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Bioloistic-mediated Transformation of Cotton (*Gossypium hirsutum* L.): Embryogenic Calli as Explant

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Abstract

Genetic transformation was carried out by using biolistic gun method. The hypocotyl derived embryogenic calli (explants) of cotton (*Gossypium hirsutum* L.) cv. Cocker-312 were transformed with a recombinant pGreen II plasmid, in which both, *bar* (selection marker) and *GUS* (*β-glucuronidase*) reporter genes were incorporated. Explants were arranged on osmoticum-containing medium (0.5M mannitol) 4 hours prior to and 16 hours after bombardment that was resulted into an increase about >80% for *GUS* stable expression. 3 days after bombardment, *GUS* assay was performed, which exhibited, 18.36±1.00 calli showed blue spots. The transformed embryogenic calli were cultured on selection medium (@ 6 mg/L basta) for 3 months. The putative transgenic plants were developed via selective somatic embryogenesis (@ 1.50 mg/L basta); maximum 27.58±1.25 somatic embryos were obtained while 17.47±1.00 embryos developed into plantlets (@ 0.75mg/L basta). In five independent experiments, up to 7.24% transformation efficiency was recorded. The presence of the transgenes was analyzed by using PCR and southern hybridization analysis. The transgenic plants were developed with in 6-7 months, but mostly transformants were abnormal in morphology.

Key words: biolistic transformation, cotton (*Gossypium hirsutum* L.), bialaphos, *GUS*, expression, embryogenic calli, somatic embryogenesis, transgene expression

Introduction

Development of a genetic transformation system for crop plants has become an important tool for the crop improvement (Raizda et al., 2001; Srivastava and Ow, 2001; Sawahel and Cove, 1992; Finer et al., 1992). The transgenic crops have been developed by using biolistic method i.e soybean (Finer and McMullen 1991), cotton (Finer and McMullen 1990; Rajasekaran et al., 1996, 2000; Sawahel, 2002), turf grass (Chai and Sticklen 1998; Zhong et al., 1993), maize (Vain et al., 1993), pineapple (Sripoaraya et al., 2001) banana (Sagi et al., 1995) and wheat (Mariana et al., 2002; Takumi and Shimada, 1996), however, there are different physical factors that involves, can be optimized, such as: pressure of helium gas, vacuum pressure, distance between the retention screen and the material to be bombarded, and also the distance between the gas chamber of high pressure and the plasmid DNA carrier membrane (Sanford, 1988; Sanford et al., 1993).

Bialaphos is a tripeptide antibiotic that is produced by *Streptomyces hygroscopicus* SF1293 (Tachibana et al., 1986a). It consists of phosphinothricin, an analog of L-glutamic acid and two L-alanine residues. Upon removal of the alanine residues by endogenous peptidases in plant cells, the resulting phosphinothricin inhibits glutamine synthetase, due to which rapid accumulation of ammonia occurs ultimately causes to cell death (Tachibana et al., 1986b, c; Krieg et al., 1990).

The bar gene isolated from *S. hygroscopicus*, encodes phosphinothricin acetyltransferase, which acetylates the amino group of phosphinothricin, abolishes its herbicidal activity (Murakami et al., 1986; Thompson et al., 1987; Botteman, 1987). Many transgenic plants that retain the *bar* gene are resistant to both bialaphos and phosphinothricin (Toki et al., 1992; Uchimiya et al., 1993; Rajasekaran et al., 1996; Sripaoraya et al., 2001). *GUS* gene (*β-Glucoronidase*), an easily assayable reporter gene used to check the transformation efficiency in cells/plants (Rajasekaran. 2000; Peng et al., 2003; Song and Yamaguchi, 2003; Chaudhary et al., 2003). Its selective agent is a

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glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide), an inactive form of the plant hormone cytokinin. This glucuronide, present in the selection medium, can be hydrolyzed by the *GUS* enzyme produced in the transformed cells, releasing active cytokinin (benzyladenine) in the medium. This cytokinin acts as a stimulator for regeneration of transformed cells while non-transformed cells are arrested in development (Joersbo and Okkels 1996; Okkels et al., 1997).

