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Establishment of *Glyoxalase I* gene transformation and regeneration of cotton (*Gossypium hirsutum* L.)

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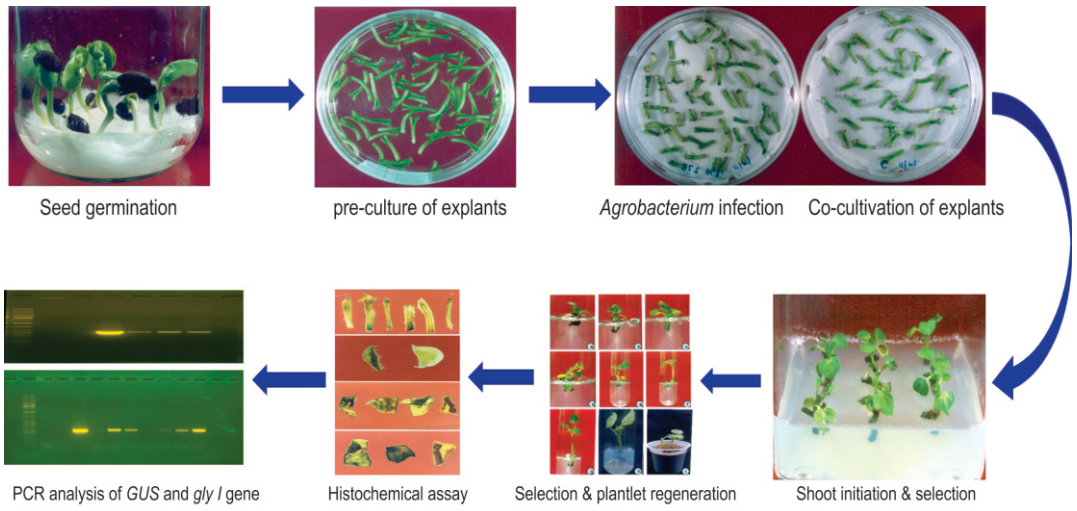
Abstract

Aim: The current study was carried out to develop transgenic cotton plantlets with *glyoxalase* I (*gly I*) gene using *Agrobacterium*-mediated transformation.

Methodology: Seeds of cotton were inoculated on MS medium and the explants such as shoot tip (3-5 mm), hypocotyl and leaf were aseptically removed from *in vitro* plantlets. The pre-cultured and infected explants with *Agrobacterium* harboring *gly I* gene and the shoot tip were inoculated on MS media for shoot initiation and subcultured on elongation medium with growth hormones, and antibiotics. Healthy and well-grown shoots were subcultured on medium with indole butyric acid (IBA) (0.3 mgl^{-1}) for root formation and the plantlets were hardened in plastic cups with sterile soil. The putative transgenic plantlets were analyzed histochemically for gus gene and the PCR analysis was performed for *gly 1* gene.



Results: The transformation efficiency of cotton ranged 48.57 to 64.53 %. The regenerated plantlets showed the presence of gus gene in terms of blue coloration in shoots, whole leaf and leaf segments. The PCR was performed in putative transgenic plant lets with both *gus* gene as well as *gly I* gene primers. The PCR results showed the presence of 1031 bp DNA band with *gus* gene primers and 800 bp DNA band with the *gly I* gene primers.

Interpretation: The current study has proven the reproducible procedure for the *Agrobacterium*-mediated gene transfer and regeneration of Indian cotton varieties. The PCR results revealed the presence of *glyoxalase I* gene in the transformants.

Key words: Cotton varieties, *Glyoxalase I* gene, PCR analysis, Regeneration, Transformation

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Introduction

Cotton also known as white gold, is the backbone of world markets and it is deliberated as the highest value-driven industrial cash crop in India, besides being the foremost fiber and oilseed crop in several countries. In native practice and as a commercial product on world market, cotton plays pivotal role as significant source for agricultural industry, which eventually boosts Indian economy. The seeds are the source of cooking oil and the remaining meal with higher amount of essential amino acids used for cattle feed or organic fertilizers. Furthermore, several consumer products are regularly developed as byproducts of cotton industry as additional goods to fiber, which is used extensively in textile industries (Pandey, 1998; Shivagaje *et al.,* 2004).

