Molecular cloning and functional characterization of a DREB1/CBF-like gene (GhDREB1L) from cotton

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The transcription factors DREBs/CBFs play important roles in the regulation of plant resistance to environmental stresses and are quite useful for generating transgenic plants tolerant to these stresses. In the present work, a cDNA encoding DREB1/CBF-like protein (GhDREB1L) from cotton was isolated, and its sequence features, DNA binding preference, and expression patterns of the transcripts were also characterized. GhDREB1L contained one conserved AP2/ERF domain and its amino acid sequence was similar to the DREB1/CBF group of the DREB family from other plants. The DNA-binding domain of GhDREB1L was successfully expressed as a fusion protein in *Escherichia coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography. Electrophoretic mobility shift assay revealed that the purified GhDREB1L fusion protein had a specific binding activity with the previously characterized DRE element (core sequence, ACCGAC) and also with the DRE-like sequence (core sequence, GCCGAC) in the promoter of the dehydration-responsive late embryogenesis-abundant gene LEA D113. Semi-quantitative RT-PCR showed that GhDREB1L was induced in the cotton cotyledons by low temperature, as well as drought and NaCl treatments. These results suggested that the novel cotton GhDREB1L might play an important role in response to low temperature as well as drought and high salinity through binding to the DRE cis-element.

cotton, DREB1/CBF, cold, dehydration, LEA D113

Cold stress is one of the major environmental stresses that affects plant growth and limits crop productivity and quality. Many plants evolved various adaptive mechanisms to survive this adverse environmental stress[1]. At present, the better understood regulation system is the DREB1/CBF (dehydration-responsive element-binding factor 1/C-repeat binding factor) cold response pathway[2]. The DREB1/CBF-type genes were first characterized in *Arabidopsis*[3,4], and subsequently, found in other plants, including wheat, rye, tomato and rape[5,6], maize[7,8], barley[9,10] and rice[11]. In *Arabidopsis*, the DREB1/CBFs are the members of a superfamily with 147 AP2/ERF domain-containing proteins[12] and are characterized by the presence of the DREB1/ CBF signature sequences[5]. These two motifs, PKK/ RPAGR- XKFXETRHP and DSAWR, flank the AP2/ERF domain and are thought to impart specificity and targeting of DREB1/CBFs to their cognate cis-acting DNA binding site[5].

This type of the DREB1/CBF gene was first identified to be induced strongly by low temperature. Within 15 min of cold stress, transcripts of *DREB1A/CBF3*, *DREB1B/CBF1*, and *DREB1C/CBF2* were accumulated[3,4]. However, recent work demonstrated that some DREB1/CBF genes possess different expression-induced patterns[11,13,14]. The *Arabidopsis* CBF4 gene and the hot pepper Ca-DREBLP1 gene were both induced by dehy-

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Hydration, but were not responsive to exposure to cold stress\cite{13,14}. OsDREB1A and HvCBF7 were induced by cold as well as salt treatment\cite{11,15}, while HvCBF1 and LeCBF1 were induced under both cold and drought conditions\cite{15,16}. These findings suggested that the DREB1/CBF type proteins participated in multiple environmental pathways in addition to cold stress.

Ectopic expression of several DREB1/CBF-type genes in transgenic plants indicated that many target genes that harbor DRE/CRT elements within their promoters were activated and these transgenic plants showed freezing, drought and high salinity tolerance\cite{17,18}. Therefore, DREB1/CBF-type genes represented a critical component in the signal transduction of cold and osmotic stresses.

Cotton is one of the most important economic crops in the world. Its growth and productivity are severely affected by environmental stresses such as drought, cold and high salinity. We previously reported the characterization of one DRE-binding protein, GhDBP1, from cotton\cite{19}. Phylogenetic analysis showed that GhDBP1 was not classified to the DREB1/CBF group, and the transient expression experiment suggested that GhDBP1 was a transcriptional repressor. There is no report about the characterization of DREB1/CBF-type genes in cotton to date. As the enhanced stress tolerance in this plant was of great importance and DREB1/CBF proteins were quite useful for generating transgenic plants tolerant to low temperature, high salt and drought stresses\cite{17,18}, we focused on examining whether there was a DREB1/CBF-type gene in cotton. In this study, a cDNA encoding the cotton DREB1/CBF gene was isolated from cotton leaves. EMSA showed that GhDREB1L interacted with the DRE element (core sequence, A/GCCGAC). Its transcripts were greatly induced by cold as well as drought and high salinity stresses. These findings provided an important insight into the molecular adaptation mechanisms of environmental stress in cotton and knowledge about these mechanisms was also crucial for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops.

