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Transformation of Iranian Cotton Varieties Using Shoot Apex

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ABSTRACT

The optimization of gene transfer into Iranian cotton commercial varieties by shoot apices is presented. Shoot apices of two Iranian varieties, 'Sahel' and 'Varamin' was used as explants for *Agrobacterium*-mediated transformation. Three *Agrobacterium* strains, harboring the plasmid vector pBI121 containing the β-glucuronidase (*gus*) gene, were used under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The neomycin phosphotransferase (*nptII*) gene was used as the selectable marker. Inoculated shoot apices were placed onto cotton co-cultivation medium. Shoot regeneration was achieved within 3-4 weeks on MS basal medium supplemented with modified B_5 vitamins. Transformed shoot apices were selected on selective medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime. Putative transgenic shoot apices were subsequently regenerated on half-strength agar-solidified MS basal medium supplemented with 0.1 mg l^{-1} indole-3-butyric acid (IBA) and modified B_5 vitamins. The presence of *gus* and *nptII* genes in the transgenic plants was verified by histochemical GUS assay and PCR analysis, respectively. The transformation frequency of 'Sahel' and 'Varamin' using LBA4404 strain was 3.7 and 5.5%, respectively. The χ^2 test of T_1 transgenic cotton plants in greenhouse conditions indicated that the inheritance of the *gus* gene followed a Mendelian ratio for a single gene (3:1). Our investigations on T_1 lines confirmed the stability of the *gus* gene and its expression.

Keywords: *Agrobacterium tumefaciens*, *gus* gene, *nptII* gene

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is the world's most important source of natural fiber with approximate annual plantation of 35 million ha worldwide (Wilkins *et al*. 2000). It is a crop of significant value throughout the world because it is not only a source of natural fiber, but is also considered as an oilseed crop. Because of its high economic importance, considerable attention has been paid to improving cotton plants by conventional plant breeding methods (Satyavathi *et al.* 2002). Although significant progress has been made in cotton breeding programs, traditional breeding techniques have several limitations. A limited gene pool, crossing barriers, inefficient selection and lengthy procedure are among these limitations. Due to the recent advances in plant transformation technology it is now possible to deliver and express various genes in many agriculturally important species, including cotton. Cotton has attracted much interest in the field of gene transfer with the aim of introducing agronomically interesting new traits (Wilkins *et al*. 2000). Development of an efficient and reproducible transformation technology provides a valuable method for introduction of agronomical useful genes into cotton. It also helps in the study of gene function and regulation. Although transformation rates have been significantly improved since the first report of success in the transformation of cotton (Firoozabady *et al.* 1987; Umbeck *et al.* 1987), fine tuning of the available protocols for desired varieties is still needed. Transformation efficiency is influenced by several factors, including the plant cultivar, kind of explant, *Agrobacterium* strain, inclusion of phenolic compounds (e.g. acetosyringone) in the co-cultivation medium, wounding treatment of the target tissue, plant growth regulators, appropriate selection of transformed cells and light and temperature (Sunilkumar and Rathore 2001; Zambre *et al*. 2003; Olhoft *et al*. 2003; Tzfira *et al*. 2006; Karami 2008). Tissue culture is a major prerequisite for the production of transgenic plants. In the published protocols of *Agrobacterium*-mediated transformation of cotton, hypocotyls and embryonic suspension culture cells have been used as explants (Rajasekaran *et al.* 1996; Tohidfar *et al.* 2005, 2008). The limitations of these explant types are their low regeneration rate and genotype-dependence limiting application to a selected group of cultivated varieties. Cotton crop has been difficult to manipulate with high efficiency since the tissue culture method used for regeneration was by indirect transformation via callus. As only 'Coker' varieties were found to respond better to gene transfer, most of the desirable genes are introduced initially into 'Coker' and back crossed into other varieties later. Several generations of backcrossing and selection are required to identify lines suitable for commercialization (Satyavathi *et al.* 2002). Aside from genotype limitation, many plants regenerated from cotton callus have exhibited extensive phenotypic abnormalities, cytogenetic changes and somaclonal variation (Bao *et al*. 2001; Labra *et al*. 2001; Nakano *et al*. 2005). Callus-induced genetic damage is observed commonly among regenerated plants. So development of tissue culture protocols to induced efficient proliferation in a genotype-independent manner is desirable for genetic transformation of cotton. Shoot tips are capable of regenerating cells of the shoot apical meristem which could serve as targets for genetic transformation (Dutt *et al.* 2007). Compared with somatic cell culture, shoot apex culture is an easier method to obtain regenerative mature plants rapidly unlike other transformation protocols developed with calli and protoplasts culture which involve several rounds of subculture, lot of chemicals, money and man power (Zhang *et al.* 2000; Arockiasamy and Ignacimuthu 2007). Theoretically, the advantage of shoot apex explants over other regeneration systems is that plants are obtained from any genotype (Zapata *et al.* 1999a, 1999b). Shoot meristem and apex cultures became popular in the ornamental nursery industry after the discovery that rapidly growing shoots of many virus infected clones could be free

