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SHORT COMMUNICATION

Genome-wide identification and expression analysis of *DREB* genes in alfalfa (*Medicago sativa*) in response to cold stress

Song Sheng[a#], Xinyu Guo[a#], Changzheng Wu[a#], Yucheng Xiang[a], Shuhui Duan[b], Weiqin Yang[a], Wenrui Le[a], Fengchun Cao[a], and Laihua Liu[a]

[a]College of Resources and Environmental Sciences, Key Lab of Plant-Soil Interaction, MOE, Center for Resources, Environment and Food Security, China Agricultural University, Beijing, China; [b]Hunan Tobacco Science Institute, Changsha, China

ABSTRACT

Dehydration-responsive element-binding proteins (DREBs) belong to members of the AP2/ERF transcription factor superfamily, which has been reported to involve various abiotic-stress responses and tolerance in plants. However, research on the *DREB*-family is still limited in alfalfa (*Medicago sativa* L.), a forage legume cultivated worldwide. The recent genome-sequence release of the alfalfa cultivar "XinJiangDaYe" allowed us to identify 172 *DREBs* by a multi-step homolog search. The phylogenetic analysis indicated that such *MsDREB*s could be classified into 5 groups, namely A-1 (56 members), A-2 (39), A-3 (3), A-4 (61) and 13 (A-5 (13), thus adding substantial new members to the *DREB*-family in alfalfa. Furthermore, a comprehensive survey in *silico* of conserved motif, gene structure, molecular weight, and isoelectric point (pI) as well as gene expression was conducted. The resulting data showed that, for cold-stress response, 33 differentially expressed *MsDREB*s were identified with a threshold of Log2-fold > 1, and most of which were transcriptionally upregulated within 48 h during a cold treatment(s). Moreover, the expression profiling of *MsDREB*s from two ecotypes of alfalfa subspecies i.e. *M. sativa* ssp. *falcata* (F56, from a colder region of Central Asia) and *M. sativa* ssp. *sativa* (B47, from Near East) revealed that most of the cold-stress responsive *MsDREBs* exhibited a significantly lower expression in F56, leading to a proposal of the existence of a distinct mechanism(s) for cold tolerance regulated by DREB-related action, which would have been evolved in alfalfa with a genotypic specificity. Additionally, by examining the transcriptome of a freezing-tolerance species (*M. sativa* cv. Zhaodong), eight *DREBs* were found to be implicated in a long-term freezing-stress adaptation with a great potential. Taken together, the current genome-wide identification in alfalfa points to the importance of some *MsDREB*s in the cold-stress response, providing some promising molecular targets to be functionally characterized for the improvement of cold tolerance in crops including alfalfa.

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1. Introduction

Cultivated alfalfa (*Medicago sativa* L.) represents one of the most important legume forages. Besides their role serving as a livestock feed, such legume plants can also be directly applied as an organic fertilizer to not only provide soil nitrogen (N) derived from symbiotic N-fixation but also to improve soil quality. However, as observed in many other crops, the growth of the cultivated alfalfa suffers often from diverse abiotic stresses, among which a low temperature resulted from e.g. 'late-spring freezing' or an abnormally sharp temperature change is a critical factor limiting severely a higher biomass production and quality of alfalfa.[1–3]

To cope with a severe challenge of a detrimental temperature (e.g. more than 5 degree lower than that required for an optimal growth) that may frequently occur in ecosystems, plants have evolved complex and diverse systems to improve their cold-stress resilience, for instance by shifting from a growth to dormant phase or changing morphological characteristics to synchronize with climatic rhythms. At a molecular and/or physiological level, under low temperature or cold conditions, endogenous regulating systems modify the membrane properties e.g. fluidity through alteration of lipid content and composition, misfolding of proteins, increase in anti-oxidative system activities as well as operating other essential strategies to maintain plants in a relatively inactive status.[4,5] In general, such mechanisms are initiated by a cold induced transient increase in a cytosolic concentration of Ca^{2+}, which is mediated by Ca^{2+}-permeable channels and detected by numerous receptors and/or kinase proteins. In addition, the primary cold-stress signal modifies also some second messengers or other signal molecules, such as cyclic nucleotides (cAMP and cGMP), amino acids (e.g. Glu and Met) and various reactive oxygen species (ROS), which may trigger the expression of transcription factors (TFs) required for activation of the cold acclimation regulatory networks at the transcriptome level.[6,7]

The CBF (C-repeat binding factor) and DREB family have been documented to play important roles in response to drought, low-temperature, and high-salt stress in many plant

CONTACT Laihua Liu LL1025@cau.edu.cn College of Resources and Environmental Sciences, Key Lab of Plant-Soil Interaction, MOE, China Agricultural University, 100193, Beijing, China

[#]Both authors contributed equally to this work.

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species. Liu et al. reported that DREB1 and DREB2 induced cold- and drought-responsive gene expressions,[8] and this signal transduction pathway is essential for plant response to cold stress.[9] Other studies have indicated that many other TFs (e.g. PIF, MPK, EIN, and CRPK) are also involved in the DREB-dependent signaling processes, emphasizing the importance of DREB proteins in plant cold acclimation.[10] Indeed, transgenic approaches with a manipulation of *DREBs*' expression in crops such as tobacco, barley, tomato and *Medicago Truncatula* can significantly improve plant growth and tolerance to cold stress.[11–14] To date, the putative sequences of *DREBs* and their AP2/ERF (APETALA 2/ethylene-responsive element) superfamily genes have been identified in many plant species, including *Arabidopsis*, wild cane, mulberry, wheat and mungbean. For alfalfa plant, although Jin et al.[15] identified 59 DREB/CBF-like proteins, the authors were not able to use more complete genome sequence data, which have been released only recently from the cultivar "Zhongmu No. 1" and "XinJiangDaYe". As reported for these two cultivars, the 816-Mb high-quality chromosome-level haploid genome sequence (n = 8) and a length of 2738 Mb for 32 (4 n = 32) chromosomes have newly assembled, respectively.[16,17] This may indicate that a large complex genome of autotetraploid alfalfa cultivar would potentially possess more genes due to an evolutional duplication event(s).

