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Agrobacterium-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants

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Abstract. Transgenic peanut (*Arachis hypogaea* L.) plants have been produced using an *Agrobacterium*-mediated transformation system. Zygotic embryo axes from mature seed were cocultured with *Agrobacterium tumefaciens* strain EHA101 harboring a binary vector that contained the genes for the scorable marker *B*-glucuronidase (GUS) and the selectable marker neomycin phosphotransferase II. Nine percent of the germinated seedlings were GUS+. Polymerase chain reaction analysis confirmed that GUS+ shoots and T_1 progeny contained T-DNA. Molecular characterization of one primary transformant and its T_1 and T_2 progeny plants established that T-DNA was integrated into the host genome.

Introduction

The transfer of new genetic material into the genome of peanut, *Arachis hypogaea* L., has been achieved with *Agrobacterium tumefaciens* and particle bombardment. Study with *A. tumefaciens* has confirmed the expression and integration of T-DNA within resulting tumor cells from inoculated plants (Lacorte et al. 1991) and regenerable explant tissues (Mansur et al. 1993). Franklin et al. (1993) obtained stably transformed callus lines from seedling hypocotyl explants cocultivated with *Agrobacterium*. Likewise, transgenic peanut callus originating from immature leaf tissue has also been reported using biolistic bombardment (Clemente et al. 1992). In this system, multiple inserts were present in all transformed cell lines tested. More recently, Ozias-Akins et al. (1993) regenerated transgenic plants from embryogenic callus transformed via particle bombardment. Approximately 1% of the bombarded calli produced stably transformed cell lines.

A potential limitation of these methods is the requirement for regenerable peanut callus. The production of callus and subsequent whole plant regeneration has been found to vary among peanut genotypes (Baker and Wetzstein 1994, Ozias-Akins et al. 1992, Sellars et al. 1990). In addition, somaclonal variation events generated through tissue culture may induce unwanted mutation in regenerants. Transformation procedures that avoid tissue culture would, therefore, be advantageous under such circumstances. Recently, transgenic peanut plants have been obtained via the particle bombardment of shoot meristems excised from mature embryonic axes (Brar et al. 1994). This system did not utilize tissue culture, and resulted in the direct development of transgenic shoots from bombarded meristems.

In this report, we present a method for peanut transformation that involves *A. tumefaciens*-mediated transformation of zygotic embryo axes of mature seed and their subsequent germination and development into transgenic plants. This method, which does not require sophisticated tissue culture techniques, is based on a report of *in planta* transformation of *Arabidopsis thaliana* seeds by Feldmann and Marks (1987). Transformants were selected in the progenies of T_0 *Arabidopsis* plants cocultured with *Agrobacterium* during seed imbibition.

Materials and methods

Plant materials. Seed of *Arachis hypogaea* L. cultivars Florigiant, Florunner, NC-7, and 435 were obtained from a seed collection maintained by the University of Florida.

Bacterial strains and plasmids. A variety of wild type *Agrobacterium tumefaciens* strains were used to test the susceptibility of several peanut genotypes to infection. Seedling hypocotyls were injected with overnight bacterial cultures. Inoculation sites were covered with plastic for 8 d to prevent desiccation. Twenty eight plants of each cultivar were inoculated with each strain of bacterium. Frequency of tumor formation and sizes were scored 30 d after inoculation. For transformation experiments, the engineered *Agrobacterium* strain EHA101 (Hood et al. 1986b), was used with the binary vector plasmid pMON9793 (Monsanto Co., St. Louis,

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MO). Plasmid pMON9793 (Fig. 1A) is a derivative of pMON505 in which a chimeric gene containing a mannopine synthase promoter, the coding region for *B*-glucuronidase (GUS) (Jefferson 1987), and the nopaline synthase (NOS) 3' polyadenylation signal was cloned into the multilinker (Rogers et al. 1987). Plasmid pMON9793 also contains a chimeric neomycin phosphotransferase II (NPTII) gene with the NOS promoter and NOS 3' polyadenylation signal. The efficacy of the *Agrobacterium*-vector plasmid combination used in these experiments was verified by transformation of tobacco prior to experiments with peanut.

Cultures of *Agrobacterium* strains were initiated from glycerol stocks and grown overnight at 28°C in liquid LB medium containing the appropriate antibiotics, to late or post log phase. Bacteria were then collected by centrifugation for 10 min at 1000 x g and resuspended in Murashige and Skoog (1962) (MS) liquid medium.