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of virus and used to produce virus-free germplasm (Gould *et al.* 1998). Over time it was observed that the incidence of genetic mutations and somaclonal variation was low in plants regenerated from shoot apices. One of the reasons for this low mutation frequency may be the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures (Hirochika 1993). With the development of a shoot apex-based cotton regeneration system, it has been possible to improve the transformation rate. To date, the meristem-based transformation method has been used successfully in *Agrobacterium*-mediated transformation of grapevine (Dutt *et al.* 2007), chickpea (Singh *et al*. 2009), jujube (Gu *et al*. 2008), squash (Shah *et al*. 2008) cotton (Zapata *et al.* 1999a; Majeed *et al*. 2000; Satyavathi *et al.* 2002; Jiang *et al*. 2004; Lv *et al.* 2004) and rice (Arockiasamy and Ignacimuthu 2007). Cotton is an important fiber crop in Iran and is cultivated on 150,000-200,000 ha.

This study will present the optimization of shoot apex based *Agrobacterium*-mediated cotton transformation. In order to transfer the genes from *Bacillus thuringiensis* into Iranian cotton varieties, the present study describes the development of an *Agrobacterium*-mediated cotton transformation system using shoot apices as explants.

MATERIALS AND METHODS

Plant materials

Seeds of two Iranian commercial varieties ('Sahel' and 'Varamin') were obtained from the Cotton Research Institute of Iran. For seed sterilization, seeds were placed in 15% (w/v) $HgCl_2$ solution for 2 min and washed subsequently at least tree times with sterile water. The seeds were placed in 90% ethanol and shaken for 10 s, then washed three times with sterile double-distilled water. Three seeds per test tube were placed on 10 ml of MS (Murashige and Skoog 1962) basal medium consisting of 30 gl^{-1} sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Seeds were maintained in the dark at room temperature (25 ± 2°C) for 3-4 days.

Isolation and preparation of shoot apices

Shoot apices were isolated from 3-5 day old seedlings with the aid of a dissecting microscope as described by Zapata *et al.* (1999a). One cotyledon was removed by pushing down on it until it snapped off to expose the shoot apex, then another cotyledon was removed and shoot apices were excised from hypocotyls by cutting at the base of the apex. The unexpanded and primordial leaves were left in place to supply hormones and other growth factors (Shabde and Murashige 1977; Smith and Murashige 1982) (**Fig. 1A-C**). Shoot apices of both cultivars were pre-cultured in Petri dishes for 2 days on hormone-free MS medium (MS0)(Duchefa) supplemented with modified B_5 vitamins (100 mg l^{-1} *myo*-inositol, 0.5 mg l^{-1} thiamin-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl) (Duchefa), 3% sucrose and 2 gl^{-1} phytagel (Sigma-Aldrich, St. Louis, USA) prior to co-cultivation with bacterial culture. Seeds and all *in vitro* plant materials were incubated in the dark at room temperature (25 ± 2°C) according to Zapata *et al.* (1999a).

