Theor Appl Genet (1999) 98: 252–256

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Transformation of a Texas cotton cultivar by using *Agrobacterium* and the shoot apex

Received: 1 June 1998 / Accepted: 28 July 1998

Abstract Transgenic cotton (*Gossypium hirsutum* L.) plants of a Texas cultivar CUBQHRPIS were obtained using *Agrobacterium*-mediated transformation coupled with the use of shoot-apex explants. After inoculation with *A. tumefaciens* strain LBA 4404 containing the pBI121 plasmid, regeneration of primary plants was carried out in a medium containing kanamycin (100 $mg l^{-1}$). Progeny obtained by selfing were germinated in the greenhouse and selected for expression of the T-DNA marker gene encoding neomycin phosphotransferase II (*NPTII*) by painting kanamycin (2%) on the leaves. Plants that survived the leaf painting were analyzed by DNA blots. Evidence for integration of the transgene (*GUS*) was observed in two successive generations from the regenerants (T_0). The transformed plants appeared to have more than one copy of the T-DNA.

Key words *Gossypium hirsutum* · Transformation · Kanamycin

Introduction

Cotton is the most important source of natural fiber. There are four cultivated cotton species, two diploids (*Gossypium arboreum*, *Gossypium herbaceum*, 2n = 26) and two tetraploids (*Gossypium hirsutum*, *Gossypium barbadense*). Cotton has been estimated to contribute $5.5 billion to the USA economy with over 180 million people worldwide depending on the crop for their livelihood. Conventional breeding programs have produced steady improvements in many agronomic traits, but the potential benefits of cotton genetic-engineering have been slow in coming (Bayley et al. 1992; John and Stewart 1992).

Genetic-engineering has enabled the isolation, amplification, and in vitro manipulation of genes. For a number of technical and practical reasons, resistance to herbicides was among the first traits introduced into crop plants (Mazur and Falco 1989). Other characteristics of agronomic importance for cotton production are those related to insect resistance and fiber-quality modification (John and Keller 1996).

Even though more than 30 crop species have been genetically engineered, the technology for most species is inefficient and hampered by cultivar restrictions for in vitro regeneration. Low transformation frequencies and ambiguities of the integration events, such as gene copy numbers and position effects (John and Stewart 1992), influence the levels of gene expression and regulation.

In this report, we describe the application of an *Agrobacterium*-mediated transformation system to a Texas cotton cultivar. The approach combined the use of the shoot-apex method developed in this laboratory and the ability of *Agrobacterium* to infect and deliver T-DNA into these cells (Ulian et al. 1988). Theoretically, the advantage of the shoot-apex explant over other regeneration systems is that plants may be obtained from any genotype rather than from only those that regenerate from callus culture. To-date, meristem-based methods have been used successfully in *Agrobacterium*-mediated transformation of petunia (Ulian et al. 1988), pea (Hussey et al. 1989), sunflower (Bidney et al. 1992; Schrammeijer et al. 1990), corn (Gould et al. 1991), banana (May et al. 1995), tobacco (Zimmerman and Scorza 1996), and rice (Park et al. 1996). Since there have been difficulties in recovering plants from *Agrobacterium*-infected meristems (John and Stewart

Communicated by P. M. A. Tigerstedt

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1992), it was important to establish and improve the cotton transformation system. We show that T_1 and T_2 progeny from transgenic cotton plants were tolerant to high levels of kanamycin applied to leaf tissues. Direct evidence of integrated foreign DNA was provided by Southern hybridization.

Materials and methods

Plant material

Cotton (*G. hirsutum* L.) seeds from the cultivar CUBQHRPIS were kindly provided by Dr. Kamal El-Zik (Cotton Research Program, Texas A&M University). Seeds were surface-sterilized with concentrated sulfuric acid (1 h), 50% Clorox (1 h) in a rotary shaker at 50 rpm and rinsed at least three times with sterile double-distilled water. The seeds were then placed on 0.15% (W/V) gelrite-solidified medium, pH 5.7, containing the Murashige and Skoog (MS) inorganic salts (Murashige and Skoog 1962), and 2% of sucrose in order to germinate. Seeds were incubated at 28°C in the dark for approximately 3–4 days.

Bacterial strain and plasmid

Agrobacterium tumefaciens LBA 4404 (octopine strain), together with the plasmid pBI121, was used in this study (Fig. 1). This plasmid was introduced into the *Agrobacterium* LBA 4404 strain by electroporation. The vector construct contained a *GUS* (*β*-glucuronidase) gene driven by a CaMV 35S promoter, and a *NPTII* (neomycin phosphotransferase) gene driven by a Nos promoter, which confers resistance to kanamycin (Kn). This construct was obtained from Dr. Rod Wing (Clemson University, S.C.).

