International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue4, 2014, p635-648
http://www.bipublication.com

Cotton Transgenic Plants with Dre-Binding Transcription Factor Gene (*DREB 1A*) Confers Enhanced Tolerance to Drought

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[Received 06/09/2014, Accepted-15/10/2014]

ABSTRACT:

Drought stress is a major limiting factor that affects the plant vegetative growth and the yield. Cotton transgenic plants tolerant to drought were developed through *Agrobacterium* mediated transformation in *Gossypium hirsutum* elite genotype LRK 516 with *DREB 1A* gene. Cottonseeds were sterilized and grown in half MS medium and embryonic axes were excised and as explants for transformation. The putative transformants were grown in the MS medium containing 50µg/ml kanamycin. The shoots were transformed in the MS medium containing auxin 1mg/lit and cytokinin 1mg/lit for rooting. The regenerated plants were then hardened in the sterilized soilrite mixture pots. The molecular confirmation of the transgenics were done for the presence of the gene by PCR using specific primers for *DREB 1A*, *npt* II and Southern blot analysis. The mRNA from the transgenic plants were isolated and cDNA was synthesized using reverse transcriptase-PCR (RT-PCR). The cDNA was used as template and amplified with *npt* II primer and *DREB 1A* gene specific primer. Drought stress was subjected to the transgenic plants by withholding for 15 days during squaring stage and physiological and biochemical studies were carried in the transgenic's after they started growing under receding moisture. Gene expression of candidate genes for drought tolerance, transgenic and non transgenic plants were grown in the pots and drought stress was subjected to the plants during square formation stage (55–60 days old plants) and boll developmental stage without watering for maximum 15 days for drought stress treatment. Fresh young leaf from the main terminal branch and root samples were collected from drought stressed plant at square formation and boll developmental stage and used for RNA isolation and real time PCR studies. Transgenic events of *G. hirsutum* variety LRK 516 carrying *DREB 1A* gene were analyzed for gene expression by Real-time quantitative RT-PCR revealed that the transcripts were accumulated at high levels during square formation stage showed an average of 5 fold increase and the proline content was also found 3-fold increase during stress condition in transgenic plant when compared to the wild type and the drought tolerant variety 28 I.

Key words: *G. hirsutum*, *Agrobacterium* mediated transformation, *DREB 1A*, real time PCR gene expression, abiotic stress, drought tolerance.

[I] INTRODUCTION:

Drought stress related gene expression has been regulated by transcription factors. Dehydration responsive element binding (*DREB 1A*) plays a key role and are important in the ABA-independent drought tolerant pathways and induce the expression of stress response genes. Transcription factors play critical roles in the regulation of cellular and physical changes in

response to environmental stresses in plants [1]. Transcription factors were grouped into families according to their DNA binding domain. The CBF/DREB (cis binding factor/dehydration responsive element binding) regulon is mainly involved in cold stress response and is conserved throughout the plant kingdom, including in plants that do not cold-acclimate (e.g. cotton, tomato and rice) .A novel cis-acting element was identified, in addition to the ABA-responsive element (ABRE) and present in the promoter of the RESPONSIVE TO DEHYDRATION 29A (RD29A), a gene induced by drought, high salinity and cold [11]. The transcription factor that bind to core motif of cis-acting element CCGAC were named CRT-binding factors or DRE-binding proteins 1(CBF/DREB 1) [15, 25]. The gene expression is quickly and transiently induced by cold stress, and in turn CBF/DREB1 transcription factors activate the several other gene expression (e.g. proteins involved in osmoprotectant and antioxidant production).

Drought stress has many physiological effects on the plants reduction in vegetative growth, leaf expansion and transpiration. ABA concentration increases and stomata close to prevent the transpirational water loss and decline in Rubisco activity that results in limited photosynthesis [2,4].The intracellular CO_2 level also decline and results in the generation of ROS components that lead to photo-oxidation finally cause extensive peroxidation and de-esterification of membrane lipids which leads to denaturation of proteins and nucleic acid mutation [3]. Crop plants tend to protect the membranes as well as macromolecules by synthesis of large number of osmolyte's [32]. The compatible solutes/osmolytes maintain cell turgor and thus the driving gradient for water uptake. The compatible solutes act as free-radical scavengers or chemical chaperones by directly stabilizing cell membranes and/or proteins [10].

Four independent signaling pathways function in the induction and expression of stress-inducible genes in which two are ABA dependent [38,39] and two are ABA independent [35,40]. Dehydration responsive element (DRE) or C-repeat (CRT) exists in *DREB1A*, as a DRE/CRT-binding transcription factor and up-regulate the gene expression. Consequently, over-expression of *DREB1A* gene will significantly introduce expression of dehydration responsive genes and it can results in high tolerance to drought stress in transgenic plants. Gene expression studies in plants include precise quantification of mRNAs expressed in various situations, such as the effects of drought at different developmental stages, and in different tissues and cells. The quantification of mRNAs and the expression can be studied by RT-qPCR [5]which has emerged as an important technique to compare the expression profiles of target genes in several species, tissues and treatments, and also to validate high-throughput gene-expression profiles[8]. Reverse transcription (RT) followed by a polymerase chain reaction (PCR) represents the most powerful technology to amplify and detect trace amounts of mRNA [26,27].This is one of the methodologies used to determine expression levels by RT-qPCR comparing the gene of interest with reference genes, whose expression does not change under different experimental conditions. qRT-PCR is the most sensitive and most reliable method, in particular for low abundant transcripts in tissues with low RNA concentrations, partly degraded RNA, and from limited tissue sample [16] Real-time quantitative RT-PCR studies revealed that the transcripts of all the three *GhPIP* genes were accumulated at high levels and were remarkably up-regulated or down-regulated under different stresses such as NaCl, cold, PEG (polyethylene glycol)treatments [9] . Gene expression of Barley transcript profiles were reported under dehydration shock and drought stress treatments [27].

