Development of Drought Tolerant Transgenic Cotton through *Agrobacterium* Mediated Transformation

Sarosha Nasreen[1], J. Amudha[2], S. S. Pandey[3]

Rajiv Gandhi Biotech Centre, Rashtrasant Tukdoji Maharaj Nagpur University, L.I.T. Premises, Nagpur-440 033, Central Institute for Cotton Research, Post Box No: 2, Shankar Nagar (PO), Nagpur, 440 010

Abstract: ***The growth and productivity of cotton (Gossypiumhirsutum L.) are adversely affected by abiotic stresses such as dehydration. Plants adaptation to these stresses is controlled by cascades of molecular networks, including the dehydration responsive element binding (DREB) transcription factors, which specifically interact with C-repeat/DRE (A/GCCGAC), play an important role in plant environmental stress tolerance by controlling the expression of many stress related genes. In this work, an optimized cotton regeneration system from shoot apices was developed. The hypocotyl explants were transformed with Agrobacterium tumefaciens strain LBA4404 harboring the recombinant binary vector containing the DREB gene and Neomycin phosphotransferase (nptII) gene as a selectable marker under the control of CaMV 35S promoter. New putative plantlets were subsequently regenerated and confirmed by Polymerase chain reaction and southern blot analysis.***

Keywords: Abiotic stress, DREB, *Gossypiumhirsutum*. Stress tolerance. Transgenic plants

1. Introduction

Cotton (*Gossypium*spp.) a crop of global importance so called as cash crop. Worldwide area is estimated as 34.5 million hectares (85.3 million acres), in 2018-19 it was 4% and the highest in 8 years. The world yield is also forecast higher in 2019-20, at 791kilograms per hectare (706 pounds per acre) (www.indoasiancommodities.com).Cotton being an excellent natural source of textile fibre, cattle feed and edible oil it is cultivated in more than 90 countries (http://www.isaaa.org) all around the world. Its production is adversely affected by biotic and abiotic stresses, viral and fungal infections, and insect predation.Major concern all over the world has been to protect it from these stresses mainly drought, a single major problem for cotton and other crops causing yield damage to the extent of 67%. Critical effortsfor enhancement of improved quality tolerant to drought and for increasing the protein content of the cottonseed oil is achieved with improved productivity by genetically modifying cotton plants.

There are many study reports on the *in-vitro* regeneration of cotton pertains to varieties of *G. hirsutum* which are not highly cultivated due to many stresses including drought. This *in-vitro* regeneration of the Indian cotton cultivars has beenof great importance. A very crucial prerequisite for the production of these transformed plants is the development of optimized regeneration protocols for cotton cultivars. In past two decades, an extensive research efforts have been madeusing different advanced biotechnological techniques. Various protocols have been explored for the transformation of cotton plants. For transgenes to be delivered in the cultured plant tissues of cotton, two methodologies are most efficient namely particle bombardment (McCabe and Martinell 1993, Finer and McMullen 1990; Rajasekaran et al. 2000) and co-cultivation with *Agrobacterium tumefaciens* (Firoozabady et al. 1987; Umbeck et al. 1987). Both these methodologies produced transgenic plants, with different degrees of efficiency. Other methods have also been reported such as meristem transformation (Gould et al. 1991; Zapata et al. 1999), ovule culture (McStewart and Hsu 1977; Thengane et al. 1986), protoplast culture (Peeters et al. 1994), somatic embryogenesis (Shoemaker et al. 1986; Trolinder and Good in 1988; Finer 1988). Advancements have been made for transformation, regeneration, and genetic enhancement of cotton, especially in *Gossypiumhirsutum*. Some very crucial genes conferring agronomic advantages have been integrated inthe plant through *Agrobacterium* mediated process or through particle gun method, also the plants were regenerated through callus-based somatic embryogenesis (Firoozabady et al. 1987; Umbeck et al. 1987; Perlak et al. 1990; Finer and Mc Mullen 1990; Bayley et al. 1992; Thomas et al. 1995; Rajasekaran et al. 1996; Zhao et al. 2006).

