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USE OF GREEN FLUORESCENT PROTEIN AS A NON-DESTRUCTIVE MARKER FOR PEANUT GENETIC TRANSFORMATION

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Summary

The ability to non-destructively visualize transient and stable gene expression has made green fluorescent protein (GFP) a most efficient reporter gene for routine plant transformation studies. We have assessed two fluorescent protein mutants, enhanced GFP (EGFP) and enhanced yellow fluorescent protein (EYFP), under the control of the CaMV35S promoter, for their transient expression efficiencies after particle bombardment of embryogenic cultures of the peanut cultivar, Georgia Green. A third construct (p524EGFP.1) that expressed EGFP from a double 35S promoter with an AMV enhancer sequence also was compared. The brightest and most dense fluorescent signals observed during transient expression were from p524EGFP.1 and EYFP. Optimized bombardment conditions consisted of 0.6 μm diameter gold particles, 12 410 kPa bombardment pressure, 95 kPa vacuum pressure, and pretreatment with 0.4 *M* mannitol. Bombardments with p524EGFP.1 produced tissue sectors expressing GFP that could be visually selected under the fluorescence microscope over multiple subcultures. Embryogenic lines selected for GFP expression initially may have been chimeric since quantitative analysis of expression sometimes showed an increase when GFP-expressing lines, that also contained a hygromycin-resistance gene, subsequently were cultured on hygromycin. Transformed peanut plants expressing GFP were obtained from lines selected either visually or on hygromycin. Integration of the *gfp* gene in the genomic DNA of regenerated plants was confirmed by Southern blot hybridization and transmission to progeny.

Key words: Arachis hypogaea; genetic engineering; groundnut; microprojectile bombardment; transgenic.

Introduction

Peanut (*Arachis hypogaea* L.) is grown in tropical and warmer temperate regions throughout the world. The crop provides an excellent source of protein and oil for human consumption. Poor yields of peanut in developing countries are largely due to factors such as erratic rainfall, low residual moisture, lack of high-yielding adapted cultivars, damage by pests and diseases, poor agronomic practices, and low-input farming (Nageshwara Rao and Nigam, 2003). Losses due to biotic factors include various diseases caused by fungal pathogens, viruses, bacteria, and nematodes (Sharma et al., 2001). Conventional breeding has led to genetic gains in yield and the development of disease-tolerant cultivars (Knauft and Ozias-Akins, 1995). Although the genetic variability within wild species includes many valuable disease resistance traits, wide hybridization between wild and cultivated *Arachis* spp. has been limited due to cross incompatibility, a low frequency of hybrid seed production, and linkage drag (Stalker and Simpson, 1995).

Plant transformation is now a core research tool in plant biology and a practical tool for cultivar improvement. Transformation of peanut has been accomplished via biolistics (Ozias-Akins et al., 1993) and *Agrobacterium* (Cheng et al., 1996), and a number of potentially useful genes, e.g., insect resistance (*cryIAc*) and tomato spotted wilt virus resistance (NP-gene), have been introduced into the crop plant (Ozias-Akins and Gill, 2001). Peanut transformation with the biolistic approach is reproducible and less genotype-dependent than *Agrobacterium* transformation. However, increasing the transformation frequency of peanut and reducing the time to transgenic plant recovery remain important goals for both biological and direct DNA delivery methods.

The availability of a suitable reporter gene is useful for recovering transformation events from low-efficiency transformation systems. An optimal selectable marker gene for plant transformation would be one that is visually detectable without the addition of substrates and whose analysis is non-destructive. In addition, the gene product should not be detrimental to transformed cell growth or plant development, nor should it provide any selective advantage to weedy relatives with which a transformed crop may out-cross (Kaeppler et al., 2000). Although there is no single gene that can yet function as a negative or positive selectable marker and as a non-destructive reporter, reporter genes have been successfully used, in some cases, for visual selection of transgenic tissues. Reporter genes include luciferase, β-glucuronidase (*gus*), green fluorescent protein (*gfp*), and regulators of anthocyanin biosynthesis (Ludwig et al., 1990; de Ruijter et al., 2003); however, both

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luciferase and GUS assays require the addition of a substrate at optimized levels to detect enzyme activity. Also, GUS assays are destructive and some plant tissues express an endogenous GUS-like activity that interferes with the detection of transgene-encoded GUS (Hodal et al., 1992; Hansch et al., 1995). Selection for anthocyanin production overcomes the disadvantage of substrate addition, but expression of anthocyanin during regeneration can be toxic (Bower et al., 1996) or may depend on tissue-specific anthocyanin promoters or tissue pigmentation.

Green fluorescent protein (GFP) causes bioluminescence in the Pacific Northwest Jellyfish, *Aequorea victoria*, and the gene was cloned and used as a transgene reporter (Chalfie et al., 1994). GFP has emerged as a reporter system of broad utility because it is non-destructive and requires no exogenous substrate to fluoresce. Mutants to enhance the stability, intrinsic brightness, and spectral characteristics of fluorescent protein have been produced (Tsien, 1998). For optimizing plant transformation protocols, early and strong expression of a reporter gene can be useful, and GFP has become a widely tested reporter (Niedz et al., 1995; Elliott et al., 1999; Jordan, 2000). We therefore evaluated two fluorescent protein mutants for their transient expression efficiencies after particle bombardment of embryogenic cultures of the peanut cultivar, Georgia Green. We also present evidence for stable transformation using GFP as a selectable marker as well as a reporter gene when combined with hygromycin selection.