Fig. 1 Shoot apex isolation scheme for cotton (*Gossypium hirsutum* L.). Cotyledon removing by pushing down until it snaps off to expose shoot apex (**A**), removing the second cotyledon (**B**), cutting shoot apex and removing from seedling (**C**).

Agrobacterium strains and plasmids

Three *Agrobacterium* strains (LBA4404, C58 and EHA101) harboring the binary plasmid pBI121 (Clontech, Washington, DC) were used as the vector system for transformation. These strains were obtained kindly by Dr. Malboobi from the National Center for Genetic Engineering and Biotechnology of Iran. The strains were cultured on LB-agar containing rifampicin (Rif) as selectable agent (75 mg l^{-1}) at 28°C. This plasmid contained the β-glucuronidase (GUS) gene under control of the cauliflower mosaic virus (CaMV35S) promoter as the reporter gene and nopaline synthase (NOS) terminator sequences. The neomycin phosphotransferase (*nptII*) gene under the control of the NOS promoter and terminator sequences was used as the selectable marker (**Fig. 2**).

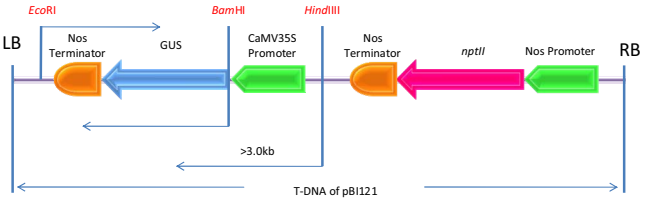

Fig. 2 Chimeric gene map of binary vector pBI121 carrying the *gus* gene and *nptII* gene driven by CaMV35S promoter (P35S). LB, Left border; RB, Right border; *nptII*, neomycin phosphotransferase; *gus*, β-glucuronidase; Pnos, nopalin synthase promoter; Tnos, nopalin synthase terminator.

Agrobacterium co-cultivation and transgenic plants regeneration

Bacteria were maintained on agar-solidified LB medium (containing 10 gl^{-1} Bacto Tryptone, 5 gl^{-1} yeast extract and 10 gl^{-1} NaCl) containing 50 mg l^{-1} kanamycin (Kan) and 25 mg l^{-1} rifampicin (Rif) (Sigma-Aldrich). For inoculation one single colony was grown overnight on 5 ml of liquid LB medium at 28°C with appropriate antibiotics (50 mg l^{-1} Kan and 100 mg l^{-1} Rif), and incubated in a 100 ml Erlenmeyer flask overnight on a shaker set for 180 to 200 rpm at 28°C (Sambrook and Russel 2001). Then 1 ml of the overnight culture was withdrawn and used to inoculate 25 ml of LB medium without antibiotics. Acetosyringone (AS) (Sigma-Aldrich) was added to the culture at a final concentration of 100 µM. After incubation for 3 to 4 hrs at 28°C with shaking at 200 rpm, those cultures were diluted with additional LB medium (containing 100 µM AS) to a concentration (OD_{600} = 0.6) for transformation. The shoot tip explants taken from 3–5 day old seedlings were pre-cultured for 2 days on hormone-free MS medium with modified B5 vitamins (MS0) in the dark at room temperature (25 ± 2°C) prior to infection and co-cultivation with bacterial culture. A number of shoot apices were randomly distributed into two independent treatments, one with *Agrobacterium* co-cultivation and one without. Shoot apices were inoculated by placing two drops of *Agrobacterium* solution onto each shoot apex in co-culture medium (MS0) and incubating at 28°C in the dark for 2 to 3 days (Zapata *et al*. 1999a). After co-cultivation, explants were cultured on selective medium consisting of MS0 with 200 mg l^{-1} cefatoxime (Sigma-Aldrich) and 50 mg l^{-1} Kan, the latter to inhibit *Agrobacterium* growth. The Petri dishes were incubated at 28°C under an 18-hr photoperiod using fluorescent lamps with 90 µmol/s/m^2 intensity and sub-cultured every 3 weeks. The process was repeated until controls, not co-cultivated with *Agrobacterium*, completely died. Shoot apices not inoculated with *Agrobacterium* were plated onto selection medium as a negative control. The green, healthy shoots were subjected to two to three more passages of selection by repeated excision of growing shoots and subcultured onto shoot proliferation medium. Healthy, elongated shoots with two leaves and 2 cm in height were rooted in half-strength MS medium containing 0.1 mg l^{-1} IBA and 50 mg l^{-1} Kan (MS1). After 40 days, plantlets containing 4 leaves, and after rooting, were transferred to soilrite in plastic cups covered with polythene bags (one plant per cup). After 1 week of hardening, the plantlets were transferred to pots containing 1: 1 soil: sand (Zapata *et al*. 1999a).

