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Regeneration of transgenic peanut plants from stably transformed embryogenic callus

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Abstract

Embryogenic tissue cultures of *Arachis hypogaea* L. (peanut or groundnut), have been transformed via microprojectile bombardment. We introduced a gene (*hph*) conferring resistance to the antibiotic hygromycin under the control of the CaMV 35S promoter. Selection for resistant callus was initiated 4–5 weeks post-bombardment on medium containing 10–20 mg/l hygromycin. Twelve percent of the bombardments resulted in recovery of a transgenic cell line. An average of two transgenic embryogenic cell lines was isolated per bombardment experiment over 4–6 months of continuous selection. Each bombardment experiment consisted of 11–19 plates, and each plate contained approximately fourteen 25 mm^2 embryogenic callus pieces. Thus, nearly 1% of the bombarded callus pieces produced a stably transformed cell line. Over 100 plants have been regenerated collectively from all of the transformed cell lines. The presence and integration of foreign DNA in hygromycin-resistant callus lines and regenerated plants has been confirmed by polymerase chain reaction amplification of a defined portion of the chimeric gene and by Southern hybridization analysis. Hygromycin resistance was expressed in leaflets from transformed plants which remained green when cultured on basal medium containing hygromycin. Leaflets from control, non-transformed plants turned brown within 3 weeks on the hygromycin-containing medium.

Key words: Arachis hypogaea; Groundnut; Hygromycin resistance; Plant transformation

1. Introduction

Arachis hypogaea L. is a legume crop that is grown primarily for its seed which are used for human consumption and oil extraction. Commercial production of this sub-tropical crop is confined to the southern regions of the United States; however, on a global scale, peanut production has a significant impact in tropical and sub-tropical regions of North and South America, Africa, and Asia. Disease pressures vary among continents

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and adapted germplasm, but production and quality can be severely limited under less than ideal growing conditions. Genetic engineering is one means of transferring individual genes for pest resistance or other traits into elite germplasm of a cultivated species [1,2]. It is a more rapid alternative to, but not a complete substitute for, incorporation of desirable traits from the readily accessible gene pool of a species.

Transgenic peanut callus has been produced via *Agrobacterium*- and biolistic mediated transformation [3,4]. Regeneration of transgenic peanut plants has not yet been reported. As a recipient tissue for foreign DNA, we have chosen to use a callus culture system, i.e., repetitive embryogenesis, that has the advantages of long-term maintenance, and thus constant availability, as well as application to multiple genotypes [5]. Stable transformation effected by microprojectile bombardment has been successful in a number of plants, but most commonly with cell suspension cultures or explanted tissues such as leaf pieces, rather than callus cultures. Microprojectile bombardment uses high velocity particles to penetrate cell walls and to deliver DNA into intact plant cells [6]. This circumvents the host-range limitations of *Agrobacterium*, and the difficulties associated with plant regeneration from protoplasts. Microprojectile bombardment has been used successfully to transform tobacco [7], soybean [8], cotton [9], maize [10], papaya [11], cranberry [12], and wheat [13]. In several of these studies, fertile plants have been regenerated [14]. The biolistic method was considered to be the most effective approach to the transformation of peanut given the potential for problems associated with genotype- or tissue-*Agrobacterium* strain specificity reported for many legumes [15]. The present study was designed to develop a transformation system for peanut that potentially would be applicable to a wide range of genotypes. Introduction of specific genes that may impact crop production can now be initiated.

