

Genome identification of the DREB gene family in *Lycium barbarum* and expression analysis in response to leaf blight infection

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Research Article

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Abstract

Background

To analyze the characteristics of members of the dehydration responsive element binding protein (DREB) transcription factor family in *Lycium barbarum* and their response patterns during *L. barbarum* leaf blight stress, bioinformatics methods were used to conduct a genome-wide identification of the DREB family members in the *L. barbarum* genome, and systematically analyze the physical and chemical characteristics of proteins, gene structures, phylogenetic evolution, collinearity, and expression patterns of family members under *Alternaria tenuis* Nees infection.

Results

A total of 16 non-redundant LbDREB members were identified in the whole genome of *L. barbarum*, all of which were hydrophilic proteins. They were unevenly distributed on 10 chromosomes of *L. barbarum*, encoding 174–503 amino acids, with a relative molecular mass from 19.29–57.93 kDa and a theoretical isoelectric point from 4.61–9.66. Phylogenetic analysis showed that the 16 genes could be divided into 6 subgroups (A1–A6), all of which contained one AP2 conserved domain. Subcellular prediction showed that the vast majority of LbDREB members were located in the nucleus and cytoplasm, and a small number were located in the mitochondria. Sequence lengths of LbDREB members varied greatly, ranging from 348–3530 bp, and seven pairs of collinear genes were detected. The ratios of non-synonymous mutations (Ka) to synonymous mutations (Ks) (Ka/Ks ratios) were all < 1, indicating that the LbDREB family tended to purify selection during evolution. The 16 LbDREB members showed significantly different expression characteristics at 0, 24, 48, 72, and 120 h after *L. barbarum* leaf blight pathogen infection. The overall expression level was highest at 120 h of the infection period, and all 16 members are upregulated in expression.

Conclusion

The results indicated that the LbDREB gene may play an important role in the response of *L. barbarum* to leaf blight, and provide a reference for further clarification of the functional mechanism of the DREB transcription factor members in *L. barbarum*.

Background

Transcription factors are a class of proteins that bind specifically to cis-acting elements in the promoter region of genes, and thus effectively regulate gene transcription activity via this mechanism. Over the duration of plant growth and development, transcription factors play a central role in plant response mechanisms to various biotic and abiotic stressors, such as pathogen infection, insect feeding, drought, and high and low temperatures^[1–5]. Analyzing the structural features of DNA-binding domains in plant transcription factors enables these factors to be classified into multiple families, such as myeloblastosis (MYB); NAM, ATAF1/2, and CUC2 (NAC), WRKY transcription factor family (WRKY), ethylene-responsive factor and APETALA2 (AP2/ERF), and basic-leucine zipper (bZIP), all of which play key roles in plant response to biotic stressors^[2, 3].

The dehydration responsive element binding protein (DREB) is a crucial member of the AP2/ERF superfamily. The constituent members of this family typically contain a conserved AP2 domain consisting of a sequence approximately 60 amino acids in length ^[5]. DREB and the ERF gene family (which also belongs to the AP2/ERF superfamily) can be differentiated by comparing the composition of specific amino acid residues in the AP2 domain, even though both contain only one AP2 domain. In the AP2 amino acid domain, the key amino acid positions 14 and 19 are critical for DREB family members, carrying valine (V) and glutamic acid (E), respectively; whereas, ERF family members carry alanine (A) and aspartic acid (D), respectively, at the same AP2 positions ^[5, 6]. It is this type of amino acid variation at key sites that weakens their binding efficiency to cis-acting elements in the promoter regions of downstream genes. DREB transcription factors can specifically bind to the core sequence ACCGAC/GCCGAC of the dehydration response element/C-repeat (DRE/CRT) cis-acting element in the promoter region of downstream genes and activate downstream gene expression through construction of a regulatory network, thereby participating in plant response to low temperatures, drought, high salinity, and other abiotic stressors. However, differences have been found in the cis-acting elements that bind to different members of the ERF family compared to DREB elements, which have led to functional differences ^[3, 5, 7].

Based on their structural and functional features, the DREB family can be further divided into six subgroups (A1–A6), with each subgroup fulfilling different functions ^[8]. Members of subgroup A1 (also known as the DREB1/CBF family) are associated with plant responses to cold stress ^[9]. In *Arabidopsis thaliana*, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 genes are able to identify low-temperature stress. By upregulating expression of these genes, the transgenic plant *A. thaliana* showed significant improvements in its tolerance for low temperatures, drought, and high salinity stress; conversely, silencing of the *DREB1A/CBF3* and *DREB1B/CBF1* genes attenuated its cold tolerance ^[10]. In addition, members of subgroups A2–A6 are closely associated with biotic stress responses and plant growth. These gene families have been detected in a variety of plants, such as *A. thaliana*, wheat (*Triticum aestivum*), strawberries (*Fragaria vesca*), tomatoes (*Solanum lycopersicum*), walnuts (*Juglans regia*), and grapes (*Vitis vinifera*). Hence, a comprehensive study on the functions of each member in these families is crucial to understanding the mechanism of DREB family members found in *L. barbarum* (LbDREB) gene expression in *L. barbarum* and establishing a basis for its application in plant breeding for stress tolerance ^[11–14].

As a branch of the AP2/ERF family, the DREB gene family is mainly involved in plant response to abiotic stressors, and is also of crucial significance to biotic stresses. In this field of biotic stress regulation, maize *DREB1A* has been shown to reduce the level of active salicylic acid (SA) by inhibiting the SA synthesis gene (*ZmSARD1*) and activating the SA inactivation gene (*ZmSAGT*), thereby negatively regulating plant blight resistance ^[15]. In moso bamboo, *PeDREB28* can bind to the promoter of the abscisic acid (ABA) receptor gene (*DlaPYL3*) to regulate the ABA signaling pathway and hence indirectly affect disease resistance ^[16]. In terms of abiotic stress regulation, 23 DREB members in *Hibiscus cannabinus* were significantly upregulated under salinity stress, which implied that DREB members are able to activate osmoregulatory genes (e.g., LEA proteins) in order to enhance salt tolerance and maintain leaf health, thereby reducing the chances of pathogen infection ^[17]. In moso bamboo, *PeDREB28* expression was significantly elevated under drought and low-temperature stress, and enhanced resistance by regulating the ABA signaling pathway and redox-related genes, thereby indirectly inhibiting pathogen colonization ^[16].

Lycium barbarum is a multi-stemmed shrub of the family Solanaceae Juss and genus *Lycium*, mainly found in northwestern China. Its fruits are oval in shape and usually red or orange–red in color ^[18]. *Lycium barbarum* is rich

in nutrients, containing a variety of vitamins (e.g., vitamins C, B1, and B2) and exhibiting significant effects in antioxidation and immune function enhancement. Furthermore, *L. barbarum* contains an abundance of minerals, including iron, zinc, and selenium, which are essential to the normal physiological functions of the body. In particular, *L. barbarum* polysaccharides have shown immune regulation, anti-tumor, blood glucose-lowering, blood lipid-lowering, and other biological activities ^[19]. In *Shennong Bencaojing* (*Divine Farmer's Classic of Materia Medica*), *L. barbarum* polysaccharides are classified as a high-grade medicine, and are described as being able to “strengthen the muscles and bones, lighten the body and prolong life, and withstand the cold and heat, if administered long-term.” The continuous discovery of the numerous health effects of *L. barbarum* has been accompanied by growing concerns over its quality, which has attracted widespread attention. Hence, examining the expressions of adversity genes in *L. barbarum* under biotic and abiotic stresses is profoundly significant for enhancing the quality of *L. barbarum*. *Lycium* leaf blight is a major fungal disease in primary *L. barbarum* production areas, with an incidence rate of 60–80% in the hot and humid season. It can cause severe damage by triggering premature senescence of affected leaves, reducing fruit yield, and degrading fruit quality, directly contributing to economic losses of 30–50% ^[20]. This disease is mainly caused by the pathogen *Alternaria tenuis* Nees ^[20], which is a broad-spectrum parasitic fungus ^[21] that can cause wheat leaf blight, eggplant early blight, northern corn leaf blight, *Alternaria brassica* leaf spot, and other diseases of major economic and food crops. Although *Lycium* leaf blight poses a substantial threat to the industry, its pathogenic mechanism remains poorly understood. Therefore, the aim of this study was to establish a theoretical basis for identifying leaf blight-resistant DREB transcription factors in *L. barbarum* through genome-wide analysis, bioinformatics analysis, and examination of DREB expression patterns under leaf blight infection. In addition, this study provides a genetic reserve for improving the disease resistance and quality of *L. barbarum*.

