TRANFORMATION OF SOYBEAN VIA PARTICLE BOMBARDMENT OF EMBRYOGENIC SUSPENSION CULTURE TISSUE

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(Received 2 April 1991; accepted 30 July 1991; editor R. S. Herick)

SUMMARY

Embryogenic suspension culture tissue of soybean (Glycine max Merrill.) was bombarded with particles coated with plasmid DNAs encoding hygromycin resistance and b-glucuronidase (GUS). One to two weeks after bombardment, embryogenic tissue was plated in a liquid proliferation medium containing hygromycin. Four to six weeks after bombardment, leathery 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, twisted, 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 patterns of the introduced DNA varied among independently transformed clones. Hybridization analysis of DNA from progeny plants showed genetic linkage of multiple copies of transformed DNA. An average of three transgenic clones were obtained per bombardment making this procedure very suitable for transformation of soybeans.

Keywords: 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 mesenchymal 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.

McClure et al. (1988) obtained transgenic soybean via particle bombardment of the shoot apex. In this report, only 1 regeneration event was expressed the gene for kanamycin resistance out of the 389 regenerated plants. In 2 subsequent papers (Christou et al., 1989; Yang and Christou, 1990), a total of 7 transgenic soybean lines were evaluated. Shoot apex transformation is labor-intensive because the embryogenic tissue is difficult to target 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 oats (Finer and McNullen, 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 plasm.

In an attempt to develop a routine transformation procedure for soybeans we subjected embryogenic suspension culture tissue to particle bombardment. Single bombardments of embryogenic soybean tissue with DNA encoding the b-glucuronidase (GUS) gene (Jefferson, 1987) yielded at least 800 GUS-expressing cotyledon. 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.

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“Faucey”) were initiated and maintained in the 10A40N medium supple- mented with 0.5 mM asparagine as described previously (Faeur and Naga- sawa, 1989). For subculture, two clumps of embryogenic tissue, 4 mm in diameter, were transferred to 35 ml of 10A40N medium in a 125-ml delmoflask. High quality embryogenic material was selectively subcul- tured monthly at this low inoculum density.

Riuality. For histologic studies, proliferating embryogenic tissue was fixed in a formaldehyde solution, dehydrated in an ethanol series, and embedded in paraffin wax (Spurr, 1969) according to Faeur (1988). Sec- tions were cut to 0.75 mm on a 3-µm microtome, mounted on glass slides, and stained with toluidine blue for viewing.

Preparation of DNA and nuclease. The plasmid pUC9 (Faeur and McMillan, 1990) was made by subcloning the GUS gene as a HindIII/ EcoRI fragment from pBI212 (Jefferson, 1987) into pUC19. The effi- ciency of particle bombardment was initially monitored using pUC19 and counting the number of tiles showing transient expression of the GUS gene, 3 days after bombardment. The plasmid pCB709 (Borstenstern et al., 1987) contains the amorphous/polysaccharide transformation-type IV (Agrobacterium) gene (Giri and Duran, 1981) flanked by a CaMV 35S promoter and terminator. The Agrobacterium gene encodes a protein that detoxifies the antibiotic hygromycin- B. The plasmid pFG1 (Fig. 1), which encodes for both hygromycin resis- tance and GUS activity, was constructed by simultaneously ligating the HindIII/EcoRI fragment from pCB709 (hygromycin) and the HindIII/EcoRI fragment from pBI212 (GUS) into EcoRI/KpnI cut pCB19. All DNA constructions were isolated after transformation into the Escherichia coli strain MM106, and plasmid DNA was purified by standard procedures (Maniatis et al., 1982).

DNA was precipitated onto 1.1-µm (average diameter) tungsten pellets using a CaCl2 precipitation procedure (Faeur and McMillan, 1990). In cases of co-transformation using both pCB709 and pUC19, the two plas- mids were mixed at 1:9 (pCB709:pUC19) before CaCl2 precipitation. The ratio was previously determined to the highest levels of coopera- tion in hygromycin-resistant transformed cotton tissue (Faeur and McMil- lane unpublished). The pellet mixture containing the precipitated DNA was gently resuspended after precipitation, and 2 µl was removed for bombard- ment.