2. Materials and methods

2.1. Plant material, culture initiation and maintenance

Peanut plants (cultivars Toalson and Florunner) were grown in the greenhouse in 15-inch diameter pots containing a 1:1 (v/v) mixture of potting mix and sand or field soil. Immature pods from self pollinations were harvested 3–4 weeks after the pegs had penetrated the soil. Pods were scrubbed in a mild detergent solution and rinsed for 1 min in 70% (v/v) ethanol followed by deionized water. Immature seeds were removed from the surface-cleaned pods and placed in water until all had been collected. The seeds were surface sterilized by sequential treatment for 1 min in 70% ethanol, 20 min in 1% (w/v) NaOCl, and 4 rinses with sterile deionized water. The immature embryo was removed from the seed and the 2 cotyledons were separated from the shoot-root axis. All 3 embryo parts were cultured on one half of a 10 cm plastic culture dish with the abaxial surface of the cotyledons in contact with the medium. The initiation medium was composed of Murashige and Skoog (MS) [16] salts and vitamins, 3% sucrose, 0.5 mg/l picloram, and 0.8% agar. The pH was adjusted to 5.8 prior to autoclaving. After initiation of somatic embryos and embryogenic callus, long-term maintenance of embryogenic callus took place by subculture at 4-week intervals on the same medium as above except with 3 mg/l picloram and 1 g/l filter-sterilized glutamine (hereafter referred to as maintenance medium). All embryogenic cultures were grown in the dark at 28°C.

2.2. Plasmid constructs and particle preparation

Plasmid pH602 was developed as a 'micro Ti' binary vector for use in *Agrobacterium tumefaciens*-mediated gene transfer. The pH602 vector is based on pH575 [17], but permits selection of transformed plant cells on medium containing hygromycin. This was accomplished by replacing the kanamycin resistance cassette of pH575 with the *hph* [18] coding region under the control of the cauliflower mosaic virus 35S promoter and the TDNA ORF25/26 polyadenylation sequence [19]. Plasmid pRT99gus [20], which contains the β-glucuronidase (GUS) gene behind the CaMV 35S promoter, was used to optimize bombardment conditions. Plasmid DNA was isolated from recombinant *E. coli* by the alkaline lysis method [21] and was purified on a cesium chloride gra-

dient. Supercoiled DNA was adsorbed onto gold particles according to the manufacturer's protocol (Bio-Rad, Richmond, CA).

2.3. Microprojectile bombardment

Embryogenic cultures were bombarded approximately 2 weeks after each transfer when it was assumed that the cultures would be between the lag and stationary phases of growth. Microprojectile bombardment was performed using a prototype model of the PDS 1000/He apparatus (Bio-Rad). Each bombardment delivered approximately 1 μg DNA (1.25 μg DNA for experiment 219) and 600 μg of microcarriers. Gold particles 1.0 μm in diameter were accelerated using 1800 psi of helium (1550 psi for experiment 219) under a vacuum of 71 cm Hg. The sample platform was placed 5 cm below the launch assembly. Each experiment included one plate bombarded with pRT99gus and the remaining plates with pH602, which allowed us to determine instrument performance during each experiment.

2.4. Transient expression and selection of stably transformed callus

Transient GUS expression was assayed 24 h after bombardment by staining embryogenic callus pieces in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% Triton X-100, and 1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for 5–8 h at 37°C [22,23]. GUS-positive foci (sites of GUS activity whether single cell or group of cells) from 3–4 callus pieces were counted in each of 6 experiments with cultivar Toalson (experiments began in November 1991 and labelled 265,266,270,272, 277,285). One experiment (219) had been initiated several months earlier. Callus bombarded with a selectable marker gene was maintained for one subculture period under non-selective conditions. Tissues then were placed under selection for one or two subcultures on agar maintenance medium containing either 5 or 10 mg/l hygromycin. Surviving embryogenic callus from each experiment (except 219) was split into two sets. One set continued to be transferred every 4 weeks onto agar maintenance medium with 10 mg/l hygromycin. The other set was transferred to 125 ml Erlenmeyer flasks containing 25 ml liquid maintenance medium plus 20 mg/l hygromycin. Liquid cultures were shaken continuously at 130 rev./min, and selection medium was replaced every 2 weeks. Embryogenic cultures under selection were monitored periodically. Actively proliferating callus was transferred to fresh medium in a separate flask and labelled at that time as a cell line. Cell lines were increased on both liquid and solid media.

2.5. DNA analysis

DNA was isolated from fresh or frozen (−80°C) tissue following the method of Doyle and Doyle [24]. Large quantities of tissue (0.5–10 g) were ground in liquid nitrogen with a mortar and pestle. Small quantities of tissue (< 300 mg) were homogenized in a microcentrifuge tube with a pellet pestle. Vacuum-dried precipitated DNA was redissolved in TE (containing 10 mM Tris and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8) and treated with 10 μg/ml RNase.

