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Agrobacterium-mediated genetic transformation method of groundnut (*Arachis hypogaea* L.) for functional studies and precision breeding

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Many groundnut varieties are known to be recalcitrant to *Agrobacterium*-mediated transformation. Establishing a successful method for *Agrobacterium*-mediated groundnut transformation is an essential first step toward developing new technologies for precision breeding. In this study, the potential for *Agrobacterium*-mediated gene transfer was evaluated in three groundnut varieties ICGV90704, ICGV12991, and JL24 using cotyledon explants and four *Agrobacterium* strains (AGL0, EHA105, C58, and LBA4404) harboring the plasmid vector pCAMBIA2301. Selection of transformed plants and assessment of regeneration potential were performed on media supplemented with 100 mg/L and 150 mg/L kanamycin. Expression of the β-glucuronidase (gusA) gene in regenerated plants was verified by histochemical staining. The presence and integration of the transgene in putative transformants were confirmed by polymerase chain reaction (PCR) and Southern blot analysis, respectively. Inoculation of explants with *Agrobacterium* strain AGL0 resulted in the regeneration of transgenic shoots in all three groundnut varieties. Variety had a significant effect ($p < 0.05$) on transformation efficiency. The highest transformation efficiency (1.33%) was obtained in variety ICGV90704 inoculated with *Agrobacterium tumefaciens* strains AGL0 and EHA105, and in variety JL24 inoculated with strain AGL0. Histochemical staining confirmed gusA expression in regenerated plants, while PCR and Southern blot analyses verified the presence and stable integration of the transgene. The transformation protocol established in this study could be used to investigate gene function and introduce important agronomic traits into groundnut varieties through modern biotechnological approaches, including genome editing.

Key words: *Agrobacterium*-mediated transformation, groundnut, cotyledon explants, *gus*A expression.

INTRODUCTION

Cultivated groundnut (*Arachis hypogaea* L.) is an important legume crop that provides food, edible vegetable oil, fodder, and household income (Song et al., 2023). Groundnut seeds contain 48–50% oil rich in omega-3 fatty acids, 26–28% protein, dietary fibre, essential minerals (Ca, P, Mg, Zn, and Fe), vitamins (B complex,

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E, and K), and beneficial phytochemicals, thereby playing a vital role in reducing malnutrition (King et al., 2008; Janila et al., 2013). Groundnut haulms also serve as nutrient-rich forage for livestock (Song et al., 2023). In addition, the crop improves soil fertility through biological nitrogen fixation (Nelwamondo et al., 2024), benefiting subsequent crops. Groundnuts are grown in more than 108 countries across Africa, Asia, and North and South America, with a total annual production of 53.93 million tons in 2021 (Food and Agriculture Organization Corporate Statistical Database [FAOSTAT], 2024). The majority of production occurs in Asia (55.7%) and Africa (33.9%), predominantly under rain-fed conditions (FAOSTAT, 2024).

However, groundnut productivity in Africa remains low at 0.94 t/ha, compared to 2.45 t/ha in Asia (Fulkah et al., 2024). In tropical and subtropical regions of Africa, yields continue to decline, with farmers achieving less than half of the crop's potential (FAOSTAT, 2024).

Several abiotic and biotic factors constrain groundnut production, reduce productivity, and negatively affect seed quality. Many African groundnut germplasm lines are highly susceptible to groundnut rosette disease (GRD), root-knot nematode (*Meloidogyne arenaria*), early leaf spot (ELS), and rust, all of which contribute to major yield losses (Izge et al., 2007; Gaurav et al., 2015). Post-harvest contamination by *Aspergillus flavus* and *Aspergillus parasiticus* is also a serious challenge, as these fungi produce aflatoxins that compromise food safety and market value (Wu et al., 2011).

Conventional breeding has significantly enhanced groundnut productivity; however, further improvement is limited by the narrow genetic base and low genetic variability of cultivated germplasm (Janila et al., 2013). Interspecific hybridization is constrained by the crop's self-pollinating nature and varying ploidy levels (Reddy et al., 1993; Pandey et al., 2012). Moreover, groundnut exhibits low natural outcrossing rates (0–8%), resulting in infrequent transfer of pest and disease resistance traits through natural hybridization (Knauft and Ozia-Akin, 1995; Reddy et al., 1993).

