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Evaluation of peanut (*Arachis hypogaea* L.) leaflets from mature zygotic embryos as recipient tissue for biolistic gene transfer

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Leaflets from mature peanut embryos are a useful recipient tissue for biolistic DNA transfer. Fertile plants were regenerated from leaflets from genotypes representing all botanical types of peanut. Regeneration frequency was strongly influenced by genotype. NPT II and GUS chimaeric gene fusions, driven by the CaMV 35S promoter, were expressed transiently following biolistic delivery to unexpanded leaflets. Bombardment conditions affecting transient expression frequency were determined using a prototype of the Bio Rad PDS 1000/He helium-powered particle acceleration apparatus. Stably transformed calli were derived routinely from leaflet tissue bombarded with the NPT II gene and subsequently cultured on kanamycin. Several plants have been regenerated from treated explants under kanamycin selection. Thus far, none of these has been stably transformed. The occurrence of escapes suggests that kanamycin is an inefficient selective agent for the recovery of transgenic peanuts from this explant. Experiments designed to regenerate plants using published regeneration protocols from stably transformed calli, devoid of primary explant tissue, have been unsuccessful.

Keywords: *Arachis hypogaea*; groundnut; microprojectile; peanut; transformation

Introduction

A gene transfer system for peanut (groundnut, *Arachis hypogaea* L.) would be useful to augment the genetic stocks available for breeding this crop. However, no procedure for transformation of this species has been described. Although certain *Agrobacterium* strains can infect peanut and mediate gene transfer (Dong *et al.*, 1990; Lacorte *et al.*, 1991), no transgenic plants have been derived by this procedure. Microprojectile bombardment (Klein *et al.*, 1987; Sanford, 1988), which has been used successfully to achieve transformation in a number of the most recalcitrant species (Christou *et al.*, 1988, 1989; McCabe *et al.*, 1988; Finer *et al.*, 1990; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Tomes *et al.*, 1990; Robertson *et al.*, 1992), is an attractive alternative for peanut.

The choice of an explant tissue which is both suitable for the bombardment process and which responds favourably in culture is a critical factor in the development of biolistic transformation protocols. Fertile peanut plants have been regenerated from immature embryos (Ozias-Akins, 1989; Sellars *et al.*, 1990), cotyledons (Illingworth, 1968; Atreya *et al.*, 1984; Bahatia *et al.*, 1985; Hazra *et al.*, 1989), embryonic axes (Atreya *et al.*, 1984), and unexpanded leaflets from mature zygotic embryos (Bajaj *et al.*, 1981; Mroginski *et al.*, 1981; Pittman *et al.*, 1983; Seitz *et al.*, 1987; McKently *et al.*, 1990, 1991). Embryonic leaflets are especially appealing for use in the microprojectile bombardment because they have a planar geometry, which facilitates bombardment and selection. In this report, we evaluate the performance of leaflets from mature zygotic embryos as a recipient tissue for biolistic transfer of exogenous DNA to peanut.

Materials and methods

Explant preparation and culture

Mature dry seeds were disinfected with 10% Clorox® bleach (0.5% sodium hypochlorite) for four minutes, rinsed in sterile deionized water and transferred to sterile

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Magenta® boxes half-filled with moistened vermiculite. Seeds were allowed to imbibe for four to eight days at 27° C under low light. After imbibition, seeds were again disinfected for one minute in 10% bleach and rinsed in two changes of sterile water. Unexpanded leaflets, 3–5 mm long, were harvested from leaves of the primary shoot axis. Typically, 6–8 leaflets of the appropriate size could be obtained from each embryo.

Leaflet culture experiments were based on the protocols of Mroginski *et al.* (1981) in which plants were recovered through primary organogenesis from unexpanded leaflets cultured on MS media (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 3.0% sucrose, and various concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BAP). Three botanically diverse genotypes were evaluated: UPL Pn 4 (*A. hypogaea* var. *fastigiata* (Valencia)), Tamnut 74 (*A. hypogaea* var. *vulgaris* (Spanish)) and NC7 (*A. hypogaea* var. *hypogaea* (Virginia)). The culture response of embryonic leaflets was evaluated over a 0–7.5 mg l^{-1} range of NAA and 2–16 mg l^{-1} range of BAP, in a total of 23 growth regulator combinations. Experiments were arranged in a completely random design with 3 replications. Each replication included 4 or 8 leaflets, with replicate size constant within each experiment.

After three weeks in culture, callus and rooting responses were visually assessed and assigned ratings based on a scale from 1 to 3, with 1 representing no response, and 3 indicating the greatest response. The number of buds formed on each explant was also recorded at three weeks. Shoots were excised and placed on rooting medium (MS salts, B5 vitamins, 3% sucrose with 1 mg l^{-1} NAA). Plants were transferred to soil after 2–3 weeks on rooting medium. The total number of plants which rooted and were capable of sustained growth in soil was recorded for 6 of the 23 growth regulator ratios tested.