In the present study, we identified 172 MsDREB proteins containing the AP2 domain with highly conserved amino acid residues (i.e. 14th valine (V) and 19th glutamic acid (E)) from *M. sativa* "XinJiangDaYe" genome sequencing dataset. Phylogenetic and conserved motif analysis could classify these genes into 5 subfamilies (A-1 to A-5), but not including A-6 subcluster. Gene structure, conserved motifs, molecular weight, and isoelectric points (pIs) were simultaneously investigated. Furthermore, we analyzed the expression pattern of *MsDREB* genes in response to cold stress (22°C to 4°C) using RNA-seq datasets from possible websites; 33 core *MsDREBs* were found to be significantly upregulated during cold treatment, and such *MsDREBs* were further characterized as low-expression genes in cold tolerance alfalfa cultivar "*sativa*" but not in "*falcata*". Additionally, a transcriptomic analysis with alfalfa cultivar Zhaodong during its long-term cold acclimation unraveled certain core *MsDREB* genes that may be involved in cold/freezing tolerance.

2. Materials and methods

2.1 Homologs sequence identification and characterization of DREB family in alfalfa

For comprehensive identification and analysis of *DREB* gene family in alfalfa, sequences of putative *DREB* genes with 14thV and 19thE were extracted from National Center for Biotechnological Information (NCBI, https://www.ncbi.nlm.nih.gov/) GenBank via BLAST search against *Arabidopsis* DREBs from the Arabidopsis International Resource (TAIR, https://www.arabidopsis.org/). The draft genome data and annotation information of alfalfa cultivar "XinJiangDaYe" were download from the figshare data repository released on 20–07-2019 (https://figshare.com/projects/whole_genome_sequencing_and_assembly_of_Medicago_sativa/66380). *MsDREB* genes were first searched by using TBtools "BLAST GUI Wrapper"[17] to obtain candidate *DREB*-family members. Second, the HMM (Hidden Markov Model) profile PF00847 (AP2 domain) was retrieved from the Pfam database (https://pfam.sanger.ac.uk/) to identify the putative *DREB* genes from previous BLAST filtered candidates. Basic parameters were predicted using the ProtParam tool (https://web.expasy.org/protparam).[18]

2.2 Phylogenetic analysis of DREB proteins

To analyze the sequence feature of DREBs, 208 putative DREB homologs (36 from *A. thaliana* and 172 from *M. sativa*) were loaded in the MEGAX version 10.2.6 (https://www.megasoftware.net) using default "ClustalW" parameters for the sequence alignment. Phylogenetic trees were then constructed with default "Neighbor-joining" algorithm parameters, and the best '.nwk' phylogenetic tree was made by iTol (https://itol.embl.de).[19]

2.3 Analysis of conserved motifs (MEME), gene structure, and domains (NCBI CDD) with MsDREB genes

Conserved motifs of MsDREBs proteins were predicted using the MEME suite version 5.4.1 (https://meme-suite.org/meme/index.html)[20] with default parameters, except the "motifs should find", which was set to 10. The coding sequences (CDS) and the structure of all genes were graphically displayed with TBtools function "Gene Structure View". The gene structure information of "XinJiangDaYe" was downloaded from figshare.com (https://figshare.com/articles/dataset/genome_fasta_sequence_and_annotation_files/12327602?file=22771946).

2.4 Expression analysis of MsDREB genes during plant development and in response to cold stress

The raw RNA sequencing data was obtained from NCBI Sequencing Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) through the SRA toolkits "prefetch" (version 2.8.0), SRP055547 for development in two alfalfa cultivars, SRP144299 for cold stress and SRP060503.[21–23] Raw data (raw reads) in fastq format were first qualified with FastQC program for Q20, Q30, GC-content and sequence duplication level; the data were then processed in Hisat2 version 2.2.1[24] for read alignment to the genome of alfalfa "XinJiangDaYe".[17] The reads were subjected to fragments per kilobase of transcript per million fragments mapped (FPKM) conversion to obtain the expression value of genes and transcripts. In-house R scripts were used to analyze gene expression and generate heatmaps. Transcripts (with a fold change (FC) ≥ 2 and an adjusted p-value < 0.05) were identified as differentially expressed genes (DEGs) with DEseq2. A heat map was created using ggplot2, reshape2, and dplyr packages in R version 4.1.2.[25]

3. Results and discussion

3.1 Genome-wide identification and phylogenetic analysis of DREB family genes in M. sativa

To identify the *MsDREB* genes in the autotetraploid alfalfa *M. sativa* genome, the BLAST search was performed by using TBtools with the dataset of cultivar "XinJiangDaYe", query data of known Arabidopsis DREBs with conserved AP2 domain containing 14th valine and 19th glutamic acid residue were processed at an E-value cutoff of 1E-5. Initially, 616 candidate proteins carrying AP2 domain were identified by HMM PF00847; MEGAX alignment was used to verify the conserved AP2 domain and AA residues. A total of 172 MsDREBs with their peptide length ranging from 79 to 505 AA residues were eventually characterized. The calculated molecular weight (MW) of the MsDREBs is in a range of 8.68 kDa to 56.56 kDa, and their isoelectric point (pI) was predicted to be from 4.55 to 10.92 (Table S1). Herein, we have completely surveyed and characterized in *M. sativa* a large DREB family containing much more members (172) than that of previously reported (59 DREB/CBFs) by Jin et al. (2019).[15]

To explore the phylogenetic relationship of *MsDREB* genes, 172 MsDREBs and 36 AtDREBs were aligned and processed in MEGAX using ClustalW with proper default parameters (The protein sequences are given in Table S2).[26] Unrooted trees were constructed using the neighbor-joining (NJ) method with specific default settings. The result showed that the *MsDREB* genes could be separated into 5 groups (except for the A-6 subfamily with distinct sequence motifs, see below), namely A-1 (with 56 members), A-2 (39), A-3 (3), A-4 (61) and A-5 (13) (Figure 1). Concerning again the previously BLAST-predicted 616 MsDREB proteins, we found that most members of A-6