Table 1 The survival frequency of cotton shoot apices after co-cultivation with 3 *Agrobacterium* strains in selective medium.

Variety	Strain	Sample	No. of explants	No. of explants on selection medium	Transformation (Survival frequency) (%)
Varamin	LBA4404	Co-cultivation	120	36	30
		Control	20	0	-
	EHA101	Co-cultivation	164	8	4.8
		Control	27	0	-
	C58	Co-cultivation	150	11	7
		Control	25	0	-
Sahel	LBA4404	Co-cultivation	144	27	18.7
		Control	24	0	-
	EHA101	Co-cultivation	135	5	3.7
		Control	22	0	-
	C58	Co-cultivation	145	13	8.9
		Control	24	0	-

*Expressed as the percentage of explants that were survival in medium containing Kan (50 mg l^{-1})

β-*Glucuronidase* (GUS) histochemical assay

GUS activity was used to distinguish transformed from untransformed tissues by blue coloration due to the presence of GUS enzyme that converts the substrate 5-bromo 4-chloro-3-indolyl glucuronide (X-gluc)(Cinagen Co., Tehran, Iran) into an insoluble precipitate as described by (Jefferson 1987; Kosugi *et al.* 1990). Young leaves of T_0 (*in vitro*) and T_1 (greenhouse) transgenic plants were also collected for GUS staining to confirm the transformation event. Leaf discs from putative transgenic shoots or rooted plantlets (one leaf from each plantlet) from T_0 and T_1 generations, when with about 4 leaves, were tested for histochemical GUS expression in X-gluc solution containing 25 mg l^{-1} X-gluc, 10 mM EDTA, 100 mM NaH_2PO_4, 0.1% Triton X-100 and 50% methanol, pH 8.0 at 37°C. After overnight incubation, chlorophyll was extracted by soaking the tissues for several hours in 70% EtOH. Then the explants were observed under a Nikon stereomicroscope and photographed. The number of leaf discs that were stained with blue spots was recorded.

Polymerase Chain Reaction (PCR) analysis

PCR amplification of the *nptII* and *gus* genes using specific primers was carried out to check the presence of the transgene in the plant genome. Genomic DNA was isolated from young leaves at 4 leaves age by following the protocol of Li *et al*. (2001). The DNA samples were tested for the presence of the T-DNA region using a pair of *nptII*- and *gus*-specific primers (Cinagen Co.) (*nptII* F: 5′-GAA CAA GAT GGA TTG CAC GC-3′ and *nptII* R: 5′-GAA GAA CTC GTC AAG AAG GC-3′; *gus* F: 5′-GCC ATT TGA AGC CGA TGT CAC GCC-3′ and *gus* R: 5'-CGT TGT ACA GTT CTT TCG GCT TGT-3′) (Zapata *et al*. 1999a) to amplify the 785 bp *nptII* fragments and 800 bp *gus* fragments. The 25 µL reaction mixture consisted of 2.5 µl 10X PCR reaction buffer (50 mM Tris pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 10 mM dNTP mix from Pharmacia Biotech); 60 ng of each primer DNA; 0.5 U of Ampli*Taq* DNA polymerase (Cinagen Co.); and 15 ng of genomic DNA as template. PCR was carried out in a thermal cycler (iCycler, Bio Rad Laboratories) under the following conditions: 94°C for 2 min, then 35 cycles of 94°C for 1 min (denaturation), 55°C (*nptII* gene) and 60°C (*gus* gene) for 1 min (annealing), 72°C for 3 min (extension); 5 min at 72°C (final extension). PCR products were separated on a 1.0% agarose (Cinagen Co.) gel. The gel was detected by ethidium bromide (0.5 µg/ml final concentration) staining and visualized under UV (ultraviolet) light in a gel documentation system (Biometra, Germany).