Genetic constructions

Optimization of bombardment parameters and bombardments for recovery of stable transformants were carried out using a single plasmid, pRT 99 GUS (Topfer *et al.*, 1988). This vector, 6.7 kilobase pairs (kb) in length, carries two chimaeric genes, one encoding neomycin phosphotransferase (NPT II) (Beck *et al.*, 1982), the other encoding β-glucuronidase (GUS) (Jefferson *et al.*, 1987). Both genes are driven by identical cauliflower mosaic virus 35S (CaMV 35S) promoter sequences. Both are terminated by nopaline synthase poly-adenylation sequences.

Plasmid DNA used in bombardment work was prepared as described by Birnboim and Doly (1979), followed by two equilibrium centrifugation in caesium chloride–ethidium bromide gradients (Sambrook *et al.*, 1989), and quantified by fluorometry in a Hoefer Mod. TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA), using the manufacturer's protocols.

Microprojectile bombardment

All bombardments with tungsten particles were carried out with a prototype of the PDS-1000 particle acceleration apparatus (Bio-Rad Corporation, Richmond, CA, USA). Tungsten particles were prepared as described previously (Klein *et al.*, 1987). Each bombardment delivered approximately 0.36 μg DNA on approximately 214 μg of tungsten particles.

Particles were accelerated in the PDS-1000 with standard (grey) DuPont accelerator cartridges and DuPont macroprojectiles and stopping plates. Macroprojectile diameter varied somewhat between production lots, causing unavoidable variation in microprojectile velocity between treatments. DuPont 'red seals', which seal the acceleration tube to prevent loss of gases around the macroprojectile, were used to reduce this variation. A sixty mesh stainless steel screen was placed approximately 3.5 cm below the stopping plate when red seals were used. This screen lessened tissue damage and aided in microprojectile dispersal (Gordon-Kamm *et al.*, 1990). The distance between the stopping plate and the tissue was 11 cm. Bombardment was carried out under a vacuum of 71 cm Hg.

Gold particles were accelerated with a prototype model of the PDS-1000/He apparatus (Bio-Rad Corporation, Richmond, CA), in which motive force for particle acceleration is provided by the explosive release of compressed helium. Gold particles were prepared following the manufacturer's protocol. Each bombardment with the PDS-1000/He delivered approximately 0.83 μg DNA and 500 μg of gold particles. In experiments designed to test the effect of increasing the ratio of DNA to particle mass, each bombardment delivered 0.83, 1.25, or 1.67 μg of DNA, while particle mass was held constant at 500 μg.

All bombardments in the PDS 1000/He were made with the same apparatus configuration. The launch assembly was placed 5 mm below the rupture disc retaining nut. The distance between the macrocarrier holder and stopping screen was 12 mm. Bombardment distance was either 7.9 cm or 4.7 cm from the launch assembly to the sample platform. Samples were bombarded under a vacuum of 71 cm Hg. Tests of DNA load, particle size and multiple bombardment effects were made at a rupture disc pressure of 1800 psi. DNA load, rupture disc pressure, and multiple bombardment effects were tested using 1.0 μm gold particles.

Molecular and biochemical testing of transgenic tissues

Putatively transformed calli were tested for the presence of the NPT enzyme by a commercial enzyme-linked immunosorbent assay (ELISA), using extracts from 75–150 mg of callus tissue according to the manufacturer's protocols (5′–3′, Inc., 117 Brandywine Parkway, West Chester, PA 19380, USA). Assays were standardized against authentic NPT II enzyme.

β-Glucuronidase histochemical and fluorometric assays were carried out essentially as described by Jefferson *et al.* (1987). The histochemical assay substrate solution contained 0.3% (w/v) of 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) and 0.35% (v/v) Triton X-100. For the fluorometric assay, samples of approximately 75–115 mg were homogenized on ice, and protein concentrations were adjusted so that all isolates were assayed at the same total protein concentration. Fluorescence was determined in a Hoefer Mod. TKO 100 fluorometer. Production of 4-methyl umbelliferone (4-Mu), expressed as picamoles of 4-Mu per minute per microgram of total protein, was standardized against fluorescence of a known solution of 4-Mu.

Total DNA was extracted from calli as described (Doyle and Doyle, 1989) for use in both polymerase chain reaction (PCR) and DNA hybridization analyses. PCR amplification was used initially to test putative transgenics for the presence of DNA sequences from the introduced plasmid. Oligonucleotide primers used to detect pRT 99 GUS sequences in putative transformant tissues were described by Robertson *et al.* (1992). One pair of 25 nucleotide (nt) primers was designed to amplify a 792 bp fragment from the NPT II coding sequence of pRT99 GUS. Another pair of 25 nt primers amplified the 1800 bp GUS open reading frame (ORF).

Samples of 50–100 ng of DNA were subjected to amplification in a Perkin Elmer Cetus PCR thermal cycler (Perkin Elmer Instrument Division, Norwalk, CT). Samples were denatured at 94° C for 5 min, followed by 30 cycles of 2 min at 92° C, 1 min at 59° C, and 2 min at 72° C.