Materials and Methods

Plant materials and explant preparation for bombardment. Embryogenic cultures of peanut were initiated from mature zygotic embryos (McKently et al., 1991) of the commercial cultivars, Georgia Green or Marc I. Seeds were disinfected by shaking for 2 × 30 min in 20% Clorox (1.05% sodium hypochlorite), followed by four rinses in sterile deionized water. The plumule part of the embryo axis was excised and cultured on embryo induction medium (EIM) which consisted of FN Lite medium (Samoylov et al., 1998) containing 12.42 μ*M* picloram and 2.4% sucrose. The pH was adjusted to 5.8 prior to autoclaving at 121°C, 103 kPa, for 20 min, and the medium was solidified with 7 g l^{-1} agar (A1296, Sigma, St. Louis, MO). After initiation of somatic embryos, embryogenic cultures were maintained by removing the loose callus and subculturing clusters of embryos at 4-wk intervals onto embryo maintenance medium (EMM). EMM had the same composition as EIM except it also contained 6.85 m*M* filter-sterilized glutamine. Cultures were maintained in the dark at 26 ± 2°C, and bombardments were conducted approximately 2 wk after subculture.

Plasmid sources and constructs. For construction of pEGFP-C and pEYFP-C plasmids, an expression vector, designated as p35N-23, was assembled as follows. The CaMV 35S promoter was inserted into the (blunted) *Bgl* II and *Xba* I sites of pLitmus-28 (New England Biolabs, Beverly, MA). The NOS terminator was inserted into *Sac* I and *Kpn* I sites. A synthetic polylinker (5′-*Avr* II-*Bam* HI-*Sac* II-*Pst* I-*Hin*dIII-*Mlu* I-*Sal* I-*Aat* II-*Xho* I-*Fse* I-*Sac* I-3′) was inserted into the *Avr* II and *Sac* I sites. The coding regions of two different fluorescent proteins (FP), EGFPC and EY (enhanced yellow) FPC, were amplified from the corresponding plasmids (BD Biosciences Clontech, Palo Alto, CA) with primers containing a 5′ *Mlu* I site and a 3′ *Aat* II site using *Pfu* polymerase (Stratagene, La Jolla, CA). Following PCR amplification, FP inserts were digested with *Mlu* I and *Aat* II and inserted into the respective sites of p35N-23 to yield pEGFP-C and pEYFP-C.

Plasmid p524EGFP.1 (Fleming et al., 2000), with the Clontech version of EGFP driven by a double CaMV 35S, AMV-enhanced promoter sequence (Datla et al., 1993), was tested for both transient expression and stable transformation. Embryogenic cultures were bombarded with p524EGFP.1 (4.8 kb) (1) alone, when visual selection for transgenic tissues was carried out, (2) in combination with a 6.1 kb plasmid containing a hygromycin resistance gene driven by a CaMV35S promoter and CaMV35S terminator, when hygromycin was used to select transgenic tissues, or (3) in combination with a 4.7 kb plasmid containing a mercury-resistance gene (*merB*; Bizily et al., 1999) driven by a potato ubiquitin (*UBI3*) promoter (Garbarino and Belknap, 1994). Co-bombardments used a 1:1 molar ratio of plasmid DNAs. Lines isolated from experiment J14 had been bombarded with EGFP and the hygromycin resistance gene in the same plasmid, pUHN/EGFP-C. This plasmid contained the EGFP cassette from pEGFP-C ligated into a plasmid (pUHN-4) that contained a hygromycin resistance gene under the control of the *UBI3* promoter.

Plasmid DNA isolation and microprojectile bombardment. Plasmid DNAs were isolated using the Qiagen Plasmid Maxi/Midi Kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. DNA concentration was estimated by both UV spectrophotometry and quantitative analysis on an agarose gel. Two weeks after subculture to fresh medium, 20 clusters of somatic embryos, with three to five embryos per cluster, were arranged in the central 2-cm area of a Petri dish. Tissues were desiccated 2–3 h prior to bombardment by uncovering the plates and exposing them to airflow in the laminar hood or by transfer to EMM supplemented with 0.4 *M* mannitol. Mannitol-treated tissues were kept in the same medium for 18–24 h after bombardment. Microprojectile bombardment was carried out using a PDS 1000/helium-driven apparatus (Bio-Rad, Hercules, CA) and previously published procedures for adsorption of DNA onto gold particles (Singsit et al., 1997). Bombardment pressures were adjusted to 9308 kPa (1350 psi), 10 690 kPa (1550 psi), or 12 410 kPa (1800 psi) and vacuum pressures to 91–95 kPa (27–28 in Hg). Bombardment was carried out with gold microcarriers of 0.6, 0.75, or 1.0 μm diameter. For stable transformation experiments, all bombardments were carried out with 0.6 μm gold microcarriers, 12 410 kPa helium pressure, 95 kPa vacuum, and 0.4 *M* mannitol as an osmotic treatment.

Transient expression. Transient expression of two different fluorescent proteins (EGFP and EYFP) and two EGFP constructs (pEGFP-C and p524EGFP.1) was evaluated by observation of bombarded tissues using a Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light source, a 480 ± 30 nm excitation filter, and a 515 nm long-pass emission filter (Chroma Technology, Brattleboro, VT). To collect quantitative data for transient GFP expression, tissues were photographed 48 h after bombardment with a Zeiss Axiocam digital camera and a magnification of 40 ×. Fluorescent spots from a 1 mm^2 area were counted for three different pieces of tissue per shot and three plates for each treatment. A minimum of 9–10 counts were collected for each variable and each experiment was repeated three times. Data were subjected to ANOVA using SAS version 8.2 (SAS Institute, Inc., Cary, NC) and Student's *t*-test was used to find the significant differences in transient expression.