Transformation. Seeds from the peanut cultivar Florigiant were harvested from greenhouse-grown plants at the R8 stage (Boote 1982), in which the inner pericarp and testa are colored. Fruits were soaked in water for 2 h and then washed to remove surface debris. Seeds were removed from the pods, and the embryos (2-2.4 cm) were separated from the seed coats. The embryos were surface sterilized in 2.6% sodium hypochlorite (NaOCl) for 12 min. One cotyledon was removed, and the embryo axes 8-10 mm long with single cotyledons (EAC) were further sterilized in 1.6% NaOCl for 3 min, 70% ethyl alcohol for 1 min, and then rinsed 3 times with sterile deionized water. The embryo axes (EA) of the EACs were wounded in 3 locations with a no. 11 scalpel blade. The cuts, 2 mm deep, were made through the epicotyl, the 2 axillary buds, and down the hypocotyledonary axis. The wounded EACs were placed directly in an overnight culture of *Agrobacterium* strain EHA101 in MS liquid medium supplemented with 5 mg/l 6-benzylaminopurine, and were cocultivated for 72 h at 26-28°C in the dark. The bacterial cell concentration of the medium at the onset of coculture was 50 x 10^6 cells/ml. After coculture the explants were washed and decontaminated in 200 µg/ml carbenicillin, and then planted in autoclaved soil mix for germination. *Agrobacterium* contamination was detected by plating explants on solid LB selection medium and incubating at 28°C for 90 d. For selection experiments, treated and control explants were plated on MS medium supplemented with the kanamycin concentrations 0, 50, 100, 150, 200, 250, and 300 µg/ml.

Analysis of putatively transformed tissue. GUS enzyme activity was assessed histochemically in freehand leaf sections and EAs as described by Jefferson (1987). Tissue was stained for 3 h at 37°C. Extended staining periods of 30 h or longer resulted in false positives. Transformed tobacco and nontransformed peanut tissue were included as positive and negative controls, respectively, with each set of assays. After clearing and fixing the tissue with FAA (10% formalin, 5% glacial acetic acid, 42.5% ethanol), it was possible to unambiguously detect even small GUS+ sectors.

PCR was used to detect specific DNA sequences from leaf and embryo tissue. DNA was isolated from fresh material using the Dellaporta et al. (1983) procedure. PCRs were performed in a total reaction volume of 50 µl and contained 0.1 µg DNA, 1.25 mM dNTPs, 1 µM of each primer, 25 mM $MgCl_2$, 1X Stoffel buffer, and 2.5 units of Taq DNA polymerase (AmpliTaq DNA polymerase, Perkin-Elmer Cetus). Reaction mixtures were covered with mineral oil and placed in a thermal cycler (Hybaid). Samples were heated to 94°C for 2 min and then subjected to 30 cycles of 1 min at 94°, 0.5 min at 55°, and 1 min at 72°. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Primers 5'ATCACGCAGTTCAACGCTGAC3' and 5'TTGGGCAGGCCAGCGTATCGT3' were used to amplify a 420 bp fragment of the GUS gene, and primers 5'TCACTGAAGCGGGAAGGGACT3' and 5'CATCGCCATGGGTCACGACGA3' were used to amplify a 320 bp fragment of the NPTII gene.

For Southern analysis, purified DNA (10 µg) was digested with 20 units of the appropriate endonuclease, electrophoresed on 0.8% agarose gel, and blotted (Southern 1975) on Immobilon-S membrane. The probe was isolated as an 827 bp ClaI to EcoRI fragment from pMON9793 and purified via agarose gel electrophoresis. Probe labelling and DNA hybridization and detection were done using the protocol and components of the PolarPlex Chemiluminescent Blotting Kit (Millipore Corp., Marlborough, MA). Hybridized DNA fragments were visualized using Cronex medical X-ray film (DuPont, Wilmington, DE).

