Transformation of Egyptian Cotton Tissue (*Gossypium barbadense*) Using *Agrobacterium tumefaciens*

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ABSTRACT

Callus induction from hypocotyl and cotyledonary explants of Egyptian cotton (Gossypium barbadense), extra-long staple (Giza 45, Giza 77) and long staple (Giza 85), were evaluated for in vitro callus induction and maintenance utilising two media formulations (G2) and (CI). Giza 85 produced significantly better callus than Giza 45 and Giza 77. However, induction of callus was genotype dependent and highly variable, not only among varieties, but also among explants of the same variety. Attempts to control tissue contamination and decay of calli were successful. Explants from three varieties were tested for regeneration using G2 and CI media containing different levels of kanamycin to test for tolerance level in callus tissue. Callus induction and growth were inhibited at 25 mg/L kanamycin. Cotyledon and hypocotyl tissues of the three varieties were transformed successfully, and transformation efficiency varied by variety and type of explant. Cotyledon and hypocotyl explants from 12-day-old aseptically germinated seedlings were inoculated with a non-oncogenic Agrobacterium tumefaciens strain LBA4404 carrying the binary plasmid vector pBI121 with a chimeric nopaline synthase (NOS) promoter driving the neomycin phosphotransferase (NPT II) gene and a CaMV35S promoter driving the β-glucuronidase (GUS) gene. After three days co-cultivation, explants were placed on callus induction medium containing 25 mg/L kanamycin for selection of transformed tissue. The presence of (NPT II) in crude cellular extracts from embryogenic callus tissue was detected by ELISA test. Expression of the GUS gene was confirmed histochemically followed by dot blot analyses to screen for DNA integration using an Xba I- EcoR1 fragment containing GUS gene coding sequences as probe.

Introduction

A major obstacle to the application of *Agrobacterium*-mediated transformation to cotton (*Gossypium* spp. L.) has been the absence of a high-efficiency plant regeneration method (Davidonis *et al.*, 1983; Shoemaker *et al.*, 1986; Gawel *et al.*, 1986; Trolinder and Goodin, 1987). A second barrier to cotton transformation has been the optimization of methods for using a selectable marker such as kanamycin resistance. In a system such as tobacco, transformed tissue at any stage of development can be selected on high levels of kanamycin (up to 1000 mg/L), while in cotton such levels are toxic. Conversely, several factors can lead to survival of non-transformed cotton calli, or to chimeric calli consisting of predominantly non-transformed tissues. These factors include low kanamycin levels and selection in later stages of callus proliferation. This paper reports the transformation of cotyledon and hypocotyl explants and the induction and maintenance of callus cultures from three Egyptian cotton (*G. barbadense* L.) varieties. Callus tissues were maintained and subjected to molecular and biochemical analysis to confirm integration and expression of foreign gene and the absence of *Agrobacterium* cells in the transformed callus cultures.

Material and Methods

Plant material. Acid delinted seeds of *G. barbadense* (cvs. Giza 45, Giza 77 and Giza 85) were washed twice with sterile distilled water, rinsed with 40% commercial bleach containing two drops of detergent for 20 min., then rinsed three times and soaked overnight in sterile distilled water. After removing the seed testa, the disinfected seeds were germinated aseptically on half strength MS basal medium (Murashige and Skoog, 1962) solidified with 0.2% phytagel and incubated at 28^{o}C with a 16-h photoperiod at 90 μE m^{-2} s^{-1}.

Bacterial strains and plasmids. The disarmed *A. tumefaciens* strain LBA4404, in conjunction with a binary vector plasmid pBI121 (Hoekema *et al.*, 1983; Jefferson *et al.*, 1987) were used in the transformation process. The *Agrobacterium* cells were grown overnight at 28^{o}C in 2XYT minimal liquid media containing 16g/L bacto-trypton, 5 g/L NaCl, 25 mg/mL streptomycin, 50 mg/mL rifampicin and 50 mg/mL kanamycin (all from Sigma). The bacterial cells were washed and resuspended in cotton callus induction medium before inoculation.

