

## TRANSFORMATION OF SOYBEAN VIA PARTICLE BOMBARDMENT OF EMBRYOGENIC SUSPENSION CULTURE TISSUE

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### SUMMARY

Embryogenic suspension culture tissue of soybean (*Glycine max* Merrill.) was bombarded with particles coated with plasmid DNAs encoding hygromycin resistance and  $\beta$ -glucuronidase (GUS). One to two weeks after bombardment, embryogenic tissue was placed in a liquid proliferation medium containing hygromycin. Four to six weeks after bombardment, lobes of yellow-green, hygromycin-resistant tissue, which began as outgrowths on brown clumps of hygromycin-sensitive tissue, were isolated and cultured to give rise to clones of transgenic embryogenic material. In vivo GUS assays of hygromycin-resistant clones showed that the early outgrowths could be negative, sectored, or positive for GUS activity. Transgenic, fertile plants could be routinely produced from the proliferating transgenic embryogenic clones. Southern hybridization analyses confirmed stable transformation and indicated that both copy number and integration pattern of the introduced DNA varied among independently transformed clones. Hybridization analysis of DNA from progeny plants showed genetic linkage of multiple copies of introduced DNA. An average of three transgenic clones were obtained per bombardment making this procedure very suitable for transformation of soybean.

*Key words:* soybean; *Glycine max*; transformation; particle bombardment.

### INTRODUCTION

Particle bombardment can be an efficient method for plant cell transformation, leading to the production of transgenic plants. The main advantage of particle bombardment over other transformation techniques is that intact plant tissues are targeted and protoplasts and *Agrobacterium* are not required. The practicality of particle bombardment for the generation of transgenic plants capable of transmitting the introduced DNA to progeny depends on the ability to identify and target either meristematic or embryogenic cells that will give rise to germline tissue.

Using tobacco leaves as the target tissue, Klein et al. (1988) obtained transgenic plants via particle bombardment. Kanamycin-resistant calli were initially obtained from bombarded leaf tissue after selection. Transgenic plants were then regenerated from these kanamycin-resistant calli. Although this approach is feasible with plants that are amenable to regeneration from calli, it is not currently practical for the majority of plant species.

McCabe et al. (1988) obtained transgenic soybean via particle bombardment of the shoot apex. In this report, only 1 regenerant expressed the gene for kanamycin resistance out of the 389 regenerated plants. In two subsequent reports (Christou et al., 1989; Yang and Christou, 1990), a total of seven transgenic soybean lines were evaluated. Shoot apex transformation is labor-intensive because the meristematic tissue is difficult to target and, without selection, a large number of plants must be regenerated and analyzed. In

addition, the primary transgenic plants obtained via shoot apex transformation are most often chimeric.

Particle bombardment of embryogenic suspension culture cells has been used to generate transgenic plants of cotton (Finer and McMullen, 1990) and maize (Gordon-Kamm et al., 1990; Fromm et al., 1990). Embryogenic cultures may be the best target tissue for transformation via particle bombardment because in a properly grown embryogenic suspension culture the majority of the cells in these cultures should be competent to form embryos and plants.

In an attempt to develop a routine transformation procedure for soybean we subjected embryogenic suspension culture tissue to particle bombardment. Single bombardments of embryogenic soybean tissue with DNA encoding the  $\beta$ -glucuronidase (GUS) gene (Jefferson, 1987) yielded an average of 709 GUS-expressing foci. Bombardments using a hygromycin-resistance gene gave rise to approximately three stable independent embryogenic clones, which were isolated and further propagated to give rise to large quantities of embryogenic material and transgenic plants. The feasibility and efficiency of this system is based on surface proliferation of high quality, highly embryogenic suspension cultures of soybean (Finer and Nagasawa, 1988), and effective selection for hygromycin resistance after bombardment.

### MATERIALS AND METHODS

*Initiation and maintenance of embryogenic suspension cultures.* Embryogenic suspension cultures of soybean (*Glycine max* Merrill. cv.

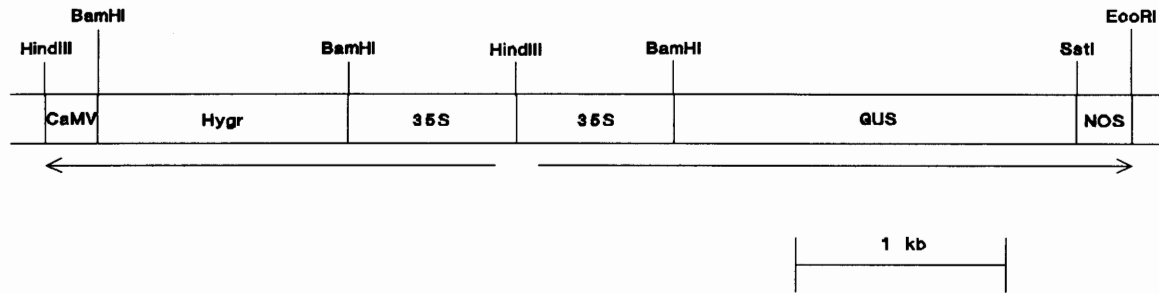


FIG. 1. Plasmid map of pHG1. pHG1 contains both the hygromycin-resistance gene and the GUS gene in pUC119. The hygromycin-resistance coding region is flanked by the CaMV 35S promoter and terminator and the GUS coding region is flanked by the CaMV 35S promoter and nopaline synthase terminator.