DNA extracted from each callus line was digested with *Hin* dIII and separated by agarose gel electrophoresis prior to transfer to MSI nylon membranes (Fisher Scientific). Subsequent hybridization analysis parameters were based on a protocol by Sambrook *et al.* (1989). Blots were probed with GUS or NPT II coding sequences, labelled with ^{32}P-dCTP by random primed synthesis (Sambrook *et al.*, 1989).

Estimation of stable transformation frequency

Large-scale bombardment experiments were carried out with two peanut genotypes, UPL PN 4 and Tamnut 74, to estimate the frequency at which stably transformed tissues could be recovered from bombarded leaflets, and to determine the relationship between transient expression frequency and stable transformation efficiency. All bombardments in these experiments were carried out at a bombardment distance of 4.7 cm from the launch assembly with 1.0 μm gold particles (500 μg per bombardment), 1.67 μg DNA, at a rupture disc pressure of 1800 psi. Explants were bombarded once. Each experiment included 19 to 22 plates, with 24 leaflets per plate.

Twenty-four hours after bombardment, nine leaflets from each plate were assayed for transient GUS expression. The remaining 15 leaflets were transferred to medium containing 50 mg l^{-1} of kanamycin, 2 mg l^{-1} NAA and 4 mg l^{-1} BAP. Explants from each bombarded plate were distributed among three plates of selective medium. Leaflets on selection were subcultured at two-week intervals. The frequency at which kanamycin resistant calli were recovered was determined for each genotype across experiments. Samples of callus lines obtained from genotype UPL PN 4 during these experiments were subsequently characterized.

Results

Culture of embryonic leaflets

Culture response of leaflets on 6 of the 23 growth regulator ratios tested are summarized in Table 1. Culture response varied among genotypes tested. BAP levels of from 10 to 16 mg l^{-1}, in combination with NAA levels of less than 5 mg l^{-1} were generally detrimental to culture response (data not shown). Root and callus proliferation of genotypes Tamnut 74 and NC 7 exceeded that of UPL PN 4 (within range of ratios listed in Table 1), while bud formation in UPL PN 4 and Tamnut 74 was superior to that of NC 7. UPL PN 4 produced shoots at a higher frequency than either of the other genotypes after three weeks in culture. Genotype effect was the only statistically significant parameter governing bud production frequency (differentiated by orthogonal contrasts $p < 0.05$). Within a genotype, the effect of the ratio of growth regulators on bud production was not significant (p=0.05). A BAP level of 4 mg l^{-1}, in general, was superior to 2 mg l^{-1} across the three genotypes evaluated. No growth regulator ratio appeared optimal across the genotypes. NAA levels of 1 or 2 mg l^{-1} and a BAP level of 4 mg l^{-1} were utilized in subsequent bombardment experiments. All plants regenerated from cultured material were fertile. While no plants were recovered from cultures of NC 7 in these experiments, plants of this genotype were recovered from bombarded leaflets in later experiments.

Transient expression and optimization of bombardment conditions

Plasmid DNA of pRT 99 GUS was precipitated onto tungsten particles 1.1 μm in diameter (DuPont M17), and delivered to the test tissue by a single bombardment with the PDS-1000 (gunpowder) apparatus. Bombarded leaflets were subjected to GUS histochemical assays after 24 h. GUS-positive cell foci were observed across the entire bombarded surface in embryonic leaflets from four-day-old germlings, indicating successful transfer of exogenous DNA into this tissue. This result also indicates that the physiological condition of the tissue at this stage of development permits expression of chimaeric genes with

Table 1. Response of embryonic leaflets cultured on media with various levels of NAA and BAP. Regeneration frequencies are the combined results of 2 experiments, each arranged in a completely random design with either 3 or 4 replications. NAA:BAP ratio is expressed in mg l^{-1}. 'Bud production' represents total number of leaflets with buds per total number of leaflets cultured. 'Plant production' represents the total number of mature plants (i.e. rooted and capable of sustained growth in soil) regenerated per leaflet cultured

Genotype	*NAA: BAP ratio*	*Shoot production* %	*Plant production* %
UPL PN-4	0.8:2	5.5	0
	1.6:2	20.0	8.3
	2:2	21.4	12.1
	0.8:4	25.0	7.1
	1.6:4	17.8	0
	2:4	15.9	12.1
Tamnut-74	0.8:2	16.6	3.7
	1.6:2	0.0	0
	2:2	7.2	0
	0.8:4	14.6	5.9
	1.6:4	11.1	3.0
	2:4	11.7	0
NC-7	0.8:2	0	0
	1.6:2	0	0
	2:2	0	0
	0.8:4	3.0	0
	1.6:4	0	0
	2:4	5.6	0

the 35S promoter, suggesting that such constructs should be useful for recovering transformants.

Limited information is available on the relationship between transiently expressing cells and the formation of stable integration events, although the frequencies of these events are thought to be positively correlated (Fromm *et al.*, 1990). Therefore, experiments designed to maximize the frequency of GUS transient expression events were performed. Several variables were evaluated to determine optimum conditions for treatment of leaflets with the PDS-1000 (gunpowder) apparatus. These included particle size, microprojectile travel distance, incorporation of mesh screens between tissue and stopping plate, and multiple bombardments.