Callus initiation and maintenance. Cotyledon pieces (0.5 cm^{2} surface area) and hypocotyl sections were

aseptically excised from sterile 12-days-old seedlings. Approximately one-half of the explants were cultured on G2 medium [MS medium (Murashige and Skoog, 1962) supplemented with 100 mg/L myo-inositol, 0.4 mg/L thiamin-HCl, 5 mg/L 6-γ,γ-dimethylallylamino-purine (2iP), 0.1 mg/L α-naphthalene acetic acid (NAA), 3% (w/v) glucose, pH 5.9, 100 mg/L ascorbic acid and 100 mg/L citric acid] (citric and ascorbic acids were not necessary for callus induction but they greatly reduced phenolic compounds). The medium was solidified with 0.2 % phytagel, and tissues were incubated at 28$_o$C with a 16-h photoperiod at 90 μE m^{-2} s^{-1}. The other half of the explants were cultured on CI media [MS media supplemented with 100 mg/L *myo-i*nositol, B5 vitamins (Gamborg, 1968), 0.1 mg/L 2,4-D and 0.1 mg/L kinetin. The medium, solidified with 0.2% phytagel, also contained 0.75 g/L $MgCl_2$, pH 5.8, 100 mg/L ascorbic acid and 100 mg/L citric acid]. Tissues were incubated as described above and explants were monitored and scored for callus induction. The resulting callus tissues were maintained on hormone free medium under the same incubation conditions.

Transformation and selection. Overnight cultures of bacteria were centrifuged and resuspended to a concentration of ≈10^8 cells/mL). Cotyledon pieces (≈0.5 cm^2 surface area) and hypocotyl sections were cut with a dull blade (to maximize wounding) from sterile 12-days-old seedlings. The explants were dipped in the *A. tumefaciens* suspension in Petri dishes and gently shaken for a few seconds to ensure contact of all explant edges with the bacterial suspension. The explants were then blotted and dried with sterile filter paper (Whatman No. 1) to remove excess bacteria, and cultured on callus induction medium. After three days co-cultivation, explants were transferred and incubated in Petri plates containing the same medium supplemented with 500 mg/L carbenicillin to control bacteria and 15-35 mg/L kanamycin sulfate. After 3-4 weeks, microcalli were excised from the original explants, transferred to fresh kanamycin-containing medium and incubated under lower light intensity. After two to three weeks, calli were placed and maintained on embryogenic medium (callus induction media without hormone) under kanamycin selection.

Neomycin Phosphotransferase II (NPT-II) Assay. The presence of the NPT-II protein in transformed embryogenic callus was detected by a sandwich immunoassay with an NPT-II ELISA Kit (5'- 3', Inc., Boulder CO, USA). Transformed cell extract, non-transformed cell extract as control, and pure NPT-II protein concentrate as standard, were added to the wells. Callus extracts were made by grinding each tissue in 3-4 ml cold 0.25 M Tris-HCl, pH 7.8, containing 1 mM phenylmethylsulfonylfluoride per gram of callus tissue. Extracts were centrifuged (7500 x g, 4$_o$C, 30 min.) and 200 ml of diluted extract were loaded in the microwell ELISA plate. The NPT-II levels were determined and expressed as described in the kit manual.

β-Glucuronidase (GUS) Assay. GUS activity in the transformed embryogenic callus tissue was determined by histochemical assay (Jefferson, 1987). Callus tissues were incubated overnight at 37$_o$C in GUS buffer containing 50 mM sodium phosphate buffer pH 7, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na_2EDTA and 1 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc). After incubation, the callus tissues were plasmolysed using 95 % ethanol and scored for intensity of blue colour.

