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Agrobacterium-mediated Genetic Transformation of Bentazon Resistant Gene (*Cyp81A6*) in Cotton

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Abstract: Present study reports the *Agrobacterium*-mediated genetic transformation of bentazon resistant gene (*Cyp81A6*) in cotton genotypes (Coker 312 and Coker 201). Main aims were to genetically transform *Cyp81A6* in cotton genome and to perform non-PCR and PCR-based confirmation studies regarding its stable transformation. The embryogenic calli (EC) were first inoculated with the *Agrobacterium tumefaciens*strain LBA4404 carrying foreign gene (*Cyp81A6*) and were transferred to the selection medium having kanamycin (100 mg/L) for 4 weeks. Embryogenic calli were stunted in growth in both cultivars when transferred to selective MS medium. The kanamycin resistant (Knr) calli grew well after few sub-culturings. Knr and benr assays showed greater percentage of Knrembryogenic calli. Also EC of Coker 312 showed greater resistance than Coker 201. Both genotypes profusely produced tulip shaped somatic embryos; however, their number was more in Coker 312 as compared with Coker 201. Molecular analysis of putative transgenic calli confirmed the presence of *Cyp81A6* in the genomes of Coker 312 and Coker 201. Present study reveals that both cotton genotypes were efficient in integration of the gene and grew well on normal and selective MS media.

Keywords: *Agrobacterium*; Bentazon; Cyp81A6; Embryogenic calli

Introduction

Cotton (*Gossypium* spp.) is an important textile fiber crop, which is grown on a wide acreage in most part of the world. It has been widely exploited for both fundamental and applied studies in the areas of weed and pest management. The past two decades have seen greater boost in genetically modified cotton, which has significantly reduced reliance on pesticides by 80% as compared with the areas under conventional cotton varieties. Likewise, many other countries such as China, US, India, cotton is considered as backbone in Pakistan economy because of its major share in GDP (Economic Survey of Pakistan, 20011-12). In Pakistan, insect resistant cotton was grown on 2.4 million hectares out of 2.8 million hectares allocated land

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(James, 2011). Among them, transgenic cotton expressing insecticidal proteins from *B. thuriengenesi s*(Bt) has been one of the most rapidly adopted GM crops in the world (James, 2002; Barwale et al., 2004; Dong et al., 2005) containing cry gene(s) such as cry1Ac, cry1Ac + cry2Ab or cry1Ac + cry1F. Cotton genetic transformation success stories depend upon somatic embryogenesis, genotype-dependent regeneration (Wilkins et al., 2000) and technique used for transformation. *Agrobacterium*-mediated genetic transformation is the most preferred and powerful tool to introduce foreign DNA in host plant species (Wilkins et al., 2000). It has become a powerful tool in functional genomics as it can assess gene function by generating gain-of- function or loss-of-function mutants.

Embryogenic calli are most frequent target for transformation of many woody plant species including cotton. It is the most common technique to transform targeted gene with help of soil dwelling bacterium (*Agrobacterium tumefaciens*) (Daud et al., 2009). Umbeck et al. (1987) were the first to transform cotton cultivar Coker 312 using *Agrobacterium tumefaciens*strain LBA4404. Firoozabady et al. (1987) transformed the Kanamycin resistance gene (*npt*II) in the genome of cotton cultivar (Coker 210). They were able to stably transform genes of interest and to confirm their presence using molecular analysis. CryIA(b) gene from *Bacillus thuringiensis* (Bt) was the first agronomically important gene, which was transformed into Coker 312 using *Agrobacterium* strain A208 (Perlak et al., 1990). Assays such as insect feeding bioassays and immunological (Western blotting) analysis confirmed the expression of Bt gene as a single dominant Mendelian trait, and the phenotype appeared normal. Since then both insect-resistant (Hussain, 2002; Rashid et al., 2008; Bakhsh, 2010) or herbicide-resistant (Daud et al., 2009) transgenic cotton plants have been developed using *Agrobacterium* (Umbeck et al., 1987; Rajasekaran et al., 1996), particle bombardment (Finer and McMullen 1990; McCabe and Martinell, 1993), or pollen tube pathway (Zhang et al., 2000) techniques. Bentazon (trade name Basagran), is used to control broadleaf weeds and sedges in most plant species including rice, cotton, maize. It disrupts photosynthesis of the target plant by blocking electron transfer in photosystem II (Forthoffer et al., 2001). The main objectives of the present study were to develop resistance against bentazon through *Agrobacterium* mediated transformation technique, to compare the transformation efficiency among the cotton genotypes and moreover, to confirm the transformation event through molecular analysis.