1 Material and methods

1.1 Plant materials and stress treatments

Cotton (Gossypium hirsutum cv. Zhongmian 35) plants were grown in controlled environment rooms at 25°C with a 16-h light and 8-h dark cycle. On 2-week-old seedlings, low temperature treatments were performed by transferring plants to a growth chamber set to 4°C for different periods of time under the light and photoperiodic conditions described above. Dehydration was induced by removing plants from the plots and then placing them on a dry filter paper. Salinity stress treatment was applied by submerging the roots of the plants in a water solution of 400 mmol/L NaCl. All samples harvested for nucleic acid extraction were weighed, immediately frozen in liquid nitrogen, and stored frozen at −80°C until extracted.

1.2 Isolation of the gene encoding DRE-binding transcription factor from cotton

Bioinformatics methods and the RACE-PCR procedure were combined to clone the full-length cDNA of DRE-binding protein from cotton. First, we searched the cotton EST database using the tblastn program (provided by the National Center for Biotechnology Information, NCBI) with the amino acid sequences of the AP2/ERF domain of Arabidopsis DRE-binding transcription factor DREB1A (AB007787)\cite{4}, and found one cotton EST (DT561036) with deduced protein sequences highly homologous with that of the DREB1A transcription factor. According to this EST sequence, the primers:

\[
\begin{align*}
3'\text{GSP1:} & \text{5'}-\text{TATTTTCCCTTGCATTTCCA-3'} \\
3'\text{GSP2:} & \text{5'}-\text{GTTTAACTGAAACTTAAACTG-3'}
\end{align*}
\]

were designed to isolate the 3’ end of this DRE-binding protein cDNA from cotton using a 3’-full RACE kit (TakaRa, Dalian, China). The PCR-amplified products were cloned into the pMD18-T vector (TakaRa, Dalian, China) and sequenced. According to the 3’-RACE results, a pair of primers:

\[
\begin{align*}
P1/3'\text{GSP2:} & \text{5'}-\text{GTTTTACCTGAACTTAAACTG-3'} \\
P2: & \text{5'}-\text{ACAGCAGCCTGATACAATC-3'}
\end{align*}
\]

were designed and used to amplify the fragments containing the open reading frames.

DNA sequence data were assembled and analyzed using the DNAMAN analysis program (Lynnon Biosoft, USA). Database searches were performed with the NCBI BLAST search program. Alignment of the GhDREB1L protein with other structurally related AP2/ERF proteins was performed using Clustal W software\cite{20} and further adjusted by the GenDoc program\cite{21}.

1.3 Expression and purification of the recombinant protein

For expression of the DNA-binding domain of GhDREB1L protein, the coding region was PCR ampli-
fied and inserted into the *Bam*H I and *Eco*RI sites of vector pET32a (+) (Novagen, Germany). The forward and reverse primers used for the amplification were 5′-CGGGATCCCTCCGATGGCTATGTGATG-3′ and 5′-CGGAATTCTTACTAAGCGTGTCGACAG-3′, respectively. The expression of the recombinant protein in *Escherichia coli* BL21 (DE3) and its affinity purification were performed according to the methods described previously.

### 1.4 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were conducted as described previously. The following DNA fragments were used in EMSAs: rd29A-DRE, 5′ upstream region of the *Arabidopsis* rd29A gene (nucleotide –258 to –234, 5′-AAAAAGTTATTAGAGGATGTT-3′); D113-DREL, DRE-like sequence of 5′ upstream region of the cotton *LEA D113* gene (nucleotide –306 to –282, 5′-GAACAACTTATTAGGGATGCTA-3′). The labeled probes (ca 0.02 pmole) were incubated with 500 ng of purified His tag fusion protein in 20 μL 1× binding buffer (25 mmol/L HEPES/KOH pH 7.9, 50 mmol/L KCl, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 5% glycerol, 1 μg mL⁻¹ BSA) for 30 min on ice supplemented with poly (dI-dC)-poly (dI-dC) (Amersham Biosciences, USA). The resulting DNA-protein complexes were loaded on 0.5× Tris-Borate-EDTA, 6% (30:0.8, acrylamide-bis) polyacrylamide gel. After electrophoresis at 10 V·cm⁻¹, the gel was subsequently dried and visualized by autoradiography. For competition experiments, unlabeled competitors were incubated with the fusion protein at room temperature for 30 min prior to the addition of labeled probes.

