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Full Length Article

Identification, Characterization, Homology Modeling and Protein-Protein Interactions of Cotton (*Gossypium arboreum*) DREB Gene

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Abstract

Drought is one of the important factors, reducing the yield in cotton. Among drought responsive genes, genes encoding for dehydration-responsive element binding (DREB) proteins play a major role in overcoming various types of stresses. The genome of *Gossypium arboreum* L. contains many important genes combating biotic and abiotic stresses that can be explored to identify these genes. For amplification of DREB gene from *G. arboreum*, primers were designed based on the conserved regions of GhDREB. For the development of cDNA libraries, RNA was isolated from cotton leaves after giving water stress treatment for 0, 30, 60, 90, 120 and 150 min. Results indicated that the expression of DREB gene was maximum after 90 min of water stress. The DREB gene amplified in *G. arboreum* was cloned, sequenced and designated as GaDREB. Phylogenetic analysis of GaDREB using twenty previously reported DREB gene sequences showed its highest homology with *Salicornia bigelivoii* DREB gene. Conserved domain analysis of the GaDREB explicated a single AP2 domain in it. Protein 3D models were determined using MODELLER 9.10 and Ramachandran plot indicated that 98% of the residues were found in the favored region. Furthermore, the protein-protein interactions showed that DREB interacted with the NAC, MYB, AREB, ABRE and DRE/CRT, which are involved in various drought stress responses via ABA independent pathway. Present study validated the involvement of the gene in conferring the drought tolerance and its interaction with the other drought responsive genes. This study will further facilitate the utilization of GaDREB gene for improving the drought tolerance in tetraploid cultivated cotton species.

Keywords: ABA independent pathway; Computational tools; Cotton; Drought stress; Improved crops

Introduction

Although cotton is mainly grown in dry environment; however, increasing scarcity of water has further become a major threat to sustainable cotton production (Ahmad *et al*., 2009; Ullah *et al*., 2017). Increased drought and high temperature conditions in Pakistan has dropped the cotton yield by 34% *i.e.,* from 9.68 M bales to 14.4 M bales (Ullah *et al*., 2017). *G. arboreum* (Desi cotton) is considered as diploid ancestor of today's cultivated cotton, owns many auspicious traits like tolerance to drought and resistance to diseases caused by insect pests like leaf curl (Wheeler *et al*., 1999; Rahman *et al*., 2002, 2005) root rot, infestation of aphids and bollworms (Mehetre *et al*., 2003).

Exposure to drought stress cause disturbance of water potential and membrane integrity, loss of turgor, and denaturation of proteins in plants (Ingram and Bartels, 1996; Osakabe *et al*., 2014). Understanding the sensing of stress and signal transduction pathways is the basis of deciphering these pathways and to genetically improve the plants against stresses through biotechnology. The basis of stress tolerance in plants is very complex (Ahmad *et al*., 2009). In plants responses to several environmental stimuli have been studied at different levels such as morphological, physiological, metabolic and biochemical (Tran and Mochida, 2010). Phytohormone abscisic acid (ABA) is involved in expression regulation of many genes and related physiological process thus playing significant role in responding to the abiotic stress (Nakashima *et al*., 2014). ABA-dependent or ABA-independent pathways lead to activation of several metabolic and physiological responses against environmental stresses (Yamaguchi-Shinozaki and Shinozaki, 2006).

Stress can activate genes, which contribute to enhance the tolerance mechanism (Seki *et al*., 2003; Zhang *et al*., 2004). These genes may also be involved in the signaling

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cascade and activate other transcription factors after activation or they may activate further functional or regulatory genes through their binding sites (Tran and Mochida, 2010). Multiple evidences have revealed that dehydration responsive element binding protein (DREB) plays an important role in the abiotic stress response pathway (Nakashima *et al*., 2014). The DREB proteins have an ERF/AP2 DNA-binding conserved domain. Many plants including *Arabidopsis*, tomato, tobacco, rice and maize contain DREB proteins (Agarwal *et al*., 2006). DREB family of transcription factors contains a unique conserved region, which make their interaction with downstream genes possible via ABA-independent pathway. DRE is also involved in ABA-dependent stress response regulation, which indicates towards a further cross talk between the ABA dependent and ABA-independent signal transduction pathways. This communication enlightens the role of ABA in stress signal transduction (Agarwal *et al*., 2006; Nakashima *et al*., 2014).