The particle bombardment method is not only a costly process but also the transformation efficiency reported was very negligible as 1 transgenic plant per 1,000 bombarded explants (McCabe and Martinell 1993). Bombardment provides a means of introduction of foreign genes into an elite variety of cotton. Particle bombardment procedures involving somatic embryogenesis utilizes the embryogenic cell suspensions for the bombardments. Different varieties like Coker have been transformed by this procedure (Rajasekaran et al. 1996, 2000). However, the maintenance as well as cryopreservation of embryogenic suspensions are skilled procedures and have notbeen widely used by many laboratories (John 1997).

Moreover, the *Agrobacterium*-mediated transformation procedures, which uses hypocotyls or cotyledonary leaves as explants for transformation is economical, rapid and much less laborious than conventional methods. In *Agrobacterium*- mediated transformation the regeneration via somatic embryogenesis has been a very common method and successfully applied for the development of transgenic cotton. This methodology involves the transformation of regenerable cells or callus tissues that were derived from the

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explants with integrated genes, and the regeneration of putative transgenic plants through somatic embryogenesis. The regenerating embryogenic line, once established, can be routinely sub-cultured and used for multiple transformations with a short culture period, while efficiently converting higher number of transformation events into transgenic regenerants. An independent transgenic line is generated from each transformed cell. These regenerable tissues possess higher transformation frequencies with reduced overall time period significantly. Although it is a multi-step laborious process, consuming around a 10–12 month period (Mishra et al. 2003), but is an efficient method which begins with co-cultivation of cotyledonary or hypocotyl explants with *Agrobacterium*, followed by production and maintenance of several hundreds of new putative plants derived from an independent transformation event on an optimized antibiotic selection medium.

The meristem transformation method of a genotype-independent plant is extremely laborious but generates a high frequency of chimeras (McCabe and Martinell1993; John 1997; Keller et al. 1997; Satyavathi et al.2002). Meristematic zone contains a limited number of cells so a large number of meristems have to be transformed, within these, regenerable cells are even less abundant, therefore the explants responding on selection medium is less (Firoozabady et al. 1987; Sunilkumar and Rathore 2001).

The transformed status of the plants can be analyzed only in the next generation if the cultivar is not amenable to induction of multiple shoots and axillary branching (John 1997). The regeneration of normal plants in cotton have been done with the development of an efficient procedure from somatic embryos and by subjecting these embryogeniccalli to nutritional stress as well as dehydration (Kumria et al. 2003). *Agrobacterium*- mediated transformation, using embryogeniccalli of cotton as an explant, to introduce the gene was observed in many other plants like tobacco with a stably integrated gene (Selvapandiyan et al. 1998) indicating that embryogenic callus provides a large population of embryogenic competent cells that are extremely amenable for transformation.

A variety of different hormonal regimes were used to cope-up most of the difficulties in obtaining transgenic cotton, associated largely with its regeneration. This will be beneficial to improve the transformation efficiencies so that a large number of independent transgenic callus lines can be obtained from which good regenerable lines can be selected. In this study we have examined several factors that can significantly affect the efficiencies of transformation and regeneration for better results.

In our study, we described a simple, rapid, and efficient protocol for transformation and regeneration of transgenic diploid cotton plants (*G. hirusutum*) using meristematic tissues of shoot tips as a source of explants. This is the first report of transformation of a DREB gene into an elite variety of diploid cotton (*G. hirusutum*).

