Transformation of peanut with a soybean \textit{vspB} promoter-\textit{uidA} chimeric gene. I. Optimization of a transformation system and analysis of GUS expression in primary transgenic tissues and plants

Aiming Wang, Hanli Fan, Chong Singsit and Peggy Ozias-Akins


Direct DNA delivery via microprojectile bombardment has become an established approach for gene transfer into peanut (\textit{Arachis hypogaea} L.). To optimize our transformation protocol and to simultaneously explore the function of a heterologous promoter whose activity is developmentally regulated, embryogenic cultures from three peanut cultivars were bombarded with two plasmid constructs containing a \textit{uidA} gene controlled by either a soybean vegetative storage protein gene promoter or a cauliflower mosaic virus 35S promoter. We found that GUS transient expression was useful to predict stable transformation and confirmed that image analysis could provide a quick and efficient method for semi-quantitation of transient expression. One hundred and sixty hygromycin-resistant cell lines were recovered from and maintained on selective medium, and those tested by Southern blot analysis showed integration of the foreign gene. Over 200 transgenic plants were regenerated from 38 cell lines. More than 100 plants from 32 cell lines flowered and 79 plants from 19 cell lines produced pods. Over 1 000 R\(_{1}\) seeds were harvested. Analysis of expression in primary transgenic plants showed that GUS expression driven by the \textit{vspB} promoter was modulated by chemical and positional information.

Key words – \textit{Arachis hypogaea}, biolistic, \(\beta\)-glucuronidase, groundnut, GUS, microprojectile bombardment, peanut, promoter, transformation, transgene, vegetative storage protein.

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Introduction

\textit{Arachis hypogaea} L., peanut or groundnut, is an important commercial crop in the US and other warm-temperate to sub-tropical parts of the world. Production and quality can be severely impacted under less than ideal growing conditions due to climatic factors, pests and diseases. Genetic engineering has emerged as a prospective way to reduce certain pest problems such as insect infestation (Braun et al. 1991, Fraley 1992, Singsit et al. 1997). To realize the potential of gene transfer in peanut, a genotype-independent, repetitive embryogenesis culture system for the species has been established (Ozias-Akins et al. 1992). This system is amenable to transformation using microprojectile bombardment and selection for hygromycin resistance, and provides prolific plant regeneration (Ozias-Akins et al. 1993). Using the published transformation method, we
have introduced a selectable marker gene, hygromycin phosphotransferase, and an insect resistance gene, *Bacillus thuringiensis cryIA(c)*, into peanut plants (Ozias-Akins et al. 1993, Singsit et al. 1997). Transgenic plants expressing the *cryIA(c)* gene have demonstrated efficacy of the insecticidal crystal protein against the lesser cornstalk borer, *Eusarcoma longissima* (Singsit et al. 1997). In spite of these significant advances, genetic transformation of peanut needs to become more efficient to reduce the time and costs associated with introduction of additional genes. Alternative methods for stable transformation of peanut do not offer greater efficiency, genotype independence, or readily available technology (Brar et al. 1994, Cheng et al. 1996). For future commercialization, it also will be necessary to produce large numbers of primary transgenic plants in order to allow selection of plants which retain all desirable agronomic characteristics and do not display position effects on transgene expression due to insertion site or somaclonal variation arising through tissue culture. To improve the transformation system for peanut, we analyzed a number of factors which had been considered critical for optimizing DNA delivery and expression (Klein et al. 1988, Wang et al. 1988, Taylor and Vasili 1991).