Results and Discussion

Our objective was to develop an *Agrobacterium*-based transformation system similar to the seed infection procedure described by Feldmann and Marks (1987). Accordingly, peanut genotypes were first evaluated for susceptibility to various wild-type *A. tumefaciens* strains by determining tumor induction frequencies and growth at plant stem inoculation sites. Four peanut cultivars and 5 bacterial strains were evaluated for optimum host-pathogen interaction for subsequent transformation study. Susceptibility response was determined by scoring the number of peanut seedlings which produced tumors and their relative size. Variations in cultivar-strain compatibility were determined (Table 1), and the Florigiant-A281 combination was selected for further study. Strain A281, which contains the oncogenic Ti plasmid pTiBo542 (Hood et al. 1984), has previously been identified as virulent in peanut (Lacorte et al. 1991) and in other legume crops such as soybean (Byrne et al. 1987), alfalfa (Hood et al. 1986a), and pea (Hobbs et al. 1989). EHA101, a nononcogenic derivative of this strain, was used in combination with the binary plasmid pMON9793 for the transformation experiments.

Table 1. Response of peanut cultivars to inoculation with *Agrobacterium tumefaciens* strains.

Peanut Cultivar	*A. tumefaciens* strain and plant response				
	B6	T37	A281	A518	C58
Florigiant(Vi)[a]	32[b]++[c]	0	67 +++	38 +	54 ++
Florunner(Ru)	11 +	14 +	61 ++	22 +	46 +
NC-7(Vi)	42 +	4 +	60 ++	38 +	54 +
435(Sp)	0	0	22 ++	43 +	13 +

[a] Market type classification: Vi = Virginia, Ru = Runner, Sp = Spanish (Knauft and Gorbet 1989).
[b] Percentage of seedlings forming tumors, n = 28.
[c] Tumor sizes: + = 1 mm, ++ = 2-5 mm, +++ = 6-10 mm.

To develop a potential selection protocol, the sensitivity of peanut zygotic embryo axes with single attached cotyledons (EAC) to kanamycin was initially investigated. Nontreated EACs were placed on MS basal medium supplemented with 0-300 µg/ml kanamycin. Germination did not occur on medium containing more than 150 µg/ml kanamycin, while on antibiotic-free medium the frequency of embryo germination was 72%. Frequencies of 71, 15, and 8% germination were obtained on 50, 100, and 150 µg/ml kanamycin, respectively.

Subsequent selection experiments involved placing cocultivated and control EACs on MS medium supplemented with 50-150 µg/ml kanamycin for germination. Control explants were wounded and cultured in the absence

of *Agrobacterium*. Neither the treated nor the control EACs germinated in the presence of 100 and 150 µg/ml concentration treatments. This reduction in germination, as compared to the earlier experiments described above, was most likely due to a loss of vigor or viability of embryo tissues as a result of the physical wounding and/or bacterial infection. A similar reduction of soybean seed germination was reported following cocultivation procedures and infection with *A. tumefaciens* (Chee et al. 1989). Peanut germination did occur, however, on the 50 µg/ml concentration at a frequency of 20% for both inoculated and control EAs. To assess transformation, the seedlings were assayed for GUS expression by X-gluc histochemical staining. One seedling from the treated group expressed GUS, while all other treated and control seedlings were GUS-. Kanamycin selection was not an efficient indicator of transformation in this particular system with peanut, as both control and treated axes were affected similarly. Therefore, transformation experiments were done without selection.

For transformation, EACs were isolated from the seed of mature peanut plants and wounded as described in the Materials and methods. Approximately 800 EACs were cocultivated, decontaminated, and then planted in autoclaved soil mix. To test for residual *Agrobacteria*, a portion were plated as described, with no observed bacterial growth after 90 d. In order to determine the efficacy of transformation, a portion of the decontaminated embryo axes (EA) were isolated from the EAC units and analyzed by Southern blotting to detect the presence of the expression vector in the tissue. High molecular weight DNA was purified from transformed and control embryo cells, and digested with 4 restriction enzyme combinations, EcoRI, BamHI, ClaI, and EcoRI-ClaI. Blots of the digests were probed with the biotinylated 827 bp MAS promoter of the pMON9793 plasmid. The putatively transformed EAs have the same digest patterns as the plasmid (Fig. 1B). Although this indicates the presence of the plasmid within the embryo tissues, it does not indicate the integration of vector DNA in the peanut genome, as bacterial cells may have been present within the apoplast or vascular system of the EAs.