Several abiotic stresses, such as drought, extreme temperature and nutrient deficiency have potential negative impacts on the vegetative and reproductive growth, which subsequently leads to reduction on agronomical characters of cotton (John, 1997). Due to many incompatibility barriers, it is difficult to exploit the presently available germplasm, though several cotton varieties have been developed for different climatic conditions using conventional methods. Alternatively, the induction of variation and development of plants using plant biotechnological tools such as genetic engineering and molecular breeding has enabled the production of desirable crops for both vegetative as well as reproductive traits, and to cope up with a number of biotic and abiotic stresses (Gasser and Fraley, 1992). Extensive research program has been carried out on cotton germplasms, and subsequently genetic transformation with a variety of potential genes via direct injection of DNA into cotton immature embryos, *Agrobacterium*-mediated transformation, particle bombardment and combination of *Agrobacterium*-mediated with particle bombardment methods (Zhou *et al.,* 1983; Finer and Mullen, 1990; Rajasekharan *et al.,* 1996; Majeed *et al.,* 2000; Satyavathi *et al.,* 2002; Katageri *et al.,* 2007). Cotton crop is highly sensitive to salt stress leading to drastic decrease in the plant productivity (Bhomkar *et al.,* 2008).

A vast tract of agricultural land in India is presently non-arable due to excessive salinity and an effort to develop salt-tolerant *Gossypium hirsutum* is a prime target for ensuring increased productivity (Zhang *et al.,* 2014). Stress response in plants are generally complex and involve expression of many genes. The enzymes glyoxalase I and II are universally present in all organisms and play a vital role in the formation of 2-hydroxyacids using gulathione from 2-oxoaldehydes (Thornalley, 1993). Furthermore, overexpression of *gly I* gene in tobacco results in substantial tolerance of transgenic plants to high salinity and methylglyoxal stress, as related to the untransformed plants (Veena *et al.,* 1999). Higher salt concentrations in soil is one of the utmost severe factors amongst various abiotic stresses, restraining the productivity of agricultural and horticultural crops worldwide. In this context, the development of cotton varieties for improved abiotic stress tolerance, especially for salinity is essential for countries where cotton is cultivated as cash crop, Therefore, the establishment of efficient and reproducible protocol for developing transgenic cotton plantlets is indispensable. The current study was designed for *Agrobacterium*-mediated genetic transformation with *glyoxalase I* (*gly I*) gene for Indian cotton varieties.

Materials and Methods

Cotton (*Gossypium hirsutum* L.) varieties, MCU 5, MCU 12, MCU 13 were acquired from the Department of Plant Breeding and Genetics, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu. The study was performed at Plant Genetic Manipulation Lab, School of Life Sciences, Jawaharlal Nehru University, New Delhi.

Seed germination and culture conditions: Seeds of cotton varieties were treated with Bavistin (5%) for 20 min and meticulously rinsed with water and surface sterilized using mercuric chloride (0.1% w/v) for 7 min, and washed five times with sterilized water. The seeds were germinated aseptically on semisolid Murashige and Skoog (1962) medium (MS) in culture bottles (6.5×11cm) for germination. The shoot apices (3-5 mm) were removed carefully using sterile scalpel from *in-vitro* seedlings (5-day old) and inoculated on MS medium augmented with BAP and NAA, phytagel and/or agar. The seeds were maintained at 25 ± 2 ºC and 16/8-hr photoperiod with 50 µE m^{-2} s^{-1} fluorescence intensity.

Direct organogenesis: Soaked seeds and seedlings were used as source of explants such as embryo axes shoot apex, cotyledons and hypocotyls. Based on these primary experiments, the shoot tip explants were selected for further experiments. The shoot explants which responded were subcultured on same medium appended with GA_3 (1.0 mg l^{-1}) for proliferation and growth. Healthy shoot explants (5-6 cm) were further transplanted to half-strength MS medium fortified with IBA (0.5 mg l^{-1}) for root initiation.

Vector and bacterial strain: *Agrobacterium* strain (GV 3101) harboring *glyoxalase I* gene was used for transformation of explants. The *gly I* construct was obtained from the International Centre for Generic Engineering and Biotechnology, New Delhi, India. The isolation of plasmid DNA from *E. coli* was performed and the recombinant plasmids with *gly I* gene were moved into *Agrobacterium* (GV 3101) (Sambrook *et al.,* 1989). This construct included *npt II* (neomycin phosphotransferase gene) as plant selectable marker for kanamycin resistance, and gus (*β*-glucuronidase) as reporter gene regulated by 35S CaMV promoter.