"Fayette") were initiated and maintained in the 10A40N medium supplemented with 5 mM asparagine as described previously (Finer and Nagasawa, 1988). For subculture, two clumps of embryogenic tissue, 4 mm in diameter, were transferred to 35 ml of 10A40N medium in a 125-ml deLong flask. High quality embryogenic material was selectively subcultured monthly at this low inoculum density.

**Histology.** For histologic studies, proliferating embryogenic tissue was fixed in a glutaraldehyde solution, dehydrated in an ethanol series, and embedded in Spurr's resin (Spurr, 1969) according to Finer (1988). Sections were cut to 0.75  $\mu$ m on a JB-4 microtome, mounted on glass slides, and stained with toluidine blue for viewing.

**Preparation of DNA and tungsten pellets.** The plasmid pUCGUS (Finer and McMullen, 1990) was made by subcloning the GUS gene as a *HindIII*/*EcoRI* fragment from pBI121 (Jefferson, 1987) into pUC119. The efficiency of particle bombardment was initially monitored using pUCGUS and counting the number of foci showing transient expression of the GUS gene, 3 days after bombardment. The plasmid pCIB709 (Rothstein et al., 1987) contains the aminoglycoside phosphotransferase type IV (AphIV) gene (Gritz and Davies, 1983) flanked by a CaMV 35S promoter and terminator. The AphIV gene encodes a protein that detoxifies the antibiotic hygromycin-B. The plasmid pHG1 (Fig. 1), which encodes for both hygromycin resistance and GUS activity, was constructed by simultaneously ligating the *HindIII*/*KpnI* fragment from pCIB709 (hygromycin) and the *HindIII*/*EcoRI* fragment from pBI121 (GUS) into *EcoRI*/*KpnI* cut pUC119. All DNA constructions were isolated after transformation into the *Escherichia coli* strain MV1190, and plasmid DNA was purified by standard procedures (Maniatis et al., 1982).

DNA was precipitated onto 1.1  $\mu$ m (average diameter) tungsten pellets using a  $\text{CaCl}_2$  precipitation procedure (Finer and McMullen, 1990). In cases of cotransformation using both pCIB709 and pUCGUS, the two plasmids were mixed at 1:9 (pCIB709:pUCGUS) before  $\text{CaCl}_2$  precipitation. This ratio was previously determined to give the highest levels of coexpression in hygromycin-resistant transformed cotton tissue (Finer and McMullen, unpublished). The pellet mixture containing the precipitated DNA was gently resuspended after precipitation, and 2  $\mu$ l was removed for bombardment.

**Preparation of plant tissue for bombardment.** Approximately 1 g of embryogenic suspension culture tissue (taken 3 wk after subculture) was transferred to a 3.5-cm-diameter petri dish. The tissue was centered in the dish, the excess liquid medium was removed with a pipette, and a sterile 500- $\mu$ m pore size nylon screen (Tetko Inc., Elmsford, NY) was placed over the embryonic tissue. Open petri dishes were placed in a laminar-flow hood for 10 to 15 min to evaporate residual liquid medium from the tissue. The 3.5-cm petri dish was placed in the center of a 9-cm petri dish immediately before bombardment. Bombardments were performed using a DuPont Biologics Particle Delivery System (model BPG). Each sample of embryogenic soybean tissue was bombarded once.

**Selection for transgenic clones.** Bombarded tissues were resuspended in the 10A40N maintenance medium. One to two weeks after bombardment the clumps of embryogenic tissue were resuspended in fresh 10A40N medium containing 50  $\mu$ g/ml hygromycin (10A40N-Hyg). Hygromycin (Calbiochem, LaJolla, CA) was filter-sterilized before addition to liquid media.

The 10A40N-Hyg medium was replaced with fresh antibiotic-containing medium weekly for 3 additional weeks.

Six to eight weeks after the initial bombardment, brown clumps of tissue that contained yellow-green lobes of embryogenic tissue were removed and separately subcultured in 10A40N-Hyg. After 3 to 4 mo. of maintenance in 10A40N-Hyg, proliferating embryogenic tissues were maintained by standard subculture in 10A40N without added antibiotic. Embryogenic tissues were periodically removed from 10A40N-Hyg and 10A40N for embryo development and Southern hybridization analyses.