For *in vitro* selection of transformed tissue, a limited number of selectable markers are available but after the development of transformants they are useless. Meanwhile the *bar* gene is not only agronomically important, can acts as a selection marker (D' Halluin et al., 1992; Tachibana et al., 1986b; Thompson et al., 1987; Toki et al., 1992; Wagdy 2002; Park and Park 2002).

During this study the *bar* gene was used as a selectable marker (herbicide resistant gene) for the *in vitro* selection of the transformants, so in this manuscript an optimized concentration of the bialaphas for the selection of herbicide-tolerant transgenic callus, selective embryogenesis and the development of putative transgenic plants in cotton (*Gossypium hirsutum* L.) cv. Cocker-312 is described. A rapid biolistic-mediated gene delivery procedure by employing on two months old hypocotyl derived calli that were used as target tissue.

Materials and methods

Plant materials and callus induction

The mature seeds were delinted with commercial H_2SO_4, then surface sterilized with 30% commercial bleach [5.25% (v/v) sodium hypochlorite] by stirring with magnet stirrer for 30 min. They were then rinsed three times with sterilized distilled water. The surface sterilized seeds were germinated on MS_0 [MS, Murashige and Skoog (1962) slats with B5 (Gamberg et al., 1968) vitamins] medium supplemented with 3% glucose and semi-solidified with 3.60g/L phytagel (Sigma, St Louis, MO, USA). For seed germination, the culture was placed under dark conditions at 28℃ ± 1 for 48 hrs. After radical emergence from the seeds, culture was shifted to growth room. Hypocotyls (3-5mm) sections were used as explants for callus induction, by culturing on MS2 (MS0; 0.1 mg/L 2,4-D; 0.5 mg/L KT; 0.75 mg/L $MgCl_2$; 3% glucose) medium. Each experiment have five replicates.

Plasmid DNA

A recombinant pGreen II plasmid (Roger et al, 2000) was used for the transformation of the two months old embrygenic calli of cotton (*Gossypium hirsutum* L.) cv. Cocker-312. In which both genes i.e *bar* as a selectable marker (pBARGRG1 provided by Dr. Brunelli) and *GUS* as a reporter gene (pJIT166, Sainsbury Lab) were incorporated. The *bar* (herbicide resistant) gene expressed under *Pnos* promoter and terminated by *Tnos* while the *GUS* (*β-glucuronidase*) reporter gene regulated by 2x35S promoters and terminated with *T35S*. The plasmid DNA was isolated on a large scale and purified by Cs-EtBr density gradient centrifugation following standard protocols (Sambrook et al., 1989).

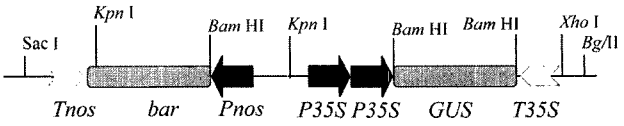

Figure 1. Schematic representation of a recombinant pGreenII plasmid.

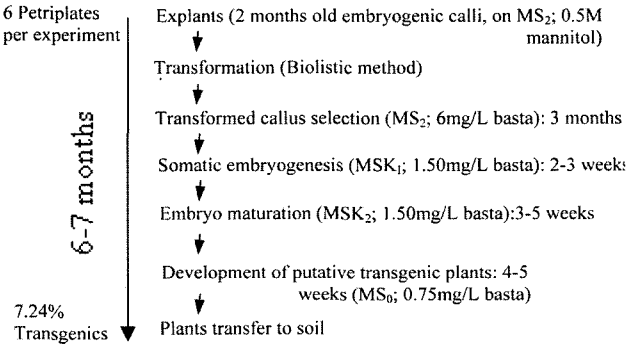

Figure 2. Flow chart: biolistic gun mediated transformation of cotton (*Gossypium hirsutum* L.) cv. Cocker-312.