Methods

Experimental Materials

Test strains and vectors: *Escherichia coli* DH5α and a cloning vector TSINGKE TSV-007S pClone007 Simple Vector Kit were purchased from Tsingke Biotech Co., Ltd.

Test plants: *L. barbarum* plants were obtained from Jingyuan County, Gansu Province.

Main reagents and culture media: The nucleic acid extraction system consisted of TRizol Total RNA Extraction Reagent (Tsingke Biotech) and a FastKing Genomic DNA Dispelling RT SuperMix system (Tiangen Biotech). The gene amplification system consisted of the PrimeSTAR® GXL High-Fidelity DNA Polymerase (Tsingke Biotech) and Ex Taq® Conventional DNA Polymerase (TaKaRa Bio). The nucleic acid analysis system consisted of SYBR® Green Pro Taq HS Real-Time Quantitative Detection Premix (AG Bio) and 2000 bp DNA Molecular Weight Marker (Biomed). The gene cloning system consisted of a *KpnI/XbaI* double digestion system (TaKaRa Bio) with a plasmid rapid extraction kit (Tiangen Biotech) and a DNA fragment purification kit (Tsingke Biotech). The reverse transcription system consisted of a PrimeScript™ II First Strand cDNA Synthesis Kit (Tsingke Biotech) and Perfect Real-Time RT dedicated reaction system. The microbial culture system consisted of Luria–Bertani (LB) agar medium containing 50 µg/mL ampicillin. All reagents were prepared according to the manufacturer's standard operating procedures.

Identification of DREB family members in *L. barbarum*

Whole-genome sequence data of *L. barbarum* was obtained from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>), and DREB family sequence data for *A. thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), wheat (*T. aestivum*), grape (*V. vinifera*), walnut (*J. regia*), and poplar (*Populus euphratica*) were obtained from the NCBI (<https://www.ncbi.nlm.nih.gov/>), TAIR (<https://www.arabidopsis.org/>), and Ensembl Plants (<https://plants.ensembl.org/index.html>) databases ^[22, 23]. Systematic screening of LbDREB was performed using two strategies. First, based on the DREB homologous sequences of six plants, potential DREB family members were identified in the *L. barbarum* protein sequence files using BLAST analysis. Second, the Hidden Markov Model (HMM) of the AP2 domain (PF00847) was obtained from the pfam (<http://pfam.xfam.org/>) database and a search was performed on the protein sequences of the *L. barbarum* genome using TBtools (v2.210) to further screen for DREB family members. To ensure accuracy and reliability of the screening results, the intersection of results obtained using these two methods was taken. The AP2 domain of the candidate genes were then further verified using the Batch CD-Search tool of the NCBI-CDD database (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) to ensure that all sequences were DREB family members.

Cloning of DREB members in *L. barbarum*

Total RNA was extracted from *L. barbarum* leaves using the TRIzol method. The RNA sample was then diluted in 40 µL of nuclease-free water. The concentration of RNA samples was accurately measured using an Unano-2000 Microvolume Nucleic Acid Analyzer, and their integrity was then assessed using agarose gel electrophoresis. Finally, total RNA samples collected from *L. barbarum* leaves were stored in a refrigerator at -80°C to preserve important materials for subsequent experimental studies.

Total RNA extracted from *L. barbarum* leaves was used as a template to perform cDNA synthesis. The manufacturer's instructions for the TaKaRa PrimeScript™ II 1st Strand cDNA Synthesis Kit were strictly followed during the reverse transcription process and system construction. Prepared cDNA samples were then transferred to a -20°C refrigerator.

Specific primers were designed using Primer 5.0 based on the full-length sequences of the *LbDREB1–LbDREB16* genes in the GenBank nucleic acid sequence database as standard sequences, and completed by Xi'an Tsingke Biotech, Co., Ltd. Subsequent procedures were performed within a 25 µL polymerase chain reaction (PCR) system, which comprised 16.3 µL of ddH₂O, 2.0 µL of cDNA, 2.5 µL of buffer solution, and 2.0 µL of dNTP Mix. In addition, 1.0 µL each of upstream and downstream primers were added, along with 0.2 µL of Taq enzyme. For this reaction, the pre-denaturation step was set at 94°C for 5 min, followed by cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 60 s, with a total of 30 cycles. Finally, an extension phase was carried out at 72°C for 10 min, after which the samples were stored at 4°C. The products amplified using the PCR reaction were separated by 2.0% agarose gel electrophoresis. Images of the separated products were then captured using a gel imager, which were recorded in detail and photographed.

Gel extraction was performed for specific bands containing the target sequence. The recovered purified product was then mixed with a cloning vector TSINGKE TSV-007S pClone007 Simple Vector Kit at a volume ratio of 1:4, followed by ligation in a metal bath at 25°C for 5 min. DH5α (100 µL) was added to the ligation product. After standing on ice for 25 min, the product was heat-shocked in a water bath at 42°C for 60 s, and immediately immersed in an ice bath for 2 min. Then, 1000 µL of LB culture broth was added, mixed well, and shaken at 37°C for 1 h at 200 rpm. Bacterial solution (100 µL) was spread onto LB solid medium and incubated for 14 h. Single

colonies were placed in 5 mL of LB liquid medium, to which 5 μ L of Amp was added, followed by incubation for 16 h at 37°C with shaking. Finally, the cultured bacterial solution was used as a template for further PCR experiments involving bacterial solutions. Three bacterial solutions with positive PCR results were screened and transported to Sangon Biotech Co., Ltd. for sequencing.

Analysis of physicochemical properties and subcellular localization of DREB family proteins in *L. barbarum*

Physicochemical properties of the 16 DREB family protein sequences screened from the dataset were comprehensively analyzed by accessing the ProtParam tool (ExPASy ProtParam tool) on the ExPASy server (www.expasy.org). This analysis involved several key indicators, such as amino acid chain length, relative molecular mass, isoelectric point, and protein stability coefficient [24]. In addition, predictions were made for the subcellular localization of DREB proteins using the online analysis tool Cell-PLoc (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>) [25, 26]. Multiple sequence alignment of LbDREB proteins was performed using DNAMAN software.

Phylogenetic tree construction and chromosomal localization of the DREB family in *L. barbarum*

Phylogenetic tree analysis was performed on the 16 LbDREB protein sequences and the DREB family members of *A. thaliana*, walnut, grape, and poplar, followed by construction of a dendrogram using the neighbor-joining algorithm in MEGA (v11.0). In addition, 1000 repetitions of the bootstrap test were executed to enhance the reliability of the data. Default values were retained for all parameters during the construction process [27]. The online tool itol (<https://itol.embl.de/>) was used to beautify the phylogenetic tree and analyze the phylogenetic relationship of the LbDREB family with *A. thaliana* homologous genes [25]. Candidate gene IDs were also screened from the *L. barbarum* genome annotation file, and TBtools (v2.210) was employed to obtain information on *L. barbarum* chromosome length and chromosomal location of the LbDREB gene. The screened gene IDs were visualized and localized on the *L. barbarum* chromosome to facilitate subsequent analysis of biological functions.

