Induction of Multiple Shoot Differentiation and Increasing Transformation Frequency in Shoot Tips of Egyptian Cotton (*Gossypium barbadense*)

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ABSTRACT

Multiple shoot differentiation and regeneration from cotton (Gossypium barbadense L.) shoot tips of aseptically-grown seedlings was developed on Murashige and Skoog media supplemented with 100 mg/L myo-inositol, 1.0 mg/L thiamin-HCL, 2% glucose, 1X Gamborg vitamin, 100 mg/L citric acid, 100 mg/L ascorbic acid, 0.1 mg/L kinetin, various levels of BA and TDZ, and 0.2% phytagel. Multiple shoot differentiation and regeneration was genotype independent when tested on four Egyptian cottons, Giza 45 and Giza 87 as extra long staple varieties, and Giza 85 and Giza 89 as long staple varieties. Plant regeneration required a specific concentration of BA and TDZ depending on the origin of the meristems. The frequency of explants forming multiple shoots ranged from 40 % to 65 % among the genotypes and the number of shoots ranged from 2 to 15 per responding explant. More than 80% of the shoots could be rooted. The frequency of genotype-independent transformation of Egyptian cotton varieties by Agrobacterium was increased by microprojectile wounding of the apical meristem. Bombardment was with tungsten (0.5 μm) particles and four rupture disk pressures, 650 psi, 900 psi, 1300 psi and 2000 psi. Increasing rupture disk pressure produced a higher transformation frequency by A. tumefaciens but decreased the percentage of recovered plantlets. Regenerated plants were phenotypically normal and all of the mature plants initiated flowers and set variable R1 seeds.

Introduction

Egyptian cotton (*Gossypium barbadense* L.) plays a major strategic role in the cultivation of newly reclaimed areas in Egypt as an oil and fiber crop. It is an attractive crop for genetic engineering because of its worldwide importance and its unique fiber characteristics that dominate the other cotton species. Cotton has been transformed using *Agrobacterium* (Horsch *et al.*, 1985; Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987) and particle bombardment (Finer and McMullen, 1990). Stabilized shoot culture can provide plant material for rapid clonal propagation of elite germplasm (Lloyd and McCown, 1980). In addition, stabilized shoot cultures have been valuable for *Agrobacterium*-mediated transformation (McCown, 1987). Transformation by engineered *Agrobacterium* strains can be conveniently performed in some systems using wounded materials. Phenolic plant metabolites released from damaged cells are required for the activation of the virulence functions within the Ti plasmid of *Agrobacterium* cells. A recent development in transformation technology has been the advent of DNA delivery into cells by particle bombardment. Ideally, particles should enter the cells in a manner that will preserve DNA integrity. In practice, the high-velocity impact of dense particles on plant tissues will generate a large array of macro- and microwounds. Excessive wounding of the target tissue is detrimental to particle plasmid methods of stable transformation. However, such wounded tissue may provide numerous sites over the surface area to induce *Agrobacterium* aggregation, attachment and plant cell transformation. In this work we attempt to produce multiple shoots from shoot tips of aseptically-grown seedlings and wound apical meristems by microprojectile bombardment prior to *A. tumefaciens*-mediated transformation in order to enhance the transformation frequency and increase the number of transformed plantlets.

Materials and Methods

Plant material. Acid delinted seeds of *G. barbadense* (Giza 45, Giza 87 as extra long stable varieties; Giza 89 and Giza 85 as long stable varieties) were washed twice with sterile distilled water, rinsed with 40% commercial bleach containing two drops of detergent (soap) 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 of 90 μE m^{-2} s^{-1}.

