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Characterization of Transgenic Cotton (*Gossypium hirsutum* L.) Lines against Moisture Stress Carrying *At*DREB1A Gene

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A B S T R A C T

Drought tolerance is accompanied by number of traits and they are regulated by the many genes, hence targeting single gene against moisture stress will not be effective in crop improvement. Use of transcription factors (TFs) to target corresponding multigene is current trend. As per the many research findings, dehydration responsive element binding proteins (DREB) are reported as an important TFs that known to induces number of abiotic stress-related genes and impart the stress tolerance in plants. With this perspective, cotton variety Coker-312 was introduced with *AtDREB1A* gene and in the present study, transgenic cotton lines of T_2 generation were evaluated for moisture stress resistance at Agriculture Research Station, Dharwad. Segregating T_2 lines were screened with the help of PCR using gene specific primer and during moisture stress, *DREB1A* gene and its target gene fold change was studied using real time PCR (RT-PCR). Higher accumulation of the osmo-protectants like proline, reducing sugar was observed in the transgenic lines as compared to the non-transgenic lines under moisture stress.

Introduction

Gossypium hirsutum L., is new world cotton, belongs to the family *malvaceae*, with the chromosome number of 2n=4X=52 (Fryxell 1979, 1992; Greever *et al.*, 1989; Fryxell *et al.*, 1992). Among all the agricultural crops, cotton is having the highest number of consumers all over the globe. Out of total cotton growing area only 53% of the cotton field is irrigated which is meeting the 73% of the total world cotton production, remaining 47% of the land contributing only 27% (Soth *et al.*, 1999; Bremen, press release, 2017).

This huge gap is majorly due to adverse environment condition leading to the drought, high salinity and cold stress as a result of climatic change.

Drought is one of the important abiotic stress limiting the plant growth and crop productivity globally (Malik *et al.*, 2006). There is only 0.007 per cent of fresh-water resources in the world surface which could be utilized by human beings (Zhang, 2003; Freshwater Crisis., 2016)., Cotton requires well distributed minimum annual rainfall of 50 cm through-out its growing season (Handbook of Agriculture, ICAR, 2006). If any moisture stress occurred at pre-flowering stage has been proven that increase in subsequent rate of flowering and yield (Singh 1975). But drought during the early stages the vegetative growth, at flowering and post flowering stage results in square and boll drop (Krieg 2000).

Even though cotton is a drought tolerant crop due to its very deep root system, it is highly sensitive to water stress between 45 to 60 days after sowing, which is coinciding with the peak square and boll formation stages (Oosterhuis, 1990). Thus, the enhanced abiotic stress tolerance is of greater importance.

The basis of drought tolerance is a complex and driven by diverse drought adaptive mechanisms, which are normally under multigenic control (Blum, 2005; Pinto *et al.*, 2010). Targeting single gene to overcome this problem will be waste of time, resources and money; hence the strategy of utilizing transcription factors (TFs) would find to be solution for this (Bhatnagar-Mathur *et al.*, 2007). TFs recognize the specific DNA sequences in the regulatory regions of target genes and lead to the activation of downstream genes (Latchman, 1997; Riechmann and Meyerowitz, 1998; Wang *et al.*, 2005). One relevant class of transcription factors with respect to abiotic stress is the dehydration-responsive element-binding proteins (DREBs), which are transcriptionally regulated by the water deficit (Liu *et al.*, 1998; Behnam *et al.*, 2006). DREBs/CBFs (C-repeat binding factors) play a very important role in abiotic stress responses and have ability to regulate a many number of target/stress-responsive genes, hence they have become popular targets for genetic engineering to improve abiotic stress tolerance in various plant species (Khan, 2011; Lopato and Langridge 2011).

Screening the plants for moisture stress is challenging because selecting the parameter itself is a complicated. As per the literature moisture stress reduce the plant height (Saimaneera *et al.*, 1997; Du *et al.*, 2008), increases the flower drop, square shedding, boll abortions and lead to biochemical changes like accumulation of osmo-protectants (Gerik *et al.*, 1996; Pettigrew, 2004). These are some parameters were considered for the evaluation. In the present study transgenic cotton lines of T_2 generation, containing *AtDREB1A* gene were evaluated for moisture stress in transgenic green house at Agriculture Research Station, Dharwad, Karnataka, India.