Analysis of gene structure, conserved motifs, and promoter cis-acting elements of DREB genes in *L. barbarum*

The 16 members of the LbDREB family were analyzed for conserved motifs using the MEME online platform (<http://meme-suite.org/tools/meme>). In this analysis, the number of conserved motifs was set to 10 and other parameters were set to default values, in order to analyze the similarity and functional features of the DREB family [4]. The structure of the 16 screened LbDREB genes was visualized using the Visualize Gene Structure feature of TBtools (v2.210). Finally, a 2000-bp sequence fragment upstream of the DREB gene in the genome was extracted and uploaded to the Plant Care database (<http://bioinformatics.psb.ugent.be/webtools/plantcare.html/>) for analysis of cis-acting elements [4, 28]. Finally, relevant response element tags were screened from the database, merged, and visualized in TBtools (v2.210) for further mining of their possible biological functions.

Analysis of collinearity and ratios of non-synonymous mutations (Ka) to synonymous mutations (Ks) (Ka/Ks) of the DREB family in *L. barbarum*

Collinearity analysis and visualization were performed intra-specifically within *L. barbarum* and inter-specifically between *L. barbarum* and *A. thaliana* using TBtools (v2.210). Defaults settings were used for all parameters. The whole *L. barbarum* genome was subjected to BLAST analysis, and gene pairs were screened based on E -value $< 1e^{-5}$ and Score ≥ 500 . In addition, CDS sequences of LbDREB gene pairs were extracted, and the Simple Ka/Ks Calculator tool was employed to accurately calculate Ks and Ka among gene pairs.

Expression analysis of DREB family in response to leaf blight in *L. barbarum*

Using healthy *L. barbarum* plants as materials, mature leaves of the third node in the middle of the plant were selected and inoculated with the *Lycium* leaf blight pathogen *A. tenuis*. Leaf samples were collected at 0, 24, 48, 72, and 120 h after inoculation, and stored in an ultra-low temperature refrigerator at -80°C after flash-freezing with liquid nitrogen. Three biological replicates were collected for each time point.

Total leaf RNA was extracted using a TRizol kit. cDNA was prepared in strict accordance with the operating instructions of a FastKing gDNA Disppelling RT SuperMix (TIANGEN) Kit, and the template was diluted 5-fold for subsequent quantitative detection.

Gene expression analysis was performed using the SYBR Green Type I double-stranded DNA-conjugated fluorescence quantitative detection system (AG Bio). The cDNA synthesized by reverse transcription was used as the template, and the β -actin gene of *L. barbarum* was used as the endogenous control. A real-time fluorescence quantitative detection system was constructed based on specific amplification primers (Table 1) designed for the conserved region of the LbDREB gene. The experimental setup was based on a three-level replication strategy, whereby three groups of biological parallels were set up for each sample, with each group containing four technical replicates. The standard reaction system was configured according to international real-time quantification specifications. The 20 μ L standard reaction system comprised 10 μ L of 2 $\times$ SYBR Green Type I premix (with ROX passive reference dye), 150 ng of cDNA template, 0.4 μ L of 10 μ M Primer F, 0.4 μ L of 10 μ M Primer R, and 8.2 μ L of RNase free water. The specific conditions of the PCR reaction were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 1 min, denaturation at 95°C for 15 s, denaturation at 95°C for 30 s, and annealing at 60°C for 40 times; each cycle consisted of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Finally, the melting curve was analyzed at 95°C. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with all C_t values of the control set to 1.

Table 1
Primers for qPCR detection of the DREB gene family in *L. barbarum*

Name of primer	Sequence	Annealing temperature (°C)	Reference gene/Literature
<i>LbDREB1-F</i>	CTCGAGCTCATGATGTTGCTG	59	LOC132599422
<i>LbDREB1-R</i>	TGCTTGAATGTCACGAGGGTT	60	
<i>LbDREB2-F</i>	GCAACTGGGGCGGTTTATTC	59	LOC132603229
<i>LbDREB2-R</i>	TCCTGGATCTCCCACTACCG	60	
<i>LbDREB3-F</i>	AGTTCTGGGGCGAACAGTAG	59	LOC132605424
<i>LbDREB3-R</i>	CGGACACCCATTTTCCGCTA	60	
<i>LbDREB4-F</i>	GTCCAATTTGCCTTGGATCTC	57	LOC132606766
<i>LbDREB4-R</i>	TTCTCAGGACCGCCTTTACC	59	
<i>LbDREB5-F</i>	GGGCAACTACATCTGCGTCT	60	LOC132609115
<i>LbDREB5-R</i>	TCAATGGTGTGCCAGCTTCA	60	
<i>LbDREB6-F</i>	ATCCGTGAACCTCGTAAGCG	60	LOC132611090
<i>LbDREB6-R</i>	GGACGAAGGGTTTGTGTGGA	60	
<i>LbDREB7-F</i>	TTGTTAGTCGGTGATGGCGG	60	LOC132618216
<i>LbDREB7-R</i>	CAGTGAGCTTTGGGTAGCGT	60	
<i>LbDREB8-F</i>	TCGAGCTTATGATACGGCGG	59	LOC132622643
<i>LbDREB8-R</i>	TGACGGGCTTGAAGTCTCAC	59	
<i>LbDREB9-F</i>	AAGTAAGCCCGGACCGTATG	59	LOC132626396
<i>LbDREB9-R</i>	CGCAGTTTGGCATGTGAGTT	59	
<i>LbDREB10-F</i>	TCTTCTCACAATTTTCAAAGGAGAA	57	LOC132630927
<i>LbDREB10-R</i>	CTTTCCCAAGTTCTTTGCCT	58	
<i>LbDREB11-F</i>	TGAGACAAAGGCCATCAGGC	60	LOC132631427
<i>LbDREB11-R</i>	TTCTTCCACGAAGGAGACGTG	60	
<i>LbDREB12-F</i>	CGTTTCCCACACCCGAAATG	59	LOC132634093
<i>LbDREB12-R</i>	CTCGTGGCAGGAACAGGTAG	60	
<i>LbDREB13-F</i>	TTTGTTAGGCACTTTCGCC	59	LOC132636814
<i>LbDREB13-R</i>	GAAATGCCCGAAACCAACGA	59	
<i>LbDREB14-F</i>	CGCCTCTGAGCGAGATAGTG	60	LOC132639570
<i>LbDREB14-R</i>	GCAAGCTGTCGAAACTGCAT	59	
<i>LbDREB15-F</i>	AGCGAAAGACGAGGCTAAGG	59	LOC132640420

Name of primer	Sequence	Annealing temperature (°C)	Reference gene/Literature
<i>LbDREB15</i> -R	TAGGCAGCACTCTGCATTCTG	60	LOC132645867
<i>LbDREB16</i> -F	AAAGTCACGCATTTGGCTCG	59	
<i>LbDREB16</i> -R	TTCACCCAACTCATCCGACC	59	
<i>Actin</i> -F	CAATCGGGTATTTCAAGGTCAAC	60	Zhang et al. [29]
<i>Actin</i> -R	GAGCAGTGTTTCCCAGCATTG	60	

Results

Identification of DREB family members in *L. barbarum*

By subjecting the obtained *L. barbarum* genome sequence data to HMM analysis and local BLASTP alignment, DREB family genes were obtained using HMM analysis. After removing sequences without conserved domains, a total of 16 LbDREB family members (*LbDREB1*–*LbDREB16*) containing one AP2 domain were obtained, and GenBank accession no. were PX233497–PX233512.