Polymerase chain reaction (PCR) amplification of a portion of the chimeric hygromycin phosphotransferase (*hph*) gene was carried out with published primer sequences [25]: one 27-nucleotide primer was specific for the CaMV 35S promoter (5′-ATATCTCCACTGACGTAAGGGATGACG-3′) and the second 26-nucleotide primer was homologous to a portion of the *hph* coding region (5′-GAATTCCCCAATGTCAAGCACTTCCG-3′). The amplification reactions were carried out using a Perkin-Elmer Cetus DNA thermal cycler under the following conditions: 94°C for 30 s (denaturation), 45°C for 30 s (annealing), 72°C for 60 s (extension), for 30 cycles, followed by a 4°C soak cycle until recovery. The amplified products were assayed by electrophoresis in 1.4% agarose (Seakem HGT) gels in 1× TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.2). A few of the cell lines and one of the plants that showed a positive PCR reaction for presence of foreign DNA were subjected to Southern hybridization analysis. DNA (10 μg) was digested with *Hin*dIII, which recognizes three sites in the pH602 plasmid (Fig. 1). Digested (10 μg) and undigested (5 μg) DNAs were subjected to electrophoresis overnight at 25 V on a 0.8% agarose (Seakem HGT) gel in 1× TBE buffer, and

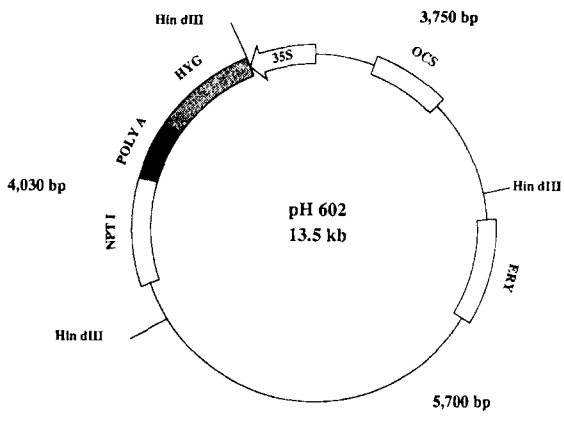

Fig. 1. Map of plasmid pH602. *Hin*dIII sites are indicated along with sizes of *Hin*dIII fragments. The 3.75-kb *Hin*dIII fragment contains the CaMV 35S promoter plus flanking DNA, and the 4.03 kilobase fragment contains the coding and 3′ regions of the hygromycin resistance gene plus flanking DNA.

then transferred by the capillary method [26] to a nylon membrane (GeneScreen Plus). The blot was hybridized with two random primed [27] ^{32}P-labelled *Hin*dIII fragments from pH602 plasmid that contained portions of the chimeric *hph* gene (Fig. 1).

2.6. Regeneration of transgenic plants

Transformed somatic embryos were transferred for regeneration onto MS media containing either 1 mg/l α-naphthaleneacetic acid (NAA) or 0.5 mg/l benzylaminopurine (BAP) + 0.5 mg/l kinetin + 0.5 mg/l zeatin + 0.1 mg/l NAA + 500 mg/l casein hydrolysate. After 4 weeks the material was transferred to B5 medium [28] supplemented with 0.1 mg/l BAP, 0.1 mg/l NAA, and 25 μM $AgNO_3$. Shoots elongated during the next 4 weeks on MS (2% sucrose) + 3 mg/l BAP + 1 mg/l gibberellic acid (GA_3). All regeneration steps were carried out in the light (average of 100 $\mu E/m^{-2}s^{-1}$ from cool white fluorescent lamps for a 16-h per day photoperiod) at 28°C. Regenerated shoots were tested with PCR-for the presence of the *hph* gene. Expression of hygromycin resistance was confirmed for the shoots by incubating 3 replicate leaf samples in wells of microtitre dishes containing basal MS medium plus 30 mg/l hygromycin. After 3 weeks in the light, leaf pieces that remained green were scored as hygromycin resistant. Fresh pollen from greenhouse-grown transgenic plants was stained with 1% acetocarmine to estimate viability.

