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Transformation of Soybean Embryogenic Cultures by Microprojectile Bombardment

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Abstract

Regenerable embryogenic suspension cultures of soybean (*Glycine max* [L.] Merrill, cv. Jack) were transformed by microprojectile bombardment with a β-glucuronidase (*gus*) gene and a hygromycin phosphotransferase (*hpt*) gene under control of the cauliflower mosaic virus (CaMV) 35S promoter on different plasmids mixed together. Many independent transgenic clones were obtained by selection for hygromycin resistance. GUS activity was detected in 45% of the transgenic clones showing that cotransformation occurred at high frequency. Stable integration of both the *gus* reporter gene and the *hpt* selectable marker were further confirmed by polymerase chain reaction (PCR) amplification of both genes using double primer sets together in the same reaction. Southern blot hybridization analysis also showed the presence of the foreign genes in genomic DNA. The frequency of embryo development was increased and the time span required for embryo development was reduced by the use of a liquid medium to induce embryo development. An average of 3645 GUS-positive spots and ten transgenic clones were produced per bombardment by this procedure giving a 0.27% ratio of stable to transient expression. The procedure described here can be used for modification of agronomic traits and for study of gene regulation in soybean.

1. Introduction

Soybean transformation has been difficult, but several methods have been recently developed using *Agrobacterium* [1-3], microprojectile bombardment [4-7], or electroporation. None of the methods are ideal at present [8]. The *A. tumefaciens* system is low in frequency, very genotype-dependent and produces chimeric plants.

Transgenic soybean plants have also been produced by microprojectile bombardment of shoot tips [4, 6] and embryogenic suspension cultures [5-7]. Sato *et al.* [6] compared *gus* gene expression transformed by microprojectile bombardment in two soybean regeneration systems, organogenesis and embyogenesis. Microprojectile bombardment using a Bio-Rad Biolistic PDS-1000 helium gun delivered particles into the first 2 cell layers of either shoot tips or somatic embryos. Histological analysis showed that shoot organogenesis from shoot tips involves a multicellular origin, but that embryogenesis involves a single cell origin of somatic embryos from the epidermal cells. Thus, bombardment of shoot tips produced chimeric plants with GUS-positive sectors, whereas bombardment of embryogenic suspension cultures produced nonchimeric, GUS-positive regenerated plants. This microprojectile method requires considerable labor and costly equipment. Furthermore, embryogenic suspension cultures have to be prepared first, which requires time and skill for initiation [8]. Nonetheless, the use of embryogenic suspension cultures has the merit of a higher transformation frequency, less genotype-specificity and less chimeric plant production due to the surface origin of embryos and the more rigorous selection in liquid medium [5].

In the first soybean transformation work with a gunpowder-powered Dupont Biolistics Particle Delivery System (Model BPG) with embryogenic suspension cultures, Finer and McMullen [5] obtained an average of 3 transgenic clones per bombardment after hygromycin selection. In the second report, Finer *et al.* [9] showed that each bombardment produced an average of 11.5 stably-transformed hygromycin-resistant clones using a home-made Particle Inflow Gun (PIG), a flowing helium device. The number of clones obtained using the PIG was more than 3 times that obtained using the gunpower version of the DNA particle gun. When GUS activity was measured histochemically, the use of the pressurized-helium powered PIG increased transient *gus* gene expression by 2.5 times from an average of 709 positive foci with the gunpowder version to 1,812 foci with the PIG.

In this report, we describe a reproducible, high frequency transformation protocol by DNA particle

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bombardment of soybean embryogenic suspension cultures using the Bio-Rad Biolistic PDS-1000 helium gun. We also discuss the use of liquid medium for embryo development and the PCR analysis of transgenic clones using double primer sets. Cotransformation with genes on separate plasmids is also demonstrated.

2. Materials and Methods

2. 1 Plant Materials

Soybean (*Glycine max* [L.] Merrill cv. Jack) plants were grown either under greenhouse conditions with 16-hr photoperiods or in the field.

