DEVELOPMENT OF TRANSGENIC EGYPTIAN COTTON (*GOSSYPIUM BARBADENSE)* VARIETIES FROM MERISTEMATIC TISSUE

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Abstract

A variety-independent protocol was developed for transformation and regeneration of Egyptian cotton plants (*Gossypium barbadense L.*) from meristemaic tissue of excised embryonic axes and from the shoot apical meristem of seedlings using the Bio-Rad PDS/1000/He gene gun. Tungsten beads coated with plasmid DNA (pBI221) carrying the coding sequence for the GUS protein were used to deliver the gene directly into the meristematic tissue. Transgenic cotton plants have been obtained using techniques of shoot meristem transformation and direct regeneration of shoots and roots. Plants derived from this process were screened for integration of the GUS gene into the plant cell genome using dot blot technique, genomic Southern analysis, immuno blot hybridization and histochemical assay. This work represents the first report for shoot meristems transformation and regeneration of commercial Egyptian cotton varieties, *Gossypium barbadense,* (Giza 45, Giza 87 and Giza 88 as extra-long staple varieties and Giza 85, Giza 89 and Giza 86 as long staple varieties) using biolistic gun transformation. Molecular and genetic characterization of primary transformants and their progeny proved that GUS gene was successfully integrated in plant cell genome.

Introduction

Egyptian cotton is an attractive crop for genetic engineering because of its worldwide importance. Transformation of cotton plants with desired genes is the focus of many plant genetic engineering research programs. Several techniques to introduce foreign genes into plants have been developed. The lack of a routine, repeatable, genotypic- independent regeneration process from plant cell culture systems for agriculturally important crops is a major obstacle in the application of genetic engineering technology. Members of the *Gossypium* genus, as well as members of other important crop families, are severely limited in their regeneration in vitro from protoplast, callus or leaf tissues. The literature concerning in vitro establishment of plants from the shoot meristems spans nearly 35 years, beginning with the work of Morel and Martin (1952) and Morel (1972). The terms "meristem" and "shoot tip" culture have often been indiscriminately interchanged. Regeneration of shoot meristems is widely recognized as the method of choice in the regeneration of virus-free germplasm and apparently can be used with all plant species (Murashige 1974). Therefore, adaptation of this classic procedure to transformation studies can eliminate many of the present restrictions as to species and varieties that result from the lack of an appropriate in vitro regeneration method. In addition, somaclonal variation can be minimized in cotton plants regenerated from shoot apex explants. This phenomenon is undesirable where it is essential to maintain the original genetic integrity of transformed plants. Particle bombardment is a rapid method for delivery of DNA to plant cells for both gene expression and stable transformation studies (Klein *et al.* 1987, Klein *et al*. 1988, Boynton *et al*. 1988, Johnston *et al*, 1988). It confers no direct effect on the chromosomal integration mechanism and offers an alternative in situ transformation method of meristematic tissue, bypassing conventional plant regeneration from callus (Finer and McMullen 1990, Sanford *et al.* 1987). The nature of the integration event is due to microprojectile penetration into the nuclear membrane and DNA deposition directly within the nucleus. This work discusses the development of transgenic Egyptian cotton varieties from meristematic tissue explants from shoot apices and embryonic axes and detection of gene integration into transformed plants.

Materials and Methods

Plant Materials

Egyptian cotton seeds *Gossypium barbadense* L. cv. Giza 45, Giza 87 and Giza 88 as extra-long-staple, and Giza 85, Giza 89 and Giza 86 as long-staple varieties) were acid delinted using concentrated sulfuric acid and neutralized with sodium bicarbonate for 10 min. Seeds were rinsed twice with sterile distilled water, soaked in a solution of 40% commercial bleach containing two drops of detergent (soap) for 20 min. Washed three times and soaked overnight in sterile distilled water. After removing the seed testa, one-half of the disinfected seeds were germinated aseptically on half strength MS basal medium (Murashige and Skoog 1962) solidified with 0.2% phytagel. Seeds were incubated at 28°C with a 16-h photoperiod 90 μE m-2 s-1 and subjected to shoot apical meristem dissection. The other half of seeds were used to excise embryonic axes.