Given these constraints, advanced breeding approaches such as gene editing could play a critical role in developing improved groundnut varieties resistant to abiotic and biotic stresses, as has been demonstrated in other crops. However, the application of such technologies requires an efficient genetic transformation system for regenerating transgenic plants. *Agrobacterium tumefaciens*-mediated transformation is preferred for gene delivery in many plant species due to advantages such as single-copy gene integration, ability to transfer large DNA fragments with minimal rearrangements, stable transgene expression, and low equipment cost (Gelvin, 2003; Xia et al., 2020). The first successful *Agrobacterium*-mediated transformation of groundnut was reported by Lacorte et al. (1991). Since then, various *Agrobacterium* strains, including agropine, octopine, and nopaline genotypes have been used in groundnut transformation studies (Gaurav et al., 2015). Although several strains have individually been tested, no studies have compared the susceptibility of multiple groundnut varieties to different *Agrobacterium* strains or evaluated their relative transformation efficiencies.

Such studies are essential for accelerating groundnut genetic improvement through modern biotechnologies. The present study hypothesized that the transformation efficiency of three groundnut varieties would differ depending on the A. tumefaciens strain used, and that certain strains might be more effective for specific varieties. Therefore, the objective of this study was to evaluate the ability of four *A. tumefaciens* strains to genetically transform three farmer-preferred groundnut varieties in Eastern and Southern Africa. The transformation techniques developed here provide a basis for introducing valuable traits and studying gene function through genome editing technologies.

MATERIALS AND METHODS

Plant and explants preparation

Three groundnut varieties (JL 24, ICGV 90704, and ICGV 12991) commonly grown in Eastern and Southern Africa were used in this study (Table 1). Smallholder farmers particularly value varieties that possess key traits such as insect and disease resistance, drought tolerance, early maturity, and high yield. The three varieties selected for this study are widely adapted across several countries in Eastern and Southern Africa and exhibit desirable agronomic characteristics, including high yield and early maturity (Table 1). However, they are susceptible to aflatoxin accumulation, making genetic engineering for aflatoxin reduction a priority. Mature, dry seeds were obtained from the Kenya Agricultural and Livestock Research Organization (KALRO), Non-Ruminant Research Institute, Kakamega, and stored in polythene bags at 4ºC until use. For surface sterilization, seeds were rinsed in 70% ethanol for 1 min, followed by treatment with sodium hypochlorite (NaOCl) containing three drops of Tween 20. Sterilization conditions were as follows: 1.5% NaOCl for 10 min for variety ICGV 90704, 3.0% NaOCl for 20 minutes for variety ICGV 12991, and 3.0% NaOCl for 10 min for variety JL 24. Seeds were then rinsed three times with sterile double-distilled water and soaked in sterile water for 2 h. After soaking, the seed coats and embryos were surgically removed, and each cotyledon was cut vertically into halves to produce the explants used for subsequent experiments.

Determination of selection pressure

Sensitivity of cotyledon explants to kanamycin was assessed to determine the optimal concentration for selecting transformed tissues (Sandhya et al., 2022). Sterilized and prepared cotyledon explants were cultured on shoot induction medium (SIM) [Murashige and Skoog (MS) macro- and microelements supplemented with B5 vitamins (4.4 g/l), sucrose (30 g/l), plant agar (8 g/l), 20 µM BAP, and 10 µM 2,4-D; pH 5.8] containing 0, 25, 50, 75, 100, 125, 150, or 175 mg/l kanamycin. After 8 weeks, the explants were evaluated for shoot-bud induction to determine shoot proliferation and regeneration rates. Three replicates of 30 cotyledon explants were used for each kanamycin concentration. The experiment was repeated three times.

Table 1. Agronomic traits of the selected three groundnut varieties used in this study.

Groundnut variety	Agronomic traits and response to various diseases	A country where the variety is highly cultivated
ICGV 90704	Rosette resistant, high yielding, medium duration, *Aphis sp*. susceptible, low oil content, and good for relish, high aflatoxin	Mozambique, Malawi, Mozambique, Uganda, Kenya, Zambia, India
ICGV 12991	Rosette resistant, aphid resistant, short duration, tolerant to drought; high aflatoxin	Kenya, Mozambique, Zambia, Uganda, Malawi
JL 24	Early maturity, short duration, wide adaptability, drought-resistant, high yielding, non-dormant, good taste, good for blanching (confectionery); high aflatoxin	Malawi, Mali, Democratic Republic of Congo, Zambia, South Africa, Zimbabwe, Uganda, Kenya, Philippines, India