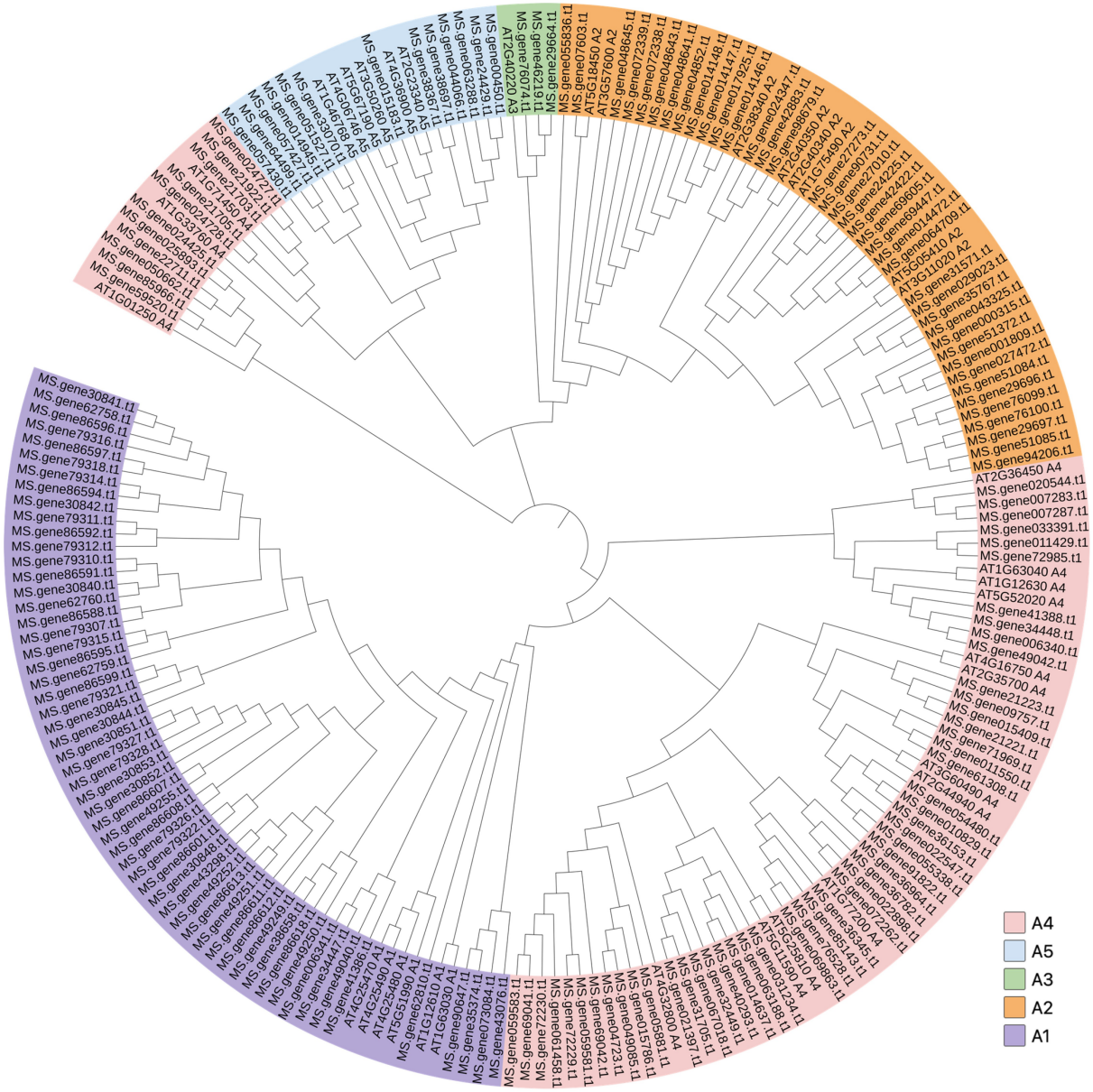

Figure 1. Phylogenetic tree of DREB proteins from *Arabidopsis thaliana* and *Medicago sativa* L. Evolutionary relationships were constructed using the neighbor joining (NJ) method (See Materials and Methods). The shaded color indicates the different subfamily.

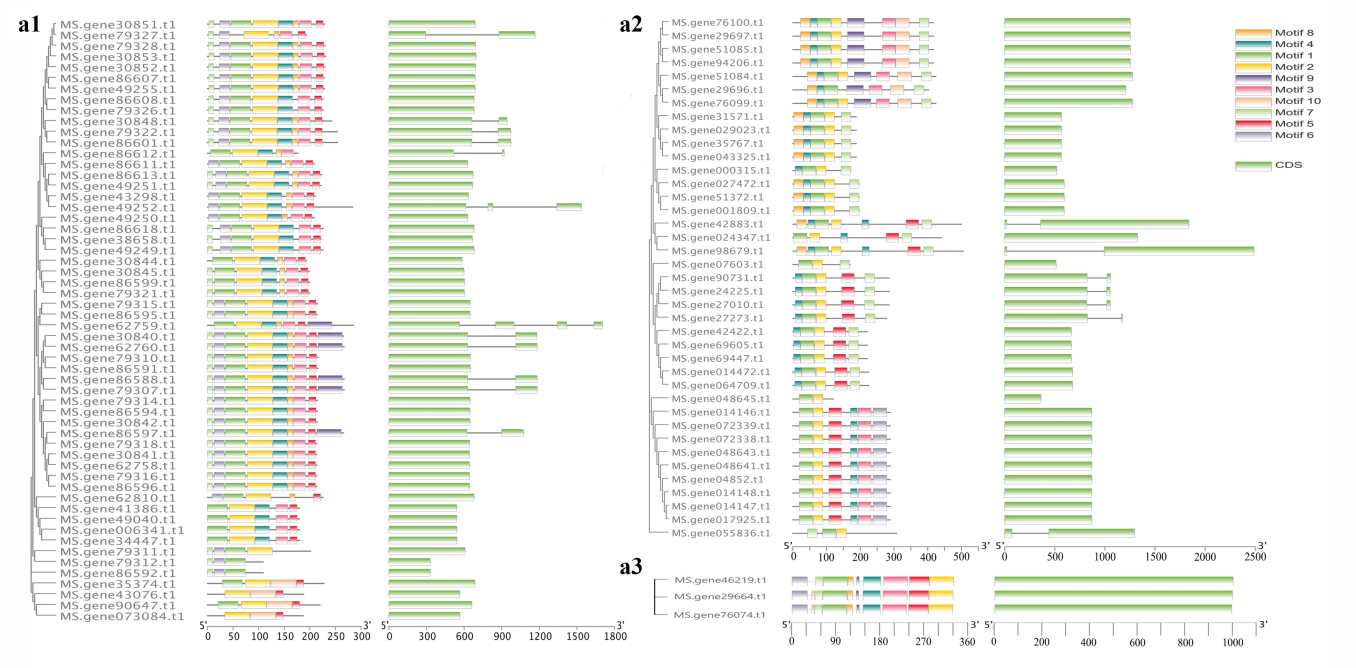

Figure 2. Analysis of the conserved motifs and gene structure of A1-3 subfamily.