Plasmid DNA

The plasmid pBI221 was employed as the source of exogenous DNA in biolistic Gun transformation. The plasmid contained the β-glucuronidase, or *gus* A gene under the regulatory control of the CaMV 35S promoter and the nopaline synthase (*nos*) gene terminating sequence (Jefferson 1987).

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Explant Preparation

Shoot meristem explants of the 5-day-old seedlings were isolated with the aid of a dissecting microscope. The seedling apex was exposed by pushing down on one cotyledon until it broke away, exposing the seedling shoot apex. The unexpanded and primordial leaves were left in place to supply hormones and other growth factors (Smith & Murashige, 1970). The germinated shoot apex consists of the meristematic dome and more than two primordial leaves (Figure. 1A,B). Embryonic axes were prepared after dissection of seeds aseptically. Samples were prepared for bombardment by placing 50 to 70 meristematic sections in each plate. Apical meristem and embryonic axes were exposed separately in the center of a 100x15 mm Petri dish containing 20 ml media supplemented with 100mg/L i-inositol, 1.0mg/L thiamine-HCL, 2% glucose, 1X Gambourg vitamin (Sigma), 100mg/L citric acid, 100mg/L ascorbic acid, 0.1mg/L kinetin, pH 5.9, and 0.2% phytagel (Gould *et al.* 1991). Tissue sections were stored at 30 °C in moderate light and were bombarded within 3 to 24 hours after dissection.

Biolistic Gun Transformation

The procedures for preparation of the micro and macro carriers were described by Heiser (1993). Microcarriers homogenized in ethanol suspension were applied to the macrocarrier and allowed to dry before used for bombardment. Dissected tissues were bombarded using 0.5 μm tungsten powder, 90-110 Kg/cm2 Microcarrier helium pressure, 6 cm target distance and 25 μl tungsten/DNA complex per bombardment event. Each sample plate was subjected to two constitutive bombardment events.

Regeneration of Bombarded Explant

After bombardment, plates were wrapped with parafilm and incubated overnight at 30 °C in the dark to heal the bombarded tissue. The bombarded tissues were cultured on shoot regeneration media containing MS basal salt supplemented with 100mg/L *myo*-inositol, 1.0mg/L thiamine-HCL, 2% glucose, 1X Gambourg vitamin (Sigma), 100mg/L citric acid, 100mg/L ascorbic acid, 0.1mg/L kinetin and 0.2% phytagel (Gould *et al.* 1991). Shoot apices and embryonic axes were cultured on this media for one week and transferred to kinetin-free media and incubated at 28°C with a 16-h photoperiod 90 μE m-2 s-1 for 2 to 3 weeks. Regenerated shoots were subcultured on half-strength MS media containing 0.3% activated charcoal for root formation. MS media were supplemented with 100mg/L indoleacetic acid (IAA) to favor development of root (Chlan *et al.* 1995). When an extensive root system had formed, plantlets were transferred to 10-cm pots containing 1:1:1 mixture of sterile topsoil:sand:peat., transferred to a Conviron unit adjusted to 28°C with a 16-h photoperiod 90 μE m-2 s-1 , watered thoroughly with half-strength MS solution and covered with plastic bag. As plants adapt to soil, and 7-10 healthy true leaves were formed, the plastic bags were removed and the plants were transferred to a greenhouse adjusted at 28°C with a 16-h photoperiod and 90 % humidity.

Screening for Transformed Plants

Screening for regenerated transformants (R0) was conducted using GUS staining histochemical assay and dot blot hybridization.

β-Glucuronidase (GUS) Assay Using Bistochemical Test

GUS activity in the transformed plantlets was determined by histochemical assay (Momtaz and Madkour, 1993). Explants from tramsformants were incubated overnight at 37°C in GUS buffer (Jefferson. 1987). After incubation, the tested parts were treated with 95 % ethanol and scored for intensity of blue color.