Shoot-apex isolation, co-cultivation and regeneration

The shoot-apex isolation and plant-regeneration techniques were performed as described by Ulian et al. (1988). The shoot apex was isolated with the aid of a dissecting microscope (see Fig. 2 A) and was then placed in MS inorganic salts (Murashige and Skoog 1962) supplemented with 100 $mg\,l^{-1}$ of myo-inositol, 0.5 $mg\,l^{-1}$ of thiamine·HCl, 0.5 $mg\,l^{-1}$ of nicotinic acid, 0.5 $mg\,l^{-1}$ of pyridoxine·HCl, 3% sucrose, and 0.15% (W/V) gelrite at pH 5.7. A single *Agrobacterium* colony grown on LB solid medium with Kn (50 $mg\,l^{-1}$) was cultured for 48 h, then transferred to LB liquid medium with Kn at 28°C on a rotary shaker (200 rpm) overnight until an OD_{600} of 0.8–0.9 was reached. Equal numbers of shoot apices were randomly distributed to two independent treatments, one with *Agrobacterium* co-cultivation and one with no *Agrobacterium*. Shoot apices were inoculated by placing two drops of 5 µl onto each shoot apex and incubating at 28°C under dark conditions for approximately 48 h. After 2 days of co-cultivation, shoots were transferred to the shoot-apex medium described above adding 250 $mg\,l^{-1}$ of clavamox, to inhibit *Agrobacterium* growth, and Kn (100 $mg\,l^{-1}$). After 3 weeks (see Fig. 2 B), the surviving shoot apices were transferred to fresh medium. The process was repeated until controls, not co-cultivated with *Agrobacterium*, were totally dead, usually around 1.5 months. After this period the shoot apices that survived were transferred to the same medium without kanamycin in order to root the plants (see Fig. 2 C). Rooted plants (T_0) were then transferred to soil and grown to maturity in a greenhouse.

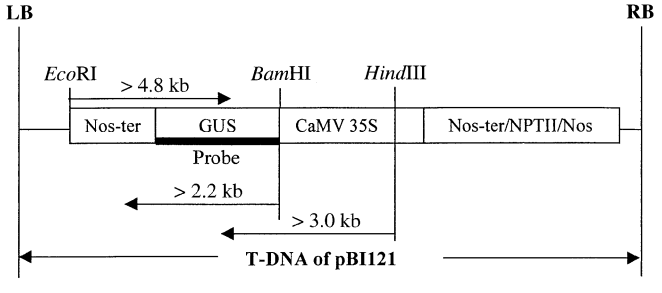

Fig. 1 Schematic representation of plasmid pBI121. pBI121 contains the Nos-NPTII-Nos-ter-CaMV35S-GUS-Nos-ter chimeric expression cassette. Abbreviations: *Nos* nopaline synthase promoter; *NPTII* neomycin phosphotransferase; *Nos-ter* 3′ signal of nopaline synthase; *CaMV35S* 35S promoter; *GUS* *β*-glucuronnidase

Analysis of transgene expression in regenerated plants and progeny

Initially, experiments to determine the kill-curve of isolated non-transformed shoot apices on kanamycin-containing medium were carried out. Survival rates of in vitro shoot apices on Kn were established on MS inorganic salts supplemented with 100 $mg\,l^{-1}$ of myo-inositol, 0.5 $mg\,l^{-1}$ of thiamine·HCl, 0.5 $mg\,l^{-1}$ of nicotinic acid, 0.5 $mg\,l^{-1}$ of pyridoxine·HCl, 3% sucrose, and 0.15% (W/V) gelrite at pH 5.7. Five different concentrations of kanamycin were used (0, 25, 50, 100 and 200 $mg\,l^{-1}$). A total of 50 shoot apices were assigned to each treatment. Cultures were maintained on the culture shelf at room temperature using a photoperiod of 16 h light/8 h dark. Survival rates were recorded 4 weeks after plating (see Table 1), and the lowest level of Kn which killed all shoot apices (100 $mg\,l^{-1}$) was used to select transgenic plants.

Kanamycin leaf-test

In the transgenic plants, expression of the transgene (*NPTII*) was analyzed by first establishing the lowest concentration of Kn that would kill untransformed plants. Leaves of control plants were painted with a cotton swab when they had two totally opened true leaves using 0, 0.1, 1, 2, or 3% (W/V) of Kn. The lowest level (2%) that caused damage to the controls was used to evaluate for resistance to kanamycin in the greenhouse (data not shown). Plants were evaluated for resistance 7 days after leaf application of kanamycin.