As a resistance strategy, transgenes can be expressed constitutively or can be designed to respond to developmental, chemical, or environmental cues for expression that is controlled to provide optimal exposure of the target pest to the gene product. This approach should reduce the constant exposure of a pest to toxic gene products and might reduce the probability for the pest to develop resistance. The widely used cauliflower mosaic virus (CaMV) 35S promoter not only is less effective in some species (Christensen et al. 1992, Taylor et al. 1993), but also expresses any gene under its control in a nominally constitutive pattern. Gene plant promoters are being isolated and dissected with regard to specific response elements. One promoter upon which we have focused the present work is derived from a soybean vegetative storage protein (*vsp*) gene (Mason et al. 1988, Rhee and Staswick 1992). This gene is developmentally regulated as its product accumulates to high levels in actively growing tissues such as the soybean pod wall and the hypocotyl of germinating seeds. The *vsp* gene expression is modulated by carbohydrates, wounding, methyl jasmonate, phosphate, auxin, and water deficit. Extrapolating from the pattern of *vsp* gene expression in soybean, expression of the Bt *cryIA(c)* gene controlled by the *vsp* promoter in peanut potentially could provide high levels of the gene product in young pods, which is the peanut plant part that suffers the most damage from lesser cornstalk borer (LCB) feeding. During scarification of the pod wall, the LCB also inoculates the damaged tissues with spores of *Aspergillus flavus* or *parasiticus* (Lynch and Wilson 1991), two fungal species which produce afla-toxin. By targeting expression of a resistance gene to its most likely site of action, it should be possible to minimize the diversion of plant resources to transgene products and reduce the potential for negative effects on insect resistance management while maintaining effective control of the target pest. With this future resistance management strategy in mind, we initially tested the activity of the *vspB* promoter, when fused with the *uidA* coding region, in peanut.

**Abbreviations** — CaMV, cauliflower mosaic virus; *hph*, hygromycin phosphotransferase gene; JA, jasmonic acid; LCB, lesser cornstalk borer; MS, Murashige and Skoog basal medium; MU, 4-methyl umbelliferone; MUG, 4-methylumbelliferone; *vsp*, vegetative storage protein; *uidA*, β-glucuronidase gene.

### Materials and methods

#### Plant material

Embryogenic cultures were initiated from three peanut cultivars, Georgia Runner, Florunner, and MARC-1. The immature seeds were surface sterilized and embryos were dissected and cultured as described by Ozias-Akins (1989) and Ozias-Akins et al. (1993). The embryogenic tissues were increased and maintained by monthly transfers on Murashige and Skoog (1962) basal medium (MS) containing 3 mg l⁻¹ picloram and 1 g l⁻¹ filter-sterilized glutamine. Fourteen-day-old subcultures were used in all bombardment experiments unless otherwise stated.

#### Plasmid sources and constructions

Two plasmid constructs, pMOG617 and pxVGH were used for bombardments. pMOG617 (MOGEN International rv, Eisteinweg 97, 2333 CB Leiden, The Netherlands) contained a β-glucuronidase (*uidA*) gene and a hygromycin phosphotransferase (*hph*) gene, both under control of the CaMV 35S promoter (*size = 8.2 kb*). Plasmid pxVGH was constructed by excising the *vsp-uidA* cassette from pBI101-X (Mason et al. 1993; provided by John Mullet, Texas A&M, College Station, TX, USA) with *XbaI* and *EcoRI*. The 35S-*hph* cassette was excised from pCB13lo-* (David Ow, Plant Gene Expression Center, Albany, CA, USA) using HindIII and *XbaI*. The two gene cassettes were ligated simultaneously into pUC19 which had been double digested with HindIII and EcoRI (Fig. 1; plasmid size = 9.6 kb).

#### Microprojectile bombardment

The DNA/microprojectile mixture was prepared using plasmid DNAs purified with Qiagen columns (Qiagen, Chatsworth, CA, USA). Microprojectile bombardment was carried out using a PDS 1000/helium-driven apparatus (Bio-Rad, Hercules, CA, USA) and previously
published procedures for adsorption of DNA onto gold particles (Singsit et al. 1997). The vacuum chamber was adjusted to 93 kPa and 12.4 MPa rupture discs were used. The sample platform was positioned 5, 8, or 11 cm below the launch assembly in one experiment and 5 cm in all other experiments. Plasmid DNA amounts per bombardment ranged from 0.17 to 1.5 µg in one experiment, and were kept constant at 1.17 µg per bombardment in all other experiments. Sixteen tissue pieces, selected 14 days after subculture to fresh medium, were repositioned within a 2.5-cm diameter area in the center of the plate except as otherwise indicated in one experiment. The plasmid DNA and host genotypes were varied over experiments since genotype was shown to have no significant effect on transient expression in these experiments.

