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Peanut (*Arachis hypogaea* L.)

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Summary

Arachis hypogea (peanut, groundnut), an annual oil seed belonging to the Leguminosae family and the Papillionacea subfamily, is a legume native to South America but now grown in diverse environments in six continents between latitudes 40°N and 40°S. *Arachis hypogea* can grow in a wide range of climatic conditions. The low yields of this crop are mainly attributed to unreliable rainfall patterns with frequent droughts, lack of high-yielding adapted cultivars, damage by diseases and pests, poor agronomic practices, and limited use of inputs. Genetic engineering approaches have been shown to be comparatively fast, leading to better isolation and cloning of desired traits for combating the various biotic and abiotic stresses. This chapter describes an *Agrobacterium*-mediated transformation protocol in peanut using the cotyledon system. The system described here is potentially applicable to a vast range of genotypes with a high transformation frequency of >70% based on the preliminary molecular data, indicating the production of a large number of independently transformed transgenic plants. The method reported here provides opportunities for crop improvement of this important legume crop via genetic transformation.

Key Words: *Agrobacterium tumefaciens*; *Arachis hypogaea*; genetic transformation; groundnut; Indian peanut clump virus; legume; peanut; shoot regeneration; transgenic plants.

1. Introduction

Legumes are important sources of dietary protein and fats in developing countries of the semiarid tropics, where peanut, or groundnut (*Arachis hypogaea* L.), is one of the important food legume crops ***(1)***. There are several constraints to the productivity of the peanut crop that result in great economic losses annually ***(2)***. Conventional plant breeding techniques and methodologies have not been successful in imparting resistance against various biotic and abiotic stresses owing to species barriers in the natural system. However,

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genetic engineering approaches have been shown to be comparatively fast, leading to better isolation and cloning of desired traits for combating biotic and abiotic stresses. The genetic transformation approach allows for the introduction of novel genes for disease and pest resistance, viral resistance, abiotic stress tolerance, and nutritional improvement that are not normally accessible by conventional breeding, i.e., are limited by sexual incompatibility ***(3)***. Effective regeneration and transformation systems are the prerequisites for a successful genetic transformation. Stable engineered resistance requires the production of numerous independent transformants to allow the selection of those with the appropriate level of gene expression ***(2)***. There are numerous reports of tissue culture and regeneration of peanut from various explants ***(5–9)***. Regeneration via somatic embryogenesis has also been reported as one of the promising methods for transformation studies in peanut ***(10–13)***. A direct regeneration system in peanut has an advantage, owing to the rapidity of morphogenesis and no requirement for frequent subculture; in addition, *de novo* production of shoot primordia is extremely rapid and initially synchronous with the period of cellular differentiation ***(3)***. Such a regeneration system favors easy accessibility of *Agrobacterium* to the meristematic cells, which are mainly surface cells during the initial cocultivation for genetic transformation.

Transformation of plants involves the stable introduction of DNA sequences, usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation efficiencies are frequently directly related to the tissue culture response, and therefore highly regenerative cultures are often transformation competent.

Peanut transformation, like all other transformation systems, relies on some common key elements. The major components for the development of transgenic plants are: (1) the development of reliable tissue culture regeneration systems, (2) preparation of gene constructs and transformation with suitable vectors, (3) efficient techniques of transformation for the introduction of genes into the crop plants, (4) recovery and multiplication of transgenic plants, (5) molecular and genetic characterization of transgenic plants for stable and efficient gene expression, (6) transfer of genes to elite cultivars by conventional breeding methods if required, and (7) evaluation of transgenic plants for their effectiveness in alleviating biotic and abiotic stresses without being an environmental biohazard ***(14)***.

Here we describe an efficient transformation system of peanut with high transformation frequency based on an earlier published procedure ***(4)*** using cotyledon explants forming adventitious shoot buds in different peanut genotypes. This transformation protocol has been optimized for the development of a transgenic peanut capable of producing fertile plants by *Agrobacterium tumefaciens*-mediated transformation that is potentially applicable to a wide

range of peanut genotypes. The protocol uses cotyledon explants from pre-soaked mature seeds that can produce multiple adventitious buds that are highly amenable to *A. tumefaciens* infection and result in large numbers of transgenic events from a given experiment. The system results in the production of a large percentage (55% of the putative transgenic plants are positive for gene integration) of transgenic plants.