2. Materials and Methods

Plant seed material

Plant seed material of *G. hirsutum* variety LRA 5166, a popular commercial cultivar with highly desirable agronomic traits, but susceptible to many biotic and abiotic stresses was used in this study. Cotton seeds were delinted with concentrated sulfuric acid (100 ml/kg) then were disinfected by Clorox method. The seeds were treated with 70% ethanol for 2 minutes prior to a20 minute exposure to 10% Clorox ® (5.25% sodium hypochlorite (NaOcCl) solution with few drops of Tween 20 per 100 ml, and rinsed thrice with steriled double distilled water. The flask of seeds was agitated on orbital shaker for 30 min at 120 rpm followed by three washes of autoclaved double-distilled water. Seeds were then treated with 0.1% mercuric chloride solution (0.1% w/v) for 10 min followed by washing again with autoclaved double-distilled water. After surface disinfection, the seeds were air dried in front of flame in laminar, then around 50 treated seeds were placed on seed germination medium. This was replicated three times. The seed germination medium contained 4.3g Murashige and Skoog (MS) salts (Sigma) (Murashige and Skoog, 1962) per litre, along with 5% sucrose and 0.6% agar (Sigma, USA) supplemented with 100 mg/Lmyo-inositol, 10 mg/L thiamine.The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC for 20 minutes on which15 to 20 seeds were placed in each flask. The seeds were incubated in the dark at 25ºC for 2 days, then were shifted in light with pre-decided16:8 h photoperiod. Upcomings from incubation, the number of elongated shoots were counted. The fungal infection and / or bacterial growth contamination was observed visually.

Selection of Shoot Apex as Explant

Shoot apices were isolated from 7 to 11days old seedlings with the aid of a dissecting microscope. The seedling shoot apex was exposed by cutting down one cotyledon.The cotyledonary leaves were gently removed from the seedlings with the help of sterile forceps, and a “V”-shaped oblique excision obtained described by Gould and Magallanes-Cedeno (1998) was used for culturing. All extraneous tissues surrounding the meristematic dome were removed for efficient *Agrobacterium* infection.Hypocotyl segments of 7 mm excised from 8 days old seedlings wereused for transformation and regeneration studies.

Bacterial Culture

The *Agrobacterium tumefaciens* strain LBA4404 harboringp Cambia 1300 with neomycin phosphotransferase *(nptII)* as a selection marker (Ooms et al. 1982) was grown overnight at 28°C in medium (pH 7.0) supplemented with 50 µg/mL kanamycin used for transformation and regeneration experiments. The binary vectors were mobilized into *Agrobacterium* by the heat shock method (An et al.1988). The bacterial culture was maintained on YEM medium plates containing rifampicin (10 mg/L) and 70 mg/L kanamycin. For inoculation of explants, a single colony of bacterial cells was grown overnight in liquid YEM broth at 28°C with continuous agitation.Around 10single colonies were inoculated individually in 10 mLYEM medium containing rifampicin (10 mg/L) and kanamycin (70 mg/L) in tubes and were grown for36 h at 28°C with 150 rpm

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shaking. Cells from 5tubes were pooled, harvested by centrifugation, and resuspended again in 50 ml of pre-induction medium (2%glucose, 7.5 mM MES, 2 mM sodium phosphate buffer pH 5.6, AB salts (Chilton et al. 1974) containing100 μMacetosyringone (Aldrich). The culture was again made grown for 24 h at 28◦C on ashaker at 150 rpm. Additional acetosyringone was added to the culture at a final concentration of 100 μM just prior to co-cultivation of explants began.

Transformation and Co-cultivation of explants by *Agrobacterium* Infection

The selected explants were inoculated with an overnight-grown culture of *A. tumefaciens* harbouring DREB gene at 28°C with continuous agitation. Agro-infection of explants can be done by different methods. For instance in one method, the explants are kept in 50 mL of half-strength MS medium inoculated with 500µl of bacterial cells and supplemented with acetosyringone for an efficient integration of the gene into the explant. These were gently agitated on an orbital shaker at 120 rpm for 45 min. In another method the shoot-tip explants, were inoculated in flask containing a semi-solid half-strength MS medium for overnight to find out if any contamination occurs. Around 5µl/explant of the bacterial culture, grown in half-strength MS medium, supplemented with 100 µMacetosyringone, were dispensed over explants drop-by-drop (Gene-Ghould method). These were sealed with Para-film and incubated at 28°C.