Materials and Methods

Genetic material and planting material

Agrobacterium strain LBA-4404 harbouring *AtDREB1A* gene (Fig. 1) was obtained from national research centre on plant biotechnology (NRCPB), New Delhi under Indo-US collaborative research programme and used to generate transgenic Coker-312 lines at Agricultural Research Station Dharwad, University of Agricultural Science, Dharwad. Transgenic Coker-312 lines were maintained in the transgenic greenhouse and in the present study, thus obtained T_2 generation 17 lines were evaluated for moisture stress resistance.

Experimental design

T_2 generation seeds of each lines were sown as two sets with five replications in the polythene bags and maintained the same amount of soil and soil moisture levels in the transgenic greenhouse. All the germinated transgenic seedlings were screened for positive plants and retained only positive plants for further study. Along with these, two set of non-transgenic plants were maintained as a negative control (NC). Among the two sets (5 plants/lines/set), one set was introduced with moisture stress by withholding the irrigation 45 DAS, while another set was continued to irrigate (Fig. 2A). Physio-morphological, biochemical and molecular parameters were recorded at 45DAS and 75DAS from both normal and stress induced condition. Moisture stress condition was confirmed by measuring the relative-water content and soil moisture content in regular interval. The plants were considered as experiencing moisture stress when their relative water content and soil moisture content was about to half of the normal condition and started showing wilting symptoms (Fig. 2B).

Molecular characterization

15 DAS young leaves were collected separately from each germinated cotton seedlings, with the proper label and DNA was isolated using CTAB method. PCR screening was carried out using gene specific primer (Forward primer TAGGCTCCGATTACGAG TCTTCGG; Reverse primer GCATACGTCG TCATCATCGCCGTCG) (Fig. 3) with programme of initial denaturation temperature 94^0C for 300sec. followed by 34 cycles of denaturation temperature 94^0C for 30sec, annealing temperature 64 ^{0}C 30 sec, extension 72 ^{0}C for 30 sec. PCR products were run in the 2 per cent agarose gel-electrophoresis with 1X TAE buffer. Based on the presence or absence of the band (600bp) separated positive (transgenic) and negative (non-transgenic) plants.

Primer designing

AtDREB1A gene (RNA) sequence of *Arabidopsis thaliana* and one of its target gene *LEA* protein gene (RNA) sequence of cotton was downloaded from NCBI. Primers were designed using IDT- PrimerQuest Tool with melting temperature (Tm) of 50-60^{0}C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC%) 40-60% and PCR amplicon size of 120-200 base pair (bp). Primers specificity was checked using blast.

Sample collection, RNA isolation and cDNA synthesis

After the induction of moisture stress (75DAS) leaf samples were collected in aluminium foil with proper label and immediately immersed in liquid nitrogen. Immediate after the samples collection, RNA was isolated using SIGMA spectrum^{TM} Total Plant RNA Kit. DNase (Invitrogen) treatment was given to remove genomic DNA, quality and quantity was checked using nanodrop as well as gel electrophoresis. Then equal quantity (1µg) of RNA was taken to synthesise cDNA (Invitrogen; cDNA synthesis kit) as per the kit protocol and quality was checked on the gel electrophoresis.

Relative expression analysis by real time PCR

Relative expression level of DREB gene and its target gene was estimated using real-time polymerase chain reaction (RT-PCR). RT-PCR programme was having initial denaturation temperature 94^0C for 10 min. followed by 40 cycles with denaturation temperature 94^0C for 15sec, annealing 57^0C (*DREB* gene : DREB-FGGAGAAACTCCGG TAAGT; DREB-R: CGAGTCAGCGAAA

TTGAG) (*LEA* gene: LEA-F:AAAGGCA AGCAAACAAATTT AAGAA; LEA-R:AAA CGCAACCTGAAACAAACA) for 25sec. Ubiquitin gene was used as an internal control. Relative fold change was calculated using $2^{-(\Delta\Delta CT)}$ method (Datta *et al.,* 2012; Livak *et al.,* 2001). ΔCT was calculated by subtracting internal control (Ubiquitin) from *AtDREB1A* CT in the given sample both in irrigated and stressed condition. The ΔΔCT value was calculated subtracting irrigated plant sample of same line, was used as a calibrator.