The structural and functional relationships of transcription factors are significant to know the molecular mechanism of stress tolerance in crop plants (Garg *et al*., 2008). Bioinformatics leads to structure/function prediction of genes by understanding the physicochemical properties (Nawaz *et al*., 2014). DREB gene from *G. arboreum* could be helpful in enhancing drought tolerance in present day cotton. However, DREBs have not been identified and characterized in *G. arboreum*. Hence, in the current study, we identified, amplified and sequenced DREB gene from *G. arboreum*. The sequence was further analyzed using computational tools for molecular characterization, homology modeling and protein-protein interaction. The results of the study would facilitate the utilization of the gene for developing drought tolerant crops. Involvement of this gene in drought tolerance mechanism by computational tools has further been validated in this study.

Materials and Methods

Plant Material and Stress Treatment

Seeds of cotton (*G. arboreum*) var. FDH-786 sourced from PGMB Lab, National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan were grown in composite soil in green house of NIBGE, Faisalabad, Pakistan. The temperature of green house was set at 25 ± 2°C during the day and night, while humidity was 50%. Water deficit treatment for 0, 30, 60, 90, 120 and 150 min was applied to one-month old seedlings by withholding irrigation until 15% weight loss of plants. Control plants were grown in the soil with sufficient water supply until collection of samples. Samples from leaves, stem and roots were collected for RNA isolation.

RNA Isolation

Total RNA from stressed and control plant tissues were harvested following Verwoerd *et al.* (1989) with some modifications. Quantity and quality of RNA was estimated using a spectrophotometer (NanoDrop-2000, Thermo Scientific, USA). For the development of cDNA libraries, RNA was isolated from leaves of one-month old seedling treated with water stress.

Primer Designing

A list of DREB genes was selected from other plant species and BLAST was run with cotton gene sequences from *G. hirsutum* to find the conserved regions of gene. The regions which were found conserved among cotton ESTs and previously identified genes in other species were considered for primer designing. Three primers Fa 5′-TGGAGCTAGGTGATTGTTG-3′, Fb 5′-GGGTCATGGAGCTAGGTGATTGTTG-3′ and R 5′-GGTCAAGGAATTAGGAGTA-3′ from gene sequence GhDREB (Accession no: AF509502.1), were used in the PCR.

Synthesis of cDNA and PCR Amplification

For making cDNA libraries, RNA obtained from leaf tissues of *G. arboreum* at different time intervals (0, 30, 60, 90, 120 and 150 min) of drought stress treatment were used. For cDNA synthesis, initially 12 μL reactions mix containing 1 μg/5 μL of total RNA, 1 μL of Oligo (dt) primer and 6 μL of DEPC treated water was incubated at 70^{o}C for 5 min and immediately chilled on ice. The 4 μL of 5x reaction buffer, 2 μL of 10 m*M* dNTP mixture and 1 μL of Riblock ^{TM} Ribonuclease inhibitor were added to the mixture by, mixing them gently. The mixture was briefly centrifuged and incubated at 37^{o}C for 5 min followed by addition of 1 μL of revert-Aid ^{TM} M-MuLV Reverse Transcriptase (Fermentas). After incubation of mixture at 42^{o}C for 60 min the reaction was stopped by heating at 70^{o}C for 10 min and keeping on ice for chilling. Further, gene specific primers were used to amplify single stranded cDNA from the library to double stranded cDNA.

A total volume of 25 μL was used for the PCR amplifications which contained 12.5 μL of 2X BioMix PCR master mix (Bioline, UK,), 1.0 μL of cDNA and 0.75 μL of 10.0 μ*M* forward and reverse primer (Invitrogen, UK). GeneAmp PCR system 9700 (Applied Biosystems) was used to carry out amplifications using the following programme: 5 min at 94^{o}C; followed by 35 cycles of 45 s at 94^{o}C, 60 s at 60^{o}C, and 90 s at 72^{o}C; and finally 7 min at 72^{o}C for the final elongation.

Cloning of the Amplified Genes

Gel electrophoresis using 1.0% (w/v) agarose gels accompanied with ethidium bromide (0.5 μg mL^{-1}) for visualizing the DNA bands was performed to visualize and analyze amplified products. Single band of estimated size was eluted from the agarose gel using GeneJET gel extraction kit (Thermo Scientific). Gel purified PCR

products were ligated in TA cloning plasmid vector pTZ57R (InsTAclone PCR Cloning Kit, Thermo Scientific) according to the manufacturer instructions and transformed into *E. coli* cells using heat shock method and transformed colonies were identified using blue white selection.

