

Genome-wide identification and expression analysis of the DREB gene family in Foxtail Millet (*Setaria italica* L.)

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Abstract

Background

Dehydration response element binding factors (DREBs) represent a family of plant-specific transcription factors involved in regulating plant responses to various abiotic stresses.

Results

This study identified and analyzed members of the *SiDREB* gene family for physicochemical properties, phylogeny, and structure of the encoded proteins. The expression pattern of the DREB transcription factor in foxtail millet under stress was analyzed by combining the qRT-PCR data of foxtail millet after low temperature, ABA, and osmotic stress (20% PEG 6000). There were 56 *SiDREB* genes, divided into 6 subgroups, which were located on 9 chromosomes of foxtail millet. Chromosomal localisation showed that the *SiDREB* genes were unevenly distributed on 9 chromosomes. Further qRT-PCR results revealed that 19 *SiDREB* genes have been identified as playing a role in the response to abiotic stress and ABA.

Conclusions

The results of this study laid a foundation for further study on the function of *DREB* genes in foxtail millet and will be beneficial to the genetic improvement of foxtail millet.

Background

In the natural environment, plants often face multiple abiotic stresses simultaneously. Abiotic stresses, including drought [1], high and low temperature [2, 3], salinity [4], and flood [5] have significant impacts on plant growth and development[6]. Plants regulate the expression of stress-related downstream genes in response to external signals by transcription factors (TFs) and cis-regulatory element (CRE) interactions [7–9]. At present, many transcription factors of stress response in plants, such as MYB [10], AP2/ERF [11], WRKY [12], and bZIP [13], are important transcription factors related to stress. Among the transcription factors, AP2/ERF is a plant-specific superfamily of plant-specific transcription factors whose members all contain one or more AP2/ERF structures consisting of 60–70 highly conserved amino acids [14]. At present, the number of AP2 domains and the degree of sequence similarity between AP2/ERF superfamily members are used to classify members of this superfamily, it can be divided into five categories, namely AP2, ERF, DREB, RAV, and Soloist families [15–18]. The DREB transcription factors contain a large number of promoter elements that play important roles in plant response to adversity stress [17].

The common feature of DREB transcription factors is that they contain a conserved AP2/ERF domain, which consists of 60–70 amino acid residues [19]. DREB subfamily has 6 groups, namely A1 ~ A6 [20].

Each subfamily plays different roles in plants. At present, *DREB* genes have been identified from the genomes of rice [21], wheat [22], maize [17], soybean [23], moso bamboo [24], and other plants. It has been shown that the DREB subfamily can regulate abiotic stress responses such as drought and low temperature in plants. For example, *AtDREB1A* plays an important role in drought stress response [25]. *VvDREB2A* was found to take part in the reaction to low temperature [26] and *ZmDREB2A* was associated with drought and heat stress response in maize seedlings [27].

Foxtail millet (*Setaria italica* L.) is an annual C4 plant of *Setaria* in Gramineae, and one of the most important food crops in northern China [28, 29]. Foxtail millet has the advantages of drought resistance, barren resistance, and wide adaptability [30]. Generally, foxtail millet is a crop with significant nutritional benefits. It has emerged as an exemplary model for investigating the response to adversity stress in many scholarly contexts [31, 32]. However, it is still unclear what the mechanism of foxtail millet response to adversity stress is. Some DREB family members have been identified in foxtail millet, but the mechanistic analyses of *SiDREB* gene expression levels and abiotic stress are not comprehensive [18]. In the present study, combining the bioinformatics analysis, we have identified and analyzed the DREB family of genes in foxtail millet and analyzed their structures. Combined with qRT-PCR data and systematic analyses of their expression patterns under low temperature, ABA, and osmotic stress were performed. The findings of this study contribute to the advancement of the theory of foxtail millet response to adversity stress and are significant for the subsequent development of a molecular breeding system for foxtail millet.

Results

Identification of SiDREBs and phylogenetic analysis

The whole genome of foxtail millet identified 56 DREB family members, which were named SiDREB1-SiDREB56, respectively (Fig. 1; Table S1). To accurately classify the DREB family members in foxtail millet, the protein sequences of 55 DREB family members in *Arabidopsis thaliana* were aligned with those of 56 DREB family members in foxtail millet and a phylogenetic tree was constructed. The 56 phylogenetic trees of *SiDREB* genes in foxtail millet could be divided into 6 subfamilies (A1-A6). The *SiDREB* genes were unevenly distributed among 6 subfamilies. Among them, subfamilies A4 had the most *SiDREB* genes, subfamilies A1 had only seven *SiDREB* genes, and subfamilies A3 had no *SiDREB* genes.

Chromosome localization results showed that 56 *SiDREB* genes were distributed on 9 chromosomes. By comparison, it was found that the length of amino acids encoded by SiDREBs was 172 aa (*SiDREB40*) ~ 460 aa (*SiDREB03*), the molecular weight (MW) was 18,292.24 Da (*SiDREB40*) ~ 49,022.91 Da (*SiDREB03*), and the fat coefficient was 48 (*SiDREB43*) ~ 81.79 (*SiDREB49*). The value of the theoretical isoelectric point (pI) ranged from 4.26 (*SiDREB10*) to 11.72 (*SiDREB42*).

The isoelectric point of 9 proteins was greater than 7, which indicated that only 16% of SiDREB proteins belong to basic proteins. The instability index of proteins changed from 35.31 (*SiDREB50*) to 86.46 (*SiDREB30*), and only the SiDREB50 protein has a stability index of less than 40. All proteins were hydrophilic, with the maximum value of *SiDREB36* being – 0.104 and the minimum value of *SiDREB19* being – 0.738. Detailed data of 56 SiDREB protein sequences were listed (Table S2).