Therefore, a portion of the EAs were also assayed for GUS activity. After a 3-h staining period at 37°C, 9% were GUS+. In contrast, the control EAs, which resulted from similar wounding and coculture in medium minus *Agrobacterium*, showed no blue coloration. GUS+ staining should be a reliable indicator of transformation, as this *Agrobacterium* strain/vector plasmid combination EHA101/pMON9793 does not stain positive for GUS. This allows for the specific detection of GUS activity derived from early transformation events in tissue that may still have residual *Agrobacterium* cells. In addition, staining incubation periods restricted to 3 h eliminated false positives as described. Thus, the GUS enzyme activity observed in the EAs was most likely due to the transfer of the pMON9793 plasmid from viable *Agrobacteria* into peanut cells adjacent to wound sites.

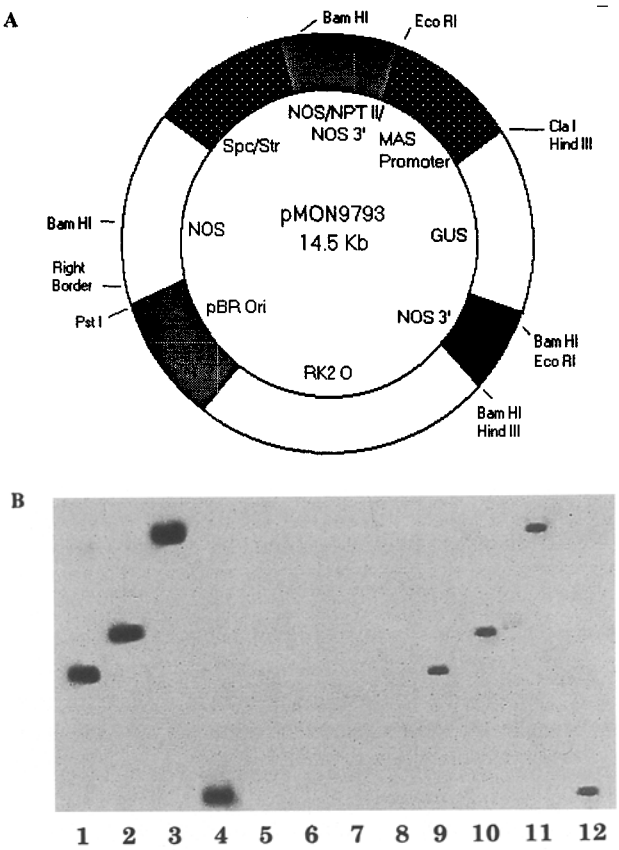

Fig.1. Map of the binary vector, pMON9793, and analysis of peanut DNA. (A) pMON9793. The plasmid contains 2 chimeric genes: the coding region for *B*-glucuronidase (GUS) with a mannopine synthase promoter (MAS) and nopaline synthase (NOS) 3' polyadenylation signal, and the coding region for neomycin phosphotransferase II (NPTII) with a NOS promoter and polyadenylation signal. The restriction sites shown were used in Southern analysis. (B) Autoradiograph from Southern blots of plasmid and genomic DNAs. Hybridization was with a biotinylated probe corresponding to the 827 bp MAS promoter coding region from pMON9793. Fragment sizes correlate with plasmid map above. Lanes: 1-4, plasmid digested with EcoRI *(1.8 kb)*, BamHI *(5 kb)*, ClaI *(14.5 kb)*, EcoRI-ClaI *(827 bp)*, respectively; 5-8, nontransformed control peanut DNA digested with EcoRI, BamHI, ClaI, EcoRI-ClaI; 9-12, DNA of putatively transformed embryo axes digested with EcoRI, BamHI, ClaI, EcoRI-ClaI.

Identification of transformed T_0 peanut plants

Approximately 20% of the EACs survived the physical wounding and *Agrobacterium* infection, and germinated. Identification of peanut seedlings which were putatively transformed was accomplished by assaying for GUS enzyme activity. At the 5-leaf stage, each of the 4 leaflets of each leaf were evaluated. From a total of 120 T_0 plants tested, 11 (9%) expressed GUS in one or more leaflets. Cross sections of these leaves revealed GUS activity in the epidermis, cortex, and vascular tissues. PCR analysis confirmed that GUS+ leaf tissue contained a specific

GUS gene fragment present in the pMON9793 plasmid (data not shown). In contrast, all control leaf samples were GUS- and lacked the PCR product.