2. 2 Initiation and maintenance of embryogenic suspension cultures

Pods were removed when seeds were 3 to 7 mm long and surface-sterilized for 15 min. in 100 m*l* of 20% Clorox (final concentration 1.05% sodium hypochlorite) plus 2 drops of Tween 20. After rinsing 3 times in sterile water, immature cotyledons of 60 to 80 mg fresh weight were excised from the pods. After removing the end of the cotyledon containing the embryonic axis, the abaxial surface of each cotyledon half was placed on a solid medium containing MS salts [10], B5 vitamins [11], 3% sucrose, 20 or 40 mg/l 2, 4-D, and 0.8% Bactoagar (pH 5.7 to 5.8). Cultures were incubated at 26±2°C with 16-hr photoperiods at 50-60μEm^{-2} s^{-1}. After three to six weeks globular-stage somatic embryos were transferred to 30 m*l* of liquid 10A40N medium [12] supplemented with 15 mM glutamine in a 125 m*l* Erlenmeyer flask. Embryogenic suspension cultures were maintained on a rotary shaker at 130 rpm. The cultures were routinely subcultured every month and 2-4 clumps of embryogenic tissue, 3-4 mm in diameter, were transferred to 30 ml of fresh 10A40N medium for subculture.

2. 3 Particle bombardment and plant transformation

A Biolistic PDS-1000 helium gun (Bio-Rad, Richmond, CA) was used for DNA particle bombardment. About 0.6-0.8 g of embryogenic suspension cultures were placed in the center of a 100-mm-diameter Petri dish and the excess liquid was removed. Five μg of plasmid DNA was precipitated as described in the owner's manual and the precipitates were then resuspended in 60 μl of ethanol. For each bombardment, 15 μl of this mixture was loaded on a macrocarrier and allowed to air dry. A 1100 psi burst pressure rupture disk was used and the sample chamber vacuum was 28 inches of mercury during the bombardment. The macrocarrier and microcarrier travel distances were 0.6 and 12 cm, respectively. Bombarded tissues were used for either transient gene expression assays or stable transformation. Plasmids used in this work included: pUCGUS which contains the CaMV 35S promoter fused to β-glucuronidase (*gus*) gene [13]; pHygr which contains the CaMV 35S promoter linked to the hygromycin phosphotransferase (*hpt*) gene [9]; pHPT-1 which contains the CaMV 35S promoter driving the *hpt* gene. The pHPT-1 plasmid was made by subcloning the *hpt* gene as a *Hin*dIII fragment from pZA300 (from J.H. Zhou and Allen Atherly, Iowa State University) into the pGEM-3Zf(+) vector. All plasmids were purified by QIAGEN plasmid kit (QIAGEN, Chatsworth, CA) and were redissolved in TE buffer. To obtain transgenic soybean clones, 5 μg of 2 plasmid DNAs were mixed at 8: 1 or 9: 1 (*gus* containing plasmid: *hpt* containing plasmid) ratio before precipitation. The precipitated DNAs were bombarded into embryogenic suspension cultures for transformation and the cultures were then resuspended in the 10A40N liquid medium. Two weeks after bombardment, the cultures were placed in the same medium but containing 25 to 30 mg/l hygromycin B (Sigma, St. Louis, MO). The medium was changed at weekly intervals. Six to nine weeks after hygromycin selection, yellow-green outgrowths from the brown-black dead clumps were separated and individually transferred for further proliferation into 30 ml of fresh 10A40N medium without hygromycin. Putative transgenic clones were harvested for histochemical GUS assay and were further analyzed by polymerase chain reaction (PCR) amplification and Southern blot hybridization analysis.

2. 4 Development and germination of somatic embryos

Embryogenic clumps, 2-3 mm in diameter, were transferred to either liquid medium containing MS salts, B5 vitamins, 6% maltose (pH 5.7), or solid medium of the same composition but solidified with 0.2% Gelrite. After two and four weeks of growth in the liquid or solid embryo development medium, respectively, the developing embryos were manually separated and cultured as individual embryos in the fresh solid medium. Individual embyros were kept in Petri dishes for 4 weeks and a portion of the mature embryos were desiccated for 2 to 10 days in empty Petri dishes. Embryos not desiccated were maintained on the same medium. Embryo germination was initiated from the desiccated embryos by transferring to a solid medium containing MS salts, B5 vitamins, 3% sucrose, and 0.2% Gelrite (pH 5.7). After shoot and root development, the plantlets were transferred to Magenta boxes containing the same medium for further growth. The regenerated plants with well-developed root systems were then transferred to peat pots (A. H. Hummert Seed Co., St. Louis, MO) containing

a sterilized 3: 1 soil and vermiculite mixture. Potted plants were placed inside sterile Magenta boxes with couplers. After complete acclimatization by gradual opening the lid of the container for 2 weeks, the plants were transferred to the pots and these pots were kept in a large growth chamber.

