

Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment*

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ABSTRACT

Embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.) were subjected to particle bombardment, where high density particles carrying plasmid DNA were accelerated towards the embryogenic plant cells. The plasmid DNA coating the particles encoded hygromycin resistance. One to two weeks following bombardment, embryogenic cotton cells were placed in proliferation medium containing 100 µg/ml hygromycin. Clumps of tissue which grew in the presence of hygromycin were subcultured at low density into fresh hygromycin-containing proliferation medium. Following sequential transfer of embryogenic tissue to development and then germination media, plants were recovered from transgenic embryogenic tissue. Southern hybridization confirmed the presence of the hygromycin resistance gene in embryogenic suspension culture tissue and regenerated plants.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, GUS = β-glucuronidase, Aph IV = aminoglycoside phosphotransferase type IV

INTRODUCTION

Before gene transfer in certain crop plants becomes routine, efficient transformation systems must first be developed. *Agrobacterium*-mediated transformation is the most commonly used method for gene transfer in plants (Horsch *et al.*, 1985). Although *Agrobacterium* has been used successfully for transformation of a number of different plant species, difficulties exist due to limited host range, low efficiency of transformation, problems with removal of *Agrobacterium* following transformation, and manipulations of DNA in wide host range plasmids. Electroporation of protoplasts has also been used successfully for production of transgenic plants (Paszowski *et al.*, 1984). Electroporation avoids the limited host range barriers of *Agrobacterium*, but requires the use of protoplast-to-whole plant systems.

Recently, transformation of plant cells (Klein *et al.*, 1988a) and shoot tips (McCabe *et al.*, 1988) has been reported using particle bombardment. The principle of particle bombardment is the acceleration of small particles carrying DNA towards plant cells. Following penetration of the plant cells by the particles, the DNA disassociates from the particles and can then be expressed. Crops which typically have been difficult to transform using conventional *Agrobacterium*-mediated and protoplast transformation techniques may be better suited for transformation via particle bombardment. In this paper, we report on the transformation of

embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.) using particle bombardment. This is the first published report of recovery of transgenic plants using particle bombardment-mediated transformation of embryogenic tissues.

MATERIALS AND METHODS

Initiation and Maintenance of Embryogenic Suspension Cultures: Embryogenic suspension cultures of cotton (*Gossypium hirsutum* L. cv. 'Coker 310') were initiated as described previously (Finer, 1988). Following initiation of the embryogenic suspension cultures in a medium containing 0.5 mg/l picloram, the tissue was then transferred to and maintained in the cotton embryo proliferation medium (CEPM) which contained 5 mg/l 2,4-D instead of picloram. Following a one month lag period, cultures were subcultured weekly. For subculture, 0.5 ml packed cell volume of proliferating embryogenic tissue was transferred to 35 ml of fresh CEPM medium. To transfer the embryogenic tissue, 10 ml of the one week old suspension cultures was taken up in a 10 ml wide-mouth pipet. The tip of the pipet was placed squarely on the bottom of the flask and the medium was blown out until air bubbles escaped, leaving approximately 0.5 ml packed cell volume (as measured by reading the calibrations on the pipet). To resuspend and transfer the tissue, fresh liquid medium was taken up into the pipet and the medium and cells were pipetted out.

Preparation of DNA and Tungsten Pellets: The plasmid pUCGUS was made by subcloning the GUS gene as a HindIII/EcoRI fragment from pBI121 (Jefferson *et al.*, 1987) into pUC119. The efficiency of particle bombardment was initially monitored using pUCGUS and counting the number of cells showing transient expression of the GUS gene (Jefferson, 1987). The plasmid pCIB709 (Rothstein *et al.*, 1987; Horn *et al.*, 1988) was used for all long-term transformation experiments. This plasmid contains the AphIV gene (Gritz and Davies, 1983) flanked by a CaMV 35S promoter and terminator. The AphIV gene encodes a protein which modifies and detoxifies the antibiotic hygromycin B. DNA was precipitated onto 1.1 µm (avg. diameter) tungsten pellets using a modified CaCl₂ precipitation procedure (Klein *et al.*, 1988a). For precipitation of DNA, 5 µl of undigested plasmid DNA (1 µg/µl) was added to 25 µl of 100 mg/ml tungsten pellets in water. Next, 25 µl of 2.5 M CaCl₂ was added to the suspension, followed by 10 µl of 0.1 M spermidine. After allowing the pellets to settle for 5 minutes, 50 µl of the supernatant was removed. The concentrated pellet mixture was gently resuspended and 2 µl was removed for bombardment.