3. Results

3.1. Transient expression

One–two-year-old embryogenic cultures maintained on picloram-amended medium consisted of somatic embryos at various stages of development and dedifferentiation, plus embryogenic callus with the potential for somatic embryo formation and development into plants (Fig. 2a). Transient GUS expression assayed 24 h after bombardment with pRT99gus-coated microprojectiles was routinely high in embryogenic callus (Fig. 2b). In one experiment, the number of GUS-positive foci was 6 536 per 43 ~ 25mm^2 embryogenic callus pieces or an average of 152 foci per callus, with as many as 500 GUS-positive foci on some callus pieces. Less mature somatic embryos, i.e., globular to early torpedo-shaped stages, tended to give a lower density of GUS positive foci than somatic embryos with differentiating cotyledons and apical meristem regions. Underlying non-embryogenic callus that had been transferred to separate plates for maximum exposure to microprojectiles gave almost undetectable levels of GUS expression (average of 4 foci per callus piece). Bombardment conditions for experiments involving a selectable marker were defined by optimization of transient expression of GUS in embryogenic callus. Each bombardment experiment was monitored by histochemically assaying GUS expression 24 h after bombardment in a few callus pieces bombarded separately with pRT99gus. Although the GUS assay was not a control for the condition of the particle preparation carrying the selectable marker gene, it did indicate uniformity of instrument performance across most experiments. The number of GUS-positive foci in each experiment was within the previously determined range for expression we had observed during optimization of bombardment conditions (Table 1).