Chromosomal distribution and collinearity analysis of DREB gene families

The results of chromosome distribution indicated that the distribution of SiDREB family gene members across foxtail millet chromosomes shows uneven patterns (Fig. 2; Table S3). Among them, it is most distributed on chromosome III with 12 *SiDREB* genes. Only 1 *SiDREB* gene was distributed on chromosome VIII, which was the least distributed chromosome. Given the significant influence of tandem repeats and fragment repeats on the evolution and proliferation of plant gene families[33], then we analyzed the *SiDREB* gene replication events in the foxtail millet genome. The results showed that 13 gene pairs on 9 chromosomes containing the *SiDREB* gene were tandem replication types. Specifically, 2 pairs of tandem duplication in the A1 class, 6 pairs in the A2 class, 3 pairs in the A4 class, and 2 pairs in the A5 class. In addition, this study predicted the collateral homology of genes and analyzed the collinearity of the DREB gene in foxtail millet. It was found that the family had obvious duplication, which indicated that it might have doubled during the evolution of foxtail millet (Figure S1). Further analysis of the evolution of the identified *SiDREB* gene shows that some members of the *DREB* gene family have collinear relationship: there are 21 collinear gene pairs between 55 Arabidopsis *DREB* genes and 56 Arabidopsis *DREB* genes, which are distributed on different chromosomes of Arabidopsis thaliana, indicating that the sources of these 21 pairs of genes are similar and their DREB proteins are closely related (Figure S2).

Gene structure and motif composition of SiDREBs

The conserved sequences of 56 *SiDREB* genes in foxtail millet were analyzed by MEME online software, and 10 conserved motifs (named motif1-10) were found (Fig. 3A, B). The length of each conserved motif varies from 15 to 50 amino acids (Figure S3). The phylogenetic tree of the protein sequence of *SiDREB* showed that these proteins could be divided into six categories.

In these six subfamilies, it was found that all the *SiDREB* genes contained AP2 domains, among which A1 contained seven proteins, and the number of conserved motifs detected was mostly three, only the *SiDREB39* protein contained four conserved motifs, mainly motif 1, motif 2 and motif 6 (Fig. 3C). At the same time, it is also found that motif 1, motif 2, and motif 6 always appear adjacent to each other in this class, and motif 6 and motif 2 precede motif 1, and only one Motif 7 is found in this subfamily. The A2 subfamily contains 11 proteins, 9 of which have the same conserved motifs and the most conserved motifs, while only motif 1, motif 2, and motif 6 are found in *SiDREB16* and *SiDREB37*. A3 has no protein; Fourteen proteins in the A4 subfamily all contain motif 1, motif 2, motif 6, and motif 7, and this subfamily

contains four motifs on average. The conserved motifs of A5 subfamily members are motif 1, motif 2, and motif 6. The number of A6 subfamily proteins is the same as that of the A2 subfamily, and the number of conserved motifs varies from 1 to 4. Motif 8 is only found in the A6 subfamily. These conserved motifs may be related to the growth of foxtail millet.

Gene structure analysis showed that all *SiDREB* genes had exon structure, and most of the exon groups in *SiDREB* gene structure were mainly concentrated in one (Fig. 3D). Only *SiDREB04*, *SiDREB05*, and *SiDREB06* in the A2 subfamily, *SiDREB17* and *SiDREB42* in the A4 subfamily, and *SiDREB16*, *SiDREB19*, *SiDREB23*, and *SiDREB37* in A6 subfamily contained two or more exons. 50% of the *SiDREB* genes (28 / 56) did not contain untranslated regions (UTR). *SiDREB04* and *SiDREB38* only contained upstream regulatory regions. *SiDREB01*, *SiDREB02*, *SiDREB06*, *SiDREB08*, *SiDREB28*, *SiDREB36*, *SiDREB37*, *SiDREB39*, *SiDREB44* and *SiDREB53* contained downstream regulatory regions. The remaining 16 members have neither upstream nor downstream regulatory regions. The protein members of the same family and their coding genes exhibit a high degree of structural and compositional similarity, which lends support to the reliability of phylogenetic development.

The Analysis of cis-acting elements of SiDREB promoter

To further investigate the regulatory mechanism of *SiDREBs* in abiotic stress, the promoter region (2 000 bp) of the *SiDREB* genes were analyzed for cis-acting elements (Fig. 4; Table S4). The results demonstrated that the promoter region of 56 foxtail millet *SiDREB* genes exhibited the presence of 20 distinct cis-acting elements, including low temperature, anaerobic induction, defense and stress responsiveness elements, light responsiveness elements, hormone response elements related to IAA, ABA, MeJA, GA and SA. As well as elements related to growth regulation and circadian rhythms, including cell cycle regulatory elements, seed-specific regulatory elements, and endosperm expression elements, and it also included a few wound-responsive elements (Fig. 4; Table S4). Among them, all *SiDREB* gene promoters contain defense and stress responsive elements, and hormone responsive elements were widely found in SiDREB family members. Furthermore, except *SiDREB20*, *SiDREB21*, *SiDREB22*, *SiDREB24*, *SiDREB25*, *SiDREB26*, *SiDREB39*, and *SiDREB54*, all the other DREB family members of foxtail millet have been observed to contain ABRE elements within their promoter regions. It is hypothesized that members of the DREB family may contribute to the stress response of foxtail millet via the ABA-dependent pathway.

Analysis of tissue expression pattern of DREB gene in foxtail millet

Different genes play different functions in different tissues of plants. By detecting the expression of 26 *SiDREB* genes in 21 tissues, the function of *SiDREB* genes was further studied (Fig. 5; Table S5). The results showed that there were some differences in the expression of *SiDREB* gene in the same kind of different organs. In addition, many *SiDREB* genes were highly expressed in neck-panicle-internodes and stem-top-second. Some *SiDREB* genes had a high abundance of transcripts in foxtail millet. *SiDREB09*

and *SiDREB12* were strongly expressed in various parts. However, the transcription abundance of *SiDREB19* gene was very low. In addition, the expression of the *DREBs* gene was different in different tissues. The expression of some *SiDREB* genes varied with different genes and tissues, showing tissue specificity. Such as *SiDREB07*, *SiDREB08*, *SiDREB47*, and *SiDREB48*, which were highly expressed in neck-panicle-internodes and stem-top-second of foxtail millet during the grain filling stage. The expression of *SiDREB52* was relatively high in developing spikelets and seeds, especially in spikelets. The expression of *SiDREB54* in 21 tissues was slightly or even not expressed. Additionally, there were significant differences in the expression of the same gene in different tissues and organs at the same growth stage. *SiDREB09* was highly expressed in leaves and roots and almost not expressed in immature seed S5, and *SiDREB12* expression was high in leaves and stems. During the grain filling stage, *SiDREB09* demonstrated notable expression in leaves and roots, whereas *SiDREB12* displayed particularly high expression in flag leaves. The results of this study elucidated the intricate role of *SiDREB* genes in the growth and development of foxtail millet, highlighting their tissue-specific expression patterns.