Shoot multiplication and transformation. Shoot tip explants were isolated from 5-day-old seedlings. The seedling shoot tip was exposed by pushing down on one cotyledon until it broke away, exposing the seedling shoot apex. The unexpanded and primordial leaves were left

Proceedings of the World Cotton Research Conference-2. Athens, Greece, September 6-12, 1998. pp.320-325.

in place to supply hormones and other growth factors (Smith and Murashige, 1970). The germinated shoot apices consisted of the meristematic dome and more than two primordial leaves. The disarmed *A. tumefaciens* strain LBA4404, in conjunction with a binary vector plasmid pBI121, was used for transformation of shoot tips (Hoekema *et al.*, 1983; Jefferson *et al.*, 1987).

The transformed shoot tips were cultured on MS (Murashige and Skoog, 1962) media containing 100 mg/L *myo*-inositol, 1.0 mg/L thiamin-HCL, 2% glucose, 1X Gamborg vitamin (Sigma), 100 mg/L citric acid, 100 mg/L ascorbic acid, 0.1 mg/L kinetin, and 0.2% phytagel. The pH of the medium was adjusted to 5.8. Combinations of Benzyladenine (BA) and N-phenyl-N'-1, 2, 3-thiadiazol-5-urea (thidiazuron or TDZ) (Reddy *et al.*, 1994; Gary and Stephen, 1990; Michael *et al.*, 1991; Yu and Barbara, 1993; Heng *et al.*, 1992; Austin *et al.*, 1991) were used for multiple shoot induction. The medium was supplied with TDZ (0 mg/L, 10 mg/L, 20 mg/L and 50 mg/L) in combination with BA (0 mg/L, 2 mg/L, 5 mg/L and 10 mg/L). Hormones were added after sterilization of the media, and the shoot tip cultures were incubated at 28^{O}C with a 16-h photoperiod 90 μE m^{-2} s^{-1} and subcultured weekly.

Rooting of shoots. Shoots produced per responding shoot tip explant were rooted on half-strength MS (Murashige and Skoog, 1962) medium containing 0.3% activated charcoal. Two to three weeks later, at least two roots were formed. Shoots were transferred to MS medium supplemented with 100 mg/L IAA to favor extensive root formation.

Agrobacterium *transformation of shoot tip.* Prior to *Agrobacterium* transformation, isolated shoot tip explants were wounded by bombardment with a particle gun (Biorad PDS 1000/He biolistic system). Controls consisted of explants not bombarded before *Agrobacterium* inoculation. Bombardment was with tungsten particles (0.5 μm) and four rupture disk pressures (450 psi, 650 psi, 1300 psi and 2000 psi). In each treatment 15-20 shoot tips were arranged around the perimeter of a 1 cm circle in a 60 mm X 20 mm Petri plate. Microprojectile preparation was according to manufacturer's instructions. Bombardment was at a fixed distance of six cm from the macrocarrier disk. Mechanical wounding of shoot tips was used as a control for the wounding process. *Agrobacterium* cultures were incubated overnight at 28^{o}C in 2XYT minimal liquid medium containing 16 g/L bacto-trypton, 5 g/L NaCl, 25 μg/mL Streptomycin, 50 μg/mL rifampicin and 50 μg/mL kanamycin (all from Sigma). For shoot tip transformation, bacteria were centrifuged and resuspended to a concentration of ≈10_8 cells/mL in liquid shoot induction medium as described, except phytagel was omitted and pH was 5.9. Bombarded and non-bombarded shoot tip explants were dipped in an *A. tumefaciens* suspension in Petri dishes and gently shaken for a few seconds to ensure full contact with the bacterial cultures. The explants were then blotted and dried with sterile filter paper (Whatman No. 1) to remove excess bacteria, and cultured on shoot induction medium solidified with 0.2% phytagel. After three days cocultivation, explants were transferred to the same medium supplemented with 500 mg/L carbenicillin to control bacterial growth. After 2-3 weeks incubation, the recovered plantlets were recorded and the transformation frequency was determined for each rupture disk pressure used.