Materials and Methods

Plant Materials and Embryogenic Callus Induction

Seeds of cotton cultivars (Coker 312 and Coker 201) were surface sterilized first with 70 % (v/v) ethyl alcohol for 3 min followed by 0.1 % (w/v) aqueous mercuric chloride solution for 10 min and washed subsequently with sterilized distilled water **(**Table 1**)**. The sterile seeds were then inoculated on MSB0 (Murashige and Skoog 1962) basal medium supplemented with 1.5 % (w/v) glucose and 0.25 % (w/v) phytagel for germination. Seeds were cultured at 28 □ 2 °C in the dark for 3 days and then transferred to the culture room (28 □ 2 °C) under a 14: 10 day: night photoperiod with light provided by cool-white fluorescent lamps at an irradiation of 135lmol/m2/s) for 5–7 days. Hypocotyls were excised from aseptic seedlings and cut into 5–7 mm segments. Callus induction was carried out on MSB1 [MS inorganic salts and B5 (Gamborg et al., 1968) vitamins] medium supplemented with 3% (w/v) glucose, 0.25%

(w/v) phytagel, 1.0 mg/l 2, 4-D and 0.5 mg/l kinetin (KT) in a 100-ml Erlenmeyer flask (Fig. 1A and B). When the induced calli were of nearly pea size, they were transferred to MSB2 (MSB supplemented with 3 % (w/v) glucose, 0.25 % (w/v) phytagel, 0.1 % (w/v) glutamine, and 0.5 % (w/v) asparagines 0.1 mg/l IBA, 0.15 mg/l KT). Each light-yellow, fresh callus was picked out and subcultured on MSB2. An embryogenic callus with high proliferation was obtained after about three or four rounds of subculture (sub-cultured monthly). After removing embryos, the embryogenic calli were used as explants for transformation (Fig. 1C and D).

Agrobacterium tumefaciens Strain and Plasmid Vectors

The *Agrobacterium tumefaciens* disarmed helper strain LBA4404 (Ooms et al., 1982) harboring p35S81A6 and pD35S81A6 with *npt*II as a selection marker was used in the present cotton genetic transformation and regeneration experiments (Fig. 2). The binary vector p35S81A6 or pD35S81A6 harbors a herbicide resistant gene (named as *Cyp81A6*) kindly provided by Pan et al. (2006), which is driven by either single or double CaMV35S promoter. The plasmid pC450-2 carries the *npt*II gene as selectable marker gene. The binary vectors were mobilized into the *Agrobacterium* by the heat shock method. The *Agrobacterium* strains were grown on LB medium plates containing rifampicin (25 mg/l) and kanamycin (50 mg/l) and the resultant colonies were stored at 4 °C.

Transformation and Plant Regeneration Process

Inoculation, Co-culture and Selection Processes

Embryogenic calli were first inoculated in LB liquid medium for 15 min, blotted dry on sterile filter paper, and transferred to co-culture medium (MSB2) having *Agrobacterium*. After 48–56 h of co-cultivation at 22 °C in the dark, the infected embryogenic calli were transferred to selection medium, MSB1 supplemented with 0.1 mg/l IBA, 0.15 mg/l KT, 100 mg/l kanamycin, and 500 mg/l cefotaxime (referred to as MSB3). After 4–5 weeks of culture, the calli were carefully transferred to fresh MSB3 medium for continuous selection and proliferation. After three rounds of selection on MSB3, the rapidly proliferating colonies (putative transgenic colonies) were subcultured on MSB4 medium; MSB2 supplemented with 0.5 mg/l IBA and 0.2 mg/l KT, for proliferation. The Knr calli re-differentiated and formed embryos about 30 days after being cultured on MSB4. Somatic embryos (about 3 mm in size) were transferred into a 100 ml Erlenmeyer flask containing MSB2 medium for maturation and conversion. Germinated embryos were transferred to seedling and rooting media (Referred as MSR1 and MSR2). When plantlets grew to 8–10 cm in height, they were transferred to pots (30 cm in diameter, 40 cm high) with soil and sand (1: 1) in a greenhouse for further growth.