Plasmid DNA Isolation and Genes Sequencing

Plasmid was isolated by AxyPrep Plasmid Miniprep Kit. Selected plasmids were subjected to PCR for confirmation of insert. The cloned fragments in pTZ57R plasmid vector were commercially sequenced. After sequencing, the homology searches for genes were performed to find homology at NCBI/EMBL database by BLAST search software.

Sequence Analysis and 3D Protein Structure Prediction of DREB

DREB gene sequence was retrieved in different species by using NCBI web portal. Multiple sequence alignment was carried out using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) online web server. The results of alignment were downloaded and saved in form of alignment and cladograms.

Conserved Domain Analysis of DREB was done by using Conserved Domain online tool available at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Amino acid sequence of DREB was retrieved by translating the newly sequenced gene by JustBio translator tool (http://www.justbio.com/). Further 3D structure prediction of this amino acid sequence was performed using MODELLER 9.10, a python based protein modeling software. A suitable template structure was obtained using NCBI BLASTp server based on the lowest e-value and highest similarity. After prediction, the quality and reliability of 3D structure was determined using different confirmation softwares. To ensure the quality of structures, Swiss model online server (http://swissmodel.expasy.org/workspace) and MOLProbity evaluation (http://molprobity.biochem.duke.edu/) were used. The minimum DOPE score was used to select best structure. The Ramachandran plot was determined based on the model.

Protein-protein Interaction

On the basis of previous literature and published articles, 68 drought responsive genes were selected from various signaling pathways. FASTA format of drought responsive genes was obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The genes were translated using the procedure previously described. 3D structures were predicted for the 68 genes by using the Phyre2. Protein encoded by DREB gene was used as receptor and proteins encoded by 68 drought responsive genes were used as ligands in protein-protein interaction studies. The interactions were studied using ClusPro (http://cluspro.bu.edu/login.php). Furthermore, Pymol software was used to analyze the protein-protein interactions.

Results

Difficulty in isolating a fine quality RNA from *G. arboreum* was attributed to the enormous occurrence of phenols and polysaccharides in this plant failing all orthodox methods. Consequently, RNA was isolated from the leaves, stems and root tissues of *G. arboreum* plants (after imposing water deficit) by modifying the method of Verwoerd *et al.* (1989) which resulted in the isolation of good quality RNA with separate 28S and 18S ribosomal RNA bands (Fig. 1A). The amount of RNA obtained from leaf tissue was comparatively higher than stem and root and for further analysis RNA from leaves was used.

Primer sets designed from GhDREB (Accession no: AF509502.1) were used for the amplification of cDNA in *G. arboreum*. After the successful amplification of GhDREB gene as a control, PCR conditions were optimized for amplification of the gene from the cDNA synthesized from the RNA captured from *G. arboreum* after applying water deficit stress at various time intervals (*i.e.*, for 0, 30, 60, 90, 120 and 150 min). After amplification of the gene, the band intensity explicated that the expression of DREB gene was maximum after 90 min of water stress, which demonstrated the role of this gene in water stress response (Fig. 1B).

After amplification and purification of the amplicon of desired length, it was cloned into ptz57RT vector and sequenced commercially and the gene sequence was searched for homology by BLAST (Altschul *et al*., 1997). DREB gene isolated from *G. arboreum* was found 99% similar to *G. hirsutum* DREB1 gene accession number AY174160.1. The gene sequence of DREB gene derived from *G. arboreum* was submitted to NCBI. The NCBI Accession number of newly identified GaDREB gene sequence is KP297804.

Sequence Analysis of DREB

GaDREB gene sequence was used for multiple sequence alignment with retrieved DREB gene sequences from multiple plant species. The phylogram (Fig. 2) showed the homology of DREB gene among the different species. GaDREB gene sequences showed its highest homology with *Salicornia bigelivoii*. Conserved domain analysis of the GaDREB was performed and single domain AP2 was predicted.

3D Protein Structure Prediction of DREB

Amino acid sequence of GaDREB (Accession no: KP297804) was retrieved by translating the newly sequenced gene sequence by JustBio translator tool.