Detection of marker genes in regenerated plantlets. The presence of the NPT-II protein in transformed plantlets was detected by a sandwich immunoassay ELISA using an NPT-II ELISA Kit (5'-3', Inc., Boulder CO, USA). Transformed cell extract, non-transformed cell extract as negative control, and pure NPT II protein concentrate as positive control, were added to the wells. The NPT-II levels were expressed and determined according to kit instructions.

Genomic DNA was isolated from transformed and non-transformed tissue using a modification of Paterson *et al.* (1993). Two to three μg of undigested DNA from each sample were transferred as dots to a Boehringer Mannheim nylon membrane. The membrane was hybridized using DIG DNA Labelling and Detection Kit (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, hybridised membrane was subjected to immunological detection as described in the kit manual. The hybridization probe was prepared from the plasmid pBI221 (Clontech) which contains coding sequences of the GUS gene. The probe was labelled as described in the kit manual.

Results and Discussion

Shoot tip transformation and multiplication. Shoot meristem culture was first described by Morel (1972) for clonal propagation and virus eradication. Theoretically, tissues of the apical meristem are best suited for plant propagation and 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 the present study, isolated shoot tips, containing a meristematic dome, leaf primordia and unexpanded leaves, were multiplied on media containing two plant hormones (BA and TDZ) at different concentrations. For direct shoot multiplication from single shoot tip explants of Egyptian cotton, a new method was applied using TDZ as a cytokinin-active urea derivative. Shoots that were considered to be non-responsive to usual methods were

successfully multiplied and regenerated using thidiazuron (Beattie and Garrett, 1995; Logan and Stewart, 1995; Szasz *et al.,* 1995). Adenine and phenylurea-type cytokinins such as TDZ have a common binding site in the plant cell that influences endogenous levels of both auxin and cytokinins (Murthy *et al.,* 1995). Egyptian cotton shoot proliferation from shoot tip explants which were transferred from medium containing BA to media containing combinations of BA and/or TDZ increased with increasing concentrations of TDZ in the first subculture, whereas shoot formation in the second and third subculture depended on the BA concentration. When shoot tips were transferred from media with BA to media with TDZ, the time for multiple shoot formation, as well as shoot size, indicated that the combined effect of BA and TDZ is expressed only during the early phase of the subculture. The frequency of multiple shoots ranged from 40 % to 65 % among the four genotypes on media containing combinations of BA and/or TDZ (Figure 1).

Efficiency of shoot multiplication in seedling shoot tip cultures of four Egyptian cotton varieties in response to combinations of TDZ and BA is given in Table 1. Increasing the concentration of TDZ and BA increased the percent of multiple shoot production. The number of secondary shoots ranged from 2 to 15 per responding shoot tip explant (Figure 2a,b). Multiple shoot production was genotype independent.

Rooting and plant recovery. Rooting of shoots and plant recovery from the shoot tip explants was direct and simple. More than 80% of the shoots could be rooted spontaneously on half-strength MS (Murashige and Skoog, 1962) media containing 0.3% activated charcoal. After two to three weeks, two roots were formed (Figure 2d). Shoots were transferred to MS media supplemented with 100 mg/L IAA to favour extensive formation of roots. By allowing shoots to form roots spontaneously as the shoots enlarged in culture (at least 1.0 cm), the frequency of rooting was improved, however, the frequency may be cultivar dependent. Cultivars giving the best response to this rooting treatment were Giza 87 and Giza 45. Induction of multiple shoots from transformed shoot tips improved the transformation frequency. However, increasing the number of multiplied shoots resulted in increased deformity in recovered plantlets.