2. 5 Histochemical GUS assay

DNA bombarded cultures and transgenic clones were analyzed for GUS activity according to Jefferson *et al.* [14] using 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-gluc) (Jersey Lab Supply, Livingston, NJ) as substrate. For transient gene expression assays, the bombarded embryogenic suspension cultures were cultured for 48 to 60 hr in the liquid 10A40N medium and samples were incubated at 37°C in the GUS assay buffer with the addition of 1 mg/l X-gluc for 24 to 36 hr and the tissues were then stored at 4°C for up to 72 hr. Photographs were taken using an Olympus photographic system model PM-10ADS connected to an Olympus SZH stereo microscope with Kodak Ektachrome tungsten 160 slide film.

2. 6 Genomic DNA isolation, polymerase chain reaction (PCR) and Southern blot hybridization analysis

Total genomic DNA from embryogenic suspension cultures was purified as described by the procedure of Lindstrom *et al.* [15]. To test for the presence of the *gus* gene in the genomic DNA of GUS expressing embryogenic lines, 500 ng of genomic DNA was amplified by PCR using the primer set, GUS5 (5′-ATGTTACGTCCTGTAGAAACCCCA-3′) and GUS6R (5′-TCATTGTTTGCCTCCCTGCTGCGG-3′), which yields a 1.8 kb *gus* fragment. The presence of the *hpt* gene was also tested by PCR with the primer set, HPT2 (5′-CCTGAACTCACCGCGACG-3′) and HPT5R (5′-AAGACCAATGCGGAGCATATAC-3′), which yields a 0.81 kb internal *hpt* fragment. Amplifications were performed in 25 μl of each reaction with *Taq* DNA polymerase (GIBCO BRL, Gaithersburg, MD) using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). Each PCR cycle consisted of 20 sec. denaturation step at 96°C, 1 min. annealing step at 36°C, and 2 min. extension step at 72°C. For the first cycle only, the duration of the denaturation step was 2 min. and for the final cycle only, the duration of the extension step was 7 min. DNA was amplified for 40 cycles. After PCR reaction, samples were loaded on a 0.7% agarose gel. PCR products were then transferred onto a Zeta-Probe GT membrane (Bio-Rad, Richmond, CA) and hybridized at 68°C with α ^{32}P-labeled *gus* or *hpt* probe according to the manufacturer's instructions with modifications. After overnight hybridization, the membrane filters were washed two times at 55°C for 30 min. each in 20 mM Na_2HPO_4 plus 5% SDS (pH 7. 2) and 20 mM Na_2HPO_4 plus 1% SDS (pH 7.2). The 1. 8 kb *Not*I *gus* fragment from pGLeGUS-7 [16] and the 1.0 kb *Bam*HI *hpt* fragment from pHygr were purified by QIAEX gel extraction kit (QIAGEN, Chatsworth, CA) and labeled with α-^{32}P-dATP using random primers. For direct analysis of genomic DNA, 10 μg of total genomic DNA isolated from each clone, digested with *Bam*HI and *Sst*I, was used for electrophoresis on a 0.7% agarose gel. After blotting, the Zeta-Probe GT membrane was hybridized with a radiolabeled *gus* probe.

3. Results and Discussion

3. 1 Bombardment and selection for transgenic clones

To evaluate the efficiency of particle delivery by measurement of transient GUS expression, cells from soybean embryogenic suspension cultures were bombarded with tungsten microprojectiles coated with the plasmid pUCGUS that contains a gene driven by a CaMV 35S promoter. Forty eight hours after the introduction of pUCGUS, a high frequency of GUS expression was detected with an average of 3645 GUS-positive blue foci obtained from a single shot (**Fig. 1-A**).

