic segregation, suggesting incorporation of a single copy. Nevertheless, there were no Southern data available for any of these three lines of each variety. Single-copy integration was demonstrated by the data, which fit the monohybrid ratio. However, a southern analysis revealed that plant number B-43 possesses T-DNA in two copies. The progeny of plants B-12 and P-17 were seen to exhibit a predominance of expression specific to their roots and leaves. Both the parent and offspring plants had tissue-specific *GUS* expression patterns that were largely comparable. PCR was used to confirm that the progenies' *GUS* and *EPSPS* gene segregation.

Southern hybridization and PCR analysis

Approximately 10 T_0 putative transformants had the fusion gene detected by PCR with *EPSPS* gene-specific primers. The PCR reaction's amplicon integrity was confirmed by probing the findings with a radio-labeled *GUS* fragment and transferring them onto nylon membranes for Southern hybridization. This experiment confirmed that the transgenic plants were designated as T_0. The Southern PCR results from the twelve T_0 plants are displayed in (*Figure 8)*. To determine the integrated fusion gene's inheritance pattern, PCR screening was done on T_1 progenies. The amplification of the *EPSPS* and *nptII* genes in the progeny of five T_0 plants verified by PCR is displayed in (*Figure 7A)*. The results of the examination of southern hybridization showed the number of copies and integration of the nptII gene in the progeny of the five T0 plants that were examined. When a *GUS* or *nptII* fragment is used to probe the genomic DNA of T_1 plants carrying *Hind-III*, which does not make internal cuts in the pRD400*GUS* fusion gene's T-DNA region, distinct restriction patterns should be obtained; the copy number and band count should coincide. When the nptII fragment was used to probe the offspring of the five *GUS* or T_0 plants verified by PCR, a southern study showed

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distinct banding patterns. Plant C-43a had two copy insertions, whereas plants C-17a, C-4a, C-53a, and C-1a had independent single copy insertions (*Figure 6*). The fact that the hybridization bands surpassed T-DNA suggests that T-DNA has been incorporated into the plant genome and that plants originated as a consequence of a separate transformation event.

Figure 5. *Transformants observation using the GUS test. Due to the GUS histochemical analysis, the severed half embryo showed a blue hue. Bacterial proliferation shown under a stereo microscope (Olympus, Japan) as a result of high density of bacteria in the GUS-positive explants and co-cultivation media. Bard-479 and. Potohar*