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Preparation of Plant Tissue for Bombardment: Approximately 0.5 ml packed cell volume of embryogenic suspension culture tissue (taken four days after subculture) was transferred and dispersed in a 3.5 cm diameter Petri dish. The liquid medium was removed with a pipet and the tissue was covered with a sterile 500 μ m pore size nylon screen. Open Petri dishes, containing the tissue which was covered with nylon mesh, were placed in a laminar-flow hood for 10-15 minutes to facilitate partial drying of the surface of the tissue. The 3.5 cm Petri dish was placed in the center of a 9 cm Petri dish immediately prior to bombardment. Bombardments were performed using a DuPont Biolistics™ Particle Delivery System (Model BPG). Each Petri dish containing plant tissue was bombarded once.

Selection for Transgenic Clones: Bombarded embryogenic cotton tissues were resuspended in CEPMHyg medium. Three days following bombardment (one week following the previous subculture), the suspension cultures were subcultured as described above for routine maintenance. One week following this subculture, the proliferating cultures were subcultured into CEPM medium containing 100 μ g/ml hygromycin (CEPMHyg). Hygromycin (Calbiochem) was filter-sterilized prior to addition to media. The CEPMHyg medium was replaced with fresh CEPMHyg medium after one additional week.

Four to six weeks following the initial bombardment, clumps of yellow embryogenic tissue (0.5-1.5 mm diameter) were selected and placed in 125 ml deLong flasks containing 30 ml of CEPMHyg. In the initial experiments, 5-10 hygromycin-resistant clumps of embryogenic tissue were pooled and placed in a single flask to establish hygromycin-resistant cell lines. In subsequent experiments, single clumps of hygromycin-resistant tissue were placed individually into flasks to establish true clones of transgenic tissue. After 1-2 months of further growth of selected tissues, proliferating embryogenic tissue could be maintained by standard subculture in CEPMHyg. Embryogenic tissues were periodically removed from CEPMHyg for embryo development and Southern hybridization analyses.

Embryo Development and Germination: For embryo development, clumps of hygromycin-resistant embryogenic tissues were placed on a medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 3% sucrose, 50 mM glutamine, 100 μ g/ml hygromycin and 0.8% agar (pH 5.7). Mature embryos, which were obtained after 4 weeks on this medium, were then placed on germination medium (Finer, 1988). The germination medium contained modified MS salts (no NH_4NO_3 and 2X KNO_3), B5 vitamins, 3% sucrose, and 0.2% Gelrite (pH 5.7). After root and shoot elongation, the plantlets were transferred to pots containing a 1:1:1 mixture of vermiculite, top soil, and peat, and covered with beakers. Plantlets were gradually exposed to ambient humidity over a two week period and placed in the greenhouse.

DNA Extraction and Southern Hybridization Analysis: DNA was extracted from proliferating embryogenic tissue using the CTAB procedure (Saghai-Marouf *et al.*, 1984). For extraction of DNA from leaf tissue, nuclei were first prepared (G. Anderson and G. Galau, pers comm). For isolation of nuclei, 0.5 g of leaf tissue was ground to a powder in liquid nitrogen and placed in 10 ml of cold homogenization buffer (HB; 10 mM Tris buffer (pH 9.5), 10 mM Na_2EDTA , 80 mM KCl, 0.5 M sucrose, 0.05% β -mercaptoethanol, 0.25% Triton X-100, 4 mM spermidine). Nuclei in HB were sequentially filtered through 500 and 100 μ m nylon filters, pelleted at 1,000xg, and washed 2x in HB by centrifugation and resuspension. Partially purified nuclei were then resuspended in HB, layered over HB containing 61% sucrose, and again centrifuged at 1,000xg. Pelleted nuclei were then used for DNA extraction using the miniprep procedure (Dellaporta *et al.*, 1983).