Transformation and co-cultivation: *Agrobacterium* strain cultures in MS with BAP (0.1 mg l^{-1}) + acetosyringone (2.0 mg l^{-1}) were taken in 150 ml conical flasks. Pre-cultured shoot tips on liquid MS medium for 2 days were infected with *Agrobacterium* and incubated in a rotary shaker (100 rpm) at 28ºC for 20 min. The infected shoot tip explants were transferred to sterile petri plates

with moist filter paper soaked in BAP (0.1 mgl^{-1}) + acetosyringone (2 mgl^{-1}) and incubated at 25 ± 2ºC for 2 days as co-cultivation period. Uninfected shoot tip explants were processed and maintained as control.

Selection and plant regeneration: The co-cultivated shoot tips were rinsed once with sterile distilled water, followed by washes with cefotaxime (400 mg l^{-1}) and then washed with sterile water and subcultured on shoot initiation medium (MS) comprising NAA (0.00, 0.10, 0.50, 1.00 mg l^{-1}) and BAP (0.05, 0.10, 0.20, 0.30 mg l^{-1}) respectively in different combinations with kanamycin (75 mgl^{-1}) and cefotaxime (200 mg l^{-1}). Proliferation of shoots was achieved with BAP (0.01 mg l^{-1}), NAA (0.1 mg l^{-1}) and GA3 (1.0 mg l^{-1}) and the elongated shoots were subcultured thrice for selection.

The proliferated shoots on the antibiotic (kanamycin) selection medium were taken to be putative cotton transformants. Kanamycin-resistant, healthy and elongated (< 4 cm) shoots were transferred on medium with IBA (0.3 mgl^{-1}), kanamycin (75 mgl^{-1}) and cefotaxime (200 mgl^{-1}) for root induction. The well-rooted plantlets were hardened in plastic pots using autoclaved vermiculite. Initially, the plantlets were irrigated with tap water and after one week with Hoagland's solution for further growth and development.

Histochemical assay for GUS: *β*-glucuronidase activity was accomplished histochemically using putative transgenic and control shoot and leaf tissues (Jefferson *et al.,* 1987). Shoots and leaves from putative transformed and control plantlets were rinsed with water and incubated in GUS staining solution [1 mM sodium phosphate buffer pH 7.0, potassium ferricyanide and ferrocyanide (0.1 M), disodium EDTA (0.5 M) and X-gluc (1.0 mg l^{-1})]. After overnight incubation at 37ºC, chlorophyll was removed by immersing the tissues in 70% ethanol. These were observed and photographed. Transformation efficiency was evaluated as total number of GUS-positive shoots with total number of leaves/ shoot apex.

PCR analysis for *gus* and *gly I* gene: DNA was isolated from control and putative transgenic plantlets by CTAB method (Paterson *et al.,* 1993). PCR was performed for *uidA* (GUS) gene using specific primer sequences (5' – 3') GCC ATT TGA AGC CGA TGT CAC GCC and GTA TCG GTG TGA TGA GCG TCG CAG AAC. PCR was accomplished in 25 µl of reaction mixture comprising of 10µ reaction buffer, 4.0µl DNA (~100 ng), 2.5µl dNTPs, 1.0µl forward and reverse primers, 0.4µl Taq DNA polymerase and autoclaved distilled water. PCR was carried out in a thermal cycler under following conditions: 94°C for 5 min as preheating, 94°C for denaturing for 30 sec, 58°C annealing for 30 sec, 72°C synthesis for 1 min and 5 min at 72°C as final extension. Similarly, PCR amplifications of *gly I* gene was performed using specific primers sequences (5' – 3') CGG GGT ACC ATG GCG TCG GAA GCG AAG and TGC TCT AGA GCT CTC AAG CGT TTCC. PCR was executed in 25µl of reaction mixture consisting of 10µ reaction buffer, 1.0µl DNA (~100 ng), 2.5µl dNTPs, 1.0µl of *Gly I* primers, 0.4µl Taq DNA polymerase and autoclaved distilled water. PCR was performed in a thermal cycler under following conditions: 94°C for 5 min as preheating, 94°C for denaturing for 30 sec, 56°C annealing for 30 sec, 72°C synthesis for 1 min and 5 min at 72°C as final extension. PCR products of gus and *gly I* gene were electrophoresed on agarose gel (1.0 %) and photographed under ultraviolet light.