**Embryo development and germination.** For embryo development, clumps of hygromycin-resistant embryogenic tissues were placed at 23°C on the embryo development medium, which contained MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 6% maltose, and 0.2% gelrite (pH 5.7). One month after plating, the developing embryos were cultured as individual embryos, 25 per 9-cm petri dish in fresh embryo development medium. After an additional 4 wk, the mature embryos were placed in dry petri dishes for 2 to 3 days. After the desiccation treatment, the embryos were transferred to a medium containing MS salts, B5 vitamins, 3% sucrose, and 0.2% Gelrite (pH 5.7). After root and shoot elongation, plantlets were transferred to pots containing a 1:1:1 mixture of vermiculite, topsoil, and peat, and maintained under high humidity. Plantlets were gradually exposed to ambient humidity over a 2-wk period and placed in the greenhouse.

**$\beta$ -glucuronidase analysis, DNA extraction, and Southern hybridization analysis.** GUS assays were performed on embryogenic soybean tissue and leaf tissue according to Jefferson (1987). DNA was extracted from embryogenic tissue and leaves using the CTAB procedure (Saghai-Marouf et al., 1984). DNAs from pCIB709-transformed cultures and plants were digested with *HindIII*, which cleaves pCIB709 once, just upstream from the CaMV 35S promoter. DNAs from pHG1-transformed cultures were digested with *SstI*, which cleaves pHG1 once at the 3' end of the GUS gene (Fig. 1). To determine if the hygromycin' and GUS expression units were intact, DNAs from pHG1-transformed tissues were digested with either *HindIII* (restriction sites flank the intact hygromycin' expression unit) or *HindIII*/*EcoRI* (restriction sites flank the intact GUS expression unit). Digested DNAs were electrophoresed on a 0.8% agarose gel. The DNA in the gels was treated with 0.2 N HCl, twice for 15 min followed with 0.5 M NaOH/0.1 M 1.5 M NaCl, twice for 30 min, and finally 1 M  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ /0.1 M NaOH, for 40 min. The DNA was transferred (Vollrath et al., 1988) to nylon membranes (Zetaprobe-BioRad, Richmond, CA) overnight by capillary transfer using 1 M  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ /0.1 M NaOH. The membranes were baked at 80°C for 2 h under vacuum and then prehybridized for 4 to 6 h at 65°C in 50 mM tris, pH 8.0, 5 $\times$  standard saline citrate (SSC), 2 $\times$  Denhardt's, 10 mM  $\text{Na}_2\text{EDTA}$ , 0.2% sodium dodecyl sulfate (SDS), and 62.5  $\mu$ g/ml salmon sperm DNA.

The *BamHI* fragment from pCIB709 (containing the hygromycin-resistance structural gene) or the *BamHI*/*SstI* fragment from pUCGUS (containing the GUS structural gene) were random-prime labeled (Feinberg and Vogelstein, 1983) and used for hybridization. Membranes were hybridized in the same solution as above but containing labeled probe (0.5 to 2  $\times$  10<sup>6</sup> cpm/ml) and 10% sodium dextran sulfate. After hybridization at 65°C for 24 to 48 h, the membranes were first washed 5 times in 2 $\times$  SSC/0.1%

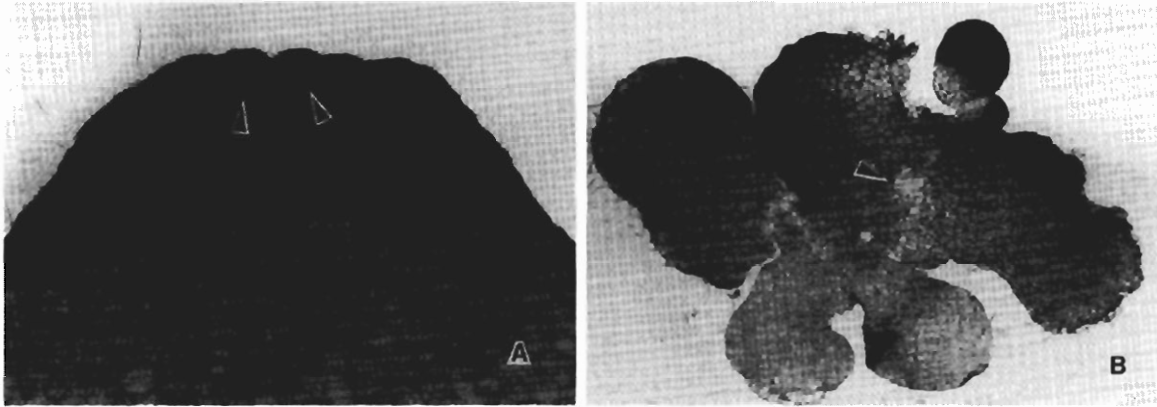


FIG. 2. A, thin section of embryogenic suspension culture material showing embryo initials (arrows) located on surface of lobe.  $\times 223$ . B, thin section of clump showing disintegration of internal tissue (arrow).  $\times 38$ .

SDS at 65° C and then washed 5 times in 0.1 $\times$  SSC/0.1% SDS at 65° C. Hybridization was visualized by exposure of the membranes to Kodak XAR-5 film at -70° C with intensifying screens.

