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 pBI21 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 B₅ 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 B₅ 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 χ² test of T₁ 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 T₁ lines confirmed the stability of the gus gene and its expression.

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. acetosyringeone) 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; Olhoff et al. 2003; Tzfira et al. 2006; Karami 2008). Tissue culture is a major prerequisite for the production of transgenic plants.

Keywords: Agrobacterium tumefaciens, gus gene, nptII gene

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, cytonomic 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 andIgnacimuthu 2007). Theoreticaly, 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.
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) HgCl2 solution for 2 min and washed subsequently at least three 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 g/l 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 (Shahde 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 B5 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 g l-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).

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).

Bacteria were maintained on agar-solidified LB medium (containing 10 g l-1 Bacto Tryptone, 5 g l-1 yeast extract and 10 g l-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 inoculated with additional LB medium (containing 100 μM AS) to a concentration (OD600 = 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 cefotaxime (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/m² 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).

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).

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, nopaline synthase promoter; Tnos, nopaline synthase terminator.

Agrobacterium co-cultivation and transgenic plants regeneration

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).
EDTA, 100 mM NaH2PO4, 0.1% Triton X-100 and 50% methanol, documentation system (Biometra, Germany).

The gel was detected by ethidium bromide (0.5 /g541g/ml final concentration) staining and visualized under UV (ultraviolet) light in a gel documentation system. 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) into an insoluble precipitate as described by (Jefferson 1987; Kosugi et al. 1990). Young leaves of T0 (in vitro) and T1 (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 T0 and T1 generations, when with about 4 leaves, were tested for histochemical GUS expression in X-gluc solution containing 25 mg 1 /g2371 X-gluc, 10 mM EDTA, 100 mM NaH2PO4, 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.

**RESULTS**

**Transformation and interaction of Agrobacterium strains with cotton genotypes**

For transformation, hormone-free MS medium (MS0) containing B5 vitamins for subsequent shoot proliferation, was routinely used. A concentration of 50 mg 1 /g2371 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 1 /g2371 IBA and 50 mg 1 /g2371 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).

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

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

*Expressed as the percentage of explants that were survival in medium containing Kan (50 mg /g2371)

**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) into an insoluble precipitate as described by (Jefferson 1987; Kosugi et al. 1990). Young leaves of T0 (in vitro) and T1 (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 T0 and T1 generations, when with about 4 leaves, were tested for histochemical GUS expression in X-gluc solution containing 25 mg 1 /g2371 X-gluc, 10 mM EDTA, 100 mM NaH2PO4, 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.

**Analysis of T1 plants**

Transgenic cotton lines (T0) were successfully self-pollinated using bags and T1 seeds were produced. PCR analysis and GUS assay were carried out for T1 progeny as mentioned above for T0 plantlets.

**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).
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** \( \beta \)-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

<table>
<thead>
<tr>
<th>Variety</th>
<th>Strain</th>
<th>Putative transformed plants</th>
<th>No. of GUS-positive explants</th>
<th>Transformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varamin</td>
<td>LBA4404</td>
<td>36</td>
<td>2</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C58</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sahel</td>
<td>LBA4404</td>
<td>27</td>
<td>1</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C58</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

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

**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 T1 plants**

The inheritance of the introduced genes in the T1 generation of both varieties was studied using GUS histochemical analysis. The χ² test in T1 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 the second generation. Fig. 8b shows PCR analysis of some of the NPTII-positive lines in the T1 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, involving 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 manyfold. 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 somatic embryos (Wilkins et al., 1997). 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 excessive somaclonal variation (Srivatankul et al., 2001; Vahabi 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 ‘Yasmine’.

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 transformation. 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 genotype of the transformed shoots of T0; and T1 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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