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Naipeng Ren

College of Agriculture, Heilongjiang Bayi Agricultural University

Hui Zang

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University

Yifu Xuan

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University

Jielin Liu

Grassland Research Institute of Heilongjiang Academy of Agricultural Sciences

Xiangping Liu

Lxp110@hotmail.com

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University

Guoliang Li

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University

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The alfalfa AP2/ERF transcription factor *MsCBF4* enhances cold tolerance in *Arabidopsis thaliana*

Nai-Peng Ren¹, Hiu Zang², Yi-Fu Xuan², Jie-Lin Liu³, Xiang-Ping Liu^{2*} and Guo-Liang Li^{2*}

¹ College of Agriculture, Heilongjiang Bayi Agricultural University, Daqing 163319, China

² College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing 163319, China

³ Grassland Research Institute of Heilongjiang Academy of Agricultural Sciences, Harbin 150086, China

*Correspondence

XiangPing Liu

Lxp110@hotmail.com

Guoliang Li

liguoliang3885@163.com

Abstract

Background: Alfalfa (*Medicago sativa* L.) is globally important forage crop. The survival and consequently the productivity of this leguminous crop is determined by the capacity of the plant to tolerate cold stress, particularly at higher latitudes. However, the molecular mechanisms of how alfalfa defends against cold stress remain unclear.

Results: In this study, we cloned the candidate gene *MsCBF4* from the cold-tolerant alfalfa genotype “Dongnong NO.1”. This gene belongs to the APETALA2/Ethylene response factor (AP2/ERF) family of

transcription factors (TFs), which serve as transcriptional activators with a transcription activation domain located at the C-terminal. The protein encoded by *MsCBF4* is localized in the cell nucleus. *MsCBF4* is induced by cold and expressed in roots, stems, leaves, flowers, and pods, with high expression levels in root and leaf tissues. *Arabidopsis thaliana* lines that overexpress *MsCBF4* demonstrate enhanced cold tolerance during both the seedling and mature stages, and the expression of the gene is thought to improve the physiological scavenging of reactive oxygen species (ROSs), inhibiting cell membrane damage. Furthermore, under freezing stress, the expression levels of key genes involved in abiotic stress resistance are upregulated in transgenic *Arabidopsis thaliana*. Herein, we describe *MsCBF4* is positively regulated by *MsERF6*.

Conclusion: These findings suggest that *MsCBF4* can enhance cold tolerance in *Arabidopsis thaliana* by alleviating oxidative stress, protecting membrane integrity, and upregulating stress-responsive genes.

Keywords: Alfalfa, *MsCBF4*, Cold stress, Antioxidant system, *Arabidopsis thaliana*

Background

Freezing stress is a type of abiotic stress faced by plants, occurring at temperatures below freezing point ($<0^{\circ}\text{C}$). Freezing may damage plant tissue directly, leading to the dehydration of protoplasm, damage to subcellular fine structures, and often such damage is irreversible and leads to plant death [1, 2]. Alfalfa (*Medicago sativa* L.) is a high-quality perennial forage crop of the legume family that is widely cultivated around the world [3]. However, the extreme winter weather conditions in northeastern China are harsh, making plants susceptible to freezing stress. This stress not only reduces crop productivity but also significantly decreases the likelihood that crop plants may overwinter [4]. Under prolonged freezing stress, alfalfa has evolved a series of coping mechanisms, including cold acclimation processes that involve sensing signals and initiating preparations [5], as well as physiological, biochemical, and molecular responses [6]. Transcription factors (TFs) play a key role in mediating this series of gene expression regulatory mechanisms and are important in alfalfa's response to cold stress [7]. Among these, TFs of the APETALA2/Ethylene response factor (AP2/ERF) family are essential for enhancing the cold tolerance of plants [8].

The AP2/ERF family is widely involved in the biological and abiotic stress processes of plants and is one of the largest families of plant-specific transcription factors [8]. They typically contain one or two highly conserved AP2 domains, consisting of 60 to 70 amino acids (AA), which form a typical helix-turn-helix structure and regulate the expression of downstream target genes through specific binding to DNA [9, 10]. As an important member of the AP2/ERF family, the C-repeat Binding Factor (CBF) is a core gene regulating the responses of plants to cold stress [11]. During the cold acclimation process of blue gum eucalyptus (*Eucalyptus globulus*), the genes *EglCBF1a*, *EglCBF1c*, and *EglCBF1d* play

significant roles, and correspondingly frost-tolerant phenotypes have been observed in transgenic *Arabidopsis thaliana* [12]. For *Malus baccata*, overexpression of *MbCBF2* in transgenic *Arabidopsis thaliana* consequently increased the expression of downstream genes (*AtCOR15a*, *AtRD29A/B*, *AtCOR6.6/47*, *AtCAT1*, *AtP5CS*, and *AtPIF1/4*), and enhanced tolerance to low-temperature stress [13]. Several CBF gene sequences responsive to low temperatures have been identified in both *Medicago falcata* and *Medicago truncatula* [14]. Studies have also shown that nine differentially expressed CBF TFs are homologous to the CBF gene at the Mt-FTQTL6 locus, and it is theorized these CBF TFs may be responsible for conferring frost tolerance to *Medicago truncatula* [15]. Although CBF genes induced by cold stress have been identified in various species, their role in cold tolerance remains poorly understood in alfalfa.

In this study, we investigated the cold-tolerant alfalfa genotype “Dongnong NO.1” using RNA-Seq data. From this analysis, we cloned the coding sequence (CDS) and promoter region of the significantly upregulated gene *MsCBF4*. We then characterized *MsCBF4* through bioinformatics analysis, gene feature annotation, and by examining its tissue-specific and spatiotemporal expression patterns. To assess its functional role, we evaluated the performance of transgenic *Arabidopsis thaliana* expressing *MsCBF4* under freezing stress. Promoter activity was validated using β -glucuronidase (GUS) staining, while yeast one-hybrid (Y1H) and dual-luciferase reporter (Dual-LUC) assays revealed that the *MsERF6* protein regulates the *MsCBF4* promoter. Together, these findings provide a theoretical foundation for understanding cold tolerance in alfalfa and support the development of new cold-resistant germplasm.

Results

MsCBF4 cloning and sequence analysis

Based on previous transcriptomic sequencing results, we observed that the gene Ms.gene006341.t1 in the “Dongnong NO.1” genotype was significantly upregulated at 2 hours, 4 hours, and 6 hours during freezing treatment (Table S1). Based on the homologous sequence of *MtCBF4* in the *Medicago truncatula* genome, the corresponding gene in alfalfa was annotated as *MsCBF4*. The open reading frame (ORF) of *MsCBF4* was subsequently cloned from the cold-tolerant genotype “Dongnong NO.1”. The ORF is 543 bp in length (Fig. 1A) and encodes a 180-amino acid protein containing a conserved AP2/ERF domain of 58 amino acids (Fig. 1F). The predicted molecular weight of *MsCBF4* is 20.44 kDa, with a theoretical pI of 5.76. The instability index is 64.25, meaning *MsCBF4* qualifies as an unstable protein. Further, *MsCBF4* is a hydrophilic protein, as it lacks transmembrane domains and signal peptides (Table S2). A phylogenetic analysis was conducted, and it is clear *MsCBF4* exhibits a high degree of homology with *MtCBF4* from *Medicago truncatula* (Fig. 1B). Additionally, the protein sequences encoded by these genes all contain a common AP2/ERF conserved domain (Fig. 1C). Structural predictions of the *MsCBF4* protein indicate that its secondary and tertiary structures are analogous to those of AP2/ERF family transcription factors. The secondary structure consists of 60.56% α -helices, 25.56% β -sheets, 6.67% β -turns, and 7.22% random coils (Fig. 1D, E).