### RESULTS

**Proliferating somatic embryo origin.** To determine the proper target tissue for particle bombardment of soybean, histologic studies were performed. Examination of proliferating embryogenic tissues revealed that embryos originated from surface and possibly adjacent subsurface tissues (Fig. 2 A, arrows). Embryo initials, which consisted of as few as two to four cells, could be detected over most of the surface of the embryogenic tissue. Embryogenic tissue in suspension culture never progressed beyond the globular stage. New initials were formed on the surface of the older globular embryos, and cells in the center of the clumps became vacuolated and disrupted (Fig. 2 B, arrow) as the clumps of tissue grew larger. Some of the larger clumps (>4 mm) seemed to be hollow, apparently due to extensive disintegration of internal tissues. Growth of proliferating cultures therefore resulted in a concentric layering of embryos. The agitation of the cultures together with disintegration of internal tissues caused breakage of larger clumps, resulting in production of smaller clumps of tissues. The surface origin of embryo initials suggested that these cultures may be amenable to transformation via particle bombardment, where surface tissues are targeted.

**Bombardment and selection for transgenic material.** To evaluate the efficiency of particle delivery, transient expression of the GUS gene was measured after bombardment with particles coated with pUCGUS. For production of stably transformed clones, pHG1 or either PCIB709 alone or in combination with pUCGUS was used for bombardments.

In early attempts to obtain stably transformed hygromycin-resistant clones, the bombarded soybean tissue was permitted 2 wk recovery (growth in liquid culture without selection) before placement of that tissue in hygromycin-containing medium. In later experiments, the 2 wk recovery period was reduced to 1 wk with no apparent decrease in transformation efficiency.

Two to three days after placement of the soybean tissue in the hygromycin-containing medium, the tissue and medium started to darken, apparently due to production of secondary compounds as a

stress response to antibiotic selection. To prevent detrimental effects from exposure of transformed hygromycin-resistant tissue to these secondary compounds, the medium was replaced with fresh hygromycin-containing medium every week for 4 wk. After 4 wk of selection, the medium remained clear.

One month to six weeks after bombardment, hygromycin-resistant embryogenic soybean tissue could be visually selected and separately cultured for establishment of individual clones. Hygromycin-resistant tissues could be identified as yellow-green outgrowths from the white-brown hygromycin-sensitive clumps (Fig. 3 A). A single clump of tissue containing one hygromycin-resistant lobe could be used to establish a prolific, embryogenic culture after 1 to 2 mo. of subsequent culture.

In vivo GUS assays of early selected clones (yellow-green outgrowth on hygromycin-sensitive tissue transformed with either pHG1 or cotransformed with pCIB709 and pUCGUS) showed that the outgrowths were either negative or possibly sectored (Fig. 3 B) for GUS activity. Upon closer examination of these early selected, GUS-sectored clones both before and after in vivo GUS assay, it was clear that in most cases the lobes of embryogenic soybean tissue that were not GUS-positive were white and therefore not viable before the assay (Fig. 3 C,D, arrows). After 2 to 3 mo. of further proliferation in hygromycin-containing liquid medium, most of the clones displayed solid GUS activity while some others were still either sectored or negative for GUS activity (Fig. 3 E).

Particle bombardment of embryogenic soybean cells with pUCGUS gave rise to an average of 709 GUS-positive foci 3 days after bombardment. An average of approximately three stably transformed clones were obtained from each separate bombardment using pCIB709, indicating a transient-to-stable conversion frequency of approximately 0.4%.

**Regeneration of transgenic plants.** Somatic embryos from hygromycin-resistant suspension cultures were permitted to develop for 1 mo. on the embryo development medium. An average of 25 embryos were produced from each 4-mm clump of proliferating embryogenic tissue (Fig. 3 F). It was possible to produce large numbers of embryos that germinated at a frequency of approximately 20% after a 2- to 3-day desiccation treatment. The desiccation treatment resulted in a 20% reduction in fresh weight of the embryos and increased the germination frequency from less than