Statistical Analyses: The experiments were carried out with 25 explants from three varieties in triplicates. The analysis of variance (ANOVA) was performed to calculate mean and standard deviation and related using Duncan's Multiple Range Test for significance (DMRT).

Results and Discussion

Improvement of cotton crop for fibre, seed oil content, increased gossypol content in vegetative parts and reduction of gossypol content in reproductive parts, abiotic stress tolerance as well as disease resistance are priority areas of research in cotton breeding programs. Induction of genetic variations in cotton using conventional breeding is restricted majorly by dearth of useful variation and time intense process. Currently, genetic engineering is a fascinating technique, which requires effective *in-vitro* regeneration methods to develop transgenic plants with improved agronomical characters. Effective regeneration protocols have been established for various commercial crops. However, only few regeneration protocols are available for Indian cotton varieties since they are recalcitrant. Although cotton continues to remain a recalcitrant crop, transgenic plants of several cotton varieties have been successfully developed to enhance disease resistance (Perlak *et al.,* 1990) and abiotic stress tolerance (John, 1997; Rajasekharan *et al.,* 1996).

There are very few reports available on the development of transgenic Indian cotton varieties (Kumar *et al.,* 1998; Ganesan *et al.,* 2009; Sumithra *et al.,* 2010a, b; Sangannavar *et al.,* 2011a, b; Sangannavar *et al.,* 2016) for both biotic as well as abiotic stress tolerance. Several factors can have a profound influence on gene transfer of a given plant species using *Agrobacterium tumefaciens*. Therefore, in this study we have evaluated transformation efficiencies and established gene transfer methods for Indian cotton towards the improvement of abiotic stress tolerance. Amongst different explants, the shoot apex with trimmed cotyledon exhibited maximum response for shoot induction. Subsequently, these shoots were excised and transferred onto MS medium augmented with BAP (0.1 mg l^{-1}) and NAA (0.1 mg l^{-1}) for shoot proliferation.

The growing shoots were subcultured 2-3 times on above mentioned medium where shoot proliferation was achieved. Shoot induction was achieved with differential percentage of responses which ranged between 52.14 and 98.20 with BAP and NAA (0.1 mg l^{-1}). Induction of multiple shoots was noted during second subculture with 1-4 multiple shoots. MCU 5 variety showed maximum percentage of shoot initiation amongst the three varieties tested (Table 1 and 2). The shoots were cultured on

Table 1: Comparison of effect of auxin with cytokinin on percent shoot responses and frequency of shoots per explant in shoot tip culture of cotton varieties

Hormones (mg l^{-1})		Cotton varieties		
NAA	**BAP**	**MCU 5**	**MCU 11**	**MCU 13**
0.00	0.05	52.50 (3)^{bc}	50.17 (2)^{d}	53.45 (2)^{bc}
	0.10	54.32 (1)^{b}	56.18 (1)^{c}	52.56 (2)^{c}
	0.20	64.38 (1)^{a}	62.32 (2)^{a}	60.23 (1)^{a}
	0.30	52.14 (1)^{c}	58.64 (1)^{b}	53.58 (1)^{b}
0.10	0.05	73.21 (2)^{d}	64.32 (2)^{d}	66.48 (2)^{c}
	0.10	98.20 (3)^{a}	94.84 (2)^{a}	90.26 (2)^{a}
	0.20	84.32 (2)^{bc}	82.17 (1)^{b}	85.12 (1)^{ab}
	0.30	86.60 (1)^{b}	80.16 (1)^{bc}	79.23 (1)^{b}
0.50	0.05	70.67 (2)^{c}	58.17 (2)^{d}	63.29 (2)^{c}
	0.10	66.42 (2)^{d}	63.17 (1)^{c}	68.10 (1)^{b}
	0.20	78.43 (1)^{a}	76.80 (2)^{a}	82.49 (1)^{a}
	0.30	74.10 (1)^{b}	72.17 (1)^{ab}	66.39 (1)^{bc}
1.00	0.05	82.89 (2)^{c}	60.17 (2)^{d}	66.28 (2)^{cd}
	0.10	78.16 (2)^{d}	70.16 (2)^{c}	69.12 (1)^{c}
	0.20	88.23 (1)^{a}	78.68 (1)^{b}	80.20 (1)^{a}
	0.30	86.42 (1)^{ab}	88.28 (1)^{a}	78.56 (1)^{ab}