Physio-morphological and biochemical parameter

Leaf water potential was collected using PSY1Stem Psychrometer; canopy temperature was collected using hand-held infrared thermometer; stomatal conductance, transpiration rate, photosynthesis rate was collected using Infrared Gas Analyser (IRGA) Portable photosynthesis system LI-6400 (LICOR 6400, Lincoln Nebraska, USA). Plant height, number of monopodial branches per plant, number of sympodial branches per plant, number of bolls per plant, number of bolls harvested per plant, boll weight, seed index was taken manually. Fibre quality like ginning out turn, lint index, fibre strength, fibre length, fibre fineness, fibre uniformity (%) were analysed using High Volume Instrument (HVI) at Central Institute for Research on Cotton Technology (CIRCOT), Regional Quality Evaluation Unit situated at ARS, Dharwad farm.

Reducing sugar was estimated by using Dinitro Salicylic Acid (DNS) method (Miller 1959), proline estimation was done using Ninhydrin method (Bates *et al.,* 1973) and chlorophyll content was recorded using SPAD 502 Plus Chlorophyll meter. All the collected data was statistically analysed using two factorial CRD design.

Results and Discussion

Molecular characterization

AtDREB1A gene relative expression was analysed by comparing stressed plants with irrigated lines. Expression was up regulated in twelve lines and down regulated in five lines. The cotton plant also carries indigenous *DREB* gene. But their identity is less than 30 % when both are aligned in NCBI BLAST and primer was highly specific to the *AtDREB1A.* Relatively higher expression was observed in transgenic lines GM-23, GM-10, GM-28, GM-14 and GM-11 compared to its control (irrigated lines) and it was ranged between -1.62 to 10.38 (Table 1). Relatively higher expression of *LEA* protein gene was recorded in stressed plant compared to irrigated plants. *LEA* protein gene fold expression was ranged from -1.11 to 29.91. Higher level of expression was recorded in GM-28 (30.424 fold than irrigated) followed by GM-23 (30.1 fold than irrigated), GM-6 (26.61 fold than irrigated) and GM-10 (21.92 fold than irrigated). Down regulation of both genes were observed in stressed NC compare to irrigated. When gene expression was compared with negative control, that also shown relatively higher expression in transgenic plants.

Biochemical and physio-morphological parameter

After the moisture stress induction, transgenic lines were recorded significantly lower leaf water potential and significantly higher relative water content compared to non-transgenic line. There was significant difference found in relative water content between irrigated and drought induced plant, also there was significant reduction in the photosynthetic rate was found in stressed plants compared to irrigated set. It was up-to 46.96 per cent reduction recorded in GM-21.

Whereas GM-14, GM-23, and GM-11 lines showed slight increased rate of photosynthesis compared to its irrigated plants indicating stable in both conditions. Plant height was significantly increased in many transgenic lines (stressed plants) compared to negative control. After the recovery from moisture stress, stressed transgenic plant lines were shown significantly increased plant height compared to regularly irrigated lines. Under moisture stressed condition, boll shedding per cent was significantly higher compared to irrigated condition. Relatively less boll shedding per cent was recorded in the transgenic lines, such as 23.64 % in GM-5 whereas, in non-transgenic plant it has shown 47.73 per cent reduction (Table 2). Transgenic lines recorded significantly higher boll weight, boll count (Table 3), lower shedding per cent compared to non- transgenic plant under moisture stress.

As for as fibre quality is considered no significant difference was observed with respect to irrigated and drought condition in transgenic and non-transgenic lines. Except uniformity index, significant difference between the lines was not found with respect to fibre length, fibre strength and fibre fitness is considered. But relatively higher uniformity index values were recorded in transgenic lines compared to NC under moisture stress.

After the induction of stress (75 DAS) transgenic lines were shown significant reduction in chlorophyll content compared to control condition, but few transgenic lines maintained the same level of chlorophyll content in both sets, that indicating stable in both conditions. The lines GM-19, GM-9 and GM-23 recorded not only less per cent of reduction, but also were able to maintain higher level of chlorophyll content compared to negative control. Transgenic lines accumulated significantly higher proline content compared to non-transgenic lines during severe moisture stress. Reducing sugar content also increased in the transgenic lines compared to non-transgenic.