DNA Isolation and Dot Blot Hybridization

Explants from each boll of transformants were used for genomic DNA isolation using a modified method of Paterson et al. 1993. About 0.05 gram of tissue was ground using liquid nitrogen. Total DNA was extracted by adding 0.5 ml. of cotton DNA extraction buffer (63.8 gm/L Sorbitol, 12.1 gm/L Tris-HCl, 1.63 gm/L EDTA and 10 μl β-mercapto ethanol. The mixture was incubated in ice for 15 minutes and centrifuged at 300 g, 4°C for 15 min. The nuclear pellet was resuspended in 5 ml extraction buffer, 2 ml 5 % Sarkosyl and 5 ml nuclear lysis buffer (0.2 M Tris-HCl, 50 mM EDTA, 2 % cetyltrimethylammonium bromide (CTAB) and 2 M NaCl), and incubated at 60°C for 20 minutes. The mixture was subjected to chloroform-isoamyl extraction. DNA was precipitated with 0.6 volume of isopropanol. Two to three μg of undigested DNA from each explant was immobilized as dots to Boehringer Mannheim nylon membrane. The hybridization probe was prepared from a purified Hind III-EcoR1 fragment containing coding sequences of GUS gene using DIG DNA labeling and detection kit. The hybridized DNA probe was detected colorimetrically as described in the kit manual.

Progeny Analysis

R1 seeds recovered from boll-positive R0 plants were germinated and grown in the greenhouse. Leafs were taken from each plant and analyzed for GUS activity using genomic Southern hybridization and immuno blot analysis.

Genomic Southern Blot Analysis

Total DNA samples from R1 transformed plants were isolated from 1 to 2 gm of young fresh tissue using modified method of Paterson *et al.* 1993. DNA samples were quantified using Beckman DU 7400 spectrophotometer. About 5 μg of DNA were digested with Hind III restriction endonuclease under the condition recommended by the supplier (Promega, Madison, WI), size fractionated on a 0.8 % agarose gel, then immobilized on MSI neutral charge nylon membrane. The hybridization probe was prepared from

a purified HindIII-EcoR1 fragment from pBI221 plasmid containing GUS gene using non-radioactive NEBlot-phototope labeling and detection system. The membrane was exposed on autoradiograph.

Immuno Dot Blot Analysis

Total leaf protein from R1 transformed plants were extracted and analyzed for β-glucuronidase content using dot blot techniques. Dot blot analysis was performed using the BIO-RAD Mini Trans-Blot apparatus according to the manufacturer's instructions. Nitrocellulose membranes were incubated in 1μg/ml anti-*GUS* antibody (Promega Madison, WI, USA) for 30 min followed by incubation in goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI, USA). β-Glucuronidase enzyme was detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates (Promega, Madison, WI, USA).