Embryonic leaflets from genotypes representing all the botanical types of peanut (Tamnut 74, UPL PN 4, Florunner and NC 7) were capable of transient GUS expression. Data generated with the PDS-1000 exhibited large experimental errors typical of the gunpowder-driven apparatus. Only experiments evaluating the effect of multiple bombardments per plate gave statistically significant results. The frequency of GUS expressing foci on leaflets bombarded twice was significantly lower than on leaflets bombarded once (data not shown).

Optimization experiments with the prototype PDS-1000/He displayed a marked decrease in shot-to-shot variation, and apparent increase in mean frequency of GUS positive foci. Experiments evaluating rupture disc burst pressure, particle size, multiple bombardments and DNA load produced statistically significant results (Table 2). In general, the best transient expression results were observed with 1× bombardment, 1.25 μg DNA per bombardment event, utilizing the 1.0 μm gold particles.

Selection of stably transformed calli

Subsets of bombarded leaflets from various optimization experiments were placed on MS medium with 50 mg l^{-1} of kanamycin sulfate, from 1 to 16 days following bombardment. This level of antibiotic prevented shoot formation and significantly retarded growth of callus on untreated leaflets. Leaflets were placed so that the abaxial (bombarded) surface of the leaflet was in contact with the medium. Leaflets were transferred to fresh selective medium at 14-day intervals. Rapidly growing chlorophyllous cell masses were observed on leaflets after 1 to 4 months in culture. These cell masses were dissected from the explant and cultured on MS medium with 50 mg l^{-1} of kanamycin. A total of eight kanamycin-resistant cell

Table 2. Effect of DNA load, particle size and bombardment number on frequency of transiently expressing cell foci observed after bombardment of leaflets (genotype NC 7) with pRT99 GUS. 'GUS' values represent the mean number of blue staining cell foci (ca 1–10 cells per focus) observed on each leaflet following GUS histochemical assays (N equals the number of leaflets assayed). Numbers in parenthesis are the standard deviations. Numbers followed by the same letter, within an experiment, are not significantly different at $p = 0.05$ level. All experiments were arranged in a complete random design with 4–5 replications.

Parameter tested	*GUS*	*N*
DNA load/bombardment		
0.83 µg	27.8 (32.4) B	55
1.25 µg	50.0 (43.0) A	55
1.67 µg	45.4 (41.2) A	57
Particle size (gold)		
1.0 µm	33.0 (31.6) C	27
1.6 µm	4.5 (5.9) D	27
Multiple bombardments		
1×	15.6 (24.5) E	36
2×	3.9 (4.7) F	32
3×	8.1 (9.6) F	34

masses were recovered in this way from the optimization experiments (PDS-1000) and were subsequently characterized.

Analysis of transformed tissues

Eight putatively transformed callus lines, obtained from the subset of leaflets placed on selection from the optimization experiments (PDS-1000), were grown on four levels of kanamycin (0, 25, 50, 100 mg l^{-1}, or 0, 50, 100, 200 mg l^{-1} of kanamycin sulfate, in two different experiments), and the mass of each culture was measured at weekly intervals. The growth rate of each callus line, at each kanamycin concentration, could then be determined. The resulting growth rates were then differentiated within a callus line across kanamycin levels by orthogonal contrasts, and orthogonal polynomials were used to separate the linear and curvilinear components of the 'kanamycin' and 'time' main effects. These statistical analyses were conducted to test the hypothesis that growth rate of a transgenic callus line was independent of the level of antibiotic applied to the culture. The growth rates of transgenic callus lines were not significantly reduced at kanamycin levels up to 200 mg l^{-1} (Fig. 1). An exception was callus line P1E173, in which a significant reduction in growth rate occurred at the 100 mg l^{-1} level in the second of two trials. The non-transgenic control callus displayed a significant reduction in growth rate in both trials at 50 mg l^{-1} kanamycin. The linear component of the kanamycin main effect on growth rate within the non-transgenic callus line accounts for the majority of the sum of squares, indicating a direct inverse relationship between kanamycin levels and growth rate of untransformed peanut tissue.

Putatively transformed calli were assayed for the presence of NPT II and GUS gene products (Table 3). The eight callus lines tested were all positive for NPT II, although NPT II levels varied among callus lines. Callus line P26E146 produced the highest level of NPT II (3.80 ng NPT II per µg total protein) while P1E173 produced the least NPT II (0.11 ng NPT II per µg total protein). Three of the eight callus lines tested were also positive for GUS as determined by fluorometric assays. Two callus lines, P14E146 and P37E146, maintained constant levels of GUS expression, while GUS expression fluctuated over time in line P1E173. Callus line P42E146 had a GUS activity of only 0.007 nmol 4-Mu min^{-1} μg^{-1} protein (approximately three times that of the untransformed control). Lines P14E146, P37E146 and P1E173 reacted positively (i.e. stained blue) in the GUS histochemical assay. Neither line P42E146 nor any of the other lines gave a positive reaction in this assay.