Dot blot analysis. Genomic DNA was isolated from transformed and non-transformed embryogenic callus tissue using a modification of Paterson *et al.* (1993). Callus tissues were weighed and ground in liquid nitrogen, and the ground tissues transferred to Nalgen centrifuge bottles containing 5x cold extraction buffer (63.8 gm/L sorbitol, 12.1 gm/L Tris-HCl, 1.63 gm/L EDTA and 10ml β-mercapto ethanol). The mixture was incubated in ice for 15 min. and centrifuged at 300 g, 4$_o$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 and 2 M NaCl). The mixture was incubated at 60$_o$C for 20 min. followed by chloroform-isoamyl extraction and DNA precipitation with 0.6 volume isopropanol. Two to three mg of undigested DNA from each callus sample were transferred as dots to Boehringer Mannheim nylon membrane. The membrane was hybridised using DIG DNA Labelling and Detection Kit (Random primed DNA labelling with digoxigenin-dUTP, alkali-label and detection of hybrids by enzyme immunoassay, Boehringer Mannheim, Cat. No. 1093 657). The hybridized nylon membrane was washed twice for 5 min. in 2X SSC buffer, 0.1 % SDS at room temperature and twice for 15 min. in 0.1X SSC buffer, 0.1 % SDS at 68$_o$C under constant agitation. The washed hybridized membrane was subjected to immunological detection as described in the kit manual. The hybridization probe was prepared from a purified Xba I- EcoR1 fragment containing coding sequences of the GUS gene from plasmid pBI221 (Clontech). The probe was labelled as described in the kit manual.

Results and Discussion

The current strategy to overcome genotype dependent regeneration is to move transferred genes from transformed regenerable cultivars into the other commercial cultivars by recurrent backcross with desired cultivars to regain the traits of the targeted cultivar. A more efficient method would be to transform the desired cultivar directly. The lack of a routine regeneration and transformation process, especially in Egyptian cotton cultivars, is a major obstacle in the application of genetic engineering technology. Recent developments and advancements in the fields of biochemistry, molecular biology and

genetic engineering make it possible to adapt this technology for Egyptian cotton improvement.

Callus Initiation and Maintenance. Explants of 12-day-old seedlings formed callus readily on the two media formulations (G2 and CI), however, callus induction differed according to medium used. In G2 medium containing 2iP and NAA, both cotyledon and hypocotyl segments produced microcalli after 12-15 days incubation. On CI medium, containing 2,4-D and kinetin, hypocotyl segments produced callus after 25-30 days incubation period while cotyledonary explants enlarged but failed to produce microcalli. These results support using G2 medium in *Agrobacterium*-mediated transformation because rapid callus initiation (on media containing high 2iP) was important for the recovery of high numbers of transformed microcalli at the periphery of the inoculated tissues. This could be due to a better recovery of transformed cells on these media, or high competency of dividing cells for transformation with *A. tumefaciens*.

Callus morphology did not differ on the two media. On both hormone-free media calli tended to be friable cream-coloured and granular. However, the time frame for initiation differed for the media. On G2 medium, callus initiation occurred five weeks after transfer to hormone-free medium and embryogenic calli developed 7-9 weeks after transfer, whereas on CI medium, callus tissue developed nine weeks after transfer to hormone-free media. CI medium resulted in a higher percentage of embryogenic calli (73%), but tissues became brown earlier. G2 medium produced 60% embryogenic calli.

Kanamycin Sensitivity of Explant Tissue. Callus induction and microcallus growth on medium containing kanamycin at concentrations ranging from 0-25 mg/L were evaluated. Based on these experiments, kanamycin of 25 mg/L was chosen for selection of transformed callus from cotyledons and hypocotyls tissues.

Plant Cell Transformation and Selection. Following co-cultivation with *A. tumefaciens*, callus induction media (G2) containing 500 mg/L carbenicillin and 25 mg/L kanamycin allowed transformed tissues to form kanamycin-resistant microcalli at wound sites after 12-15 days. No callus from control tissue initiated at this level of kanamycin in the same time period. Five weeks after inoculation, about 1 to 3 kanamycin-resistant calli were obtained per cotyledon segment and about 1 to 2 kanamycin-resistant calli per hypocotyl segment (Table 1). A total of 499 putative transformed calli were tested for NPT-II production. On lower levels of kanamycin (15 mg/L), some nontransformed calli proliferated very slowly but eventually turned brown and died. The percentage of kanamycin-resistant and NPT-II positive calli was higher when higher levels of kanamycin (25 or 35 mg/L) were used for selection.