All plant growth regulators and amino acids were added prior to autoclaving, but the antibiotics were filter-sterilized. All media mentioned above were adjusted to pH 5.8 prior to autoclaving, except the co-culture medium, which was adjusted to pH 5.6. Cultures were maintained in a room at 28 ± 2 °C under a 16: 8 day: night photoperiod with light provided by cool-white fluorescent lamps at an irradiation of 50 μmol m^{-2} s^{-1}.

Kanamycin and Bentazon assays

In order to confirm the transformation event, kanamycin and bentazon assay was done. After three rounds of selection of EC on MSB3 medium, the highly proliferating, fresh and yellow

colored masses of the EC of both cultivars were transferred to two different types of media. Medium MSB3-1 was having kanamycin @ 100 mg/l and medium MSB3-2 was having kanamycin and bentazon.

Table 1. Media used in embryogenic callus induction and transformation of Coker 312 and Coker 201. MS: Murashige and Skoog, 1962; MSB: Murashige and Skoog + Gamborg 1968.

Media Name	**Event**	**Composition**
MSB0	Seed Germination	1.5%Glucose;0.25% Phytagel
MSB1	Callus Induction	1mg/L 2,4-D; 0.5mg/L KT; 3% Glucose; 0.25% Phytagel
MSB2	Callus Proliferation	0.1mg/L IBA; 0.15mg/L KT; 0.1% Glutamine; 0.5% Asparagin; 3% Glucose; 0.25% Phytagel
MSB2	Co-culture	MSB2
MSB3	Selection	MSB2+ 100mg/L Kanamycin; 500mg/L Cefotaxime
MSB4	Somatic embryos induction	MSB2 + 0.5mg/L IBA; 0.2mg/L KT
MSR1	Seedling Development	1/2MS; 0.25% Phytagel; 3% Glucose
MSR2	Rooting	1/2MS; 0.3% Phytagel; 3% Glucose

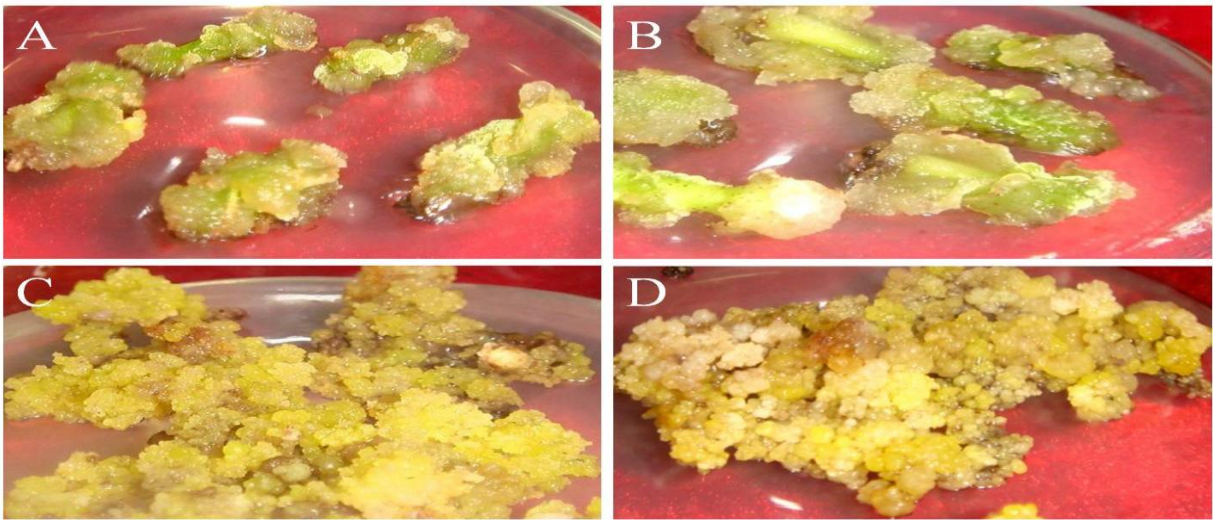

Fig. 1. Callus induction process in Coker 312 (A) and Coker 201 (B) on MSB1. Embryogeniccalli of Coker 312 (C) and Coker 201 (D) induced on MSB2, which were used in genetic transformation.