Selection of transgenic lines. Cultures were visually monitored every 2–3 d and subcultured after 3–4 wk onto the same medium. For visual selection, small pieces of embryogenic callus that showed green fluorescence were separated and cultured in fresh medium. In the case of co-bombardment and negative selection, hygromycin selection was begun 3–4 d after bombardment by transferring callus from a single plate into 20 ml of liquid EMM supplemented with 20 mg l^{-1} hygromycin in a 125-ml, wide-mouth, transparent polypropylene jar (Nalgene, Rochester, NY), or phenylmercuric acetate (PMA) selection was begun 1 mo. after bombardment on agar medium containing 1 μ*M* PMA. Cultures in liquid medium were incubated at 130 rpm in the dark, and the hygromycin-containing medium was changed every 2 wk. After 8–10 wk of selection, tissues that showed new growth were removed from liquid and transferred to agar-solidified medium containing hygromycin. Putative transgenic lines were observed under a fluorescence microscope for expression of GFP.

Regeneration of transgenic lines. Embryogenic tissues stably transformed with *gfp* from both visual and hygromycin selection were subcultured onto MS medium (Murashige and Skoog, 1962) containing 5.37 μ*M* naphthaleneacetic acid (NAA) and 2% sucrose and kept in the dark for the first 3 wk followed by a week in the light. Embryos having a well-developed shoot–root axis were then transferred to MS medium with 2% sucrose containing 13.32 μ*M* 6-benzylaminopurine (BA) and 2.89 μ*M* gibberellic acid (GA_3) or only 9.08 μ*M* thidiazuron (TDZ) under a daily photoperiod of 16 h light (100 μmol m^{-2} s^{-1}) and 8 h dark. Elongated shoots were rooted on basal MS medium or MS containing 5.4 μ*M* NAA.

DNA isolation. DNA was extracted from embryogenic callus and leaf tissue according to Murray and Thompson (1980). Briefly, tissue (50–100 mg) was homogenized in a 1.5 ml microcentrifuge tube in 700 μl of 2 × cetyltrimethylammonium bromide (CTAB) buffer [2% CTAB, 5% polyvinyl-pyrrolidone (PVP-40), 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl, pH 8.0]

with 2% mercaptoethanol (freshly added), incubated in a 65°C water bath for 15 min, and extracted once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with isopropanol and pellets were resuspended in 50 μl of sterile deionized water and treated with 0.5 μl of RNase A (2 μg ml^{-1}) for 1 h at room temperature. DNA was quantified with a spectrophotometer.

PCR analysis. Putative transformants were analyzed for the presence of the *gfp* or *merB* genes by PCR. A 548-bp fragment from the open reading frame of *gfp* was amplified using 20-nucleotide sense (EGFPS1: 5′-AAG GGC GAG GAG CTG TTC AC) and antisense (EGFPAS1: 5′-TTC TGC TGG TAG TGG TCG GC) primers. A 465-bp fragment from the open reading frame of *merB* was amplified using 24-nucleotide sense (merB_S1: 5′-AGA ACT TCT CAC TTC GGT CAA TCG) and antisense (merB_A1: 5′-TGT ACA TGG CAA CAG AAG GAC TGA) primers. Amplification was carried out under the following conditions: 95°C for 60 s, 60°C for 60 s, 72°C for 60 s, for 40 cycles followed by an extension of 10 min at 72°C and 4°C until recovery. The amplified product was detected by electrophoresis in 1% agarose gels in 1 × TBE (0.089 *M* Tris, 0.089 *M* boric acid, 0.002 *M* EDTA, pH 8.2).

Southern blot analysis. Approximately 10 μg of DNA was digested with *Hin*dIII, which cuts only once in each plasmid used for stable transformation. Digested DNA was subjected to electrophoresis overnight at 25 V on a 0.8% agarose gel (Seakem HGT) in 1 × TBE buffer. DNA was blotted to GreenScreen Plus nylon membrane (PerkinElmer Life Sciences, Boston, MA) using 0.4 *N* NaOH. Blots were hybridized with ^{32}P-labeled PCR products, exposed to phosphor screens overnight, and read in a Cyclone (Packard, Meriden, CT).

Fluorometric quantification of GFP. The visually and hygromycin-selected transgenic lines were subjected to fluorometric quantification of GFP. Extraction of protein was carried out according to Remans et al. (1999), and fluorescence was read using a Fluorocount microplate fluorometer (Packard). Tissue of transformed lines (50 mg) was ground in 1 ml of protein extraction buffer (10 m*M* Tris-EDTA, pH 8.0; 0.02% sodium azide). Debris was removed by centrifugation for 10 min at 10 600 × *g*. Extraction buffer alone was used to zero the fluorometer at 485 nm excitation and 530 nm emission wavelengths. The linearity of fluorescence was tested using a dilution series (50–200 μl of sample in 200 μl of total volume) of the brightest fluorescent plant sample. Relative fluorescence units (RFU) were recorded for 100 μl of each plant sample. Three replicate samples from each line were assayed. Student's *t*-test was used to determine significant differences between treatments.

Results and Discussion

Transient expression of fluorescent proteins in peanut. Among the two fluorescent proteins and three constructs (pEYFP-C, pEGFP-C, and p524EGFP.1), the latter was found to exhibit the brightest transient expression as detected through the microscope, followed by pEYFP-C. The quantitative data for number of fluorescent spots showed that transient expression was highest for p524EGFP.1 and was significantly different from the other two constructs (Table 1). The same fluorescent protein coding sequence was present in p524EGFP.1 and pEGFP-C, thus the lower expression level from pEGFP-C could have been due to a non-enhanced CaMV 35S promoter or its design as a fusion construct. Interestingly, for p524EGFP.1, more than 10% of the transiently expressed spots were found to persist even 10 d after bombardment.