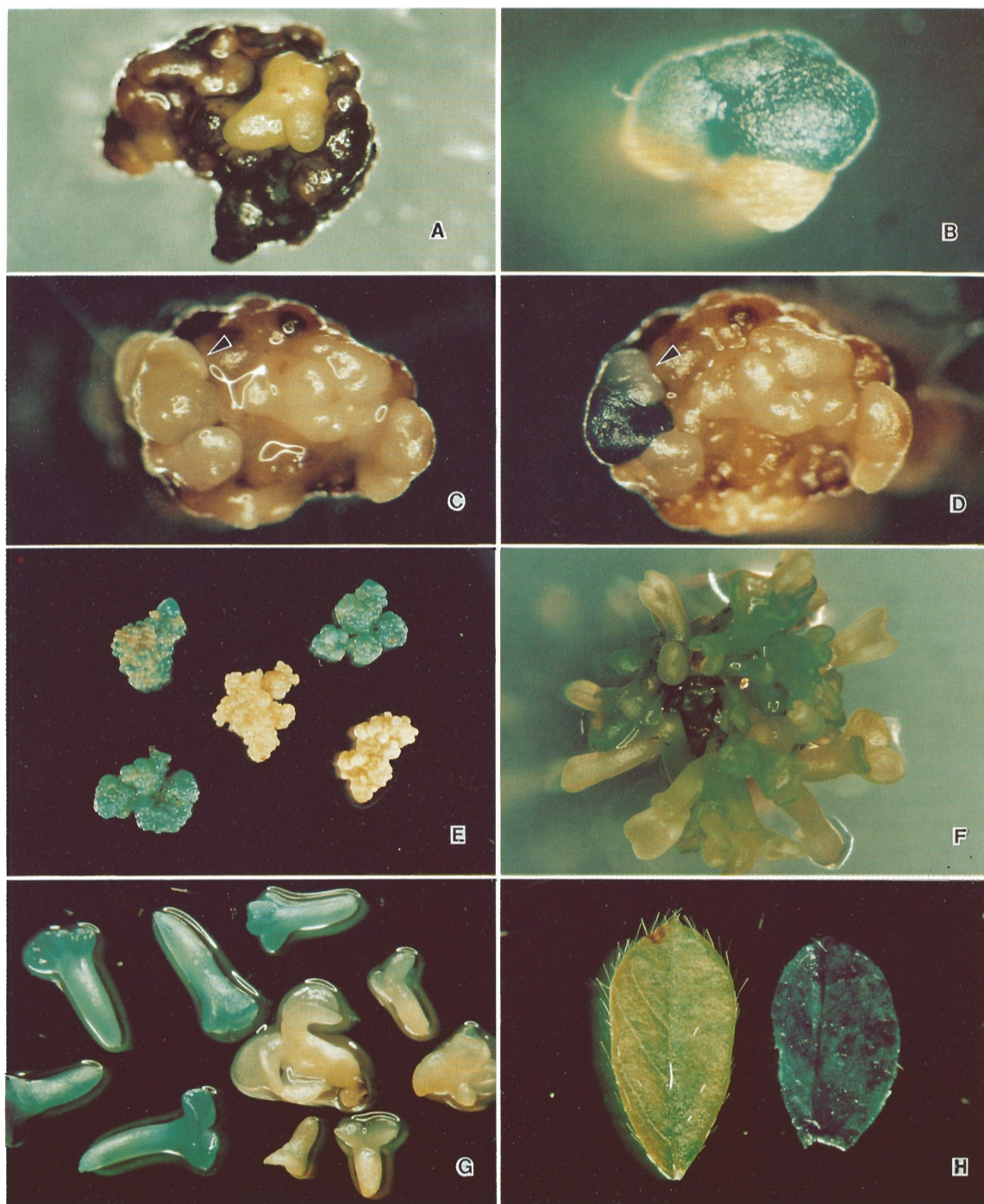


FIG. 3. *A*, outgrowth of yellow-green, hygromycin-resistant lobe of embryogenic tissue from brown, hygromycin-sensitive clump 6 wk after bombardment.  $\times 5$ . *B*, early selected hygromycin-resistant tissue showing apparent sectorial GUS activity. Tissue was assayed 6 wk after bombardment.  $\times 28$ . *C, D*, outgrowth of hygromycin-resistant tissue on white-brown hygromycin-sensitive clump before and after GUS *in vivo* activity stain. Note that sectors that are not GUS-positive (*D*, arrow) do not seem viable in the prestained tissue (*C*, arrow).  $\times 14$ . *E*, GUS assay for four pHG1-transformed clones of soybean and control tissue (center). Clone 5 (top left) is sectorial for GUS activity; clones 7 (top right) and 10 (bottom left) are solid positive; clone 11 (bottom right) is negative.  $\times 2.3$ . *F*, embryo development 1 mo. after plating pCIB709-transformed soybean tissue on embryo development medium.  $\times 7$ . *G*, GUS activity in pHG1-transformed developing soybean embryos (left) and nontransformed embryos (right).  $\times 1.4$ . *H*, GUS activity in a leaf from a pCIB709-transformed plant (left) and a pHG1-transformed plant (right).  $\times 3.5$ .

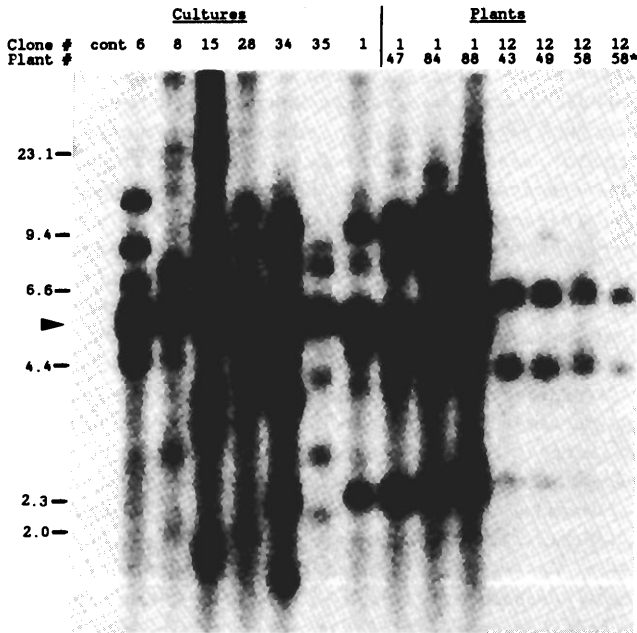


FIG. 4. Southern hybridization analysis of pCIB709-transformed cultures, regenerated plants, and one progeny plant (asterisk). Arrow indicates unit plasmid length of 4.9 kb. All DNAs were digested with *HindIII* and DNAs were hybridized to the coding region for hygromycin' (*BamHI* fragment) from pCIB709.