subfamily contained also the AP2 domain, but the conserved 14th V and 19th E were mutated to other amino acids. To date, proteins with the AP2 domain consisting of 14th V and 19th E have been more likely characterized as DREBs, but those with an alanine (A)- and aspartate (D)-containing AP2 motif are assigned to ERF proteins.[27] Because the specific AP2 recognizes and binds to the DRE (dehydration-responsive element) *cis*-regulatory sequence in regulatory networks, such a AP2 domain represents a typical feature for *DREB* genes. Thus, in this study, we further used the 172 *MsDREB*-gene datasets for the preliminary analysis of alfalfa plant in response to cold stress.

3.2 Conserved motifs and gene structures

To explore the distribution and structural diversification of conserved motifs of MsDREB proteins, we processed a maximum of 10 motifs *in silico* prediction using MEME online tools and renamed the motifs using a number from 1 to 10 (Figure 2 and Figure S1). Only the A-3 subfamily has all 10 motifs, and the others share parts of the motifs. However, the motif diversity is relatively conserved in all groups, with slight differences among subfamilies. The motif 1 was characterized in all members of *MsDREBs*. Gene structure analysis showed that the number of exons in *MsDREB* genes varied from 1 to 4 (Figure 2 and Figure S1). The 'gene62759' processes 4 exons and large intron regions. In summary, a large variation of motif numbers and content among different subfamilies of MsDREBs was revealed, different from their gene structures with a less variation, for which 137 out of 172 *MsDREB* genes had only one exon or without an intron.

3.3 Expression patterns of 33 MsDREB genes in response to cold stress

To know if or how the transcription of *DREB* genes is responsive to cold stress, a publicly available RNA-seq dataset related to time-depend (0 h (CK), 2 h, 6 h, 24 h, 48 h) cold-stress (4°C) treatments of *M. sativa* was analyzed.[22] The expression pattern of all 172 *MsDREBs* was obtained from the dataset; only 33 genes were identified as DEGs at a threshold of '|Log2fold| > 1' (*p-value* < 0.05) using the data from the CK as a reference (Figure 3). Almost all *DREBs* were continuously induced at the transcriptional level during the tested cold stress, except for gene022547 (showing a downregulation after 24 h and 48 h cold treatment) that was also clustered separately in the phylogenetic tree (Figure 3). The 32 *MsDREBs* were divided into 3 subclades; genes in the clade I (7 members) were transcriptionally upregulated after 24 h cold treatment, the mRNA level of clade II (17 members) increased initially but relatively declined after 24 h, and the expression of clade III (8 members) was significantly upregulated, with more than 10-fold higher than that of those in the clade I and II (Figure 4). It has been known that *DREB* family genes are regulated by various types of TFs.[28] In *Arabidopsis*, CAMTA (calmodulin-binding transcription activator) are Ca^{2+}-dependent trans-activator.[29] Previous studies described that CAMTA3 positively regulated DREB by binding to the *cis*-element CM2 motif of *CBF2* under cold stress and the expression of *CBF* family genes were significantly downregulated up to approximately 50% in *camta1 camta2* double mutant.[30] As a cytosolic oscillation of Ca^{2+} often reflects an early event in the cold stress response, our results suggest that clade II/III *DREB* genes would be involved in the rapid action at a low temperature, in which such genes were markedly upregulated during first 2 h cold treatment (Figure 4). Interestingly, the