Southern-analysis of progeny plants

Cotton genomic DNA was extracted from 1 g of young leaf tissue of progeny (T_1 and T_2) and control plants, in alcohol, according to the extraction protocol developed by Murray and Pitas (1996). Ten micrograms of DNA undigested or digested with two restriction enzymes, *Eco*RI or *Hin*dIII (New England Biolabs, Beverly, Mass.), was loaded onto the gel. The DNA was separated in a 0.9% (w/v) agarose gel by electrophoresis and blotted onto a nylon membrane (Zeta-Probe GT membrane, BIO-RAD, Richmond, Calif.) according to the manufacturer's instruction. DNA was fixed to the membrane by baking at 80°C for 30 min. Hybridization was carried out using DNA fragments labelled with ^{32}P-dCTP using a random-primers DNA labelling system (Gibco BRL, Gaithersburg, Md.).

The probe employed to detect the *GUS* gene was made from a 2.2 kb *Bam*HI and *Eco*RI restriction fragment of plasmid pBI121 isolated from *Escherichia coli*. The membranes were pre-hybridized overnight at 65°C in 7% SDS and 0.25 M Na_2HPO_4 and hybridized overnight at 65°C in the same solution. After overnight hybridization, membranes were washed twice for 30 min each with 20 mM Na_2HPO_4 and 5% SDS at 65°C, then washed twice for 30 min with

20 mM Na_2HPO_4 and 1% SDS at 65°C. Membranes were exposed to X-ray film at −70°C.

Results

Shoot-apex isolation, co-cultivation and regeneration of transgenic plants

Experiments to determine the survival rates of isolated non-transformed shoot apices on the media containing Kn, showed that no untransformed shoot apices survived on 100 mg l^{-1}; therefore, 100 mg l^{-1} of Kn was used for selection during all subsequent experiments (Table 1). Experiments with *Agrobacterium* showed that after 10–14 days of shoot-apex culture on selection medium, leaves from the shoot apices which were co-cultivated with the *Agrobacterium* remained green. No axillary bud outgrowths were formed.

Out of a total of 1010 *Agrobacterium*-treated shoot apices placed on Kn selection, 8 (0.8%) regenerated plants (T_0), grew, and were transferred to the soil, reaching maturity after approximately 4 months (Table 2). In contrast, for the 150 apices not treated with *Agrobacterium*, all died on Kn selection. Rooting of the transformed shoot apices occurred when they were transferred from Kn selection medium to medium with no Kn (Fig. 2 C).

These primary plants were initially tested using the Kn leaf-spotting test on the upper leaves. Since the upper leaves were drastically damaged, compromising the survival of the plants, and since we wanted to check the transmission of the gene into the germline, the bottom leaves were not spotted with Kn, and plants were allowed to mature and set seed for progeny analysis.

Analysis of transgene expression in the progeny

Seeds (T_1) from each of the eight individual primary plants were harvested and tested for kanamycin resistance by evaluating leaf yellowing after Kn leaf spotting (Fig. 3) 1-week later. Kanamycin resistance activity in the leaves was variable. Not all the progeny plants had the same level of damage compared to the control (Table 3). Plants that were resistant to Kn were tested by Southern-blot analysis.

DNA-blot analysis of progeny plants

Some of the plants surviving the leaf test for Kn resistance were tested for the presence of the *GUS* gene by DNA-blot analysis. Table 3 shows that only three primary plants produced progeny, T1, that were Southern-positive. The results of plant T1 that was resistant to kanamycin are shown in Fig. 4. Hybridization of undigested DNA indicated that the introduced T-DNA was integrated into high-molecular-weight DNA. Digestion of genomic DNA with *Eco*RI produced three hybridizing bands that were different in mobility from that of the digestion with *Hin*dIII, which again indicates genomic integration of the T-DNA. Furthermore, since the T-DNA of pBI121 has single *Eco*RI and *Hin*dIII sites, the presence of these hybridizing fragments suggests three independent insertions of the transforming T-DNA into the genome of this plant.

Seeds (T2) from a T1 plant were grown, and a DNA-blot analysis of some of the progeny was carried out. All these plants had the same hybridization pattern of DNA as in the T1 plant (Fig. 4). The fact that kanamycin-resistant plants could be recovered, and that most of the plants tested from resistant plants were *GUS* positive in the DNA blot, strongly suggested the stable integration of the *NPTII* gene and the *GUS* gene.