Analysis of physicochemical properties and prediction of subcellular localization of DREB family proteins in *L. barbarum*

Based on the BLAST alignment and HMM analysis of DREB homologous sequences in 6 plants, 16 DREB protein sequences were obtained using TBtools (v2.210) based on the BLAST alignment of *L. barbarum* genome protein sequences. Protein physicochemical properties of the 16 screened DREB family member sequences were further analyzed using the ProtParam tool on the ExPasy server (www.expasy.org). The results indicated that the amino acid length of LbDREB proteins ranged between 174–503, the relative molecular mass ranged between 19.29–57.93 kDa, the theoretical isoelectric point ranged between 4.61–9.66, the aliphatic index ranged between 49.67–80.73, and the grand average of hydrophobicity ranged from –0.89 to –0.41 (Table 2). Among the 16 DREB proteins, all were classified as hydrophilic proteins, and 3 were classified as stable proteins and the remaining as unstable proteins. Next, the characteristic attributes of DREB proteins were predicted using the Cell-PLoc online tool (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>). Based on the prediction results, we found that DREB proteins were mainly located in the cytoplasm and nucleus, with only two (*LbDREB14* and *LbDREB16*) found in mitochondria.

Multiple sequence alignment was performed on the 16 LbDREB protein sequences using DNAMAN to clarify the structural properties of LbDREB transcription factors (Fig. 1). Our study identified one amino acid sequence that was highly conserved among the different members of the LbDREB gene family, and contained one AP2 domain. This domain was composed of > 60 amino acid residues. Among them, amino acid residues at positions 14 and 19 of this domain were valine (V14) and glutamic acid (E19), respectively, which are essential for cis-acting element recognition of DREB transcription factors. In the DREB family, valine (V14) was generally more conserved than glutamate (E19), and the structural properties of LbDREB genes were consistent with those of the DREB family in other species.

Table 2
Information and characteristics of the DREB gene family of *Lycium barbarum*

Sequence ID	Protein ID	Amino acid length	Molecular mass (kDa)	pI	Instability index	Aliphatic index	GRAVY	Subcellular localization
<i>LbDREB1</i>	XP_060168778	179	20.07	5.93	54.15	80.73	-0.47	N
<i>LbDREB2</i>	XP_060172202	302	33.23	6.33	54.85	57.52	-0.75	N
<i>LbDREB3</i>	XP_060174560	188	20.05	4.68	53.69	68.19	-0.48	N
<i>LbDREB4</i>	XP_060176381	503	57.93	4.69	64.30	65.29	-0.79	N
<i>LbDREB5</i>	XP_060178952	348	38.39	4.97	48.33	60.60	-0.76	N
<i>LbDREB6</i>	XP_060181480	331	36.50	5.96	55.81	49.67	-0.83	N
<i>LbDREB7</i>	XP_060189258	177	19.57	8.90	39.54	66.72	-0.76	N
<i>LbDREB8</i>	XP_060193270	174	19.29	7.72	45.26	63.39	-0.76	N
<i>LbDREB9</i>	XP_060197238	255	28.30	9.66	36.62	67.33	-0.80	E and N
<i>LbDREB10</i>	XP_060202491	391	44.79	5.15	60.12	67.54	-0.84	N
<i>LbDREB11</i>	XP_060202985	176	19.91	8.84	57.46	74.26	-0.78	N
<i>LbDREB12</i>	XP_060206378	202	21.66	4.80	51.45	70.69	-0.41	N
<i>LbDREB13</i>	XP_060209827	391	42.42	4.61	61.86	66.32	-0.48	N
<i>LbDREB14</i>	XP_060211992	191	21.66	5.28	57.51	60.26	-0.74	E, M and N
<i>LbDREB15</i>	XP_060212995	366	41.15	4.85	42.92	54.45	-0.89	N
Note: N represents the cell nucleus; E represents the extracell; M represents the mitochondrion.								

Phylogenetic analysis and chromosomal localization of the DREB family in *L. barbarum*

To further demonstrate the evolutionary relationships among these gene family members, we constructed a phylogenetic tree based on the screened *LbDREB* members, as well as those reported in *A. thaliana*, poplar (*P. euphratica*), grape (*V. vinifera*), and walnut (*J. regia*). As shown in Fig. 2, based on the phylogenetic results and combined with the grouping of DREB genes in *A. thaliana*, the *LbDREB* family members were categorized into six subgroups (A1–A6). Subgroups A1 and A6 were absent in *L. barbarum*; whereas, subgroups A2, A3, A4, and A5 contained 6, 2, 6, and 2 DREB family members, respectively, as well as 17, 2, 38, and 18 DREB family members, respectively, from other species.

The *L. barbarum* reference genome on the NCBI database (<https://www.ncbi.nlm.nih.gov/n>) was downloaded as the basis for chromosome-level visualization of the 16 DREB genes using TBTools (v2.210). As shown in Fig. 3, these 16 genes were distributed across 10 different chromosomes. The largest number of *LbDREB* genes were distributed on chromosome 3 (3 in total). Chromosomes 1, 5, 7, and 8 each contained two *LbDREB* genes. Chromosomes 2, 4, 6, 9, and 10 only contained one *LbDREB* gene each, namely, *LbDREB9*, *LbDREB13*, *LbDREB1*,

LbDREB7, and *LbDREB8*, respectively. Furthermore, their distribution was independent of chromosome length and density, showing a relatively even distribution of genes across each chromosome.

Conserved motifs and gene structure analysis of the DREB gene family in *L. barbarum*

The conserved motifs of the 16 DREB proteins were analyzed in detail through the online website MEME (<http://meme-suite.org/tools/meme>). A total of 10 conserved motifs (Motifs 1–10) were predicted (Fig. 4). Among them, Motifs 1 and 2 were prevalent among DREB family members and their sequences were highly conserved; hence, they can be regarded as the two most central protein motifs in this family. The two conserved motifs of the LbDREB protein contained approximately 60 amino acid residues, which belong to the AP2 domain, playing a key role in DNA recognition and binding (Fig. 5). In shown in Fig. 5, the C-terminus of the RAYD conserved element of Motif 2 contained 39 amino acid residues, which can facilitate binding of regulatory transcription factors to cis-acting elements. These conserved motifs may be involved in regulating the transcriptional activity of DREB proteins, which in turn can affect plant response to adversity. Further structural analysis of genes in the LbDREB family showed that eight LbDREB genes (50%) did not contain introns, seven LbDREB genes (43.75%) contained one intron, and only *LbDREB9* contained two introns, suggesting that the LbDREB gene structure was relatively conserved.

Analysis of promoter cis-acting elements of the DREB gene family in *L. barbarum*

The 2000 bp cis-acting elements in the upstream promoter region of the 16 LbDREB genes were analyzed using the Plant Care online database. As shown in Fig. 6, these elements mainly included the following categories in response to abiotic and biotic stressors: auxin-responsive elements, which play a key role in defense and stress responses, and; gibberellin- and SA-responsive elements, each of which correspond to the signaling processes of different plant hormones. Cis-acting elements that play a key role in the ABA signaling pathway were also identified. These include cis-regulatory elements critical to induction of anaerobic conditions, as well as those that modulate light responsiveness. In addition, MYB binding sites associated with drought inducibility were also included, as well as cis-acting elements involved in low-temperature responsiveness and wound repair processes.

Collinearity analysis of DREB family members in *L. barbarum*

A total of seven pairs of segmental duplication genes were detected from the LbDREB family (Fig. 7), of which one pair (*LbDREB7* and *LbDREB8*) was in subgroup A5, two pairs (*LbDREB4–LbDREB10* and *LbDREB5–LbDREB15*) were in subgroup A2, and four pairs (*LbDREB3–LbDREB12*, *LbDREB11–LbDREB13*, *LbDREB11–LbDREB16*, and *LbDREB13–LbDREB16*) were in subgroup A4. Genes exhibiting collinear relationships accounted for 81.25% of the LbDREB genes.