Expression analysis of DREB gene family in foxtail millet under ABA and abiotic stress

To detect the response pattern of the *SiDREB* gene family to abiotic stress, the relative expression of this family gene in foxtail millet leaves under low temperature (4°C), 100 µM ABA, and osmotic stress (20% PEG 6000) was analyzed by qRT-PCR technology. Subsequently, we constructed a gene expression heat map (Fig. 6). Generally, under hormone treatment and stress, the expression of *SiDREB* genes did not show consistent characteristics. Under low temperature stress, *SiDREB48* and *SiDREB53* gene expression decreased obviously, and the expression level of *SiDREB08*, *SiDREB09*, *SiDREB11*, *SiDREB13*, *SiDREB14*, *SiDREB15*, *SiDREB28*, *SiDREB33*, *SiDREB35*, *SiDREB39*, *SiDREB41*, *SiDREB45*, *SiDREB47*, and *SiDREB52* were remarkably increased. The expression levels of *SiDREB11* and *SiDREB47* increased first and then decreased. The *SiDREB08*, *SiDREB09*, *SiDREB11*, *SiDREB14*, *SiDREB15*, *SiDREB41*, and *SiDREB47* genes reached a high level at 3 h. The expression of *SiDREB39* was the highest at 72 h, which was about 25.5 fold that at 0 h. The expression peak of *SiDREB13*, *SiDREB33*, *SiDREB45*, and *SiDREB52* appeared at 96 h (Fig. 6A).

After ABA treatment, the expression of *SiDREB09*, *SiDREB33*, and *SiDREB41* were decreased. The expression of genes *SiDREB07*, *SiDREB08*, *SiDREB09*, *SiDREB15*, *SiDREB28*, *SiDREB35*, and *SiDREB39* exhibited notable enhancement, and the expression was exceeded 0 h. At 6 hours, the expression peaks of *SiDREB07* and *SiDREB39* appeared. The expression levels of *SiDREB13*, *SiDREB14*, *SiDREB18*, and *SiDREB45* genes were the highest at 48 h, and their expression levels were 20.0 times, 15.0 times, 2.7 times, and 4.9 times higher than those of 0 h, respectively. The expression peak of *SiDREB53* appeared at 96 h (Fig. 6B). Furthermore, they could respond to osmotic stress. For example, the expression of *SiDREB08*, *SiDREB09*, *SiDREB13*, *SiDREB15*, *SiDREB35*, and *SiDREB52* could be up-regulated by osmotic stress. The expression of *SiDREB09*, *SiDREB15*, *SiDREB28*, and *SiDREB47* were highest at 3 h. The expression peaks for *SiDREB08*, *SiDREB39*, *SiDREB41*, and *SiDREB45* appeared at 12 h. The expression

levels of *SiDREB07*, *SiDREB14*, *SiDREB18*, and *SiDREB19* increased and then decreased. The expression of *SiDREB11*, *SiDREB13*, and *SiDREB52* changed significantly at 96 hours (Fig. 6C). Some *SiDREBs* genes showed down-regulated expression patterns under stress, and the expression patterns of the *SiDREB* genes differ in response to various forms of stress, likely due to their involvement in disparate defence mechanisms against these stressors.

Discussion

Characterization and analysis of DREB Genes in foxtail millet

Extreme environmental stresses, such as low temperature, drought, and salt stress, can adversely affect the growth and ultimate yield of plants and change the balance of the whole ecosystem [34, 35]. During long-term evolution, plants can initiate the expression of relevant genes through TFs and participate in the regulation of stress responses [9, 36]. The DREB transcription factor can perceive stress signals and activate the expression of stress response genes [37, 38]. The identification of *DREB* genes in foxtail millet helps analyze the important function of the DREB family in foxtail millet response to abiotic stress. This study screened members of the DREB TF family in foxtail millet, identified *DREB* genes, and studied its chromosome location, phylogenetic tree, structural characteristics, relative expression patterns in different tissues, and abiotic stress expression patterns. This study identified and screened 56 *SiDREB* members in the whole genome of foxtail millet. Based on the classification method of *AtDREB* genes in *Arabidopsis thaliana*, 56 genes were split into 6 categories (Fig. 1). The number of DREB gene families in foxtail millet was comparable to that observed in maize [17], wheat [39], rice [40] and potatoes [41]. The number of *DREB* genes in foxtail millet was less than that obtained in previous studies [42]. The difference may be caused by different sequencing versions and different screening thresholds for family members. All 56 *SiDREB* genes were located on 9 chromosomes, but their distribution was not uniform. Among them, the number of members on chromosome 3 was the most, and the number of members on chromosome 8 was the least, there were 12 and 1, respectively. 13 genes were clustered on chromosomes (Fig. 2). The two main ways by which gene families are amplified are fragment replication and tandem replication [33].

Similar to the *Arabidopsis DREB* gene subfamily [20], *SiDREB* genes can also be divided into six groups, A1 to A6, each group has certain specificity in its gene structure and motif composition (Fig. 3A). Gene structure analysis revealed that the majority of *SiDREB* genes exhibited a lack of intron-containing sequences [43]. In the A4 group, only 2 of the 17 members contain introns, but in the A2 group, 4 of the 11 members contain introns (Fig. 3D). This was the same as previously reported in gramineous plants such as barley [44] and maize [45].

The number of introns is related to plant response to various stresses, and a small number of introns contributes to the rapid expression of genes [46, 47]. Therefore, this gene structure of the *SiDREB* family may contribute to the stress resistance of foxtail millet. Conversely, the presence of introns can lead to

alternative splicing, which may delay the response to abiotic stress. According to the 10 conserved motifs identified by the SiDREB protein sequence, it was found that all *SiDREB* genes contain the AP2 domain (Fig. 3C). There is a certain degree of difference between the motifs in different subgroups [48]. For example, the A2 subfamily contains 11 proteins, of which 9 proteins have the same conserved motifs and have the most conserved motifs, while only motif 1, motif 2, and motif 6 were found in SiDREB16 and SiDREB37, A3 had no protein (Fig. 3A, B). These structural differences may be one of the reasons for the functional differentiation of DREB family proteins.