1% to 20%. Developing embryos (Fig. 3 G) and leaf tissue from regenerated plants (Fig. 3 H) expressed GUS activity. Plants have been recovered from 12 different transgenic clones, and the first plants were recovered 9 mo. after bombardment. The time required for recovery of transgenic plants will be reduced once development and germination conditions are further refined.

**Analysis of PCIB709 clones.** The presence of the introduced hygromycin-resistance gene in pCIB709-transformed soybean cultures, regenerated plants, and progeny was confirmed by Southern hybridization analyses (Figs. 4 and 5). All plants regenerated from the same clone gave the same hybridization pattern (Fig. 4), indicating that the introduced DNAs were not rearranged or modified during the regeneration process and that regenerated plants were of unicellular origin. The introduced DNA was transmitted to progeny (Fig. 4), with the same hybridization pattern as the parent plant. Eight of ten progeny plants from clone 12 assayed to date received the introduced DNA and all eight plants yielded both main hybridization signals (data not shown). This indicates that the two major hybridization fragments in the original clone 12 transformation event were integrated in a genetically linked manner.

All cultures selected for hygromycin resistance and assayed by Southern analysis (> 50) have contained introduced copies of the hygromycin-resistance gene. After digestion of DNA from transgenic tissue with *HindIII*, which cleaves pCIB709 once, most clones exhibited a strong hybridization to unit plasmid length (4.9 kb) DNA (Fig. 4). This pattern suggests the formation of concatemers with copies arranged in a head-to-tail orientation. Additional hybridization signals may represent alternate arrangement of some copies in concatemers, rearrangement of copies, independent integration at sites other than the concatemers, partial copies, or

plant-plasmid DNA borders. The intensity and patterns of the hybridization signals to the introduced hygromycin resistance gene varied greatly among the different cultures, indicating differences in copy number and gene arrangement within the transformed cells.

**Analysis of pHG1 clones.** Figure 3 E illustrates *in vivo* GUS activity in nontransformed embryogenic soybean tissue (center) and four pHG1-transformed clones. After digestion with the appropriate restriction enzymes (see Methods), DNAs from clone 5 (sectored GUS activity), clone 10 (solid GUS activity), and clone 11 (no GUS activity) gave hybridization signals corresponding to the size of the intact expression unit for the introduced GUS gene (Fig. 5, top). Clone 7, which expressed solid *in vivo* GUS activity, did not contain the intact expression unit. All hybridization signals from clone 7 DNA digested with *EcoRI/HindIII* were larger than the expected 2.9 kb intact expression unit. Additional hybridization analyses indicated interruption of or modification to the 3' end of the GUS gene (data not shown).

After digestion with *HindIII*, all four pHG1-transformed clones showed a hybridization signal at 2.3 kb which represents the intact expression unit for the hygromycin-resistance gene (Fig. 5, bottom). All of the clones also showed additional fragments that may represent partial copies or plant-plasmid DNA borders. Digestion of DNA from four pHG1-transformed clones with *SstI*, which cleaves pHG1 once at the 3' end of the GUS coding unit, and probing with either the *BamHI* fragment from pCIB709 or the *BamHI/SstI* fragment from pUCGUS did not release a high copy number of unit plasmid length DNA as observed with *HindIII*-digested pCIB709-transformed soybean (Fig. 4) and cotton (Finer and McMullen, 1990). Head-to-tail concatemer formation, which occurs in most pCIB709-transformants, does not occur at similar frequencies in pHG1-transformed soybean. In addition, pHG1-transformed clones contained fewer copies of introduced plasmid than pCIB709-transformed tissues.

## DISCUSSION

**Embryo origin and suitability of the culture for transformation.** Histologic examination of soybean embryogenic suspension culture tissue indicated that embryo initials were formed from surface or the adjacent subsurface tissues. New embryogenic structures were formed on top of older, apparently globular-staged embryos, with the involvement of essentially all of the surface in embryo initial formation. Surface proliferation has been reported earlier for proliferative embryogenic soybean tissue maintained on an agar-solidified medium (Finer, 1988). Even though the clumps of embryogenic soybean tissue are large relative to other embryogenic suspension culture systems, this tissue is suitable for transformation due to surface origin of embryos. Other transformation methods including *Agrobacterium* (McGranahan et al., 1988), silicon carbide fibers (Kaepler et al., 1990), and UV-laser microbeam (Weber et al., 1989) may also be employed as long as the surface tissue is the target for the specific transformation protocol.