Histochemical GUS assay

Transient expression was determined 24 h after bombardment by a histochemical, colorimetric assay according to Jefferson (1987). The assay solution contained 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100, and 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl β-d-glucuronide (X-gluc) in 50 mM phosphate buffer, pH 7.0. Three tissue pieces were randomly collected from each plate and placed in a microcentrifuge tube containing 250 µl staining solution. The assay tubes were incubated for 20 h at 37°C. The sample buffer then was discarded and replaced with 500 µl of 70% (v/v) ethanol before visual examination, image analysis, and photography. GUS-positive foci that consisted of one or more contiguous blue-stained cells were counted manually and by image analysis as described by Anderson et al. (1994). Data collection and analysis of GUS expression also followed their description.

For analysis of GUS expression in plant organs, tissues were fixed immediately after harvest in 0.3% (w/v) paraformaldehyde, 0.3 M mannitol buffered with 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.6. Tissues then were washed three times in assay buffer and stained as described above. To assay GUS expression in stably transformed cell lines, two pieces of tissue were randomly collected from each cell line and incubated in the assay solution which was of the same composition as above except that 0.01 M borate buffer (pH = 7.0) was substituted for phosphate buffer.

GUS quantification

GUS extraction buffer (200 µl) that was composed of 50 mM NaHPO₄, 10 mM 2-mercaptoethanol, 10 mM Na₂EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (v/v) Triton X-100 was added to each of the tubes containing three pieces of transformed tissue or about 50 mg leaf, stem or root tissues which were collected from PCR-positive plants. The samples were homogenized for 30 s on ice and extracts were clarified by centrifugation. The reactions were started by adding 75 µl GUS assay buffer, which contained 2 mM 4-methylumbelliferyl β-d-glucuronide (MUG) in extraction buffer, to a 50-µl aliquot of each of the extracts. The mixture was vortexed and incubated at 37°C. Each of the reactions was stopped after 15 min by adding 50 µl of the reaction to 950 µl of stop buffer (0.2 M Na₂CO₃) and vortexing. Further dilutions were performed as needed to obtain a 2-ml sample with the appropriate concentration for fluorometric analysis. The amount of fluorescence was determined using a Hoefer TKO 100 fluorometer (Pharmacia, Piscataway, NJ, USA). The amount of protein per sample was determined using the Bio-Rad protein assay kit and bovine serum albumin as the standard.

Selection and regeneration of hygromycin-resistant cell lines

Selection was initiated 24 or 72 h post-bombardment in all experiments except one where time points of 24, 96, and 168 h were tested. Bombarded cultures were transferred to liquid selection medium (same as maintenance medium) containing 10 mg l⁻¹ hygromycin for 1 week, followed by same medium but with 20 mg l⁻¹ hygromycin for subsequent weekly subculture. During liquid selection, the culture was shaken at 110 rpm in the dark. After 6 weeks, antibiotic-resistant embryogenic tissues were carefully transferred to solid maintenance medium containing 20 mg l⁻¹ hygromycin and were subcultured onto fresh medium at 30-day intervals. Regeneration was initiated according to Ozias-Akins (1989) and Ozias-Akins et al. (1993) after putative transformed cell lines had proliferated.