The expressions of SiDREB genes in foxtail millet

The promoter region of *SiDREBs* contains numerous cis-acting elements related to hormone or adversity response. The presence of light responsive elements in all 56 genes of the foxtail millet DREB family suggested that this gene family might play an important role in photosynthesis (Fig. 4; Table S4). Signaling pathways in plant photoreceptors are essential for plant adaptation to the surrounding environment [49]. The activity of the DREB pathway in *Arabidopsis thaliana* was influenced by photoperiod [50]. This pathway was inhibited by phytochrome B (phyB) and light receptor interaction factors PIF 4 and PIF 7 in warm seasons with long days [51, 52]. As daylight hours decreased, the inhibition was relieved, resulting in enhanced activity of the DREB pathway and an increase in cold tolerance in plants [53]. Additionally, the promoter region of *SiDREBs* not only contains the most basic response elements of plant life activities but also contains various elements that respond to adversity stress and plant hormones, such as light response element, auxin responsiveness, abscisic acid response element, low temperature, and anaerobic induction. These results showed that DREB TFs were related to plant growth, development, and stress resistance. Similarly, in potatoes [54] and *Brassica rapa* [55], the DREB transcription factor is involved in coping with different types of environmental stress factors.

Considering that the gene expression pattern can reflect its function, our study investigated how *DREB* gene family expression changes across different foxtail millet tissues as the plant matures. The expression patterns of *the DREBs* gene in different tissues of foxtail millet showed that *SiDREBs* were expressed in roots, stems, leaves, and ears, but there were differences in expression levels in different tissues. For example, *SiDREB52* was highly expressed in developing spikelets. The specific expressions of *SiDREB07*, *SiDREB13*, and *SiDREB35* were high in the tissues of foxtail millet during the grain-filling stage. The expression of *SiDREB12* in all tissues was high, while *SiDREB30*, *SiDREB33*, and *SiDREB41* had lower expression levels (Fig. 5). This might be due to differences in the functionality of *SiDREBs* and the inconsistencies in the period of functionality. In chrysanthemum, *DmDREBa* and *DmDREBb* played a role in the aboveground part [56].

Due to their sessile nature, plants are often exposed to adverse conditions [1–4, 6]. These abiotic stresses are major factors limiting plant geographical distribution and crop yield. The DREB gene family has been demonstrated to enhance the resilience of plants to abiotic stress. For example, transgenic plants overexpressing *OsDREB1A* [57] and *OsDREB1F* [58] were significantly more tolerant to drought and freeze stresses. Expression of *LsDREB2A* was significantly up-regulated under salt and hyperosmotic

stresses, and overexpressed in transgenic plants, which improved the tolerance to high salt [59]. In foxtail millet, the majority of DREB gene family members also participate in controlling responses to non-biological stresses. In this study, qRT-PCR was used to detect the expression of the *DREB* gene in foxtail millet under three different stresses (Fig. 6). Results from treatments with low temperature, osmotic stress, and ABA for 24 hours and 48 hours indicate that some *SiDREB* genes play active roles in the abiotic stress and ABA response in Jingu21 seedlings. Among them, the expression levels of *SiDREB08*, *SiDREB09*, *SiDREB11*, *SiDREB13*, *SiDREB14*, *SiDREB15*, *SiDREB28*, *SiDREB33*, *SiDREB35*, *SiDREB39*, *SiDREB41*, *SiDREB45*, *SiDREB47*, and *SiDREB52* were significantly induced under cold stress. Moreover, more than half of the *DREB* genes have LTR elements in their promoters, which are predicted to respond to low-temperature stress. Additionally, similar to reports on *DREB* genes in rice [60] and tomato [61], DREB transcription factors in foxtail millet were also significantly induced by exogenously applied ABA (Fig. 6).

Conclusions

In our study, foxtail millet was taken as the research object, based on bioinformatics analysis, 56 *DREBs* gene family members were finally identified, and their chromosome position, phylogenetic relationship, domain conservation, and tissue-specific expression and expression patterns under low temperature, ABA and osmotic stress were studied. The results presented here provide an important theoretical basis for further in-depth study of the function of the *DREB* gene family in foxtail millet. However, there are still many issues that require further investigation, including but not limited to the specific biological functions of *SiDREB* family proteins, the precise regulatory mode of plant hormones on *SiDREB* gene family, and the molecular mechanism of *SiDREB* protein participation in stress response to adversity.

Materials and Methods

Identification and physicochemical properties characterisation of the *SiDREB* genes, construction of phylogenetic tree, and chromosome localization

Download the *Setaria italica* genome and protein sequence from the National Genome Science Data Center (<https://www.ncbi.nlm.nih.gov/genome/?term=Setaria+italica+>, accessed on 14 August 2021). The Hidden Markov Model (HMM) of the conserved domain of AP2 (PF00847) was obtained from the Pfam website (<http://pfam.xfam.org/>, accessed on 7 June 2022), and was searched from the local protein database by using HMMER software. The conserved domain of the DREB protein sequence was identified on the NCBI-CDD website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 7 June 2022), and the candidate protein containing the DREB domain was obtained. The phylogenetic tree of the DREBs gene family in foxtail millet and *Arabidopsis thaliana* was constructed by MEGA 7.0 software, and the verification parameter Bootstrap was set to 1000. The DREBs protein sequence was obtained from the Arabidopsis information resources website (tair, <http://www.arabidopsis.org>, accessed on 27 October 2020). The online website Expasy ProtParam

(<https://web.expasy.org/protparam/>) was used to analyze the protein characteristics of foxtail millet DREB.

Gene duplication and collinearity analysis

Using TBtools software to locate the members of the DREB family on chromosomes through visual drawing, so that the results can be presented. TBtools was used to draw a visual map of the collinear relationship between genes. The collinearity analysis between foxtail millet and *Arabidopsis thaliana* DREBs was constructed by using MCScanX and TBtools.

Structural analysis and Motif analysis of SiDREB genes

According to the gene position information, the scaffold position and exon-intron structure of DREB were drawn with the help of TBtools software. The determination of conserved motifs in SiDREB proteins was performed using the MEME online programme (<http://meme-suite.org/tools/meme>, accessed on 17 April 2023).

Analysis of cis-acting elements of the promoter region of the SiDREB gene

The promoter region sequence of 2000bp upstream of *DREB* gene coding sequence (CDS) in foxtail millet was obtained by TBtools software. The cis-acting elements in the promoter region were predicted using the online Plant CARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 6 April 2023) and then used TBtools tools to visualize the data.