**Selection for embryogenic, transgenic tissue.** The ability of embryogenic suspension culture tissue of soybean to survive and proliferate at low inoculum density in liquid culture (Finer and Nagasawa, 1988) allowed for the selection of transgenic clones. For routine subculture of nontransformed embryogenic cultures, approximately 30 mg of tissue was transferred to 35 ml of fresh medium. For selection after bombardment, only a few cells out of

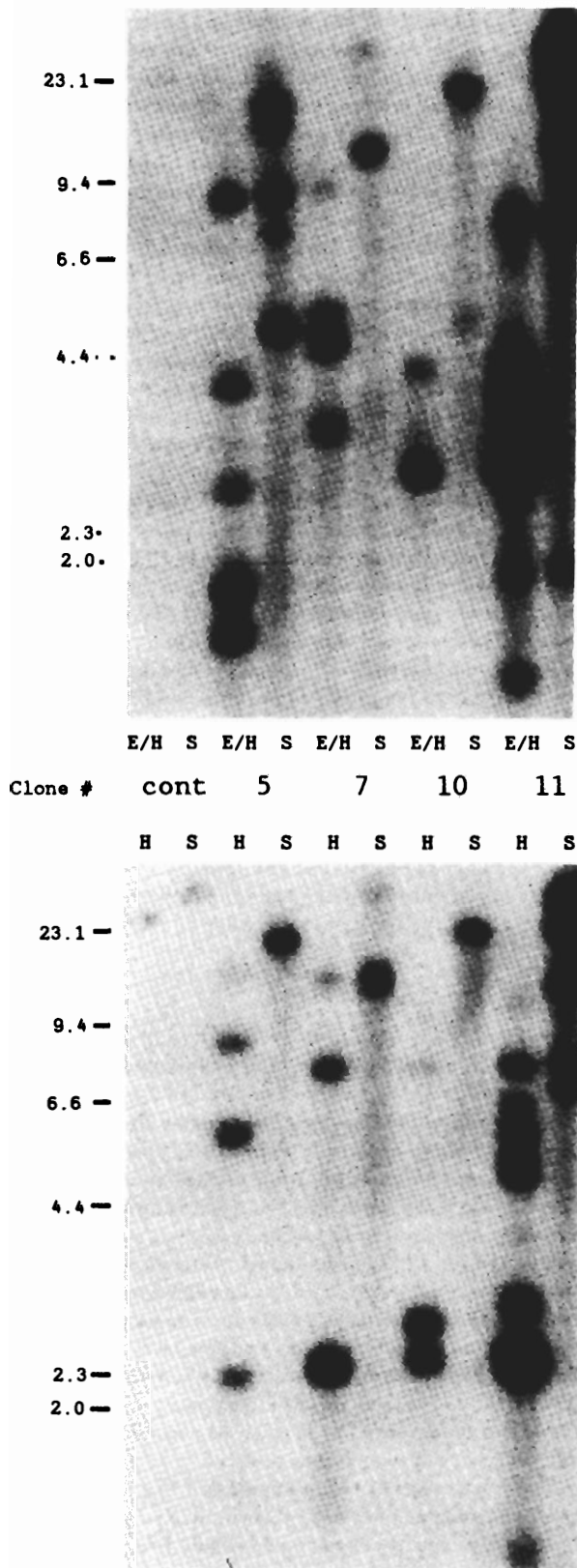


FIG. 5. Southern hybridization analysis of pHG1-transformed soybean cultures. DNAs in the top panel were digested with either *EcoRI/HindIII* (E/H) which releases the intact expression unit for the GUS gene as a 2.9 kb

approximately 1 g of embryogenic tissue were able to survive and proliferate in 35 ml of the hygromycin-containing medium. It is difficult to assess the contribution of the surrounding, nontransformed tissue to the transgenic proembryo during the early stages of selection. A certain level of organization may be necessary for proliferation or maintenance of the embryogenic state of these cultures. The concentration of hygromycin that was used for selection did not kill the cultures immediately and may have initially permitted outgrowth of transgenic material from supporting nontransformed tissues. Once the transformed embryogenic tissue was of sufficient size and organization, the tissue could assume independent growth in selective media.

A similar selection scheme has been successfully used for particle bombardment-mediated transformation of embryogenic suspension cultures of cotton (Finer and McMullen, 1990), where individual clumps of yellow, hygromycin-resistant transgenic tissue were isolated from the white hygromycin-sensitive clumps. After particle bombardment of embryogenic suspension cultures of maize, Gordon-Kamm et al. (1990) performed selections using a solid support system. The use of liquid systems for selection after transformation may be beneficial where extensive contact of the selective agent with the cells is desirable. The medium can be replaced easily in liquid culture, and selection is generally more rigorous due to the high medium-to-tissue ratio. In addition, growth of transgenic cells at low density during selection may actually be beneficial in selecting cells that are both transgenic and embryogenic. Suspension cultures can often be enriched for embryogenic cells by low density subculture where only embryogenic cells can survive and proliferate (Finer, 1988; Nagasawa and Finer, 1988; Finer et al., 1989).