DNAs were digested with HindIII, which cuts pCIB709 once just upstream from the CaMV 35S promoter. For digestion of DNA extracted from leaf tissue, spermidine was added to the enzyme mix to a final concentration of 5 mM. Digested and undigested DNAs were electrophoresed on a 0.8% agarose gel. The DNA in the gels was treated with 0.2 N HCl, 2x for 15 min followed with 0.5 M NaOH/0.1 M 1.5 M NaCl, 2x 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) 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 and then prehybridized for 4-6 hr at 65°C in 50 mM Tris pH 8.0, 5x SSC, 2x Denhardt's, 10 mM Na_2EDTA , 0.2% SDS, and 62.5 μ g/ml salmon sperm DNA.

The BamHI fragment from pCIB709 (containing the coding region of the hygromycin resistance gene) was random-prime labeled (Feinberg and Vogelstein, 1983) and used for hybridization. Membranes were hybridized in the same solution as above but containing labeled probe (0.5-2 x 10⁶ cpm/ml) and 10% sodium dextran sulfate. After hybridization at 65°C for 24-48 hr, the membranes were first washed 5x in 2x SSC/0.1% SDS at 65°C and then washed 5x in 0.1% 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 AND DISCUSSION

Bombardment: The preparation of plant tissue and pellets reported here differs somewhat from a previously reported procedure (Klein *et al.*, 1988a). Problems initially were encountered with clumping of pellets prior to bombardment and severe damage to the tissue and culture plate during the actual bombardment. Pellets did not adhere to each other as tightly if they were permitted to settle out prior to bombardment rather than being subjected to centrifugation. In addition, loss of plant tissues was avoided if the cultures were covered with the nylon screen. The nylon screen helped to retain the tissue in the bombarded dish and may have lessened the damaging impact from the particle suspension. As the open area of the screen was only 57%, approximately 43% of the particles were retained by the screen, resulting in darkening of the center of the screens with repeated usage.

Selection for Transgenic Clones: One month following bombardment, tissues which were resistant to hygromycin could be visually selected and removed for further culture. Embryogenic cotton tissue which was hygromycin-sensitive turned white after a 3 week exposure to 100 μ g/ml hygromycin, whereas hygromycin-resistant embryogenic cotton tissue was yellow-green if viewed under a dissecting microscope. Using an inverted microscope, hygromycin-sensitive tissues appeared dense while resistant, viable clumps were translucent and characteristically yellow-brown (Fig. 1).

Individual clumps of hygromycin-resistant embryogenic tissue could be easily identified and separately cultured. A single clump, less than 0.5 mm in diameter could be used to establish a prolific, embryogenic culture after 1-2 months of subsequent culture. Low inoculum subculture of embryogenic suspension culture tissue of cotton has been previously reported (Finer, 1988). The ability of embryogenic tissue of cotton to survive and proliferate at low inoculum densities permitted the survival and regrowth of small amounts of transgenic tissue under hygromycin selection.

An average of approximately 30 stable transgenic clones were obtained from each separate bombardment using pCIB709. Particle bombardment of cotton cells with pUGGUS gave rise to an average of 4,351 cells per bombardment which expressed GUS transiently. These 30 clones represent a transient-to-stable conversion frequency of approximately 0.7%. A