Analysis of T_1 plants

Transgenic cotton lines (T_0) were successfully self-pollinated using bags and T_1 seeds were produced. PCR analysis and GUS assay were carried out for T_1 progeny as mentioned above for T_0 plantlets.

RESULTS

Transformation and interaction of *Agrobacterium* strains with cotton genotypes

For transformation, hormone-free MS medium (MS_0) containing B_5 vitamins for subsequent shoot proliferation, was routinely used. A concentration of 50 mg l^{-1} Kan was chosen for selection of transformants (data not shown). The same conditions were routinely used for transformation studies in both varieties. In order to increase the transformation efficiency, we evaluated the transformation efficiency of three different *Agrobacterium* strains (LBA4404, C58 and EHA101). For both studied varieties, 'Sahel' and 'Varamin', the efficiency of transformation with strain LBA4404 was more than the two other strains (**Table 1**). A total of 858 shoot tip explants were co-cultivated in the two varieties (**Table 1**). The survival percentage of treated 'Sahel' and 'Varamin' was 10.6 and 12.6%, respectively. In contrast, for the 240 apices not treated with *Agrobacterium*, all died on Kan selection medium (**Table 1**). The frequency of Kan-resistant shoot apices in 'Varamin' and 'Sahel' was 30 and 18.7%, respectively (**Table 1**). Under Kan selection pressure, most of the untransformed shoots appeared to become bleached (**Fig. 3A, 3B**) and some of the shoots that were initially green gradually became bleached. Although the explants remained green, some of the shoots did not grow more than 1 cm. Shoot apices were sub-cultured every three weeks. Shoots measuring 2–4 cm were transferred to rooting medium. Putative transgenic shoots from the selection medium were rooted on MS1 (half strength MS medium containing 0.1 mg l^{-1} IBA and 50 mg l^{-1} Kan). Root initiation was observed in 40-45 days and sufficient roots were produced in 2-3 months and were transferred to soil (**Figs. 4, 5**).

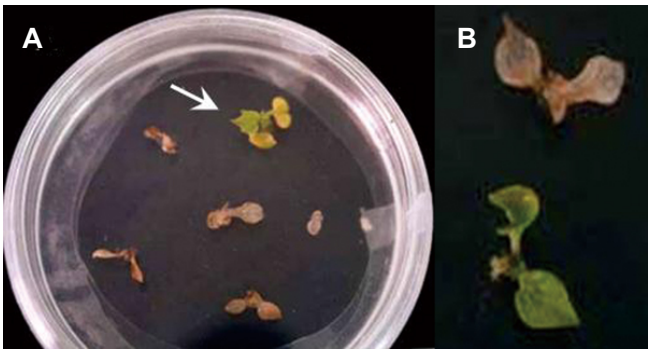

Fig. 3 Putative transgenic plants. Shoot apex regenerated on selective medium (Survival) (**A**) Shoot apex after 3 weeks on selection medium (**B**). Bottom: survival, Top: bleached (control).

Fig. 4 Elongated shoots were induced to form roots on half strength MS medium supplemented with 0.1 mg l^{-1} IBA.

Fig. 5 Regenerated plants in soil.