Agrobacterium strains, plasmid vector, and preparation of bacterial culture

Preparation of *Agrobacterium* cultures followed the method of Anuradha et al. (2006). Four *A. tumefaciens* strains (C58, EHA105, LBA 4404, and AGL0) harboring the binary vector pCAMBIA2301 were used. The pCAMBIA2301 vector carries the cauliflower mosaic virus (CaMV 35S) promoter, the neomycin phosphotransferase (nptII) selectable marker gene, and the β-glucuronidase (gusA) reporter gene. The pCAMBIA2301 plasmid DNA was introduced into the four *Agrobacterium* strains by electroporation. Glycerol stocks stored at –80ºC were streaked onto yeast extract peptone (YEP) plates containing 50 mg/l kanamycin, 50 mg/l rifampicin, and 100 mg/l streptomycin. Single colonies were used to inoculate 2.5 ml YEP liquid medium (Sambrook et al., 1989). Cultures were grown for 48 h at 28ºC with shaking at 200 rpm. To prepare inoculum, 25 ml of YEP medium containing the same antibiotics was inoculated with the starter culture and incubated at 28ºC with shaking (200 rpm) until the OD600 reached 1.0. The culture was centrifuged at 3500 rpm for 15 minutes, the supernatant discarded, and the pellet resuspended in 25 ml half-strength MS medium with vitamins, 3% (w/v) sucrose, and 100 µM filter-sterilized acetosyringone (Sigma Chemical Co.). The bacterial suspension was incubated at 25ºC for 2 h at 100 rpm. The optical density (OD600) of the suspension was adjusted to 0.5 before use in transformation experiments.

Agrobacterium infection and Co-cultivation of cotyledon explants

A 3 ml aliquot of *Agrobacterium* suspension was poured into a sterile Petri dish, and cotyledon explants were immersed with their cut edges in the suspension for 1 min. Explants were blotted dry on autoclaved Whatman filter paper and placed on SIM with the cut edges in contact with the medium (seven explants per plate). Co-cultivation was carried out for 72 h on SIM supplemented with 100 µM filter-sterilized acetosyringone. After co-cultivation, inoculated and non-inoculated explants were washed four times with liquid SIM containing 250 mg/l filter-sterilized cefotaxime. Explants were then blotted dry on sterile filter paper and transferred to SIM containing 250 mg/l cefotaxime for a 1-week recovery period.

Selection and regeneration of putatively transformed shoots

Following the recovery phase, explants were transferred to SIM containing 250 mg/l cefotaxime and 100 mg/l kanamycin for four weeks, with subculturing onto fresh medium after two weeks (first selection, S1). Explants producing shoot buds were transferred to shoot elongation medium (SEM) [modified MS macro- and microelements with B5 vitamins (4.4 g/l), sucrose (30 g/l), plant agar (8 g/l), and 2 µM BAP; pH 5.8] supplemented with 250 mg/l cefotaxime and 125 mg/l kanamycin for two weeks (second selection, S2). Elongated shoots (3 to 4 cm) were transferred to SEM containing 250 mg/l cefotaxime and 150 mg/l kanamycin for the third selection (S3). Shoots that survived selection were moved to antibiotic-free root induction medium (RIM) for two weeks. Well-rooted shoots were transplanted into 10 cm pots containing sterile peat moss and maintained in a greenhouse. After three weeks, the plants were transferred into larger 20 cm pots filled with a soil–sand mixture (1:1) and grown to maturity in a containment greenhouse.

Histochemical GUS assays

Histochemical analysis of β-glucuronidase (gusA) expression was conducted on leaves of kanamycin-resistant in vitro plants using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as the substrate (Jefferson et al., 1987). Leaves were placed in microtubes containing fixative for 1 h, washed three times with 50 mM NaH_2PO_4 (pH 7.0), and then incubated in 300 µl X-Gluc solution at 37ºC for 24 h. After staining, leaves were washed in 70% ethanol and examined under a stereomicroscope. Leaves from non-transformed plants served as negative controls.

Molecular analysis of transformants

Genomic DNA extraction and polymerase chain reaction (PCR) analysis

To verify the presence of transgenes in the transformants, all shoots with well-developed roots on rooting medium containing the selection agent were analyzed. Genomic DNA was extracted from fresh leaves of putatively transformed and untransformed control plants using the DNeasy Plant Mini Kit (Qiagen, GmbH, Germany), following the manufacturer's instructions. PCR amplification using gusA- and nptII-specific primers was performed to confirm the presence of the transgenes. The gusA gene-specific primers were: forward 5'-AAAGTGTGGGTCAATAATCAGG-3' and reverse 5'-ATGGATTCCGGCATAGTTAAAG-3'. The nptII gene-specific primers were: forward 5'-GGGTGGAGAGGCTATTCGGCTATGA-3' and reverse 5'-ATTCGGCAAGCAGGCATCGC-3'. PCR reactions were