***MsCBF4* transcriptional activation activity and subcellular localization**

Our analysis makes it clear *MsCBF4* protein is localized in the nucleus, chloroplasts, and cytoplasm (Table S2). After transferring pCAMBIA1302-*MsCBF4*: GFP into *Agrobacterium* strain GV3101, transient expression was achieved on the back of tobacco leaves. Fluorescence distribution results showed that the green fluorescence of pCAMBIA1302-GFP was localized in the cytoplasm and nucleus, while the green fluorescence of *MsCBF4*-GFP was only detected in the nucleus and overlapped with the

AHL22-mRFP marker, evidence the MsCBF4 protein is localized in the nucleus (Fig. 2A). Through yeast two-hybrid (Y2H) analysis, it was found that Y2H yeast cells transformed with pGBKT7 could only grow on SD/-Trp medium, while the positive controls pGBKT7-*GmbZIP63* and pGBKT7-*MsCBF4* Y2H yeast cells could grow normally on all three nutrient-deficient media and turned blue on SD/-Trp/-His/-Ade medium supplemented with X- α -gal (Fig. 2C). The pGBKT7-*MsCBF4* fragment was cleaved into three segments: N-terminal, domain, and C-terminal (Fig. 2B). It was found that only the sequence located at the C-terminal of *MsCBF4* could grow normally (Fig. 2C). These results indicate that *MsCBF4* is a transcription activator with an active region located at the C-terminal.

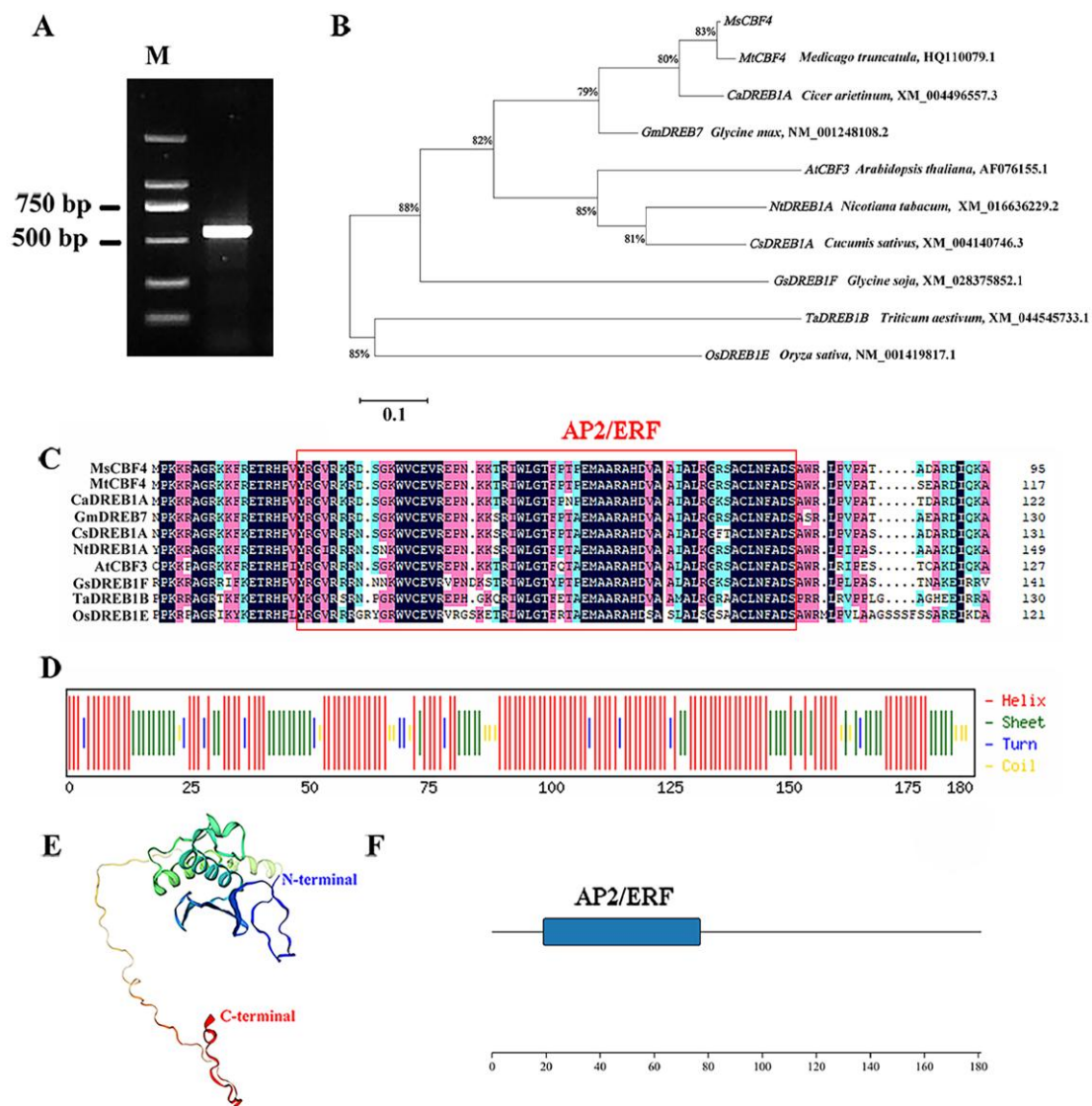


Fig. 1 Cloning and sequence analysis of *MsCBF4*. **A** ORF sequence cloning. **B** Phylogenetic analysis. **C** AA sequence alignment of *MsCBF4* with other homologous proteins. **D** Protein secondary structure. **E** Protein tertiary structure. **F** Conservative domains of *MsCBF4*.

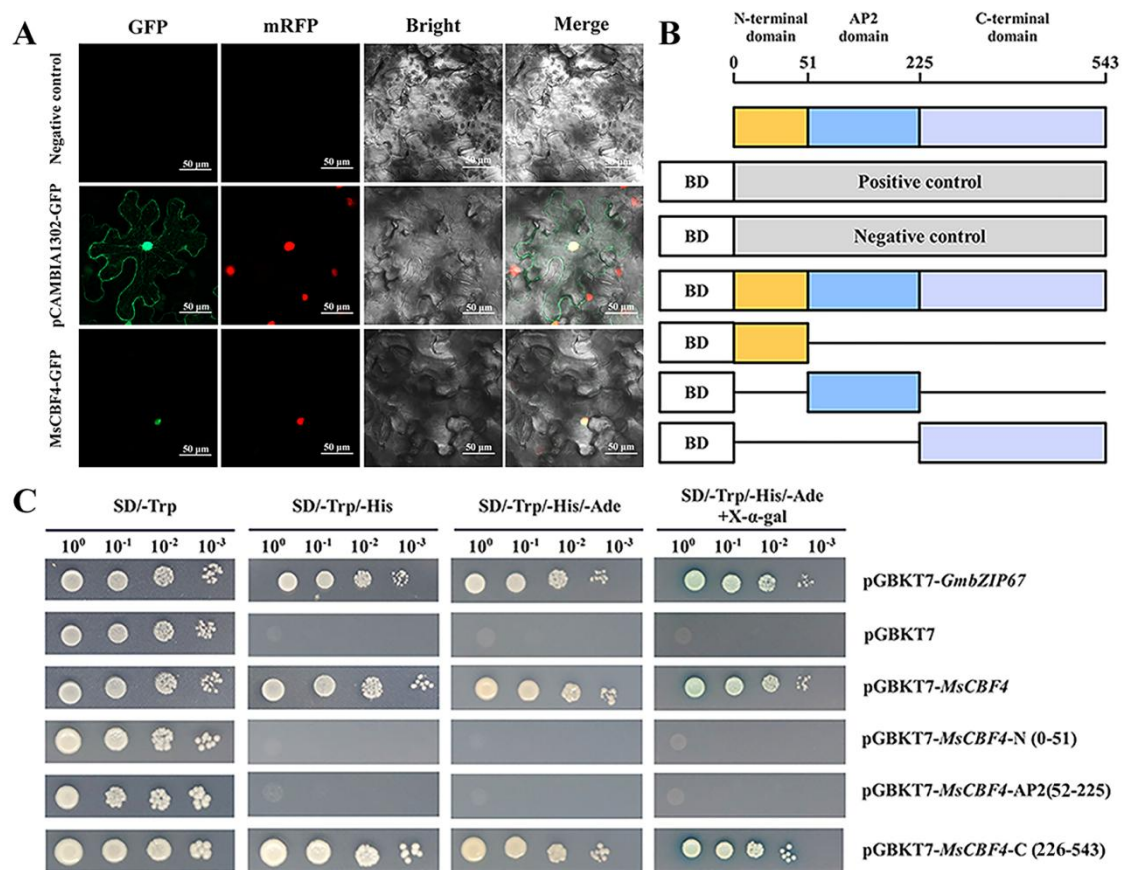


Fig. 2 Subcellular localization and transcriptional activation analysis of *MsCBF4*. **A** Subcellular localization of *MsCBF4*. mRFP indicates the location of the cell nucleus, scale bar = 50 μ m. **B** Schematic diagram of the truncated full-length sequence of pGBKT7-*MsCBF4*. **C** Transcription activation analysis of pGBKT7-*MsCBF4* in transformed Y2H yeast strains.