Confirmation of transformation event

1. GUS histochemical analysis

Stable expression of the *gus* gene was determined by histochemical analysis of leaf samples of mature transgenic plants established in the greenhouse. Histochemical staining revealed that, out of a total of 100 putative transgenic plants, the leaves of 3 (3%) transgenic plants were strongly positive for GUS activity; transformation frequency of plantlets obtained from LBA4404 strain in 'Varamin' and 'Sahel' was 5.5 and 3.7%, respectively (**Table 2; Fig. 6A, 6B**), suggesting that an integrated *gus* gene was expressed at a high level under the control of the 35S promoter of cauliflower mosaic. Leaves from untransformed plant (control) showed no GUS activity in the histochemical analysis (**Fig. 6A, 6B**). Young leaves from the shoots regenerated from the putative transgenic plants gave consistent GUS expression while older leaves stained exclusively along the wounded edge of the leaf blade.

Table 2 β-Glucuronidase expression in leaf discs of cotton (*G. hirsutum* L.) cultivars 'Varamin' and 'Sahel'. The survival of shoots was tested after 3 and 12 weeks of culture

Variety	Strain	Putative transformed plants	No. of GUS-positive explants	Transformation (%)
Varamin	LBA4404	36	2	5.5
	EHA101	8	0	0
	C58	11	0	0
Sahel	LBA4404	27	1	3.7
	EHA101	5	0	0
	C58	13	0	0
Total	-	100	3	3

*Transformation efficiency was determined as the percentage of explants that showing GUS expression on the basis of leaf discs assay

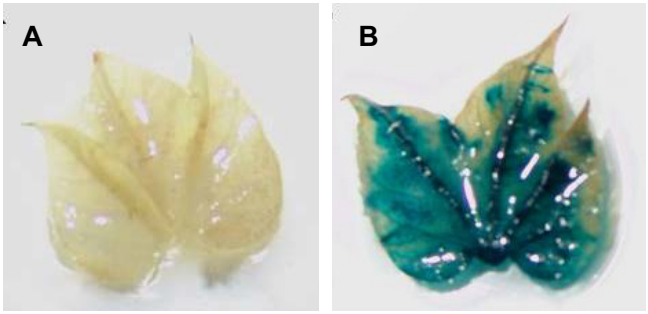

Fig. 6 Histochemical staining of cotton leaf ('Varamin'). Leaf from control plant (not treated with *Agrobacterium* (**A**), Leaf from putative transgenic plant derived from transformation with strain LBA4404 (**B**).

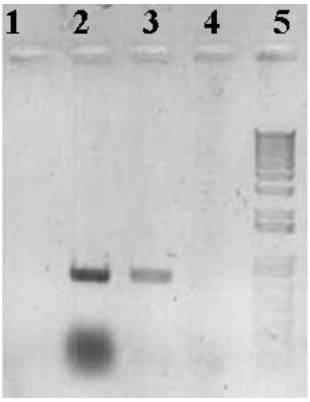

Fig. 7 PCR analysis of DNA isolated from leaves of independent transformants cotton (var. 'Varamin') using specific primer pairs for amplification of 800 bp *gus* gene in agarose gel. Sterile water (Lane 1); DNA from plasmid pBI121 (positive control) (Lane 2); DNA sample from putative transgenic plant (var. 'Varamin') (Lane 3); DNA sample from non-transgenic control plant (Lane 4); 1 Kb plus DNA ladder (Lane 5).