2. Materials

2.1. Plant Tissue Culture

1. Healthy peanut seeds of variety JL24 obtained from the gene bank of the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT).
2. 70% Ethanol.
3. 0.1% (w/v) Aqueous mercuric chloride.
4. MS medium ***(15)***: MS inorganic salts and MS organic constituents:
   a. Major salts (50X): prepare stock solutions of the major salts of MS medium.
      i. NH_4NO_3: dissolve 33 g of NH_4NO_3 in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
      ii. KNO_3: dissolve 38 g of KNO_3 in 400 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 20 mL of the stock solution for preparing 1 L of the medium.
      iii. KH_2PO_4: dissolve 3.40 g of KH_2PO_4 in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
      iv. $CaCl_2$: dissolve 8.80 g of $CaCl_2$ in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
      v. $MgSO_4 \cdot 7\ H_2O$: dissolve 7.40 g of $MgSO_4 \cdot 7\ H_2O$ in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
   b. Minor salts (100X): weigh the required quantities of the minor salts (83 mg KI, 2230 mg $MnSO_4 \cdot H_2O$, 860 mg $ZnSO_4 \cdot 7H_2O$, 25 mg $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg $CuSO_4 \cdot 5H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$) and dissolve in 100 mL of sterile distilled water. Store the stock 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
   c. Iron (100X)
      i. $Na_2EDTA \cdot 2H_2O$: dissolve 3.73 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
      ii. $FeSO_4 \cdot 7H_2O$: dissolve 2.78 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.

OR

iii. $FeNa_2EDTA$: dissolve 2 g of the chemical in 500 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium

5. B5 organic constituents ***(16)***: dissolve 50 mg nicotinic acid, 50 mg pyridoxine monohydrochloride, and 50 mg thiamine hydrochloride in 250 mL of sterile distilled water. Use 10 mL of this stock for preparation of 1 L medium.
6. Modified MS medium (MMS): MS inorganic salts, B5 organic constituents, 3% sucrose, and 0.8% Difco Bacto agar. The pH of the medium is adjusted to 5.8 prior to autoclaving.
7. Benzyl adenine (BA; Sigma): 1 m*M* stock. Dissolve 22.5 mg of BA in 1 mL of 1 *N* NaOH and then bring the volume to 100 mL with autoclaved water. Stock solutions are stored at 4°C.
8. Kinetin (Sigma): 1 m*M* stock. Dissolve 21.5 mg of kinetin in 1 mL of 1 *N* NaOH and then make up the volume to 100 mL with autoclaved water. Stock solutions are stored at 4°C.
9. β-naphthalene acetic acid (NAA, Sigma): 1 m*M* stock. Dissolve 18.6 mg of NAA in a few drops of ethanol and then bring the volume to 100 mL using autoclaved distilled water. Stock solutions are stored in single aliquots at 4°C.
10. 2,4-Dichlorophenoxyacetic acid (2,4-D, Sigma): 1 m*M* stock. Dissolve 22.1 mg of 2,4-D in a few drops of ethanol, bring the volume to 100 mL with autoclaved distilled water, and store in single aliquots at 4°C.
11. Cefotaxime: 125 mg/mL stock. Dissolve the chemical in distilled water and filter-sterilize the stock prior to use.
12. Kanamycin monosulfate (Sigma): 125 mg/mL stock. Dissolve the kanamycin powder in water, filter-sterilize, and store in aliquots at –20°C for no more than 15 d.
13. Shoot induction medium (SIM): MMS plus 20 μ*M* BA and 10 μ*M* 2,4-D. Pour the medium into 90 × 16-mm sterile disposable plastic Petri plates. Store in a cool place for no more than 15 to 20 d.
14. Shoot elongation medium 1 (SEM): MMS plus 2 μ*M* BA. Pour the medium into 150 × 25-mm-long test tubes plugged with nonabsorbent cotton plugs wrapped in one layer of cheesecloth. Store in a cool place for no more than 15 to 20 d.
15. Root induction medium (RIM): MMS plus 5 μ*M* NAA. Pour the medium in 150 × 25-mm-long test tubes plugged with nonabsorbent cotton plugs wrapped in one layer of cheesecloth. Store in a cool place for no more than 15 to 20 d.