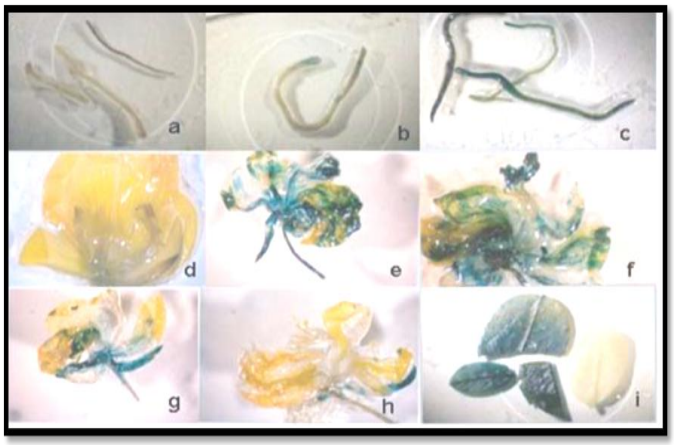

Figure 6. *(A) Within the synthetic T-DNA region, there is promoter-less GUS::EPSPS bi-functional fusion gene, the translational enhancer sequence of the cauliflower mosaic virus (CMV), the distinct restriction sites, the three boundaries are the Nopaline synthase terminator (NOSTER), the left border (LB), and the right border (RB). (B) A. hypogaea CV Bard-479 showed evidence of CN-driven transformation and regeneration mediated by Agrobacterium. (a) Simulated CN explants treated with 175 mg/l of kanamycin following three weeks of induction of numerous buds. (b) SIM cultures treated with 175 mg/l of kanamycin that had developed into fully green numerous shoots after six weeks. (c) Control cultures that were fully bleached on SIM using 175 mg/l of kanamycin. (d) Firmly anchored shot on BlackBerry. (e) Soil for plant hardening: a mixture of vermiculite. (f) Plant adaptation to the environment of a greenhouse. (C) Different T0 plants exhibited GUS expression in different plant parts. (a) Control roots Bard-479. (b) Expression of GUS at the root tip. (c) Expression of GUS in roots. (d) Control flower Bard-479. (e and f) GUS expression is seen throughout the C-1 plant's whole flower, exhibiting discolored floral parts. (g) Plant C-2's pedicel and keel petals stained with GUS. (h) Plant C-13 only expresses GUS on the keel petal. (i) Compared to the bleached control leaf, GUS expression was found in the leaves*

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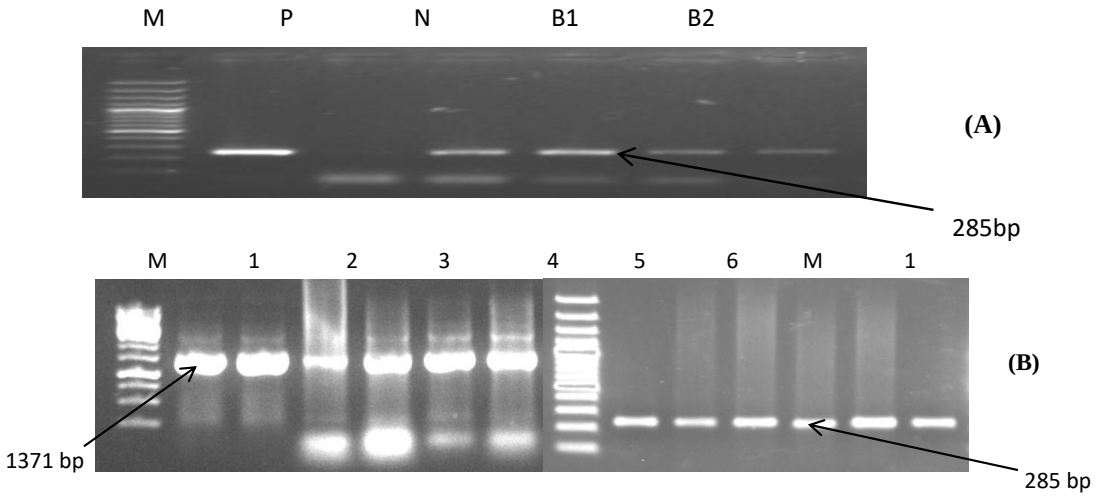

Figure 7. *(A) GUS is the probe for the T_0 transformants' PCR Southern. Lane 1. Marker means ladder 100 bp Lane 2 means P, indicates positive control the bi-functional fusion gene GUS::EPSPS serving as a constructive measure; Lane 3 N is the Negative control plant. The possible transformants for T_0 are displayed in Lanes 4 and 5 for Bard-479 and lane 6 and 7 for Potohar. (B) NptII (left) and EPSPS (right) primers are used to evaluate T_1 transformants. DNA marker at 1 kb in Lane 1 and lane 2, 3 and 4 for Bard-479 and lane 5, 6 and 7 for Potohar and Lane 8 Marker 100 bp and Lane 9, 10 and 11 for Bard-479 and 12, 13 and 14 for Potohar. Displays the DNA of the transformed plants*