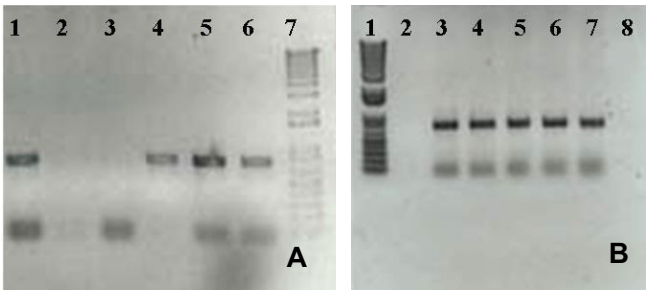

Fig. 8 PCR analysis of DNA isolated from leaves of independent trasformants of the two cotton varieties ('Varamin' and 'Sahel') using specific primer pairs for amplification of 785 bp *nptII* gene in agarose gel. T_o progeny Lanes: DNA from plasmid pBI121 (positive control) (Lane 1); sterile water (Lane 2); DNA sample from non transgenic control plant (Lane 3); DNA sample from putative transgenic plant (var. 'Varamin') (Lanes 4, 5); DNA sample from putative transgenic plant (var. 'Sahel') (Lane 6); 1 Kb plus DNA ladder (Lane 7) (**A**). T_1 progeny selected from the T_0 transformant: 1Kb plus DNA Ladder (Lane 1); DNA sample from nontransgenic control plan (Lane 2); DNA from transgenic lines (Lanes 3, 4 and 6, 7); DNA from plasmid pBI121 (positive control) (Lane 5) (**B**).

2. PCR analysis

PCR analysis was used for those shoots which showed resistance to Kan and GUS activity. Total genomic DNA was extracted from putative transgenic plants, a non-transgenic control plant, and plasmid pBI121 (isolated from *Agrobacterium* strain LBA4404) and used as template for PCR amplification of the *gus* and *nptII* genes, respectively (**Figs. 7, 8a**). Three putative transgenic plants showed positive results after amplification of the predicted 800-bp fragment of the *gus* gene (**Fig. 7**) and the 785-bp fragment of

the *nptII* gene (**Fig. 8a**). Amplification of these fragments was not observed in non-transformed (control) plants (**Fig. 8a**).

Analysis of T_1 plants

The inheritance of the introduced genes in the T_1 generation of both varieties was studied using GUS histochemical analysis. The χ^2 test in T_1 transgenic cotton indicated that the inheritance of the *gus* gene followed a Mendelian ratio for a single gene (3: 1). The results confirmed the stability of the *gus* gene in th second generation. **Fig. 8b** shows PCR analysis of some of the NPTII-positive lines in the T_1 progeny of both varieties selected on medium containing Kan.

DISCUSSION

The transformation efficiency can be increased by manipulating either the explant and/or the transformation method. Various protocols have been explored for the transformation of cotton such as shoot apex transformation (Ganesan *et al*. 2009), particle bombardment (Rajasekaran *et al*. 2000) and *Agrobacterium*-mediated transformation (Tohidfar *et al*. 2008). Of these methods, only the *Agrobacterium*-mediated method is routinely used in cotton transformation studies (e.g. Tohidfar *et al*. 2005, 2008).

Agrobacterium-mediated transformation *via* somatic embryogenesis is labor intensive, involviong work over a 10-12 month period. In this procedure, the transformation efficiencies are generally low due to the low frequency of embryogenesis and the difficulty in germination of transformed embryos (Wilkins *et al.* 2000). Compared with somatic embryogenesis, shoot tip-mediated regeneration is an easy and less time-consuming process (Ganesan *et al.* 2009). In recent years, there has been increasing focus on the use of meristems and shoot apices as the sources of tissue explants for transgenic cotton production. Previously, it was shown that shoot tips are capable of regenerating cells of the shoot apical meristem that could serve as targets for genetic transformation (Dutt *et al*. 2007). Similarly, this has been reported in sugar beet (Yang *et al*. 2005), maize (Zhang *et al*. 2005), and cotton (Majeed *et al*. 2000; Satyavathi *et al*. 2002; Lv *et al*. 2004). The advantages of shoot tip culture over other regeneration systems are many fold. Shoot regeneration from shoot tips is direct, relatively simple and needs less time to regenerate a large number of plants (Nasir *et al.* 1997). Theoretically, the advantage of shoot apex explants over other regeneration systems is that plants may be obtained from any genotype rather than from only those that regenerate from callus culture (Zapata *et al.* 1999a). Another major advantage using shoot apex explants is that they can be rapidly regenerated into mature plants unlike other transformation protocols developed with calli and protoplast culture which involve several rounds of subculture, many chemicals, money and man power (Arockiasamy and Ignacimuthu 2007). It is also reported that transgenic plants derived from calli and protoplasts showed extensive DNA changes, demonstrating the occurrence of somaclonal variation (Bao *et al*. 2001; Labra *et al*. 2001; Nakano *et al*. 2005). However, plants regenerated from shoot apices are true to phenotype with a low incidence of somaclonal variation, mutation and chromosomal abnormalities (Bajaj 1998). Shoot apices have been used in either *Agrobacterium*-mediated transformation or particle bombardment for many plant species (Gould and Magallanes-Cedeno 1998; Zapata *et al.* 1999; Srivatanakul *et al.* 2001; Cho *et al.* 2003; Goldman *et al.* 2004).