Expression pattern analysis of SiDREB

The gene expression data of various tissues, including roots, stems, flowers, and leaves of foxtail millet, were obtained from the Multi-omics database for *Setaria italica* (<http://foxtail-millet.biocloud.net/home>, accessed on 17 April 2023). A heat map was constructed and displayed by the visualization software TBtools, based on the expression differences of genes in plant tissues and organs.

Plant materials and stress treatments

12-day-old foxtail millet seedlings of Jingu 21 with consistent growth were selected for stress treatment, including low temperature, drought stress, and ABA treatment, and each treatment was set with 3 independent replicates. The specific operations of each treatment are as follows: placing the seedlings used for low-temperature treatment in an incubator at 4°C, using PEG-6000 as a drought stress agent, 20% PEG-6000 solution was prepared to irrigate foxtail millet seedlings for drought treatment, and 100 µM ABA solution was prepared to treat the seedlings. After 0 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h, respectively, leaf tissue samples were pooled, frozen with liquid nitrogen, and stored in an ultra-low temperature refrigerator.

RNA extraction and quantitative real-time PCR analysis

qRT-PCR was used to accurately detect the relative expression of the target gene. At least 3 replicates were set for each experiment. The specific steps of the experiment were as follows: (1) Prepare the reaction system: the amplification system was 20 μ L, including 2 $\times$ SYBR Green Pro Taq HS premix 10 μ L, forward/reverse primers 0.4 μ L, cDNA 2 μ L, RNase-free water 7.2 μ L. Among them, *SiActin* (SETIT_026509mg) was the reference gene. (2) Design test procedure: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 59°C for 30 s. (3) Result analysis: Calculate the expression of each gene by $2^{-\Delta\Delta C_t}$ and TBtools was used as the gene expression map. The specific primers of 19 SiDREBs and the internal reference used are shown in Table S6.

Data availability

All data for the conclusions of this article can be found in this article and its supplements.

Abbreviations

ABA	Abscisic acid
DREBs	Dehydration response element binding factors
TFs	Transcription factors
CRE	Cis-regulatory element
pI	Isoelectric point
UTR	Untranslated region
IAA	Auxin
MeJA	Methyl Jasmonate
GA	Gibberellins
SA	Salicylic Acid
LTR	light response elements
qRT-PCR	Quantitative real-time polymerase chain reaction

Declarations

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Contributions

YZ, XL and LZ contributed to the conception of the experiment. YZ, MC, XL and XY made significant contributions to the analysis of the data and preparation of the manuscript. XL, XY, SD and JW reviewed the draft of the paper. XL, SD and JW helped to revise the paper. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional information

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Figures

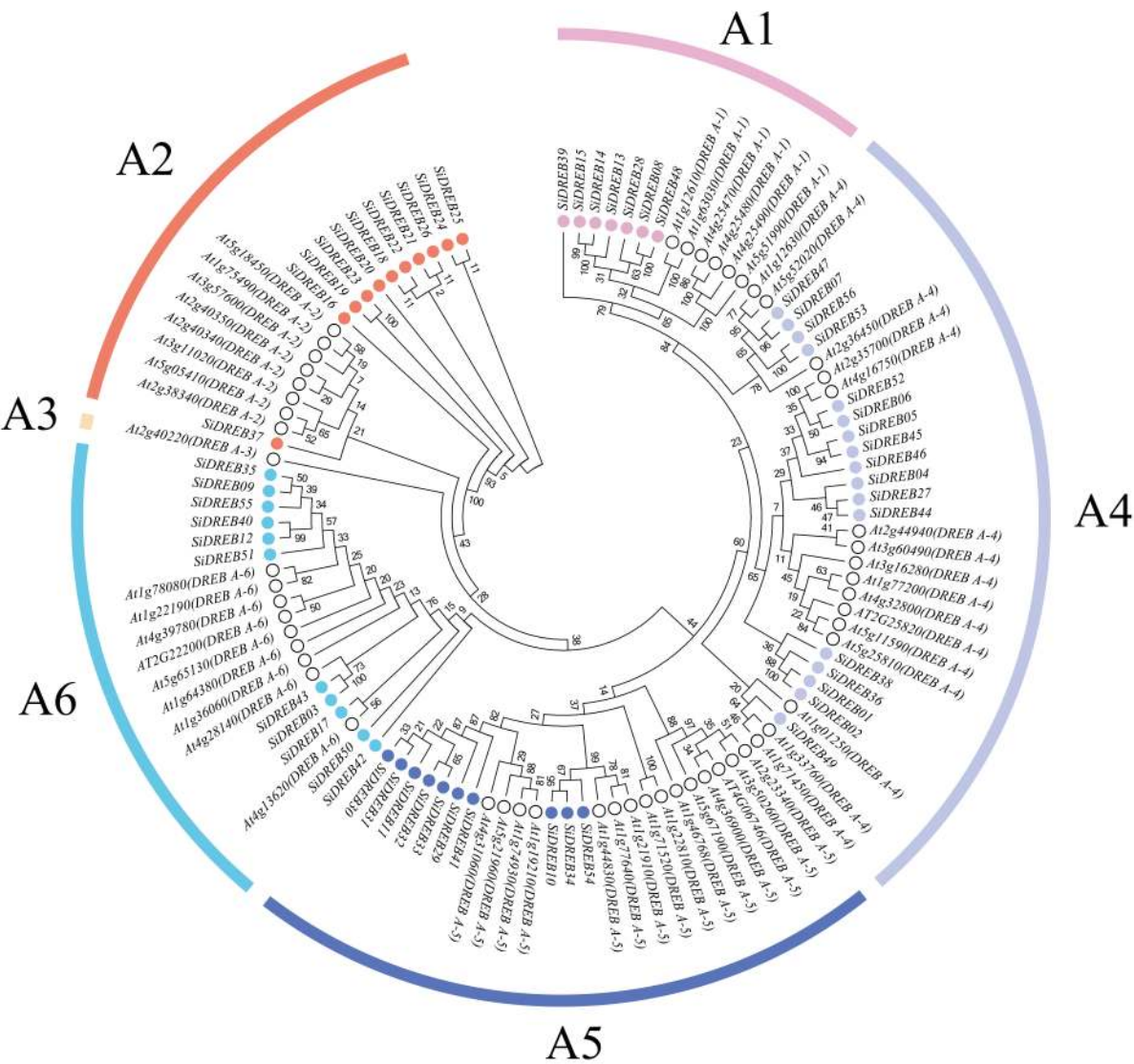


Figure 1

Phylogeny and distribution of DREB proteins from foxtail millet and Arabidopsis thaliana. The DREB proteins from the Arabidopsis thaliana were marked with black circles and the DREB proteins from the foxtail millet were marked with different colors.