Preparation of plant tissue for transformation. 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 covered in the dish, the excess liquid medium was removed with a pipette, and a sterile 500-kd filter (Millipore, Bedford, MA) was placed over the embryogenic tissue. Open petri dishes were placed in a laminar-flow hood for 10 to 12 min to remove any 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. Bombardment was performed using a DauPuit/Hoe- bischer Particle Delivery System (model BPH-6). 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 med- ium containing 50 µg/ml hygromycin (10A40N-Hyg). Hygromycin (Cal- biochem, 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 leaves of embryonic tissue were removed and separately subcultured in 10A40N-Hyg. After 5 to 6 mo. of maintenance in 10A40N-Hyg, proliferating embryogenic tissues were maintained by stan- dard subculture in 10A40N without added antibiotic. Embryogenic tissues were periodically removed from 10A40N-Hyg and 10A40N for embryo development and Southern hybridization analysis.

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 0.8% gelrite (pH 5.7). One month after plating, the developing embryos were cultured as individual embryos, 25 per 9-cm petri dish in fresh pro- vention medium. After an additional 8 wk, the mature embryos were placed in dry petri dishes for 2 to 3 days. After the desiccation treat- ment, the embryos were transferred to a medium containing MS salts, B5 vitamins, 3% sucrose, and 0.2% Colchic (pH 5.7). After root and shoot elongation, plantlets were transferred to pots containing a 1:1 mixture of vermiculite, topsoil, and peat, and maintained under high humidity. Plant- let roots were gradually exposed to ambient humidity over a 2- to 3 wk period and placed in the greenhouse.

β-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 (Bhatia and Marcello, 1989). DNAs from pCB709-transformed cultures and plants were digested with HindIII, which cleaves pCB709 once, just upstream from the CaMV 35S promoter. DNAs from pCB19-transformed cultures were dig- ested with SalI, which cleaves pCB19 once at the 3' end of the GUS gene (Fig. 1). To determine if the hygromycin- and GUS expression units were intact, DNAs from pCB19-transformed tissues were digested with both HindIII/HindIII (restriction sites flanking the intact hygromycin/gene expression units) or HindIII/HindIII (restriction sites flank the intact GUS expression unit) di- gested DNAs were electrophoresed on a 0.8% agarose gel. The DNA is- gated was treated with 0.2 N HCl for 15 min followed with 0.5 M NaOH/0.5 M NaCl twice for 30 min, and finally 1 M NaCl/0.1 M Na2CO3 for 20 min. The DNA was transferred (Volkmann et al., 1985) to nitrocellulose membranes (Zierke-Ernsfeld, Richmond, CA) overnight by capil- lary transfer using 0.5 M NaCl/10 mM sodium phosphate (pH 7.6) at 0°C for 20 h under vacuum and then probed with 4 to 6 h at 65°C in 5× sodium phosphate, pH 7.0, 0.05% sodium dodecyl sulfate (SDS), 0.2% sodium dodecyl sulfate, 10× sodium phosphate, 0.1 M NaCl, and 0.1% sodium dodecyl sulfate.

The resulting fragment from pCB709 (containing the hygromycin-resis- tance structure) was probed at 10°C with a DNA fragment from pUC19 (contain- ing the GUS structural gene) were random-primed labeled (Feinberg and Vogelstein, 1983) and used for hybridization. Membranes were hybridized in the same solution as above but contained 9-cm petri dish in 2× SSC/0.1% SDS and washed at 80°C for 2 h under vacuum and then probed with 4 to 6 h at 65°C in 1× sodium phosphate, pH 7.0, 0.05% sodium dodecyl sulfate, 0.2% sodium dodecyl sulfate (SDS), and 62.5 µg/ml sodium apate at room temperature.
RESULTS

Proliferating somatic embryogeny 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 subepidermal tissues (Fig. 2 A, arrow). Embryogenic 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 resided in a concentric layering of embryos. The agitation of the cultures together with disintegration of internal tissues caused breakdown of larger clumps, resulting in production of smaller clumps of tissue. The surface origin of embryogenic 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 pCBT09 alone or in combination with pUCGUS was used for bombardment.

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 an 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-hygroycin-sensitive clumps (Fig. 3 A). A single clamp of tissue containing one hygromycin-resistant lobe could be used to establish a prolific, embryogenic culture after 1 to 2 mo. in subculture.