[II] MATERIALS AND METHODS:

Seeds of cotton variety LRK 516 were obtained from the gene bank, Central Institute for Cotton Research, Nagpur.

2.1 Bacterial strain and vector

Agrobacterium mediated transformation with pCambia 2300 - Prd29: DREB1a: nos gene constructs were used for transformation (Fig 1). The gene construct has *npt II* gene as selection

marker in the T-DNA region driven by Cauliflower mosaic virus (35S CaMV) promoter and NOS-terminator in the vector system. Bacterial culture was maintained on YEMA medium (1.0% w/v Yeast extract, 1.0%w/v Mannitol, 0.1%w/v Sodium chloride, 0.2% w/v Magnesium sulphate, pH-7.0) containing 50mg/l kanamycin and 25mg/l rifampicin. One single colony was inoculated and grown overnight in liquid YEMA at 28ºC with antibiotics.

2.2 Transformation of cotton plants:

Elite cotton variety LRK 516 was raised aseptically on half- Murashige and Skoog (MS) medium. The embryonic axes were excised and trimmed from both the sides and used for co-cultivation with *A.tumefaciens*. The explants were co-cultivated in the half-MS liquid medium with actively growing culture of 1.0 OD *A.tumefaciens* and 100mM acetosyringone. After overnight co-cultivation, shoots were decontaminated in the half MS medium containing cefotaxime 250 mg/l. The explants were then transferred to selection medium containing kinetin 0.1mg/l, BAP 0.1mg/1 and kanamycin 50mg/l. The kanamycin resistant putative shoots were sub-cultured in a media containing 0.1 mg/l BAP for root induction. The regenerated plants were rinsed well and transferred to pots containing peat, soil and sand in 1:1:1 ratio. Plants were covered with plastic bags and then to a pot with soil for hardening for 15 days before transferring to the greenhouse under natural condition.

2.3 Screening for transformed plants using PCR

Genomic DNA was isolated by the method of Paterson *et al*.,(1993) from the young leaves of T_0 plants. The template DNA was used for PCR amplification with the DREB 1A gene Specific primer (5'-3') F-AAGAAGTTTCGTGAGACTCG and R-CTTCTGCCATATTAGCCAAC, *npt II* specific primer F-GAGGCTAATTCGGCTATGACTG and R- ATCGGGAGAGGCGATACCGTA was carried out to check the presence of the transgene. PCR was performed in 20µl (total volume) reaction mixture containing 1.0 µl of 100ng DNA, 2.0 µl 10X reaction buffer, 2.0 µl of 10mM dNTP's, 1.0 µl of 100nM of each forward and reverse primer, 3.0 µl of 25mM $MgCl_2$ and 0.5 µl of 1U of *Taq* DNA polymerase. The following PCR conditions of 94ºC for 5min, then 35 cycles of 94ºC for 30sec, 56ºC for 1min, 72ºC for 1 min and 5 min of final extension at 72ºC was maintained in a thermo cycler (BIOMETRA). The amplified products were resolved on 1.5 % w/v agarose gel and documented.

2.4 RT-PCR

The mRNA was isolated using mRNA capture kit (Roche, Germany) from young leaves of T_0 plants. The mRNA was used for cDNA synthesis by Transcriptor high fidelity c DNA kit (Roche, Germany).

2.5 Southern hybridization of transformed plants

To confirm the gene integration Southern blotting method was used in the transgenic plants (T_0 plants). Genomic DNA was isolated from the leaves of the T_0 plants. For Southern hybridization 10µg of total genomic DNA from the putative transgenics was digested with *Bam HI* and resolved on 0.8% agarose. The probe was labeled with non- radioactive DIG labeling kit (Roche, Germany).

2.6 RNA dot blot analysis

The independent events of T1 plants were grown in the poly house for best event selection and gene expression analysis under drought stress condition. RNA was isolated from the young leaves of the independent transgenic events and blotted on the nitrocellulose membrane and with the specific *DREB 1A* probe.

2.7 Drought stress treatment to transgenic plants and inheritance studies:

To evaluate the gene expression of candidate genes for drought tolerance, transgenic and non transgenic plants were grown in the pots and drought stress was applied to the plants during square formation stage (55–60 days old plants) and boll developmental stage without watering for maximum 15 days for drought stress treatments.

2.8 Physiological studies

The reducing sugars, amino acids and proline [1] were quantified at regular interval using

standard procedures. The chlorophyll content of the transgenics were recorded.The leaf discs from non-stressed plants of both transformed and non-transformed were placed on PEG medium with varying degrees of stress (0, 0.4, 0.6, and 0.8 MPa). The biochemical changes induced due to stress was quantified at 0, 7 & 15 days after inoculation.

2.9 Total RNA extraction and cDNA synthesis

Total RNA was extracted using Sigma Spectrum Total RNA isolation kit. Fresh young leaf and root samples were taken from the main terminal branch of stressed plant at square formation and boll developmental stage and used for RNA isolation. RNA was quantified with the help of Nanodrop ND-1000 spectrophotometer by measuring absorbance at 260 nm and280 nm wavelength. RNA quality was checked by resolving the sample on 1% agarose gel. cDNA was synthesized by using Agilent cDNA synthesis kit.

2.10 Quantitative real-time PCR

The primers used for real-time PCR for *DREB*1A were F-CGATATTTGAGATGCCGAGTT and R-CGCCGTCGACTTCATGATTA. Real-time PCR reactions were carried out with Stratagene machine in 96-well plate using the Agilent SYBR Green Master Mix. 1ug of total RNA was used to prepare cDNA. The reaction conditions for genes *DREB1A, GADPH* genes were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s, and final elongation step at 72 °C for 10 min. Cotton glyceraldehyde-3-phosphate dehydrogenase (GAPDH) house-keeping gene was used as a control. Gene specific primers were designed using BIOEDIT software.