3.2. Stable transformation

Embryogenic callus was bombarded with plas-

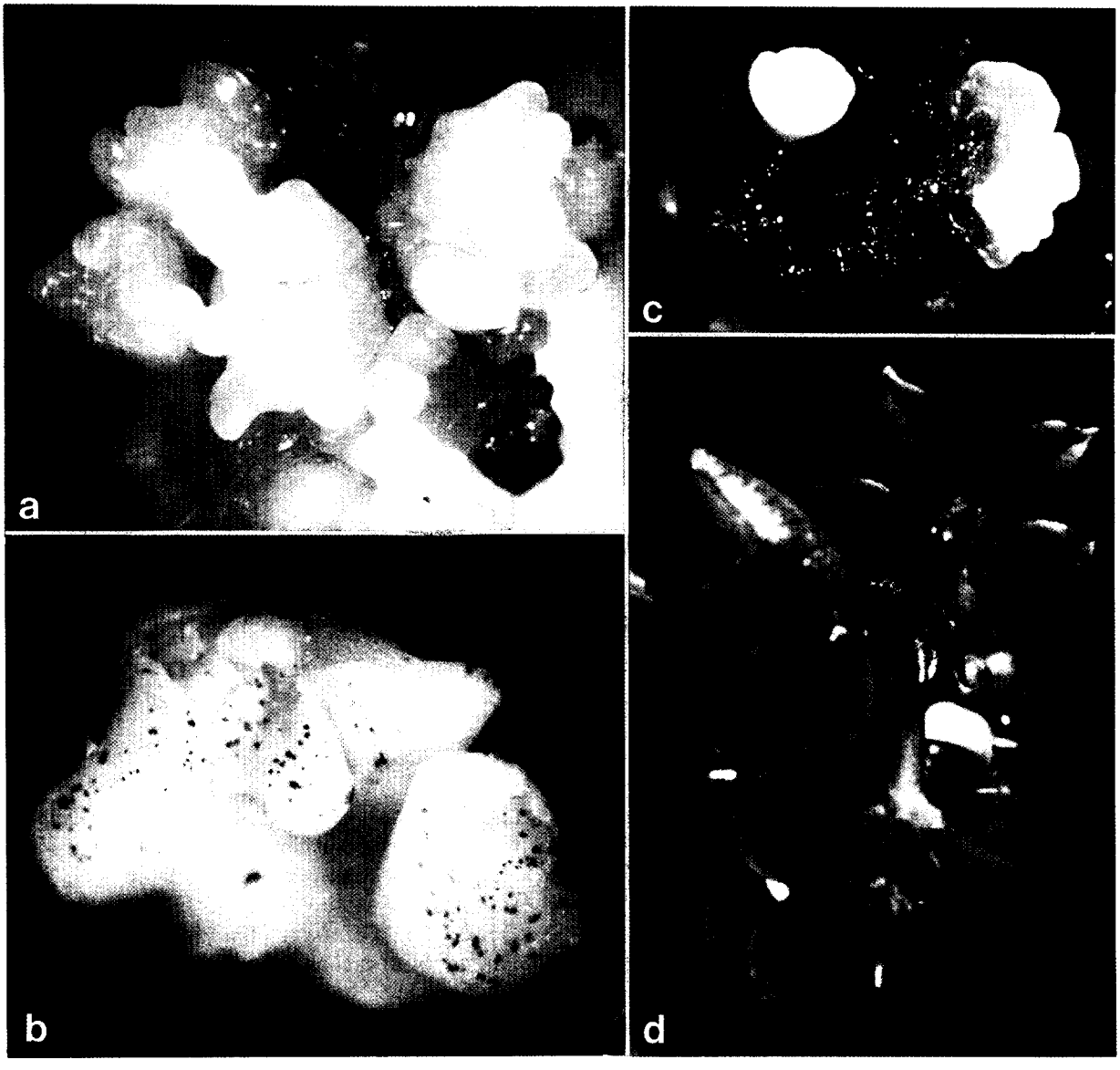

Fig. 2. (a) Embryogenic callus of *A. hypogaea* 'Toalson' that shows somatic embryos at various stages of development. (b) Transient GUS expression in embryogenic callus 24 h post-bombardment. (c) Selection of a hygromycin-resistant cell line on agar medium (experiment 219). (d) Hygromycin-resistant plantlet regenerated from stably transformed embryogenic cell line 270-T1.

mid pH602 containing a hygromycin resistance gene under the control of the CaMV 35S promoter. Bombarded tissues were not immediately placed under selection, but were grown for one subculture period (4 weeks) on maintenance medium. Tissues were first exposed to low levels of hygromycin (5–10 mg/l) approximately 5 weeks post-bombardment. Hygromycin levels were increased to 20 mg/l in subsequent subcultures. Selection for hygromycin-resistant cell lines was successful using both solid and liquid selection media (Fig. 2c). However, selection in liquid medi-

Table 1
Transgenic cell lines recovered from microprojectile bombarded embryogenic callus of peanut

Experiment number	Number of bombardments[a]	Number of callus pieces tested for transient GUS	Numbers of GUS-positive foci	Nomenclature of Hyg^r cell lines recovered	Number of weeks under selection	Amplification of 35S:*hph*[b]	Southern hybridization[c]
219	11	0	nt	219-T1	32	+	+
				219-T5	26	+	+
265	18	3	3-1000	265-T1	30	+	+
266	15	4	28-237	266-T1	16	+	nt
270	19	3	43-66	270-T1	12	+	+
				270-T2	14	+	+
				270-T3	32	+	nt
272	18	2	258-> 1000	272-T1	28	+	nt
				272-T2	28	+	nt
277	19	3	50–163	277-T1	14	+	+
				277-T2	24	+	nt
285	14	1	10	285-T1	16	+	+
				285-T2	16	+	nt
				285-T3	16	+	nt

[a]Each bombardment consisted of a single culture dish.
[b]PCR amplification of a 380 bp region of the chimeric hygromycin resistance gene from genomic DNA of a putatively transformed cell line.
[c]Integration of plasmid DNA into the genome of the recipient tissue was confirmed by hybridization of genomic DNA with a portion of the plasmid (+; nt = not tested).

um was more efficient and faster for eliminating non-transformed tissues. In experiments where selection in solid and liquid media was carried out in parallel, only selection in liquid resulted in recovery of transgenic cell lines. Transformed callus usually appeared as a rapidly-growing mass of tissue 3–4 months after initiation of the liquid cultures.