Agrobacterium transformation of shoot tips. The shoot meristem-based method can be applied to plant transformation, either by particle bombardment (Christou, 1989) or *Agrobacterium*-mediated gene transfer. Despite the popular belief that meristematic tissues of plants cannot be used in *Agrobacterium*-mediated transformation, there is no direct evidence to support this view. To date, meristem-based methods have been used successfully in *Agrobacterium*-mediated transformation of petunia (Ulian *et al.,* 1988), pea (Hussey *et al.,* 1989) and sunflower (Schrammeijer *et al.,* 1990). Despite the recalcitrance of the *Gossypium* genus, the use of shoot apical meristem can make a wider range of cotton germplasm accessible to improvement by the currently available transformation methods. This report describes a protocol to increase transformation frequency by wounding of apical meristems by microprojectile bombardment prior to application of *A. tumefaciens*. Transformation frequency differed according to disk rupture pressure and variety (Figure 3). Bombarded and non-bombarded shoot tip explants inoculated with *A. tumefaciens* initiated transformed kanamycin-resistant plantlets after 2-3 weeks. The probability of DNA delivered to meristem cells increased with increasing the disk rupture pressure and led to high transformation frequency. However, the percent of recovered plantlets decreased with increasing rupture disk pressure. Microprojectile bombardment as a mean of inducing a wound response in plant tissue is an effective method for improving transformation frequencies by *A. tumefaciens*. The wounds created by the impact of particles on a shoot tip explant produced sites conducive to *Agrobacterium* infection over a large surface area. It is recommended to use 650 psi rupture disk pressure to obtain a reasonable transformation frequency and a good frequency of recovered plantlets.

Acknowledgment

The author acknowledges the assistance of the GERT staff, Ayman Diab, Mona Sadek, Nahla Sherif, and the trust, support and help of the Ministry of Agriculture in this work, specifically H.E. Prof. Wally, Prof. Mamdouh Sharaf El-Dine, Dr. Ahmed Momtaz and Prof. Saad Nassar.

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Table 1. Relative efficiency of shoot multiplication in seedling shoot-tip cultures of four Egyptian cotton varieties.

TDZ	10	20	50	0	0	0	10	20	50	10	20	50	10	20	50
BA (mg/l)	0	0	0	2	5	10	2	2	2	5	5	5	10	10	10
Giza 45	-	+	++	-	-	+	-	+	+	-	-	+++	-	+	+++
Giza 87	-	+	++	-	-	+	-	+	+	-	-	+++	-	+	+++
Giza 85	-	+	++	-	-	+	-	+	+	-	-	+++	-	+	+++
Giza 89	-	+	++	-	-	+	-	+	+	-	-	+++	-	+	+++

(-) no response
(+) 2-5 shoot per responding explant
(++) 5-10 shoot per responding explant
(+++) 10-15 shoot per responding explant

Figure 1. Effect of different concentrations and combinations of TDZ and BA on multiple shoot induction of *Gossypium barbadense* Giza 45.