During drought conditions, plants produce osmolytes such as free proline and various soluble sugars as osmo-protectants in stress tolerant plants (Igarashi *et al.*, 1997; Ishitani *et al.,* 1996; Taji *et al.,* 2002). Before the induction of moisture stress and recovery from the moisture stress there was no significant increase or decrease in reducing sugar level in the two blocks. But during moisture stress higher accumulation of reducing sugar was observed. It acts as an osmo-protectant. Along with this, proline is also one of the known osmolyte which accumulate in plants under abiotic stress conditions (Zhao *et al.,* 2007). It also functions as a sink for energy to regulate redox potentials, such as a hydroxy radical scavenger, and as a solute that protects macromolecules against denaturation (Kishor *et al.,* 1995).

Transgenic plants have been shown to accumulate higher levels of proline content compared to non-transgenic plant. Over expression of *DREB 1A* improved the drought tolerance in transgenic plant and after stress, alleviation of the solute content by increase in reducing sugar and proline when compared to control plants (Amuda *et al.,* 2014). Relative water content was found higher in the transgenic plants compared to non-transgenic plant. As a result, leaf water potential was comparatively low in transgenic. Transgenic lines retained higher leaf chlorophyll levels even under moisture stress (Chen *et al.,* 2009).

Table.1 Relative fold change of *AtDREB1A* and its target (*LEA* protein) gene in misture stressed transgenic lines by comparing with irrigated transgenic line

Lines	Relative fold change of DREB Gene	Relative fold change of LEA protein gene
GM3	2.630	9.099
GM5	4.091	2.138
GM6	3.232	26.620
GM9	-1.621	-1.003
GM10	8.461	21.921
GM13	1.364	6.206
GM14	5.613	1.404
GM15	1.579	1.048
GM19	2.136	1.059
GM 23	10.383	30.101
GM 30	3.492	12.457
GM 11	4.568	22.100
GM 28	5.824	30.425
GM 31	1.212	2.428
GM 34	-1.055	-1.109
GM 22	-1.088	1.675
GM21	-1.308	4.602
NC	-1.933	-1.923

NC: Negative control

Table.2 Differences in the transgenic lines for Boll shedding per cent

Lines	Irrigated	Drought	Mean
GM3	15.00	37.73	26.36
GM5	15.56	23.64	19.60
GM6	10.27	34.40	22.33
GM9	3.57	24.75	14.16
GM10	5.90	34.83	20.37
GM13	11.56	31.30	21.43
GM14	15.26	29.82	22.54
GM15	23.38	35.71	29.55
GM19	13.38	35.42	24.40
GM 23	12.22	29.46	20.84
GM 30	28.21	32.05	30.13
GM 11	23.30	25.19	24.25
GM 28	16.78	37.09	26.94
GM 31	15.00	35.12	25.06
GM 34	17.71	25.00	21.35
GM 22	20.83	29.67	25.25
GM21	33.64	35.90	34.77
NC	39.23	47.73	43.48
	Lines	**Condition**	**Interaction**
S.Em. ±	5.12	1.71	7.23
C.D. @ 5 %	NS	6.56	NS

NC: Negative control NS:Non significant

Fig.1 Plasmid Construct of *Agrobacterium* strain LBA-4404 harbouring binary vector pCambia 2300, carrying *AtDREB1a* gene linked to the rd29 promoter, nopaline synthase (nos) terminator I and *npt*-II gene under the control of 35S promoter and 35S polyA terminator was used in transformation studies. *npt*-II is the selectable marker.