2.2. Bacterial Culture

1. *Agrobacterium* strain and vector: disarmed *A. tumefaciens* strain C58, harboring the plasmid pROKII:IPCVcp containing the coat protein gene and pBI121 containing *uid*A (GUS) as the reporter gene linked to the CaMV 35S promoter, with both the plasmids having the *nptII* gene as a selectable marker under the control of the NOS promoter and polyA sequences within the T-DNA borders.

2. Luria-Bertani medium (LB): 1% Bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, 1.5% agar dissolved in deionized water. The pH is adjusted to 7.0 with 5 *N* NaOH (approx 0.2 mL) prior to autoclaving.
3. Yeast extract medium (YEB): 0.5% Bacto-peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose, 0.05% $MgSO_4 \cdot 7H_2O$. The pH is adjusted to 7.0 with NaOH prior to autoclaving.
4. ½ MS containing 3% sucrose. The pH is adjusted to 5.8 before autoclaving.

2.3. Characterization of Putative Transformants

2.3.1. Histochemical Analysis

1. 0.1 *M* Sodium phosphate buffer: 0.1 *M* of monobasic sodium phosphate (13.9 g in 1000 mL), 0.1 *M* of dibasic sodium phosphate (26.85 g in 1000 mL). Add 31 mL of monobasic solution and 69 mL of the dibasic sodium phosphate to a final volume of 100 mL, pH 7.0. Store at 4°C.
2. X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) solution: dissolve 10 mg of X-gluc in 100 μL of dimethylformamide (DMF) and adjust the volume to 5 mL with 0.1 *M* sodium phosphate buffer (pH 7.0), 25 μL of 200 m*M* potassium ferrocyanide, 25 μL of 200 m*M* potassium ferricyanide, 50 μL of 1 *M* Na_2EDTA, 100 μL of 0.1% Triton-X, 200 μL of 50 mg/L sodium azide. Store in a dark bottle away from light at 4°C for immediate use.

2.3.2. Genomic DNA Extraction and Purification

1. Extraction buffer: 100 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, 500 m*M* NaCl, 10 m*M* β-mercaptoethanol,
2. 20% Sodium dodecyl sulfate (SDS).
3. 5 *M* Potassium acetate, pH 5.0.
4. Isopropanol.
5. 10 mg/mL RNAse A in 10 m*M* Tris-HCl, pH 8.0, 15 m*M* NaCl. Boil for 20 min to inactivate contaminating DNases. Store in aliquots at –20°C.
6. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
7. DEAE-cellulose suspension: 7.5% Whatman DE 52, 2 *M* NaCl, in TE buffer, pH 8.0.
8. Wash buffer: 400 m*M* NaCl, in TE buffer, pH 7.5.
9. Elution buffer: 2 *M* NaCl, in TE buffer, pH 7.5.

3. Methods

3.1. Agrobacterium *inoculum Preparation*

1. Disarmed *A. tumefaciens* strain C58, harboring binary plasmids pBI121 and pROKII:IPCV, are maintained on LB agar plates containing 50 mg/L kanamycin and 25 mg/L rifampicin, for use in transformation.
2. Grow single colonies of the strain in 20 mL of YEB supplemented with 50 mg/L kanamycin at 28°C on an orbital shaker (100 rpm) overnight or till the OD reaches 0.6.

3. Centrifuge 10 mL of bacterial cells at 600*g* for 10 min and resuspend the pellet in 30 mL of ½ strength MS-containing 3% sucrose (1:3 dilution). Store the suspension at 4°C for 1 to 2 h before cocultivation.
4. Shake the bacterial suspension and pour it into sterile Petri plates so as to make a thin film (2–3 mm) at the base of the plate.