DNA analysis

To isolate DNA for PCR analysis, a piece of tissue from each hygromycin-resistant cell line or about 50 mg leaf tissue was extracted according to the method of Doyle and Doyle (1987). PCR amplification of a portion of the chimeric hph gene was carried out with primer sequences and cycling conditions according to Ozias-Akins et al. (1993). For amplification of a portion of the CaMV 35S promoter/uidA cassette, a 21-nucleotide primer (5'-TGCCAGTTCCGTTGTTGTTGTTGTT-3') that was specific for uidA and the same 35S promoter primer as used for hph were combined, while for the usp promoter-uidA fusion, a 21-nucleotide primer (5'-AAAGAACGTCACCCCTCCCAA-3') was designed for the promoter and used in combination with the uidA primer. The amplification reactions were carried out with a Perkin-Elmer Cetus DNA thermal cycler (Foster City, CA, USA) under the following conditions: 94°C for 30 s, 45°C for 30 s, 72°C for 2 min, 35 cycles,
Tab. 1. Transient GUS expression as a function of plasmid type, genotype, and time. Image analysis quantitation of GUS activity 24 h after bombardment (for plasmid type and genotype experiments) or immediately before selection (day-selection-initiated experiment). For plasmid type, data from both genotypes were pooled. For genotype, data from both plasmids were pooled. An equal number of samples (24) for each genotype and each plasmid was used. The number of hygromycin-resistant cell lines also is reported. Values ± SD are given.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean no. GUS+ foci mm⁻²</th>
<th>Total % area stained GUS⁺</th>
<th>Average size GUS⁺ foci (mm²) × 10⁻³</th>
<th>Mean no. Hyg⁺ cell lines per bombardment</th>
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<td>Plasmid type</td>
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<td>pMOG</td>
<td>10.6 ± 7.5</td>
<td>23.5 ± 22.1</td>
<td>19.8 ± 18.5</td>
<td>3.4 ± 2.3</td>
</tr>
<tr>
<td>pxVGH</td>
<td>8.7 ± 6.8</td>
<td>3.0 ± 6.3</td>
<td>2.6 ± 3.5</td>
<td>3.1 ± 2.3</td>
</tr>
<tr>
<td>Genotype</td>
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<td></td>
<td></td>
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<td>MARC-I</td>
<td>9.2 ± 7.6</td>
<td>16.8 ± 23.3</td>
<td>14.4 ± 19.5</td>
<td>4.2 ± 2.5</td>
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<td>Georgia Runner</td>
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<td>9.7 ± 13.4</td>
<td>8.0 ± 10.2</td>
<td>2.4 ± 1.8</td>
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<td>Day selection initiated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>15.7 ± 10.3</td>
<td>13.8 ± 17.0</td>
<td>7.9 ± 8.0</td>
<td>4.5 ± 2.2</td>
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<tr>
<td>4</td>
<td>9.4 ± 5.6</td>
<td>13.0 ± 22.6</td>
<td>12.4 ± 20.7</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>7</td>
<td>6.3 ± 4.5</td>
<td>6.9 ± 11.6</td>
<td>7.7 ± 12.4</td>
<td>1.2 ± 1.3</td>
</tr>
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</table>

followed by a 4°C soak cycle until recovery. The amplified products were assayed by electrophoresis in 1.4% (w/v) agarose (Seakem HGT, FMC, Rockland, ME, USA) gels in 1× TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA, pH 8.2).

A few of the cell lines that showed a positive PCR signal for presence of foreign DNA were subjected to Southern hybridization analysis. For Southern blotting, DNA was isolated from fresh or frozen (−80°C) tissue following the method of Doyle and Doyle (1987). DNA (10 μg) was digested with EcoRI, which recognizes three sites in the pxVGH plasmid. Digested and undigested (10 μg) DNAs were subjected to electrophoresis for 24 h at 30 V on a 0.8% agarose (Seakem HGT) gel in 1× TBE buffer, then transferred by the capillary method to a nylon membrane (GeneScreen Plus, DuPont NEN, Boston, MA, USA). The blot was hybridized with a random primed [³²P]dCTP-labeled EcoRI fragment (4.6 kb) from pxVGH plasmid that contained all of the vsp-uidA gene and the 3' end of the hph gene.