The values in parentheses indicate the number of shoots per explants. 1: Single shoot; 2: two shoots; 3: three shoots or more per explant. The data on percent shoot responses and frequency of shoots were subjected to ANOVA and mean separation was carried out adopting DMRT. Mean values within a column having the same alphabet are not significantly different (p = 0.05) according to DMRT and data are mean of three replicates

Table 2: Comparison of shoot elongation of cotton varieties with 0.1 mgl^{-1} BAP and NAA and 1.0 mg l^{-1} GA

Variety	Number of shoots in elongation medium	Number of elongated shoots	Percentage of shoot elongation	Height of shoots (cm)	Number of shoots in rooting medium	Number of rooted shoots	Percent of rooting
MCU 5	98.0	80.86^{a}	82.10^{b}	7.4^{a}	80.0	65.26^{a}	81.15^{a}
MCU 12	96.0	79.64^{ab}	82.77^{ab}	6.9^{bc}	74.0	55.78^{b}	75.37^{b}
MCU 13	94.0	78.46^{b}	83.46^{a}	7.0^{b}	70.0	50.46^{c}	72.08^{c}

Data on number of elongated shoots, percentage of shoot elongation and height shoots were subjected to ANOVA and mean separation was carried out adopting DMRT. Mean values within a column having same alphabet are not significantly different (p = 0.05) according to DMRT and data are mean of three replicates

Table 3: Transformation efficiency of Indian cotton varieties

Varieties	Sample	Number of explants	Shoots on selection medium	Transformation efficiency (%)
MCU 5	I) Control	50	0	00.00
	ii) Transformed	815	526	64.53
MCU 11	I) Control	50	0	00.00
	ii) Transformed	725	409	56.41
MCU 13	I) Control	50	0	00.00
	ii) Transformed	735	357	48.57

[a]Expressed as number of explants surviving on 75 mg l^{-1} kanamycin in the medium

MS medium with activated charcoal (0.1%) for two weeks and later the shoots were transferred on charcoal free MS medium augmented with BAP, NAA (0.1 mg l^{-1}) and GA_3 (1.0 mg l^{-1}) for multiplication and shoot elongation. The responded shoots were cultured for further multiplication, after 2-3 passage of subculture, the multiple shoots were transplanted on medium appended with GA_3 (1.0 mgl^{-1}) for shoot elongation. Elongated shoots (<4 cm) were subcultured for root initiation onto medium augmented with IBA (0.3 mgl^{-1}). Number of shoot elongation ranged from 82.1 to 83.46 % in MS medium with charcoal for 2 weeks and alternate

with charcoal free MS medium with hormones (Table 2). Amongst several combinations of cytokinins and auxin (Table 1) tested for direct organogenesis, 0.1 mg l^{-1} NAA and BAP showed highest responses in shoot initiation, followed by BAP and NAA (2.0+1.0 mg l^{-1}), BAP and NAA (2.0+0.5 mg l^{-1}) and BAP (0.2 mg l^{-1}). The NAA and BAP combinations resulted in shoot initiation in all varieties and the response was higher in MCU 5 than other varieties (Table 1, 2). Comparable results were described earlier by shoot tip culture and development of plantlets (Gould *et al.,* 1991; Hemphill *et al.,* 1998; Zapata *et al.,* 1999) with lower percentages, whereas by Satyavathi *et al.* (2002) and Muthusamy *et al.* (2004) and Khatoon *et al.* (2014) described higher percentage of plantlet regeneration. Plantlets with well-established roots were transplanted in plastic cups with autoclaved soil for hardening.