DNA/microcarriers preparation and bombardment conditions

Embryogenic calli (2 months old) were placed 4hrs prior to bombordment in the center of 9 cm diameter Petri dishes containing osmoticum (MS_2; 0.5M mannitol) medium. The explants were bombarded with the Particle Delivery System (PDS-1000/He, Bio-Rad). The manufacturer's instructions were followed for coating micro-particles (gold) with the plasmid DNA. 0.30-0.36 mg (1.0 μm diameter) of gold particles was sterilized in 200 μL absolute ethanol for 6 min with vortaxing and placing on ice alternatively. After washing (2 times) with sterile water, then resuspended in 50 μL sterile water.

A mixture of, 50 μL of particle suspension; 10μL plasmid

DNA (1 μg/ μL), 50μL CaCl2 (2.5 M) and 20μL spermidine (0.1 M) was prepared. The particle/DNA suspension was vortexed and left for 10 min at room temperature. The DNA coated particles were pelleted by centrifugation at 10,000 rpm for 10 sec. The supernatant was completely removed. After washing with 200 μL absolute ethanol, the pellet was then resuspended in 50 μL absolute ethanol. 8μL of particle/ plasmid DNA mixture was placed in the center of micro-carrier. The target explants were placed at a distance of 6 cm from the stopping screen and bombarded single time at 11,00 psi helium capacity rupture disks. After bombardment, the calli were separated on the same medium and incubated in the dark room at 28℃±1 for 16 hrs.

Transformed cell selection and plant regeneration from

Sixteen hrs after bombardment, the explants were transferred to callus induction medium containing 6mg/L basta for the purpose of selective transgenic cell growth. After 3 months of callus selection and its proliferation, they were subcultured on embryo induction MSK$_I$ (MS$_2$; 1.90mg/L NH_4NO_3; 1.50 mg/L basta) medium. After 2-3 weeks the embryogenic calli was transferred to embryo maturation MSKI (MS2; 1.90mg/L KNO_3; 1.50 mg/L basta) medium. The regenerated plantlets were transferred to Magenta boxes containing root/shoot induction medium (MS$_0$; 1.50 mg/L basta). With in 2-4 weeks, roots/shoots were developed, and then the plants were shifted to soil. Note: All cultures were maintained at 28±1℃ under a 16/8 h (light/dark) photoperiod with light supplied at an intensity of 60 μE m^{-2} s^{-1}.

Histochemical *GUS* assays

Bombarded calli were spread to the surface of semisolid osmoticum medium (MS$_2$; 0.5M mannitol) for 72h then assayed for *GUS* activity by immersing them in X-Gluc solution [1.5 mM 5-bromo-3-chloro-3-indolyl-b-D-glucuronic acid (X-Gluc; Gold Biotechnology, St. Louis, USA), 20 mM Na_2HPO_4 + NaH_2PO_4 (pH 7.0), 10 mM $K_3Fe(CN)_6$, 10mM $K_4Fe(CN)_6 \cdot 3H_20$, 10 mM Na_2EDTA and 0.1% (v/v) Triton X-100 (Sigma)] for 12 h at 37℃. The number of indigo GEUs (*GUS* expression units) was counted. Clusters of GEUs were scored as single transformation events (Fig 3a). The selective well proliferating embryogenic calli and regenerated plants were also analysed for *GUS* activity.