Further experiments were conducted with p524EGFP.1 to test the effects of gold particle size, bombardment pressure, and vacuum pressure on transient expression of GFP. When three different sizes of gold particles were compared by assaying transient expression of GFP 48 h after bombardment with p524EGFP.1, there was no significant difference in the number of fluorescent spots, although there clearly was a trend for more spots with the smaller particle size (means of 78, 62, and 57 for 0.6, 0.75, and 1.0 μm particles, respectively). Gold microprojectiles, 0.6 μm in diameter, also showed less variation between samples compared with 1.0 μm particles, and the spots were more discrete. Two vacuum pressures, i.e., 91 and 95 kPa, gave significantly different ($P \leq 0.05$) levels of transient expression

TABLE 1

TRANSIENT EXPRESSION OF FLUORESCENT PROTEIN 48 H AFTER BOMBARDMENT

	Mean number of fluorescent spots	
Construct	Mannitol pretreatment	Air-dry pretreatment
p524EGFP.1	145 a	81 a
pEYFP-C	91 b	59 b
pEGFP-C	49 c	32 c

Tissues were desiccated 2–3 h prior to bombardment by uncovering the plates and exposing them to airflow in the laminar hood or by transfer to EMM supplemented with 0.4 *M* mannitol. Mannitol-treated tissues were kept in the same medium for 18–24 h after bombardment. Bombardment conditions included 12 410 kPa helium pressure, 95 kPa vacuum, and 0.6 μm gold particles.

Each mean was derived from three experiments, each consisting of nine measurements within each of three replicate plates (total of 81 data points per treatment). Means followed by the same *letter* within pretreatments are not significantly different ($P \leq 0.01$).

(means of 73 and 97 fluorescent spots, respectively). Particle delivery was more efficient at 95 kPa when tested with 12 410 kPa rupture discs and 0.6 μm gold. Among the three rupture disc pressures, the highest (12 410 kPa) resulted in the highest level of transient expression for peanut embryogenic tissues and was significantly better ($P \leq 0.001$) than the two lower pressures (means of 78, 58, and 34 for 12 410 kPa, 10 690 kPa, and 9308 kPa, respectively).

Earlier optimization of various bombardment parameters for peanut comprising distance between target tissue and the stopping plate assembly, DNA quantity per bombardment, and radius from the center of the target platform were reported using GUS (Wang et al., 1998). The present study took advantage of the non-destructive reporter GFP to examine the effect of additional parameters. Microprojectiles of smaller diameter, which resulted in a trend toward increased transient expression, probably are less damaging to embryogenic cells. Rasco-Gaunt et al. (1999) found that 0.6 μm gold particles showed less aggregation after DNA precipitation than larger particles, which probably led to more evenly dispersed expression units in bombarded wheat embryos. Consistent with our observations, they did not find a statistically significant difference among particle sizes for transient expression. Schöpke et al. (1997) reported that transient expression varied with particle size (1.0, 1.6, and 1.8–2.3 μm) and that the smallest particle size showed a significantly higher number of GUS-expressing spots after bombardment of embryogenic suspension cultures of cassava, which probably was an expected outcome given the greater number of small particles in the same mass.

Based on the highest level of transient expression, we concluded that 12 410 kPa pressure was most appropriate for embryogenic tissues of peanut. Similar conclusions were previously reached using *gus* as a reporter gene (Ozias-Akins et al., 1993). Optimum pressures vary greatly with tissue type and species (Livingstone and Birch, 1995; Schöpke et al., 1997; Rasco-Gaunt et al., 1999). As much of the overlying air is removed from the vacuum chamber of the biolistic device as is practical to reach a vacuum pressure of 91–95 kPa, which usually cannot be exceeded because of residual water vapor pressure from the biological sample itself (Sanford et al.,

1993). For our materials, both higher vacuum and bombardment pressures significantly increased transient expression, probably due to an increase in number or more even distribution of particles that impacted the tissues. Similar observations were reported for wheat (Rasco-Gaunt et al., 1999).

Transient expression was evaluated after pre-bombardment treatment of tissues with air-drying or exposure to 0.4 *M* mannitol. Pre-incubation of somatic embryos on 0.4 *M* mannitol resulted in a significant increase of transient expression over that of air-drying for all three constructs tested (Table 1; $P \leq 0.01$ for p524EGFP.1; $P \leq 0.05$ for pEGFP-C and pEYFP-C). Similarly, Livingstone and Birch (1995), who used 0.2 *M* each of sorbitol and mannitol as an osmotic pretreatment in peanut, observed a 10-fold increase in transient expression in bombarded embryos. Neither osmotic treatment nor any of the other variables that affect transient expression have been tested for their effects on stable transformation of peanut because the stable transformation frequency is too low for meaningful statistical analysis of small effects. Increased transient expression has been the justification for incorporating modifications to peanut transformation protocols where stable transformation is the objective (Livingstone and Birch, 1999). We found GFP expression in mannitol-treated cultures to persist even up to 10 d after bombardment for 10–20% of the prominent transient spots. Transient expression from air-dried tissues was reduced to 2–5% after 10 d with subsequently diminishing intensities (data not shown).