3.2. Explant Preparation and Cocultivation

1. Mature seeds of the selected peanut variety are removed from healthy pods and stored at 4 to 10°C prior to use. If the pods are not stored at low temperature, the seeds can lose their viability.
2. Surface-sterilize the seeds by rinsing in 70% ethanol for 1 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. Wash thoroughly four to six times with sterile-distilled water and soak the seeds in sterile water for 4 h before use. Unless noted otherwise, all steps are to be performed in a laminar airflow chamber.
3. Remove the seed coat surgically under aseptic conditions, remove the embryonic axis, and cut each cotyledon into vertical halves (**Fig. 1A**) to obtain the cotyledon explants (*see* **Note 1**).
4. Immerse the freshly excised cotyledons with their proximal cut ends into the bacterial suspension for a few seconds and implant on SIM with cut ends embedded in the medium for 72 h at 26 ± 1°C under continuous lighting provided by white cool fluorescent lamps of 60 μE/m^2/s light intensity (**Fig. 1B**).

3.3. Maintenance of Cultures and Regeneration of Shoots

1. Transfer the cotyledon explants cocultivated with the *Agrobacterium* solution (five explants per plate) to SIM supplemented with 250 mg/L cefotaxime for 2 wk until multiple shoots appear on at least 70% of the explants. Take the utmost care to embed the cut end of the explant (**Fig. 1C**) into the medium (*see* **Note 2**).
2. Transfer the explants bearing shoot buds (**Figs. 1D–F**) to SEM containing 250 mg/L cefotaxime and 100 mg/ L kanamycin to initiate selection and enrichment of the transformed cells for another 2 wk (*see* **Notes 3** and **4**). Cultures are maintained at 26 ± 1°C under continuous light provided by white cool fluorescent lamps with 60 μE/m^2/s light intensity.

Fig. 1. *(opposite page)* Regeneration of adventitious shoots from cotyledon explants of *A. hypogaea* L. **(A)** Embryo axis is removed surgically from the healthy groundnut seeds, and each cotyledon is then cut into vertical halves to obtain the cotyledon explants. **(B)** Cultured explants on shoot induction medium after cocultivation for 48 to 72 h. **(C)** Greening of cotyledons and initiation of shoot bud formation from the explants after 7 to 9 d of culture. **(D)** Induction of adventitious shoot buds from cotyledon explants after 2 wk of culture on SIM showing the swelling of cut ends resulting in shoot buds. **(E)** Development of multiple adventitious shoots from cut end of the cotyledon explants after 3 wk. **(F)** Cotyledon explants bearing the multiple shoot buds after 4 wk of culture.