***MsCBF4* is strongly induced by cold stress in alfalfa**

The expression of *MsCBF4* in alfalfa tissues was analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). *MsCBF4* was expressed in all tissues, with the the highest levels of expression in roots and the lowest in flowers (Fig. 3A). The expression of *MsCBF4* reached its peak after 24 hours of cold treatment, while in roots, it peaked after 12 hours and then declined (Fig. 3B). In leaves, the expression of *MsCBF4* was induced after only one hour of freezing treatment and stabilized

quickly, while in roots, expression peaked after four hours of freezing treatment (Fig. 3C). During cold exposure, the expression of *MsCBF4* was higher in roots than in leaves. Conversely, during freezing treatment, the expression of *MsCBF4* was higher in leaves than in roots. This observation suggests that *MsCBF4* is induced by cold conditions, exhibiting greater expression sensitivity in the roots during cold treatment, while demonstrating heightened sensitivity in the leaves during freezing treatment.

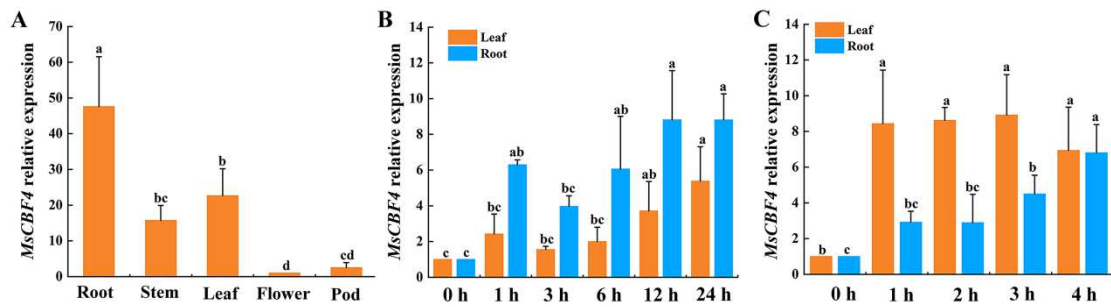


Fig. 3 Expression profiling analysis of *MsCBF4*. **A** Expression of *MsCBF4* in the roots, stems, leaves, flowers, and pods of alfalfa. **B** Expression of *MsCBF4* in the roots and leaves of alfalfa during cold treatment (4°C). **C** Expression of *MsCBF4* in the roots and leaves of alfalfa during freezing treatment (-5°C). Data are presented as mean $\pm$ SD (standard deviation) of three independent samples. Different letters indicate significant differences at the tissue (A) and time (B, C) levels with $P < 0.05$.

Overexpression of *MsCBF4* enhances cold tolerance in *Arabidopsis thaliana*

To further study how *MsCBF4* may confer cold tolerance to plants, we constructed transgenic *Arabidopsis thaliana* lines overexpressing *MsCBF4*. After screening, we were left with three homozygous lines with high transcription levels (#27, #33, and #41). qRT-PCR showed that the expression level of the *MsCBF4* gene in the overexpressed lines was significantly higher than that of the WT lines (Fig. S1). In addition, three T-DNA insertion mutant homozygous lines (*Atcbf2-1*, *Atcbf2-4*, *Atcbf2-27*) of the *AtCBF2* gene, which is homologous to *MsCBF4*, were screened using the three-primer method, and qRT-PCR

detected that the expression level of *AtCBF2* in the mutant lines was significantly lower than that of the WT lines (Fig. S2).

We subjected *Arabidopsis thaliana* seedlings of different lines to freezing treatment at -5°C. Prior to treatment, the WT lines, the overexpressing (OE) lines, and the mutant (*Atcbf2*) lines exhibited consistent phenotypes (Fig. 4A). After three days of recovery following freezing treatment, the OE lines retained most of their green coloration, whereas the WT and *Atcbf2* lines showed complete wilting and whitening of the entire plant (Fig. 4B). The survival rates of the three OE lines were significantly higher than those of the WT and *Atcbf2* lines (Fig. 4C). Furthermore, the REL of the OE lines was significantly lower than that of the WT and *Atcbf2* lines (Fig. 4D). These results indicate that *Arabidopsis thaliana* seedlings overexpressing *MsCBF4* exhibit significant cold tolerance.

Subsequently, we tested the resistance of different *Arabidopsis thaliana* lines to freezing stress during their mature stages. Under control conditions, there were no significant differences among the three lines. After treatment at -5°C for three hours and recovery at room temperature for seven days, the OE lines mostly recovered to normal growth, whereas both the WT and *Atcbf2* lines exhibited varying degrees of wilting and yellowing, with growth and survival significantly reduced when compared to the OE lines (Fig. 5A). Histochemical staining with DAB and NBT revealed that, compared with the WT and *Atcbf2* lines, the OE lines showed lighter staining after freezing stress, while the *Atcbf2* lines showed deeper staining than the WT lines (Fig. 5B, C). This indicates that the accumulation of H₂O₂ and O₂⁻ in the OE lines is lower than that in the WT and *Atcbf2* lines. Further measurements of physiological indicators related to abiotic stress revealed that there were no significant differences in physiological levels among the different lines under control conditions. After treatment, stress measures rose in all three

lines. Interestingly, the CAT, POD, and SOD activities of the OE lines were significantly higher than those of the WT and *Atcbf2* lines (Fig. 5D-F). At the same time, the accumulation of MDA and REL in the OE lines was significantly lower than that in the WT and *Atcbf2* lines (Fig. 5G, H). These results indicate that overexpression of *MsCBF4* enhances the cold tolerance of *Arabidopsis thaliana* by reducing the accumulation of reactive oxygen species (ROSs), increasing antioxidant enzyme activity, and inhibiting membrane damage.

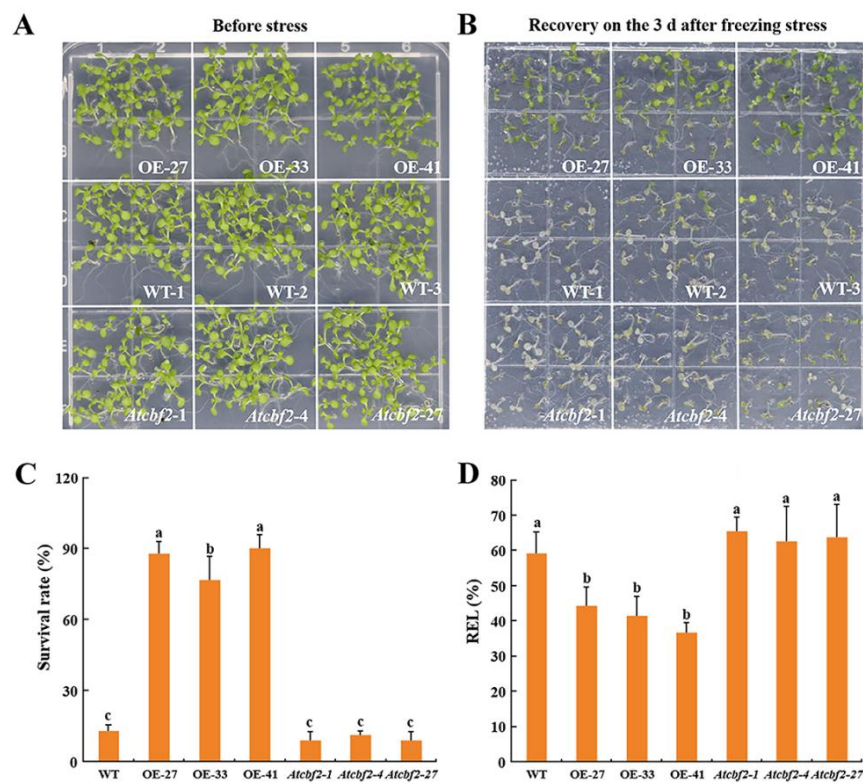


Fig. 4 Effects of freezing treatment on *Arabidopsis thaliana* seedlings. **A** Phenotypes of OE, WT, and *Atcbf2* lines before freezing treatment. **B** Phenotypes after three hours of freezing treatment at -5°C and three days of recovery at room temperature. **C** Survival rate of each line after freezing treatment. **D** REL detection in each line after freezing. Data are presented as mean $\pm$ SD of three independent

samples. Different letters indicate significant differences at the level of *Arabidopsis thaliana* lines ($P < 0.05$).