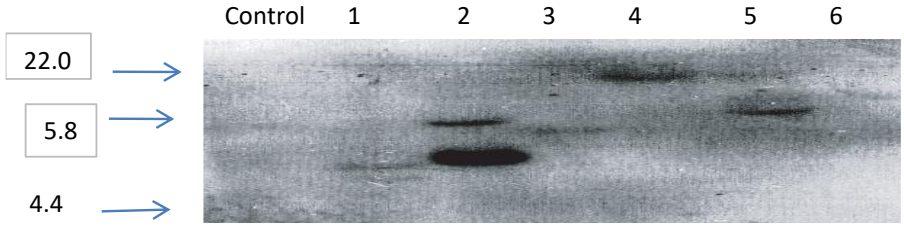

Figure 8. *T_1 transgenic peanut plants southern blot analysis to check for the EPSPS gene. Hind-III genomic DNA was broken down using an enzyme, and PCR-amplified EPSPS fragments from plasmid DNA were used to probe the DNA. Control plant, Lane 0. Plants transgenic T_1 in Lanes 1 through 6. Similar banding patterns were observed. Once the blot was stripped and the GUS fragment was used to re-probe it, showing that the fusion gene had been incorporated into the genomes of the transgenic plants (data not shown). These findings demonstrate that in the T_1 generation, the EPSPS and GUS genes co-integrate and co-segregate, respectively*

Discussion

Developing highly repeatable transformation techniques typically encounters a hurdle in plant regeneration. The efficiency of accidental branch development by organogenesis increases, if an appropriate explant is found. In the past, transgenic peanut plants were created using leaf-section explants and leaf discs; nevertheless, there was little use of these methods (Cheng et al., 1996; Eapen and George, 1994). Cotyledons were utilized as a source of explants in recent experiments to achieve an elevated transformation frequency in peanuts, the cultivar JL-24 in particular (Sharma and Anjaiah, 2000), as reported by Venkatachalam et al. (1998). On the other hand,

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compared to cotyledons and other explants documented thus far, there may be a relatively greater regeneration response from the cotyledonary nodes. Axillary meristematic cells close to where the embryonic and cotyledon axes converge are highly regenerable, making them promising candidates for gene delivery targets. In other grain legumes explored, such as soybeans (Olhoft et al., 2003) it has been demonstrated that seed maturity produces cotyledonary nodes are particularly receptive to produce transgenic plants and to induce the growth of many branches of pea (Bean et al., 1997), black gram (Saini et al., 2003), pigeon pea (Geetha et al., 1999), and mung bean (Jaiwal et al., 2001). Compared to other gene transfer methods, *Agrobacterium* -based genetic transformation produces a significant number of stable transgenic plants. The dual-purpose fusion gene promoter *GUS::nptII* tagging system confers *GUS* activity and kanamycin resistance (Datla et al., 1991). As previously demonstrated, the translational enhancer for AMV region increases the reporter gene's expression levels (Datla et al., 1993) and hence making it easier for weak promoters to recover. In addition to impairing normal gene function, downstream unforeseen introduction of a reporter gene lacking a promoter, to a promoter stimulates the reporter gene's production by successively entering the plant promoter up stream's transcriptional regulation. The introduced element serves as a marker to identify genes. This method is predicated on the idea that the explants undifferentiated cells express some level of expression for even highly regulated genes. Plant cells that undergo productive fusion events become resistant to kanamycin, which is why they have been selected (Datla et al., 1997). The recovery of altered shoots was significantly aided by the selection agent kanamycin. While certain control explants exhibited no bleaching entirely, the production of shoot buds was as much reduced as feasible. However, by cultivating the shoots on medium without the use of any selection agent, shoots with promoter-less genes placed, it is also possible to conserve weak promoters that show minimal action downstream. Some of these shoots appeared to be chlorotic, which allowed us to distinguish them from typical green shoot sand and keep them on kanamycin-free medium. A portion of the chlorotic shoots that were subsequently cultivated on kanamycin-free media demonstrated expression in the floral sections and were *GUS*-positive, which was later verified to be Southern-positive. Multiple expression patterns were found, according to *GUS* analysis. The transient *GUS* expression profile and the independent transformants' *GUS* data are consistent. Compared to the other shoot sections, there were higher *GUS* hits overall in the randomly stained leaf sections. There were more sections of explants in the cultures that are highly stained and positive for *GUS*, suggesting that the shoots emerging from those locations would transform. Our research showed that, of the 141 T_0 plants examined thus far, *GUS* expression in the leaves was more common than in the roots, stems, and flowers ($P \leq 0.05$). It was hypothesized that all plants expressing *GUS* in their leaves would also express it in the roots or stems. Lindsey et al. (1993) reported tobacco plants' roots have been shown to express uidA at a higher frequency than other plant components. The presence of more recombination loci formed by hot spots next to genes that are activated in leaf tissues may be the cause of the increased integration seen in leaves.