Putative transgenic calli were analysed by PCR for the presence of the NPT II and GUS open reading frames (ORF). They were also analysed for the presence of an intergenic region of pRT99 GUS, which included the poly-adenylation signal 3′ of NPT II, an intergenic (plasmid) sequence, and the CaMV 35S promoter of the chimaeric GUS gene. All PCR products were subsequently subjected to gel electrophoresis, transferred to nylon membranes and probed with the internal *Pst* I fragment of the NPT II cassette (NPT II ORF product) or the entire GUS cassette (GUS ORF and intergenic products) of pRT99 GUS in order to verify the authenticity of amplification products.

PCR analysis using NPT II primers and DNA from each of the calli tested amplified products of the expected size for the NPT II ORF, and these products hybridized with the internal *Pst* I fragment of the NPT II cassette (Fig. 2). PCR with GUS or intergenic primers generated products of expected sizes diagnostic for these sequences, and subsequent hybridization results confirmed the identity of these products (data not shown).

Southern blots were performed using total DNA extracted from transgenic calli to determine whether introduced sequences were integrated into the peanut genome. Probing blots with either NPT II or GUS ORF sequences resulted in hybridization with multiple digestion fragments from each sample (Fig. 3). Some of these bands, in samples from all calli tested, exceeded the molecular weights of fragments expected from the digestion of free plasmid. Hybridization patterns produced with both NPT II and GUS probes are strongly suggestive

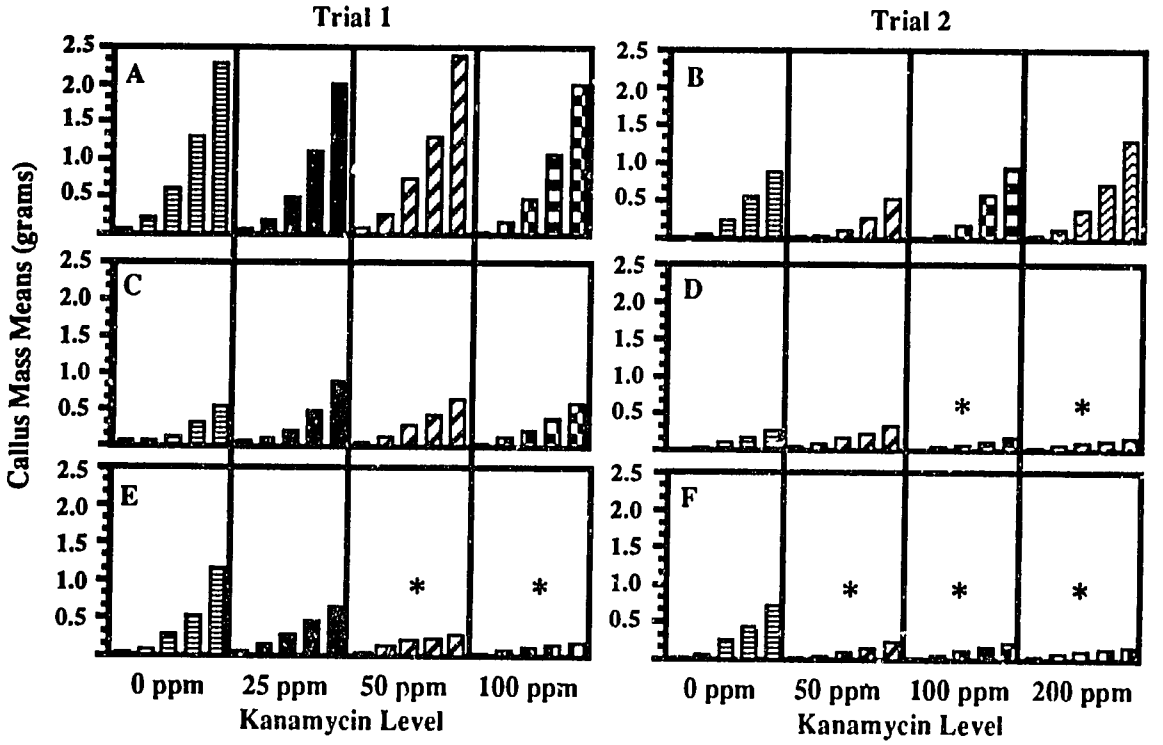

Fig. 1. Growth rates of two representative transgenic callus lines, P26E146 (A and B), P1E173 (C and D), and negative control, P13E135 (E and F) on media containing various concentrations of kanamycin. Each bar within a kanamycin level represents the mean callus mass at each time point (weekly intervals) from initial mass up to 4 weeks in culture. *Indicates significant (p=0.05) reduction in the growth rate of a callus line as compared to the same callus line under the 0 ppm kanamycin level. Growth rates were differentiated by partitioning the kanamycin main effect utilizing orthogonal contrasts (Steel *et al.*, 1980). Each trial was arranged in a completely random design with four replications per kanamycin level within each callus line.

Table 3. Results of biochemical assays of transformed and control callus lines for stable expression of NPT II and GUS. GUS activity is expressed as nanomoles 4-Mu min^{-1} μg^{-1} protein. Each value is the mean of 4-6 independent assays. Numbers in parentheses are the standard deviation. (—) indicates that the sample was assayed once. NPT II levels are expressed as ng NPT II μg^{-1} total protein. Values were tabulated by averaging 4-7 wells over 1-2 dilutions of protein extracts (protein levels ranged from 0.33-0.0023 µg total protein per well) from each callus line. P13E135 is a negative control; '--' indicates that the negative control callus line was used as a correction factor (NPT II data only) and therefore by definition is zero.