For selection, the bombarded tissues were resuspended in nonselective 10A40N liquid medium, cultured for 2 weeks, and placed into selective 10A40N liquid medium containing 25 to 30 mg/l hygromycin B [16]. The tissues gradually turned brown due to the apparent production of secondary compounds in response to hygromycin selection. The medium was replaced with fresh medium containing hygromycin every week to eliminate the detrimental effect of these secondary compounds. The hygromycin sensitivity of the embryogenic suspension cultures was related to the age of the cultures as well as the genotype; the older the cultures, the more sensitive they are to hygromycin. Four weeks after selection, hygromycin-resistant tissues could be visually identified on the surface of the bombarded tissues as yellow-green outgrowths from the brown-black dead clumps when viewed under a dissection microscope. Each bombardment produced 10±7 stable transgenic clones after hygromycin selection. This gave approximately 0.27% transient to stable conversion efficiency. After two to three weeks of further selection, individual clumps of live hygromycin-resistant embryogenic tissues, 2-3 mm in diameter (**Fig. 1-B**), were removed and separately transferred for further proliferation in 30 ml of fresh 10A40N medium without hygromycin where the transformed tissues could grow indepen-

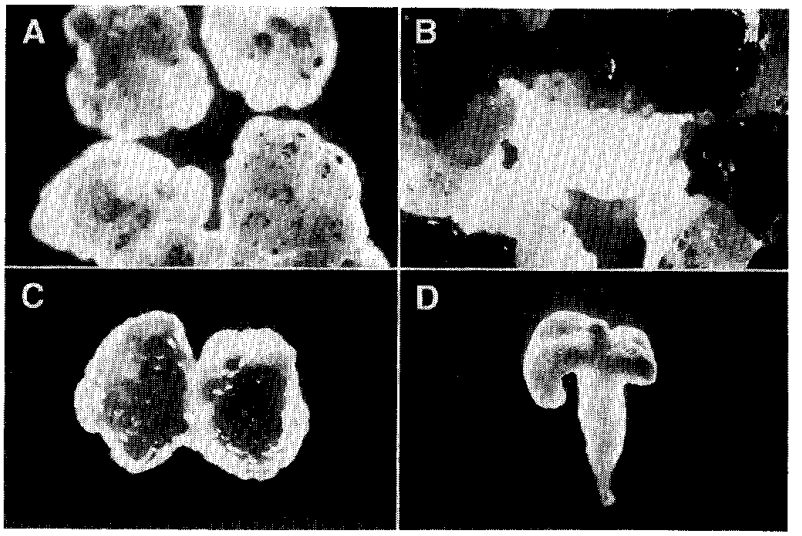

Fig. 1 Selection and GUS expression in transgenic embryogenic soybean cultures.
A. Transient *gus* gene expression 48 hrs after bombardment of embryogenic suspension cultures with the plasmid pUCGUS. The dark spots are dark blue in color.
B. Hygromycin-resistant tissues selected in 10A40N medium containing 25 to 30 mg/l hygromycin for 6 weeks. The light colored yellow-green outgrowths from the brown-black hygromycin-sensitive dead clumps can be visually identified.
C. GUS activity in transgenic embryogenic clumps. The clumps are dark blue.
D. GUS activity in a developing transgenic embryo. The embryo is blue in color.

dently. Finer and McMullen [5] also showed that a sufficient size and organization of the embryogenic tissues was required for proliferation and maintenance of the embryogenic state of the cultures. After one or two months of proliferation, the putative transgenic clones could be used for plant regeneration and for characterization by GUS assay, PCR amplification and Southern blot hybridization analysis.