Results and Discussion

To circumvent difficulties with genotype-dependent regeneration, a few investigators have reported successful transformation of cotton meristem tissue using a biolistic approach (Finer and McMullen, 1990; McCabe and Martinelli, 1993). Modification of Egyptian cotton plants using genetic engineering techniques would facilitate the rapid development of new varieties with traits such as insect, herbicide and disease resistance, environmental stress and seed quality improvement. Shoot meristem regeneration has been used successfully with many monocot and dicot families (Murashige, 1974). Theoretically, the tissues of the apical meristem are best suited for use in plant regeneration because these tissues are programmed for shoot organogenesis and do not need to differentiate to a meristematic state. In practice, this method has a low incidence of somaclonal variation in regenerated plants (Murashige, 1974). In view of the economic importance of Egyptian cotton and the potential to improve commercial cultivars by genetic transformation, a search was initiated for regeneration and proper transformation methods to achieve the most efficient protocols. Protocols for gene transfer into cotton have been reported for specific cultivars (Firoozabody *et al.* 1987, Umbeck *et al.* 1987, Finer and McMullen 1990, Cousins *et al.* 1991, Bayley *et al.* 1992). These protocols have been tested with Egyptian cotton varieties (Giza 70, Giza 75, Giza 83, Giza 45, Giza 87, Giza 88, Giza 89 and Giza 85) and significant stumbling block were faced in completing the later stage of somatic embryogenesis to be able to formulate shoots and roots (Diab 1997, Momtaz *et al*. 1998). This work has focused on the establishment of an almost universal method of plant regeneration, which is not limited to genotype. In the present study, isolated shoot apices and embryonic axes from Egyptian cotton *Gossypium barbadense* Giza 45, Giza 87 and Giza 88 as extra-long staple cultivars and Giza 85, Giza 86 and Giza 89 as long-staple cultivars were successfully transformed using biolistic gun transformation. The bombarded meristem tissue was allowed to regenerate and then subjected to molecular and genetic analysis. For the six tested varieties, the culture media used as reported by Gould *et al*. 1991 was adequate for normal shoot development. After 1-2 weeks, shoots were initiated from the shoot apices and embryonic axes. Three weeks later, elongated shoots produced 2-3 true leaves. The percent of shoot regeneration in the 6 transformed varieties ranged from 83% to 95 %. One cm or larger shoots were cultured on half-strength media containing 0.3% activated charcoal 2 to 3 weeks until at least 2 roots were formed. Shoots were then transferred to rooting media to favor formation of roots. The frequency of root induction from shooted explants ranged from 75-85 % and the frequency of recovered plants ranged from 95-100 %.

Detection and Screening of Transgenic Plants (R0)

β-Glucuronidase (GUS) Assay Using Histochemical Test

GUS histochemical assay confirmed the expression of GUS gene in transgenic Egyptian cotton varieties which were transformed using both shoot apices and embryonic axes. The results of histochemical GUS activity, as indicator for GUS gene expression are shown in figures 1C, D&E.

Dot Blot Analysis

Dot Blot analysis of total undigested DNA from transformed plant tissue, immobilized on nylon membrane and hybridized with a GUS gene probe confirmed the presence of the GUS gene in cotton genome. Genomic DNA from non-transformed tissue did not hybridize with the labeled probe. The percent of chimeric plants produced from meristematic shoot apex sections from dissected seedlings among varieties varied from 60% in the case of Giza 89, to 70 % in Giza 86. The percentage transformed bolls was calculated by dividing the number of GUS positive bolls, indicated by the dot blot analysis, over the total number of bolls produced for each variety per plant. The percent of transformed bolls ranged from 0.9 % in Giza 86 to 1.2 % in Giza 45. Embryonic axes transformation among varieties produced a range from 39.8 % in Giza 89 transformation to 63.39 % in Giza 86. The percent of transformed bolls ranged from 0.032 % in Giza 89 to 0.09 %in Giza 45 (Table 1).

Progeny Test and DNA Analysis

Germinated R1 seeds recovered from positive bolls (R0) were tested to confirm the integration of the GUS gene using Southern hybridization and immuno blot analysis.

Southern Hybridization Analysis

Southern hybridization analysis of genomic DNA of R1 plants digested with Hind III and immobilized on nylon membrane were hybridized with GUS probe indicating the integration of GUS gene through the first generation of developed transgenic cotton plants. Each of the developed

transgenic cotton plants showed GUS activity and positive hybridization to GUS gene probe (Figure, 2).

Immuno Dot Blot Analysis

The results of immuno dot blot confirmed the expression of transformed GUS gene in the shoots of the transgenic plants. It was clear that GUS gene is expressed in the first generation of the transgenic Egyptian cotton plants (Figure 3). In this work, authors described a method for transforming cotton meristem tissue using the Bio-Rad PDS/100/He Gun and regeneration of transformed plant. The results obtained clearly showed that microprojectiles can deliver DNA to cells in a form that can be integrated and expressed in the progeny of the Egyptian cotton genome.

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Table (1). GUS expression detection in transformed Egyptian cotton varieties using dot blot hybridization and PCR analysis.