Each putative independent transformant was numbered at the time of isolation and separately maintained for subsequent DNA analysis and regeneration. Fourteen hygromycin-resistant cell lines were selected from a total of seven bombardment experiments. All but 2 pairs of the cell lines were derived from separate bombardments and were undoubtedly independent transformation events. The two pairs of cell lines were sufficiently separated in integrity or time of origin to have a high probability of being independent events. Oligonucleotide primers specific to sequences in the CaMV 35S5 promoter and *hph* coding region amplified the expected 380 nucleotide fragment from each hygromycin-resistant cell line but no amplification was observed from control, non-transformed tissue (Table 1, Fig. 3). Genomic DNA from all of the cell lines tested showed integration of the foreign gene, as determined by Southern hybridization analysis (Table 1, Fig. 4). Hybridization of high molecular weight, undigested genomic DNA with the combined 3.75 and 4.03 kb *Hin*dIII fragments from pH602 showed the absence of free plasmid DNA. The hybridization pattern of *Hin*dIII-digested genomic DNA from some of the transformed cell lines indicated that the *Hin*dIII sites flanking the selectable marker gene probably were distanced from the *Hin*dIII site internal to the hygromycin resistance gene or deleted during integration of the plasmid DNA. Further molecular analysis would be required to determine the extent of rearrangement of integrated plasmid DNA.

Two experiments were conducted without selec-

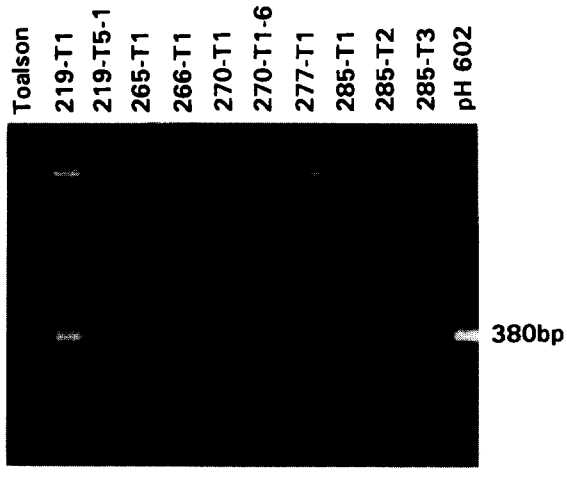

Fig. 3. An ethidium bromide-stained gel showing amplification of the expected 380 bp fragment of the chimeric hygromycin resistance gene from 8 independent transgenic cell lines (numbers correspond with those listed in Table 1) and 2 transgenic plants (219-T5-1; 270-T1-6). No amplification was observed from the control callus DNA (Toalson). Strong amplification was observed from the positive control (plasmid only) DNA. Details of reaction and electrophoresis conditions are given in Materials and methods.

tion in order to determine if selection, and thus use of a selectable marker gene, could be avoided with our transformation system. Approximately 5 weeks post-bombardment, the embryogenic calli were transferred to regeneration media. In the first experiment, calli (cultivar Toalson) were bombarded with pRT99gus so that regenerated shoots could be rapidly screened for GUS activity. Leaflets from plants developing in each of 120 plates were pooled for the GUS assay. Out of approximately 600 plants tested, none gave the blue GUS reaction product expected for a stably transformed plant. In the second experiment, cultivar Florunner calli were bombarded with pH602 and leaflets from regenerated plants were tested for hygromycin resistance. No leaflets remained green on hygromycin-containing medium (30 mg/l) after 3 weeks. No PCR amplification of the *hph* gene was obtained from DNA of plantlets whose leaflets browned more slowly on hygromycin.

3.3. Plant regeneration from stable transformants

Transgenic cell lines proliferated prior to initiation of plant regeneration so that the cell lines could be maintained as well as used for regeneration. In this manner, plants could be regenerated indefinitely from a competent cell line. The sequence of media optimal for plant regeneration had previously been determined for cultivar Toalson (Ozias-Akins, unpublished results). Approximately 3 months were needed for plant regeneration from an embryogenic cell line. Using this regeneration method (outlined in Materials and methods), all of the transformed cell lines have produced plants (Fig. 2d). At least 100 plants have been regenerated and over 80 of these plants have been transferred to soil in the greenhouse. Flowers with a high proportion of stainable pollen have been produced. Of 8 plants from cell line 270-T1 tested for amplification of the foreign gene, all 8 were positive (Fig. 3). Likewise, two plants from 219-T5, tested by the same method, also were positive. Southern hybridization analysis of one plant (Fig. 4) confirmed the positive PCR results for transformation. Leaves from the transgenic plants remained green and formed a small amount of callus after 3 weeks on medium containing 30 mg/l hygromycin, whereas all leaves from non-transformed control plants turned brown and died.