Assayed using image analysis and manual counts. The results indicated a significant correlation ($r = 0.858$, $P < 0.05$) between manual spot counts and those by image analysis. However, no significant relationship existed between the number of GUS foci measured (either manually or by image analysis) and the average staining size of GUS foci or the total percentage of stained area measured by image analysis. The correlation between total percentage of stained area by image analysis and average size of GUS foci was 0.844. Based on image analysis data for the number of GUS foci, there was no significant difference between genotypes, MARC-I and Georgia Runner, or between plasmid types, pMOG617 and pxVGH (Tab. 1). There was a significant difference, however, between pMOG617 and pxVGH with respect to total percentage of stained area and size of GUS-positive foci. GUS foci in embryogenic tissues bombarded with pMOG617 were substantially larger than those resulting from pxVGH bombardments (Tab. 1).

A decrease in GUS transient expression measured as number of GUS-positive foci was observed with an increase in response time, for data 7 days post-bombardment.
Tab. 2. Transient GUS expression as affected by bombardment parameters. Image analysis quantification of GUS activity 24 h after bombardment as a function of DNA amount per bombardment (at a 5-cm distance), distance between rupture disc and sample platform (at a DNA concentration of 1.17 μg), and radius from center of sample platform. Values are indicated ± sd.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean no. GUS+ foci mm⁻²</th>
<th>Total % area stained GUS+</th>
<th>Average size GUS+ foci (mm²) × 10⁻³</th>
</tr>
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<tr>
<td>DNA amount (μg)</td>
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<tr>
<td>0.17</td>
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<td>2.8 ± 1.6</td>
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<td>0.5</td>
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<td>4.3 ± 2.9</td>
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<td>16.7 ± 12.1</td>
<td>4.6 ± 2.7</td>
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<tr>
<td>1.17</td>
<td>35.3 ± 22.5</td>
<td>24.3 ± 19.6</td>
<td>6.0 ± 3.5</td>
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<tr>
<td>1.50</td>
<td>30.8 ± 17.2</td>
<td>27.0 ± 20.8</td>
<td>5.2 ± 2.4</td>
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<tr>
<td>Distance (cm)</td>
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<tr>
<td>5</td>
<td>13.6 ± 11.1</td>
<td>3.2 ± 3.2</td>
<td>1.8 ± 1.4</td>
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<tr>
<td>8</td>
<td>5.4 ± 8.4</td>
<td>0.4 ± 0.7</td>
<td>0.7 ± 0.5</td>
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<tr>
<td>11</td>
<td>2.0 ± 2.9</td>
<td>0.2 ± 0.6</td>
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<td>Radius (cm)</td>
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<td>0–0.8</td>
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<td>36.3 ± 34.1</td>
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<td>1.2–1.7</td>
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<tr>
<td>1.7–2.1</td>
<td>1.7 ± 2.2</td>
<td>1.3 ± 2.2</td>
<td>4.3 ± 5.5</td>
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</table>

positive foci due to the large standard deviations, although there was a distinct trend toward more foci up to 1.17 μg DNA per bombardment. Target distance from the stopping plate and radius from the center of the target platform were determined to be optimum at 5 cm and up to 1.2 cm, respectively (Tab. 2). Outside of a 1.2-cm radius, the number of GUS-positive foci declined significantly.

Confirmation of introduction of foreign genes by DNA analysis

Of the 160 hygromycin-resistant cell lines recovered, 105 were tested by PCR for amplification of a 380-bp fragment from the hph gene cassette and 99 of these were positive. All of the samples negative by PCR for the 35S-hph fragment were positive for amplification of the covalently linked, non-selected, reporter gene driven by the vsp promoter, suggesting false-negative PCR reactions in a few of the 35S-hph amplifications since all lines were hygromycin resistant and presumably contained a functional hph gene. All but two of the lines with positive amplification of the 35S-hph fragment also were positive for vsp-uidA amplification. The two lines negative for vsp-uidA amplification remained negative upon repeated assay.