Callus line	*GUS activity*	*NPT II level*
P26E146	0.002 (—)	3.80
P8E146	0.002 (—)	0.64
P14E146	2.81 (± 1.74)	3.61
P33E146	0.003 (± 0.003)	1.03
P37E146	1.24 (± 0.911)	1.67
P42E146	0.007 (—)	1.44
P11E135	0.003 (± 0.001)	0.49
P1E173	0.94 (± 1.01)	0.11
P13E135	0.002 (± 0.001)	--

of complex patterns of transgene integration, perhaps involving multiple integration events.

Estimation of stable transformation frequency

Combined results of three large-scale bombardment experiments conducted with UPL PN 4 and two of the three experiments with Tamnut 74 are given in Table 4. A total of 237 rapidly growing chlorophyllous cell masses were recovered from treated UPL PN 4 leaflets. Of these, 202 were capable of continued growth when transferred to medium containing 50 $\mu g\ l^{-1}$ of kanamycin. Two experiments with Tamnut 74 yielded 22 cell masses, 15 of which were capable of continued growth on selective medium when subcultured. Extensive necrosis of Tamnut 74 leaflet tissue, relative to that of UPL PN 4, required termination of selection experiments in Tamnut 74 approximately two weeks sooner than UPL PN 4 leaflets.

Transient GUS expression levels 24 hours after bombardment could not be correlated with recovery of stably growing callus masses within the UPL PN 4 experiments. Linear regression analysis was performed, on the combined data from the UPL PN 4 experiments, regressing mean GUS foci/(leaflet)(plate) versus recovery of stable callus masses per plate. No significant correlation

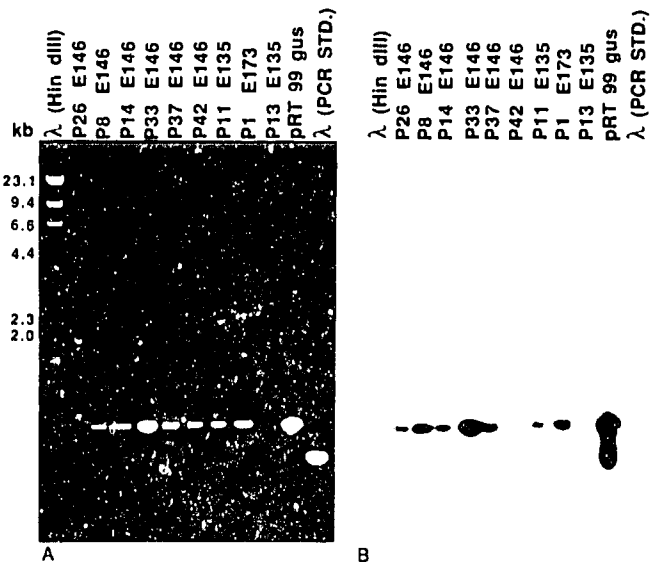

Fig. 2. PCR analysis of transformed and control callus lines. (A) PCR amplification products generated with primers npt4and npt6 (selectively amplifies NPT II ORF). (B) Hybridization analysis of PCR products from (A) probed with the internal *Pst* I fragment of the NPT II cassette. Lane 1: λ *Hin* dIII DNA; Lanes 2–9: DNA from putatively transgenic callus lines P26E146, P8E146, P14E146, P33E146, P37E146, P42E146, P11E135 and P1E173. Lane 10: p13E135 (negative control). Lane 11: pRT99-GUS (37 ng/reaction). Lane 12: 500 bp λ internal (PCR) control.

was observed (r=0.031). Combined results of mean GUS foci per leaflet, for both genotypes, are listed in Table 4.

Ten callus lines selected from each of three experiments with UPL PN 4 were subsequently characterized by biochemical assays for NPT II and GUS expression, and by PCR analysis for the presence of each of the marker gene sequences. All 30 callus lines showed a positive ELISA reaction for NPT II. Eight of these also showed GUS activity. PCR analysis revealed that all 30 lines contained the NPT II coding sequence. Twenty-eight of these lines also contained the 1800 bp GUS coding sequence.

Regeneration of plants from bombarded tissues

Ten fertile plants have been recovered from bombarded leaflets of genotypes NC 7 and from Tamnut 74 cultured under kanamycin selection for protracted periods. Selection stringency varied among experiments. Four plants were derived from bombarded leaflet tissue cultured on medium containing 1 mg l^{-1} NAA, 4 mg l^{-1} BAP and 50 mg l^{-1} kanamycin for 72 days. Responding explants were transferred to medium containing 25 mg l^{-1} kanamycin for an additional 47 days, prior to excision of shoots. Two plants were derived from bombarded leaflet tissue cultured on medium containing 2 mg l^{-1} NAA, 4 mg $^{-1}$ BAP and 50 mg l^{-1} kanamycin for 75 days prior to excising shoots. Four plants were regenerated from bombarded explants cultured on medium containing 2 mg l^{-1} NAA, 4 mg l^{-1} BAP and 50 mg l^{-1} kanamycin for approximately 100 days. Shoots were excised after elongation on non-selective medium for an additional month. In all experiments, shoots were rooted without selection.