Explants free from any contamination were selected and further co-cultivated for 7 days separately on a medium consisting of MS, 0.1 mg/L kinetin, 10 mg/Lmyoinositol, 10 mg/L thiamine, and 30 g/L glucose and solidified with 6.5 g/L phytagel.The explants were then transferred to fresh MS medium containing 50 mg/Lkanamycin and 400 mg/L ciprofloxacin antibiotics and incubated at 28°C, under a 16 h photoperiod for 15-20 days. The kanamycin resistant explants growing here represents stably transformed individual plants (Figure 1B).These individual explants were maintained on this medium for 12 weeks (16 h photoperiod) with a regular subculture to fresh medium for every four weeks. Subsequently, explants were subjected to screening on the same medium described above, but containing 30 mg/L kanamycin for first three passages of 10 days each. To promote shoot elongation, shoots (or explants with developing shoots) were transferred to MS medium supplemented with 50 mg/L kanamycin, 0.1 mg/L kinetin, and 0.1 mg/L gibberellic acid or 2 mg/L BA and 50 mg/L kanamycin for multiple shoot formation.

Shoot Elongation and Rooting Development:
The isolated shoot apices from cotton variety were placed on MS medium supplemented with 0.1mg/L Kinetin (Gould *et al.*, 1991) for two weeks to induce shoot elongation. The number of elongated shoots were recorded and then the shoots were transferred to fresh MS medium for rooting. After three weeks, the number of rooted shoots were recorded. The rooted shoots were then transferred to Magenta boxes containing MS medium and incubated in a culture chamber (28°C) for 10 to 15 days which were then transferred to the greenhouse. The shoots without root development were subjected to an IAA shock at different concentration (0.1 to 2.0 mg/ml) for one minute. These treated shoots were then transferred to fresh MS medium for another two to three weeks. The number of rooted plants was recorded and the rooted plants were transferred to Magenta boxes containing MS medium and incubated in a culture chamber for four weeks before being transferred to the greenhouse. The remaining shoots without root development were then grafted to a germinated seedling of the same variety, in the MS medium contained 4.3g/L MS salts (Sigma), and 1 ml/L MS vitamins (Sigma).

Multiple Shoot Induction
For multiple shoot induction the growing explants from kanamycin medium were selected and transferred to a medium consisting of MS base supplemented with B5 vitamins, glucose (30 g/L), myoinositol (100 mg/L), thiamine (10 mg/L), BA (2 mg/L), and kinetin (1 mg/L) for multiple shoot induction in *G. hirsutum* (Nandeshwar et al. 2002).The individual shoots with four to five nodes were individually transferred in medium meant for rooting.The plantlets with 5–7 cm long shoots along with new tender leaves and roots were transferred to MS medium composed as1/2× MS salts, 2% glucose, 0.1 mg/L thiamine,0.1 mg/L pyridoxine, 0.1 mg/L nicotinic acid, maintained at pH 5.8 solidified with 0.6% Phytagel, incubated at 25°C, under16 h photoperiod.

Hardening
The plantlets with around 10 cm long shoots with well established root system obtained by multiple shoot induction was first washed with sterile double-distilled water, the roots were then dipped in0.1% (w/v) aqueous solution of fungicide, bavastin for 2 min and transferred to liquid MS medium supplemented with 0.05 mg/L NAA and 20 g/L glucose. The plantlets were maintained at light intensity of 3,000–4,000 lux for 3-5 days with a photoperiod of 16:8 hrs. They were then transferred to plastic pots containing sterilized soil rite (Figure 3A) and kept under optimized humidity and temperature conditions in growth chambers for further10 - 15 days, then gradually hardened in growth chambers (28°C, 16 h photoperiod)for 2–3 weeks. They were finally transferred to large pots containinghorticultural grade expanded soilrite mixed with soil and sand in 40:50:10and grown to maturity in the greenhouse (Figure 3B).
Molecular Analysis of Gene Integration