[III] RESULTS:

3.1 Plant transformation and regeneration:

Embryonic axes of 5-10 mm were trimmed from 2-3 day old cotyledons and were used for co-cultivation with *A.tumefaciens*. After co-cultivation for overnight the explants were decontaminated with Carbenicillin 50ug/50 ml. The explants were inoculated and selected in MS medium with kanamycin 50 mg/l as selection marker which allows only the transformants to grow. The shoot induction was observed after 10 -15 days in the MS medium. The putatively transformed shoots were sub-cultured in the MS medium with 0.1mg/l of IAA for shoot development and then transferred to MS medium with 0.1 mg/l 2,4-D for rooting after shoots attained a height of 5-6 cm. Rooted plants were rinsed well and transferred to pots containing peat, soil and sand in 1:1:1 ratio . The transgenic plants were grown in the green house under controlled condition.

3.2 Analysis of the transgenic plants:

The PCR analysis with the *npt II* gene and *DREB 1A* gene specific primers was carried out with the genomic DNA as template of individual plants resulted in the amplification of 700bp and 540 bp product respectively (Fig 3&4). Southern hybridization was carried out in the PCR positive transgenic plants by using Roche DIG labeling kit. Genomic DNA was isolated from the transgenic plants and they were restricted with the *BamHI* enzyme. The restricted DNA was resolved on 0.8% agarose gel electrophoresis and then blotted on to nitrocellulose membrane. The *DREB 1A* gene was amplified from the gene construct with the specific primer and eluted after purification and the probe DNA was labeled with the dig labeling kit. DNA samples of the independent transgenic plants of cotton was digested with *BamHI* and hybridized with *DREB 1A* gene probe has shown the integration of single copy gene.

The independent transgenic plants were grown in the pots. RNA was isolated from the young leaves of the independent transgenic events and blotted on the nitrocellulose membrane and hybridized with the specific *DREB 1A* probe using Roche DIG labeling kit. The dot blot assay result has shown positive in the transgenic plants (Fig 5).

3.3 Physiological studies:

Physiological and biochemical constituents were quantified at regular interval. The leaves of both non-transformed and transformed plant started to droop within seven days after stress induction.

The transformed plants would show recovery during night and leaves were turgid in early part of the day. This helped the plant to recover and grow normally. The leaf water potential also was maintained at higher level due to accumulation of solutes, which was not observed in case of control plants. Under control conditions, the soluble sugar contents in leaves were similar for wild type and different transgenic plants, that is, around 2.56 mg/g FW. After the drought stress treatment, the soluble sugar levels in transgenic plants increased after 5 days, and reached 3.64 to 7.01 mg/g FW at 20 days, whereas those in control plants at the same developmental stage reached only 3.08 g/g FW at 20 days. The statistical analysis showed that soluble sugar contents of transgenic plants were significantly higher than those of the wild type plants at 5, 10, 15 and 20 days (P\0.05). The amino acid content in transgenic plants after drought treatment increased after 5 days, and ranged between 0.532 to 0.779 mg/g FW at 20 days, whereas those in wild typel plants at the same developmental stage reached only 0.750 mg/g FW at 20 days. The statistical analysis showed that proline contents of transgenic plants were significantly higher than those of the control plants at 5, 10, 15 and 20 days (P\0.05).After drought treatment, the phenol levels in transgenic plants increased after 5 days, and ranged between 2.720 to 3.139 mg/g FW at 20 days, whereas those in wild type plants at the same developmental stage reached only 2.355 mg/g FW at 20 days. The chlorophyll A&B content ranged between 0.415 to 0.693 and 0.136 to 0.221 respectively (Table 1).

3.4 Analysis of stress tolerance and physiological changes in transgenic cotton by PEG treatments

The leaf discs from non-stressed plants of both transformed and non-transformed were placed on PEG medium with varying degrees of stress (0, 0.4, 0.6, and 0.8 MPa). The biochemical changes induced due to stress was quantified at 0, 7 & 15 days after stress. The data showed that there was an inherent tolerance developed in transgenic plants due to DERB 1A. The control plants showed a immediate burst in synthesis of reducing sugars, amino acids and proline but declined by seven days, while the transgenic plants showed a gradual increase in solute accumulation and maintained high even after 7 days. The non-transformed discs produced very high phenolic's which led to death of the tissues with stress (Fig 6). Over expression of *DREB 1A* improved drought tolerance in transgenic cotton and after stress alleviation of the solute content by increase in aminoacid, reducing sugar and proline when compared to control plants (Fig 7).

3.5 Gene expression studies:

Total RNA was extracted from leaves and roots under drought stress condition at different growth stages: Square formation (after 55–60 days) and Boll formation (after 120–130 days). During Square formation stage water was withheld for 15 days. Total RNA of both leaves and roots were isolated at 0 day and 15 day (water stress). The products of Total RNA were visualized as a smear along with two distinct ribosomal RNA bands, 28S and 18S. Real-time PCR detected the expression level of foreign genes in the transgenic progeny. Different lines exhibited different gene expression in response to drought stress at the two growth stages taken here.