Selection using an embryogenic suspension culture of soybean offers a distinct advantage over the shoot tip transformation system of McCabe et al. (1988). If the bombarded apical meristem is a mixture of transformed and nontransformed cells, direct selection may not be practical. Particle bombardment-mediated transformation of the soybean shoot tip in the absence of selection most often results in chimeric plants, which may or may not pass the introduced gene to progeny depending on the placement of DNA in tissues that give rise to germ line cells. Shoot tip transformation is beneficial in cases where a shoot organogenesis system is available and an embryogenic system is not.

*$\beta$ -glucuronidase assays of early selected transgenic soybean clones.* In most cases, when early selected clones were sacrificed for GUS assays, they appeared to be either negative or sectored for GUS activity. Careful observation of the sectored early selected clones both before and after GUS assay showed that, in most cases, GUS-negative regions (Fig. 3 C,D, arrows) were not green before GUS assay and most GUS negative regions were simply not viable. After 1 to 2 mo. of proliferation of clones in hygromycin-containing medium, most transgenic clones were solid for GUS activity. However, a few clones were still sectored for GUS activity. Because the in vivo GUS assay is toxic to plant cells, it was not possible to separate GUS positive and negative sectors and follow activity in the

fragment (arrow), or *SstI* (S) which cleaves pHG1 once. DNAs in the top panel were then hybridized to the *BamHI/SstI* fragment from pUCGUS (containing the coding region for GUS). DNAs in the bottom panel were digested with either *SstI* or *HindIII* (H), which releases the hygromycin<sup>r</sup> expression unit as a 2.3 kb fragment (arrow). DNAs in the bottom panel were then hybridized to the coding region for hygromycin<sup>r</sup> (*BamHI* fragment) from pCIB709.

same piece of tissue over time. Klein et al. (1988) also described kanamycin-resistant tobacco calli which expressed GUS in sectors.

*Integration and expression of DNA in transgenic soybean.* Southern analyses demonstrated that all of the hygromycin-resistant cultures have the introduced hygromycin-resistance gene. For both cotton (Finer and McMullen, 1990) and soybean, hygromycin is a very specific and efficient antibiotic for selection of transformed, embryogenic suspension cells.

All of the soybean cultures transformed with the plasmid pCIB709 exhibited multiple hybridization signals, with the majority exhibiting a strong hybridization signal to unit plasmid length DNA. This pattern is most consistent with a head-to-tail arrangement of the introduced plasmid copies. Although the unit length fragment is shared among the different clones, the non-unit length fragments are clone specific. These fragments may represent partial plasmid pieces, plant-plasmid DNA borders, or DNA rearrangements either before or after integration. Interestingly, many of these clone-specific fragments seem to be at multicopy signal intensity. If each of these fragments is derived from a unique integration or rearrangement event as suggested by their clone specificity, the multiple copies may be arising by amplification of the chromosomal DNA after formation of the initial concatemer. The amplification of chromosomal DNA containing the hygromycin-resistance gene may occur in a manner similar to the generation of homogeneous staining regions upon selection for methotrexate resistance in mammalian tissue culture cells (Schimke et al., 1981).

Hybridization patterns for pHG1-transformed soybean cultures did not indicate formation of head-to-tail concatemers as was observed with pCIB709-transformed tissues. DNAs from pHG1-transformed tissues that were digested with *Sst*I and hybridized to either the hygromycin' or GUS coding region yielded hybridization signals larger than the 8.4 kb unit length plasmid. In three of the four cases shown in Fig. 5, pHG1 integrated at low copy number. The cause for a different integration pattern for pHG1 relative to pCIB709 is not known. Differences between pHG1 and pCIB709 include sequence duplication and the larger size of pHG1. The plasmid pHG1 is not nearly as efficient for generating hygromycin-resistant clones as either pCIB709 or pCIB709 together with pUC-GUS as a cotransformation (Finer and McMullen, unpublished).

All pHG1-transformed soybean clones contained at least one intact hygromycin expression unit, as expected for clones selected for hygromycin resistance. The situation for the GUS expression unit is more complicated. Clone 11, which had the highest copy number of intact GUS expression units was negative for GUS expression. Clone 5 contained an intact GUS expression unit and remained sectored for GUS expression after extended selection in hygromycin-containing liquid medium (Fig. 3 E). We believe that, in this clone, sectored GUS activity was not caused by cells that did or did not contain the GUS gene but was a result of differential expression of the GUS gene. Both the hygromycin-resistance gene and the GUS gene have CaMV 35S promoters. Matzke et al. (1989) have reported that the presence of duplicated 35S promoters can result in the inactivation of one of the 35S driven genes. Napoli et al. (1990) and van der Krol et al. (1990) have presented evidence that inactivation of duplicated, introduced DNA may occur as a more general phenomena that they have termed co-suppression. Such a co-suppression mechanism may result in gene suppression with time in culture. Clearly much remains to be learned about the

correlation of the structure of the introduced DNA and the resulting integration pattern and gene expression.

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