PCR analysis

Genomic DNA was isolated from the transformants and nontransformants (regenerated plants), according to the CTAB/chloroform extraction protocol (Chaudhary et al., 1999) and conducted PCR for the presence of both (*bar* and *GUS*) genes. The 0.460kb fragment of bar gene was amplified by using 5'-GGTCTGCACCATCGTCAAC-3' and 5'-GAAGT-CCAGCTG-CCAGAAAC-3' as forward and backward primers. The 1.8kb fragment of *GUS* gene amplified; the primers set utilized were 5'-ATGTT-ACGTCCTGTAGAAAC-3' as forward and 5'-CATTGTTTGCCTC- CCTGCT-3' as backward. The PCR conditions were maintained at 94℃ for 5 min, followed by 30 cycles of 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 50sec. Cycling was followed with an ending step at 72℃ for 10 min then followed by 22℃ as standing temperature. PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis

Twenty μg of plant genomic DNA (transformants and untransformants) and 1 μg of plasmid DNA were restricted with *Kpn*I and *Bam*HI enzymes (for *bar* gene) but for the *GUS* both were restricted with *Bam*HI enzyme. Same restriction reactions were carried out for plasmid to get required fragment (*GUS* and *bar*) for probe labeling. The restricted DNA was fractionated on 0.8% agarose gel, denatured, and transferred to high bond-N^+ membrane (Amersham) The membrane was pre-hybridized for 1 hr at 65℃ in a solution containing 0.25 M sodium phosphate (pH 7.4), 1 mM EDTA, 1% BSA and 7% SDS, and hybridized in the same solution with probes (both *bar* and *GUS* genes in a separate reaction) labeled with R-^{32}P-dCTP using a mega primer DNA labeling kit (Amersham). Then the membrane was washed twice with 2×SSC solution at room temperature and once with 0.1×SSC solution containing 0.1% SDS at 60℃ for 15 min each time. Auto- radiophotography were exposed at -70℃ for up to 7 days.

Result and Discussion

Cotton (*Gossypium hirsutum* L.) has been earlier transformed by particle bombardment but the transformation efficiency measured through transient assay that have remained low (usually 0.1-2.0%), for that various attempts (Finer and McMullen, 1990; Rajasekaran et al., 2000) have been made. The low success rate in cotton transformation experiments thus necessitates the use of a large number of explants for that hypocotyl derived embryogenic calli as reported by Mishra et al., (2003) Chaudhary et al., (2003) and Kumria et al., (2003), so callus was used an explant for biolistic transformation (Rajasekaran et al., 2000), while Leelavathi et al., (2004) and Haq, (2004) have also been used embryogenic calli as xplant for *Agrobacterium*-

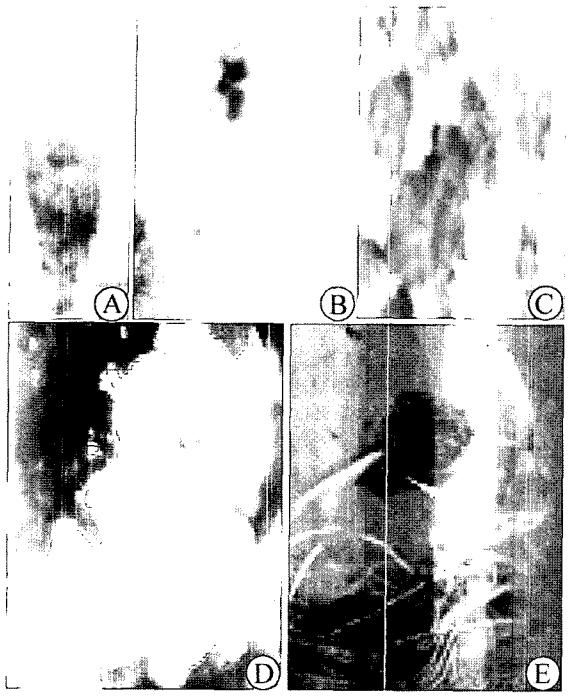

Figure 3. Histochemical analysis of *GUS* gene and and different tissue culture stages during the development of putative transgenic plants of cotton (*Gossypium hirsutum* L.) cv Cocker-312. a: *GUS* histochemical analysis, after 72hrs of biolistic shoots; b: Basta resistant, well proliferating embryogenic calli with untransformed dead cells after one month; c: *GUS* assay for basta resistant, well proliferating embryogenic calli; d: Callus on embryo induction medium (1.50mg/L basta) with developing embryoids at different developmental stages; e: *In vitro*, *GUS* assay of basta resistant putative transgenic plant on the rooting medium.

mediated transformation. Identifying explants/cultivars with a relatively high embryogenic response is one of the most critical factors for any transformation endeavour. Based on the higher regeneration response in Cocker-312 was chosen so 2 months old embryogenic calli was used as explant.