The first square is formed on the lowest reproductive branch of the plant. Gene expression of leaf samples was observed to increase at squaring stage (15.67 fold expression) of *DREB1A* in transgenic plant number 3, 7.12 and 5.68 fold change increase in transgenic plant number 2 and 1 respectively. Compared to control non-transgenic plant (under the aforesaid conditions) and a drought tolerant variety it showed 2.97 and fold change respectively of *DREB1A.* The proline content in the transgenic plants has increased upon stress treatment and ranged from 0.37 to 0.75 *uM* when compared to the wild type and 28 I. The statistical analysis showed that proline content of transgenic plants were significantly higher than those of the wild type plants (P\0.05).Gene expression of root samples observed at squaring stage were found to be 2.53, 2.0 and 0.92 for transgenic plant number 1,2 and 3 respectively. Control non-transgenic plant and a drought

tolerant variety it showed 1 and 0.33 fold change. The proline content in the transgenic plants has increased upon stress treatment and ranged from 1.26 and 1.96 *uM* when compared to the wild type and 28 I. The statistical analysis showed that proline content of transgenic plants was significantly higher than those of the wild type plants (P\0.05) (Fig 8).

Cotton bolls generally begin to open 125–130 days after sowing and irrigation during this period is thought to be critical. Transformed lines were once again subjected to drought stress (10 days) and expression of gene at boll formation for leaf samples revealed that transgenic plant number 1 on average basis showed maximum drought tolerance (10 days) showing maximum expression (32.3 fold expression) of *DREB1A* followed by transgenic plant number 3 and 2 which exhibited fold quantity change of 25.9 and 24.7 respectively. Stressed Control (non-transgenic) plant exhibited 24.1 fold quantity changes and the drought tolerant variety 25.8 fold changes. The proline content in the transgenic plants has increased upon stress treatment and ranged from 1.08 and 2.02 *uM* when compared to the wild type and 28 I. Gene expression of root samples observed at boll formation stage was found to be 5.5, 4.56 and 4.17 for transgenic plant number 1, 2 and 3 respectively. Wild type non-transgenic stressed plant and a drought tolerant stressed variety showed 1.77 and 1.11 fold change expression of *DREB1A.* In the transgenic plants the osmolite, proline content has increased on stress treatment and ranged from 0.77 and 1.99 *uM* when compared to the wild type and 28I (Fig 9). Another distinguished feature that was observed in the height of transgenic plant was more than both wild type and 28I (Fig 10).

[IV] DISCUSSION

Abiotic stresses cause accumulation of compatible solutes and antioxidants, such as sugars, proline[16,30]. There are some overlaps in the regulation pathways of gene expression between different environmental stresses [31].Because of the various adverse effects of abiotic stresses on plant growth and productivity, it is very important to improve stress tolerance of the crop plants to maintain growth, productivity and increase crop yield under stress conditions. Tolerance of plants to abiotic stresses is a multigenic trait; genes that play a role in the abiotic stress response is frequently insufficient to improve stress tolerance significantly. Transcription factors that regulate several stress-responsive genes (*DREB1*s family),have been used to manipulate plants in order to overcome the stress and maintain the function and structure of cellular components [33].

During drought conditions, plants produce osmolytes such as free proline and various soluble sugars are osmoprotectants in stress-tolerant plants [20,19,36].CBF3/DREB1A overexpressed in transgenic Arabidopsis plants produced a higher level of free proline than in control plants under stress conditions [14] and over expression of OsDREB1A in transgenic rice plants was associated with increased soluble sugars levels compared with wild-type plants under drought, high salt and low temperature conditions [21]. In our study, rd29A:: DREB 1A transgenic plants accumulated higher levels of soluble sugar than wild-type plants under drought stress conditions, suggesting that overexpression of DREB activated the expression of downstream genes involved in sugar biosynthesis, which in turn, enhanced tolerance to drought stress in transgenic plants. DREB1A gene in transgenic Arabidopsis and rice plants activated the expression of many stress-inducible genes and improved drought, high salt and freezing tolerance [25, 23, 11] . Similar results of transgenic cotton plants in our study showed increased tolerance to drought stress. CBF/DREB1 genes in Arabidopsis resulted in plants with improved survival rates when exposed to salt, drought and low temperatures [22,23].The tolerance was correlated with both altered relative abundance of transcripts encoding proteins associated with stress adaptation and increased sugar contents [15] revealing the same kind of result in cotton. When CBF/DREB1 genes from Arabidopsis were over-expressed in other plants, the result

was similar to that in Arabidopsis [17,29],revealing a conserved signaling and response mechanism even between dicots and monocots. There is improved stress tolerance by over-expression of CBF/DREB1 genes is associated with sustained photochemical efficiency and photosynthetic capacity as compared with wild-type plants [18,28,34]. CBF/DREB1 genes have been expressed in transgenic plants under the control of a stress-inducible promoter, RD29A to overcome growth retardation [24] but in our study the control plants had growth retardation than transgenic plants. Transgenic plants have also shown enhanced abiotic stress tolerance without totally compromising the yield [30]. The use of the Arabidopsis RD29A promoter is more efficient in driving the expression of CBF/DREB1 genes in dicots rather than in monocots, or at least in rice [21].The transgenic plants grew normally, suggesting that DREB genes from a monocotyledon plant might not affect the growth and development of a dicotyledonous [7] plants. The basis of underlying abnormal growth and development of transgenic plants with DREB genes might be very complicated and possibly related to a range of factors, such as gene structure, uncertain insertion sites of the exogenous genes, and instability of gene expression caused by copy numbers [12].

Prevalence of drought stress is inconsistent under field conditions and plants may perhaps be exposed to this abiotic stress at any time throughout their life. Cotton is comparatively drought-tolerant, but severe water losses can slowdown plant maturity, affecting bolls and ultimately reduce yield. This study demonstrates that cotton plants of transgenic lines of progeny transformed with *DREB1A* showed improved drought tolerance at plant squaring growth stage that is a key water stress period during plant growth. Transcription factors that regulate stress-responsive genes (e.g. the *DREB1*s family) often have been used to manipulate plants in order to have a broader response and maintain the function and structure of cellular components [33]. During drought stress, many plants produce osmolytes such as free proline and various soluble sugars, which may function as osmoprotectants in stress-tolerant plants [19,36]. DREB1A in transgenic Arabidopsis plants produced a higher level of free proline than in control plants under stress conditions[14], and OsDREB1A in transgenic rice plants was associated with increased soluble sugars levels compared with wild-type plants under drought stress[25,23,11,21].Cotton transgenic plants in our study accumulated higher levels of soluble sugar than wild-type plants under drought stress conditions due over expression of DREB activated the expression of downstream genes involved in sugar biosynthesis, which in turn, enhanced tolerance to drought stress in transgenic plants.