A low titer of bacteria ($\approx 10^8$ cells/mL) was very important for inoculation. High titers resulted in overgrowth on the explant tissues. Excision of microcalli from explants was essential to promote growth of these calli and to avoid *Agrobacterium* contamination which otherwise usually developed on the explants. To control bacterial growth, callus tissue was maintained on media containing 500 mg/L carbenicillin. Glucose was used as carbohydrate source all through out since sucrose encourages production of phenolics and delays development (Firoozabady *et al.*, 1987). When kanamycin-resistant calli were placed and maintained on embryogenic medium (induction medium without hormones) under selection and incubated at 30^oC with a 16-h photoperiod of 90 $\mu E\ m^{-2}\ s^{-1}$, 60 % of the calli became friable, cream-coloured and granular, typical of embryogenic calli (Figure 1A). Early stages of embryogenesis were also observed (Figure 1A,C and D). After ten weeks, some embryoids became compact and green but this tissue stopped at this early stage of embryogenesis and returned to undifferentiated growth. Incubation in continuous dark with frequent subculture failed to prolong embryogenesis.

Assay for NPT-II, GUS gene activity. Assay for NPT-II and GUS activities detected expression in the transformed cotton calli. A total of 499 putative transformed calli were tested for NPT-II production with the ELISA kit and were found positive (Table 1). NPT-II levels ranged from 177 to 565 pg/mL of crude cellular extract (Table 2). Relative levels of NPT-II protein in transformed callus were at least as high as the background level present in the untransformed callus. Expression of the GUS reporter gene in transformed callus was confirmed by histochemical assay (Figure 2). There were differences among transformed calli that generally can be attributed to either positional effect or copy number of the transferred gene.

In a callus transformation system, cells of *Agrobacterium* that may survive antibiotic treatment (i.e. 500 mg/L carbenicillin), but are unable to produce visible colonies, may interfere with molecular and biochemical analysis and lead to a false positive indication that stable integration and expression of the foreign gene has taken place. In order to discount such a phenomenon, two tests were conducted to verify the absence of *Agrobacterium* in the transformed callus. During tissue preparation for GUS assay, samples of transformed callus were plasmolysed with 95 % ethanol in order to verify that the blue dye indicating GUS activity was inside the cells and not produced by *Agrobacterium* in the intercellular spaces. As a further test, an inoculation loop was stabbed several times in and around putatively transformed calli (after the 4th or 5th subculture), and then incubated in liquid and solid 2XYT medium with and without appropriate levels of antibiotics (25 mg/mL streptomycin, 50 mg/mL rifampicin and 50 mg/mL kanamycin). 2XYT

medium inoculated with *A. tumefaciens* served as control. Bacterial growth was not observed in the medium inoculated with the stabbed loop from callus tissue even after several days of incubation, whereas normal bacterial growth was observed in the control. The results confirmed the absence of *Agrobacterium* in the transformed cotton callus. Dot blot analysis of total undigested DNA from transformed calli using a GUS gene probe confirmed the integration of T-DNA into the cotton genome (Figure 3). Genomic DNA from untransformed cotton calli did not hybridize with the labeled GUS gene. Results from molecular and biochemical analysis confirm stable integration and expression of the foreign gene in the callus. This study demonstrates *Agrobacterium*-mediated transformation of Egyptian cotton tissue and confirms genotype-independent *Agrobacterium* infectivity and embryogenic callus formation. The callus transformation system described here can be used for quantifying the expression of a foreign gene stably integrated into Egyptian cotton cells.

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Table 1. Transformation frequencies of cotton (G. barbadense) cotyledon and hypocotyl sections using A. tumefaciens LBA4404. Five weeks after inoculation, calli were excised and placed on fresh kanamycin-containing media, and 9 weeks after inoculation, calli were scored for kanamycin resistance and NPT II production.

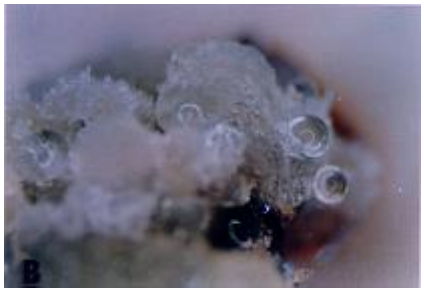
Explant	Number of section inoculated	Kanamycin mg / L	mean microcalli per section developed on kanamycin	NPTll positive callus tissue tested
cotyledon	30	15	1.6	48
	80	25	3.4	272
	34	35	1.1	37
hypocotyl	71	15	1.3	92
	168	25	0.3	50
	63	35	0	0

Table 2. β-glucuronidase activity and NPT-II levels in transgenic Egyptian cotton calli.