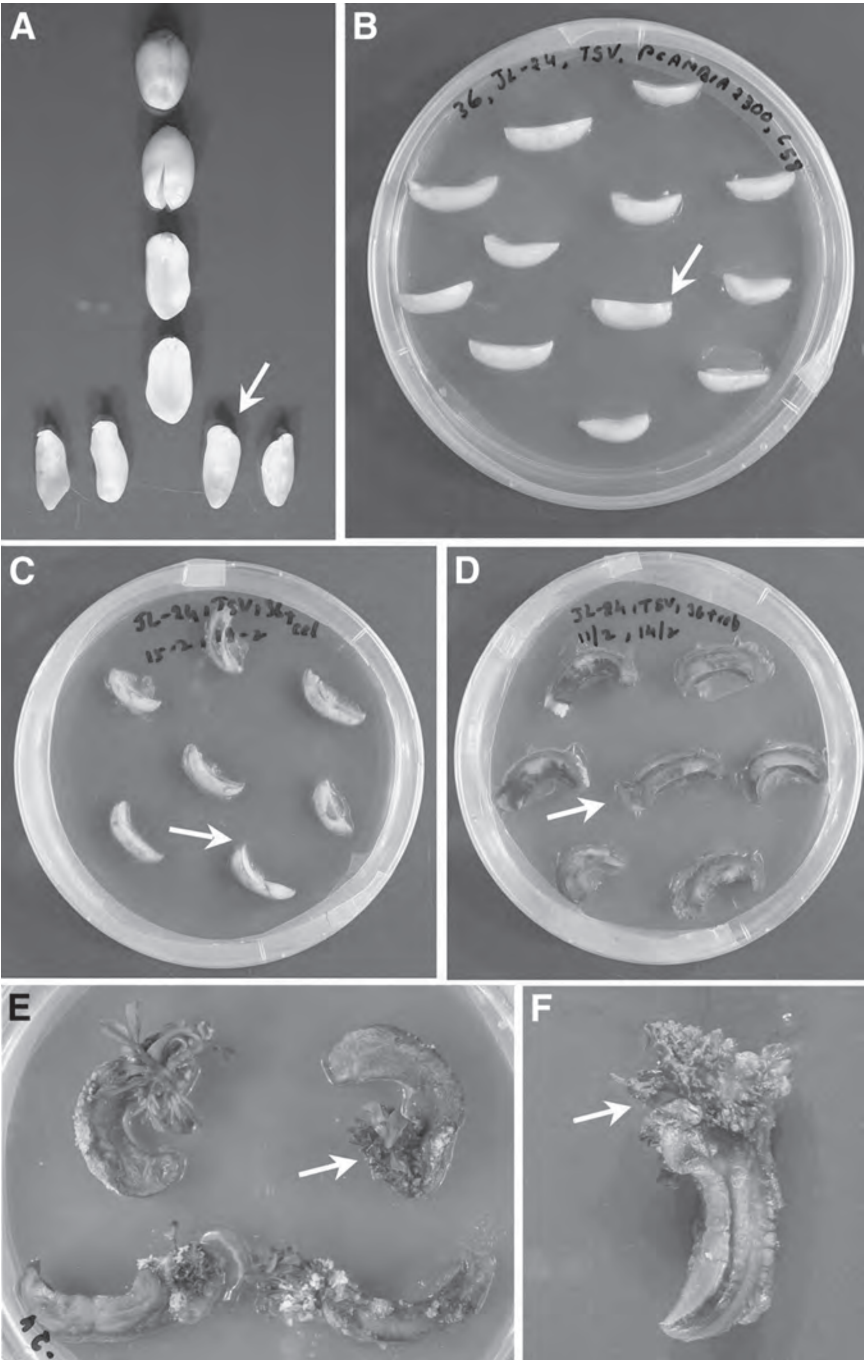

Fig. 1.

3. After 2 wk, excise the proximal parts of the explants containing multiple adventitious shoot buds (**Fig. 2A**) and transfer to SEM containing 125 mg/L kanamycin for two to three subcultures of 4 wk duration each (*see* **Notes 5** and **6**).
4. Culture the elongated shoots (3–4 cm) on RIM without any antibiotics for rooting of the shoots. It takes about 2–3 wk to obtain multiple adventitious roots on the shoots after culture on RIM (**Fig. 2C**).

3.4. Transplantation and Maintenance of Plants in the Greenhouse

1. Remove the plants gently from the culture tubes and carefully wash the medium using sterile distilled water.
2. Transfer the rooted shoots to pots containing an autoclaved sand and red soil (1:1) mixture covered with plastic bags to maintain high humidity (85%) at 25°C in a growth cabinet with a 16-h photoperiod and 60 μE/m^2/s light intensity.
3. Make small holes in the plastic bags after 7 to 8 d for acclimatization of the plants. Remove the plastic bags after 10 d.
4. After 2 wk, transfer the plants to 25-cm-diameter pots containing autoclaved field soil to a contained greenhouse with 26 to 30°C/20 to 25°C day/night temperatures and about a 10- to 12-h photoperiod for flowering and seed set.
5. The plants are irrigated with Hoagland's nutrient solution once a month and routinely irrigated with tap water whenever required.
6. All the transformed plants produced flowers and pods within 120 d and contained viable seeds. Each plant produced up to 40 pods that provided a total of up to 75 seeds per plant when grown in 12-inch-diameter pots. The flowering and maturity may depend on the selected variety.

3.5. Histochemical Localization of uidA Gene Activity in Putative Transformants

1. The primary transformants (T_0) are analyzed for the presence and expression of the introduced genes upon transfer to the greenhouse.
2. Cut free-hand sections of petiole and stem sections of putative transformants growing in vitro or in the greenhouse.
3. Add 200 μL of the X-gluc assay mixture to the tissue sample and infiltrate under vacuum for 3–5 min.
4. Incubate the samples at 37°C for 4 to 24 h in the dark.
5. Stop the reaction by removing the mixture and dehydrating the sample by sequential washing in 70 to 100% ethanol until tissue is devoid of chlorophyll.
6. Examine the tissue for staining under a microscope.