Annotation files of *L. barbarum* and *A. thaliana* were combined using TBtools (v2.210) to perform collinearity analysis between the two species. The results showed that there were 19 orthologous DREB gene pairs between *L. barbarum* and *A. thaliana* (Fig. 8), indicating that the evolutionary relationship of the DREB family was highly similar between the two.

Mutation analysis of DREB family members in *L. barbarum*

Based on calculations of amino acid non-synonymous mutations (Ka) and synonymous mutations (Ks), the Ka/Ks value was > 1 for all seven LbDREB gene pairs, suggesting that the coding of the genes was biased toward purifying selection during evolution (Table 3).

Table 3
Ka/Ks analysis of LbDREB genes in *Lycium barbarum*

Gene 1	Gene 2	Nonsynonymous mutation (Ka)	Synonymous mutation (Ks)	Ka/Ks	Effective length/bp	Average S-sites	Average N-sites
<i>LbDREB7</i>	<i>LbDREB8</i>	0.15	1.21	0.12	507	118.67	388.33
<i>LbDREB11</i>	<i>LbDREB16</i>	0.19	0.91	0.20	513	120.58	392.42
<i>LbDREB4</i>	<i>LbDREB10</i>	0.37	0.97	0.38	1035	216.17	818.83
<i>LbDREB5</i>	<i>LbDREB15</i>	0.23	0.98	0.23	981	223.25	757.75
<i>LbDREB3</i>	<i>LbDREB12</i>	0.16	1.31	0.13	546	128.00	418
<i>LbDREB11</i>	<i>LbDREB13</i>	0.34	1.89	0.18	534	124.58	409.42
<i>LbDREB13</i>	<i>LbDREB16</i>	0.35	2.12	0.17	510	118.92	391.08
<i>LbDREB7</i>	<i>LbDREB8</i>	0.15	1.22	0.20	507	118.67	388.33

Expression analysis of LbDREBs in response to leaf blight infection in *L. barbarum*

By infecting *L. barbarum* with the leaf blight pathogen, we analyzed the expression patterns of the 16 LbDREB family members that were previously identified. Gene expression characteristics of the inoculated and control groups were examined by quantitative real-time PCR at five key time points (0, 24, 48, 72, and 120 h). All family members exhibited a significant upregulation trend ($p < 0.05$) during pathogen infection; however, different members had distinct time-sequential expression characteristics.

As shown in Fig. 9, expressions of *LbDREB1*, *LbDREB6*, *LbDREB10*, *LbDREB15*, and *LbDREB16* increased sharply at 24 h of infection, with peak values reaching 4–6-fold that of the control ($p < 0.01$). This rapid response was highly consistent with the initial stage of lesion formation, suggesting that these members may have played a key role in pathogen recognition or early defense signal transduction. In the middle stage of the disease (48–72 h), *LbDREB2*, *LbDREB4*, *LbDREB5*, *LbDREB12*, *LbDREB13*, *LbDREB14*, and *LbDREB16* maintained 2–3-fold higher expression levels ($p < 0.05$), and their sustained activation patterns were synchronized with lesion expansion and spread. Of particular note was that *LbDREB8*, *LbDREB9*, and *LbDREB16* showed a second expression peak (2–3-fold, $p < 0.05$) in the late stage of disease (120 h), suggesting their specific role in systemic defense repair. In contrast, *LbDREB3*, *LbDREB7*, and *LbDREB11* were stably upregulated throughout all infection stages and exhibited statistically significant differences. Hence, these members may enhance broad-spectrum host resistance by enhancing the basal defense pathway.

Discussion

The DREB family is derived from the AP2/EREBP superfamily. The AP2/EREBP family of transcription factors is unique to plants, and its members have a conserved DNA-binding domain—the AP2/EREBP domain^[17, 30]—which is activated when plants are exposed to adverse stressors such as drought, high salinity, and low temperatures. This domain is able to bind specifically to the PUCGAC-containing DRE/CRT cis-acting element, thereby activating expression of multiple downstream resistance genes^[31, 32]. In the present study, we screened for members of the

DREB transcription factor family in *L. barbarum* and analyzed the data on protein physicochemical properties, gene structure, phylogeny, collinearity, and expression patterns of the family members under *A. tenuis* Nees infection.

A total of 16 DREB family transcription factors were downloaded and identified based on published data from the *L. barbarum* whole-genome database, and were classified into six subgroups according to the *A. thaliana* classification system [8, 14]. However, the 16 LbDREB members only formed 4 subgroups, as no LbDREB members were found in subgroups A1 or A6. One possible reason was that because *L. barbarum* is a relatively unique medicinal plant, these two subgroups were unable to adapt to environmental requirements. This could have resulted in their extinction or assimilation into other subgroups, ultimately enabling *L. barbarum* to adapt to its unique growing environment and medicinal value over the long course of evolution. Nonetheless, subgroups A2, A3, A4, and A5 still showed some similarities with DREB subfamilies of other plants [6, 33]. This suggested that the DREB gene was relatively well conserved during plant evolution and may fulfill the same function in different species. Systematic research on the evolutionary history and adaptive changes of the DREB family in *L. barbarum* will facilitate a more comprehensive understanding of its stress resistance mechanism and medicinal value.

Gene structural analysis revealed that the exon–intron structure and promoter region of LbDREB genes contained a variety of cis-acting elements [6, 34]. The structural diversity of LbDREB may be related to its function and evolution, while differences in exon–intron structure may affect regulation as well as protein structure and function [35]. Genes with more exon–intron structures may have complex expression regulation mechanisms, whereas genes with fewer exon–intron structures may be more susceptible to environmental factors [12]. Cis-acting elements in the promoter region can determine gene expression patterns, causing the latter to function differently in different tissues or stress conditions [36].

Furthermore, conserved domain analysis revealed the presence of the characteristic AP2/EREBP domain in the LbDREB protein, which has a conserved binding sequence for DNA binding [6, 37]. This suggested that the LbDREB gene may bind to DNA with specific binding sequences and regulate expression of downstream genes involved in abiotic stress responses, such as high temperature, low temperature, and high salinity. The upregulation of DREB gene expression in *L. barbarum* under abiotic stress may have been due to activation of specific transcription factors by stress signals. Hormonal signaling and biotic stressors such as diseases, pests, and weeds may also be involved in DREB gene regulation [10, 38].

Therefore, in conjunction with the expression patterns, we hypothesized that LbDREB members may participate in the response to adversity via several mechanisms. First, they may directly activate expression of stress-resistance genes rich in DRE/CRT elements [3, 5, 7]. Second, they may form a regulatory network with other transcription factors to coordinate multiple stress signaling pathways. In particular, some LbDREB genes were differentially expressed after leaf blight fungal infection, and hence may be involved in the response of *L. barbarum* to biotic stressors. This may indirectly regulate the response to adversity through mechanisms, such as hormone signaling crosstalk, maintenance of reactive oxygen species homeostasis, and induction of disease resistance-related proteins, when subjected to stress [15, 16].