Potential reporter gene expression may be improved by incorporation of foreign genes into the proliferating meristematic leaf cells' transcriptionally active chromatin. The various *GUS* expression patterns show that each plant's sequences that regulate were identified differently. The findings suggest that the kind of promoter attached to the fusion gene had an impact on the *GUS* gene's transitory expression. Younger leaves

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showed more intense blue staining than older ones did. According to segmentation ratios with chi-square analysis $P \leq 0.05$ indicates that *GUS* features for the new genes follow a 3:1 ratio. As a result, the theory that claims this distribution is meaningful and the segregation pattern is low due to chance is evaluated. It was discovered that the plants with T_1 progeny that exhibited the Mendelian inheritance pattern only had the exogenous gene in one copy. The plant C-43's progeny has two copies of the incorporated transgene are explained by the non-Mendelian segregation of *GUS* expression. Because of homology-dependent transcriptional silencing, a negative association between the quantity of expression and the copy number in plants that have been modified using constructs that contain constitutive promoters. In plants, there is no connection between low expression and high copy number transformed using promoter-less constructs, and there is no relationship between the range of T-DNA copy number and *GUS* expression patterns (Lindsey et al., 1993; Datla et al., 1993). The kind of plant regulatory sequence upstream that attaches itself to the promoter-less fusion gene may be the cause of the variation in specificity and expression levels among plants with similar copy numbers (Datla et al., 1991).

For instance, *GUS* expression was mostly limited to the leaves of plant C-43a, despite the fact that two copies of the fusion gene were found. Plant C-43a needs more research since the size 4.4 kb has a thick hybridization signal may indicate two copies of the gene. Alternatively, *GUS* staining of the root tip and leaf lamina for plant C-53, a single copy insertion was found. Plant C-1 displayed a more restricted *GUS* expression pattern than plants C-17, C-53, and C-4, despite only a single copy of T-DNA, its integrated *GUS* gene was also present in a single copy. This suggests that different roles could be played by the labeled regulatory sequences. More transcriptionally active genes are identified using methods like cDNA library screening, which produces mRNAs that are more prevalent or stable (Lindsey et al., 1993). For additional promoter analysis, a few downstream-positioned fusion genes in promoter-tagged plants of tissue-specific promoters were chosen. As far as we are aware, this is the first publication on the endeavor to identify regulatory components in peanuts, a crop that is significant globally. Currently, analysis is being conducted on a subset of promoter-tagged plants to copy the unidentified sequences encircling the T-DNA region upstream. This has an impact on the range of supporters that this approach can find. To evaluate the tagged promoter sequences' specificity, the future work plan centers on reintroducing the isolated regulatory elements into heterologous systems, such as peanuts. In light of this, this strategy presents fresh chances to build a library of promoters particular to tissues for possible use in the area of agronomic feature enhancement using plant genetic engineering.

Conclusion

The protocol for *Agrobacterium-mediated* peanut transformation presented in this study demonstrates a highly efficient system for the regeneration of adventitious shoots from explants derived from cotyledonary node of two peanut genotypes. For both genotypes, *Agrobacterium-mediated* transformation of explants of cotyledonary resulted in superior regeneration and transformation efficiencies. Furthermore, the transformation and regeneration protocols outlined here are particularly effective for peanut genotypes that mature early and produce relatively small fruits, such as Bard-479 and Potohar. One critical enhancement to the protocol involved submerging target

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tissues in *Agrobacterium* solutions, which significantly improved transformation efficiency compared to submersion alone. The development of this efficient and reliable transformation system will be indispensable for the significant advancements expected in functional genomics studies, particularly given the increasing availability of the complete genome sequence of peanut. This protocol provides a valuable tool for future research and breeding efforts aimed at improving peanut genotypes.

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