The effect of a short-term osmotic pre-conditioning (plasmolysis) of target cells or tissues on transient and stable transformation has been reported in several studies (Perl et al., 1992; Vain et al., 1993; Iglesias et al., 1994; Altpeter et al., 1996). Short-term osmotic treatments, typically for a few hours before or after bombardment, are thought to reduce turgor pressure, thereby protecting the cell membrane from disruption (Sanford et al., 1993). In addition, part of the increase in transient expression could be due to greater penetration of the tissues by the microprojectiles where more viable target cells are impacted (Kemper et al., 1996). One detrimental effect of osmotic treatment was the wide range of chromosomal variation observed in transformed callus cells of barley that was not detected in untreated controls (Choi et al., 2001).

Stable transformation of peanut with EGFP. The plasmid p524EGFP.1 facilitated the recovery of GFP-expressing transformed plants by visual selection for fluorescence. Green fluorescent spots (transient expression) were visible in peanut somatic embryos as early as 24 h after bombardment and peaked by the second day (Fig. 1*A*). More than 80% of the transient expression had disappeared by 10 d, leaving small, dimly fluorescent sectors of cells on a few embryos. Only those very prominent spots persisted up to 3 wk. After transient expression data were recorded, cultures were monitored periodically under the fluorescence microscope for larger GFP-expressing sectors. Due to the relatively small size of GFP-expressing sectors, complete separation and handling of transformed regions was almost impossible. Instead, tissues were allowed to grow without segregation for approximately 2 mo. and were separated only when they were large enough to handle without injury, i.e., without fragmenting a developing somatic embryo (Fig. 1*B*). Continuous selection of embryos showing sectors of GFP expression gradually resulted in completely transformed somatic embryos. Only two lines of transformed peanut were visually selected from six bombarded plates. At least five additional independent lines were visually selected after bombardment with pUHN/EGFP-C (data not shown). The selection is relatively inefficient, however, at less than one line per bombardment. In the two lines putatively transformed with p524EGFP.1, the presence of the *gfp* gene was confirmed by PCR analysis (data not shown). One line (P98-1) was particularly vigorous and was used for Southern blot analysis and plant regeneration. During the process of regeneration, GFP expression was more apparent in developing roots than in shoots because expression in shoot tissues was often confounded with fluorescence from chlorophyll (Fig. 1*C*, *D*) (Molinier et al., 2000).

Twenty-five cell lines were recovered from co-bombardment of six plates (experiment P104) with *gfp* and a hygromycin resistance gene and selection with hygromycin rather than visual selection. After 8–10 wk on antibiotic-containing liquid medium, 17 proliferating clusters showed GFP expression when observed under the fluorescence microscope, and DNA from these lines amplified the expected PCR product. The co-transformation frequency in this experiment was 68%, which was similar to the 57% frequency observed by Livingstone and Birch (1999) for peanut tissues co-bombarded with *luciferase* and *hph*. We did not carry out Southern blot analysis on all of these lines to test for independence of events.

Different GFP brightness classes were observed among sublines of the visually selected line P98-1 after six or seven subcultures, and the hygromycin-selected lines appeared to be brighter under the microscope than the visually selected lines (Fig. 1*E*, *F*). The presence of sectors of GFP-expressing and non-expressing tissues during visual selection, that may represent transformed and non-transformed tissues, posed problems for stable transgenic line isolation (Fig. 1*G*). Uniform expression was observed throughout all cell layers for brightly fluorescing GFP lines from hygromycin selection. Rigorous separation of transformed and non-transformed cells during visual selection was necessary to enrich for transformed cells in order to reduce the probability of regenerating chimeras.

GFP expression was quantified from the transformed P98-1 lines that had been subjectively divided into different GFP brightness classes. Control samples (non-transformed callus extract) had very low fluorescence (RFU mean ± SD of 547 ± 213) as compared to *gfp*-transformed lines. Samples from a hygromycin-selected line exhibited the brightest fluorescence, both quantitatively (146 639 ± 1765 RFU) and qualitatively, compared with nine visually selected P98-1 sublines (28 845 ± 10 946 RFU). The brightest tissues from the less bright P98-1 sublines were carefully separated and allowed to grow for 2–3 mo. with visual selection applied at each subculture. A 2-fold increase in RFU was observed only in the visually selected lines (89 067 ± 7731 RFU), whereas the RFU of the hygromycin-resistant line remained very similar over time (156 639 ± 1765 RFU). This suggests that rigorous and continuous visual selection of GFP-expressing tissues is required in order to recover homogeneous cultures of transformed cells.

Elliott et al. (1999) found that it was difficult to maintain the preferential growth of fluorescent cells in culture, even with regular selective subculturing to remove non-transformed cells. We did reproducibly demonstrate, however, that enrichment is possible through a subsequent experiment (J14) where embryogenic tissues were bombarded with one construct containing both EGFP and *hph* genes (pUHN/EGFP-C). After visual selection for 6 mo., part of each callus line was placed on hygromycin-containing medium where any residual non-transformed cells would be expected to die. Of the nine visually selected lines, five showed a significant difference