In Arabidopsis the over-expression of DREB1 genes resulted in plants with improved survival rates when exposed to drought tolerance was correlated with both altered relative abundance of transcripts encoding proteins associated with stress adaptation and increased sugar contents [22,23,15]. DREB1 genes from Arabidopsis were over-expressed in other dicots and monocots, the result was similar to that in Arabidopsis [17,29] , revealing a conserved signaling is responsible for the mechanism. Improved stress tolerance was observed by over-expression of DREB1 gene is associated with sustained photochemical efficiency and photosynthetic capacity as compared with wild-type plants [18, 34, 28] same was observed in our results. Growth retardation can be overcome when DREB1 genes have been expressed in transgenic plants under the control of a stress-inducible promoter, RD29A [24] as in our experiment the transgenic plants have good growth than the wild type. Transgenic plants have shown enhanced abiotic stress tolerance without totally compromising the yield [30] . The use of the Arabidopsis RD29A promoter is more efficient in driving the expression of DREB1 genes in dicots rather than in monocot rice [21]. *DREB1A* is a transcription factor that induced by abiotic stress, strongly up-regulates many downstream genes; result in adaptation of plants to stress conditions and exercise specific tolerance mechanisms. Many studies indicated

that over-expression of *DREB1A* in crop plants can result in high tolerance to abiotic stress and thereby increased efficiency of plants production. Taken together suggest we used gene transfer by *DREB 1A* strategy to prevent of large and widespread yield reductions under stress conditions and obtain more products in agronomic development. However *DREB1A* has a key role in tolerance to abiotic stress such as drought, salinity, heat and low temperature stresses. Given that the molecular control mechanisms of abiotic stress tolerance are similar and *DREB1A* effected on majority of them, therefore it is possible that over-expression of *DREB1A* gene leads to tolerance in transgenic plants. It is hoped in the future that these efforts will help to prevent global-scale environmental damage that is resultant from these stress.

ACKNOWLEDGEMENT: We acknowledge INDO-US collaborative project for providing fund and the gene constructs to carry out the work.

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Fig 1: Gene Construct and Restriction Map of DREB 1A

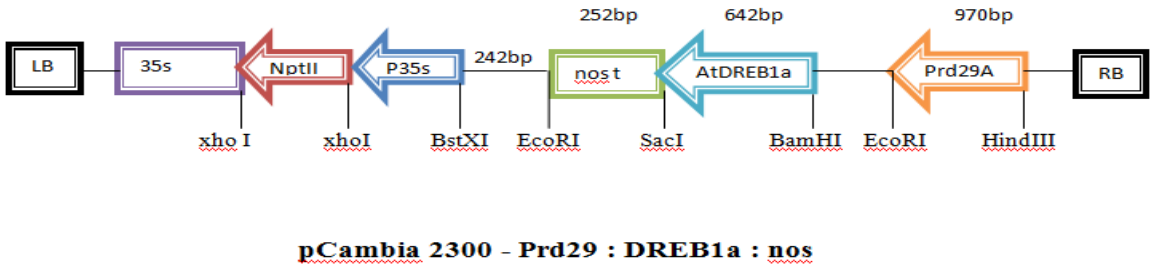
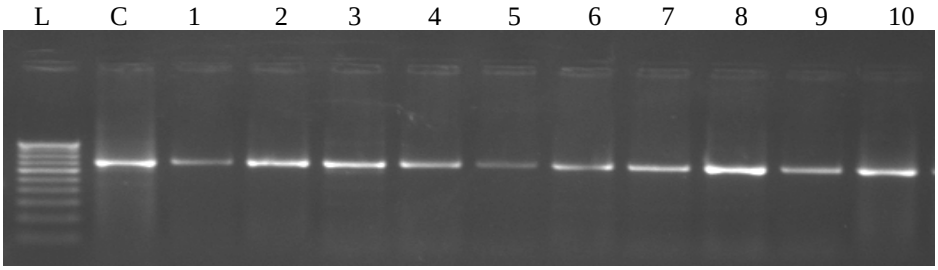


Fig 2: Selection and regeneration of putative transformants

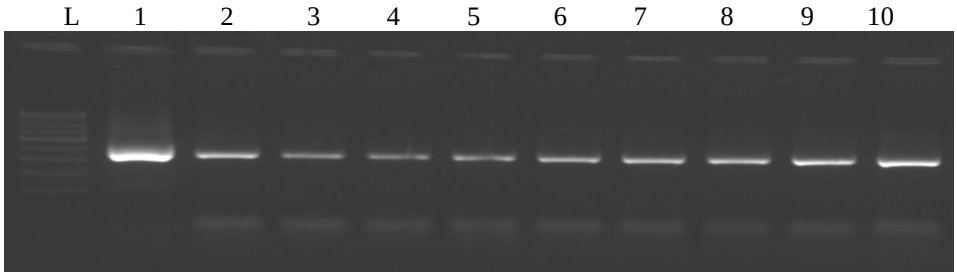
MS selection medium **Regenerated plant** **Putative transformant**