4. Discussion

While many resources have been devoted to the development of a transformation system for soybean [8] and important cereals [10, 13], much less work has been focused on the transformation of peanut. Transformation of forage legumes generally has been more straightforward than transformation of grain legumes [15]. Approaches that rely on tissue culture for the regeneration of transgenic plants must take into account the type of culture (callus, suspension or protoplast), the mode of plant regeneration (embryogenesis vs. organogenesis), the maintenance of regeneration potential over time, and the ability to select stably transformed tissue. We have developed an embryogenic culture system for multiple genotypes [5,29,30] that satisfies two important criteria: long-term preservation of regeneration capacity and

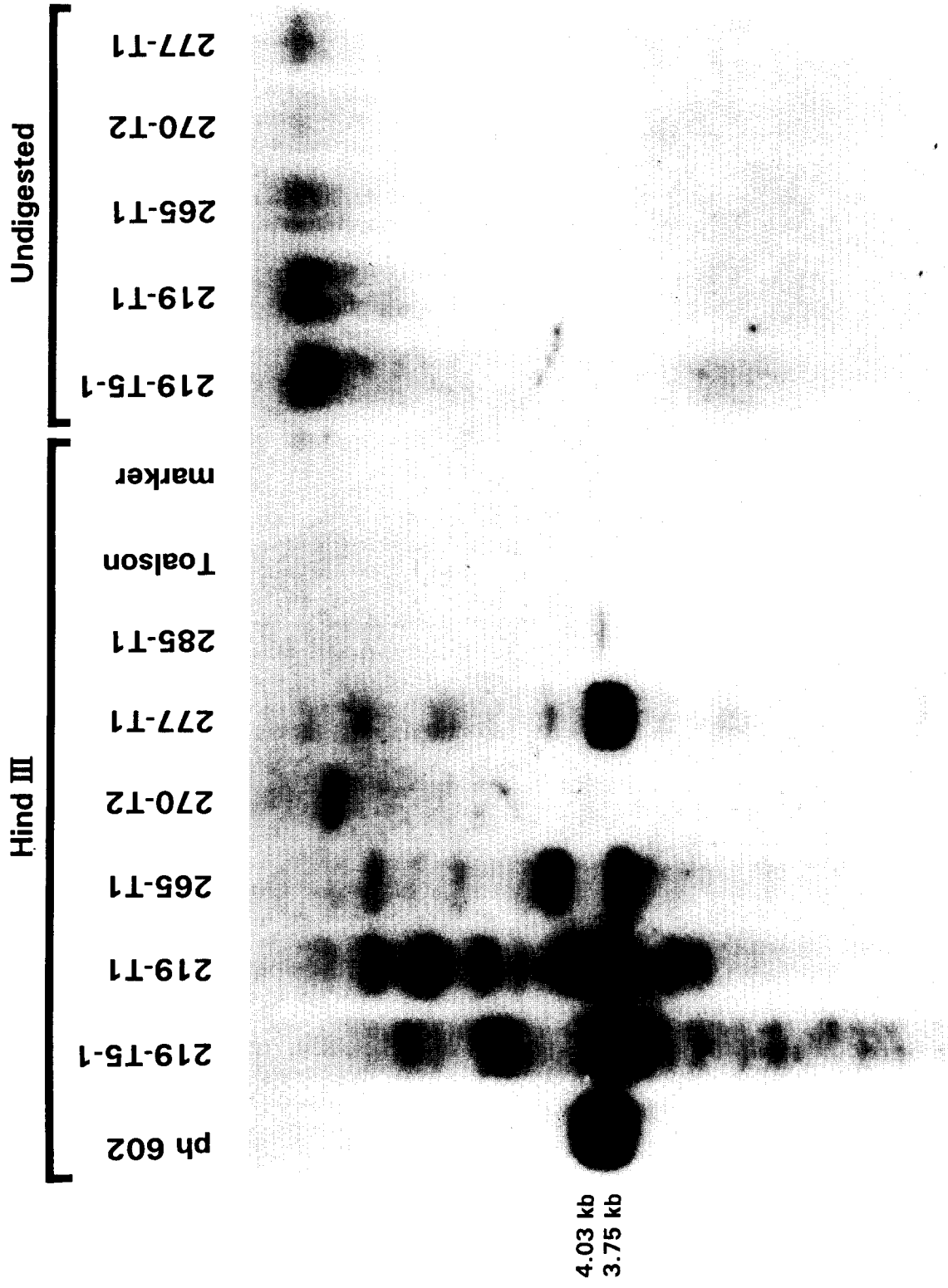

Fig. 4. An autoradiograph of *Hin*dIII-digested and undigested DNA from several transgenic cell lines (numbers correspond with those listed in Table 1) and one regenerated plant (219-T5-1) hybridized with two ^{32}P-labelled *Hin*dIII fragments (3.75 and 4.03 kb, see Fig. 1) from pH602. *Hin*dIII-digested plasmid DNA (50 pg) was loaded in the first lane (pH602). This amount of plasmid DNA is approximately 2 copies per diploid genome if the genomic DNA content peanut ($2n = 4x = 5.83$ pg) reported by Arumuganathan and Earle [34] is used to calculate copy number. Multiple insertions and rearrangements of plasmid DNA are likely, given the distribution of hybridizing fragments.

suitability for antibiotic selection as a function of introduced resistance genes.

Although transient GUS expression was used to optimize bombardment conditions, there was no clear relationship between GUS expression in an experiment and the number of stable transformants selected. For instance, the best transient GUS expression was observed in experiment 272, but 2 transformed callus lines were recovered only after an exceptionally long period of selection. Conversely, unusually low expression was found in experiment 285 where three transformed cell lines were selected relatively quickly. Since expression varied widely among callus pieces, the small number of samples from each stable transformation experiment tested for transient GUS expression may not have accurately reflected the potential overall range of GUS expression within the experiment. Also, the total number of transformed cell lines recovered was too low to allow any meaningful conclusions regarding