In vivo GUS assay of early selected clones (yellow-green outgrowth on hygromycin-sensitive tissue transformed with either pHG1 or co-transformed with pCBT09 and pUCGUS) showed that the outgrowths were either negative or possibly selected (Fig. 3 B) for GUS activity. Upon closer examination of these early selected, GUS-selected 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, arrow). 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 negative or negative for GUS activity (Fig. 3 D).

Particle bombardment of embryogenic soybean ends with pUC-GUS grown 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 pCBT09, indicating a transient-to-stable conversion frequency of approximately 0.49%.

Regeneration of transgenic plants. Somatic embryos from hygromycin-resistant suspension cultures were permitted to develop for 1 mo. on the embryogenic development medium. An average of 25 embryos were produced from each 4-mm clamp 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 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 calli of embryogenic tissue from bean hypocotyl explant at 6 weeks after bombardment. B, C. Early selected hygromycin-resistant tissue showing apparent transformed GUS activity. These were assayed in vitro after bombardment. D, E. Calli of hygromycin-resistant tissue on white-brown hygromycin-sensitive chaff before and after GUS in vivo activity stain. Note that sectors that are not GUS positive (i.e., arrow) do not stain yellow in the prestained tissue (C, arrow). X4. F. GUS assay for four pM9 transformed clones of soybean and control tissue (center). Clone 1 (top left) is tumorous for GUS activity clones 7 (top right) and 11 (bottom left) are solid positive; clone 11 (bottom right) in a separate X2.3. F. Embryo development 1 mo. after plating pC87091 transformed soybean tissue on embryo development medium. X7. G. GUS activity in pM9 transformed developing soybean embryos (left) and nontransformed embryos (right). X3.8. H. GUS activity in a leaf from a pC87091 transformed plant (left) and a pM91 transformed plant (right). X2.3.
Fig. 4. Southern hybridization analysis of pCB709-transformed cultures, regenerated plants, and one progeny plant (asterisk). Arrows indicate unit plasmid length of 4.9 kb. All DNAs were digested with HindIII and Dases were hybridized to the coding region for hygromycin (Boswell fragment) from pCB709.

1% to 20%. Developing embryos (Fig. 3 G) and leaf tissue from regenerated plants (Fig. 3 B) 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 pCB709 clones. The presence of the introduced hygromycin-resistance gene in pCB709-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 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 pCB709 once, most clones exhibited a strong hybridization to unit plasmid length (4.9 kb) DNA (Fig. 4). This pattern suggests the formation of concatamers with copies arranged in a head-to-tail orientation. Additional hybridization signals may represent alternate arrangement of some copies in concatamers, rearrangement of copies, independent integration at sites other than the concatamers, 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 pGUS clones. Figure 3 E illustrates in vivo GUS activity in nontransformed embryonic soybean tissue (center) and four pGUS-transformed clones. After digestion with the appropriate restriction enzymes (see Methods), DNAs from clone 5 (ectometric 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 pGUS-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 pGUS-transformed clones with SalI, which cleaves pGUS once at the 3' end of the GUS coding unit, and probing with either the BamHI fragment from pCB709 or the BamHI/Sall fragment from plCGUS did not release a high copy number of unit plasmid length DNA as observed with HindIII-digested pCB709-transformed soybean (Fig. 4) and cotton (Fiser and McMullen, 1990). Head-to-tail concatemer formation, which occurs in most pCB709-transformants, does not occur at similar frequencies in pGUS-transformed soybean. In addition, pGUS-transformed clones contained fewer copies of introduced plasmid than pCB709-transformed tissues.

**Discussion**

Embryo origin and suitability of the culture for transformation. Histologic examination of soybean embryonic 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-shaped 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 agar-solidified medium (Fiser, 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 origins of embryos. Other transformation methods including Agrobacterium (McGrath et al., 1988), silicon carbide fibers (Keppele 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 medium (Fiser and Naga- sawa, 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
approximately 1 g of embryonic 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 progeny during the early stages of selection. A certain level of organization may be necessary for proliferation or maintenance of the embryonic 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 embryonic 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 embryonic suspension cultures of cotton (Finer and McMullen, 1996a), where individual clumps of yellow, hygromycin-resistant transgenic tissue were isolated from the white hygromycin-sensitive clumps. After particle bombardment of embryonic suspension cultures of maize, Gordon-Kamm et al. (1990) performed selection 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, 1984; Finer et al., 1985).