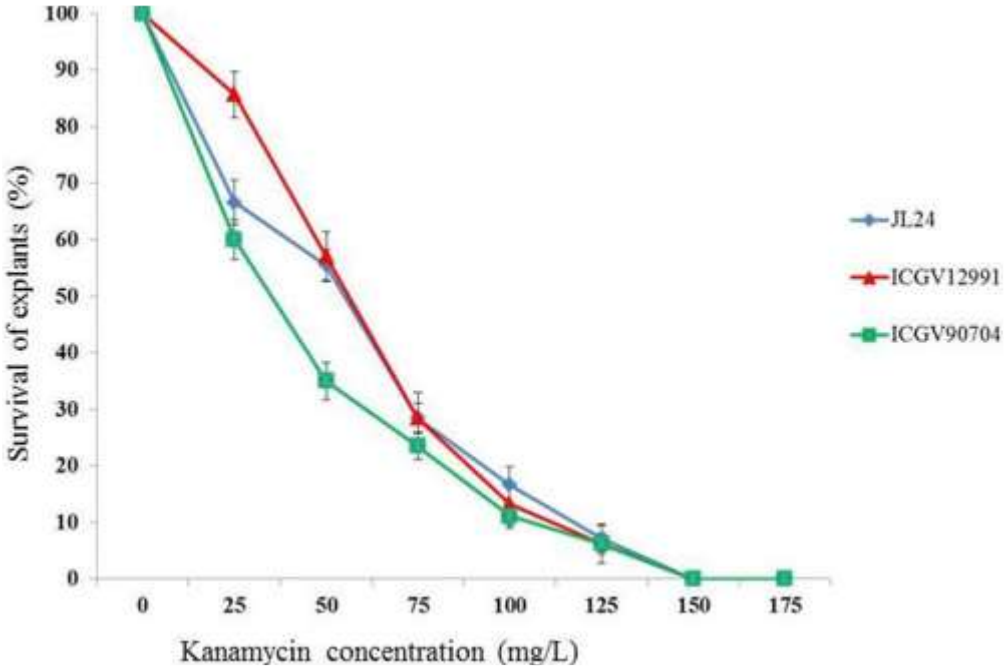

Figure 1. Effect of kanamycin on the survival of cotyledon explants from three groundnut varieties. Data was recorded after 4 weeks of culture and 20 cotyledon explants were used for each concentration with three biological replicates.

were performed in a total volume of 25 µl containing 2.5 µl of 10× PCR buffer, 1 µl of 1.5 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM of each primer, 0.20 µl of 5 U Taq DNA polymerase (Qiagen), 2 µl of DNA template (50 ng genomic DNA or 20 ng plasmid DNA), and 17.8 µl of double-distilled water. Cycling conditions were: 94 °C for 10 minutes; 35 cycles of 94ºC for 30 s, 58.5ºC (for nptII) or 60 °C (for gusA) for 30 s, and 74ºC for 30 s; followed by a final extension at 72ºC for 5 min. PCR products (10 µl) were mixed with 2 µl loading dye and electrophoresed on 1.2% agarose gels stained with 5 µg/ml ethidium bromide at 100 V. Fragment sizes were estimated using a 1 kb DNA ladder (NEB, Nebraska, USA), and bands were visualized under UV light.

Southern blot hybridization

Total genomic DNA for Southern blot analysis was extracted from leaves of PCR-positive plants and non-transformed control plants using the cetyltrimethylammonium bromide (CTAB) method (Mace et al., 2003). Approximately 20 µg of genomic DNA was digested with HindIII (3 U μg^{-1}; New England Biolabs Inc., MA, USA) at 37 °C for 12 hours. Digested DNA samples were resolved on 0.8% (w/v) agarose gels. DNA was transferred onto Hybond-N+ nylon membranes (Roche) by capillary blotting (Sambrook et al., 1989) and cross-linked using a STRATALINK™ UV cross-linker. A digoxigenin (DIG)-labeled nptII-specific probe was synthesized using the PCR DIG Probe Synthesis Kit (Roche Applied Sciences, Mannheim, Germany) and used for membrane hybridization. Detection was performed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche Applied Sciences, Mannheim, Germany).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate the response of the three genotypes to the four *A. tumefaciens* strains using SPSS version 12.0. Mean differences were evaluated at the 5% significance level using standard error (SE). Transformation efficiency (TE) for each genotype was calculated as the percentage of PCR-confirmed transformants relative to the total number of cotyledons inoculated.