Genomic DNA from hygromycin-resistant and PCR-positive cell lines transformed with pxVGH was subjected to Southern hybridization analysis. The labeled 4.6-kb EcoRI fragment from pxVGH recognized a 4.6-kb fragment in the genomic DNA of transgenic cell lines digested with EcoRI (Fig. 2). The same probe hybridized to HindIII digests of genomic DNA recognized both intact fragments (5.7 and 3.8 kb) of the plasmid (Fig. 1) in some, but not all cell lines (Fig. 2). All transgenic cell lines also produced less intense signal from DNA fragments larger and smaller than the hybridizing plasmid bands. Only a single high molecular mass band in non-digested genomic DNAs hybridized with the probe (data not shown). No hybridization was observed in the control, non-transgenic DNA lanes.

Plant regeneration from stable transformants

Over 200 plants were regenerated from 38 cell lines PCR-positive for the selectable marker gene (Tab. 3). All cell lines subjected to the regeneration protocol produced plants. Approximately 3 months were needed for plant regeneration from embryogenic cultures and about 5 additional months were required for plant development (growth, flowering, pegging and seed pod maturation) in the greenhouse. Over 50% of the plants flowered; however, only 62% of these produced

Fig. 1. Map of pxVGH containing the selectable marker gene for hygromycin resistance and the reporter gene, uidA, driven by the vspB promoter. Both genes are terminated by the nos 3' region.
Fig. 2. Southern blot of transgenic *vsp uidA* cell lines and control Florunner (FR) digested with EcoRI (RI) or HindIII (dIII) and probed with a 4.6-kb EcoRI fragment containing the *vsp uidA* cassette and the 3’ end of the hph gene. Plasmid pxVGH also is digested with EcoRI or HindIII (partial digest). Signals in lanes 5 and 13 are weak relative to the same genotypes in lanes 14 and 3, respectively, because of less DNA in lanes 5 and 13.

GUS activity in transgenic tissues and organs modulated by jasmonic acid, phosphate deficit and mannose

Although the GUS activities were different among different cell lines, the average GUS activity increased approximately 3- to 5-fold when transgenic tissues from seven lines were exposed to 50 μM JA, either in the presence or absence of 7% mannose (Fig. 3). There was no consistent induction in MS medium without phosphate or MS plus 7% mannose. In nine other cell lines tested, the basal level of GUS expression in MS medium was low and no enhancement of expression by JA was observed. Plants were regenerated from four of these lines, and the plants also showed a relatively low level of GUS expression when leaves were assayed. The expression of GUS driven by the *vspB* promoter was regulated in an organ-specific manner with low activity in roots, moderate activity in leaves, and the highest activity in stems of three primary transgenic plants (Figs 4 and 5).

Histochemical assay of GUS activity in whole organs also indicated intense staining of anthers, young peanut pegs, and developing pod walls (Fig. 5c–f). As observed with expression in cell lines, expression varied greatly among transgenic plants of independent origin even though stems from the same developmental position on each plant were sampled for the analysis (Fig. 6).

Discussion

Image analysis as a quick and efficient measurement for evaluating transient GUS expression

The *uidA* gene (Jefferson et al. 1986) routinely is fused with different promoters so that it can be used as a reporter of promoter function in transgenic plants or other organisms (Gallagher 1992). When the gene is driven by a nominally constitutive promoter, such as CaMV 35S, it is useful for measuring transient expression several hours after delivery of foreign DNA to cells and tissues. Analysis of transient GUS expression usually is accomplished by manually counting GUS-positive foci under a microscope. This procedure is
Tab. 3. Fertility of transgenic plants. *Only a selected group of hygromycin-resistant lines recovered were taken through regeneration. **One line, no flowers or pods. ***One line, flowers, but no pods. ****One line, no flowers; 2 lines, no pods. ******Transformed with pMOG. ******When multiple regenerated plants were present within a fertile line, the majority of the sister plants set seed.