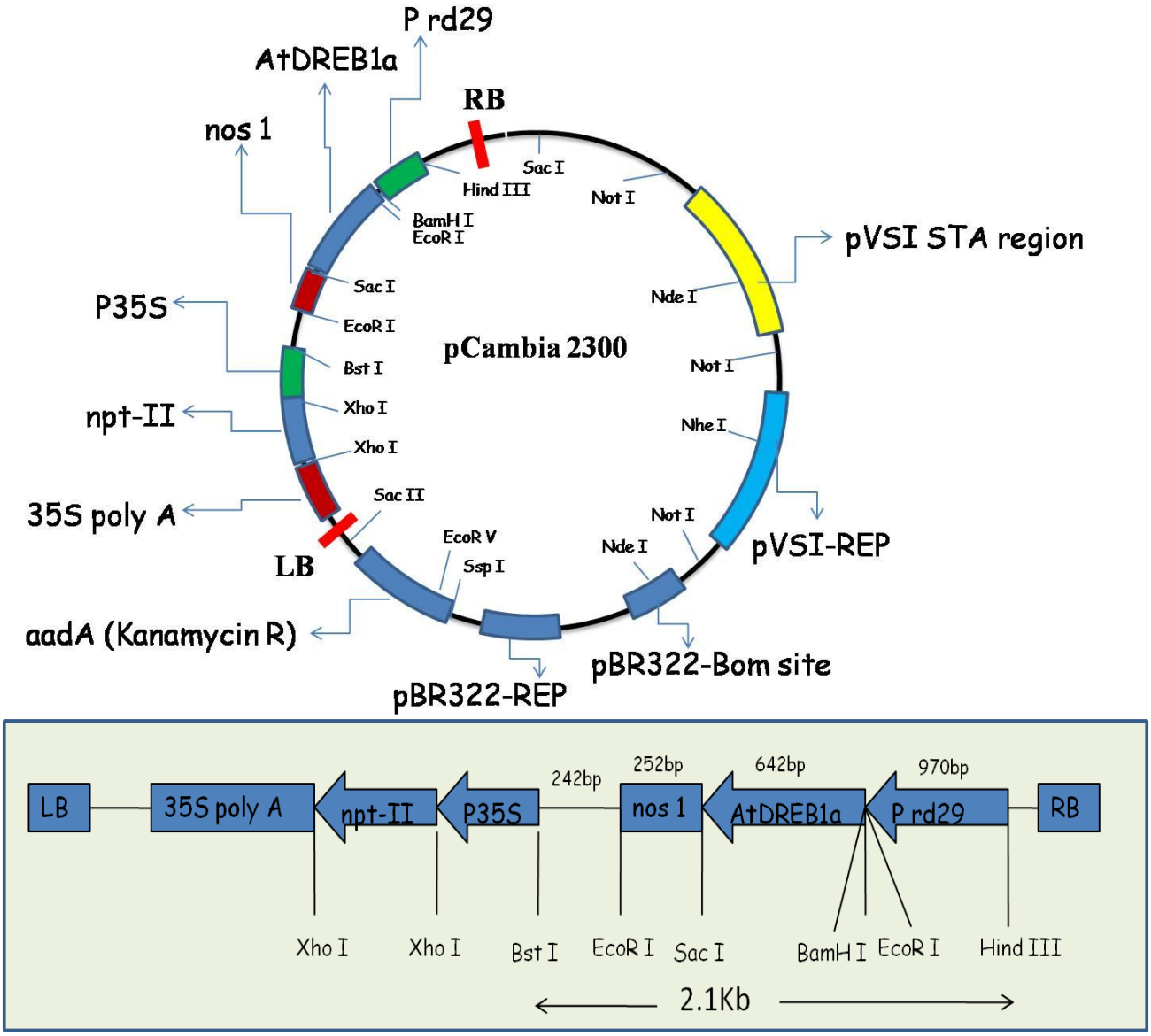
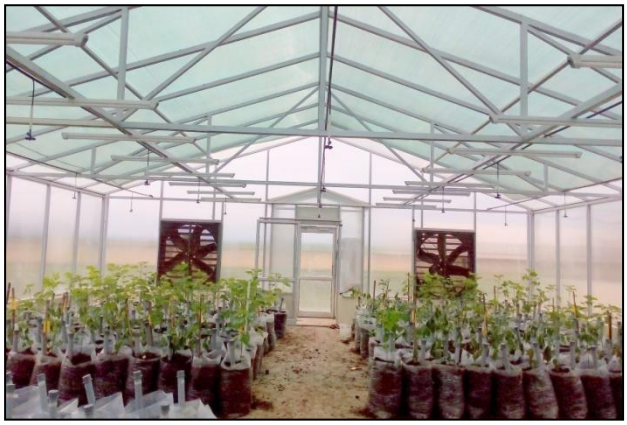


Fig.2 A. General view of plants. B. Cotton plants 75 days after sowing DAS a. nontransgenic under moisture stress b. Transgenic plant under moisture stress c. Non-transgenic under moisture stress