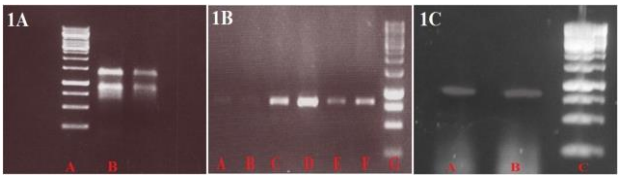

Fig. 1: (1A) RNA of *G. arboretum* (A) 1 KB Ladder (B) *G. arboreum* RNA; (1B) PCR amplifications of DREB from *G. arboreum* cDNA at different sress levels (A) at zero time, (B) at 30 min, (C) at 60 min, (D) at 120 min (E) at 150 min (F) 1KB Ladder; (1C) PCR of plasmids having inserts of *G. arboreum* and *G. hirsutum* (A) *Ga*DREB (*G. arboreum*), (B) *Gh*DREB (*G. hirsutum*), (C) 1KB Ladder

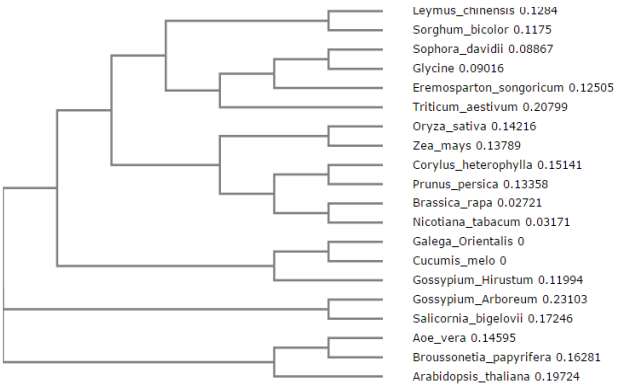

Fig. 2: Phylogram of DREB gene sequences in different plants

A BLASTp search was performed against the PDB database to find the best matching template (PDB ID: 3PTY). Modeller v9.10 was used to predict the 3D structure of GaDREB. The minimum DOPE score was used to select best structure (Fig. 3A). Ramachandran plot was determined based on the model. Ramachandran plot showed that 98% residues were in allowed regions (Fig. 3B). The model was found to be of good quality according to Ramachandran plot statistics and online server's evaluation.

Protein-Protein Interaction

Among the proteins encoded by 68 genes reported in drought stress tolerance pathways, DREB interacted with the NAC, MYB, AREB, ABRE and DRE/CRT (Fig. 4), which are members of the ABA-independent pathway of stress tolerance. Pymol was used for determination of the frequency (number) of residues of DREB protein interacting with residue of ligand protein and to get information about names of residues involved in the interaction of receptor and ligand (Table 1). Results show that DREB binds to NAC, MYB, AREB, ABRE and DRE/CRT in active binding sites.

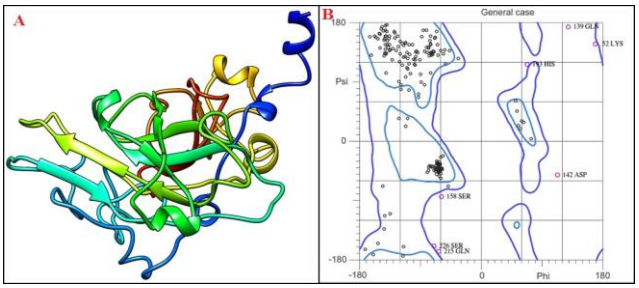

Fig. 3: (A) 3D structure of DREB gene of *G. arboretum*, (B) Ramachandran plot showing 98% residues in most favorable regions

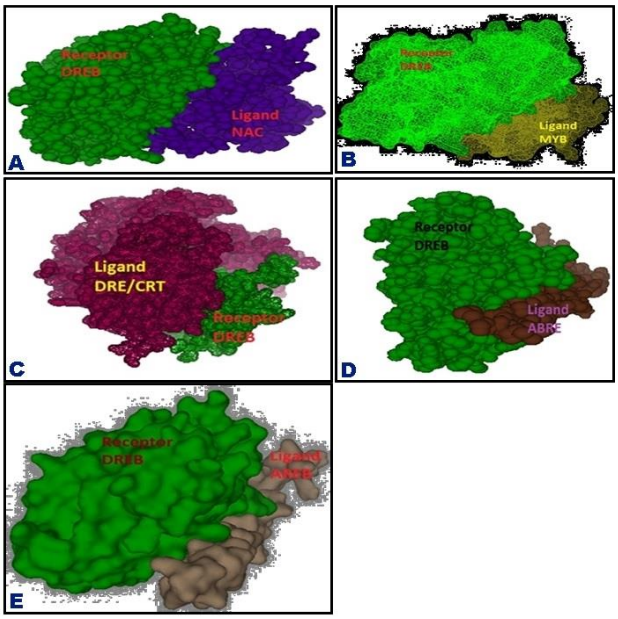

Fig. 4: Interaction studies of DREB gene with drought responsive genes in ABA-independent pathway. Interaction of DREB with: A) *NAC*, B) *MYB*, C) *DRE/CRT*, D) *ABRE* and E) A*REB*