3.6. Molecular Analysis of Putative Transformants

3.6.1. Extraction and Purification of Genomic DNA from Peanut Leaves (4)

1. Collect young leaf tissue (0.5 g) from putatively transformed peanut plants growing in a contained greenhouse. Freeze-dry the samples by immersing in liquid nitrogen and lyophilize for 10 to 15 min.

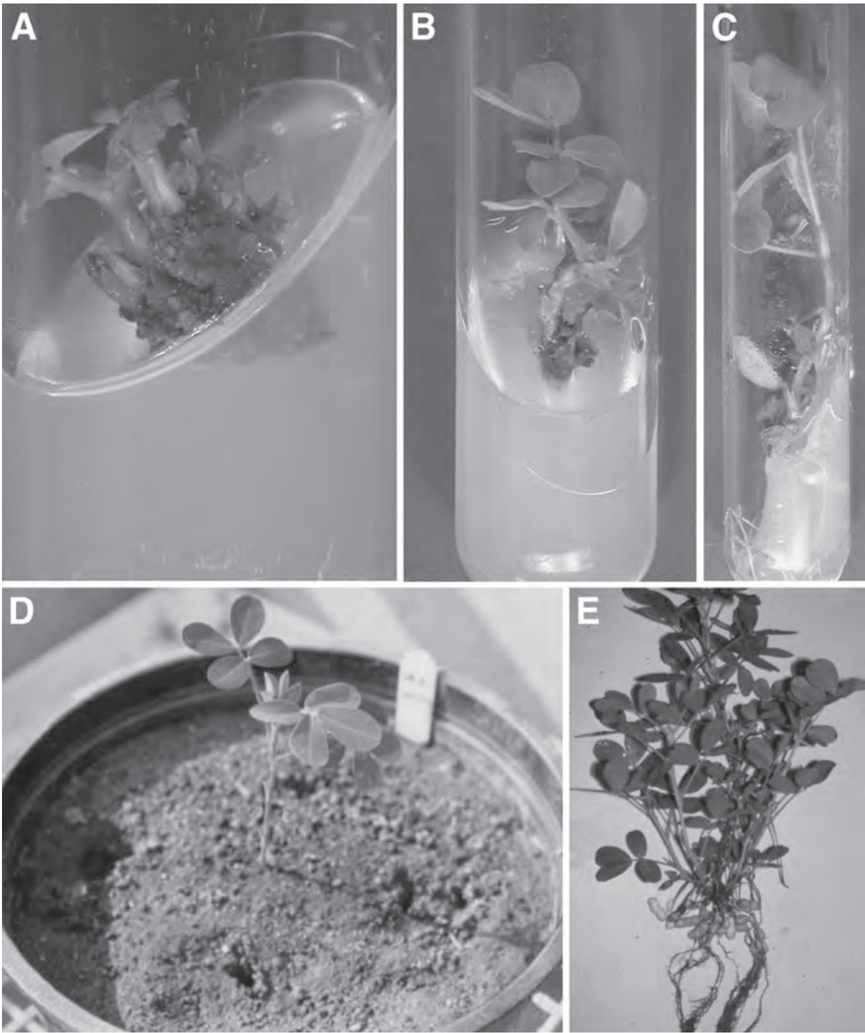

Fig. 2. Development of adventitious shoots into plants. **(A)** Development of multiple adventitious shoots after culture on shoot elongation medium (SEM) after 10 d of culture. **(B)** Elongation of a shoot cultured on SEM after 2 wk. **(C)** Production of multiple adventitious roots on elongated shoots after 2 to 3 wk of culture on root induction medium (RIM). **(D)** A rooted plant transplanted to a pot and maintained under greenhouse conditions after 2 wk of transplantation. **(E)** Mature groundnut transgenic plant with well-developed pods and seeds at the time of harvest.