frequency of stable integration events as a function of transient expression events. Timmermans et al. [31] reported that approximately 2% of transiently expressing cells became stably transformed in plated tobacco suspension cells subjected to microprojectile bombardment, and in soybean suspension cultures, a transient to stable transformation frequency of 0.4% was observed [32]. Suspension cultured cells would be more homogeneous than the highly differentiated tissues used in our experiments and might be expected to yield more stable transformants.

At the outset, we considered the heterogeneous nature and asynchronous development of our embryogenic cultures to be a likely obstacle to the isolation of non-chimeric stable transformants. The absence of escapes in experiments we have carried out thus far suggests that chimerism will probably be an infrequent event. One step that may be critical to the isolation of transformants, but which we do not yet have data to support, is the delay in applying selection after bombardment. One presumes that a single transformed cell present within an organized somatic embryo probably would not survive selection imposed only a few days after bombardment. However, if the cell were given time under non-selective conditions to divide and to produce a substantial sector within the somatic embryo, a critical mass of tissue would be more likely to survive and to proliferate under selective conditions to allow for the rescue of a resistant cell line. Fitch et al. [11] obtained transformed papaya tissues by delaying selection for 3–5 weeks after bombardment to allow the bombarded tissues time for recovery.

Provided that all regenerated plants prove to be fertile, our transformation frequency of 2 transgenic cell lines per bombardment experiment is high enough to provide sufficient transgenic cell lines to overcome possible positional effects on expression or phenotypic variability from tissue culture. A higher transformation frequency, however, would be desirable and possibly could be obtained by such treatments as osmotic shock, which has been shown to improve transient expression and stable transformation 5–7-fold in embryogenic suspension cultures of maize [33]. The transformation/regeneration system we describe provides a practical means for introducing foreign genes into peanuts. Analysis of foreign gene expression in the progeny of transformed plants will be underway shortly.

5. Acknowledgements

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