Fig 3: PCR analysis of To transgenics with *npt II* gene specific primer



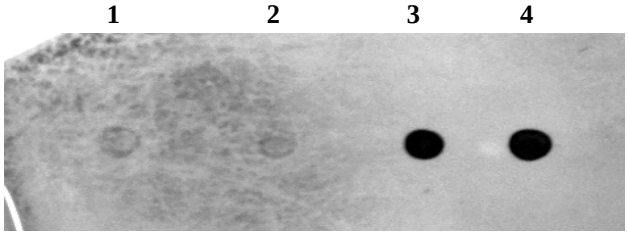
L- 100 bp ladder, C-+ve Control ,1-12: LRK 516 (*Dreb 1A)*

Fig 4: PCR analysis of putative transformants with *Dreb 1A* gene specific primer



L- 100 bp ladder,1-Control , 1-12: LRK 516 (*Dreb 1A)*

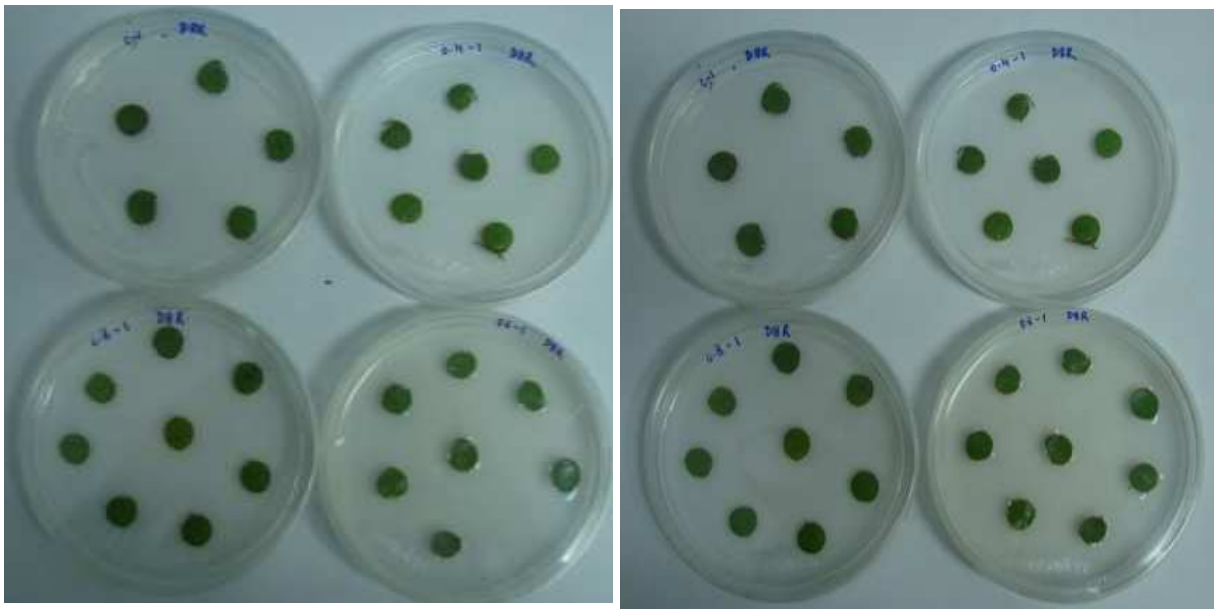
Fig 5: RNA dot blot assay of the transgenic plants.



1-4: RNA sample from independent transgenic plants (Dreb 1A)

Fig 6: Screening of transgenics with different concentrations of Poly Ethylene glycol

CONTROL 0.4 MPa CONTR 0.4 MPa



CONTROL 0.4 MPa

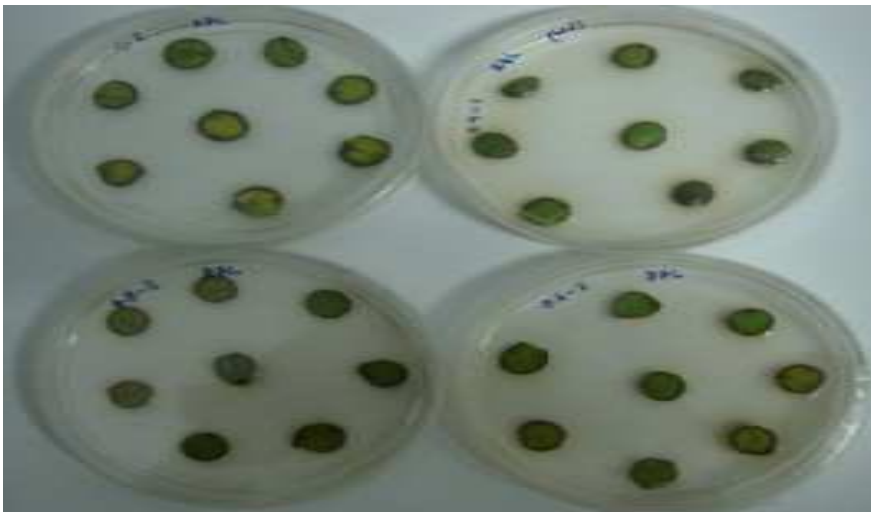
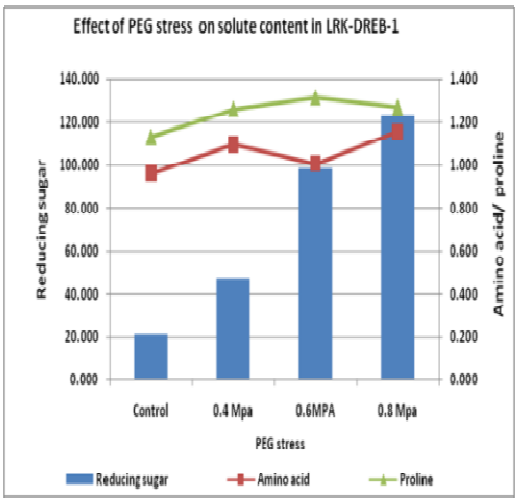