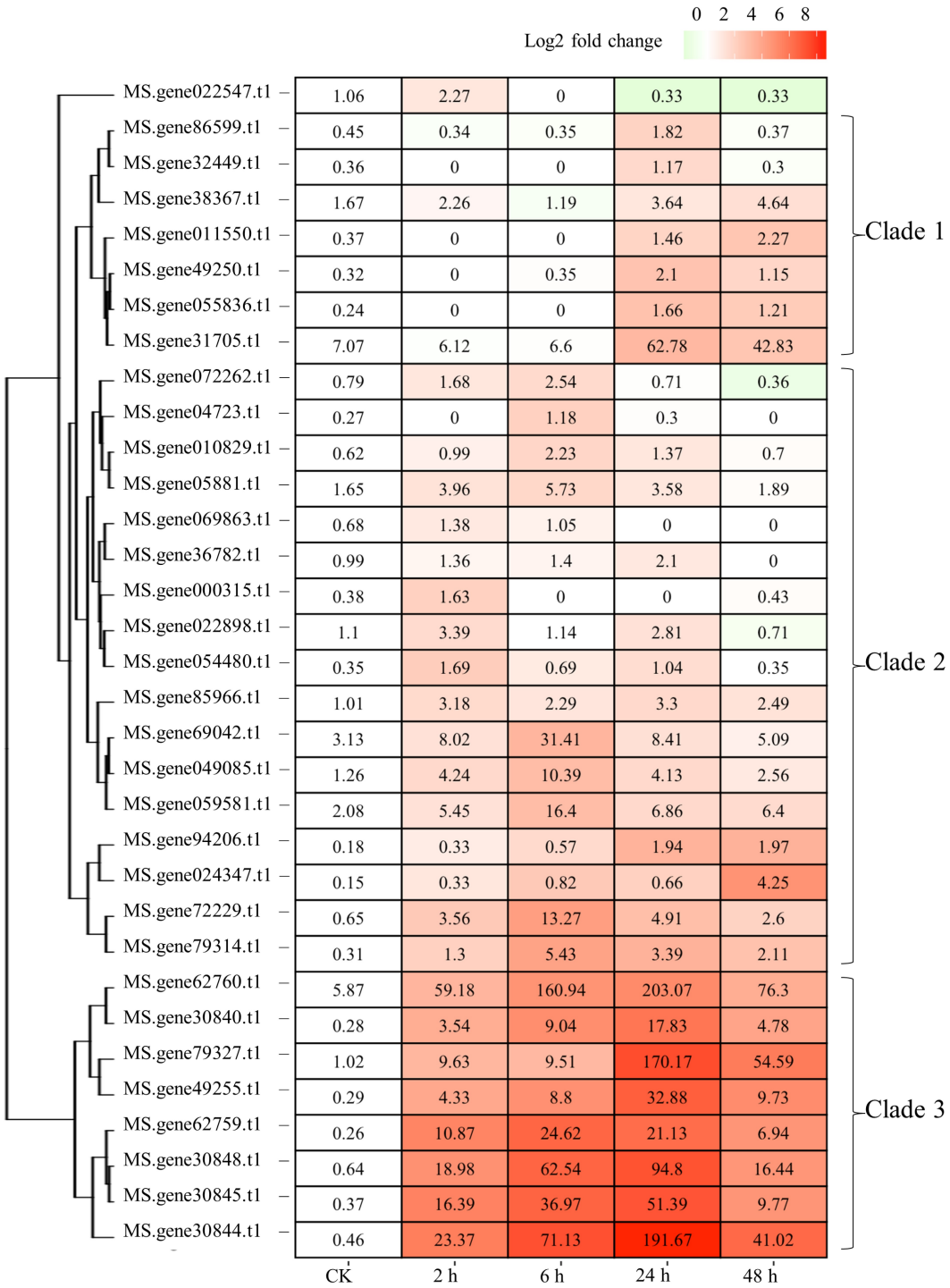

Figure 3. Clustering approach-based heatmap of *MsDREB* genes. The heatmap represents the expression pattern of genes in response to cold stress. Relative expression levels were calculated as a Log2-fold change against CK (See Materials and Methods). The red color shows an upregulation of a given gene, and the green indicates a downregulation. Labeled number in each tile is the expression level "FPKM".

regulation of *CBF1/2* expression by CAMTA3/5 is only determined in the case of a sharply decreased temperature (e.g. directly from 22°C to 4°C) but not of gradually lowering of the temperature (e.g. decreasing 3°C every 10 min).[31] Moreover, there is other evidence showing that numerous DREBs play important roles in response to a long-term cold stress, e.g. SsDREB102 and SsDREB105 for E3-mediated ubiquitination during the cold stress in *Saccharum spontaneum*.[32] In addition, the A-1 subfamily gene *CBF2* and *DREB* displayed differential expression patterns in response to cold; genes in cultivar 'Chinese Spring' were only transcriptionally induced in a relative short-term and then followed by rapid decrease in their mRNA abundance;[33] in wheat cultivar "Mironovskaya 808", the genes were shown their expression at consistently high cold-upregulation levels, similar to that of top half, most clade II *DREBs* and clade III genes[33,34] (Figure 3). For clade I *DREBs*, a comparable expression pattern was observed for wheat *DREB* genes "EVM0034637_A5" and "EVM0026054_A4";

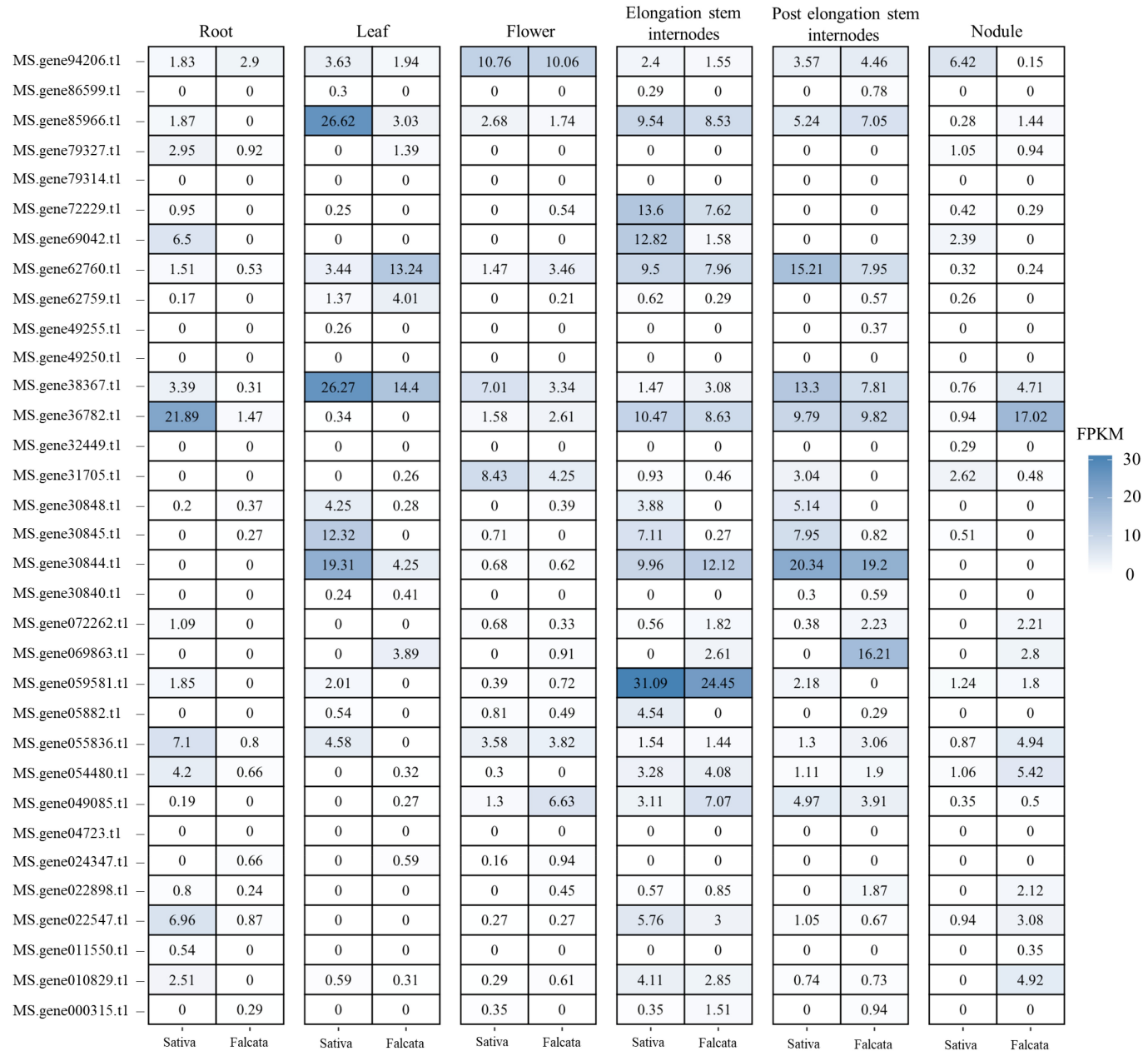

Figure 4. The expression level of *MsDREB* genes in different tissues. The expression level is shown by the intensity of blue color. Labeled number in each tile is "FPKM".