<table>
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<tr>
<th>Bombardment*</th>
<th>Resistant lines</th>
<th>Plants</th>
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<th>Plants with pods***</th>
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<td>80-9^c</td>
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<td>249</td>
<td>127</td>
<td>79</td>
<td>724</td>
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Data collection on transient GUS expression in plant tissue. To compare the results obtained by image analysis and manual methods, we collected GUS expression data from 44 embryogenic tissue samples and observed a strong correlation between the two assays. This confirms the previous report from Anderson et al. (1994) that manual and image analysis measurement of number of GUS-positive foci are highly correlated, although both studies found no correlation between number of foci (recorded by image analysis) and either percentage of area stained or average size of foci.

Factors affecting GUS transient expression and stable transformation

Regardless of biolistic devices used, the important factors that have been reported to affect transformation efficiency include momentum of the particles, the quantity of DNA that each particle can carry into the cell, the number of particles that impact the target cells per unit area, and the subsequent regeneration system (Klein et al. 1992). GUS transient activity, which may be expressed without integration of the gene into the genome, has been used to demonstrate the successful transfer of DNA into plant cells, and to preliminarily screen for the effect of vector modification on gene expression as well as bombardment parameters and target tissue variation. Klein et al. (1989) and Timmermans et al. (1990) both reported that approximately 2–5% of the cells that transiently expressed a foreign gene became stably transformed in tobacco. A lower transformation frequency of 0.4% of transient expression events in soybean suspension cultures was observed by Finer and McMullen (1991). The metabolic

Fig. 3. GUS activity in embryogenic tissues cultured in MS medium, MS plus 7% mannose (MS + MAN), MS plus 7% mannose and 50 μM jasmonic acid (MS + MAN + JA), or MS plus 50 μM jasmonic acid (MS + JA). Tissues were incubated under a 16/8 h photoperiod for 48 h.
between 10 and 15 days after subculture. Other tissue types could be used for bombardment but they must be capable of rapid cell division and competent for plant regeneration. Plant genotype (MARC-1 vs Georgia Runner) was not a source of variation with respect to either transient expression or stable transformation in the present experiments, although Anderson et al. (1994) did observe a significant difference between the Spanish genotype, Toalson, and the runner genotype, Florunner. The embryogenic cultures used as a source of tissue for bombardment are more developmentally heterogeneous in some genotypes than in others which

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Fig. 4. GUS activity ± SD in three organ types (root, stem, and leaf) from three transgenic plants (containing vsp-uidA) of
could be a partial explanation for the variation between genotypes.

Vector construction also can have an effect on transient expression (Hensgens et al. 1993, Taylor et al. 1993). We observed that DNA from pMOG in bombarded tissues yielded a slightly, but non-significantly higher number of GUS-positive foci per mm² than pxVGH. The number of hygromycin-resistant cell lines recovered for both constructs also was not significantly different, although the percentage of stained area and average size of foci were distinctly higher in pMOG-bombarded tissues. This may indicate a higher level of expression in embryogenic cultures of GUS driven by the CaMV 35S promoter when compared with the uspB promoter, which could result in more enzyme or enzyme product that could diffuse from the site of production.

The velocity of the microprojectiles was shown to be an important factor affecting the delivery of DNA (Klein et al. 1988). The final velocity of microprojectiles is related to air resistance (Morrish et al. 1993). Hence, the distance travelled in a partial vacuum will affect the velocity and thereby the penetration of particles upon impact. In our experiment, when a sample was placed at various distances from the stopping plate, the highest GUS expression was observed at a distance of 5 cm. Increasing the distance gave a correlated decrease in DNA delivery with a 3- and 7-fold drop in expression at 8 and 11 cm, respectively. Similar results have been demonstrated in maize (Klein et al. 1988) and pearl millet embryos (Taylor and Vasil 1991). We also optimized the target area (1.2-cm radius) at a given distance (5 cm) for maximized GUS transient expression. Since the DNA-coated particles were spread onto the macro-carrier film in a ring of 1-cm diameter, we would predict that most particles would be accelerated into the central part of sample. Therefore the number of GUS-positive foci is a function of particle density, but high particle density also can increase tissue damage.