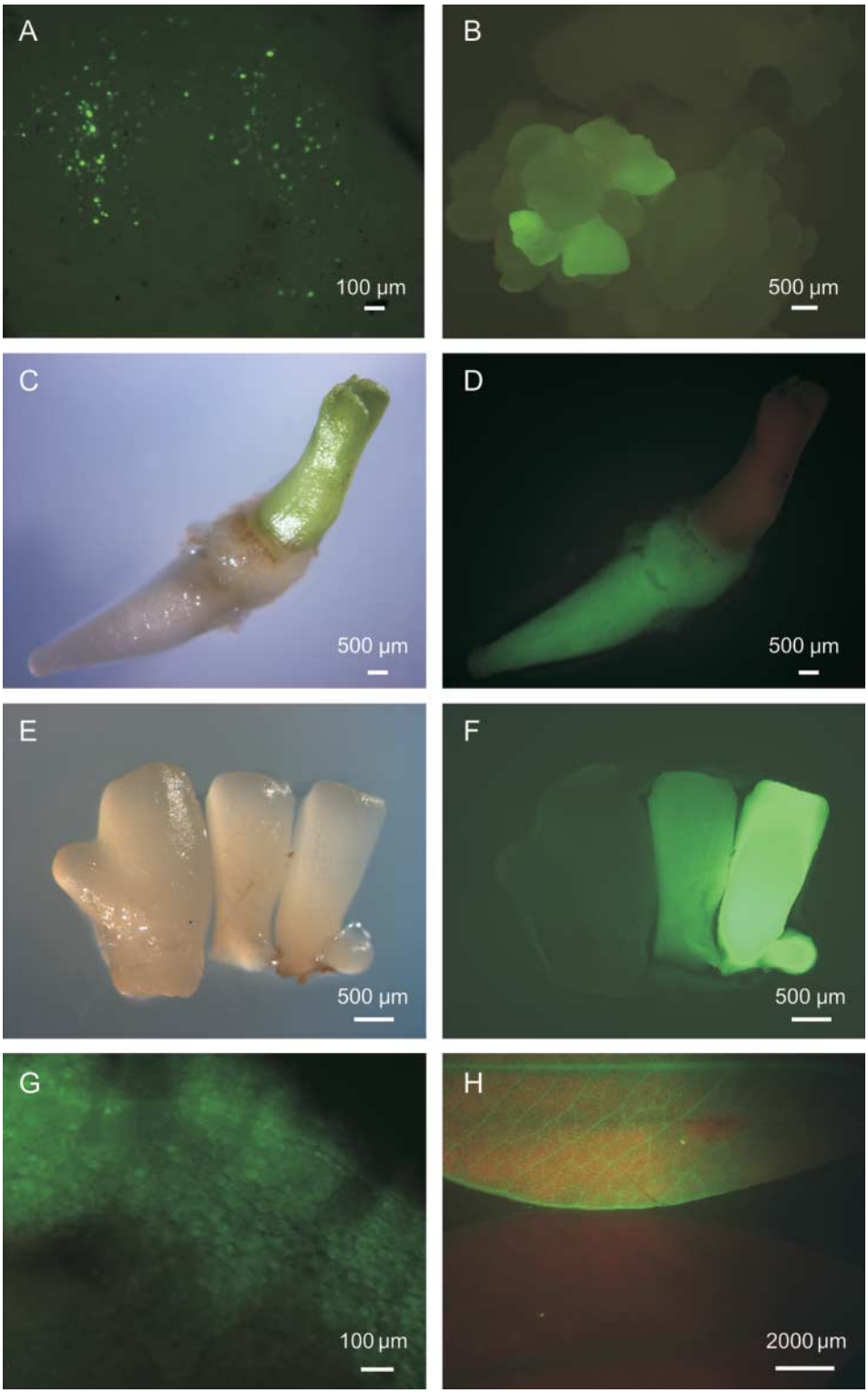

FIG. 1. GFP expression in peanut tissues bombarded with plasmid p524EGFP.1. *A*, Transient expression of *gfp* 48 h after bombardment. *B*, Initiation of stably transformed *gfp* embryos 2 mo. after bombardment. *C*, A transformed embryo rooted in 5.37 µ*M* NAA. *D*, Same as (*C*) but shows GFP expression in the radicle whereas the plumule shows a light pink color due to chlorophyll fluorescence. *E*, Multiple embryos transformed with *gfp* photographed under visual light. *F*, Multiple embryos transformed with *gfp* showing GFP expression under a fluorescence microscope (*left*, non-transformed; *middle*, visually selected; *right*, hygromycin selected). *G*, Layers of GFP transformed and non-transformed cells in visually selected lines. *H*, GFP fluorescence in a transformed (*top*) and non-transformed (*bottom*) leaf.

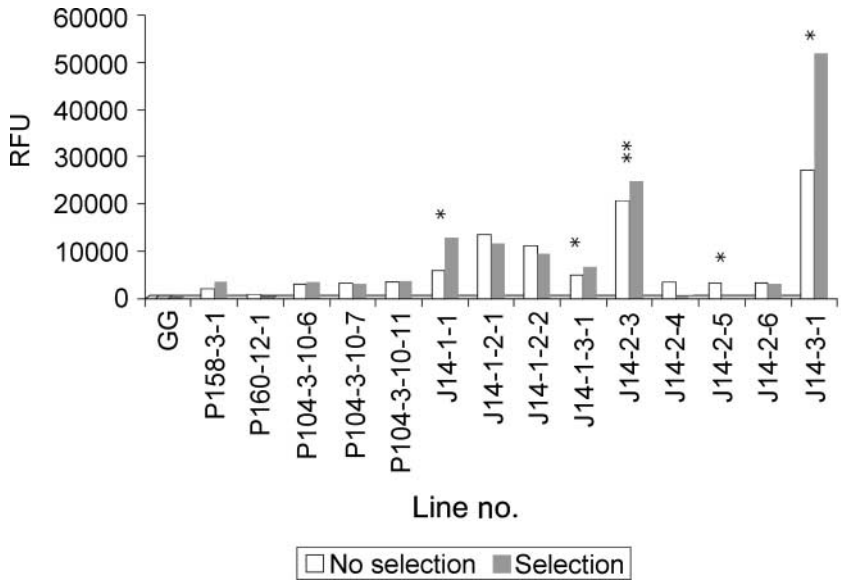

FIG. 2. Expression of GFP as relative fluorescence units (RFU) from different cell lines isolated by visual (J14 sublines) or hygromycin (P104, P158, P160) selection. Calluses were split into two parts. One part was transferred to hygromycin-containing medium (to apply hygromycin selection to visually selected lines), and the other part was maintained on medium without hygromycin (to release selection on hygromycin-selected lines). Split cultures were grown for 8 wk (subcultured at 4 wk) prior to quantitative analysis of GFP. **Asterisks* indicate significant differences at $*P < 0.05$ or $**P < 0.01$. GG, non-transformed control.

