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Original article

Characterization of *CBF1*, *CBF2*, *CBF3*, and *CBF4* genes of *Malus sieversii* and analysis of their expression in different habitats

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Summary

***Malus sieversii* is a cold-resistant rootstock. Understanding the mechanisms of its cold resistance is important for screening cold-resistant germplasm sources. *CBF* genes are closely related to cold resistance in plants. In this study, four *CBF* genes from *M. sieversii*, namely *MsCBF1*, *MsCBF2*, *MsCBF3*, and *MsCBF4*, were cloned and their sequences analyzed. The expression levels of these four *MsCBF* genes after treatment at a low temperature were compared at different time points and habitats. Our results showed that the four MsCBF proteins have a characteristic CBF protein domain and are water-soluble unstable proteins that are induced by low temperatures. The expression levels of four *MsCBF* genes were different, and their times of expression levels increased were different too. *MsCBF1* was first expressed, followed by *MsCBF2*, *MsCBF3*, and *MsCBF4*. *MsCBF1* and *MsCBF2* expression levels were lower and *MsCBF3* expression was higher after a period of 4°C treatment. Changes in *MsCBF* expression were consistent. Comprehensive comparison of the four *MsCBF* gene expression levels in *M. sieversii* in different habitats indicated that *M. sieversii* was strongest in Toli county, followed by Emin county, Gongliu county, Huocheng county, and Xinyuan county.**

Keywords
cold resistant, cold stresses, gene cloning, gene expression, multiple sequence alignment, phylogenetic tree, quantitative RT-PCR

Significance of this study

What is already known on this subject?

- It has already been known that *Malus sieversii* is resistant to cold in different habitats.

What are the new findings?

- The *CBF1*, *CBF2*, *CBF3*, and *CBF4* genes in *M. sieversii* were cloned. The four *MsCBF* gene expression levels in *M. sieversii* in different habitats were analyzed. They were induced by low temperatures. The expression levels of *MsCBF* genes and the time points of expression increase were different. *MsCBF1* was expressed first, followed by *MsCBF2*, *MsCBF3*, and *MsCBF4*. *MsCBF1* and *MsCBF2* expressions were lower and *MsCBF3* expression was higher. Changes in *MsCBF* expression levels were consistent. Comprehensive comparison of four *MsCBF* gene expression levels in *M. sieversii* in different habitats indicated that *M. sieversii* was more cold stress tolerant in Toli county, followed by Emin county, Gongliu county, Huocheng county, and Xinyuan county.

What is the expected impact on horticulture?

- On the basis of the study results, we expected to provide reference for the molecular breeding and selection of cold-resistant germplasm resources.

Introduction

Low temperatures affect the growth and development of plants (Thomashow, 1999), as well as cause damage of plant tissues (Steponkus, 2003; Uemura et al., 1995) and even plant death (Guy, 1990). In low temperature environments, plant cell membranes are subjected to low-temperature stress, activating stress signals and a series of physiological biochemical reactions and metabolic changes (Haddon et al., 1991). Plants have a low-temperature signaling system that enables them to quickly adapt to low temperatures via a number of mechanisms (Jaglo et al., 2001). One such mechanism is the abscisic acid (ABA) pathway, which includes the ABA response element and ABA response element-binding protein, which contains a bZIP motif, and the other is a non-ABA-dependent mechanism via the C-repeat binding transcription factor/dehydration-responsive element binding (CBF/DREB) pathway. *CBF* genes are induced by low temperatures (Gilmour et al., 1998). During low temperature stress signal transduction in plants, CBF transcription factors receive upstream signals and transmit to downstream signaling components (Guo et al., 2004; Sangwan et al., 2001; Tang et al., 2005). The *CBF* gene family contains *CBF1*, *CBF2*, *CBF3*, and *CBF4*. Stockinger identified *CBF1* as a transcription factor while studying cold-inducible genes in *Arabidopsis thaliana*. It is confirmed that *CBF1* bound to DNA regulatory motifs of C-repeat/dehydration-responsive element (DRE) (Stockinger et al., 1997). A few years later, *CBF2* (Xue, 2003), *CBF3* (Gilmour et al., 2000), and *CBF4* (Haake et al., 2002) were discovered in *A. thaliana.* When plants are exposed to low temperatures, the inducers of the *CBF* expression (ICE) transcription factors are activated (Badawi, 2008). ICEs induce the expression of *CBF* genes, and the CBF protein binds to C-repeat/DRE in the promoter region of downstream cold-regulated genes. This induces the expression of genes that improves the cold resistance of plants (Jaglo et al., 2001).