Fig 7: Comparative expression of transgenic and Non-transformed (NT) leaf disc to PEG stress and alleviation

Transgenic plant LRK 516 (*Dreb 1A*)



Non transformed plant LRK 516

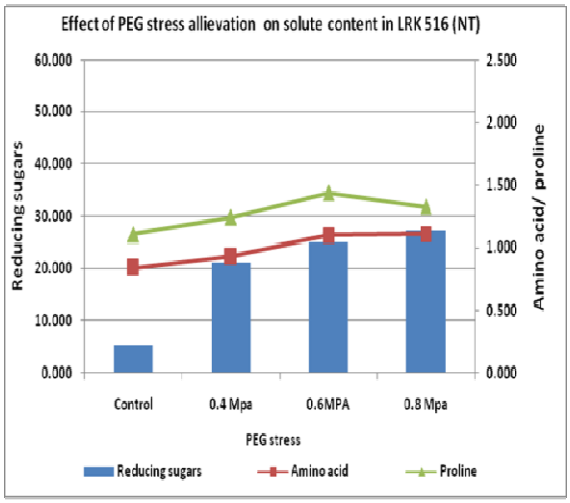
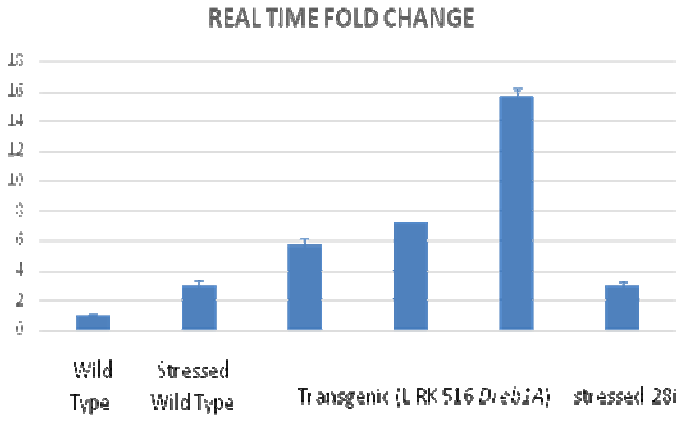
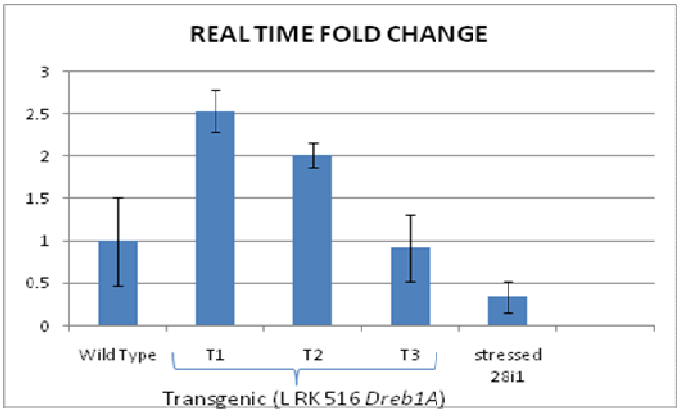


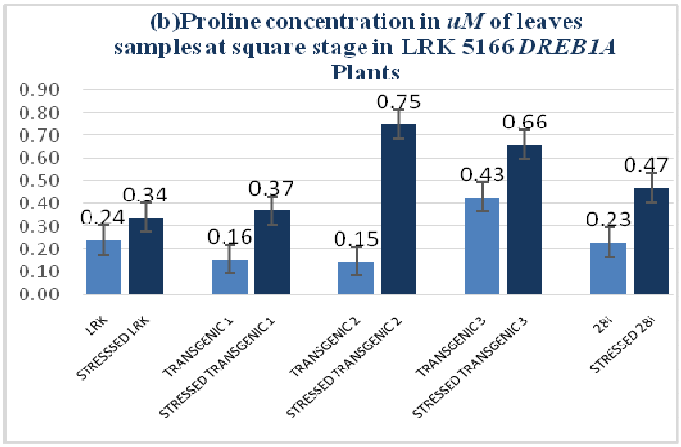
Fig 8: Relative fold gene expression of *DREB1A* in transgenic cotton plants and wild type at squaring growth stages.

(a)Real Time Fold Change data of leaves samples at square stage in LRK 5166 *DREB1A* Plants



(c)Real Time Fold Change data of root samples at square stage in LRK 5166 *DREB1A* Plants