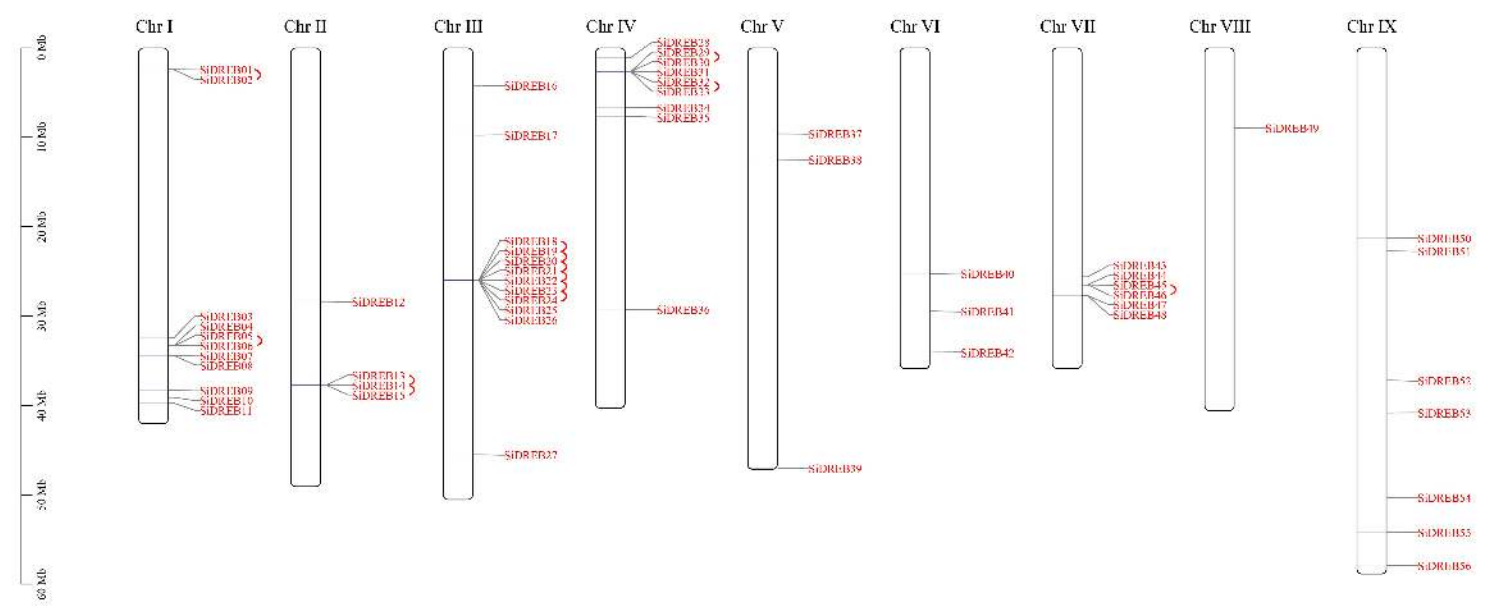


Figure 2

Chromosomal distribution of the *SiDREB* genes. Chromosome size was expressed by its relative length. The black line on the bar indicates the position of each gene.