Genomic DNA extraction from transgenic plants:
Genomic DNA was extracted following an optimized protocol. The upcoming tender leaves ofputative transgenic cotton plants were grounded in liquidnitrogen in pre-chilled pestle mortar. 0.1gm of the grounded tissue was again grinded for genomic DNA extraction in 2 mlof freshly prepared extraction buffer (100 mMTris–HCl pH 7.8, 10mM EDTA Na_2, 500mMNaCl, water) in a 2mleppendorff tube. The mixturewas again homogenized in 1mlof suspension buffer (50 mMTris–HCl pH 8.0, 10mM EDTA Na_2 and water) and 100 µl of 20% SDS and 130 µl 20% PVP was added, which was mixed by inversion and incubated at 65°C for 30 min withoccasional shaking. After this incubation tubes were brought to room temperature and 7.5 mM Ammonium acetate was added upto the rim and again kept for incubation in ice for an hour, then centrifuged for 15

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min. To the aqueous layer added 6/10th volume of prechilled isopropyl alcohol and kept for overnight incubation at -20°C for an efficient DNA precipitation. After incubation it was centrifuged and the pellet was dissolved in 25µl of TE buffer along with 10µg of RNase was added and kept at 35°C for 15 min and again centrifuged at 14000 rpm for 20 min.

For the purity of DNA extractedan equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, producing an emulsion by gently inverting the eppendorff few times and transferring the upper aqueous layer to a fresh tube following centrifugation at 15,000×g for 10 min at 4°C.Following precipitation, the DNA was spooled gently and carefully with the help of pipette after allowing the tube to stand at−20°C for 30 min. The spooled and the pelleted DNA was rinsed with 70% alcohol twice, air dried, and dissolved in 100 µl of molecular grade TE. The purity of DNA was checked by obtaining absorbance ratios at A260/A280 and A260/A230. Concentration of DNA in the sample was calculated by computing the value of absorbance at A260.

Detection and molecular confirmation of DREB gene by PCR

Standardization of PCR Conditions

In order to get a single sharp amplicon band corresponding to *DREB3* gene, itwas prerequisite to optimize PCR components and thermal profile conditions. For this the primers synthesized at SIGMA-ALDRICH Pvt. Ltd. Bangalore, was used with an efficient 2.5pM concentration. The temperature of 63.9°C was found to be the best annealing temperature. At this temperature a very sharp band was observed in Biometra machine. Reaction mixture for PCR amplification was standardized and the master mix of different components used in PCR was prepared freshly to avoid any handling errors with electronically operating pippetes for better results. The master mix (20µl) was distributed in each of the tubes. A high fidelity, proofreading TaqDNA polymerase enzyme which could amplify up to 5kb fragment, Taq B assay buffer and dNTPs from Bangalore Genie Pvt. Ltd., Bangalore, India, were used. Slight modification was done in routinely used PCR condition, as proofreading polymerase requires extra time in amplifying the target fragment. The primer pair was amplified with all genotypes and also a NTC (No template control) was kept to decipher the intensity of primer dimmers.

Presence of the transgene in transgenic cotton plant was ascertained by polymerase chain reaction (PCR) for amplification of a 630 bpdreb gene fragment using a set of designed oligonucleotide primer with the sequence
DREB-FP5' CCCTCTAGAGAATTCATGGCGAAACCCAGCAGC3'
DREB-RP5' CCCCTCGAGCGGCATTTCCGGCACATA3'.

The PCRwas performed in 20 µl master-mix volume. Each reaction consisted of 1.5 µl of 150 ng/µlpurified DNA template, 1.5 µl of 10XTaqbuffer B, 1 µl of 1mMdNTPs, 1.8 µl of 25 mM $MgCl_2$,1 µl each of 2.5 pM forward and reverse primers, 0.5 µlof 5 U/µlTaq DNA polymerase (Bangalore Genie Pvt. Ltd., Bangalore, India),and 11.2 µl sterilized molecular grade distilled water (Sigma). The PCR protocol was standardized with the initial denaturation at 94°C for 5 min followed by 36 cycles including of denaturation at 94°Cfor 45 s, primer annealing at 63.5°C for 1 min, and primer extension at 72°C for 1 min and a final extension at 72°Cfor 5 min. with a pause. Negative controls were maintained for non specific amplification, if any.