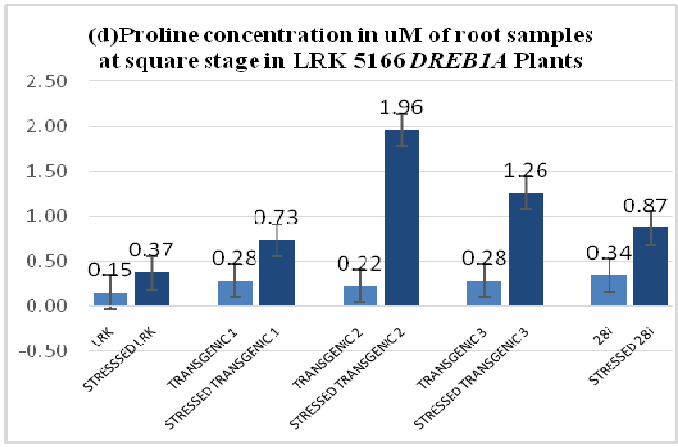
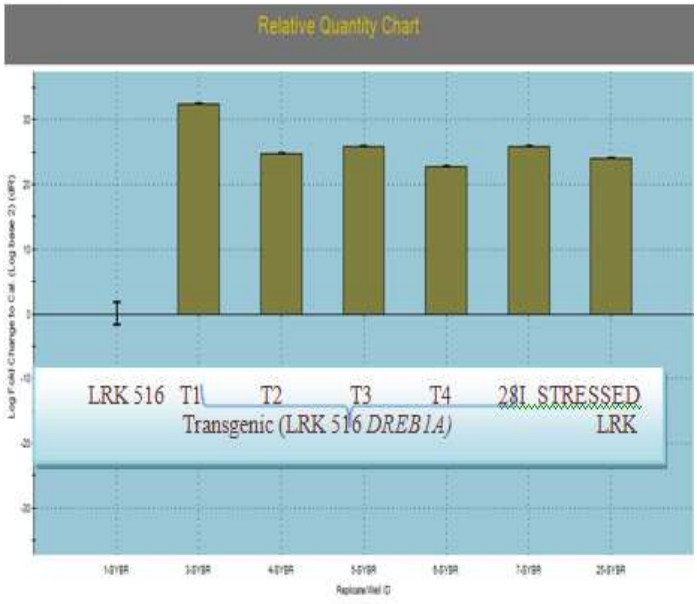
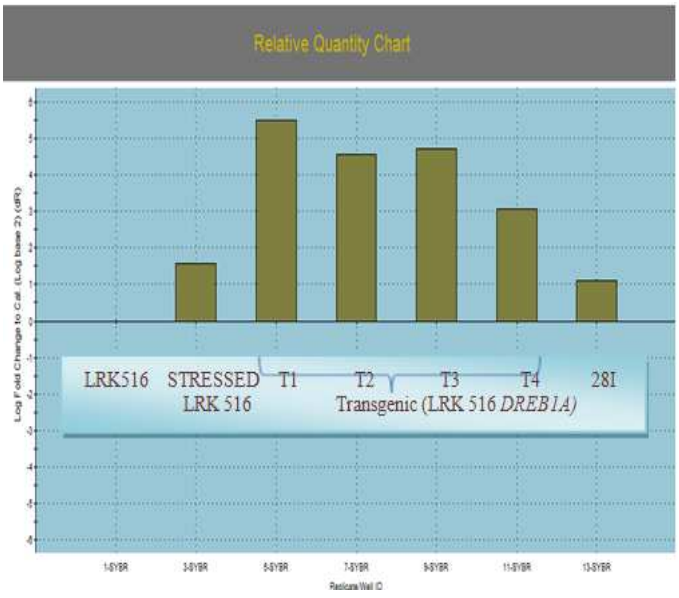


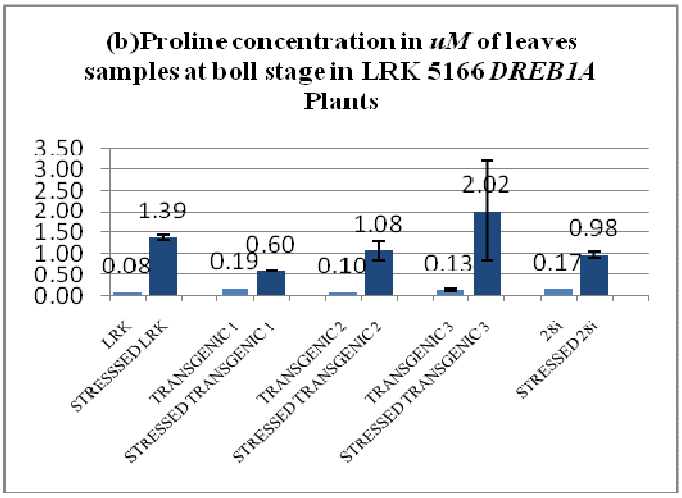
Fig.9: Relative fold expression of *DREB1A* gene in transgenic cotton plants and wild type at boll formation stage.

(a)Real Time Fold Change data of leaves samples at boll stage in LRK 5166



(c)Real Time Fold Change data of root samples at boll stage in LRK 5166 *DREB1A* Plants





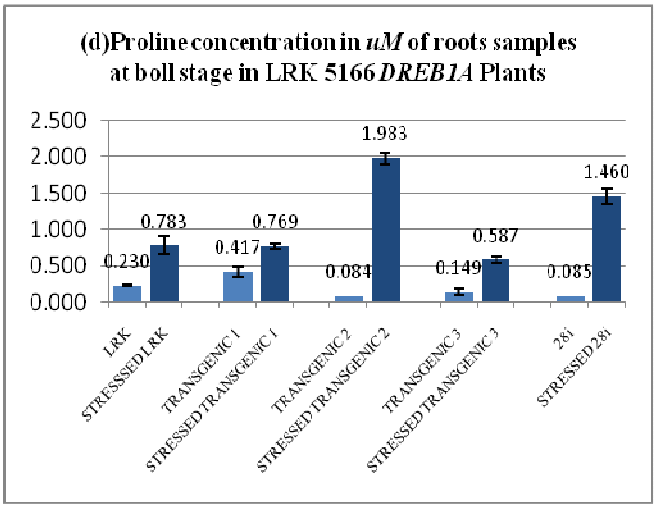


Fig 10: Height of the transgenic plant and wild type, drought tolerant variety 28 I.

Transgenic plant wild type Transgenic plant 28 I

Table 1 : Solute accumulation and chlorophyll content in Transgenic plants

Variety (LRK 516)	RS(mg/gFW)	Amino acid(mg/gFW)	Phenols mg/gFW	Chlorophyll content mg/gFW		
				A	B	Total
1	7.010	0.760	3.105	0.445	0.174	0.649
2	3.640	0.532	2.720	0.693	0.221	0.941
3	5.597	0.779	3.139	0.415	0.136	0.568
Control plant	3.080	0.750	2.355	0.536	0.236	0.800