however, their functions as well as associated regulatory networks were largely unclear.[35] Thus, it has been suggested that genetic variations may result from potent differences in the regulatory motifs present in promoters of genes in different cultivars.[36] Although many DREBs have been documented to play diverse and critical roles in plant response to abiotic stimuli including heat, drought and cold,[8,37] for example, the expression of *Arabidopsis DREB1/CBF* are induced by cold, while the transcription of *DREB2* genes are generally upregulated by dehydration, salinity and high temperature,[8,38–40] 33 out of 172 *MsDREB*s in alfalfa have been characterized here at least as cold-responsive elements, and the rest *MsDREB*s might participate in other stress regulatory networks, which may be interesting to be explored in the future.

3.4 Expression difference between M. sativa subspecies during plant development

To extend our understanding of possible biological functions of the preliminarily selected cold-responsive *MsDREB* genes, a *de novo* transcriptome data assembly from two alfalfa subspecies (*M. sativa* ssp. *sativa*-B47 from the Middle East, *M. sativa* ssp. *falcata*-F56 from colder region Central Asia) was inspected, and RNA-seq data from different tissues were analyzed.[21,41] In this study, we comprehensively examined the expression level of 33 *MsDREBs* in specific tissues including root, leaf, flower, elongation stem internode, post-elongation stem internode, and nitrogen-fixing nodule (Figure 4). In general, most *MsDREB* genes

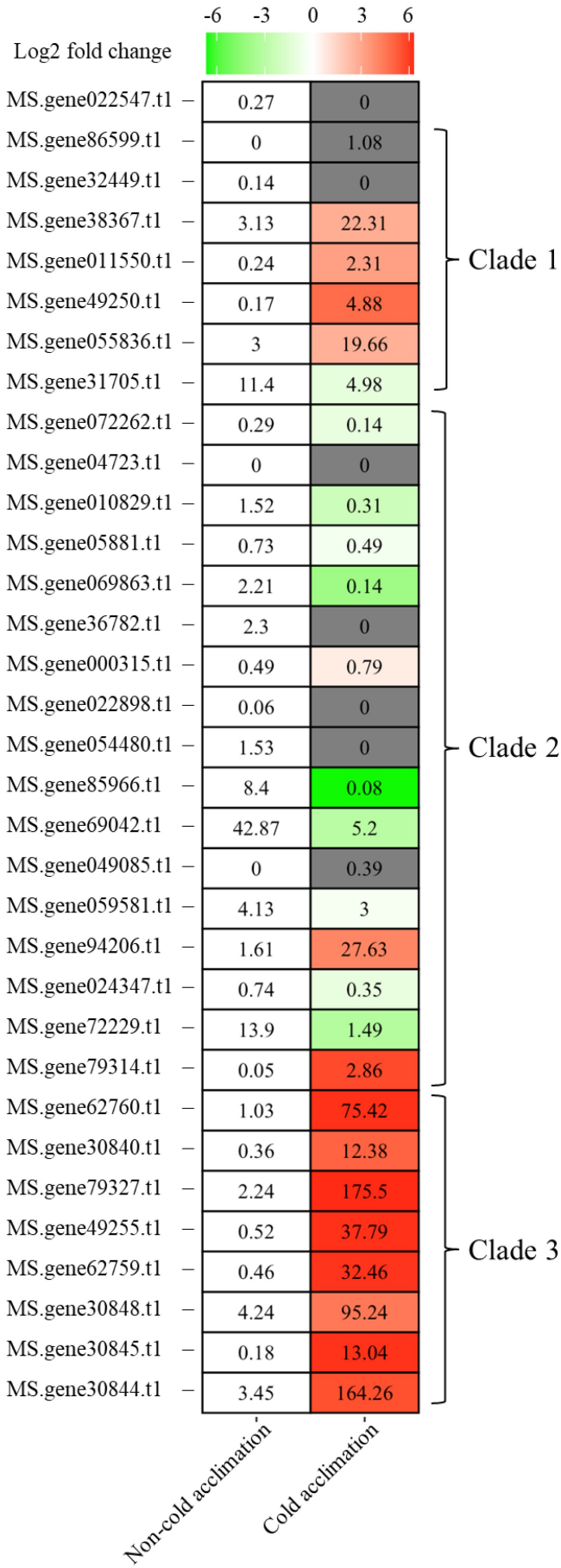

Figure 5. Clustering approach-based heatmap of *MsDREB* genes in freezing acclimation alfalfa.

showed a low or undetectable expression (FPKM = 1–0) in the cold-tolerant cultivar F56, with a respective number of 31, 25, 25, 17, 21 or 22 *MsDREBs* in the root, leaf, flower, elongation stem internode, post-elongation stem internode or nodule. Differently, numerous *MsDREBs* exhibited a relatively high transcription level in some tissues of the cold-sensitive cultivar B47, e.g. gene 85966 and gene 38367 in the leaf and gene 36782 in the root (Figure 4). These results clearly indicate a distinct and ecosystem-dependent expression pattern of 33 *MsDREBs* in B47 and F56. Being a cold area-adaptive subspecies, since some genes implicate in certain valuable agronomic traits (e.g. winter hardiness, creeping root habit) from F56 plant have already been assigned to cultivated alfalfa,[41] and since such *MsDREBs* were further characterized in this study as cold-responsive genetic components under a short-term cold stress, we assume that an existence of a cold-induced regulatory network would lead to the different cold response contributed by the action of MsDREBs in different subspecies. However, this hypothesis needs to be experimentally confirmed at a molecular-physiological level. To date, a comparison of gene expression between sensitive and tolerant cultivars has been extensively applied to assess and verify abiotic responsive regulators in many plants, e.g. cowpea, rice, and Chinese jujube under cold stress, wheat and barley subjected to various growth constraints,[42–45] resulting in a successful identification of many potential cold-stress regulatory components. Thus, we expect that those *MsDREB* genes with differential expression identified between B47 and F56 would be worthy candidates for further functional characterization.