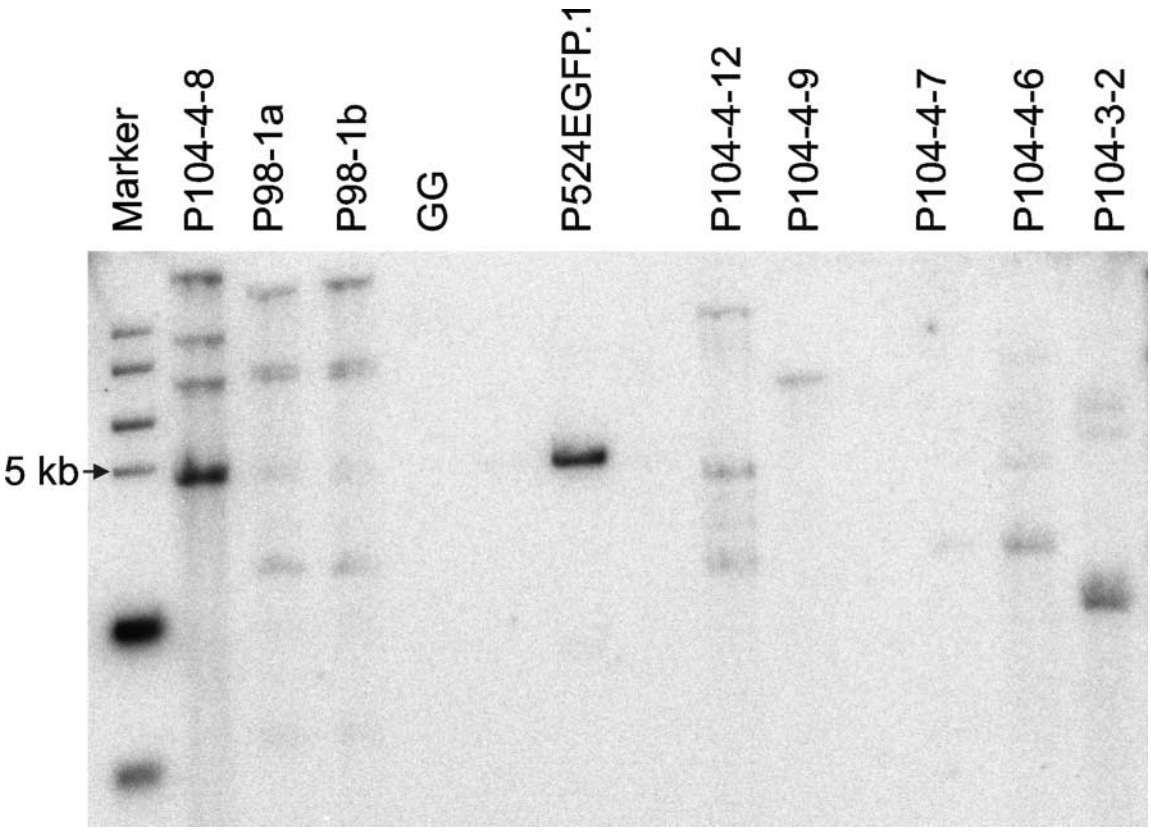

FIG. 3. Southern blot analysis of *Hin*dIII-digested DNA from transgenic cell lines (*lanes* to the right of plasmid) or regenerated plants (*lanes* between marker and plasmid) hybridized with the ^{32}P-labeled 548 bp *gfp* fragment. GG, non-transformed control Georgia Green. P542EGFP.1 was linearized by digestion with *Hin*dIII.

($P < 0.05$) between GFP expression in the selected and non-selected sublines (Fig. 2). Three showed increased and two showed decreased fluorescence on hygromycin-containing medium. Growth of the two that showed decreased fluorescence, J14-2-4 and J14-2-5, clearly was inhibited on hygromycin-containing medium suggesting that the hygromycin resistance gene was either disrupted or silenced. There was no significant difference in fluorescence when selection had been removed from previously selected hygromycin-resistant lines that expressed GFP (Fig. 2, lines P104, P158, and P160).

Plants were regenerated from P98-1 (visual) and P104 (hygromycin-selected) lines. Expression of GFP was visually confirmed at the time of shoot initiation. There was no obvious detrimental effect of GFP expression on tissue growth or regeneration since leaves from plants selected on hygromycin (P104-4) showed quantitative and qualitative GFP expression (~40 000 RFU for P104-4-2 and Fig. 1*H*, respectively). Leaves of plants from the visually selected line, P98-1, did not show GFP fluorescence that was significantly different from the non-transformed control. The *gfp* gene was transmitted to progeny of P104-4-8 as shown by PCR amplification of the gene from six of seven seeds harvested (data not shown).