3. 2 Plant regeneration

Transgenic somatic embryos developed on the liquid or solid embryo development media without growth regulators. Liquid medium enhanced the frequency of embryo development and reduced the time span required for embryo development. This might be due to a uniform supply of nutrients for tissues and dilution of 2, 4-D carried over from 10A40N medium which contains 5 mg/l 2, 4-D. It is noted that 2, 4-D is required for maintenance of an embryogenic state. An average of 85 developing embryos were produced from a single clump in liquid medium. After three weeks of growth, 8 to 10 embryogenic clumps in 30 ml liquid medium resulted in 3.5 to 6 g fresh weight of developing embryos, whereas only 0.5 to 1 g of developing embryos were produced on solid medium. Rinsing the clumps with liquid embryo development medium prior to transfer to solid embryo development medium also stimulated the growth of the embryos apparently by removing the 2, 4-D. Similarly, activated charcoal at the concentration of 0.5% (w/v) in solid medium has also been used to eliminate the carryover effect of 2, 4-D on embryo development [17]. However, culturing the tissues in liquid medium for more than 3 weeks was detrimental to further embryo development and germination due to the production of secondary compounds and/or possibly the lignification of tissues.

Embryo germination was achieved after a desiccation period of 2-10 days. The desiccation treatment could increase the germination frequency up to 80% depending upon the clone. It has also been reported that partial desiccation enhances conversion of somatic embryos of soybean [7, 18, 19]. The desiccation period has been reported to be associated with the synthesis of proteins related to the germination ability [20, 21]. All plants that were regenerated were sterile.

3. 3 Identification and molecular analysis of transgenic clones

Histochemical analysis of GUS activity in putative transgenic clones provided positive evidence of soybean transformation. GUS activity driven by CaMV 35S promoter was observed in putative transgenic clumps after hygromycin selection as shown by the dark blue color (**Fig. 1-C**). Of the 31 independent hygromycin-resistant clumps examined, 14 were positive for GUS activity, giving a 45% coexpression efficiency. Developing transgenic embryos and germinating transgenic tissues also expressed GUS activity (**Fig. 1-D**).

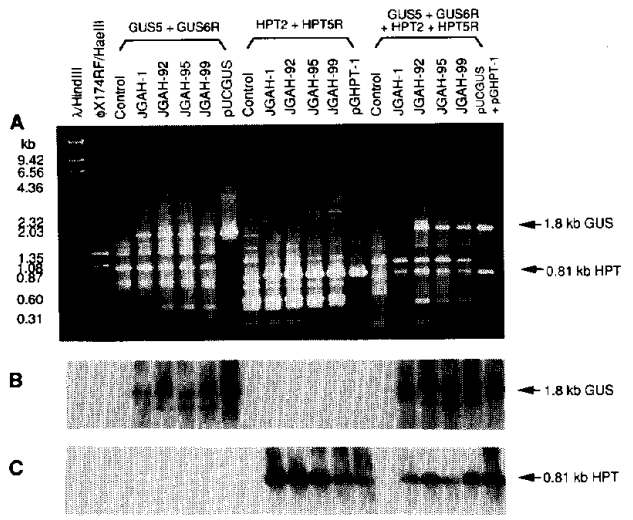

Fig. 2 PCR analysis of genomic DNAs and Southern blot hybridization analysis of PCR products from transformed soybean embryogenic clones.

Genomic DNA was amplified by PCR from a nontransformed control and four independent transgenic clones (JGAH-1, JGAH-92, JGAH-95 and JGAH-99).

A. Ethidium bromide stained gel showing PCR amplified *gus* and *hpt* fragments with two different primer sets. One set, GUS5 and GUS6R, was used to amplify the 1.8 kb *gus* fragment, and the other set, HPT2 and HPT5R, to amplify the 0.81 kb internal *hpt* fragment. The mixture of these 2 primer sets were used to amplify both fragments together in the same reaction.

B, C. Southern blot analysis of PCR products in gel A using ^{32}P-labeled *gus* (B) or *hpt* (C) probes.

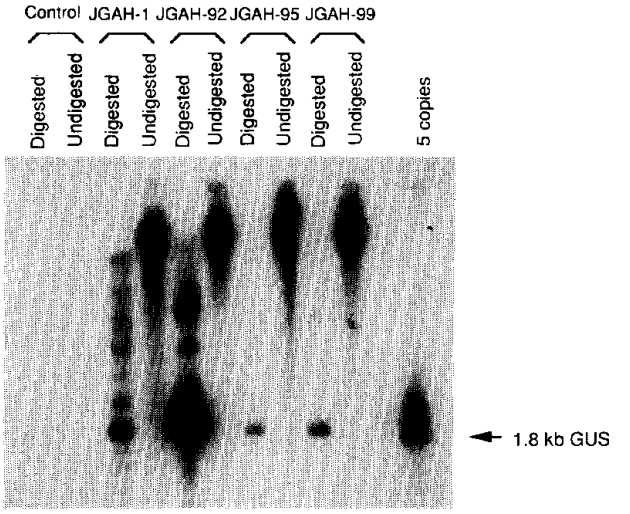

Fig. 3 Southern blot hybridization analysis of genomic DNAs from transformed soybean embryogenic clones.