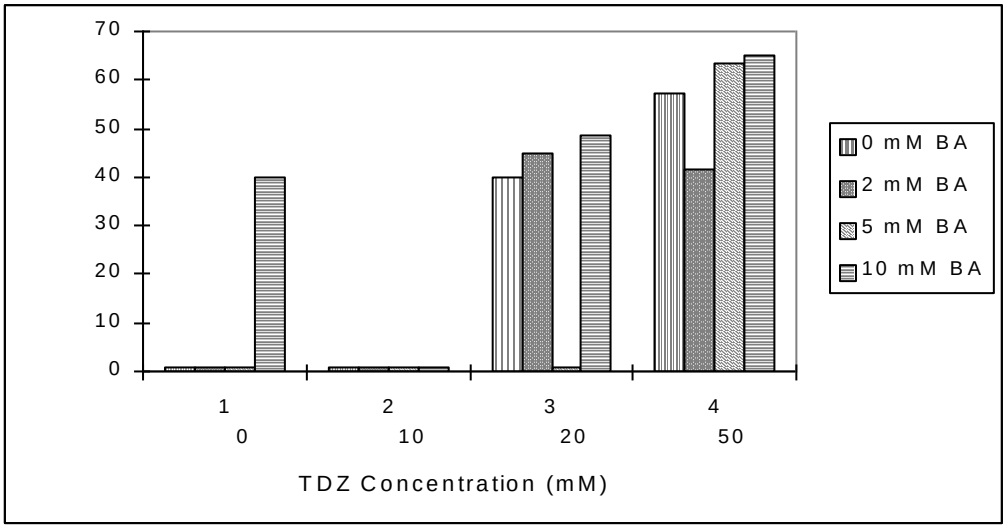

Figure 2. Regeneration and recovery of transformed plantlets produced from multiplied shoots. A, B, C, Multiplied shoots produced from shoot tip explant of *Gossypium barbadense* Giza 45 after 3, 5, and 8 weeks, respectively. D. Root produced from shoot after 2 to 3 weeks.

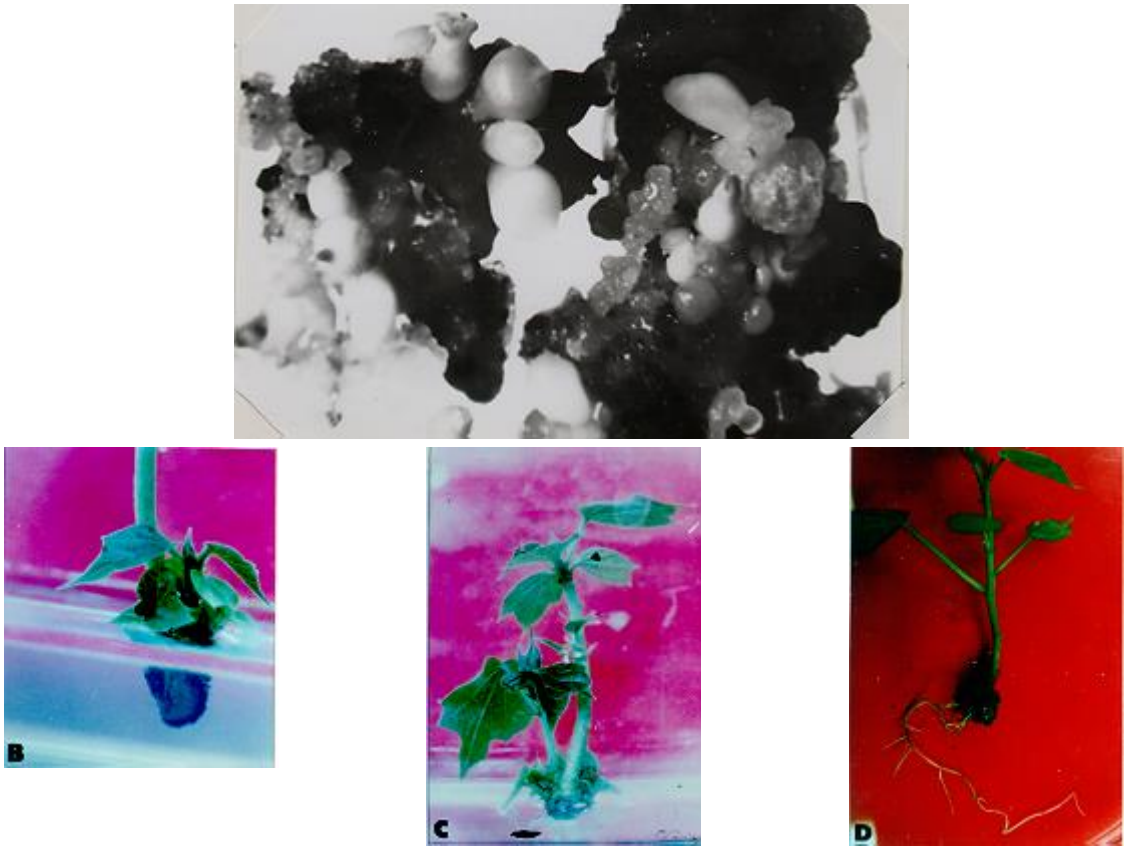

Figure 3. Histogram showing the frequency of *Agrobacterium* transformation according to different rupture disk pressure and varieties used.