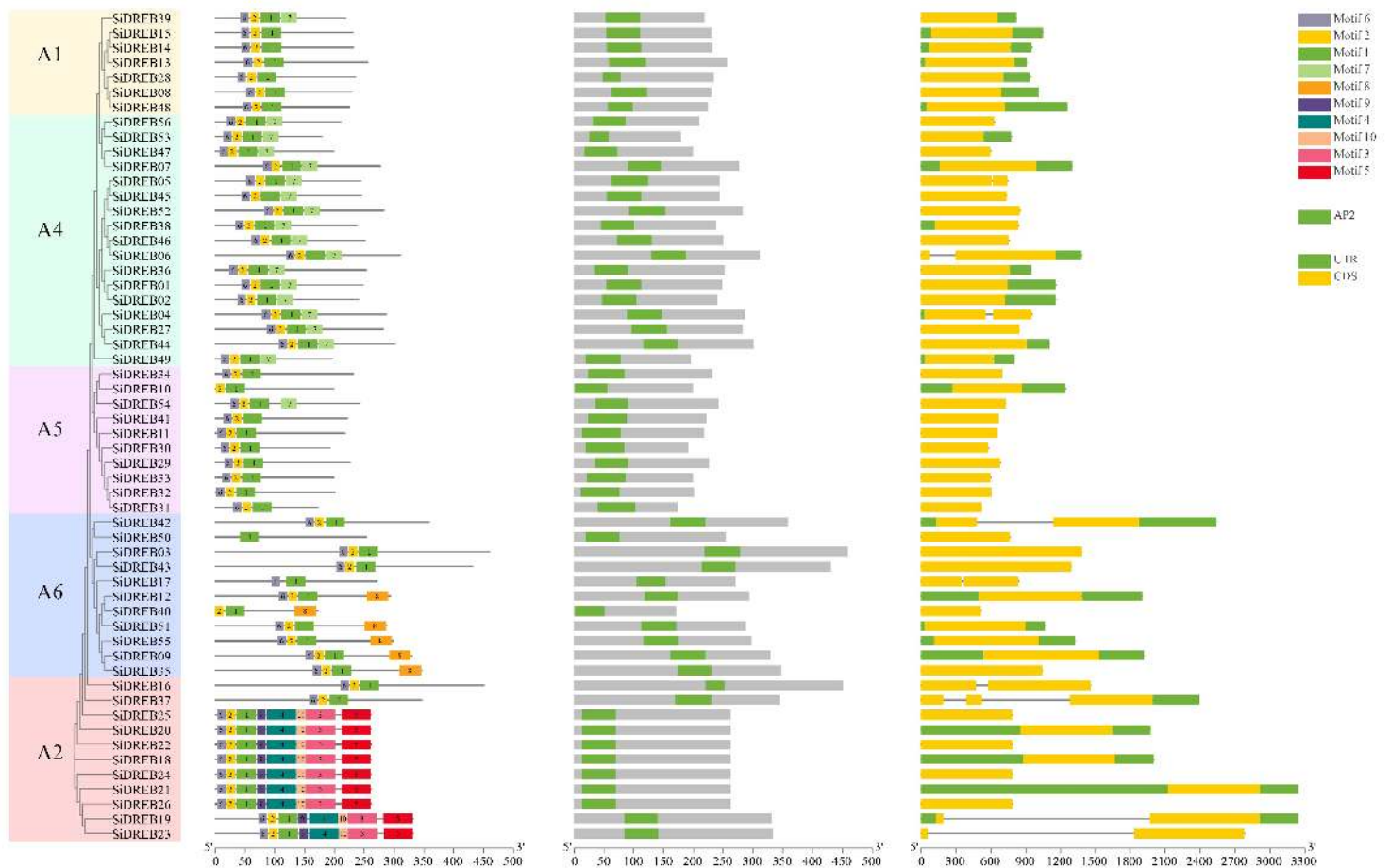


Figure 3

Evolutionary analysis, conserved motif, domains and gene structure in *SiDREBs* family. A. The phylogenetic relationship of *SiDREB* proteins in foxtail millet contains 56 DREB proteins (named *SiDREB*1 to *SiDREB*56). B. Conserved motif of *SiDREB* proteins. The conserved motifs were presented in different colored boxes. C. AP2 functional domain contained in DREB family proteins. D. Analysis of gene structure of *SiDREBs*. CDS was represented by a yellow box.

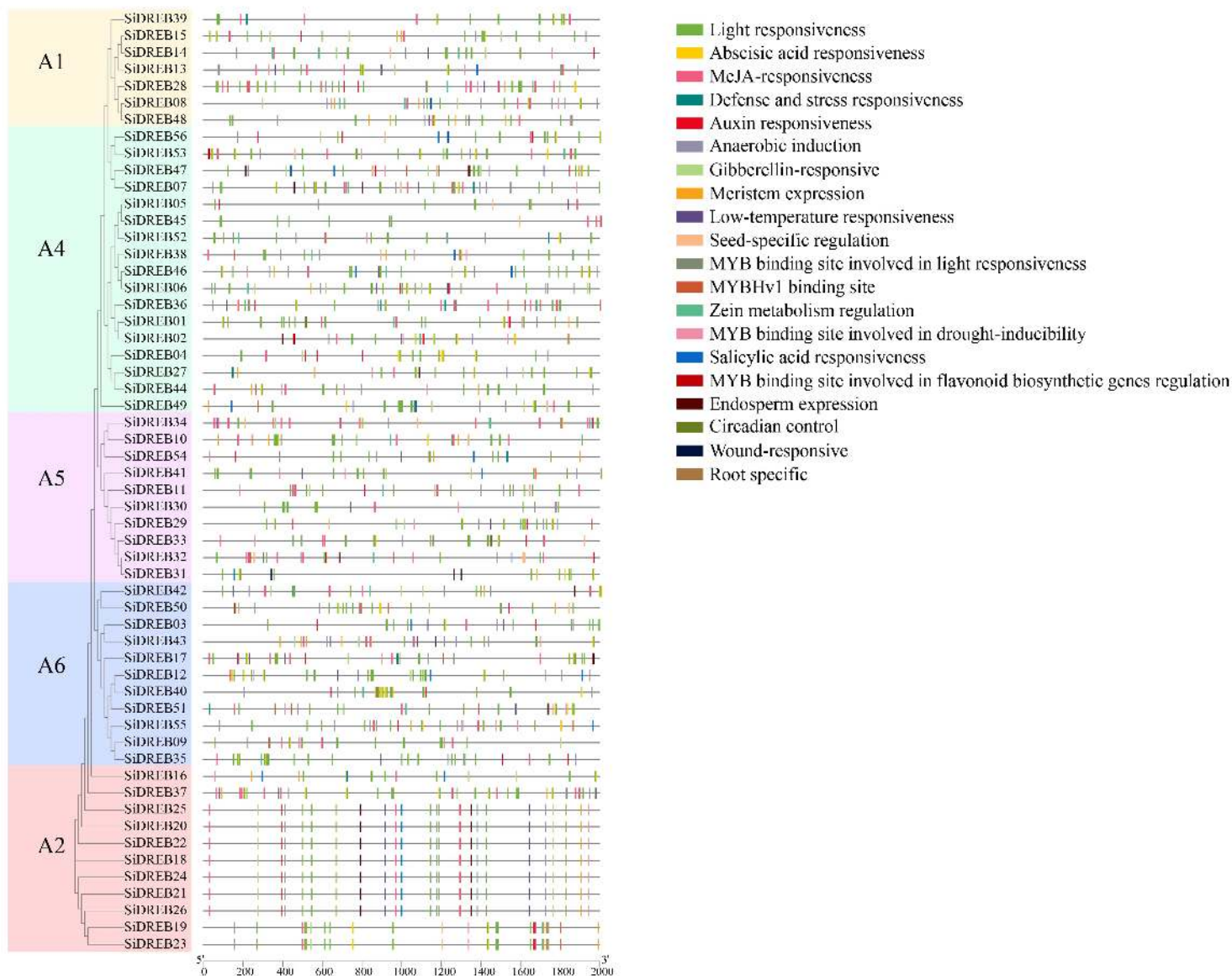


Figure 4

The cis-elements analysis in promoters of the *SiDREBs* genes. Totally 20 types of cis-acting elements were predicted.