Electrophoresis:

All the PCR amplicons were made to run on 1.5% agarose gel containing 2.5µl of ethadium bromide (10mg /ml). PCR products of 9µl was mixed with 1µl of 6X gel loading dye and loaded in the wells. The gel was run at 80V current (constant) to separate the amplified bands. The 100bp/50bp standard DNA marker (0.5-1.5µl) was also run along with the samples. The separated bands were scored and documented under UV trans illuminator using a gel documentation system (Bio-Rad, California).

At the end of PCR programme, electrophoresis was done to get highly resolved and separate amplified product. About 10 µl of PCR product from each tube along with 2 µl of loading dye was separated on 1.5 percent agarose (Invitrogen, Laboratories Pvt. Ltd.) gel along with the DNA double digested with *Hind*III/ *Eco*RI (Bangalore Genie Pvt. Ltd, Bangalore). Gel was prepared using 1x TAE buffer from 50ml TAE buffer and agarose. The electrophoresis was done at 70 V for 45 min. The gel was observed under a mid-range UV transilluminator and gel image was documented using gel documentation system (UVtec, Cambridge, England) (Figure 3A).

Detection of Transgene integrated in transformed putative cotton plants by Southern Hybridization

Southern blot analysis of genomic DNA extracted from transgenic cotton plant with dreb gene, non-transformed plants and plasmid pCambia was detected in the two transformed plants. This was consistent with the restriction map of pCambia, which has *Eco. RI* sites. No hybridization was detected in the non-transformed control plants.The total DNA (10 µg) was subjected to digestion with *Eco.RI* restriction enzyme. Following electrophoresis, the DNA was stained with ethidium bromide and examined under UV light for the band pattern. TheDNA fragments digested by *Eco.RI* were transferred on positively charged nylon membrane (Biodyne B, USA) following the transfer in alkaline solution of 0.4 N NaOH (Sambrook et al. 1989).

The DREB gene fragment present in plasmid pGemT-DREB (Promega, USA) was used as DNA probe for hybridization. The fragment was excised and purified using gel extraction kit (Qiagen, Germany) as per manufacturer's protocol. Probe was labeled with digoxigenin DNA labeling and detection kit (Roche, Germany). Genomic DNA in the blot was further hybridized with DIG labelled probe at 68°C for 24 h in hybridization chamber. The hybridized probes were detected on the nylon membrane (Figure 3B).

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3. Results

Germination of seeds into seedlings

The seeds of *G. hirsutum* cv. LRA5166 gave healthy and contamination free *in-vitro* germinated seedlings for isolation of shoot-tip explants after 7-8 days for transformation purpose. Out of 5,730 seeds of G. hirsutum cv. LRA5166cultured, 3,533 seeds were germinated. Cotton plant transformation and recovery of transgenics.

The meristematic explants 0.5-0.7 mm in diameter developed from the hypocotyl segments were co-cultivated with *Agrobacterium* culture in 10 to 15 days after incubation on MS medium containing kanamycine and phytohormones. After third passage of sub-culturing in kanamycin selection medium, the method that employed co-cultivation of 2,371inoculated explants, resulted into regeneration of 73 putative transformants. Thus second method of transformation gave efficient transgenic seedlings. In the initial cycles of passage, explants survived on kanamycin selection were large but drastic reduction in the survival of explants was observed during subsequent passages of sub-culturing. These marestemeticaxes germinated into normal seedlings and these putative independent transgenic plants with well developed leaves and root systems were transferred to soil and left to flower and set seeds under green-house conditions. The seedlings could withstand infection and about 70% of the agro-infected seedlings survived after infection and developed into plantlets. About 80% of the subjected seedlings to *Agrobacterium* infection survived which were transferred to the greenhouse. After a period of 4 months independent, putatively transformed shoots were selected out of 5,443 explants used in co-cultivation and35 plants survived, attained maturity, flowered and set seed with well developed leaves and root systems were transferred to soil under green house conditions. The untransformed controls cotton plants matured earlier.