Varieties	# of Explant bombarded		# of positive explant		% of chimeric plants		% of transformed bolls	
	SA	EA	SA	EA	SA	EA	SA	EA
Giza 45	1150	2213	785	1403	69.46	51.41	1.2	0.09
Giza 87	2130	1756	1381	778	64.98	44.30	0.48	0.067
Giza 88	950	893	592	480	62.71	53.75	0.77	0.034
Giza 89	700	834	420	332	60	39.80	0.37	0.032
Giza 86	1115	1375	772	707	70	63.39	0.9	0.022
Giza 85	2350	2586	1449	1106	61.65	42.76	0.61	0.084

SA= Shoot apix
EA= Embryonic axes

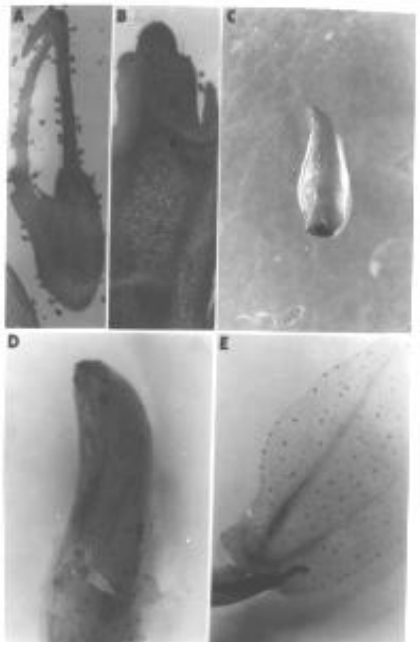

Figure 1. **A**. Longitudinal section of the cotton shoot apex which includes the meristematic dome and primordial leaves. **B**. Longitudinal section of shoot apex prepared for bombardment and shoot culture. **C**. Transformed stem section showing high level of GUS activity. **D**. {100X} **E**. Histochemical test for transformed true leaf showing GUS expression.

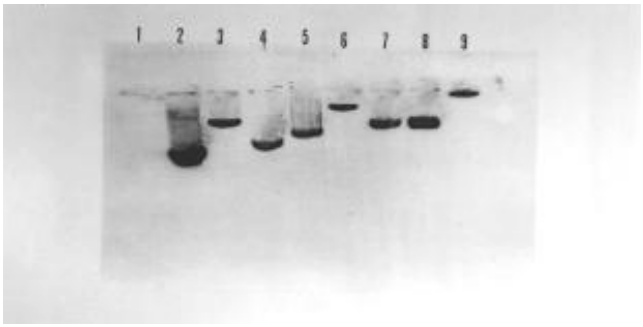

Figure 2. Southern analysis of transformed Egyption cotton. *Gossypium barbadase* (Giza 45). DNA smaples (5 µg) were digested with HindIII, resolved, hybridized and detected as described in materials and methods. **Lane 1**. Negative control (non-transformed cotton plant). **Lane 2**. Positive control (pB1221 plasmid). **Lane 3, 5, 6, 7, 8, 9**. Contain DNA from cotton plant progeny transformed by pB1221 plasmid.

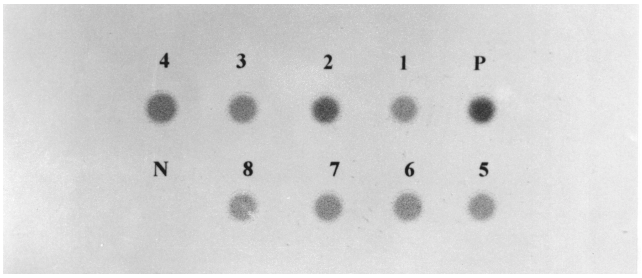

Figure 3. Detection of GUS protein using dot blot analysis in R1 cotton plant progeny transformed by pB1221 DNA containing GUS gene using Biolistic bombardment. **(p.)** Positive control (purified β-Glucuronidase enzyme). **(1-8.)**. Protein samples isolated from transformed (R1) cotton plant (Giza 45). **(n)**. Negative control (non-transformed cotton plant.