RESULTS AND DISCUSSION

Sensitivity of cotyledon explants to kanamycin

Antibiotic resistance genes such as nptII and hygromycin phosphotransferase (hpt) are commonly used as selectable markers for efficient genetic transformation of legume crops. In this study, the nptII gene which detoxifies aminoglycosides and enables the regeneration of transformed plants on medium containing kanamycin (Hsieh et al., 2017; Sabbadini et al., 2019) was used. To determine the optimal kanamycin concentration for effective selection against non-transformed tissues, cotyledon explants were cultured on medium containing different concentrations of kanamycin. As the kanamycin concentration increased, shoot bud induction decreased markedly, and all explants cultured on 150 mg/l kanamycin died (Figure 1). After 4 weeks on SIM medium containing 100 or 125 mg/l kanamycin, shoot buds and stems exhibited complete bleaching. Shoot bud induction was entirely inhibited at 150 mg/l. Because shoot buds did not form at 125 mg/l and only a few bleached buds appeared at 100 to 125 mg/l, concentrations of 100, 125,

Figure 2. *Agrobacterium*-mediated transformation of cotyledon explants of groundnuts. (A) Shoot induction of cotyledon explants of variety ICGV 90704 on SIM at 7 days after co-cultivation. (B) Elongated shoots of ICGV90704 at fourteen days after culture on selection medium. (C). Root induction of groundnut variety ICGV90704 on RIM for 2 weeks. (D) and (E) Acclimatized regenerant of ICGV 90704 variety in the glass house.

and 150 mg/l kanamycin were selected for progressive selection during the regeneration of transformed shoots.

Agrobacterium-mediated transformation system and regeneration

The co-cultivated cotyledon explants began enlarging and greening after seven days of culture on SIM medium (Figure 2A). Shoot bud induction at the proximal cut end of the cotyledons was observed after 14 days of culture on the first selection medium (S1). Proliferation of multiple shoots occurred 10–14 days after transfer to the second selection medium (S2). The shoot buds of non-transformed plants bleached after two weeks on the second selection medium. Elongated putatively transformed shoots (approximately 4 cm) (Figure 2B) were excised and transferred to the third selection medium (S3) for two weeks, during which the leaves of non-transformed plants bleached. Rooting was assessed for elongated shoots that survived the S3 medium by transferring them to RIM medium and maintaining them for four weeks. All transformed shoots developed roots, with root emergence observed two weeks after transfer and full root development achieved after four weeks (Figure 2C). The rooted shoots were subsequently hardened in the greenhouse (Figure 2D and E).

Effect of *A. tumefaciens* strains and groundnut variety on transformation efficiency

Agrobacterium-mediated transformation of plant species can be influenced by several factors, including variety, explant type, plasmid vector, *A. tumefaciens* strain, infection duration, and bacterial culture density, among

Table 2. Effects of different *Agrobacterium* strains on plant regeneration and transformation of cotyledon explants from three groundnut varieties.

Agrobacterium strain	Groundnut variety	Explants with shoots	Shoot buds in the first selection (S1)	Shoots in the second selection (S2)	Shoots in third selection (S3)	Transgenic lines[a]	Transformation efficiency (TE) (%)[b]
LBA4404	ICGV90704	31.67±0.88	85.33±12.72	35.67±6.17	1	1	0.67
	ICGV12991	32.33±0.33	63.33±1.76	12.00±1.16	1	1	0.67
	JL24	33.00±0.58	91.67±3.28	1.33±0.33	0	0	0
AGL0	ICGV90704	28.00±3.06	82.67±8.35	23.67±3.18	2	2	1.33
	ICGV12991	26.67±0.67	30.00±5.00	10.33±0.33	1	1	0.67
	JL24	33.33±0.67	64.67±1.76	3.33±1.86	2	2	1.33
C58	ICGV90704	22.00±1.16	66.33±4.06	32.33±3.84	0	0	0
	ICGV12991	32.00±0.58	56.33±1.86	8.33±1.86	1	1	0.67
	JL24	22.33±3.38	33.67±2.97	1.67±0.33	1	1	0.67
EHA105	ICGV90704	24.33±3.84	50.00±8.08	16.33±1.86	2	2	1.33
	ICGV12991	30.00±1.16	62.00±1.16	1.33±0.33	0	0	0
	JL24	29.00±2.08	55.33±3.71	2.33±0.33	0	0	0

others (Venkatachalam et al., 2000). In this study, the ability of four *A. tumefaciens* strains (LBA4404, AGL0, EHA105 and C58) to transform three groundnut varieties was evaluated, along with their respective transformation efficiencies (Table 2).

During the first selection stage (S1), more shoots were produced when cotyledon explants of varieties JL24, ICGV90704, ICGV90704, and ICGV12991 were inoculated with strains LBA4404, AGL0, C58 and EHA105, respectively. No significant differences ($p \geq 0.05$) were observed between or within varieties in shoot bud induction for explants inoculated with strains LBA4404 and EHA105. However, significant differences were detected between and within varieties infected with strains AGL0 ($p = 0.002$) and C58 ($p = 0.001$).