Seven of these plants have been subjected to biochemical and molecular analysis. All were negative in the NPT II ELISA. PCR and Southern-blot analysis failed to reveal the presence of NPT or GUS coding sequences in any of these plants. Progeny from four of the seven primary regenerants were also tested, in an attempt to detect chimaeric transgenic sectors that might have been present in the original regenerant. All were negative in both NPT II ELISA and PCR analysis.

One plant, P13E215, produced callus tissue at the base of the shoot while on rooting medium. NPT II ELISA of this callus tissue was positive for the production of NPT II, but assay of the shoot failed to detect the protein.

One of 875 leaflets in a large-scale experiment with genotype UPL PN-4 produced a shoot while under kanamycin selection. Roots never formed on this shoot, however, and it subsequently died in culture.

Attempts to regenerate plants from transgenic callus lines devoid of primary explant tissue have so far been unsuccessful. An array of culture experiments tested the effect of various combinations of growth regulators on the callus, in experiments designed to regenerate shoots. No shoot formation was observed in these callus lines, although root formation has occurred sporadically within some lines maintained on medium containing 2 mg l^{-1} NAA and 4 mg l^{-1} BAP, with 50 mg l^{-1} kanamycin.

Discussion

We report here on the feasibility of using embryonic leaflets as recipient tissue for biolistic transfer of exogenous DNA into peanut. Because biolistic transformation efficiency is dependent upon efficient regeneration, we first evaluated the regeneration response of selected genotypes. Our results are consistent with results reported by others (Mroginski *et al.*, 1981; Pittman *et al.*, 1983; McKently *et al.*, 1991). Shoot regeneration response is strongly affected by genotype, age of explants, and growth regulator concentrations. NAA and BAP were found to be effective growth regulators for regeneration of shoots from immature leaflets of all genotypes tested; however, no ratio was optimal across genotypes.

NPT II and GUS chimaeric gene fusions, driven by the CaMV 35S promoter, are expressed transiently in unexpanded leaflets, although expression declined as leaflets expanded beyond approximately 5 mm in length (data not shown). Transient expression data suggest that the planar