The embryogenic calli transformed with biolistic gun, exhibiting 17.05 blue spots/experiment were observed (Fig 3a; Table 1), however, towards achieving herbicide resistance in cotton cv. Cocker-312, the recombinant plasmid DNA was transformed in embryogenic calli on osmoticum medium (MS_2; 0.5M mannitol) by particle bombardment. The untransformed calli did not show growth on selection medium as against the bombarded calli. To eliminate the possibility of escapes the selection of transformed calli and its proliferation was attained by culturing bombarded calli for 3 months on the transgenic callus selection (MS_2; 6mg/L basta; Fig 3b) medium, then *GUS* assay showed a complete green mass (Fig 3c;d) that indicate the presence of *GUS* gene in all the selected cells. After 3 months of selection of the basta resistant embryogenic calli cultured on the selective embryo induction MSK_1 (MS_0; NH_4NO_3; 1.50mg/L basta) medium. After 2-3 weeks, the embryoids were shifted from MSK1 medium to embryo maturation MSK_2 (MS_0; KNO_3; 1.50mg/L basta; Fig 3d) medium (Kumria et al 2003; Haq and Zafar, 2004a, b; Haq, 2005). The regenerated plantlets were grown on MS medium for the root/shoot induction (MS_0; 1.50 mg/l basta; Fig 3e). Out of 747 explants of cotton cv. Cocker-312, 54 putative transgenic plants were able to recover their roots/shoots normally (Table 1 showing data for the means of each experiment). The transformation efficiency 7.24% was calculated based on the results of 5 independent experiments (Table 1). *GUS* assay was performed for the fully regenerated plants (Fig 3e).

The present results demonstrate the importance of an efficient selection regime. The use of bialaphos as the selection agent, that interferes somewhat during somatic embryogenesis and in the root/shoot development stages. To eliminate the escapes, the putative transformants were maintained on somatic embryo induction MSK_1 and embryo maturation MSK_2 supplemented with 1.50mg/l basta and root shoot medium with 0.75mg/L basta. The reason for high transformation efficiency may be attributed to optimize regeneration protocol and the effective selection procedure employed for the recovery of transformants (12 weeks after particle bombardment). As Altpeter et al., (1996) who reported transformation efficiency may be enhanced by reducing the total time taken for production of transgenic plants.

Using embryogenic calli as the target explant, Takumi and Shimada (1996) reported the positive influence of culture duration of the target tissue prior to bombardment on stable transformation efficiency. During our study, two months old hypocotyl derived embryogenic calli were cultured on fresh osmoticum (MS2; 0.5M mannitol) medium for four hours prior to particle bombardment resulting in a high transformation efficiency (up to 80 %) as depicted in terms of, the number of blue spots were counted after *GUS* assay for per experiment resistant embryogenic calli (Vain et al., 1993). The *GUS* assay was performed after 72hrs of particle bombardment (Fig 3c; Table 1).

To confirm the presence of transgenes in the bialaphos resistant transgenic plants, a number of transformants were tested by PCR amplification of genomic DNA by using a set of specific primers of both (*GUS* and *bar*) genes. The results of PCR analysis were found: The expected size for the *GUS* gene (1.80kb; Fig 4a) and the bar gene (0.46kb; Fig 4b) were detected, which were in correspondence to the plasmid control. No amplified fragment was detected in the samples containing genomic DNA isolated from an untransformed control plant.