Table 1 The survival rate of cotton shoot apices on selection medium containing kanamycin

Kanamycin (mg l^{-1})	# Shoot apices	# Surviving shoots	Survival frequency (%)
Control (−) Kn	50	30	60
25 Kn	50	30	60
50 Kn	50	15	30
100 Kn	50	0	0
200 Kn	50	0	0

Table 2 Survival of shoot apices after co-cultivation with *Agrobacterium* LBA 4404 and selection on kanamycin

Item	LBA 4404	# Shoot apices	# Surviving selection	% Established in soil
Co-cultivation	+	1010	8	0.8
Control, 100 Kn	−	150	0	

Discussion

Although cotton has been transformed by *Agrobacterium*-mediated systems, and plants have been subsequently regenerated (Firoozabady et al. 1987; Umbeck et al., 1987, 1989; Perlak et al. 1990; Bayley et al. 1992), commercially important cultivars have proven very difficult to regenerate due to the inability to generate embryogenic cell lines. Consequently, *Agrobacterium*-mediated transformation of cotton has been limited to those specific cultivars that can be regenerated in tissue culture (Chlan et al. 1995). To circumvent difficulties with cultivar-dependent regeneration, a few investigators have reported successful transformation of cotton embryogenic tissue-cultures

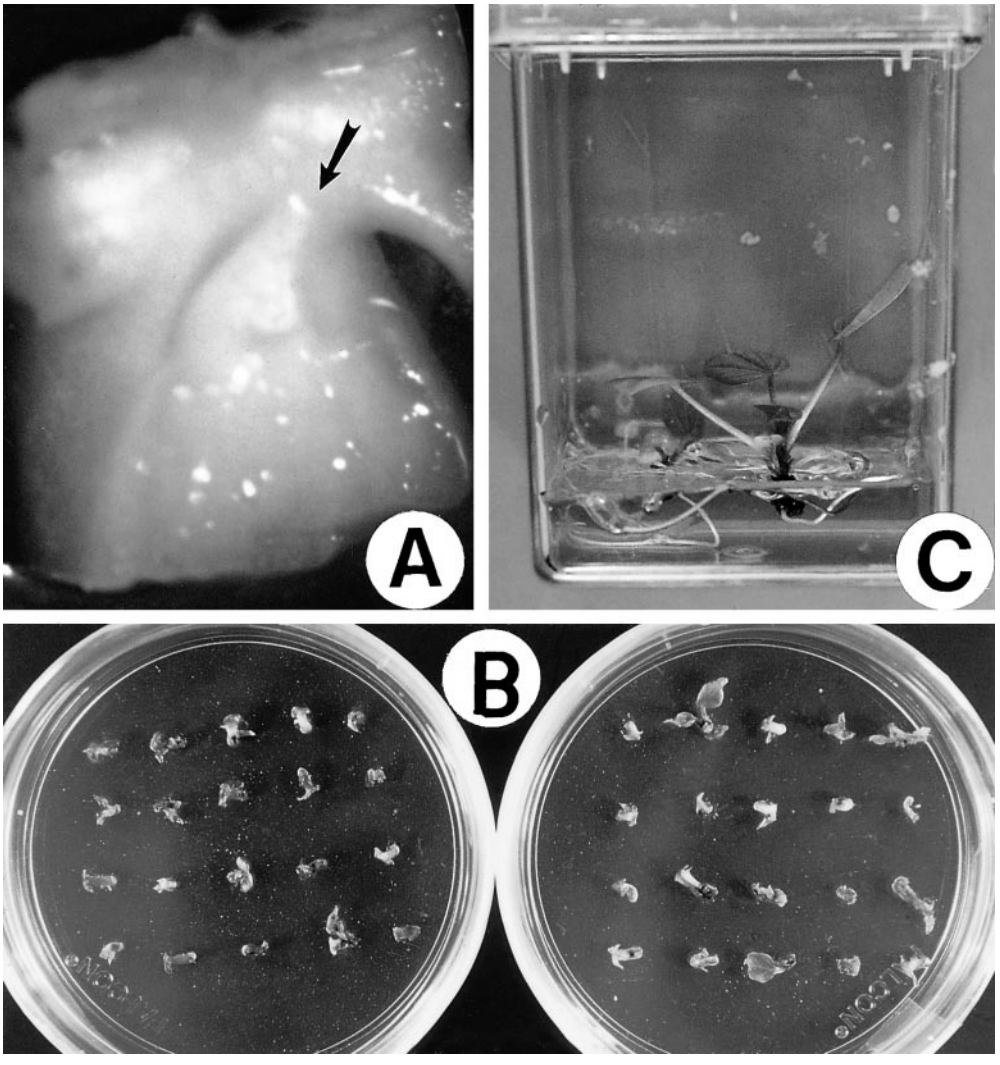

Fig. 2A–C Isolation and regeneration of the cotton shoot apex. **A** The cotton shoot apex explant. **B** Shoot apices after 3 weeks on selection medium with kanamycin (100 mg l^{-1}) showing several surviving apices with expanded leaves. **C** Cotton plant after rooting in medium without kanamycin

