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 β-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 β-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.
EcoRI

tance and GUS activity, was constructed by simultaneously ligating the

dLong flask. High quality embryogenic material was selectively subcul-

tured 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). Sec-
tions were cut to 0.75 μm 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 effi-
ciency 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 pCB709 (Rothstein et al., 1987)
contains the aminoglycoside phosphotransferase type IV (AphIV) gene
(Gritz and Davies, 1983) flanked by a CaMV 35S promoter and termina-

The AphIV gene encodes a protein that detoxifies the antibiotic hygromycin-

B. The plasmid pHG1 (Fig. 1), which encodes for both hygromycin resis-
tance and GUS activity, was constructed by simultaneously ligating the
HindIII/KpnI fragment from pCB709 (hygromycin) and the HindIII/
EcoRI fragment from pUC119 (GUS) into EcoRI/KpnI cut pUC119. All
DNA constructions were isolated after transformation into the Escherichia
coli strain M11190, 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 (Finer and McMullen, 1990). In
cases of cotransformation using both pCB709 and pUCGUS, the two plas-
mids were mixed at 1:9 (pCB709:pUCGUS) before CaCl2 precipitation.
This ratio was previously determined to give the highest levels of coexpres-
sion of the hygromycin-resistant transformed cotton tissue (Finer and McMul-
len, 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 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-μm pore size nylon screen (Tetko Inc., Elmsford, NY) was placed over
the embryogenic 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 Bio-
listics 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 me-
dium 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 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 stan-
dard 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
yygromycin-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 em-
broyo development medium. After an additional 4 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% Gelrite (pH 5.7). After root and shoot
colonization, plantlets were transferred to pots containing a 1:1:1 mixture
of vermiculite, topsoil, and peat, and maintained under high humidity. Plant-
lets were gradually exposed to ambient humidity over a 2-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 (Saghai-Maroo-
of et al., 1984). DNAs from pCIB709-transformed cultures were di-
gested with HindIII which cleaves pCB709 once, just upstream from the
CaMV 35S promoter. DNAs from pHG1-transformed cultures were di-
gested with SalI, 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). Di-
gested 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 NH4C2H5O2/
0.1 M NaOH, for 40 min. The DNA was transferred (Vollrath et al., 1988)
to nylon membranes (Zetaprobe-BioRad, Richmond, CA) overnight by capil-
lar transfer using 1 M NH4C2H5O2/0.1 M NaOH. The membranes were
digested 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× standard saline citrate (SSC), 2× Den-
hardt's, 10 mM NaeEDTA, 0.2% sodium dodecyl sulfate (SDS), and 62.5
μg/ml salmon sperm DNA.

The BanHI fragment from pCB709 (containing the hygromycin-resis-
tance structural gene) or BanHI/SalI 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 × 106
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× SSC/0.1%