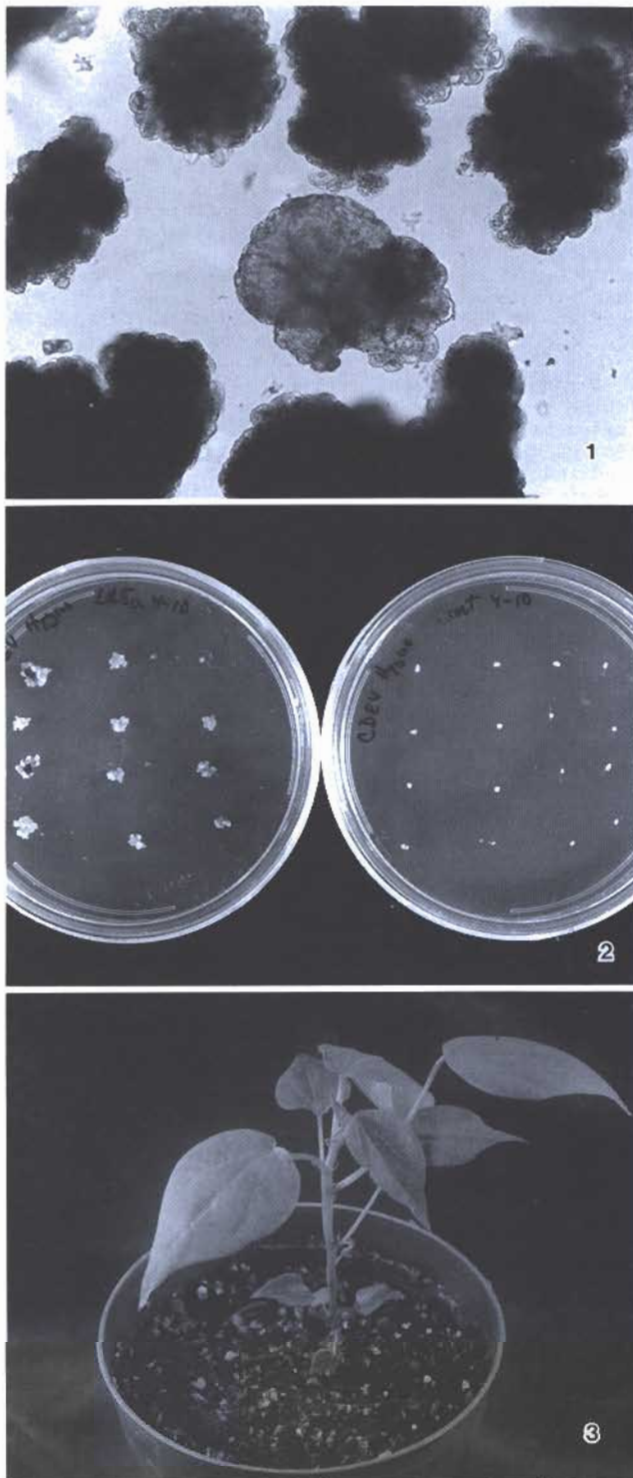


Fig. 1) Clump of selected, hygromycin-resistant, embryogenic cotton suspension culture tissue surrounded by dense, hygromycin-sensitive tissues.

Fig. 2) Hygromycin-resistant (left) and sensitive (right) cotton embryos on embryo development medium containing 100 µg/ml hygromycin.

Fig. 3) Regenerated transgenic cotton plant obtained by particle bombardment of embryogenic suspension cultures.