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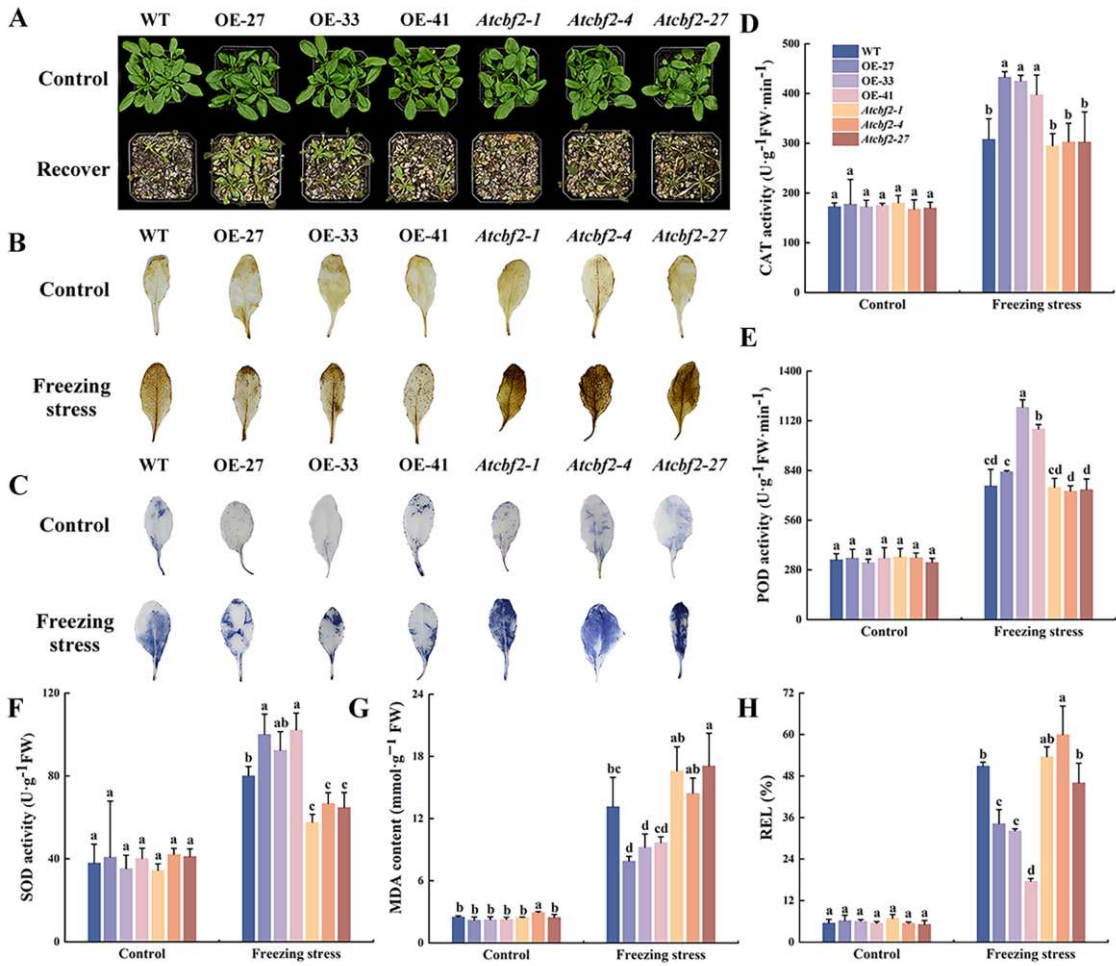


Fig. 5 Effects of freezing treatment on mature *Arabidopsis thaliana*. **A** Phenotypic changes before and after three hours of freezing treatment and seven days of recovery at room temperature. **B** DAB staining to detect H_2O_2 levels in *Arabidopsis thaliana*. **C** NBT staining to detect O_2^- levels in *Arabidopsis thaliana*. **D** CAT activity in *Arabidopsis thaliana*. **E** POD activity. **F** SOD activity. **G** MDA content. **H** REL. Data are presented as mean $\pm$ SD of three independent samples. Different letters indicate significant

differences at the $P < 0.05$ level among different *Arabidopsis thaliana* lines.

MsCBF4* induces the expression of abiotic stress-related genes in *Arabidopsis thaliana

To investigate the regulatory mechanism of *MsCBF4* expression, we analyzed the expression levels of genes associated with abiotic stress, including those involved in responses to low temperature, drought, and salinity. Under non-stress conditions, the expression levels of *AtCOR15A/B*, *AtCOR6.6/47*, *AtRD29A/B*, *AtCBL1*, *AtKIN1*, and *AtSOS2/3* were comparable. After cold treatment, the expression of these genes was significantly upregulated. In OE lines, transcript levels were markedly higher than those in WT and *Atcbf2* lines (Fig. 6). These results indicate that *MsCBF4* plays a crucial role in regulating the cold response in *Arabidopsis thaliana*.

***MsCBF4* promoter cis-acting elements and GUS activity analysis**

To investigate the function and molecular mechanism of the *MsCBF4* promoter, we cloned a 1793 bp sequence from the upstream flanking regulatory region of the ORF from the genome DNA of alfalfa “Dongnong NO.1” (Fig. 7A). Analysis of cis-acting elements in the promoter region revealed that this region contains elements associated with plant stress resistance, such as MYB, STRE, JERE, ERE, ARE, G-box, and TC-rich repeats (Fig. 7B). The activity of the *MsCBF4* promoter and its tissue expression pattern were detected using pCAMBIA3301-*MsCBF4*pro: GUS. The results showed that transgenic *Arabidopsis thaliana* seedlings, mature leaves, inflorescences, and pods were stained blue (Fig. 7C-F). This indicates that the *MsCBF4* promoter has transcriptional activity in different tissues throughout the growth and development stages of *Arabidopsis thaliana*.