Integration and expression of the DREBgene into cotton plant :

Twenty five T0 plants from kanamycin resistant medium were randomly selected, further confirmed and analyzed by PCR. 17 out of these plants were found *nptII* and specific positive. Transformants of these plants yielded a single prominent gene band specific for DREB gene with a 628 bp size (Fig 4A). As a control untransformed cotton plant was used which gave negative result for both *nptII* and DREB genes. Infected apical tissues with *Agrobacterium* resulted in the gene integration in T0 plants and based on these infected seedlings were transferred to pots in the greenhouse for further T1 generation confirmation. Fragments of PCR amplicons of first generation obtained from the T0 plants were similar. The putative transgenic cotton lines were allowed to self-pollination for setting seeds. The genomic DNA of the transgenic and non-transgenic cotton plants were extracted separately and the purity and quality of DNA were read for the intact banding pattern of DNA. The non-transformed plants showed no target DNA fragment amplified from the genomic DNA.

4. Discussion

Recent advances for an efficient transformation system in transgenic technology is essential for improvement of cotton through genetic engineering by genetic manipulation. The cotton is highly recalcitrant to regeneration by somatic embryogenesis due to number of impediment, including genotype specificity (Trolinder and Goodin1987).In our study, transgenic cotton plants obtained by regeneration system (Kumar and Pental, 1998; Zapata et al., 1998; Zhang et al., 2001).

The results of many studies revealed that EHA101 and EHA105 gave more suitable for stable transformation of cereals (Donaldson and Simmonds, 2000) and certain dicots (Rashid et al., 1996). Our study with Agrobacterium strain LBA4404used proved to be a highly efficient for cotton transformation as compared to other bacterial strains studied by Firoozabady et al.(1987), Umbeck et al. (1987) and Chen et al.(2002) on cotton transformation. Though from the first report of success in the cotton transformation rates have been significantly improved (Firoozabady et al., 1987; Umbeck et al., 1987), an efficient technique is required for cotton transformation to improve important agronomic traits via introducing the desired.

A number of transgenic seedlings were obtained from hypocotyl explants on basal MS medium supplemented with 3% sucrose, 0.5 mg/L antibiotic Kanamycin along with 0.1 mg/L 2,4-D. These explants were subcultured onto fresh MS hormone-free medium for *Agrobacterium* mediated transformation. Hormone-free MS medium containing 3% sucrose gave developing new plants when sub-cultured for twice. Co-cultivation with *Agrobacterium* of the hypocotyl explants produced an average of 85 Km-resistant putative transformed plants within 4–5 weeks as one independent transformation event. These putative plants transferred to soilrite and then soil in the greenhouse, exhibited normal morphology and flowered. An optimized concentration of 0.46µllM kinetin and 0.45 µllM 2, 4-D in MS medium was crucial for the recovery of a large number of transformed microcalli from the inoculated tissues, due to a high competency of differentiating cells for transformation with *A. tumefaciens* (Firoozabady and Galbraith, 1983, 1984). In our research, hypocotyl was used as an explants for inoculation (Firoozabady et al., 1987). Only the transformed explants infected by *Agrobacterium* harbouring kanamycin resistant gene in a kanamycin containing medium survived. These were again transferred to fresh medium to avoid *Agrobacterium* contamination which is essential for promoting growth.