The development of an efficient transformation system is an important tool for gene manipulation. Satyavathi *et al.* (2002) reported genetic transformation of two Indian genotypes of cotton using shoot apices from 3 to 5-day-old seedlings. Hence in this research we optimized a shoot apex-based *Agrobacterium*-mediated transformation system for two Iranian varieties 'Sahel' and 'Varamin'.

The use of proper concentration of antibiotics in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or plants to survive. Kan has been extensively used as a selective antibiotic in transformation experiments, mainly because several plant transformation vectors include the NPT II gene as a selectable marker. Among the reports of cotton shoot apex transformation, Katageri *et al*. (2007) co-cultivated shoot apical tissue with *Agrobacterium* and selected shoots on medium containing 100 mg l^{-1} Kan. In our study using 50 mg l^{-1} Kan selection pressure was sufficient in the selection medium, supporting the same findings by Banerjee *et al.* (2002) and Tohidfar *et al*. (2005).

Agrobacterium strains play an important role in the transformation process of cotton as they are responsible not only for infectivity but also for the efficiency of gene transferring. The use of strain LBA4404 yielded a higher degree of transformation compared to other tested strains (EHA101 and C58). Our results support the findings of other reports (Firoozabady *et al*. 1987; Umbeck *et al*. 1987; Zapata *et al*. 1999; Sunilkumar and Rathore 2001; Chen *et al*. 2002; Tohidfar *et al.* 2005) on cotton transformation with LBA4404, but the results of other studies indicated that the super-virulent strains EHA101 and EHA105 were more suitable for stable transformation of cereals (Donaldson and Simmonds 2000) and certain dicots (Rashid *et al*. 1996).

The overall transformation rate was 3% (**Table 2**), which seems comparable with other previous reports on cotton: 0.8% by Smith *et al.* (1997) and Zapata *et al.* (1999), 6.5% by Finer and McMullen (1990), 9.6% by Majeed *et al*. (2000). In our work and other studies transformation efficiency was calculated based on the transgene expression by GUS staining and not on the number of Kan-resistant shoots.

Plants were analyzed for PCR amplification of the *gus* and *nptII* genes. The presence of target bands in samples from transformed plants confirmed the integration of both genes. Amplification of these fragments was not observed in nontransformed control plants. These observations indicated that both genes had been integrated into the genome of the transformed shoots of T_0 and T_1 progeny thereby confirming transformation. Our experiments on PCR analysis proved that chimera formation was significantly reduced and the 3 randomly selected plantlets showed PCR-positive results.

The transgenic plants obtained by the present procedure were phenotypically normal and regenerated within 3-4 months compared to one year or more in an embryogenesis-based transformation system (Tohidfar *et al.* 2005). Therefore, considerable time and resources could be saved by using this method. Optimization of gene transformation in a recalcitrant system like cotton enables the production of a large number of transgenic plants and the method described here serves as a useful tool for genetic engineering of cotton for various agronomical traits like insect resistance, herbicide resistance, fiber quality, etc.

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