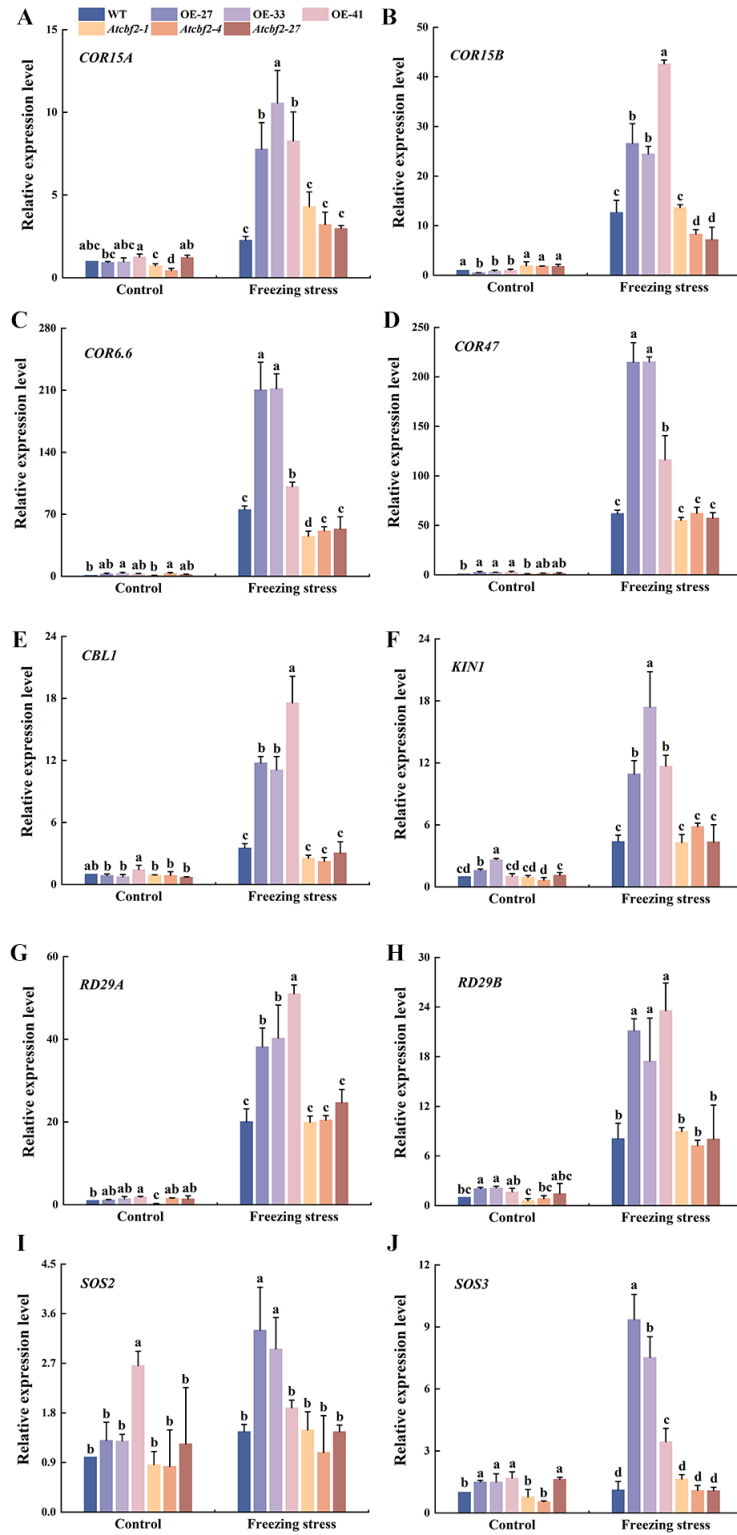


Fig. 6 Expression of key genes involved in abiotic stress response in *Arabidopsis thaliana*. **A** *COR15A*.

B *COR15B*. **C** *COR6.6*. **D** *COR47*. **E** *CBL1*. **F** *KIN1*. **G** *RD29A*. **H** *RD29B*. **I** *SOS2*. **J** *SOS3*. Data are presented as mean $\pm$ SD of three independent samples. Different letters indicate significant differences at the $P < 0.05$ level among different *Arabidopsis thaliana* lines.

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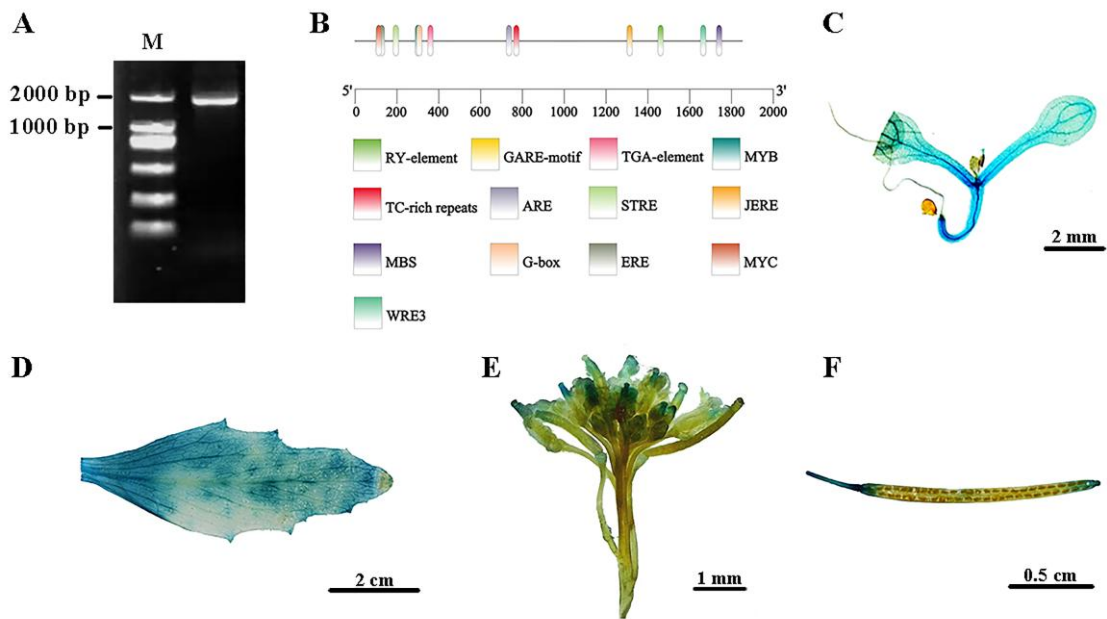


Fig. 7 Cloning and analysis of the *MsCBF4* promoter. **A** Cloning of the promoter sequence. **B** Cis-acting elements predicted in the promoter. **C** GUS staining of transgenic *Arabidopsis thaliana* seedlings. **D** Leaf, **E** inflorescence, and **F** pod tissue GUS staining of transgenic *Arabidopsis thaliana*.

172 ***MsERF6* activates the expression of the *MsCBF4* promoter**

173 To investigate the regulatory relationship between TFs and *MsCBF4* in alfalfa, we constructed a
 174 *MsCBF4*pro-pAbAi bait. In SD/-Ura medium containing 90 ng/mL Aureobasidin A (AbA), the
 175 self-activation of *MsCBF4*pro-pAbAi is inhibited (Fig. S3). By screening the *MsCBF4*pro-pAbAi using a
 176 Y1H nuclear system library, potential *MsERF6* prey proteins may be identified. The identification of such
 177 prey proteins is evidence *MsERF6* may bind to the *MsCBF4* promoter. To validate the regulatory

relationship between *MsERF6* and *MsCBF4*, we further investigated the interaction between pGADT7-*MsERF6* and *MsCBF4*pro-pAbAi using Y1H technology (Fig. 8A). All Y1H yeast cells grow normally on SD/-Leu medium. However, on SD/-Leu screening medium supplemented with 90 ng/mL AbA, only Y1H yeast cells co-transformed with the pGADT7-*MsERF6* fusion protein and *MsCBF4*pro-pAbAi grow normally compared to the negative control (Fig. 8B). This is further evidence *MsCBF4* is positively regulated by *MsERF6*.

In addition, we measured dual luciferase activity in tobacco leaves using Dual-LUC technology (Fig. 8C). When *MsCBF4*pro: LUC and 35Spro: *MsERF6* were co-expressed, the fluorescence signal on tobacco leaves was significantly enhanced, and the LUC/REN ratio was significantly increased (Fig. 8D, E). This is further evidence that *MsERF6* may bind to the promoter region of *MsCBF4* in plants and activate its transcription. Further, we observed that the expression of *MsERF6* and *MsCBF4* were both upregulated during periods of cold exposure (Fig. 8F). These results suggest that cold induces *MsERF6* and activates transcription of *MsCBF4*.