2. Homogenize, with a mortar and pestle, the dry lyophilized plant material to a fine powder.
3. Transfer the powder with a metallic spatula to a 25-mL polypropylene tube and add 15 mL of extraction buffer and 1 mL 20% SDS. Mix gently and incubate in a water bath for 15 min.
4. Bring the samples to room temperature, add 5 mL of 5 *M* potassium acetate, mix gently, and incubate on ice for 30 min.
5. Spin the tubes for 20 min at 1800*g* at room temperature.
6. Transfer the supernatant to another tube, avoiding disturbance to the interphase.
7. Precipitate the DNA with 0.6 vol of isopropanol, mix gently, and incubate for 30 min at –20°C before centrifugation at 1500*g* for 10 min.
8. Discard the supernatant and wash the pellet in 70% ethanol, followed by air-drying.
9. Add 700 µL TE buffer and 10 µL of RNase (10 mg/mL) and incubate at 37°C for 2 to 3 h.
10. Dilute the DNA with two volumes of distilled water.
11. Add to it 1 mL of DEAE-cellulose suspension with gentle mixing for 3 min so as to maximize the interaction between nucleic acids and the DEAE-cellulose matrix (*see* **Note 7**).
12. Spin the mixture for 30 s at 500*g* to allow the sedimentation of DEAE-cellulose particles, to which the nucleic acids have bound.
13. Discard the supernatant carefully and resuspend the pellet in 1.2 mL wash buffer to eliminate the proteins, polysaccharides, and secondary metabolites not bound to DEAE-cellulose.
14. Repeat this step again at least once for better results.
15. Add 0.5 mL of elution buffer to the DEAE-cellulose pellet and mix gently prior to centrifugation at 500*g* for 30 to 45 s (*see* **Note 8**).
16. Collect the supernatant in a fresh 1.5-mL microfuge tube and repeat this step once again with 0.3 mL of elution buffer.
17. Carefully pool the supernatants and precipitate using 0.6 vol of isopropanol, followed by centrifugation at 1500*g* for 10 min at room temperature.
18. Discard the supernatant, wash the pellet with 1 mL of 70% ethanol, and centrifuge at 1800*g* for 2 min.
19. Discard the supernatant and air-dry the pellet properly. Dissolve the dry pellet in 50 to 100 µL of TE buffer (pH 8.0) for long-term storage at –20°C. Typically, 0.5 g tissue yields approx 15 to 20 µg of purified DNA.

3.6.2. Characterization of Transgenic Plants

1. Carefully number each putative independent transformant arising from a treated explant and maintain separately for subsequent DNA analysis and progression, e.g., generations such as T_1, T_2, T_3, and so on.
2. Ascertain the segregation pattern of the *uid*A gene by using β-glucuronidase (GUS) expression in the T_1 and T_2 generation progenies. PCR analysis of the

introduced genes can also be carried out to confirm the segregation pattern in the progenies.

3. Examine the T-DNA insertion pattern by separately digesting the genomic DNA from each of the putative transformants by using the restriction enzyme that has a single internal site in the plasmid, and resolve on 0.8% agarose gel for transfer onto nylon membranes (HybondN+, Amersham) by a standard protocol.
4. Probe the blots with PCR-amplified fragments of the *nptII* gene labeled with a radioactive or nonradioactive detection system according to the manufacturer's instructions.

4. Notes

1. The embryonic axis should be removed surgically before the explants are prepared.
2. Care should be taken to embed the proximal cut end of the cotyledon into the medium so that it remains in contact with the medium at least for the first 2 wk of culture initiation.
3. Judicious use of selection levels is an important criterion for the recovery of transformed cells, as too high a level would be detrimental even to the transformed cells in early stages.
4. A greater number of shoots can be recovered if the explants are subcultured on SEM1 for one to two extra subcultures.
5. The bleached shoots must be removed with care at every stage of selection to reduce the number of escapes.
6. Use of kanamycin has not been considered very reliable for visual selection at a later stage since some of the nonbleached shoots have been found to be untransformed. However, it does play a selective role in suppression of the shoot bud induction from the untransformed cells.
7. The DEAE-cellulose suspension must be properly mixed with the DNA solution to maximize the interaction between the two.
8. Elution of the DNA should be done at least twice to maximize the yield of the purified DNA.

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