Analysis of the second selection stage (S2) showed significant differences between and within varieties across all four *Agrobacterium* strains: LBA4404 ($p = 0.001$), AGL0 ($p < 0.001$), C58 ($p < 0.001$), and EHA105 ($p < 0.001$). At the third selection stage (S3), no significant differences were observed between or within varieties infected with strains LBA4404 and AGL0. In contrast, significant differences were found for varieties infected with strains C58 ($p = 0.004$) and EHA105 ($p = 0.021$).

Transformation efficiency (TE), a key indicator of successful *Agrobacterium*-mediated gene transfer, was calculated based on PCR-confirmed transgenic plantlets. The highest TE (1.33%) was recorded for variety ICGV90704 infected with strains EHA105 and AGL0, and for variety JL24 infected with strain AGL0. No transformants were obtained from cotyledon explants of varieties ICGV90704, ICGV12991, or JL24 when infected with strains C58, EHA105 and LBA4404, respectively. Additionally, no transformants were recovered from JL24 explants infected with strain EHA105.

Based on the transformation efficiencies, strain EHA105 was the most effective for transforming variety ICGV90704, while AGL0 was effective for both ICGV90704 and JL24 (Table 2).

The values represent the mean ± SE of three independent experiments. A total of 50 cotyledon explants were used for each experiment, with three replications. A) Transgenic plants refer to PCR-positive lines, and the values represent independent transgenic lines regenerated from three independent experiments. B) Transformation efficiency (%) = (Number of PCR-positive plants / Total number of infected cotyledon explants) × 100. In the present study, the use of *Agrobacterium* strain AGL0 resulted in the regeneration of transgenic shoots in all three groundnut varieties, with the highest transformation efficiency observed in ICGV90704 and JL24 (Table 2). This suggests that AGL0 may be more suitable for genetic transformation across different groundnut varieties compared with strains LBA4404, EHA105 and C58. These differences may be attributed to the activity of distinct virulence genes or combinations thereof, which play key roles in the infection process of each *Agrobacterium* strain (Lacorte et al., 1991; Gustavo et al., 1998).

Several *Agrobacterium* strains, including AZ, C58, EHA101, EHA105, GV2260, GV3101, LBA4404 and AGL1, have previously been used for groundnut transformation with variable efficiencies depending on the explant type, genotype, and regeneration pathway (Gaurav et al., 2015). This is the first study to report the