3.5 Transcriptome analysis of M. sativa cv. Zhaodong *grown* adaptively under a long-term freezing condition

M. sativa cv. Zhaodong is a major forage legume cultivar that can survive in the northern China, where temperatures may reach around −30°C in winter. Song et al. (2016) provided a systemic analysis of adaptive mechanisms for frost tolerance using RNAseq assay.[23] *M. sativa* cv. Zhaodong were cultivated in September and harvested in mid-winter after freezing adaptation during a two-month temperature decrease. In this adaptation process, the antioxidant defense system was believed to confer freezing tolerance in a rapid way, and numerous potential freezing-sensing and signal transduction components were revealed.[23] Here, we focused on the expression pattern of previously identified 33 cold-tolerance related MsDREBs. As shown in Figure 5, the genes in clade 3 were differentially expressed at high transcription levels (FPKM > 10) under the long-term cold/freezing treatment as compared to that of in control samples without cold acclimation. For clade 2, the most genes, whose expressions were previously analyzed to be rapidly upregulated by cold stress but decreased after 24 hours (Figure 3), were not substantially expressed in the freezing acclimation alfalfa (Figure 5). Interestingly, transcripts of the clade 1 genes were highly accumulated in freezing acclimation plants (Figure 5). Such results suggest that the functional expression of clade one-third members would contribute to a long-term freezing fitness of alfalfa plants, while clade 2 genes might be required for an early response (e.g. within 24 h cold) to cold threat. With experimental evidence demonstrating that the overexpression of *MtCBF1* (also termed *MtDREB1C*) triggered by the CaMV 35S RNA promoter leads to a constant and remarkable enhancement of freezing tolerance of *Medicago truncatula* and Chinese rose,[14] we would like to suggest that the eight *MsDREB* genes in the clade 3 (Figure 3) would be interesting molecular targets available for the improvement of plant ability to acclimate both cold stress and long-term freezing through genetic manipulation.

In conclusion, here we have genome-widely identified in alfalfa plant 172 putative *MsDREB* genes using updated whole-genome sequence datasets, detailly characterized their genetic lineage and gene structures as well as coding-proteins that consist of a conserved AP2 domain with 14th valine and 19th glutamic acid residue. In particular, 8 out of 33 differentially expressed *MsDREBs* in the clade 3 were characterized as potential cold-stress responsive components, which may serve as crucial trans-activators participating in or controlling a signal transduction network(s) regulating growth adaption of crops including alfalfa to cold and/or long-term freezing environments. In the future study, it may be interesting and necessary to functionally appreciate such *MsDREB* genes at molecular and physiological levels. Thus, we believe that our findings with adding many new members to the DREB-family in alfalfa should provide valuable information for further profoundly understanding of *DREBs'* action in plant cold-stress behavior, assisting us to take a proper approach aiming at improving crop cold-tolerance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Abbreviations

DREB, Dehydration-responsive element-binding protein; CBF, C-repeat binding factor; CDS, coding sequences; pI, isoelectric point.

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References

1. Chamberlain CJ, Cook BI, Cortázar-Atauri IGD, et al. Rethinking false spring risk. Global Change Biol. 2019;25(7):2209–2220. doi:10.1111/gcb.14642.
2. Zhu J. Abiotic stress signaling and responses in plants. Cell. 2016;167(2):313–324. doi:10.1016/j.cell.2016.08.029.
3. Boyer JS. Plant productivity and environment. Science. 1982;218 (4571):443–448. doi:10.1126/science.218.4571.443.