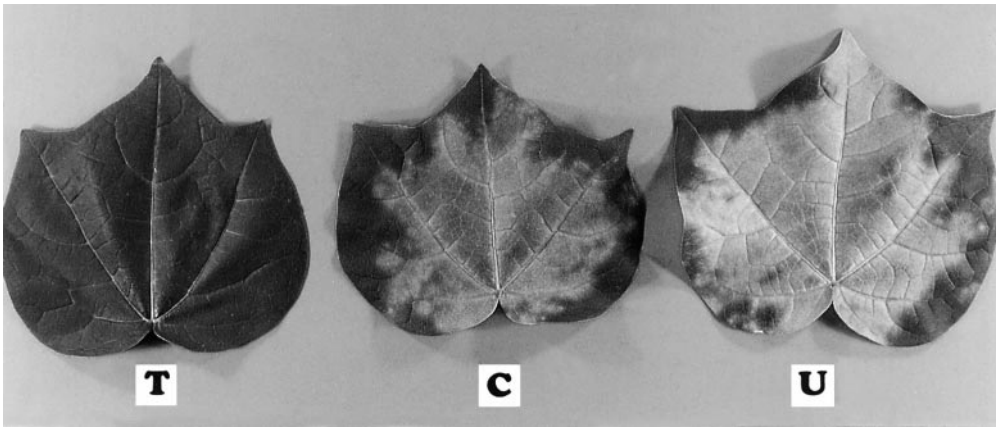

Fig. 3 Kanamycin leaf test. Progeny (T1) plants were screened in the greenhouse by the application of a 2% solution of kanamycin. *C* = control; *U* = susceptible; *T* = resistant

using the biolistic approach (Finer and McMullen 1990; McCabe and Martinell 1993). However, regeneration from suspension cultures is successful only in genotypes that regenerate well from callus tissue (John and Stewart 1992).

In conclusion, we have developed a method for the recovery of genetically transformed cotton plants via *A. tumefaciens*. The use of the shoot apex as a target tissue circumvents the problem of genotype-independent regeneration of cotton (Gould et al. 1991). Plants

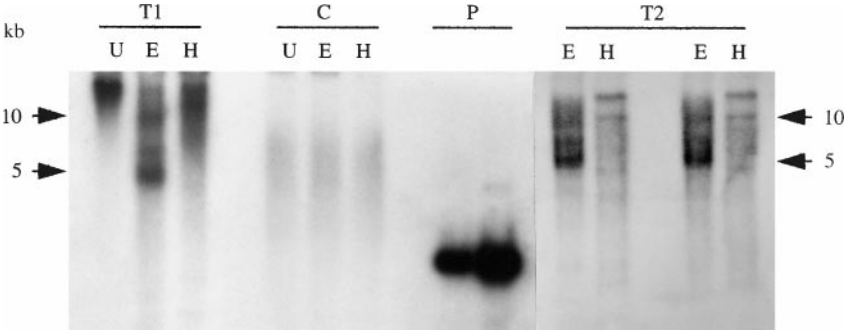

Fig. 4 Southern-blot analysis of cotton genomic DNA from one transgenic progeny plant (*T1*) and two plants, *T2*, originated from *T1*. Hybridization analysis from uncut (*U*), *Eco*RI (*E*)-and *Hin*dIII (*H*)-digested genomic DNA hybridized to a ^{32}P-labeled probe made from the 2.2-kb fragment containing the GUS gene from pBI121. *C* = Non-transgenic cotton. *P* = positive control

Table 3 Number of progeny plants (T1) resistant to kanamycin painted on leaf tissue

Primary plants	# Progeny (T1)	# Surviving leaf bioassay	Southern-positive
1	263	6	0
2	281	3	0
3	209	3	0
4	180	3	0
5	161	16	1
6	370	2	0
7	162	5	1
8	330	9	2
Control	252	0	0
Total	2208	47	4

can be obtained in a period of 3 months. Other cotton cultivars are being tested in the laboratory, and all regenerate using the shoot apex. Although the efficiency of transformation and regeneration procedures can most likely be improved, we are convinced that this method can be applied to transform cotton with genes that have economical importance.

Acknowledgments This work was supported by funds from the Eugene Butler Chair (R.H.S.). The experiments comply with the current laws of the United States in regard to work on transgenic plants.

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