Based on the results of comprehensive phylogenetic, gene structure, and expression pattern analyses, we predicted that the LbDREB genes in *L. barbarum* may have been involved in regulating expression of downstream antiretroviral genes, interacting with transcription factors and participating in biological processes, such as growth and development [6, 39]. These functional predictions provide ideas for studying the specific targets of LbDREB

genes. As a medicinal and edible plant, *L. barbarum* has a wide range of applications^[30]. Hence, studying the function and regulatory mechanism of the DREB gene family in *L. barbarum* will provide new ideas and methods for improving its resistance and quality, while also offering a theoretical basis and genetic resources for subsequent research on resistance signal transduction against *Lycium* leaf blight and breeding for disease resistance.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets presented in this study are available in the NCBI GenBank repository, GenBank accession no. PX233497–PX233512.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Yahan Chen and Wenxu Wang designed this study and completed the test operation content, Wenxu Wang and Yi Zhou identified the DREB family members in the *L. barbarum* genome, Haitao Yu and Wei Liu analyzed expression patterns of family members under *A. tenuis* Nees infection. Yahan Chen and Wenxu Wang wrote the manuscript. All the authors have read and agreed to the published version of the manuscript.

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Figures

	V14	E19		
	★	★		
LbDREB1	I YRGVRR. NVGKVVSEI	REPRK.	KSRI WLGTFTPEMAARAHDAALSI KGDAAI LNFPELASCL.	PRPVS. QNPRDI QAAATKAASM 97
LbDREB2	RFRGVRQR. SVGKVVAEI	REPRK.	RTRRWLGTFTAEDAARAYDRAAI VLYGSRAQLNLQPNT. NS.	SSSSSPNSKRSSSSSAQTLR 124
LbDREB3	VYKGI RKRKSSGKVVSEI	REPRS.	PNRI WLGTFTPEMAAVAYDVAALALKGQDAELNFPNSASSL.	PVPET. NSSRDI QAAAACAAAA 107
LbDREB4	KYRGVRQR. TVGKVVAEI	REPVYSNGQFKSNGKRL	WLGTFTAGEAAI AYDEAASVMYGSYAMLNFPDYR. VHNSLTSSLES	SEQSFVEHEDSGVDDAM 197
LbDREB5	NYRGVRQR. TVGKVVAEI	REPNR.	GSRL WLGTFTGTAI KAALAYDEAARAMYGPCARLNLPNYP.	SSRESRDDSSWATTSASDSTV 164
LbDREB6	RYRGVRQR. SVGKVVAEI	REPRK.	RTRRWLGTFTAEDAARAYDRAAI ILYGSRAQLNLQPSGNS.	NSSSSSQSSRKFTSSSSQTTLR 145
LbDREB7	I YKGI RMR. KWGKVVAEI	REPNK.	RSRI WLGSYSTPVAARAYDTAVYYLRGPARLNFPELLVG.	DGGLSDLSADSI RKKAVEVGAR 112
LbDREB8	RYKGI RMR. KWGKVVAEI	REPNK.	RSRI WLGSYSTPVAARAYDTAVYYLRGPARLNFPELLVGS.	DGGLNDLSAASI RKKAI EVGAQ 113
LbDREB9	KERQVKGK. SFSTYVSKSASVDI		NVRPVNASKKPSKNDATG. . KSTNQVGESSI VLALPARTHMP.	NCGVASSNVEGEKAKGKTKL 132
LbDREB10	KYRGVRQR. TVGKVVAEI	REPVYSNEYQSNGKRL	WLGTFTSTAI EAARVYDEAAKAMYGHDAL LNFDDYYCVQNVQLANDSS	VSI ARTTSSLESSSESSVE 169
LbDREB11	KFI GVRQR. PSGRVVAEI	KESSQ.	KLRL WLGTFDKAEAAALAYDSARLLRGRNAKTNFKKPH.	EENCSLLEKNPRLYQLLKHAI M 110
LbDREB12	VYRGI RRRKSSGKVVSEI	REPRS.	PNRI WLGTFTPEMAAVAYDVAAL ALKGPDTDLNFPNSAASL.	PVPAT. SSACDI QAAAASAAAA 119
LbDREB13	SYVGVRMR. AVGKVVSEI	REPKK.	KSRI WLGTFTPEMAARAHDAALSI KNSAI LNFQFADLL.	PRPVT. CSPRDVQAAAVKAAHM 158
LbDREB14	SYVGVRMR. DWGKVVSEI	REPRK.	KSRI WLGTFTAEMAARQHDVAALSI K. EAEVPASPPLSEI V.	ELP. NLI YDSVESAEQ 140
LbDREB15	KYRGVRQR. TVGKVVAEI	REPHR.	GNRL WLGTFTGTAI DAALAYDEAARAMYGPCARLNLPNYT.	SSKESVTDSSSLPTI SQSDSTT 158
LbDREB16	I YRGVRR. SVGKVVSEI	REPRK.	KSRI WLGTFTPEMAARAHDAALSI KKNSSI LNFPHLVNSL.	PRPI S. LSPRDVQAAAAKAAAM 101

Figure 1

Multiple sequence alignment of DREB proteins in *L. barbarum*

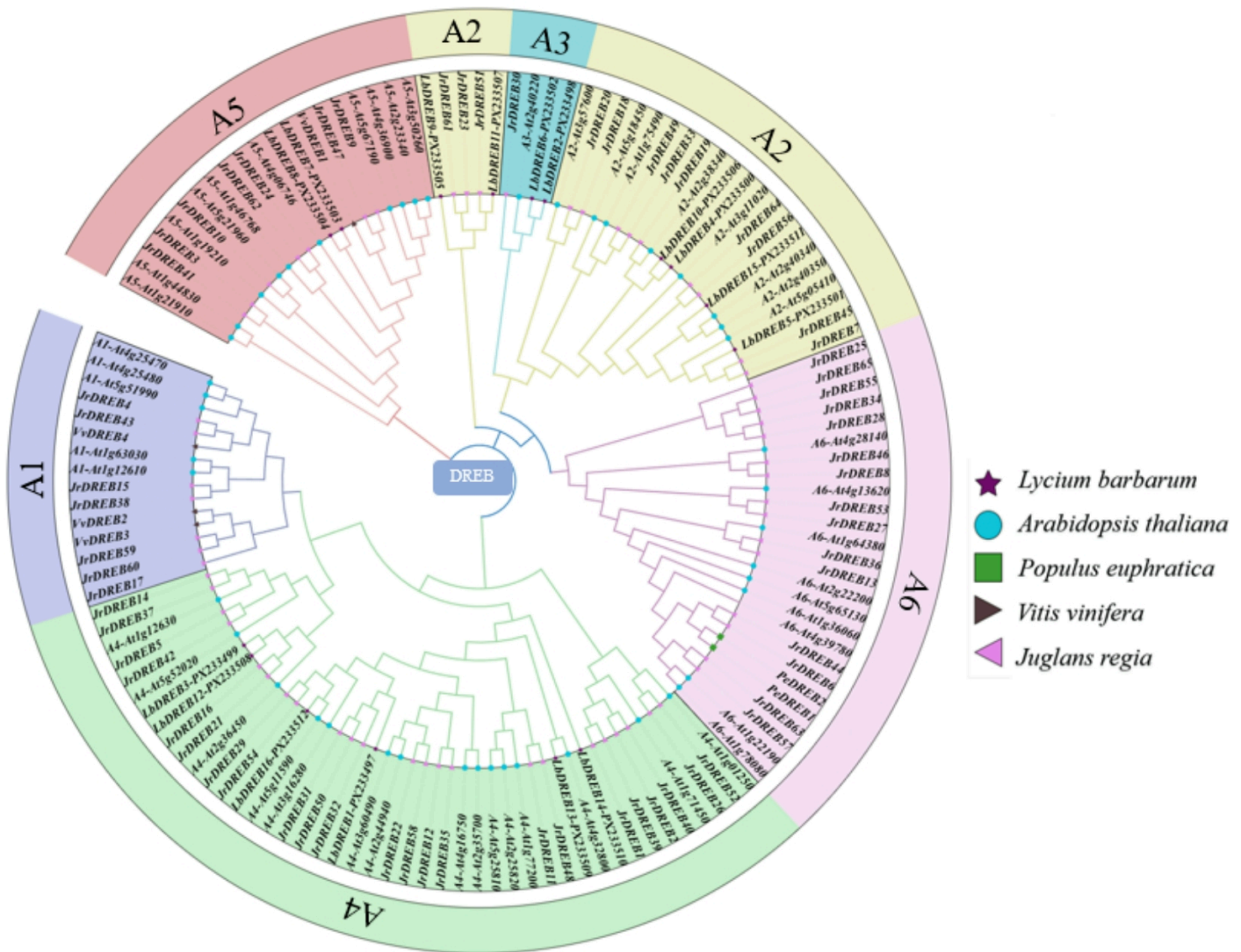


Figure 2

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Phylogenetic analysis of the DREB family in *Lycium barbarum*