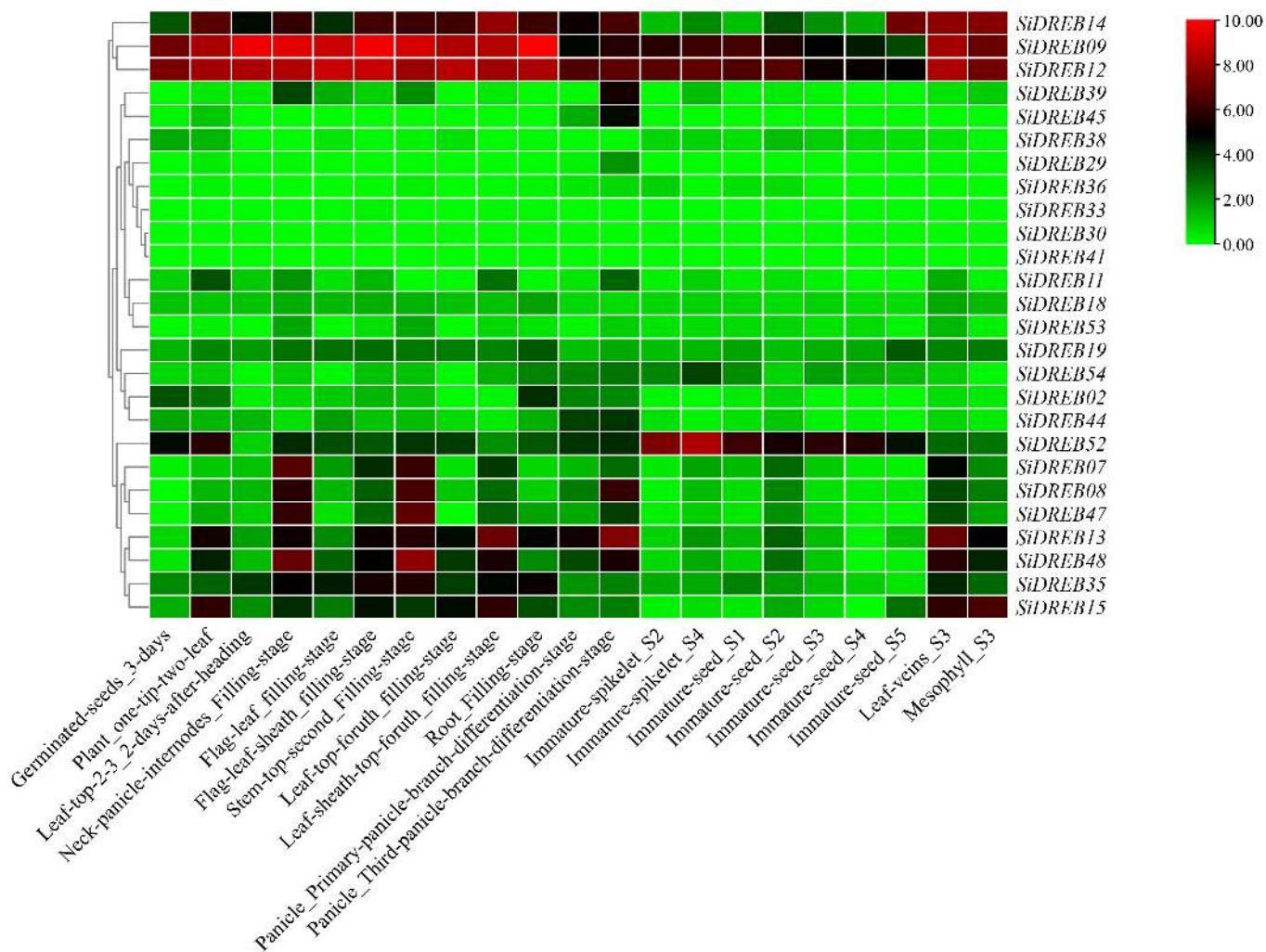


Figure 5

Relative expression profiles (FPKM value) of *DREB* genes in the different tissues of foxtail millet. Rectangle with different colors indicated that log2 expression level (FPKM).

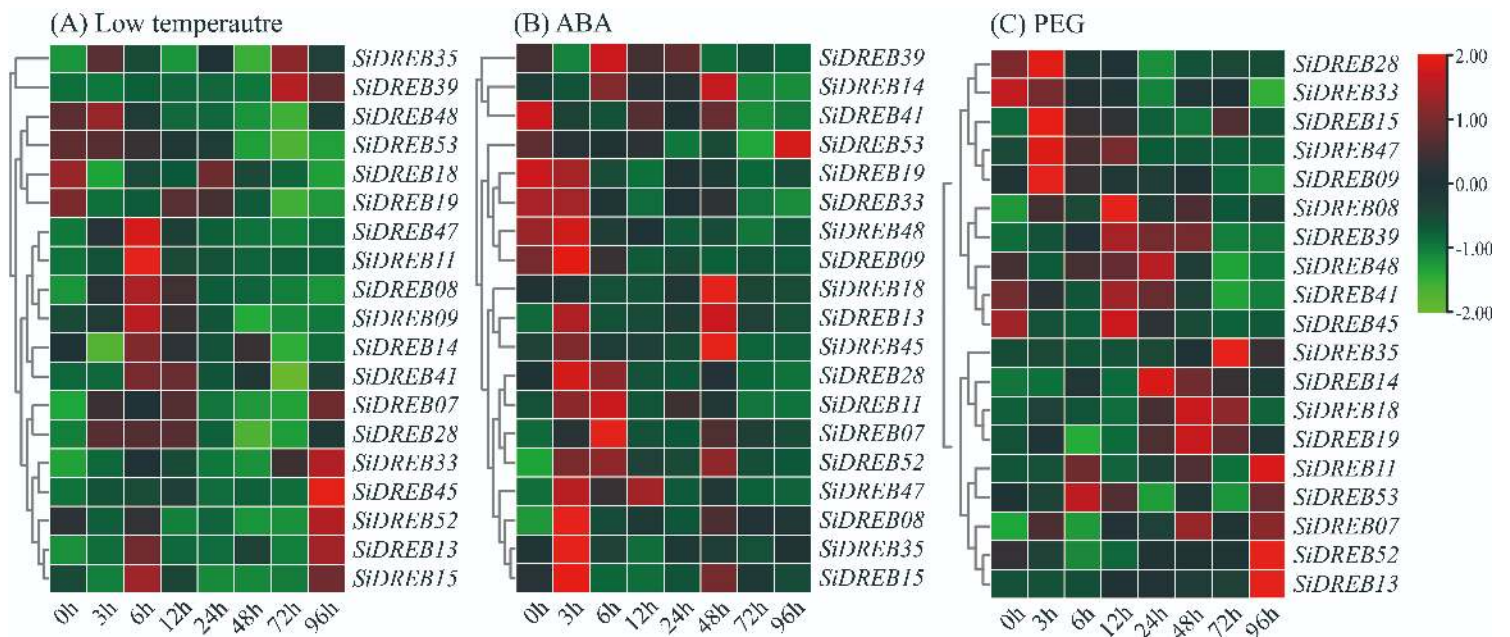


Figure 6

qRT-PCR analysis of expression patterns of 19 SiDREB genes family under cold stress (4 °C temperature) (A), 100 μM ABA (B) and osmotic (20% PEG 6000) (C).

Supplementary Files

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