A



B

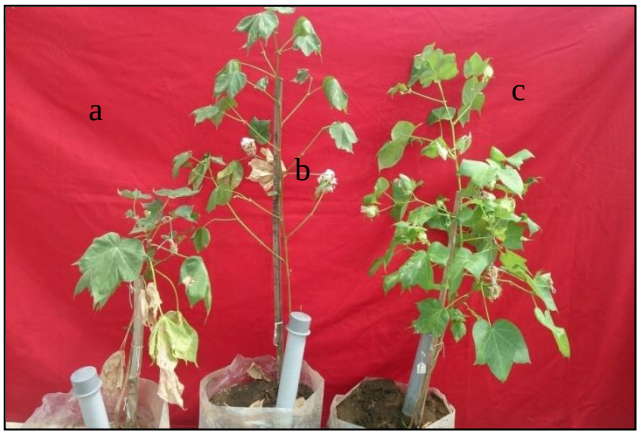


Table.3 Differences in the transgenic lines for boll count

Lines	Irrigated	Drought	Mean	% Change
GM3	8.50	6.50	7.50	7.50
GM5	8.00	8.00	8.00	0.00
GM6	13.50	10.50	12.00	22.22
GM9	14.00	10.50	12.25	25.00
GM10	16.00	10.00	13.00	37.50
GM13	23.00	10.50	16.75	54.35
GM14	14.00	13.00	13.50	7.14
GM15	9.50	9.00	9.25	5.26
GM19	16.00	10.00	13.00	37.50
GM 23	14.50	10.50	12.50	27.59
GM 30	9.00	9.50	9.25	-5.56
GM 11	11.50	12.00	11.75	-4.35
GM 28	10.00	8.50	9.25	15.00
GM 31	14.00	8.50	11.25	39.29
GM 34	11.50	6.50	9.00	43.48
GM 22	11.50	9.50	10.50	17.39
GM21	8.50	8.00	8.25	5.88
NC	8.50	6.50	7.50	23.53
	Lines	**Condition**	**Interaction**	
S.Em. ±	0.61	0.20	0.87	
C.D. @ 5 %	2.36	0.79	3.33	

NC: Negative control

Fig.3 Confirmation of transgenic plant on gel electrophoresis. B- Blank; L-Ladder 1kb; NC-Negative control; PC-Positive control;+ve – Transgenic plant

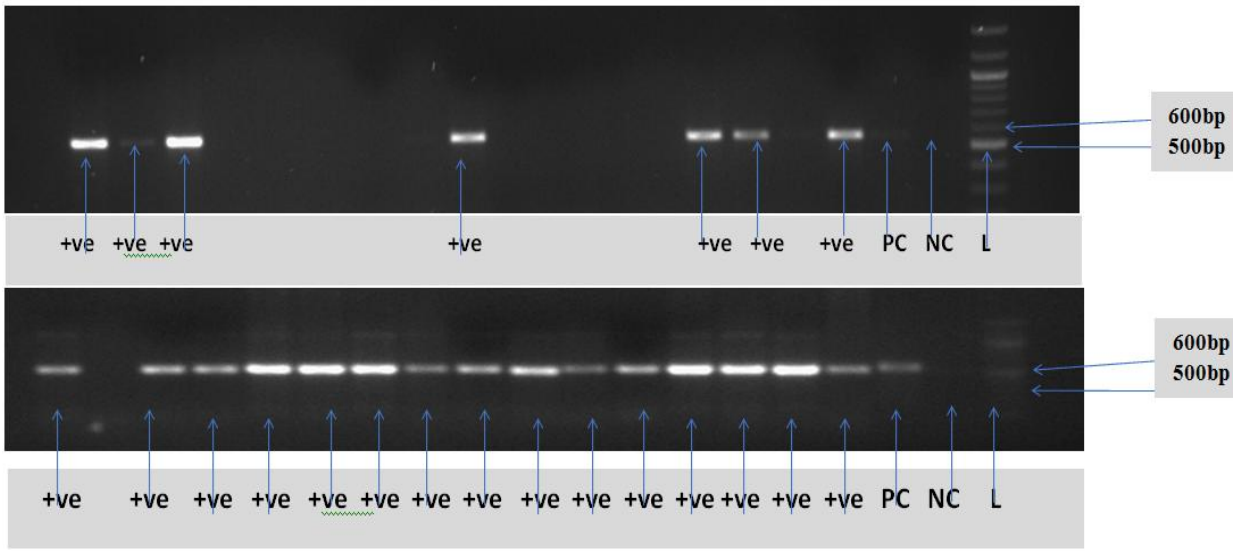
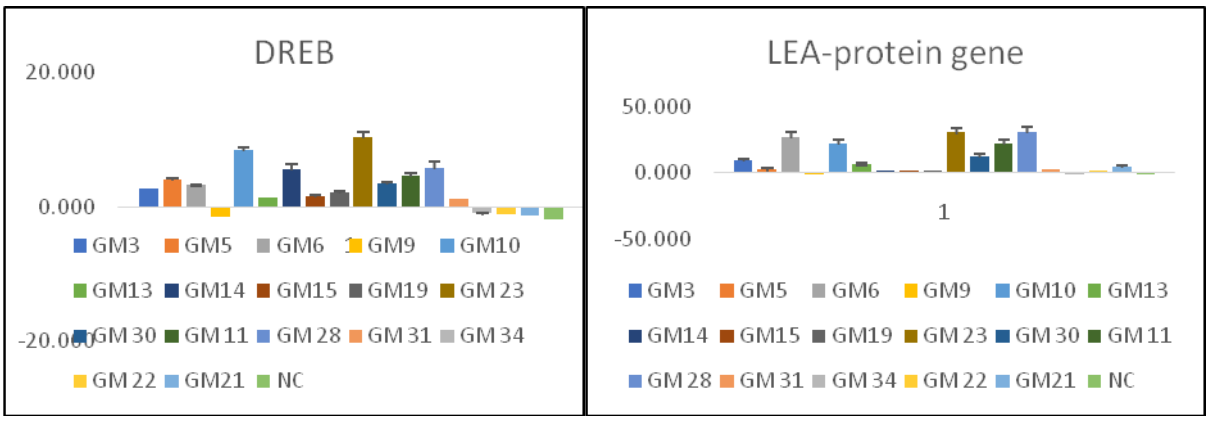