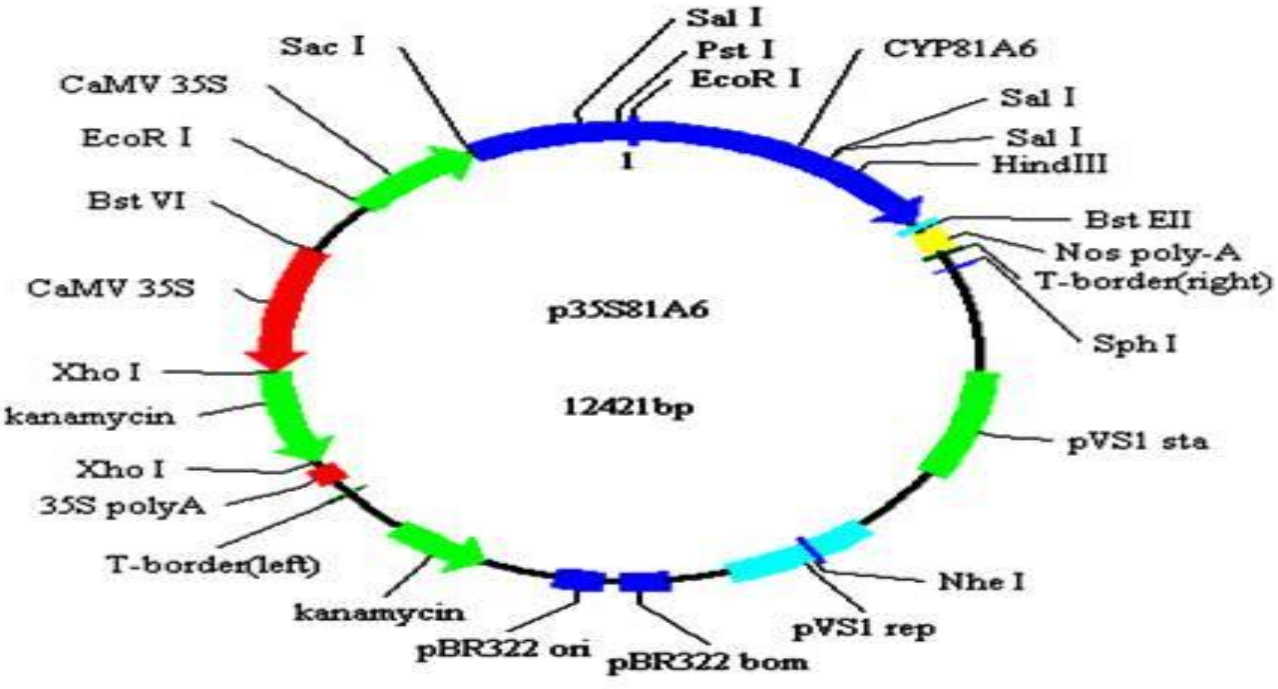

Fig. 2. Chimeric gene map of the recombinant binary vector p35S81A6 carrying the rice herbicide-resistant gene and *npt*II gene driven by CaMV35S promoter (P35S). LB: left border, RB: right border, *npt*II, neomycin phosphotransferase, Lines show restriction sites, *NOS* represents the polyA signal of CaMV35S as terminator.

PCR analysis for putative transformants

PCR analysis was carried out using specific primer pairs to amplify *npt*II and *Cyp81A6* genes in the kanamycin resistant calli. Total genomic DNA was extracted from the embryogenic calli according to Li et al. (2001). PCR reaction mixture was 25 µl consisting of 10x reaction buffers, 50 ng DNA templates, 15 mM MgCl2, 10 mMdNTPs, 50 ng of each primer and 1 unit Taq DNA polymerase. PCR was carried out in an eppendorf (Model 42102) thermal cycler using the following conditions: Initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for *npt*II and 55 °C for *Cyp81A6* gene for 1min, extension at 72 °C for 3 min and final extension at 72 °C for 5 min. PCR product was analyzed by gel electrophoresis on 1.5 % agarose gel. The forward and reverse primers used for the detection of *npt*II were respectively 5'-TCG GCT ATG ACT GGG CAC AAC AGA-3' and 5'- AAG AAG GCG ATA GAA GGC GAT GCG-3' and they were the part of the coding region of the *npt*II gene. For the detection of the *Cyp81A6* gene, primers 5'-GAA GTT CAT GCC GGA GAG-3' (forward primer) and 5'- ATT GCG GGA CTC TAA TCA TA-3' (reverse primer) and they were the part of the coding region of the *Cyp81A6* gene.