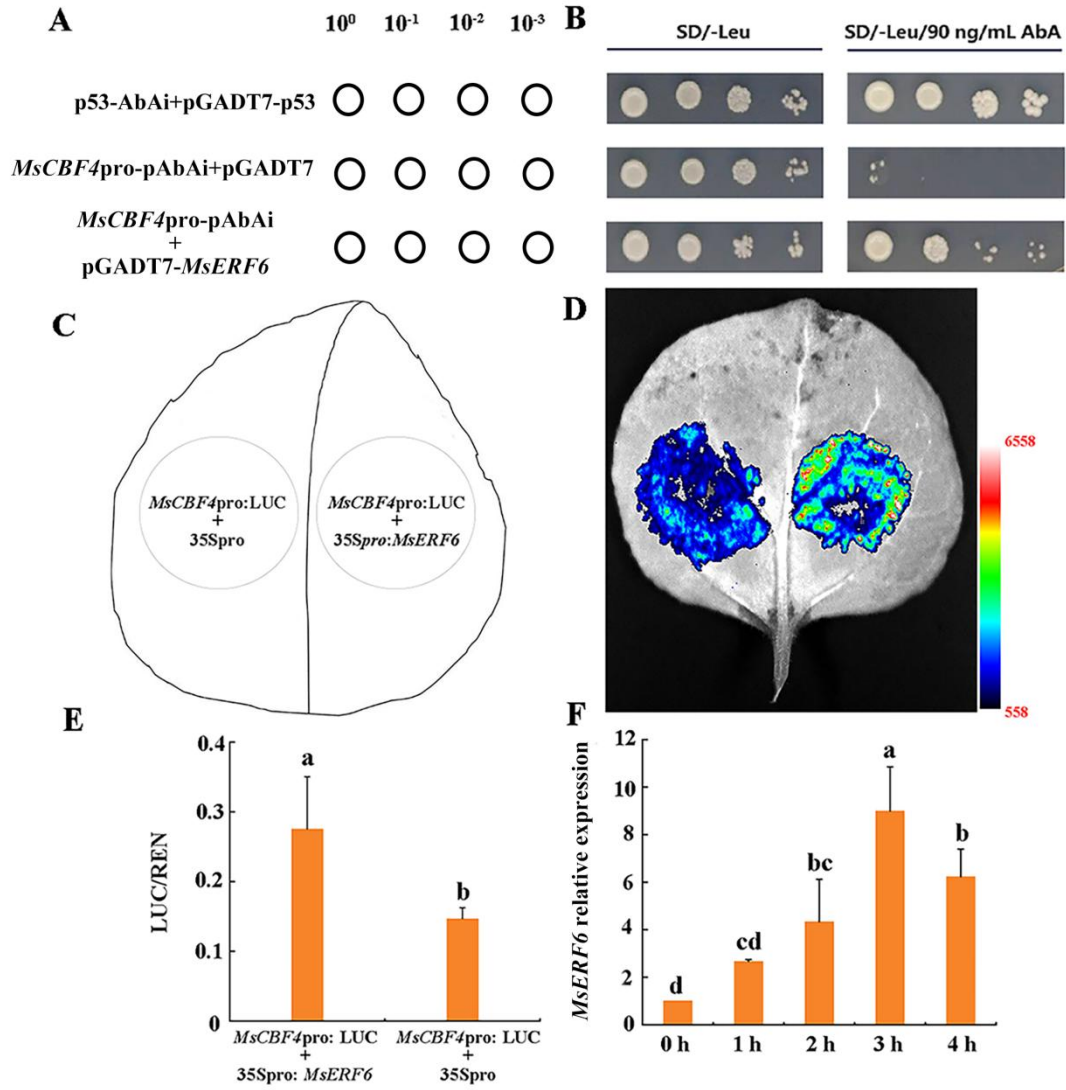


Fig. 8 *MsERF6* protein activates the *MsCBF4* promoter. **A** Schematic diagram of the Y1H technique. **B** Validation of the interaction between *MsERF6* protein and the *MsCBF4* promoter using the Y1H method. **C** Schematic diagram of the Dual-LUC technique. **D** Detection of the activating effect of *MsERF6* on the *MsCBF4* promoter using the Dual-LUC method. **E** Activity of LUC and REN. LUC is firefly luciferase, and REN is sea cucumber luciferase. **F** Dynamic expression of *MsERF6* in alfalfa leaves during freezing treatment (-5°C). Data are presented as mean $\pm$ SD of three independent samples, and different letters indicate significant differences ($P < 0.05$).

Discussion

In plants, the AP2/ERF family is a large family of proteins which may be divided into four subfamilies: AP2, ERF, DREB, and RAV, as well as a few unclassified subgroups based on the number of domains and characteristic sequences. Among these subfamilies, the AP2 subfamily is distinguished by the presence of two repeated AP2 domains, the ERF, DREB, and RAV subfamilies each contain a single AP2 domain. Members of the RAV subfamily are characterized by the presence of one B3 domain [16, 17]. In this study, the AA sequence of *MsCBF4* contains only one AP2/ERF conserved domain consisting of 58 AA. To further determine its subfamily classification, we compared the AA sequence of this AP2 domain with those of homologous genes from other species. We found that the MsCBF4 protein clusters with known DREB/CBF proteins and is closely related to the MtCBF4 protein from *Medicago truncatula*. We take this as evidence the *MsCBF4* is best considered a member of the DREB subfamily. We found that the MsCBF4 protein is localized to the cell nucleus, consistent with where most members of the AP2/ERF family TFs [18-20]. Y2H approaches confirmed that the full-length MsCBF4 protein and its C-terminal region strongly activate the expression of the GAL4 reporter gene in yeast cells, whereas the truncated form in which the C-terminal region has been removed completely loses any capacity to activate the GAL4 reporter gene. This result is highly consistent with the general characteristics of members of the AP2/ERF family. For example, *CbuERF114* in *Catalpa bungei* possesses a transcription activation domain at its C-terminal region [21], and the transcription activation activity of *TaAP2-10* in *Triticum aestivum* depends on its C-terminal region [22].

Analyzing the tissue expression patterns of target genes in plants is important for studying gene function [23]. This study revealed that *MsCBF4* may have differential levels of expression in various

tissues of alfalfa CBF genes have been reported to be important genes in plant responses to abiotic stress [11]. This study investigated changes in the expression levels of *MsCBF4* in alfalfa roots and leaves during cold treatment and freezing treatment. Cold stress upregulated the expression of this gene, evidence *MsCBF4* production may be a response of plants to low temperatures. It is worth noting that under freezing stress, the expression level of *MsCBF4* in leaves is higher than in roots, whereas under cold stress, the expression level in roots is higher than in leaves. This may be because under freezing stress, leaves directly sense the sudden drop in temperature and form ice crystals, thereby promoting the preferential expression of *MsCBF4*. In cold stress, the alfalfa root system continuously perceives low temperature signals from the soil and coordinates adaptive responses, leading to high expression of this gene [2, 24]. This may explain the differential response of *MsCBF4* in roots and leaves to different types of low temperature stress.

Previous studies have demonstrated that the overexpression of *PmhCBF* from *Prunus mume* in *Arabidopsis thaliana* enhances cold tolerance and oxidative stress resistance [25]. Similarly, the overexpression of *StCBF1* and *StCBF4* from *Solanum tuberosum* conferred cold tolerance to *Arabidopsis thaliana* [26]. Likewise, overexpression of *JcCBF2* from *Jatropha curcas* improved frost tolerance in *Arabidopsis thaliana* during the initial stages of freezing stress [27]. Our research indicates that overexpression of *MsCBF4* in alfalfa is associated with improved cold tolerance of *Arabidopsis thaliana* seedlings, and raised expression levels were associated with improved survival rates and reduced ion leakage compared to the WT and *Atcbf2* lines. Consistent with this, the visual phenotype of mature *Arabidopsis thaliana* with and without the expression of *MsCBF4* after freezing stress provided results consistent with this premise. These multiple lines of evidence suggest that overexpression of *MsCBF4*

confers higher cold tolerance to *Arabidopsis thaliana* after freezing treatment.

ROSs are chemicals produced when plants are subjected to external stress, and excess build-up of ROSs can cause damage to plant proteins, lipids, carbohydrates, DNA, and other components, leading to cell death [28]. H_2O_2 and O_2^- are examples of ROSs species which are produced during oxidative stress responses associated with cold stress in plants [29]. This study revealed through DAB and NBT staining analysis that cold stress increased levels of ROSs in mature *Arabidopsis* leaves, but the ROSs levels in the lines (where *MsCBF4* was deliberately overexpressed) were lower than those in the WT and *Atcbf2* lines. When plants are subjected to external stress, ROSs are not the only molecules produced. Plants also produce a series of antioxidant protective enzymes are produced to clear ROSs and protect plant structures from damage caused by oxygen free radicals [30]. Our study found that after freezing, the activities of antioxidant enzymes CAT, POD, and SOD were significantly increased in all lines, and these antioxidants were highest in the OE lines. Overexpression of *MsCBF4* increased the levels of antioxidant enzymes in *Arabidopsis thaliana* and inhibited the excessive accumulation of ROSs. MDA content reflects the severity of cell membrane lipid peroxidation produced by plants under adverse conditions, while REL is a key indicator of cell membrane integrity [31, 32]. In this study, there was no significant difference in MDA content or REL between any of the lines before freezing stress. After freezing treatment, MDA accumulation in the OE lines was significantly lower than that in the WT and *Atcbf2* lines. It has been reported that the overexpression of the transcription factor *VvERF63* in grapes (*Vitis vinifera*) improves cellular damage, increases antioxidant enzyme levels, and reduces reactive ROSs levels [33]. In tomatoes (*Solanum lycopersicum*), antisense *SLACS2* fruits exhibit severe cold damage, characterized by reduced antioxidant enzyme activity, elevated MDA content, and increased ion leakage

[34]. Our findings indicate that *MsCBF4* may likewise effectively enhance the cold tolerance of *Arabidopsis thaliana*.