Fortification of GA_3 (1.0 mg l^{-1}) along with BAP and NAA (0.1 mg l^{-1}) showed marginal proliferation in number of shoots and shoot elongation. Higher proportion of shoot elongation was observed in MCU 5 and the shoot height increased to 7.4 cm (Table 3). The responses of shoot initiation, elongation and rooting in all eight different genotypes were significant according to DMRT. The developmental stages of transgenic plantlets of MCU 5 were observed with reference to the control. Although the explants remained green during *in-vitro* culture, some of the shoots showed 10-15 mm. Putative transgenic shoots were transplanted on MS (½ strength) with IBA (0.3 mg l^{-1}) for root initiation. Root initiation was noted in 15-20 days of subculture and percentage of rooting differed with different genotypes (Table 2).

The range of transformation efficiency from 48.57 to 64.53 % was observed in MCU varieties. Well-rooted putative transgenic and control plants were hardened in plastic cups and molecular analysis was carried out for gus and *gly I* gene expression. The shoots, whole leaf or segments from T0 transgenic as well as control plants were stained in X-glue for histochemical GUS assay. The GUS expression was detected in shoots (Fig. 1a), whole leaf (Fig. 1b) and leaf segments (Fig. 1c-d) of putative transgenic lines whereas no GUS activity was noted in shoots, leaf and segments from control plants. Similar *gus* positive transgenic lines was reported using *gly I* gene in *Vigna mungo* (Bhomkar *et al.,* 2008). Further, PCR analysis was performed with shoots, which showed kanamycin resistance for PCR analysis of transgenic lines. Majority of the shoots from T0 plantlets exhibited amplification of 1031 kb fragment of the gus gene (Fig. 2a) and 800 kb fragment of the *gly I* gene (Fig. 2b). No PCR amplification was observed in untransformed controls plantlets.

The shoot growth of cotton varieties on selection medium showed differential transformation frequencies with in the range of 48.57-64.53 %. Our results are in conformity with 60-70% transformation efficiency in cotton (Satyavathi *et al.*, 2002). Further, the present observations corroborates with genetic transformation of cotton by Banerjee *et al.* (2002), Katageri *et al.* 2007, Ganesan *et al.* (2009), Sangannavar *et al.*

Fig. 2: Histochemical detection of GUS gene expression in shoots, whole leaf and segments: (a) control (left) and transformed shoots (right); (b) whole leaf transformed (left) and control (right); (c-d) Leaf segments showing characteristic blue coloration (left) when incubated overnight in Gus buffer at 37°C. No GUS activity seen in untransformed (control, right) tissues.

(2011a, b; 2016). In contrast, 6.5 and 9.6 % of transformation efficiency was reported in cotton (Finer and McMullen, 1990; Majeed *et al.,* 2000) and 12% of *Gly I* gene transformation efficiency was reported in *Vigna mungo* (Bhomkar *et al.,* 2008). The shoots and leaf from putative transgenic plantlets were