Table 1. Biolistic-mediated transformation efficiency (%) in cotton (*Gossypium hirsutum* L.) cv. Cocker-312.

experiments transformation %	No of explants	GUS^{+ve} calli (#)	GUS^{+ve} (%)	basta resistant calli (#)	somatic embryos (#)	Embryos regenerated (#)	Plants transfered to soil (#)	efficiency
Without osmoticum-traetment(0.5M mannitol)								
1	10	10.05±1.05		6.45±0.25	10.56±1.05	6.38±1.65	0.11±1.25	1.12
With osmoticum-traetment(0.5M mannitol)								
1	15	11.54±0.05	14.90	20.35±1.05	20.81±0.70	15.95±0.50	1.02±1.05	6.82
2	10	12.57±0.50	72.00	17.25±0.60	15.65±0.75	12.14±0.15	0.72±1.35	7.13
3	17	16.62±0.20	55.42	15.62±0.75	27.58±1.25	17.47±1.00	1.23± 2.25	7.24
4	14	18.36±1.00	81.79	18.27±1.05	12.82±0.50	10.22±2.05	0.64±2.05	4.57
5	10	13.42±1.05	62.69	16.35±0.75	24.57±1.25	8.58±1.50	0.32±1.25	3.21

NOTE: **# of explants** are the mean of five replicates, while over all 747 explants of cotton cv. Cocker-312 were used for the biolistic transformation only 54 putative transgenic plants were able to recover their roots/shoots normally.
The number of **GUS^{+ve}** were counted by numbering the blue spot on bomborded calli, after *GUS* assay by taking means of GUS^{+ve} calli in five experiments (17.05 blue spots) treated with osmoticum medium.
GUS^{+ve} (%) were measured by formula

$$\text{GUS}^{+ve}\ (\%) = \frac{(\text{The \# of GUS}^{+ve}\text{ (blue spots) calli on osmoticum medium}) - (\text{The \# of GUS}^{+ve}\text{ (blue spots) calli on without osmoticum medium})}{\text{The \# of GUS}^{+ve}\text{ (blue spots) calli on without osmoticum medium}} \times 100$$

Osmoticum (0.5M mannitol) was added to the MS_2 for abiotic stress treatment to the embrogenic calli prior to 4 hours, which is used for biolistic transformation.
Each experiment was conducted with five replicates.

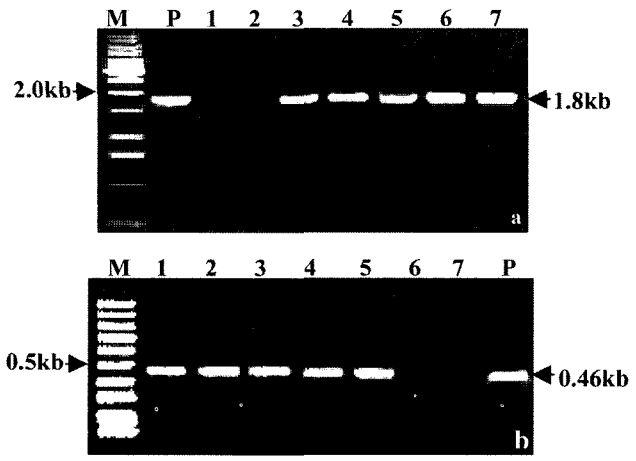

Figure 4. PCR analysis for the presence of transgenes in putative transgenic plants of cotton (*Gossypium hirsutum* L.) cv. Cocker-312. a: PCR analysis confirorming the presence of the *GUS* gene in basta resistant plants (lanes 3-7), but not in a non-transformed plant (lane 2). plasmid and Water controls are shown in lanes P and 1, respectively and M: DNA marker.; b: PCR analysis for the presence of the *bar* gene in basta tolerant plants (lanes 1-5), but not in a non-transformed plant (lane 6). Lanes 7 and P: water and plasmid controls, respectively and M: DNA Marker.