Selection using an embryonic suspension culture of soybean offers a distinct advantage over the shoot tip transformation system of McCabe et al. (1988). If the bombardmed 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.

β-glucuronidase assays of early selected transgenic soybean clones. In most cases, when early selected clones were sacrificed for GUS assays, they were allowed to be either negative or scored for GUS activity. Careful observation of the selected early selected clones both before and after GUS assay showed that, in most cases, GUS-negative regions (Fig. 3 C,D, arrows) were not seen before GUS assay and most GUS negative regions were simply not visible. After 1 to 2 mos, of proliferation of clones in hygromycin-containing medium, most transgenic clones were solid for GUS activity. However, a few clones were still selected 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 Sal I (B) which cleaves pGH1 once, DNA 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

FIG. 5. Southern hybridization analysis of pHG1-transformed soybean cultures. DNA 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

fragment (arrow), or Sal I (B) which cleaves pGH1 once, DNA in the top panel were digested with either EcoRI/HindIII (E/H) which releases the hygromycin expression unit as a 2.3 kb fragment (arrow). DNA in the bottom panel were then hybridized to the BamHI SalI fragment from pH5GUS (promoting the coding region for GUS), DNA in the bottom panel were digested with either EcoRI/HindIII (E/H) which releases the hygromycin expression unit as a 2.3 kb fragment (arrow). DNA in the bottom panel were then hybridized to the BamHI SalI fragment from pH5GUS.
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same piece of tissue over time. Klein et al. (1988) also describe transgenic tobacco cells which were used to enrich and stabilize the transgenic tobacco cells. All of the soybean cultures transformed with the plasmid pCB1709 exhibited multiple hybridization signals, with the majority exhibiting a strong hybridization signal to unit plant length DNA.

This pattern was consistent with the 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 the clone-specific fragments seem to be at a multiplicity signal intensity. If each of these fragments is derived from a unique integration or rearrangement event as suggested by their clone specificity, the multiplicity may arise 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 homogenous staining regions upon selection for methotrexate resistance in mammalian tissue culture cells (Cohen et al., 1981).

Hybridization patterns for pHC1-transformed soybean cultures did not indicate formation of head-to-tail concatamers as was observed with pCB1709-transformed tissues. DNA from pHC1-transformed soybean tissues were digested and hybridized to refer to the soybean, or GC codon ratio yielded hybridization signals larger than the 5.8 kb unit length plasmid. In three of the four plants analyzed, the 5.8 kb plasmid band was digested and hybridized to the clone number. The clone for a different integration pattern for pHC1 relative to pCB1709 in not known. Differences between pHC1 and pCB1709 including the number of copies and the larger size of pHC1. The plasmid pHC1 is not easily available for generating soybean or resistant clones as either pCB1709 or pCB1709 together with pUC-57S as a cotransformation (Fisher and McMullen, unreported).

All pHCl-transformed soybean clones contained at least one intact hygromycin expression unit, as expected for clones selected for hygromycin resistance. The situation for the 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 selected for GUS expression after extended selection in hygromycin-containing liquid medium (Fig. 3 E). We believe that, in this clone, selected 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 CmV55S promoters. Mawka et al. (1989) have reported that the presence of duplicated 35S promoters can result in the inactivation of one of the 35S driven genes. Napol 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 phenomenon that they have termed co-suppression. Such a co-suppression mechanism may result in gene suppression with the GUS gene in culture. Clearly, much remains to be learned about the correlation of the structure of the introduced DNA and the resulting integration patterns and gene expression.

ACKNOWLEDGMENTS

The authors acknowledge Barbara Norris and Mark Jones for their technical assistance. We also thank L. Hinkle and R. Gagnaire for helpful suggestions in the preparation of this manuscript. Technical research support was provided by State and Federal funds appropriated to OARDC and USDA.AS. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OARDC or USDA, and are not implied approval to the exclusion of other products that may also be suitable. OARDC Journal Article no. 66-91.

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