RESULTS

Co-culturing and Proliferation of Embryogenic Callus (EC) on Selective Medium (MSB3)

In order to enhance the attachment of bacteria to the calli, highly proliferating and yellow green embryogenic calli were co-cultured with *Agrobacterium* suspension. After co-culturing on MSB2 for 48 hours, the infected calli were transferred to selective medium (MSB3) containing either kanamycine. For about two weeks, the calli were stunted in growth and about 97% of

them died. However, approximately within one month small gall-like masses appeared on the sphere of the stunted calli in both cultivars (Fig. 3A and B). These calli were carefully subcultured on the same MSB3 medium and every subculturing process was done after 3-4 weeks. The subculturing process was done for 3 times and after every subculturing the growth of Kanamycin resistant calli progressively enhanced (data not shown).

Selection of Putative Transgenic Embryogenic Calli by Kanamycin and Bentazon Assays

Table 2 shows the kanamycin and bentazon assays. According to the table, more than 50 % of the resistant EC of both cultivars proliferated well and developed good texture as well as color in the presence of both kanamycin and bentazon. The percentage of Knrembryogenic calli was more as compared to benr calli. Moreover, the embryogenic calli of Coker 312 showed better resistance to these stresses as compared to Coker 201.

Somatic Embryos Production, their Germination, Maturation and Conversion to Plantlets

The Knr and benr EC were transferred into flasks having MSB2 for proliferation and then highly proliferating and granular calli were put on the MSB4 for somatic embryos production (Fig. 3C and D). A number of well-developed tulip-shaped somatic embryos appeared after 2-3 rounds of subculturing on the same MSB4, which later on were allowed to germinate and mature well. These matured and well-germinated embryos were transferred to MSR1 and then MSR2 (Fig. 4). After 2-3 months of subculturing, these plantlets rooted well and those that did not develop roots were grafted. Then plantlets were acclimatized under the green house conditions.

Molecular analysis of the putative transgenic embryogenic calli

The molecular analysis of the EC of Coker 312 and Coker 201 was done in order to confirm the presenceof *Cyp81A6* codingsequence (Fig. 5). The results showed the presence of the expected 150 bp fragment of the *Cyp81A6* gene and hence confirmed the presence of *Cyp81A6* gene in the recipient genome.

Table 2. Percentage of the resistant embryogenic calli on kanamycin and bentazon media.

Stress type	Genotype	No. of Calli Tested	Resistant calli	%age of resistant calli
Kanamycin	C312	156	49	50.64
	C201	142	34	45.07
Kanamycin+ Bentazon	C312	120	41	34.17
	C201	116	28	24.14

Fig. 3. Kanamycin resistant embryogenic calli of Coker 312 (A) and Coker 201 (B) on selection medium. Arrows show the kanamycin resistant embryogenic calli. Somatic embryos of Coker 312 (C) and Coker 201 (D) developed on MSB4 after a series of selection on selective medium. Arrows point to the development of somatic embryos.