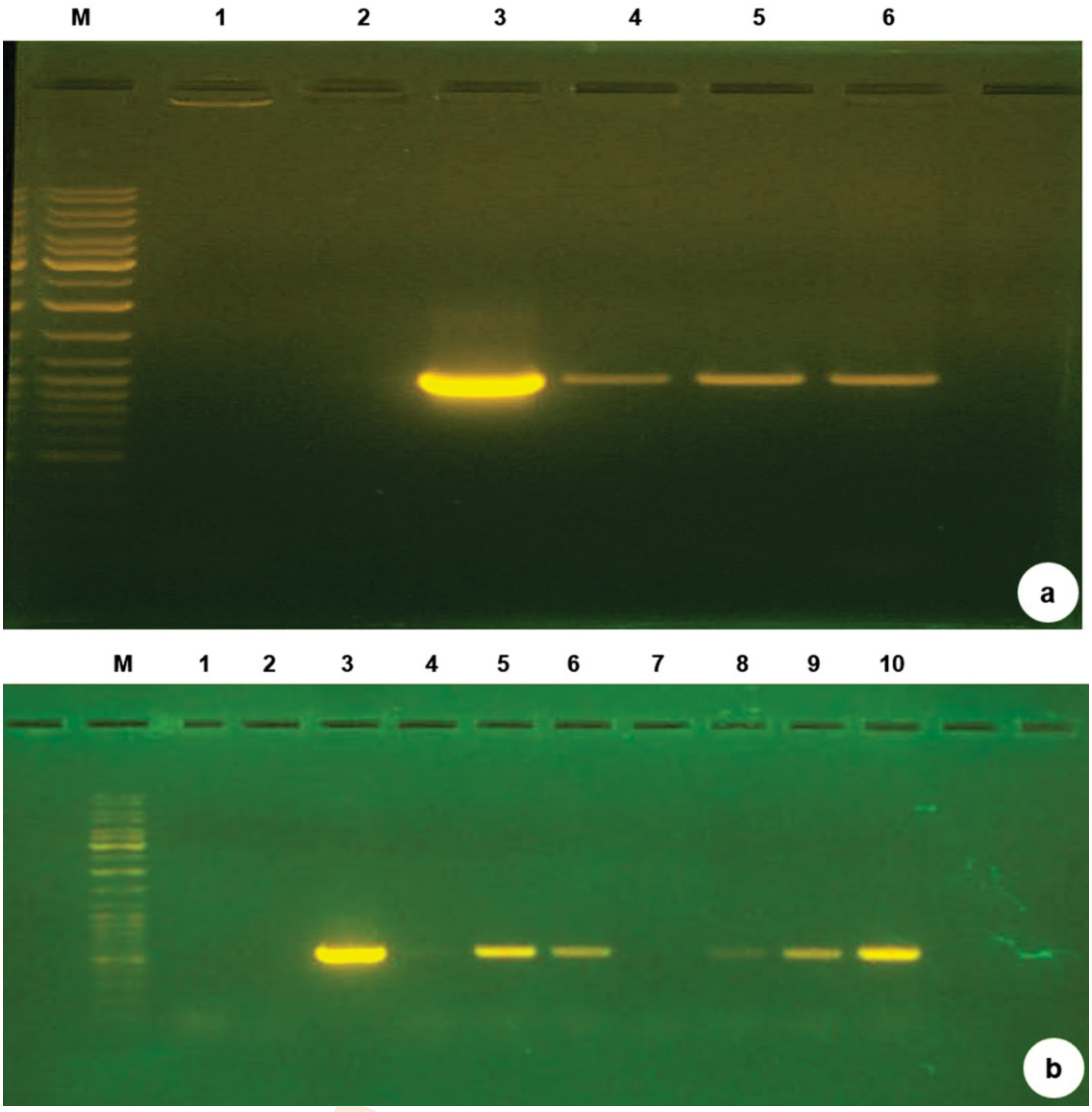

Fig. 2: PCR analysis of T0 transgenic lines of cotton with *GUS* gene specific primers; (a). Lane M: molecular size marker; lane 1: untransformed control; lane 2: negative control; lane 3: positive control (DNA from plasmid pBI-S2); lane 4-6 putative transgenic cotton and PCR analysis of T0 transgenic lines of cotton with *Gly I* gene specific primers; (b) Lane M: DNA ladder, lane 1: untransformed control; lane 2: negative control; lane 3: positive control (DNA from plasmid pBI-S2); lane 4-10 putative transgenic cotton plantlets.

positive whereas tissues form untransformed control showed negative for *GUS* and *Gly I* gene. The *GUS* positive putative transgenic plantlets were maintained, PCR was done for the occurrence of *gly I* gene and the selected putative transgenic lines showed affirmative for 0.8 kb *Gly I* gene.

The PCR results revealed the presence of *gly I* gene in transgenic lines. Further experiments will be required for the confirmation of integration of gene using Southern blots and RT-PCR, southern and northern analysis, physiological analysis and tolerance test with transgenics of cotton. Similarly, Ganesan *et al.* (2009) reported the development of Indian cotton with rice Chitinase gene (*Chi II*) for fungal resistance. Recently, Siddiqui *et al.* (2019) reported double transgenic cotton with *Cry1Ac* and *Cry2Ab* for durable resistance against bollworms. In conclusion, the effort was made in the study to transfer the gly I gene into Indian cotton varieties via *Agrobacterium*-mediated transformation.

The present study proved the possibility to transform novel Indian cotton varieties and regeneration of plantlets, and successful standardization of protocol for development of transgenic cotton with *gly I* gene for abiotic stress tolerance.

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Add-on Information

Author' contribution: A. Muthusamy: Plan of the study, experiments, data analysis, preparation of tables and figures, preparation and revision of manuscript.

Research content: The research content is original and has not been published elsewhere

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