Discussion

Like many other crop species, drought imposes the negative impact on cotton growth, which ultimately reduces the cotton yield. Excessive heat and high act salinity together synergistically with drought stress, which further impede the growth of cotton plant. Many research efforts have been made for exploring the resistance mechanisms conferring tolerance to drought (Wright and Rachaputi, 2004). Desiccation stress results in changing expression patterns of genes in plants (Chandler and Robertson, 1994). Physiology of the cotton plant is badly affected by the drought stress, which is a complex phenomenon. DREBs attained a lot of attention in the past decades, because it played an important part in plants by responding to several abiotic stresses (Khan, 2011).

Table 1: Residue interaction of proteins encoded by drought responsive genes with DREB protein. (Residues of Receptors are in interaction with residues of Ligands respectively)

Receptor	Ligand (accession No. of encoding gene)	Residue interaction	
		Receptor	Ligand
DREB	NAC (AJ401151)	ASN-63, ALA-262, LYS-192, ARG-188, SER-22, ASN-21, ALA-262	LYS-105, ARG-142, GLU-12, LEU-13, GLU-25, GLU-25, ARG-142
DREB	MYB (AY366352.1)	LYS-261, THR-56, ILE-55, ASN-63, SER-22, GLN-33, ARG-188, ARG-188, ARG-188, LYS-192, LYS-202, LYS-202,TRP-93	ASN-63, ARG-94, ARG-94, ARG-94, LYS-82, LYS-82, ILE-117, ASP-119, ARG-124, ASP-126, GLU-71, GLU-72, GLN-120
DREB	AREB (JN812059.1)	THR-6, THR-6, THR-209, THR-137, LEU-113, ASN-112	THR-387, LYS-393, GLU-389, ASN-401, LEU-404, LEU-40
DREB	ABRE (AF093546.1)	ILE-173, SER-177, VAL-109, ARG-145, ASP-142, LYS-140, LYS-140, HIS-249, THR-6, THR-6, THR-209	SER-385, ARG-392, ARG-392, GLU-401, THR-397, THR-397, ALA-402, GLN-406, GLU-413, LYS-416, GLN-415
DREB	DRE/CRT (AY502052.1)	ARG-94, ARG-94, LYS-92, ARG-94, ARG-94, ARG-89, ARG-89, ARG-89, ARG-89, ARG-196, SER-201, LYS-202, LYS-202, VAL-29, LYS-59, SER-34, SER-34, ASN-32, GLN-33	ARG-407, GLN-411, ARG-343, GLU-308, ARG-342, ARG-343, HIS-209, LEU-128, ASP-279, HIS-275, ARG-129, ARG-129, GLU-126, GLY-124, HIS-275, HIS-161, ASP-233, MET-234, LEU-203, MET-234

Keeping in view the significance of *G. arboreum* having biotic and abiotic stress tolerance, this study was designed to identify DREB gene from *G. arboreum* genome. Gene sequence of *G. hirsutum* was used as reference sequence to identify DREB gene from *G. arboreum*. Using conventional genetic tools, various drought tolerant cultivars were developed in the past. Conventional breeding being a laborious and time taking task (Iqbal *et al*., 2013) can be augmented by biotechnology and computational methods to transfigure the breeding methods. These tools were found a vital source for the depiction of drought responsive genes in plants (Kopecky *et al*., 2013). In this study, many computational tools were used to predict the occurrence of the DREB gene in *G. arboreum* and the pathway and combination of several genes involved in drought tolerance.

The expression of DREB gene was found maximum after 90 min of drought stress application on *G. arboreum* plants, which indicated the upregulation of this gene under water stress. The higher expression of DREB genes under drought conditions has also been reported in similar studies. A higher expression of mRNA of DREBs was observed after exposing *Triticum turgidum* subsp. durum to limited water stress for an extended time period, thus exhibited the up-regulation of this gene under water-limited conditions (Melloul *et al*., 2014). In another study, drought stress differentially regulated the expression level of DREB gene in wheat cultivars (Hassan *et al*., 2015).