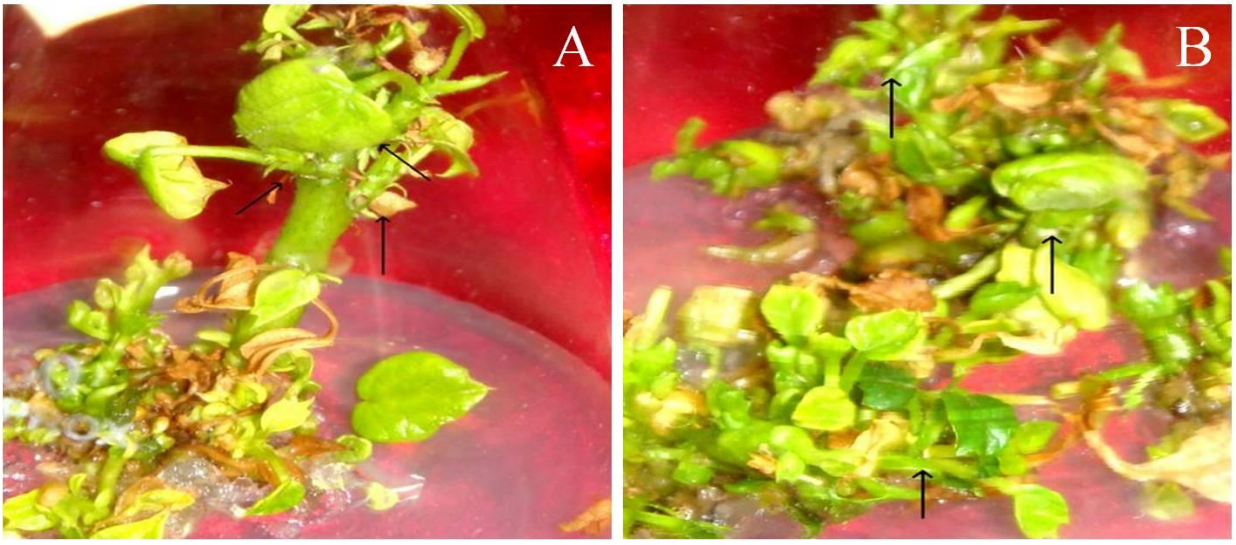

Fig. 4. Putative transgenic plantlets of Coker 312 (A) and Coker 201 (B). Arrows indicate the development of a number of shoots from the main stem.

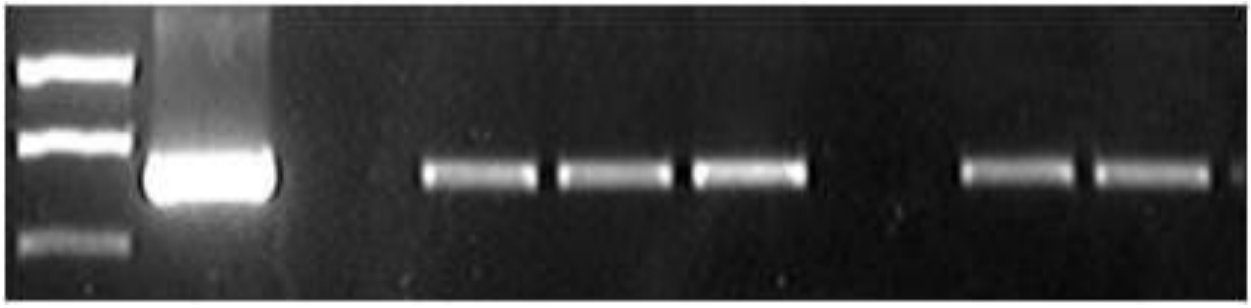

Fig. 5. PCR product of the putative non-transgenic and transgenic embryogenic calli. M: marker

DISCUSSION

To produce embryogenic calli in cotton, explants like hypocotyls and cotyledons are often used (Firoozabady et al., 1987, Umbeck et al., 1987). Using embryogenic callus in genetic transformation of cotton offers a number of advantages. For example, embryogenic callus has small size, condensed cytoplasm, and rapidly dividing cells, a huge mass of callus can be obtained in a short possible time for transformation and more importantly, the embryogenic callus is most likely obtained from single cell, so the transformants resultant from the embryogenic callus can avoid the mosaic/ somaclonal variations.

Keeping in view of the above facts, we first obtained embryogenic calli for Coker 312 and Coker 201 and then we used the resultant embryogenic calli for the transformation of the bentazon resistant gene. The results obtained from callus growth, its proliferation and molecular analysis revealed that *Cyp81A6* could be successfully integrated into the genome of both cultivars. We started our experiment by putting the co-cultured EC on selective medium having kanamycin and the survival test was done based on the percentage of surviving calli on kanamycin containing medium. We further put the surviving EC on a medium containing both kanamycin and bentazon and obtained highly proliferating resistant calli. In order to have molecular evidence regarding the successful integration of *Cyp81A6*, the PCR analysis revealed the presence of approximately 150bp segment of the integrated gene in both plasmids DNA and putative transgenic EC but the non-transformed EC were having no such band. These PCR positive and kanamycin and bentazon resistant EC were transferred to somatic embryos production, maturation and plantlet formation media. Resultantly, we could obtain the putative transgenic plantlets, which were successfully transferred to the soil.

CONCLUSIONS

We can conclude that both genotypes responded positively in some cases. Cotton is the most recalcitrant plant to tissue culture and plant regeneration via somatic embryogenesis. Successful transformation is most likely based on a number of factors, in which the type of explant is the first priority of the persons doing transformation. In the present experiment, we used EC as explants for transformation and could successfully obtain a large number of somatic embryos and regenerated putative transgenic plantlets. The EC have also been successfully used for plant genetic transformation by Scorza et al. (1996) and Martinelli and Mandolino (2001) and they were also verified to be much suitable explants by Wu et al. (2005).

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