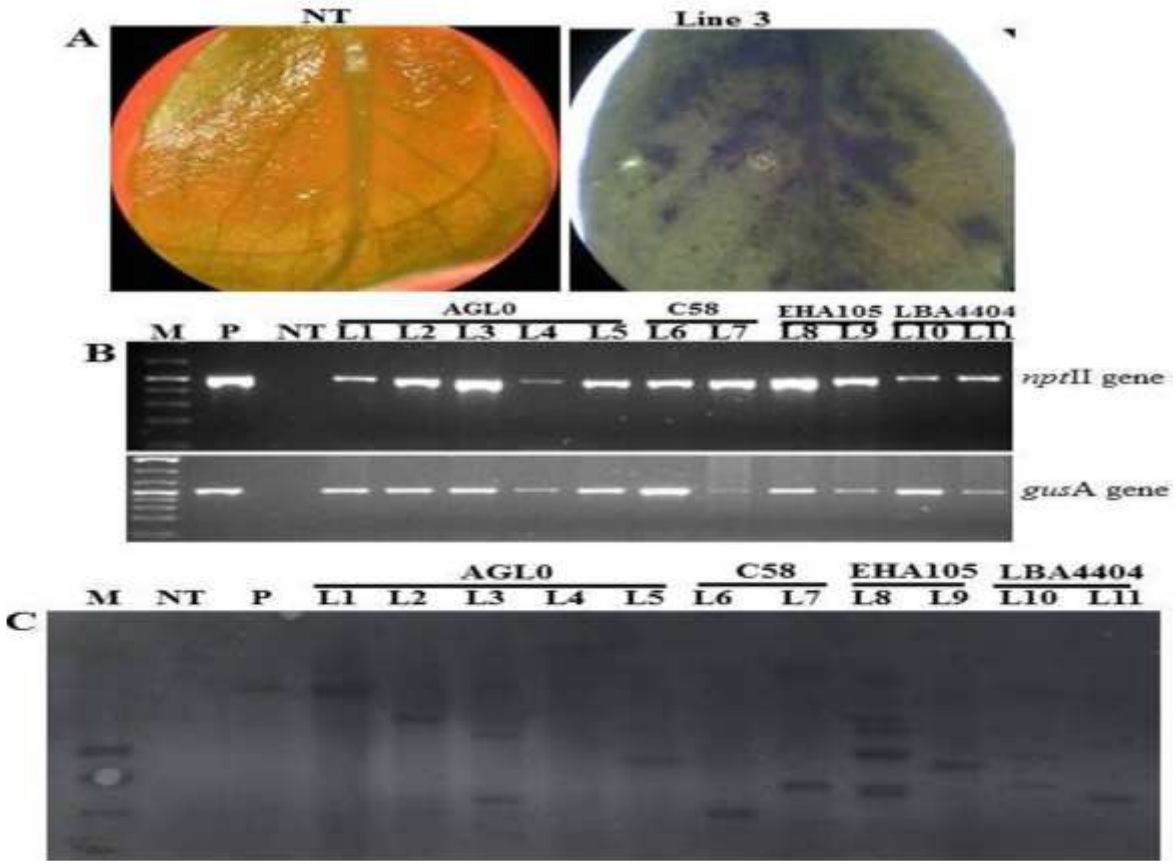

Figure 3. Histochemical *gus*A staining and molecular analysis of regenerated transgenic plants. (A) Stable *gus*A expression in leaves of transgenic and non-transformed groundnut plant leaves of variety ICGV90704. No staining in leaf NT and *gus*A expression in leaf of transgenic Line 3. (B) Electrophoretic profile of PCR amplification of *npt*II and *gus*A genes, respectively. (D) Southern blot hybridization analysis of DNA from transgenic plants. NT: Non-transformed control plant, P: DNA of plasmid pCAMBIA2301 and M: Molecular weight marker.

successful genetic transformation of groundnut using strain AGL0. The results corroborate earlier findings of AGL0 hypervirulence in other plant species (Wu et al., 2014; Cho et al., 2014). AGL0 has been used effectively in the transformation of several crops, including lily, sorghum, and maize (Wang et al., 2012; Wu et al., 2014; Cho et al., 2014; Zhi et al., 2015).

The three other strains used in this study (LBA4404, EHA105, and C58) have also been reported as efficient for genetic engineering of other groundnut genotypes (Gaurav et al., 2015) and various plant species, including mungbean, soybean and sunflower (Jaiwal et al., 2001; Lee et al., 2012; Benzle et al., 2015). These findings support the conclusion that susceptibility to *Agrobacterium* varies across plant species and genotypes, and that strain virulence plays a critical role in transformation success.

All groundnut varieties in this study were transformable with the four *Agrobacterium* strains, although with differing transformation efficiencies (Table 2). Apart from strain type, variety was an important factor influencing transformation rate. Among the varieties tested, ICGV90704 showed the highest transformation efficiency, followed by ICGV12991, while JL24 exhibited the lowest. Because uniform optical cell densities were used for inoculating all explants, differences in transformation response likely reflect genotype–strain interactions and differential susceptibility (Khanna et al., 2007). Genotypic variation in susceptibility to *A. tumefaciens* infection has been documented previously in groundnut (Lacorte et al., 1991) and in other legumes, including pigeon pea (Rathore et al., 1997), chickpea (Islam et al., 1994), pea and soybean (Byrne et al., 1987; Delzer et al., 1990; Tomasz and Jozef, 2005), and black gram (Geetha et al., 1997).

Transgenic plant confirmation by histochemical GUS assay, PCR and Southern blot analysis

Stable *gusA* gene expression was observed in the leaves of putatively transformed groundnut plants (Figure 3A),