Fitch et al. (1990) obtained transformed papaya tissues by delaying selection for 3–5 weeks after bombardment. Ozias-Akins et al. (1993) also delayed selection for 1 month after bombardment since distinct somatic embryos beyond the globular stage of development comprised the bombarded cultures. It has been speculated that bombarded tissues need time for recovery before initiation of selection. To the contrary, we obtained just as many stably transformed cell lines from experiments where selection was initiated only 1 day after bombardment vs 4 days, and significantly more transgenic cell lines were recovered when early selection was compared with selection initiated at 7 days. Perhaps the cells recovering from bombardment injury cannot compete effectively with uninjured, and thus non-transgenic, cells when grown under non-selective conditions. Exposure to a selection agent would, however, negatively affect growth of uninjured cells thereby potentially conferring a competitive advantage to bombarded cells.

GUS expression driven by the uspB promoter in response to positional and chemical factors

soybean leaves, VSPβ accumulates before flowering, declines during early seed development, and reaccumulates during seed pod maturation (Wittenbach 1982). Removal of developing pods from soybean plants or petiole girdling to block transport from leaves causes the accumulation of the VSPβ protein and its mRNA, as well as three other proteins including VSPz (Wittenbach 1983). The VSPβ protein is abundant in developing leaves, stems, pods, and flowers but rare in roots, seeds, and mature stems and leaves of soybean (Staswick 1989, Mason and Mullet 1990).

In transgenic peanut, we found that GUS expression from the vsp-uidA gene was regulated in a tissue-specific manner and was much higher in leaves and stems compared with that in roots. This pattern of expression is similar to that observed in soybean (Mason and Mullet 1990). In addition to developmental regulation, vspβ gene expression is modulated by several physiological/nutritional factors such as wounding, water deficit, sugar, methyl jasmonate, phosphate, auxin and nitrogen (Franceschi and Grimes 1991, Staswick et al. 1991, Mason et al. 1992, 1993, DeWald et al. 1994, Sadka et al. 1994). Evidence has shown that JA or methyl jasmonate as well as sugar are the primary regulators of vsp gene expression (Mason and Mullet 1990, Anderson 1991, Franceschi and Grimes 1991, Staswick et al. 1991, Mason et al. 1993). Mason et al. (1992, 1993) found that the promoter of vspβ contained jasmonate- and carbon-responsive domains which could act independently. The vspβ gene can be induced in soybean leaves and cell cultures by jasmonate at 10⁻⁶ to 10⁻⁵ M (Mason and Mullet 1990, Staswick 1990, Anderson 1991). High accumulation of vsp mRNA in soybean seedling hypocotyl hooks and low accumulation in roots is paralleled by higher concentrations of methyl jasmonate in hypocotyl hooks than in roots.

In agreement with above findings, our results showed that expression from the vsp-uidA gene in transgenic embryogenic tissues and plants of peanut was subject to tissue-specific regulation and was enhanced by the presence of JA. It perhaps is not surprising that the vsp promoter is similarly regulated in peanut and soybean since both species are legumes, and thus may have similar biological responses. The following major morphological differences between the species do exist: (1) Soybean has a specialized tissue connecting the vascular bundles in leaves called the paravascular mesophyll (Franceschi and Giaquinta 1983). This type of specialized tissue does not occur in peanut. (2) The peanut fruits are hypogal, thus they develop underground in the dark. All reproductive parts of soybean are produced above ground and in the light. Since light is known to positively affect vsp gene expression, it was important to experimentally determine the ability of this promoter to control expression in the hypogal peanut pods before using the promoter in a pest resistance strategy. The apparent high level of expression in peanut pods, tested thus far only with a histochemical assay, may be a function of the high sink capacity of this tissue combined with likely high jasmonate levels (Creelman and Mullet 1995). More extensive analysis of expression in pod tissues will be carried out with R₂ progeny. The potential for controlling expression of genes targeted to reduce aflatoxin contamination of peanut pods and seeds with promoters other than CaMV 35S may have implications for pest resistance management as well as putative silencing of multiple transgenes having sequence homology.

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References