Fig.4 In transgenic cotton, expression of *ATDREB1A* and its target (*LEA* protein) gene under moisture stress (75DAS) compared with irrigated set



There was no significant reduction in photosynthetic rate, before induction of the stress. Reduction in the photosynthesis during moisture stress is very common phenomenon and this is due to stomatal closer (Ennahli and Earl, 2005). Since moisture stress reduce the rate of photosynthesis and stimulates the ABA and ethylene production in young bolls. Establishment and pre-bloom irrigations affect total yield, but water deprivation following bloom and into boll development also affects lint quality. Even though fibre quality was not much affected by the moisture stress, yield was reduced due to flower and ball abortion and falling (Luz *et al.*, 1997). Here fibre fineness value was found high in the transgenic lines compared to non-transgenic plants.

Most of the abiotic stresses regulated by the key genes that are transcription factors. DREB is the transcription factor (DREB - dehydration-responsive element binding protein) specifically interacts with the element

DRE and induces the expression of genes involved in the stress response. Drought tolerant variety/transgenic cotton expressed 10.35 folds higher of *DREB1A* gene (GM-23) compared to irrigated plant (Amudha *et al.,* 2014). There was number of DREB target gene were identified (Seki *et al.,* 2001). *LEA* protein gene is one of the target gene of *DREB 1A*, since it carries DREB protein binding domain. *LEA* proteins involved in protecting macromolecules like enzymes, lipids and mRNAs from dehydration. *LEA* protein gene in *A. thaliana* responded to drought and cold stress treatment. This gene has a DRE core motif in the promoter region that is regulated by both *DREB1A* and *DREB2A* (Sakuma *et al.,* 2002). Through real-time PCR precise quantification of the mRNA levels of genes of interest can be done, when their expressions are compared under various conditions or treatments (Volkov *et al.,* 2003). The relative expression of both *DREB* gene and *LEA* protein gene was found to be higher in the transgenic plants compared to non-transgenic plants (Fig. 4). Even though some transgenic lines shown higher expression of DREB, performance was not as expected, the site of gene insertion also play important role in the action the gene.

Transgenic lines were positively responded to the drought resistance related parameter like, higher proline content and reducing sugar with lowered photosynthetic rate. Even though fibre quality was not much affected by the moisture stress, yield reduction was higher in non-transgenic compare to transgenic lines. Also, fibre index was significantly higher in transgenic lines compared to non-transgenic line, indicating transgenic lines exhibited fine fibre compare to non-transgenic. This indicates the mere presence of the *AtDREB 1A* gene is responsible for drought tolerance, since all the lines are of same with-respect to genotype.

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