Integration of *gfp* into genomic DNA was confirmed by Southern blot analysis. When digested with *Hin*dIII, multiple bands were observed that were both larger and smaller than the *gfp*-hybridizing fragment (4.7 kb) from linearized p524EGFP.1 (*Hin*dIII cuts once in the plasmid at the 5′ end of the promoter) (Fig. 3). The *gfp* sublines of P98-1 that showed different levels of brightness had identical integration patterns; therefore, they represent a single integration event (Fig. 3). At least four copies of *gfp*, represented by four hybridizing fragments, were present in the visually selected line, which was a similar number compared with some of the hygromycin-selected lines (Fig. 3). The number of copies of *gfp* was not strictly correlated with GFP expression levels, although the brighter hygromycin-resistant lines (P104-4-9 and P104-4-7) each had a single copy (Fig. 3).

Co-bombardment of two different plasmids can greatly reduce the time for construct development, and co-transformation of peanut is reproducible in this work and that of Livingstone and Birch (1999). Jordan (2000) also observed a high co-transformation frequency in wheat for GFP when the antibiotic resistance gene resided on a separate plasmid. Although visual selection using GFP alone was possible to attain in our work and that of others (Fleming et al., 2000; Carlson et al., 2001; Kaeppler et al., 2001), the efficiency of recovery of GFP-expressing shoots increased under antibiotic selection (Cho and Widholm, 2002; Huber et al., 2002). Similarly, Elliott et al. (1999) used *gfp* expression for the early identification of transformed sugarcane cell lines under antibiotic selection, where antibiotic selection along with visual selection enabled the removal of untransformed tissue at an early stage and facilitated the identification of transformed cell clusters. Nevertheless, we have shown that it is possible to avoid antibiotic resistance genes and use visual selection of GFP-expressing peanut to recover transgenic lines, although at a lower frequency than with antibiotic selection. Although we found that the hygromycin-selected peanut lines were initially brighter than the visually selected lines, this may have been due in part to the expression potential of these particular integration events ('position effect') rather than solely due to a mixture of transformed and non-transformed cells in the visually selected lines. A wide range of expression levels ('position effect') was observed among both hygromycin and visually selected lines. Leffel et al. (1997), while working with tobacco plants, also observed different classes of fluorescence in transformed plants, and fluorescence levels have been correlated with the amount of GFP synthesized in tobacco (Leffel et al., 1997) and canola (Richards et al., 2003).

When multiple genes have been introduced via microprojectile bombardment, application of *gfp* as a secondary marker has been useful for tracking transmission of linked genes (Harper et al., 1999). Co-bombardment of *gfp* and an untested selectable marker gene could be used in peanut to more rapidly assess the utility of the selectable marker. We recently have tested the efficacy of a mercury resistance gene (*merB*; Bizily et al., 1999) for selection using co-bombardment with *gfp* in only a 7-mo. period. *MerB* was not effective as a selectable marker gene in peanut somatic embryos since there were more than 95% escapes in lines surviving on PMA (neither *gfp* nor *merB* genes amplified; data not shown). However, we did find that two out of three lines visually selected for GFP expression (SH-21, SH-215, SH-25) also possessed the *merB* gene as shown by Southern blot analysis (Fig. 4). GFP-expressing plants and progeny have been produced from these lines, confirming results of the initial experiments. If the

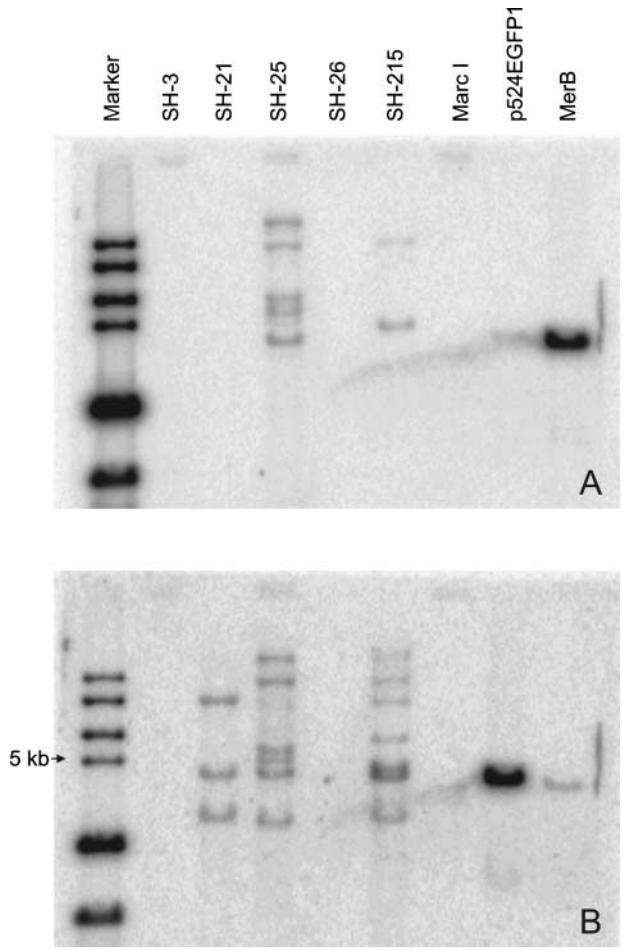

FIG. 4. Southern blot analysis of *Hin*dIII-digested DNA from transgenic cell lines hybridized with the ^{32}P-labeled 548 bp *gfp* fragment (*A*) or the 465 bp *merB* fragment (*B*). Marc I, non-transformed control. P542EGFP.1 and *merB* were linearized by digestion with *Hin*dIII. SH-3 and SH-26 represent escapes.

objective is to avoid antibiotic resistance genes as selectable markers, we have shown that selection of transgenic lines of peanut using GFP expression alone can be successful, albeit less efficient, provided that diligent observation and transfer of fluorescent transformation events is practiced.

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