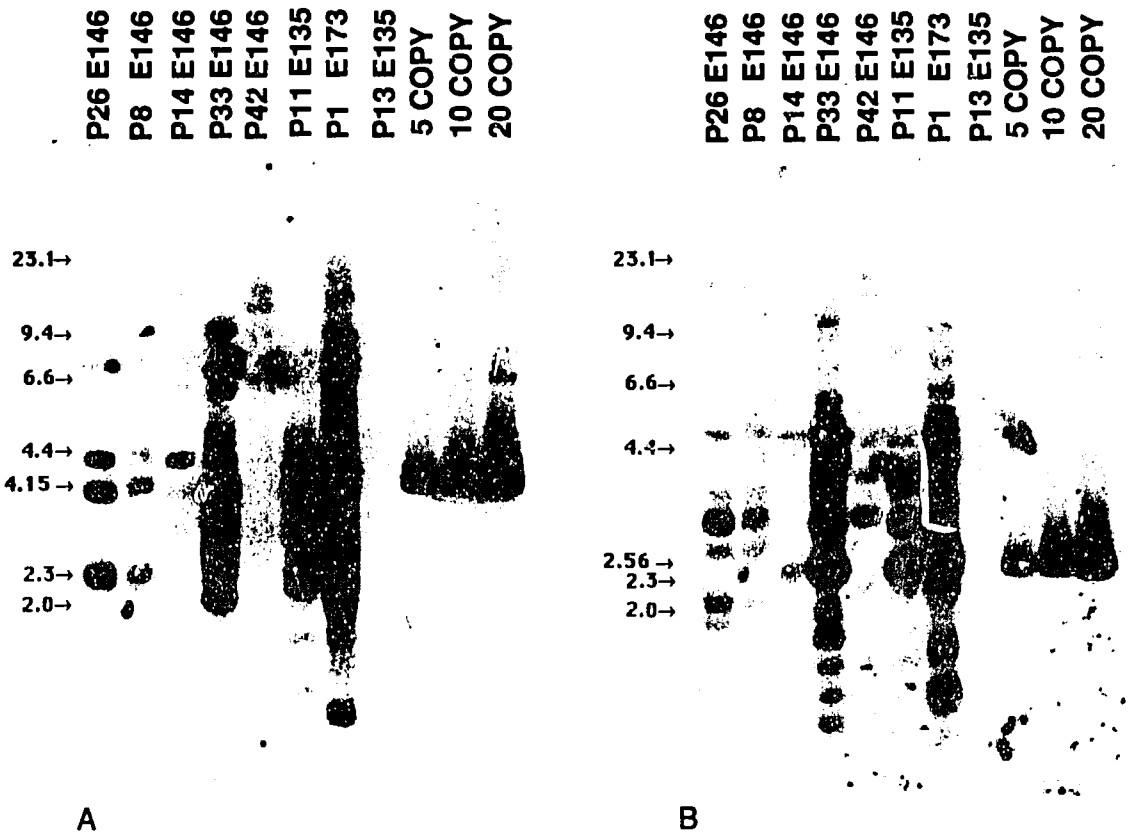

Fig. 3: Southern blot analysis of DNA from callus lines probed with the NPT II (A) and GUS (B) PCR generated ORFs. Total DNA (10 µg) from each callus line, digested with *Hin* dIII, was loaded per lane. P13E135 is untransformed callus used as negative control. Positive controls consisted of 29.9 pg (approximately 5 copies/somatic cell (Resslar *et al.*, 1981), 56.7 pg (approximately 10 copies), and 116 pg (approximately 20 copies) of *Hin* dIII digested pRT99-GUS.

Table 4. Frequency of recovery of stably growing callus masses derived from 3 experiments with UPL PN 4 and 2 experiments with Tamnut 74. Each experiment consisted of 19–22 plates with 24 leaflets per plate. Twenty-four hours after bombardment 15 leaflets from each plate were distributed onto three selective medium plates (MS salts, B5 vitamins, 2 mg l^{-1} NAA, 4 mg l^{-1} BAP with 50 mg l^{-1} kanamycin). Leaflets were subcultured at approximately two-week intervals. A chlorophyllous mass isolated from leaflet tissue was considered stably growing if it was capable of continued growth on selection for two additional subcultures (approximately four weeks) after isolation. Mean GUS foci per leaflet was determined 24 h after bombardment (see Materials and methods).

	UPL PN 4	*Tamnut 74*
Number of leaflets cultured	875	600
Number of stably growing calli	202	15
Frequency of calli per leaflet	0.231	0.025
Number of leaflets assayed for GUS transient expression	358	301
Mean GUS foci per leaflet	7.9	24.8

geometry of unexpanded leaflets presents an ideal target for efficient DNA delivery by the biolistic process.

Optimization of bombardment parameters with the PDS-1000/He prototype apparatus was highly instructive. Gold particles1.0 µm in diameter were found to produce transient expression events at significantly higher frequency than could be obtained with 1.6 µm particles. Transient expression frequency increased with increasing DNA load and with increased rupture-disc burst pressure. Frequency of transient expression events was not enhanced by multiple bombardments. Published reports on the influence of multiple bombardments on transient expression are apparently conflicting (Gordon-Kamm *et al.*, 1990; Goldfarb *et al.*, 1992). It is probable that this factor interacts strongly with other factors, such as tissue type, particle velocity and particle diameter, so this result should be viewed with caution.

Differential growth rates were established between transformed and non-transformed tissues by subjecting bombarded leaflets to a moderate level of kanamycin. Transformed calli were selected unambiguously from bombarded tissues by culturing treated explants on

medium containing 50 mg l^{-1} of the antibiotic. The rapid growth rate and green pigmentation of transgenic calli made them readily distinguishable. Quantitative analysis of growth rates of calli unequivocally demonstrated that transformed callus exhibits more rapid growth on selective media than does control tissue. This differential growth rate was sustained in most lines under increased selection stringency (Fig. 1).

Transient expression could not be correlated with recovery of stably growing callus masses. Transient expression does not appear to be a good predictor of integration events (based on recovery of callus lines). However, it is a useful indicator for the successful delivery of DNA.

All putatively transformed calli produced levels of the NPT II enzyme detectable by ELISA. All lines tested carried the NPT II coding sequence. Cotransformation also occurred with high frequency. GUS sequences were detected by DNA analysis in all but two of 38 calli tested (callus lines derived from both optimization and large scale bombardment experiments). However, only 11 of 38 lines tested expressed GUS at levels detectable by the fluorometric assay procedure. This result is not unexpected, since it is widely reported that transgene expression varies, and expression of the GUS gene was not required for recovery of the transformed calli.

Although several plants were regenerated under selection, none was found to be stably transformed. This result is puzzling, since 50 mg l^{-1} of kanamycin prevented differentiation of shoots from leaflet tissue in earlier experiments. Further, all calli recovered under selection were stably transformed. Regeneration experiments with stably transformed calli suggest, however, that these plants were regenerated from remnants of the primary explant, rather than from stably transformed callus tissue. Where stably transformed (kanamycin-resistant) callus was associated with the regenerating shoot (clearly demonstrated, in one instance), detoxification of the selective agent by the transgenic callus could have permitted the shoot to escape selection.

The occurrence of frequent escapes suggests that kanamycin is an inefficient selective agent for use with this explant. An alternative selection scheme that does not require a detoxification mechanism (e.g. methotrexate) may be more appropriate, since it would prevent cross-protection between transformed callus and untransformed shoot tissue.

Unexpanded embryonic leaflets are promising recipient explants for biolistic DNA delivery to peanut. Successful recovery of transgenic plants from this tissue, however, may require development of more efficient culture, selection and regeneration protocols for primary explants and calli.

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