To demonstrate the presence of T-DNA in the genome, Southern analyses were performed. Interestingly, not all leaflet tissue from a single identified GUS+ T_0 plant expressed GUS. This suggested chimerism or variable levels of gene expression. Genomic DNA was therefore purified from GUS+ and GUS- leaflet tissue from putative T_0 plant #11, and digested with restriction enzyme ClaI for hybridization analysis. There is a single internal ClaI site in the plasmid which linearizes the coiled DNA to a 14.5 kb sequence (Fig. 2, lane 1). The lack of a 14.5 kb hybridizing fragment in lanes 4 and 5 (plant #11) indicates the absence of free plasmid DNA or residual *Agrobacteria* in the peanut tissue. DNA from the GUS+ leaves hybridized to the MAS promoter probe, but DNA isolated from GUS- leaves did not (Fig. 2, lanes 4,5). These results indicate that only a sector of T_0 plant #11 contained T-DNA, and it was therefore a chimeric transformant. The unexpected size of the hybridizing fragment is discussed below. The amount of transformed tissue in this plant was difficult to assess, as the nature of the transformation event was unknown. It is presumed that the amount of transformed tissue in a T_0 plant depends on the number and location of viable cells initially transformed, and their subsequent dividing patterns. It is expected that the transformed status of T_0 plants resulting from this system will also vary, as the T-DNA transfer is random and not subject to any form of selection.

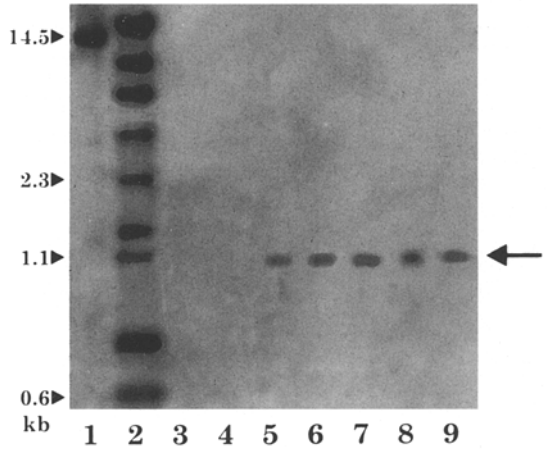

Fig.2. Autoradiograph of a Southern blot of transformed peanut plant T_0 #11 carried through 3 generations. DNA samples (10 µg) were digested with ClaI, resolved by electrophoresis and transferred to a nitrocellulose membrane. The membrane was hybridized with a biotinylated probe corresponding to the 827 bp MAS promoter coding region from pMON9793. Hybridized fragments were detected by autoradiography for 3 h. Lanes: 1, pMON9793 plasmid DNA; 2, HindIII/HaeIII digested lambda DNA marker; 3, nontransformed control peanut plant; 4, GUS- leaf tissue from T_0 #11; 5, GUS+ leaf tissue from T_0 #11; 6-8, T_1 generation plants (11-a, 11-b, 11-c); 9, T_2 generation plant (11-c-1).

The 11 putatively transformed T_0 plants were grown to maturity. Progeny seed were collected from 10 of the T_0 plants, and genomic DNA purified from the T_1 EA. These were analyzed via PCR to detect the presence of specific introduced NPTII and GUS gene sequences. Amplification was with oligonucleotide primers for a 420 bp GUS gene fragment (Jefferson et al. 1986) and a 320 bp NPTII gene fragment (Beck et al. 1982) which are present in the pMON9793 plasmid. PCR analyses revealed that EA DNAs from 9 of the 10 putative T_0 plants yielded bands of the expected sizes (Fig. 3), with positive PCR response ranging from 10-57% of the tested T_1 EAs. Nineteen percent of these T_1 EAs contained only the 420 bp GUS fragment, suggesting possible rearrangement of the plasmid and/or deletion of the NPTII coding region during those specific transformation events. The PCR method was selected to test progeny embryo axes for the presence of the NPTII and GUS marker genes because of its sensitivity for detecting minute amounts of specific DNA sequences and its efficiency for screening a large number of putative transformants (Chee et al. 1991). The analysis of EAs rather than plants of the T_1 generation appeared to be an efficient approach for determining inheritance in chimeric populations. Analysis of select tissues from a growing plant potentially allows small transformed sectors to go undetected. This is not the case with embryo axes. Although they are destructively assayed and therefore not available for subsequent inheritance study, entire genomic DNA of the progeny can be purified and analyzed.