A single domain AP2 was predicted in GaDREB, which is DNA-binding domain and is also found in other plant transcription regulators like APETALA2 and EREBP. EREBP is involved in stress response and it contains single copy of AP2 domain (Chen *et al*., 2016). APETAL-2 is involved in plant development and holds two copies of the AP2 domain (Okamuro *et al.*, 1997). Identification of AP2 domain reflects the importance of DREB in conferring stress tolerance in *G. arboreum*. Previous studies reported that ERF/AP2 DNA-binding domain is conserved and is widely distributed in many plants, including *Arabidopsis*, tomato, tobacco, rice and maize (Agarwal *et al*., 2006). DREB proteins have AP2/ERF domain and a conserved part that is accessible next to an AP2/ERF domain which helps in phosphorylation of DREB protein, consequently DREB proteins can bind with DNA and transcribe the downstream genes (Agarwal *et al.*, 2006). In homology studies GaDREB gene sequence has shown the highest homology with the DREB gene of *Salicornia bigelivoii* which is a dicotyledonous facultative halophyte (Jaradat and Shahid, 2012). *S. bigelivoii* has been found highly tolerant to salinity and slightly tolerant to drought stress (Martinez-Garcia, 2010).

The 3D structure of the protein encoded by GaDREB was determined and the Ramachandran plot showed that 98% residues were in allowed regions, which provide the authenticity of the model. Ramachandran plot provides a simple view of the conformation of a protein (Ho and Brasseur, 2005). In understanding the protein interactions, functions, and localizations, 3D structure analysis plays an important role (Parasuram *et al.*, 2010). Out of 68 gene products, DREB protein interacted with the NAC, MYB, AREB, ABRE and DRE/CRT encoded proteins (Fig. 4) which are members of the ABA-independent pathway of stress tolerance. Results show that DREB binds to NAC, MYB, AREB, ABRE and DRE/CRT in active binding sites that depict their involvement in the same pathway. DREB, AREB, ABRE and DRE/CRT work together in the *A. thaliana* to make it resistant to desiccation and salt stress (Narusaka *et al*., 2003). In a similar study in wheat, biochemical function predictions using computational tools suggested that the wheat DREB1A orthologs have similar biochemical functions and pathways to that of AtDREB1A (Kumar *et al.*, 2016). The DREBs (dehydration responsive elements binding) were also found important for the survival of plants in temperature extremes (Riechmann *et al*., 2000) and play vital role in activation of low temperature or freezing temperature and desiccation linked genes (Andeani *et al*., 2009). Freezing or low temperature stress and dehydration stress induces the *DREB1* and *DREB2* respectively, which are involved in an ABA independent pathway (Khan, 2011).

The information generated in this study has augmented the available repository of candidate genes for crop

improvement. The GaDREB reported in this study can be further utilized to make transgenic drought tolerant plants. In previous studies, many DREB1-type genes inserted into plants resulted in stress tolerant agronomic crops like in tobacco (Kasuga *et al*., 2004), tomato (Shah *et al*., 2016), wheat (Pellegrineschi *et al*., 2004), rice (Dubouzet *et al*., 2003), and potato (Pino *et al*., 2008). Furthermore, transgenic *Arabidopsis* developed through constitutive overexpression of DREB1s/CBFs, which was more tolerant to drought, freezing and high salinity (Gilmour *et al*., 1998; Jaglo-Ottosen *et al*., 1998). Drought tolerance was found to be improved by an active form of DREB2, which further trans-activate the target stress-inducible genes in transgenic *Arabidopsis* (Sakuma *et al*., 2006). Furthermore, engineering to develop stress tolerant crop varieties, DREBs are considered as candidate genes (Lata *et al.,* 2011; Melloul *et al*., 2014). AtDREB1A was also used to enhance drought stress tolerance in transgenic indica rice (Ravikumar *et al*., 2014). The findings of the present study are very promising for further utilization of the GaDREB for development of drought tolerant crops.

Conclusion

GaDREB was identified in *G. arboreum* and reported to NCBI (Accession No. KP297804). The gene was found to be upregulated under drought stress conditions. The protein-protein interaction studies showed that DREB protein interacted with a number of proteins encoded by drought responsive genes like NAC, MYB, AREB, ABRE and DRE/CRT involved in ABA-independent pathway of stress tolerance. GaDREB is a good candidate gene to develop transgenic plants having sustainable resistance against drought stress under different climatic conditions.

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