Genomic DNAs isolated from a nontransformed control and four independent transgenic clones (JGAH-1, JGAH-92, JGAH-95 and JGAH-99) were digested with *Bam*HI and *Sst*I to release an intact 1.8 kb *gus* fragment. Each lane of the gel was loaded with 10 μg of genomic DNA and the gel blot was hybridized with α ^{32}P-labeled *gus* probe. A lane designated 5 copies represent the soybean genome containing 90 pg of the 1.8 kb GUS expression unit generated from pUCGUS digested with *Bam*HI and *Sst*I.

The presence of the *gus* and *hpt* gene sequences in genomic DNA was initially assayed by PCR analysis. **Fig. 2-A** shows the results of PCR amplification of the *gus* and *hpt* genes from genomic DNAs with 2 different sets of primers. One set, GUS5 and GUS6R, was used to amplify the 1.8 kb *gus* fragment for the pUCGUS plasmid, and the other set, HPT2 and HPT5R, to ampify the 0.81 kb internal *hpt* fragment for the pHygr or pHPT-1 plasmid. All four transgenic lines, JGAH-1, JGAH-92, JGAH-95 and JGAH-99 which were identified as GUS-positive transformants after hygromycin selection, exhibited both the 1.8 kb *gus* fragments and the 0.81 kb *hpt* fragments after PCR amplification with either set of primers or with both sets in the same reaction (**Fig. 2-A**). However, neither the *gus* nor *hpt* fragments were amplified in nontransformed controls with either of the primer sets. The PCR produced many other fragments under the conditions used but Southern blot hybridization analysis of these PCR products confirmed that the correct sequences were amplified in the 4 lines tested (**Fig. 2-B** and **2-C**). As expected, the *gus* probe hybridized only to the expected *gus* fragments, and did not hybridize to the *htp* fragments amplified with the primer set, HPT2 and HPT5R (**Fig. 2-B**). Similarly, the *hpt* probe did hybridize only to the amplified *hpt* fragments, but not to the amplified *gus* fragments (**Fig. 2-C**).

Incorporation of the introduced *gus* gene into the genomic DNA of transgenic soybean clones was further confirmed by Southern blot hybridization analysis of the genomic DNA of the transgenic clones. Digestion of pUCGUS with *Bam*HI and *Sst*I generated the 1.8 kb *gus* gene [9]. Genomic DNAs from all 4 transformants that were positive for GUS activity and for PCR assay results did show hybridization with the *gus* probe in the *Bam*HI/*Sst*I digest (**Fig. 3**). Southern blot analysis of all 4 transformed lines indicated that 1-7 copies of the *gus* gene were integrated into the genome and that the introduced *gus* gene was present in undigested high molecular weight genomic DNA. Nontransformed tissue did not contain DNA which hybridized to the *gus* probe.

The transformation protocols we describe here are reproducible and extend those developed by Finer and McMullen [5] and Finer *et al.* [9] and illustrate that the method is routinely reproducible. Problems with a low frequency of plantlet regeneration and with sterility of regenerated plants were found in this study as in previous reports [5]. The rate of sterility appears to correlate with the age of the somatic embryo cultures.

Our initial cultures had been cultured for over 1.5 years at the time of bombardment with exogeneous DNA. It appears that these cultures should not be more than 1 year old at the time of bombardment. Thus, we and others suggest that cultures be continuously reinitiated (J. Finer, personal communication). Although reinitiation is labor intensive and time consuming [8]. transformation of soybean via particle bombardment of embryogenic suspension cultures provides a good system for modification of agronomic traits and for study of gene regulation. For example, we have recently found that the soybean embryogenic cultures could be used to test the function of a soybean seed-specific promoter [16].

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