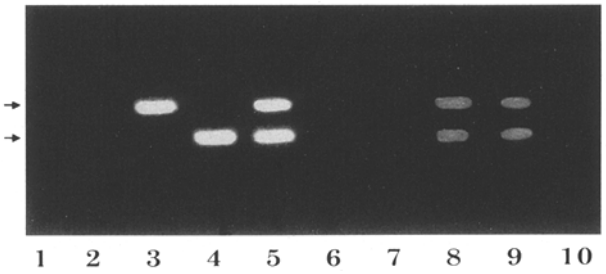

Fig.3. PCR analysis of putatively transformed T_1 embryo axes. Purified DNA was amplified with primers specific for regions of the NPTII and GUS genes contained in the T-DNA. The PCR products (indicated with arrows at appropriate 320 and 420 bp bands) were visualized on an ethidium bromide-stained 2% agarose gel. Lanes: 1, negative control-plasmid DNA without TAQ polymerase; 2, negative control-plasmid DNA with TAQ and no primers; 3, positive control-plasmid DNA with TAQ and GUS primers; 4, positive control-plasmid DNA with TAQ and NPTII primers; 5, positive control-plasmid DNA with TAQ and NPTII + GUS primers; 6-7,10, nontransformed control peanut embryo DNA; 8-9, transformed peanut embryo DNA.

Analysis of T_1 plants

Seed from transgenic T_0 plant #11 was not subjected to the PCR study, but was germinated for further

inheritance study. Twenty T_1 seedlings were analyzed histochemically for GUS expression, and 3 (15%) of the plants (11-a, 11-b and 11-c) were GUS+. Incorporation of T-DNA into the genome of these 3 plants was confirmed by Southern blot analysis. Each of the 3 plants tested showed sequences that hybridized (Fig. 2, lanes 6-8). All of the hybridizing plants showed the same 1 kb size fragment, suggesting that the 3 T_1 plants were derived from the same transformation event in the parent T_0 #11 plant and presumably from the same sectored region of that plant. Indeed, the seed for these 3 T_1 plants were collected from a single branch of T_0 plant #11. The size of the fragment indicates that the integrated vector DNA had undergone substantial rearrangement. The right border sequence of T-DNA, which is responsible for the integration of the vector into the host chromosome, is 7 kb from the ClaI site. Therefore, if the T-DNA had integrated intact within the genome of T_0 #11, the minimum DNA length after ClaI digest would be 7 kb. Since this is not the case, it appears that during this particular transformation event in T_0 #11, the plasmid lost a substantial part of its DNA which included both the NPTII and Spc/Str coding sequences. It appears that this loss may have occurred during integration into the host genomic DNA, because DNA isolated from T_0 EAs shortly after the cocultivation period showed the same digest pattern as the pure plasmid (Fig. 1B). The PCR analyses of T_1 EAs, described above, also support possible T-DNA deletion. Approximately 19% of the PCR GUS+ EAs did not contain the NPTII fragment.

Analysis of T_2 plants

The 3 T_1 progeny plants 11-a, 11-b and 11-c from transgenic T_0 plant #11 yielded 11, 18, and 9 seed, respectively. These were germinated and assayed for GUS expression. Data presented in Table 2 show an approximate 3:1 segregation ratio for the GUS gene in selfed progeny, indicating the presence of a single T-DNA locus. Genomic DNA was isolated from the leaves of 1 T_2 plant, #11-c-1, for transgenic confirmation by Southern hybridization (Fig. 2, lane 9).

Table 2. Histochemical expression of GUS activity in progeny T_2 plants of transgenic peanut T_0 plant #11.

T_1 Transgenics	No. T_2 plants Tested	GUS+	GUS-	3:1 Segregation[a] X^2	P
11-a	11	7	4	0.58	0.45
11-b	18	14	4	0.22	0.64
11-c	9	7	2	0.26	0.61

[a] Goodness of fit to 3:1 ratio tested by Chi-square analysis. Data calculated with df=1.

In conclusion, a protocol to produce transgenic peanut plants from cultured embryo axes of mature seed is described. This system circumvents tissue culture, providing wide application and potentially reducing chromosomal and developmental abnormalities among transgenics. The frequency of transformation was 9% in the T_0 generation, with 10 of 11 transgenic T_0 plants yielding transformed T_1 progeny. Putative transgenic plants were initially assessed and readily identified by GUS expression. An encouraging aspect of this work is that the tissues of the epicotyl and hypocotyledonary axes of peanut zygotic embryos were shown to be competent for transformation. Additional work to further identify competent cell types and their location could lead to more effective means for targeting transformation and minimizing chimerism in the T_0 generation.

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