### 1.5 RT-PCR analysis

RT-PCR analysis was carried out as described by Yang and Pooavaiah. The gene specific primers were: P1 (5′-GTTTAACCTGGAGCCCTAACTAG-3′) and P2 (5′-GGGAGCCGTATGTAAACCAC-3′) for *GhDREB1L*; SSU1 (5′-AATCCAAAGAAAGGAGTTAAGGAG-3′) and SSU2 (5′-GCATCACAGACCTGTTATCGAAGATG-3′) for cotton small-subunit (SSU) rRNA. PCR was performed for 29 cycles each including 40 s at 94°C, 40 s at 53°C, and 1 min at 72°C. In order to ensure the gene-specificity of these primers used, we sequenced the PCR fragments obtained with these primers and found that these fragments were indeed derived from the *GhDREB1L* transcript or cotton small-subunit (SSU) rRNA, respectively. The PCR products were then separated on a 1.5% (w/v) agarose gel. Amplification of the different RT reactions with the SSU primers resulted in bands with similar intensities between different samples, whereas their amplification with the *GhDREB1L* primer resulted in bands with different intensities between samples. This suggested that the PCR reactions were still in the linear range of amplification and did not reach the plateau level expected from deletion of the rate-limiting PCR components.

### 2 Results

#### 2.1 Cloning and sequence analysis of the *GhDREB1L* gene

One cDNA encoding DRE-binding protein was isolated from the leaves of cotton with 3 h treatment at 4°C using the bioinformatics methods and RACE-PCR procedure as described in materials and methods. This cDNA, designated *GhDREB1L*, encodes a protein of 216 amino acids with a predicted molecular mass of 24.4 kD and a theoretical isoelectric point of 8.42 (Figure 1). The sequence was submitted to the GenBank under the accession number of DQ409060. The alignment of this protein against various AP2/ERF proteins suggested that *GhDREB1L* encoded a DREB1/CBF-type protein in cotton (Figure 2). Besides the presence of putative AP2/ERF domain, GhDREB1L also contained a typical DREB1/CBF-type four-amino-acid sequence (DASW) at the end of its AP2/ERF domain and a basic amino acid stretch, KRRAGK, before the AP2/ERF domain, which had been suggested to function as a nuclear localization signal (NLS) in the DREB1/CBF-type proteins. GhDREB1L also possessed an acidic region in its C-terminal, which might act as a transcriptional regulation domain (Figure 2). In addition, GhDREB1L had two conserved residues of V14 and E19 in the AP2/ERF domain, which had been reported to be conserved in the DREB subfamily.

The sequence alignment of GhDREB1L with the other known DREB1/CBF-type proteins revealed that GhDREB1L shared an overall amino acid sequence identity of 51%, 48%, 47% and 47% with tomato *LeCBF1*, *Arabidopsis thaliana* CBF4/DREB1D, CBF1/DREB1B, and DREB1A/CFB3, respectively (Figure 2). Despite this low sequence identity among these proteins, the AP2/ERF domain of GhDREB1L shared 81%—84%
Figure 1  Nucleotide and deduced amino acid sequences of GhDREB1L cDNA. The AP2/ERF domain is underlined and the polyadenylation signal is boxed. The two GSP primers for isolation of 3′-end of GhDREB1L cDNA are indicated by arrows.

amino acid sequence identity with that of DREB1/CBF proteins. Furthermore, the two DREB1/CBF signature sequences were also conserved between these five proteins (Figure 2, underlined).

2.2 Prokaryotic expression and purification of the GhDREB1L protein

From the sequence alignment, it was supposed that the GhDREB1L protein was a DREB1/CBF-type transcription factor. Being a transcription factor, the DNA-binding activity was important to its function. So we used the EMSA method to characterize its DNA-binding activity. As the first step, the DNA binding domain (117 amino acids) of GhDREB1L was expressed as a His-tag fusion protein in E. coli. The recombinant protein was exhibited by SDS-PAGE with a molecular weight of about 34 kD (Figure 3) and found to be identical as expected. Recombinant protein was rapidly purified by simple one-step Ni-NTA affinity chromatography and the purity of the final GhDREB1L fusion protein was estimated to be more than 97% (Figure 3).