Established *in-vitro* grown shoots (8 days old) were used for shoot apices and nodal meristems which were made to grow on fresh medium for 15 to 20 days in optimized conditions. They started forming elongated shoots within 2 weeks.At 0.45 µM of BA nodal meristems gave best results while for shoot apices required a higher level of BA of 1.5µM. Explants co-cultured with *Agrobacterium* were selectedon selection medium to introduce a mild dehydration stress, as studies reported to enhance regeneration in Asparagus (Saito et al. 1991), eggplant (Saito and Nishimura 1994) and rice (Jain et al.1996). Due to the presence of excessive moisture

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resulted in less response to dehydration stress further. While the transformed lines harbouring DREB gene gave dehydration stress tolerance. The transformed plants regenerated from kanamycin containing medium revealed the molecular analysis of confirmation of inserted gene.Of the plants analyzed by PCR, 70% were found to be positive. The present study demonstrates that *Agrobacterium*-mediated putative transformed plants were well dehydration stress tolerant with a high frequency of transformation.

Acetosyringone a potent inducer of *Agrobacterium vir* genes is a phenolic compound secreted by the wounded plant tissues (Stachel *et al*. 1985). The pre-induction of *Agrobacterium* and/ or inclusion of acetosyringone in the co-cultivation medium can enhance the transformation (Yao, 2002; Samuels, 2001; Sunikumar *et al*. 1999). In our experiments, acetosyringone was included at a final concentration of 120 µM during the finalstage of *Agrobacterium* growth and during co-cultivation. Acetosyringone was completely removed for the control treatment. In this experiment, shoot apices were transferred onto a medium containing kanamycin at 0, 30, 50, 75 and 100 mg/L after pre-culturing in MS medium +0.1mg/L kinetin for 5 days. Ten shoot apices were placed in each flask and replicated four times for each concentration. Over a period of two weeks, the number of elongated shoot apices were counted and recorded each week. The control (0mg/L) grew very well in MS media. Shoot elongation was significantly decreased on MSmedia containing kanamycin. Thirty percent of shoot apices survived in MS containing 30mg/L Kanamycin after three weeks. The minimum lethal concentration to kill all the apices in three weeks was 50mg/L. The higher level of kanamycin (100 mg/L and 75 mg/L) killed all the apices within week. Therefore, a concentration of 50mg/L kanamycin was used toselect transgenic apices in this research. Under kanamycin selection pressure, most of the shoots appeared to be bleached and some of the shoots that were initially green bleached out gradually, leaving only a few green shoots. These shoots were transferred to fresh media with an interval of 10 to 15 days. After five weeks of selection, surviving shoots were transferred to MS media without kanamycin to induce rooting. Rooting of the transformed shoot occurred in kanamycin free medium. The obtained nucleotide sequences were compared with the database to confirm the similarity of the cloned insert using the BLAST software at www.tigr.org.

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A)

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B)

C)

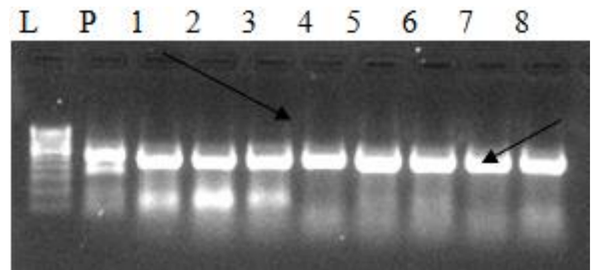
D)

Figure 1: Transformation of shoot tip explants of *G. hirsutum*cv.5166 by *Agrobacterium* containing DREB 3 gene and regeneration of transgenic plant by direct shoot organogenesis. A) Inoculation of shoot-tip explants. B) Screening of explants on kanamycin medium. c) Regeneration of putative transformed shoots on MS medium containing kanamycin. D) Regeneration of transformed Plant

A)

B)

Figure 2: A) Development of putative shoot from transformed shoottipexplant of *G. hirsutum* in soilrite. B) Transgenic plant of *G.hirsutum* in green house



A)

B)

Figure 3: a) PCR amplification of DREB3 gene fragment in transgenicG. hirsutum. Lane 1, 1 kb ladder; lane 2, positive control; lane 3-8 transformed plants b) Southern blot analysis of transformed plants for integration of the *DREB* gene. Lane 1: undigested plasmid DNA (positive control) Lane 2: DNA sample from non transformed control plant Lanes 3, 4: DNA samples from putative transformed plants.

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