Variety	Transgenic Callus #	Intensity of blue colour after staining with X-GLUC	NPT-II pg/mL of crude cellular extract
	B2 (control)	-	99
	F2	+++	565.79
Giza 85	G2	+++	540.68
	D3	++	301.01
	E3	++	312.55
	F3	++	380.09
Giza 77	G3	++	375.57
	E6	+++	550.21
	F4	++	308.8
	G4	++	302.44
	B7	+	255.64
Giza 45	C7	+	259.74
	E8	+++	567.89
	D6	+	177.01

- (negative), + (low), ++ (high), +++ (very high)

Figure 1. Embryogenesis stages of *G. barbadense* produced on maintenance media containing 25 mg/L kanamycin. (A) friable, cream-colored, granular calli developed 5 weeks after transfer to hormone-free medium; (B-D) early stages of embryogenic calli developed 7-9 weeks.



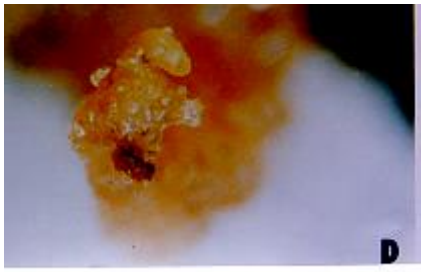


Figure 2. Histochemical staining assay of β-Glucuronidase (GUS) activity in transformed callus tissue. (A, B) GUS positive transformed callus regions regenerated from hypocotyl explant; (C, D) callus tissues showing high level of GUS expression.

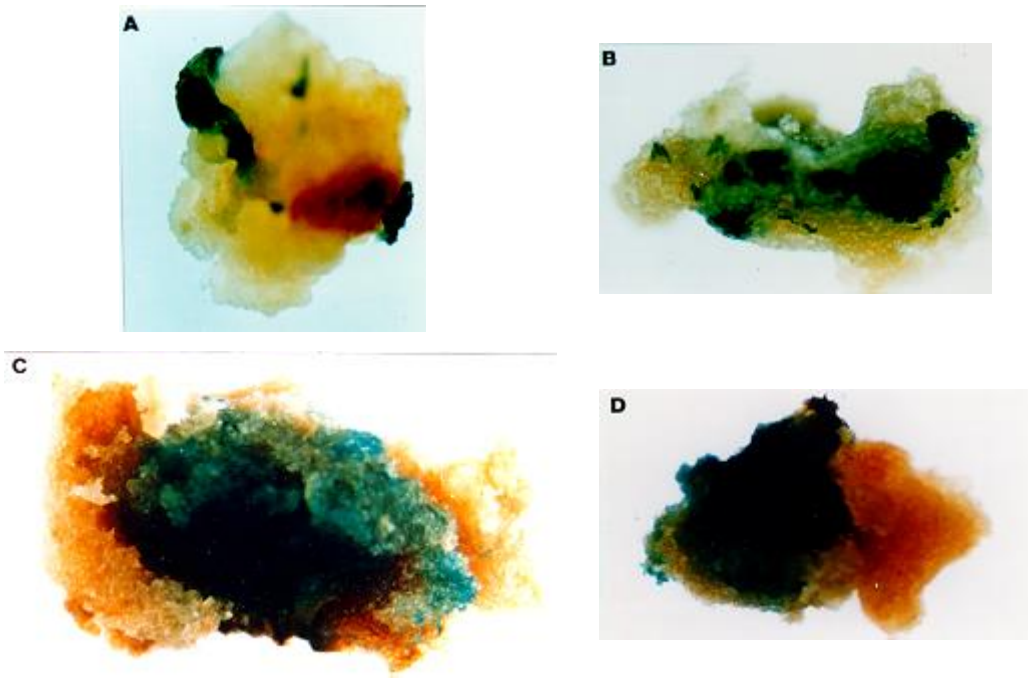


Figure 3. Dot blot assay for GUS gene integration in transgenic callus tissues from three Egyptian cotton varieties, Giza 45, Giza 77 and Giza 85. Dots A to M represent transgenic calli; P represents the positive control which was bacterial plasmid pBI121 extract. The negative control N was untransformed callus.