2.3 Specific interactions of the GhDREB1L protein with DRE cis-element

The binding ability of the GhDREB1L protein to the DRE sequences was examined using the EMSA method. A 25-bp oligonucleotide containing one copy of the DRE binding site from the Arabidopsis rd29A promoter[22] was used as probe and was shown in Figure 4(a). In the mutant versions of the oligonucleotide, the
Figure 2  Multiple alignment of the DREB1/CFB proteins. The amino acid sequences of cotton GhDREB1L (DQ409060), Arabidopsis DREB1A/CFB3 (AB007787), CBF1/DCBF1B (AB007788), CBF4/DCBF1D (AB015478) and tomato LeCBF1 (AY497899) were aligned. The consensus sequences of AP2/ERF DNA-binding domains are boxed. Two DREB1/CFB signature sequences, PKRRAGRKKFRETR and DSAW, are underlined. The valine and glutamic acid residues at positions 14 and 19 inside the AP2/ERF domain are marked by asterisks. Three glutamic acid and three aspartic acid residues in the extreme carboxyl-terminal activation domain are shown by dots. Dashes represent gaps introduced to maximize similarities.

Figure 3 SDS-PAGE analysis of the purified recombinant GhDREB1L protein. The fusion protein was overexpressed in E. coli BL21 (DE3) and purified by Ni-NTA affinity chromatography. M, low molecular mass protein marker (kD); 1—2, eluted fusion protein under 150 mmol/L imidazole, 50 mmol/L NaH2PO4 and 300 mmol/L NaCl.

-core sequence of the DRE cis-element had been changed from CCGAC to CTTTT (Figure 4(a)).

As shown in Figure 4(b), oligonucleotide rd29A-DRE was clearly shifted by the recombinant GhDREB1L protein (Figure 4(b), lanes 2—4). The specificity of the GhDREB1L protein binding to the rd29A-DRE element was also confirmed by competition assays utilizing increased quantities of the unlabelled wild-type rd29A-DRE oligonucleotide. The results showed that the intensity of the shifted band was decreased with a 50-fold excess of unlabelled wild-type probe (Figure 4(b), lane 3). However, when using the unlabelled mutant rd29A-DRE sequence, the intensity of the shifted band was not altered (Figure 4(b), lane 4). These results indicate that GhDREB1L has a specific binding activity with the rd29A-DRE sequence.

Next, we searched for some target genes of GhDREB1L in cotton plants. Previously, we demonstrated that there was a DRE-like sequence (D113-DREl) in the promoter region of the cotton late embryogenesis-abundant gene LEA D113 and this DRE-like sequence could be bound by DRE-binding protein GhDBP1[19]. The expression of LEA D113 gene could also be regulated by GhDBP1[19], suggesting the LEA D113 gene could be one target of cotton DRE-binding proteins. To test whether GhDREB1L was involved in the regulation of the LEA D113 gene, the EMSA method...
was first used to analyze whether GhDREB1L could bind to the DRE-like sequence in the promoter of the LEA D113 gene in vitro. The probe containing this DRE-like sequence as well as its mutant version was shown in Figure 4(a). The results illustrated that the GhDREB1L protein was able to bind to the D113-DRE1 sequence (Figure 4(b), lanes 5—8), suggesting that the GhDREB1L might regulate LEA D113 gene expression by binding specifically to the D113-DRE1 cis-element. It is clear that the real target genes or/and binding sites of GhDREB1L in cotton must be explored using chromatin immunoprecipitation (ChIP) or another useful technique.

2.4 Expression profiles of GhDREB1L

The expression pattern of GhDREB1L was analyzed using the semi-quantitative RT-PCR method. Using gene-specific probes, it was found that GhDREB1L had a low expression level in cotton cotyledons under normal conditions. Exposed to low temperature, its mRNA accumulated quickly and reached the maximum level at 6 h (Figure 5(a)). After 12 h of cold treatment, the transcripts of GhDREB1L began to decline. The expression profile of GhDREB1L under cold stress was similar to most DREB1/CBF-type genes from Arabidopsis as well as other plants [3—5, 7, 9, 11]. It should be noted that the expression of GhDREB1L was also induced by salt and drought treatments (Figure 5(b), (c)). By NaCl treatment, the expression profile of GhDREB1L was coincident with that induced by cold treatment (Figure 5(b)). However, in drought conditions, the transcripts of GhDREB1L were more transient than those induced by cold or NaCl treatment. They accumulated slightly at 1 h and began to decrease after 6 h and were hardly detected after 12 h treatment (Figure 5(c)).