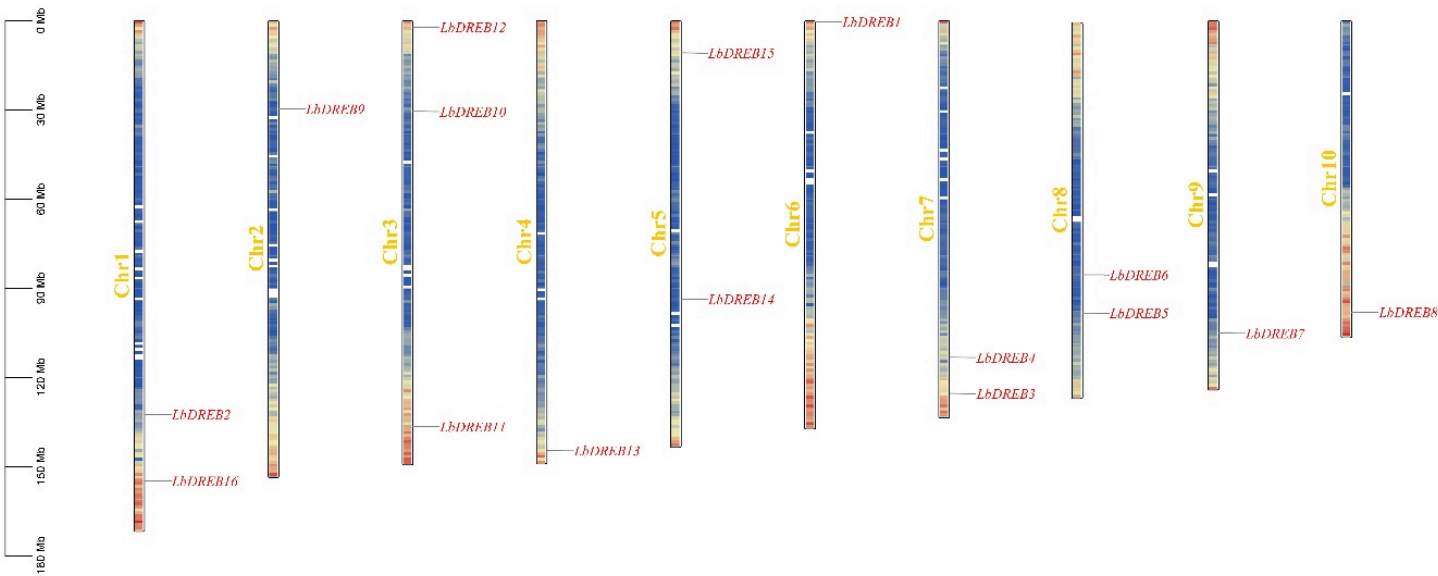


Figure 3

Distribution of DREB family members on chromosomes in *Lycium barbarum*

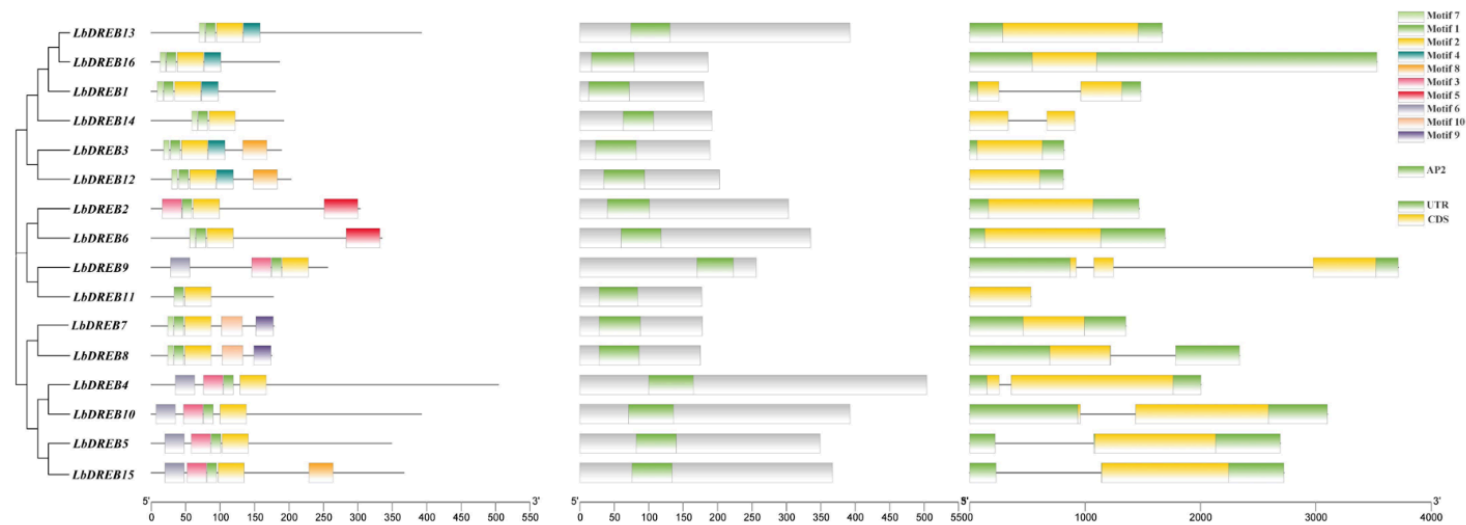


Figure 4

Conserved motifs and gene structures of the DREB gene family in *Lycium barbarum*