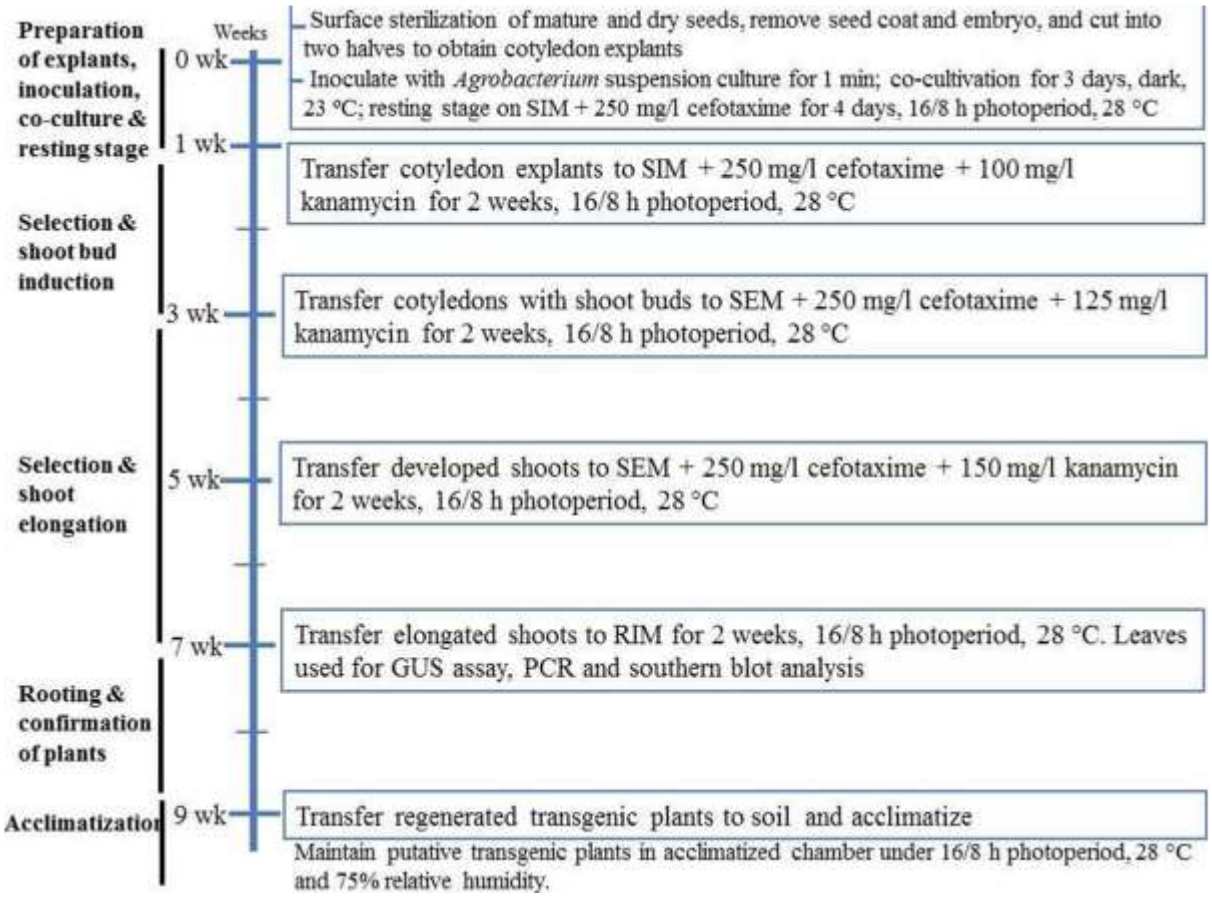

Figure 4. A flow diagram for *Agrobacterium*-mediated transformation method of groundnut varieties using cotyledon explants.

whereas no staining was detected in non-transformed control plants (Figure 3B). The presence of transgenes in the genome of putative transformants was confirmed by PCR amplification of genomic DNA using *gusA* and *nptII* gene-specific primers. Amplification of the expected 528 bp (*gusA*) and 542 bp (*nptII*) fragments verified successful integration in all transformants, while no amplification occurred in non-transformed controls. The absence of escapes among regenerated plant lines further indicates that kanamycin selection based on the *nptII* gene was effective for recovering true transgenic groundnut plants.

Southern blot analysis of PCR-positive plants confirmed the integration of T-DNA into the genome of the transgenic groundnut lines (Figure 3C). The *nptII* gene copy number varied among the transgenic lines, ranging from 1 to 5 copies.

All lines transformed with strain AGL0 exhibited fewer than two integrated T-DNA copies, whereas strain EHA105 yielded the highest copy number, with up to five integrations. These results indicate that *Agrobacterium* strains differ not only in transformation efficiency but also in the number of T-DNA copies integrated into the genome of regenerated transgenic plants. In this study, strain AGL0 achieved the highest transformation efficiency and consistently integrated low transgene copy numbers into groundnut plants. Cotyledonary nodes from mature seeds have previously been identified as highly responsive explants for multiple shoot induction and transgenic plant regeneration in several grain legumes, including soybean (Olhoft et al., 2003), mungbean (Jaiwal et al., 2001), and black Gram (Saini et al., 2003). *Agrobacterium*-mediated transformation has been shown to produce large numbers of stable transgenic plants and is preferred over other gene transfer methods (Anuradha et al., 2006).

Based on the findings of the present study, we report a simple and rapid protocol for *Agrobacterium*-mediated transformation of *A. hypogaea* using cotyledon explants. Following *Agrobacterium* infection, transgenic lines can be generated and prepared for transfer to soil in approximately nine weeks. A schematic representation of the established transformation pipeline is shown in Figure 4.

Conclusions

This study demonstrates that groundnut cotyledons can

serve as effective explants for Agrobacterium-mediated transformation. Genotypic variation and the choice of A. tumefaciens strain are critical factors influencing transformation efficiency. The results confirm that African groundnut varieties can be successfully transformed, providing a foundation for future studies aimed at introducing genes for agronomically important traits, such as drought tolerance and disease resistance, thereby complementing existing conventional breeding programs.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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