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 plated DNA were accelerated towards the embryogenic plant cells. The plated DNA coating the particles encoded henyogenin 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 10-day intervals. From 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, MS = Murashige-Skoog, APh IV = amino- glycine-phosphate transpherase 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 (Murashige 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 renewal 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 (Pryce et al., 1985; Electroporation avoids the limited host range barriers of Agrobacterium, but requires the use of protoplast-to-plant cell systems.

Recently, transformation of plant cells (Klein et al., 1988a and射手 tip (McCown et al., 1988) has been reported. The principle of particle bombardment is the acceleration of small particles carrying DNA towards plant cells. Following penetration of the plant cells by the DNA particles, the DNA is incorporated from the particles and can then be expressed. Guns which target a specific cell site to bombard 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 tissue.

MATERIALS AND METHODS

Initiation and Maintenance of Embryogenic Suspension Cultures: Embryogenic suspension cultures of cotton (Gossypium hirsutum L. cv. ‘Coker 312’) 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 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 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 ceased. Leaking, 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 Tunngate Pellets: The plasmid pPGUS was made by subcloning the GUS gene as a HindIII/SacI fragment from pGUS1 (Jefferson et al., 1987) into pGUS1.19. The efficiency of particle bombardment was initially monitored using pPGUS and counting the number of cells showing transient expression of the GUS gene (Jefferson, 1987). The plasmid pCG7097 (Reinhardt et al., 1988; Moss et al., 1988) was used for longer term reconstitution experiments. This plasmid contains the APh IV gene (Gritz and Dawes, 1983) flanked by a CaMV 35′ promoter and terminator. The APh IV gene encodes a protein which confers and confers the antibiotic hygromycin B. DNA was precipitated onto 1.0 μg (approx. 100 mg/ml) tungsten pellets using a modified CaCl2 precipitation procedure (Klein et al., 1984). For precipitation of DNA, 5 μg of undigested plasmid DNA (1 μg/μl) was added to 25 μl of 100 mg/ml tungsten pellets in water. Then, 25 μl of 2.5 M CaCl2 was added to the suspension, followed by 50 μl of 0.1 M spermidine, thus allowing the pellets to settle in 5 μl volumes. The supernatant was removed. The concentrated pellet mixture was gently resuspended and 2 μl was used for bombardment.

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Preparation of Plant Tissue for Bombardment:  
Appropriate dehydration and cryo- 
suspension culture tissue (taken four days after 
subculture) was transferred to a 3.5 cm diameter Petri 
dish. The tissue medium was replaced with a sterile  
100 μm 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 
before bombardment. Bombardments were performed 
using a DeFondeville® Particle Delivery System (Model BPC). Each Petri dish 
containing plant tissue was bombarded once.

Selection for Transgenic Clones:  
Bombarded embryonic 
cotyledon tissues were resuspended in 
CEM medium. Three days following bombardment time 
(3 days following subculture) the suspension medium was 
filtered to remove large clumps of tissue. 
In the initial experiments, a significant 
number of tissue explants were killed by 
the laser. The aliquots were screened with 
the arbitrary classification of growth 
of shoots, callus, or no growth. 

Enzyme Development and Germination:  
For enzyme development, two sets of 
embryonic tissues were placed on a medium 
containing MS salts, 3% sucrose, 50 μM cefotaxime, 
100 μg/ml kanamycin, 0.5 mg/ml 
benomyl. Calli were grown in darkness 
(Günberg et al., 1986), 35 °C, sucrose, 50 μM cefotaxime, 
100 μg/ml kanamycin, 0.5 mg/ml 
benomyl. Embryos, which were obtained after 4 weeks on this medium, were cut into small 
segments, mixed with callus, and 
transferred to petri dishes containing a 1:1 mixture of 
vegetative shoot medium and 
callus medium (Firsey, 1988). After germination, 
shoots were transferred to vigorously 
watered media for 4-6 weeks in the 
dark. Media were changed weekly. 

DNA Extraction and Southern Hybridization Analysis:  
DNA was extracted from the embryonic tissue 
using the CCB procedure (Taghizadeh-Mehrnoosh et al., 1987). For extraction of DNA from leaf tissue, 
nuclei were first prepared (G. Anderson and G. Gale, pers com). For isolation of nuclei, 0.5 g of 
leaf tissue was ground to a powder in liquid 
nitrogen. The powder was placed in 10 ml of cetylamine buffer (10 mM Tris buffer (pH 9.5), 10 mM 
NaCl, 2.5% sucrose, 0.05% Triton X-100, 4 mM spermidine) and centrifuged at 10,000 g for 10 minutes. 
The supernatant was removed and the 
residue was extracted with phenol/chloroform 
(1:1, v/v). The nucleic acid fraction was precipitated with 
ethanol, washed with 70% ethanol, and resuspended in 
water. For Southern hybridization, total DNA was 
extracted from leaf tissue, and digested with various 
genetic markers. 

RESULTS AND DISCUSSION:  
Bombardment:  
The preparation of plant tissue and 
parameters reported here differ somewhat from a 
previously reported procedure (Kleins et al., 1986). 
Problems initially were encountered with clumping of 
quantities of plant material 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 sit on ice prior to 
being placed in floating medium. This has been remedied by 
the use of a floating medium which was added to the culture 
medium. This method has been successful in 
the tissue in the all bombarded dish and may have increased 
the efficiency of bombardment. This method was developed in 
our laboratory by T. A. S. and has been successful for 
approximately 4-6 weeks/25°C in a dark incubator. 

Selection for Transgenic Clones:  
One month following 
initial bombardment, protoplasts were 
plated on medium containing 100 μM 
benomyl and 0.5 μg/ml 
to monitor growth. 

Enzyme Development and Germination:  
For enzyme development, two sets of 
embryonic tissues were placed on a medium 
containing MS salts, 3% sucrose, 50 μM cefotaxime, 
100 μg/ml kanamycin, 0.5 mg/ml 
benomyl. Calli were grown in darkness 
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dark. Media were changed weekly.
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) Schematic diagram of Southern hybridization to DNA from control (untransformed) embryogenic suspension cultures (cont.), four hygromycin-resistant embryogenic cell lines (limes 4, 7, 8, and 11), control leaf tissue (cont.), four hygromycin-resistant leaf tissue (trans). The hybridization probe was the BamHI fragment of pC18709. Materials and Methods. The DNA were either digested (S) or digested with Hinfl (H), which cleaves pC18709 (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., 1986). In that report, each bombardment of leaf tissue and suspension culture cells yielded an average of 83.3 and 16.5 GUS-expressing cells and an average of 2.3 and 4.9 kanamycin-resistant cells, respectively.

Plant regeneration: Developing embryos from transgenic embryogenic cultures 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 observed 3 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 Hinfl, which cleaves pC18709 once, many lines exhibited a strong hybridization to unit plasmid length DNA (pC18709 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 arrangements of some copies in concert. Independent integration events, partial copies, or plant-plasmid DNA borders. Hybridization of undigested DNA 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 Agrobacteri um-mediated transformation of cotton (Frouzakbady et al. 1987; Uebbeck 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 transferred 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, whitehead shoot tips were utilized as the target tissue (Molly et al., 1988). The transplanted plants were always chlorotic 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 tissue can be placed under selection pressures and continual scoring for potentially transformed tissue is possible. Selection for transgenic cultures using antibiotic resistance in liquid culture is very efficient because 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 tissue. This approach could potentially be used for the production of transgenic plants in any species where embryogenic cultures are available.

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REFERENCES