3 Discussion

In the present work, a novel cDNA of DREB1/CBF-type transcription factor, GhDREB1L, was isolated from cotton. Its protein sequence contained a typical AP2/ERF DNA-binding domain and two DREB1/CBF signature sequences (Figures 1—2). Sequence comparison analysis showed that GhDREB1L shared high amino acid identity with previously characterized DREB1/CBF transcription factors especially in the AP2/ERF domain. It was formerly reported that the 14th valine and 19th glutamic acids are important for recognition between DRE-binding transcription factors and the cis-element in
the promoter regions of their target genes\textsuperscript{[25,26]}. The former is more conservative than the latter, as the 19th glutamic acid varies slightly in the DREB subfamily. The 14th and 19th amino acids of the AP2/ERF domain of the GhDREB1L protein are valine and glutamic acid, respectively. Therefore, it was logical to propose that this protein could bind to the DRE cis-element. Then, EMSA was performed and the results showed that GhDREB1L protein successfully bound to the previously characterized DRE cis-element in sequence specificity. Similar results were also observed in its homologous proteins such as Arabidopsis DREB1, rice DREB1, and hot pepper Ca-DREBLP1\textsuperscript{[14,11,14]}. Many candidates for the DREB1/CBF target genes have been identified using Northern blot and microarray analyses\textsuperscript{[17,18,27—29]} and are classified into two groups. The first group includes effectors that are believed to function directly in stress tolerance, while the second group contains protein regulators that are involved in regulation of gene expression and signal transduction in the stress responses. The major members of the first group are LEA or LEA-like hydrophilic polypeptides thought to play a role in dehydration and freezing tolerance. LEA protein was first characterized in cotton\textsuperscript{[23]}. Recently, we showed that the cotton LEA gene (LEA D113) was greatly accumulated in cotton leaves when the cotton seedlings were exposed to various environmental stresses\textsuperscript{[30]} and the DRE-like sequence in its promoter region could be bound by DRE-binding protein GhDBP1\textsuperscript{[19]}. So it was expected that the LEA D113 gene might be the direct target of DRE-binding protein in cotton. As illustrated in Figure 4, the purified fusion GhDREB1L protein could bind to the D113-DRE1 sequence in the LEA D113 gene promoter region, suggesting that GhDREB1L (A-1 group of DREB subfamily) like transcriptional repressor GhDBP1 (A-5 group of DREB subfamily) might participate in the regulation of the LEA D113 gene in cotton plants. Like many DREB1/CBF-type genes from Arabidopsis and other plants\textsuperscript{[3—5,7,9,11]}, GhDREB1L was induced by low temperature (Figure 5(a)). In addition, the transcripts of GhDREB1L were also increased under NaCl and drought treatments (Figure 5(b), (c)). Results from several studies illustrated that some DREB1/CBF-type genes could also be induced by other abiotic stresses in addition to low temperature. For example, OsDREB1A and HvCBF7 were induced by cold as well as salt treatment\textsuperscript{[11,15]}, while HvCBF1 and LeCBF1 were induced under cold and drought conditions\textsuperscript{[15,16]}. Previous results demonstrated that LTI (low temperature induced), COR (cold regulated), RD (responsive to dehydration), and ERD (early responsive to dehydration) genes, which all encode effectors that function directly in stress tolerance, were induced by both low temperature and osmotic stress which includes drought and high salinity\textsuperscript{[1,31,32]}. And recently, some reports\textsuperscript{[11,15,16]} together with the present work showed that some DREB1/CBF-type genes, which regulated these LTI/COR/RD/ERD genes, were also induced by these two environmental stresses. These results indicated that in addition to cold stress, some DREB1/CBF\textsubscript{S} like the LTI/COR/RD/ERD genes also participated in the osmotic stress signal pathway.

In conclusion, we described here the cDNA cloning and functional characterization of a DREB1/CBF-type transcription factor gene, GhDREB1L, from cotton. It possessed one conserved AP2/ERF domain and two DREB1/CBF signature sequences. The DNA-binding domain of GhDREB1L could interact with the previously characterized DRE cis-element in vitro and could also bind to the DRE-like sequence in the LEA D113 gene promoter. In addition, GhDREB1L was induced by low temperature as well as drought and high salt stress treatment. These results provided more information about DREB1/CBF type transcription factors and an important insight into the molecular adaptation mechanisms of environmental stresses in cotton plants.

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