4. Quint M, Delker C, Franklin KA, et al. Molecular and genetic control of plant thermomorphogenesis. Nat Plants. 2016;2(1):15190. doi:10.1038/nplants.2015.190.
5. Aslam M, Fakher B, Ashraf MA, et al. Plant low-temperature stress: signaling and response. Agronomy. 2022;12(3):702. doi:10.3390/agronomy12030702.
6. Gong Z, Xiong L, Shi H, et al. Plant abiotic stress response and nutrient use efficiency. Sci China Life Sci. 2020;63:635–674.
7. Ding Y, Shi Y, Yang S. Molecular regulation of plant responses to environmental temperatures. Mol Plant. 2020;13(4):544–564. doi:10.1016/j.molp.2020.02.004.
8. Liu Q, Kasuga M, Sakuma Y, et al. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell. 1998;10(8):1391–1406. doi:10.1105/tpc.10.8.1391.
9. Thomashow MF. PLANT COLD ACCLIMATION: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol. 1999;50(1):571–599. doi:10.1146/annurev.arplant.50.1.571.
10. Shi Y, Ding Y, Yang S. Molecular regulation of CBF signaling in cold acclimation. Trends Plant Sci. 2018;23(7):623–637. doi:10.1016/j.tplants.2018.04.002.
11. Agarwal P, Agarwal PK, Joshi AJ, et al. Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. Mol Biol Rep. 2010;37(2):1125–1135. doi:10.1007/s11033-009-9885-8.
12. Morran S, Eini O, Pyvovarenko T, et al. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol J. 2011;9(2):230–249. doi:10.1111/j.1467-7652.2010.00547.x.
13. Shah SH, Ali S, Jan SA, et al. Piercing and incubation method of in planta transformation producing stable transgenic plants by over-expressing DREB1A gene in tomato (*Solanum lycopersicum* Mill. Plant Cell. 2014;120:1139–1157.
14. Chen J, Lü J, Liu R, et al. DREB1C from *Medicago truncatula* enhances freezing tolerance in transgenic *M. truncatula* and China Rose (*Rosa chinensis* Jacq. Plant Growth Regul. 2010;60(3):199–211. doi:10.1007/s10725-009-9434-4.
15. Jin X, Yin X, Ndayambaza B, et al. Genome-wide identification and expression profiling of the ERF gene family in Medicago sativa L. under various abiotic stresses. DNA Cell Biol. 2019;38(10):1056–1068. doi:10.1089/dna.2019.4881.
16. Shen, Du H, Chen Z, et al. The chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. Mol Plant. 2020;13(9):1250–1261. doi:10.1016/j.molp.2020.07.003.
17. Chen H, Zeng Y, Yang Y, et al. Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. Nat Commun. 2020;11(1):2494. doi:10.1038/s41467-020-16338-x.
18. Wilkins MR, Gasteiger E, Bairoch A, et al. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol. 1999;112:531–552. doi:10.1385/1-59259-584-7:531.
19. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 2016;44(W1):W242–W245. doi:10.1093/nar/gkw290.
20. Bailey TL, Johnson J, Grant CE, et al. The MEME Suite. Nucleic Acids Res. 2015;43(W1):W39–W49. doi:10.1093/nar/gkv416.
21. Rourke JAO, Fu F, Bucciarelli B, et al. The *Medicago sativa* gene index 1.2: a web-accessible gene expression atlas for investigating expression differences between *Medicago sativa* subspecies. BMC Genomics. 2015;16(1):502. doi:10.1186/s12864-015-1718-7.
22. Zhou Q, Luo D, Chai X, et al. Multiple Regulatory Networks Are Activated during Cold Stress in Medicago sativa L. Int J Mol Sci. 2018;19(10):3169. doi:10.3390/ijms19103169.
23. Song LL, Jiang L, Chen Y, et al. Deep-sequencing transcriptome analysis of field-grown *Medicago sativa* L. crown buds acclimated to freezing stress. Funct Integr Genomics. 2016;16(5):495–511. doi:10.1007/s10142-016-0500-5.
24. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12(4):357–360. doi:10.1038/nmeth.3317.
25. Team R. R: a language and environment for statistical computing. R foundation for statistical computing. Vienna (Austria, Computing);2010Vol. 14:12–21
26. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673–4680. doi:10.1093/nar/22.22.4673.
27. Liu YD, Zhang L, Chen LJ, et al., Genome-wide identification, expression and potential function analysis of the ERF and DREB subfamily members in tomato, Preprint (2020).
28. Ding Y, Shi Y, Yang S. Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. New Phytol. 2019;222(4):1690–1704. doi:10.1111/nph.15696.
29. Finkler A, Ashery-Padan R, Fromm H. CAMTAs: Calmodulin-binding transcription activators from plants to human. Febs Lett. 2007;581(21):3893–3898. doi:10.1016/j.febslet.2007.07.051.
30. Doherty CJ, Van Buskirk HA, Myers SJ, et al. Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. Plant Cell. 2009;21(3):972–984. doi:10.1105/tpc.108.063958.
31. Kidokoro S, Yoneda K, Takasaki H, et al. Different cold-signaling pathways function in the responses to rapid and gradual decreases in temperature. Plant Cell. 2017;29(4):760–774. doi:10.1105/tpc.16.00669.
32. Huang X, Song X, Chen R, et al. Genome-wide analysis of the DREB subfamily in *saccharum spontaneum* reveals their functional divergence during cold and drought stresses. Front Genet. 2020;10:1326. doi:10.3389/fgene.2019.01326.
33. Shen YG, Zhang WK, He SJ, et al. An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theor Appl Genet. 2003;106:923–930. doi:10.1007/s00122-002-1131-x.
34. Kume FK, Ishibashi M, et al. Differential and coordinated expression of CBF and Cor/Lea genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes Genet Syst. 2005;80(3):185–197. doi:10.1266/ggs.80.185.
35. Cao YW, Li X, et al. Characterization of the AP2/ERF transcription factor family and expression profiling of dreb subfamily under cold and osmotic stresses in *Ammopiptanthus nanus*. Plants. 2020;9(4):455. doi:10.3390/plants9040455.
36. Xu ZS, Ni ZY, Liu L, et al. Characterization of the TaAIDF a gene encoding a CRT/DRE-binding factor responsive to drought, high-salt, and cold stress in wheat. Mol Genet Geno. 2008;280(6):497–508. doi:10.1007/s00438-008-0382-x.
37. Agarwal PK, Agarwal P, Reddy MK, et al. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. 2006;25(12):1263–1274. doi:10.1007/s00299-006-0204-8.
38. Shinwari KZ, Nakashima K, Miura S, et al. DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun. 1998;250(1):161–170. doi:10.1006/bbrc.1998.9267.
39. Nakashima K, Shinwari ZK, Sakuma Y, et al. Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration-and high-salinity-responsive gene expression. Plant Mol Biol. 2000;42(4):657–665. doi:10.1023/A:1006321900483.
40. Lata C, Prasad M. Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot. 2011;62(14):4731–4748. doi:10.1093/jxb/err210.

41. Quiros CF, Bauchan GR. The genus *Medicago* and the origin of the *Medicago sativa* comp. Alfalfa and Alfalfa Improvement. 1988;29:93–124.
42. Tan H, Huang H, Tie M, et al. Transcriptome profiling of two asparagus bean (*Vigna unguiculata subsp.* sesquipedalis) cultivars differing in chilling tolerance under cold stress. PloS One. 2016;11:e151105.
43. Kumar M, Gho Y, Jung K, et al. Genome-wide identification and analysis of genes, conserved between *japonica* and *indica* rice cultivars, that respond to low-temperature stress at the vegetative growth stage. Front Plant Sci. 2017;8:1120. doi:10.3389/fpls.2017.01120.
44. Zhou H, He Y, Zhu Y, et al. Comparative transcriptome profiling reveals cold stress responsiveness in two contrasting Chinese jujube cultivars. BMC Plant Biol. 2020;20(1):240. doi:10.1186/s12870-020-02450-z.
45. Yang YF, Al-Baidhani HHJ, Harris J, et al. DREB/CBF expression in wheat and barley using the stress-inducible promoters of HD-Zip I genes: impact on plant development, stress tolerance and yield. Plant Biotech J. 2020;18(3):829–844. doi:10.1111/pbi.13252.