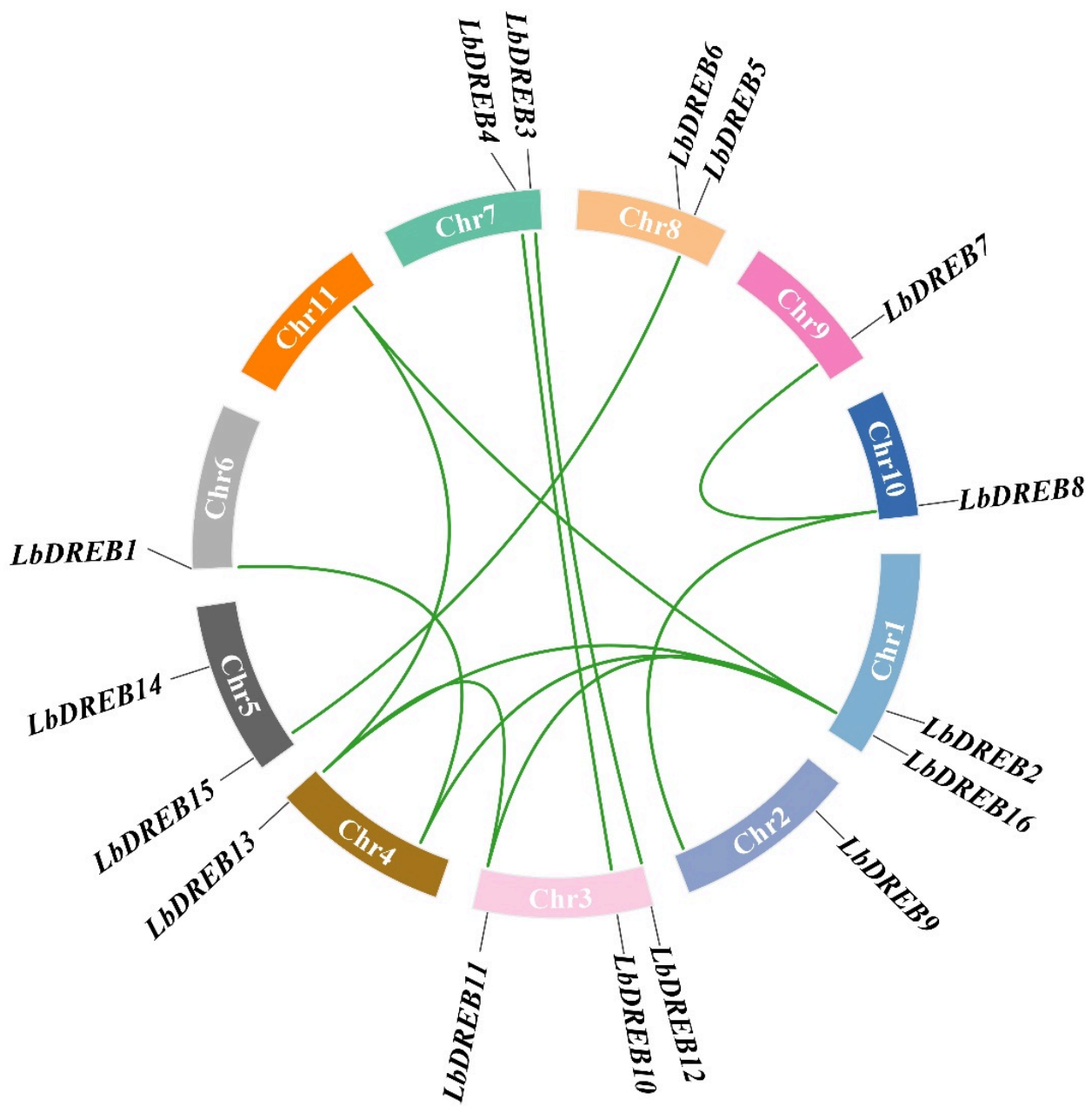


Figure 7

Collinearity analysis of DREB gene family members in *Lycium barbarum*

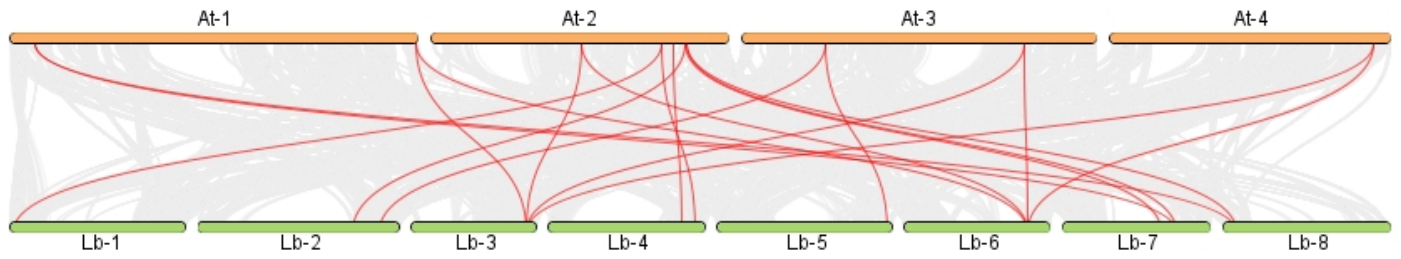


Figure 8

Collinearity analysis of DREB genes between *Lycium barbarum* and *Arabidopsis thaliana*

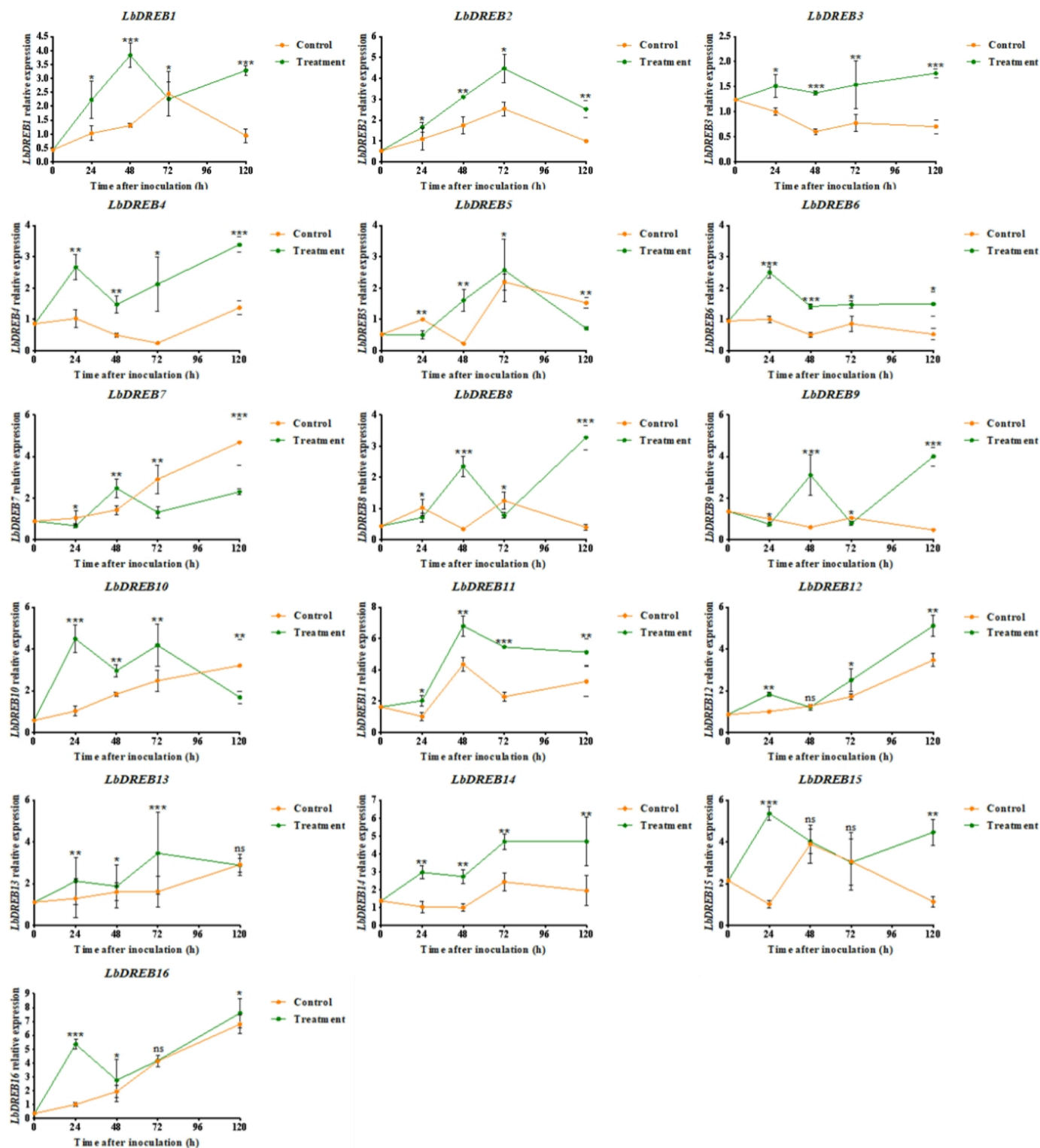


Figure 9

Analysis of DREB gene family expression patterns in *L. barbarum*

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