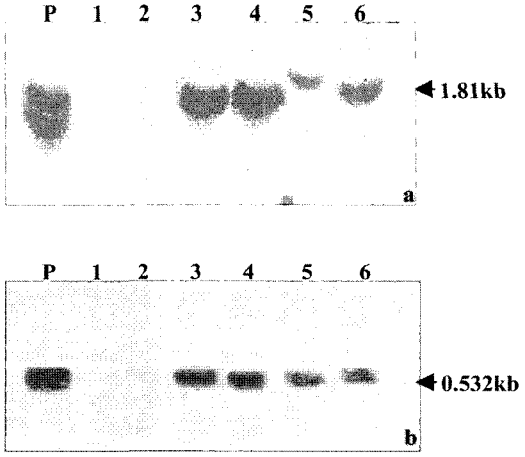

Figure 5. Southern blot analysis for the presence of transgenes in putative transgenic plants of cotton (*Gossypium hirsutum* L.) cv. Cocker-312. a: Southern blot analysis of DNA from basta resistant plants (Lanes 3-6) but no hybridization was detected in non transformed plant (Lane 2); Lane 1 for Water and P: Plasmid control for *GUS* gene; b: Southern blot analysis of DNA from basta resistant plants (Lanes 3-6) but no hybridization occur in non transformed plant (Lane 2); Lane 1: Water and P: Plasmid control for bar gene.

The transgens in the transformants of cotton (*Gossypium hirsutum* L.) cv. Cocker-312 were also confirmed by Southern analysis by using radioactively labeled gene specific probes of both (*bar* and *GUS*) genes. The genomic DNA from the plants (transformed and untransformed) and plasmid (*bar* and *GUS* genes) used in transformation were digested with *Bam*HI and *Kpn*I. The results were obtained by the detection of a ~0.532 kb hybridizing band for *bar* gene and 1.81kb for *GUS* gene (Fig 5a, b) in the basta

resistant transformed plants, which were in corresponds to the fragments (Finer and McMullen 1991; Curtis and Nam 2001; Sawahel 2002) detected in plasmid control that were used in plant transformation. So the results from PCR and southern analysis further confirm the presence of transgenes in the genome of the basta resistant transgenic plants.

Cotton is considered to be more recalcitrant, but Cocker-312 has well plant regeneration response. The embryogenic calli was used as an explant for the biolisitic mediated gene transformtion and there is no any report for *in vitro* selection of bombarded embryogenic calli on solidified medium in the presence of basta as a transgenic cell selection antibiotics.

In the present investigation, we have been able to introduce both the marker genes into cotton (*Gossypium hirsutum* L.) cv. Cocker-312 successfully with 7.24% transformation efficiency through biolistic method. The basta concentration 6mg/L was considered best for the purpose of the selection of the bombarded embryogenic calli (2 months old), on the solidified callus induction medium, but for somatic embryogenesis 1.50mg/L basta: considered the best, above to that it leads to the hardening and growth inhibition of the embryogenic calli. All the transgenic lines obtained in the present investigation displayed functional activity of the marker genes (*bar* and *GUS* genes). The genetic transformation methodologies employed are identical for other cotton varieties, thus opening the possibility of extending this system to other genotypes as well. The present efforts are thus encouraging and further in depth analysis of the integration and segregation patterns for both Cocker cultivars and other local varieties will pave a way for the possibilities of engineering cotton varieties with the genes, that are agronomical important.

Conclusions

Biolistic-mediated gene delivery system into embryogenic calli is an effective method for the production of transgenic cotton (*Gossypium hirsutum* L.) cv. Cocker-312, which has now provided a baseline for the introduction of an additional agronomically useful gene into cocker varieties and, possibly, to other cotton cultivars. The present study also emphasizes the benefits of biotechnology to cotton improvement. Commercially defined cotton production is hampered not only by pests, diseases and the necessity for chemical control of lowering, but by the difficulty of maintaining product quality (e.g. shelf-life), all of which cannot be targeted by traditional selective breeding strategies. However, the exploitation of a range of potentially useful transgenes is likely to overcome these difficulties, resulting in rapid commercial development of improved cotton cultivars. Establishment of the particle delivery system and optimized basta concentration for the selective transformed cells growth and their somatic embryogenesis in cotton will be of great value for such aspect.

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