As a TF, CBF can recognize CRT/DRE cis-acting elements in the promoters of downstream target genes, initiating transcription [35]. Previous studies have found that *DlCBF1/2/3* in longan (*Dimocarpus longan*) induces the expression of *AtRD29A*, *AtCOR15A*, *AtCOR47*, and *AtKIN1* by binding to the CRT/DRE cis-acting element, thereby promoting the cold adaptation process in *Arabidopsis thaliana* [36]. In addition, *COR6.6* and *COR15B* are also affected by cold stress responses [37, 38]. *AtCBL1*, *AtSOS2*, and *AtSOS3* participate in the SOS signaling pathway of salt stress [39]. *AtRD29B* participates in the abscisic acid (ABA) signaling pathway [40]. We detected these target genes related to abiotic stress using qRT-PCR and found that freezing stress upregulated the expression of all genes in each line, but that the expression level of each was significantly higher in the OE plants than that in the WT or *Atcbf2* lines. This indicates that *MsCBF4* may act by not only activating the CBF-COR pathway but also by indirectly enhancing tolerance to freezing by coordinating the SOS and ABA signaling pathways.

Analyzing the function of promoters is key to exploring the transcriptional regulation mechanism of TFs [41]. This study revealed the expression patterns of the *MsCBF4* promoter in different tissues during the vegetative growth stage of *Arabidopsis thaliana*, as demonstrated through GUS staining. We found that the *MsCBF4* promoter was expressed in *Arabidopsis thaliana* seedlings, mature rosette leaves, inflorescences, and pods. This indicates that the *MsCBF4* promoter is active across various tissues in *Arabidopsis thaliana*. In addition, previous studies have found that *SlERF15* in tomatoes activates its transcription by binding to the promoter of *SlCBF1* [42]. *VviERF11-c* in grapes specifically binds to the promoter of the *VvCBF* gene [43]. Further, we provided evidence *MsERF6* can bind to the *MsCBF4*

promoter and may positively regulate *MsCBF4* expression using Y1H technology and Dual-LUC technology. Analysis of the *MsCBF4* promoter sequence revealed that the cis-acting element of the *MsCBF4* promoter contains an ethylene response element ERE (ATTTTAAA). It has been reported that ERF and DREB proteins can bind to DRE/CRT or ERE elements [44], and we speculate that MsERF6 protein may interact with *MsCBF4* by binding to the ERE element in the *MsCBF4* promoter. Furthermore, through spatio-temporal expression analysis of *MsERF6*, we found that *MsERF6* expression was upregulated with increasing freezing treatment time. Our study suggests that *MsCBF4* may be a target gene of *MsERF6* and positively regulates the cold tolerance of alfalfa.

Conclusions

In summary, we cloned TF *MsCBF4* from alfalfa. The encoded protein localizes to the nucleus and exhibits transcriptional activation activity, with its C-terminal region functioning as the activation domain. *MsCBF4* is most highly expressed in roots, and its expression is markedly upregulated in both roots and leaves under cold stress. In transgenic *Arabidopsis thaliana*, *MsCBF4* enhances cold tolerance by limiting ROSs accumulation, increasing antioxidant enzyme activity, and reducing membrane damage. Furthermore, *MsCBF4* participates in the CBF-COR, SOS, and ABA signaling pathways, which collectively strengthen freezing tolerance. In addition, the *MsCBF4* promoter is active, and its expression is regulated by *MsERF6* through direct promoter binding. Collectively, these findings demonstrate that *MsCBF4* may improve cold tolerance in *Arabidopsis thaliana*.

Methods

Alfalfa cultivation and freezing treatment

The alfalfa seeds of variety “Dongnong NO.1” used in this study were provided by the Grassland

Science Laboratory of Northeast Agricultural University. To break seed dormancy in the alfalfa genotype “Dongnong NO.1,” seeds were incubated at 50°C for two hours. They were then transferred to pots containing a 2:1 mixture of nutrient soil and vermiculite and grown in a controlled environment at 25°C with a 16-hour light/8-hour dark photoperiod. At 13 weeks, healthy seedlings with uniform growth were selected, and tissues were collected from roots, stems, leaves, flowers, and pods.

Cold stress treatments were applied at the five-week growth stage. For cold treatment, plants were exposed to 4°C for 0, 1, 3, 6, 12, or 24 hours. For freezing treatment, plants were first acclimated at 4 °C for two days, after which the temperature was gradually lowered to –5°C at a rate of 3°C per hour. Plants were then subjected to freezing for 0, 1, 2, 3, or 4 hours. Root and leaf tissues were collected separately. Each treatment included three biological replicates. All samples were immediately frozen in liquid nitrogen after collection and stored at –80°C until gene expression analysis.

Gene cloning and sequence analysis

Total RNA was extracted from leaves of “Dongnong NO.1” using the RNAPrep Pure Plant Kit (TianGen, Beijing, China) following the manufacturer's instructions. cDNA was synthesized using HiScript® III RT SuperMix (Vazyme, Nanjing, China). Genomic DNA was extracted using Fast Plant Genomic DNA Extract (Coolaber, Beijing, China). Specific primers were designed based on the CDS and upstream flanking regulatory region sequence of Ms.gene006341.t1 in the genome of alfalfa genotype “XinjiangDaye” (Table S3). The target sequence was amplified using cDNA and DNA as templates. Sequences were aligned using DNAMAN (Version 6.0) and a phylogenetic tree constructed using MEGA (Version 7.0).

Bioinformatics predictions were performed using the following tools: SignalP 4.0

(<http://www.cbs.dtu.dk/services/SignalP/>, accessed May 12, 2024), TMHMM Server 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>, accessed May 12, 2024), ExPASy PROSITE (<https://prosite.expasy.org/>, accessed May 10, 2024), CELLO (<http://cello.life.nctu.edu.tw/>, accessed May 12, 2024), SOPMA (<https://npsa-prabi.ibcp.fr/>, accessed May 15, 2024), and PlantCARE (<http://bioinformatics.psb.ugent.be/>, accessed May 15, 2024).

Subcellular localization of MsCBF4 protein

Seeds of the tobacco “*Nicotiana benthamiana*” were provided by the Crop Stress Molecular Biology Lab at Heilongjiang Bayi Agricultural University. The pCAMBIA1302-GFP vector was used as the plant expression vector. The *MsCBF4* coding sequence, lacking a stop codon, was fused in-frame with mGFP5 at the 5' end to enable expression [45]. The recombinant plasmid *MsCBF4*-GFP and the marker *AHL22*-mRFP were separately introduced into *Agrobacterium tumefaciens* strain GV3101 (p19+psoup) via the heat-shock method. These two *Agrobacterium* strains were co-infiltrated into tobacco leaves, and after three days of transient expression, subcellular localization of *MsCBF4*-GFP was examined using laser confocal microscopy (Zeiss LSM 800, Germany). Excitation wavelengths were 488 nm for GFP and 587 nm for mRFP, with emission detected at 400–580 nm (GFP) and 580–620 nm (mRFP).

Verification of yeast cell *MsCBF4* transcription activation

The coding sequences of the full-length *MsCBF4* (0-543 bp), N-terminal (0-51 bp), AP2-terminal (52-225 bp), and C-terminal (226-543 bp) regions were cloned into the pGBKT7 vector. Then, the constructed vector was transformed into Y2H Gold yeast cells. The cells were cultured at 30°C for three days on SD/-Trp, SD/-Trp/-His, and SD/-Trp/-His/-Ade (X- α -gal) media, and transcription activation characteristics were observed.