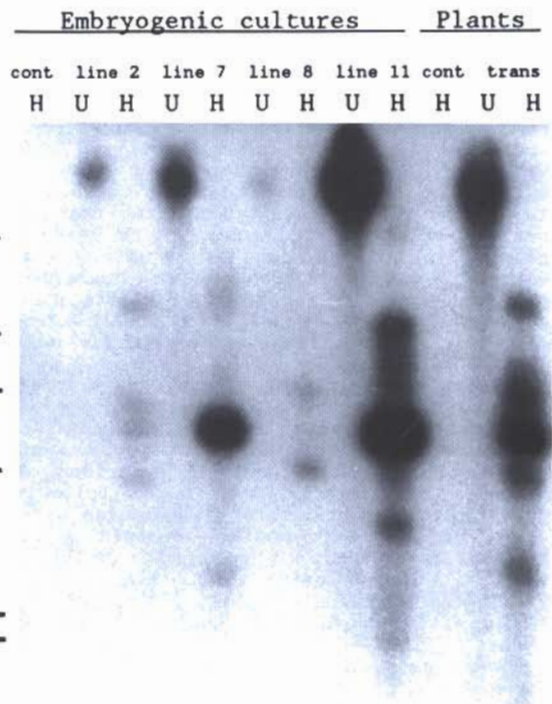


Fig. 4) Autoradiogram of Southern hybridization to DNAs from control (nontransformed) embryogenic suspension culture tissue (cont), four hygromycin-resistant embryogenic cell lines (lines 2, 7, 8, and 11), control leaf tissue (cont), and transgenic plant leaf tissue (trans). The hybridization probe was the BamHI fragment of pCIB709 as described in Materials and Methods. The DNAs were either undigested (U) or digested with HindIII (H), which cleaves pCIB709 (4.9 kb) once, upstream from the BamHI fragment.

transient-to-stable conversion frequency of 2-5% was reported for tobacco tissue bombarded with DNA encoding both GUS and kanamycin resistance (Klein *et al.*, 1988b). In that report, each bombardment of leaf tissue and suspension culture cells yielded an average of 83.3 and 143 GUS-expressing cells and an average of 2.3 and 6.9 kanamycin-resistant calli, respectively.

Plant Regeneration: Developing embryos from transgenic embryogenic clones of cotton showed no sensitivity to hygromycin (Fig. 2). Transgenic somatic embryos reached maturity in one month and were then placed on a hygromycin-free germination medium. Embryos were capable of germination in the presence of 100 µg/ml hygromycin but hygromycin addition was not considered necessary for continued selection at this point. Large amounts of embryos have been produced and thus far, plants have been recovered from 10 different transgenic clones. The first plants were recovered 5 months following bombardment (Fig. 3). This time might be reduced further once development and germination conditions are further refined.

Southern Hybridization Analysis: The presence of the introduced hygromycin-resistance gene in the hygromycin-resistant cultures and regenerated plants was confirmed by Southern analysis (Fig. 4). The intensity of the hybridization signals to the introduced hygromycin gene varied greatly between lines indicating differences in copy number in independently transformed lines. After digestion with HindIII, which cleaves pCIB709 once, many lines exhibited a strong hybridization to unit plasmid length DNA (pCIB709 is 4.9 kb). This suggests formation of concatemers of the introduced plasmid

with most copies arranged in a head-to-tail orientation. The weaker hybridization signals (Fig. 4) could represent alternate arrangement of some copies in concatemers, independent integration events, partial copies, or plant-plasmid DNA borders. Hybridization of undigested DNAs of transgenic cell lines and plants indicated that the introduced plasmid DNA was integrated into high molecular weight DNA. A detailed study of copy number and integration patterns in transgenic cotton lines and plants is in progress.

Although there are 2 reports of *Agrobacterium*-mediated transformation of cotton (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987), the time required for recovery of complete plants using *Agrobacterium* for cotton transformation was from 6-12 months. The majority of this time was necessary for initiation and proliferation of embryogenic tissue. By using cultures that were initially embryogenic for particle bombardment-mediated transformation as in this report, no time was required for conversion of the transgenic tissue to the embryogenic mode. In addition, if embryogenic cultures could be obtained from cultivars of cotton that give a low embryogenic response, these cultures could also be transformed directly using particle bombardment without the need to convert tissues to the embryogenic mode with each *Agrobacterium*-mediated transformation experiment.

In the only other report on recovery of transgenic plants via particle bombardment, soybean shoot tips were utilized as the target tissue (McCabe *et al.*, 1988). The resultant plants were always chimeric and leaf tissues from the resultant shoots and plants had to be periodically sacrificed and assayed for GUS activity. Use of embryogenic suspension cultures for particle bombardment is advantageous because transgenic, embryogenic tissues can be placed under selection pressures and continual scoring for potentially transformed tissue is not necessary. Selection for transgenic tissues using antibiotic resistance in liquid culture is very efficient and to date, all tissues that have been selected using hygromycin resistance and analyzed by Southern hybridization analysis have contained the transforming DNA.

This represents the first report of transformation of cotton using particle bombardment. It is also the first report of recovery of transgenic plants of any species via bombardment of embryogenic tissues. This approach could potentially be used for the production of transgenic plants in any species where embryogenic cultures are available.

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