Obtaining transgenic *Arabidopsis thaliana* plants and freezing stress treatment

Seeds of *Arabidopsis thaliana* “Columbia ecotype” were provided by the Crop Stress Molecular Biology Lab at Heilongjiang Bayi Agricultural University. *Arabidopsis thaliana* seeds were disinfected and placed on Murashige and Skoog (MS) medium (containing 3% sucrose, pH 5.8), followed by a vernalization treatment at 4°C for two days. After seven days of cultivation at room temperature, the seedlings were transplanted into a mixture of peat soil, perlite, and vermiculite (1:1:1) and cultivated in a growth chamber at 22°C under a 16/8 h (light/dark) cycle for six weeks. The *Agrobacterium* strain GV3101, containing the plant expression vector *MsCBF4*-GFP, was employed to infect the inflorescences of *Arabidopsis thaliana* [46]. The first batch of seeds was designated as the T₀ generation. These seeds were collected and disinfected, then sown on MS medium. After disinfection, T₀ seeds were germinated on MS medium, and T₁ seedlings were selected on medium containing 25 µg/mL kanamycin. This selection process was repeated until T₃ homozygous lines were obtained. The T-DNA insertion mutant lines *Atcbf2* of *Arabidopsis thaliana* *AtCBF2* was obtained from AraShare Science (<https://www.arashare.cn/index/>, accessed on April 10, 2025), and homozygous strains were screened using the three-primer method [47].

Wild-type (WT) plants, overexpressing T₃ lines, and homozygous mutant lines were used for stress treatments. For each line, 30 seeds were sown on MS medium, vernalized, and cultured at room temperature for five days. During the seedling stage, plants were cold-acclimated at 4°C for three days, after which the temperature was gradually reduced to –5°C at a rate of 3°C per hour. Leaf tissue was collected after three hours of freezing treatment.

To induce freezing stress in mature *Arabidopsis thaliana*, germinated seedlings were transplanted

into soil (four plants per pot) and grown for three weeks. Plants were then cold-adapted at 4°C for eight hours, followed by a gradual decrease in temperature to −5°C (3°C per hour). Leaf tissue was harvested after three hours of freezing treatment. All treatments were performed in triplicate. Collected samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Measurement of physiological and biochemical indicators

The production of O₂[−] was detected using the nitro blue tetrazolium (NBT) method, and the production of H₂O₂ was detected using the 3,3'-Diaminobenzidine (DAB) method [48]. The activities of antioxidant enzymes catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), as well as glutaraldehyde (MDA) content, were analyzed following existing methodology [49]. The determination of relative ion leakage level (REL) was conducted using the conductivity method [50].

GUS staining

The pCAMBIA1302-GFP vector was used as the plant expression vector, with the native CaMV 35S promoter replaced by the *MsCBF4* promoter and fused to the GUS reporter gene at the 5' end to enable expression [51]. *Arabidopsis thaliana* plants were transformed using *Agrobacterium tumefaciens* strain GV3101, and T₁ transgenic lines were selected on MS medium containing 25 µg/mL glufosinate. Screening was continued until stable T₃ lines were obtained.

For tissue-specific expression analysis, samples were collected from one-week-old seedlings, seven-week-old rosette leaves, inflorescences, and pods, with three biological replicates for each tissue type. GUS staining was performed using the GUS Stain Kit (Coolaber, Beijing, China) for 6 hours in the dark. After chlorophyll removal, tissues were observed under a stereomicroscope (Leica EZ4 W, Germany).

Y1H Assay

The promoter sequence of *MsCBF4* was inserted into the pAbAi vector as a bait, and the recombinant plasmid *MsCBF4*pro-pAbAi was transformed into Y1H Gold yeast cells using the LiAC-mediated competent cell preparation method to prepare competent cells [52]. The “Dongnong NO.1” nuclear system yeast library plasmid was then transformed into the prepared Y1H Gold competent cells so that upstream genes may be screened from the product. The *MsERF6* CDS was inserted into the pGADT7 vector to construct the prey, which was then transformed into the prepared Y1H Gold competent cells. The cells were cultured at 30°C for three days in SD/-Leu medium with or without AbA.

Dual-LUC Assay

The *MsCBF4* promoter was cloned into the pGreen II 0800-LUC vector to generate the reporter construct, while the *MsERF6* CDS was inserted into the pGreen II 62-SK vector to serve as the effector [53]. Both constructs were separately transformed into *Agrobacterium tumefaciens* strain GV3101 (p19+psoup) and co-infiltrated into tobacco leaves, with three biological replicates for each combination. After three days of transient expression, luminescence was visualized using a chemiluminescence imaging system (Tanon 5200, China). LUC and REN activities were quantified using the Dual Luciferase Reporter Assay Kit (Vazyme, Nanjing, China).

qRT-PCR and statistical analysis of data

qRT-PCR detection was performed following established methodology [54], with specific primers listed in Table S3. The CFX 96 Real-Time PCR Detection System (Bio-Rad, USA) was used. Relative abundance values were normalized to the internal reference gene, and the relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta C_t}$ method [55]. Each experiment included three biological

401 replicates, with each biological replicate comprising three technical replicates. All data were analyzed
402 using one-way analysis of variance (ANOVA) with SPSS software (SPSS Inc., Chicago, IL, USA, version
403 19.0), and differences were tested for significance using Duncan's multiple range test and Student's t-test
404 ($P < 0.05$). Data visualization and analysis were performed using Origin 2018 64-bit software.

Supplementary Information

The online version contains supplementary material available at

Additional file 1: Supplementary Figure S1. Identification of *MsCBF4* transgenic *Arabidopsis thaliana*.

A Hygromycin screening of seven-day-old plants of the WT and *MsCBF4* transgenic lines. **B** Agarose gel electrophoresis detection and **C** qRT-PCR detection. $P < 0.05$ were used to determine statistical significance.

Additional file 2: Supplementary Figure S2. Identification of *AtCBF2* *Arabidopsis thaliana* mutants with T-DNA insertions. (A) Schematic diagram of three-primer identification method. HZ is heterozygote, HM is homozygous lines. (B), (C), (D) Agarose gel electrophoresis detection and (E) qRT-PCR detection. 1: *Atcbf2-1* LP+BP, 2: *Atcbf2-1* BP+RP, 3: *Atcbf2-4* LP+BP, 4: *Atcbf2-4* BP+RP, 5: *Atcbf2-27* LP+BP, 6: *Atcbf2-27* BP+RP, 7: WT LP+BP, 8: WT BP+RP. $P < 0.05$ were used to determine statistical significance.

Additional file 3: Supplementary Figure S3. *MsCBF4*pro-pAbAi self-activation inhibition of Aureobasidin A (AbA) concentration screening.

Additional file 4: Supplementary Table S1. RNA-Seq results of the Ms.gene006341.t1 gene in alfalfa “Dongnong NO.1”.

Additional file 5: Supplementary Table S2. Sequence analysis of *MsCBF4*.

Additional file 6: Supplementary Table S3. Primers used in this study.

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Authors' contributions

Conceptualization, X.P.L. and N.P.R.; methodology, X.P.L. and N.P.R.; software, J.L.L.; validation,

Y.F.X.; data curation, N.P.R.; writing-original draft preparation, N.P.R.; writing-review and editing, X.P.L. and H.Z.; visualization, N.P.R.; supervision, H.Z., X.P.L. and G.L.L.; project administration, X.P.L. and G.L.L.; funding acquisition, X.P.L. and G.L.L. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The sequence data of *MsCBF4* can be obtained from the NCBI database with accession number: PRJNA1217929 (<https://www.ncbi.nlm.nih.gov/sra/>). All analyzed or generated data is included in this article. The data analyzed or generated in this study can be obtained from the corresponding author with upon reasonable request.

Declarations

Ethics approval and consent to participate

The experimental research and all plant materials (either cultivated or wild) were complied with relevant institutional, national, and international guide lines and legislation.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors claim that they have no competing interests.

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