Apples are one of the most important temperate fruits. They have a high economic value and an extremely abundant germplasm resource. In addition, they are important fruit, ornamental, and rootstock resources. The planting area and yield of Chinese apple have been ranked in forefront world-

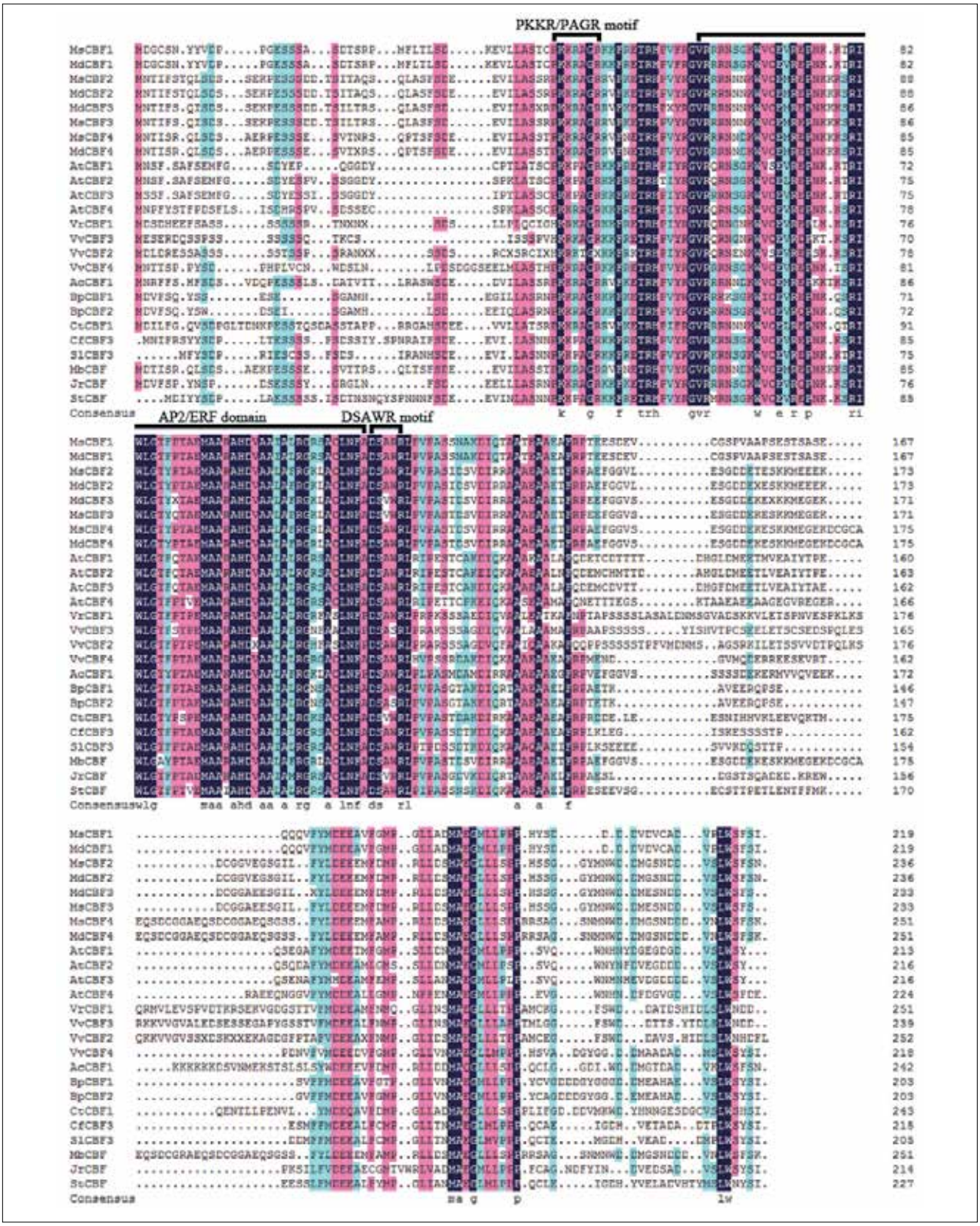


Figure 1. Multiple sequence alignment of amino acid sequences of *CBF* genes from *Malus sieversii* and other plants. Notes: *Malus domestica*: *MdCBF1* (GenBank accession no. AAZ20446), *MdCBF2* (GenBank acc. no. AGM16327), *MdCBF3* (GenBank acc. no. AGL07697), *MdCBF4* (GenBank acc. no. AGL07696); *Arabidopsis thaliana*: *AtCBF1* (GenBank acc. no. ABV27064), *AtCBF2* (GenBank acc. no. ABV27106), *AtCBF3* (GenBank acc. no. ABV27154), *AtCBF4*: (GenBank acc. no. ABV27157); *Vitis riparis*: *VrCBF1* (GenBank acc. no. AIL00558); *Vitis vinifera*: *VvCBF2* (GenBank acc. no. AIL00643), *VvCBF3* (GenBank acc. no. AIL00727), *VvCBF4* (GenBank acc. no. AIL00828); *Amygdalus communis*: *AcCBF1* (GenBank acc. no. AIW60866); *Betulaplaty phylla*: *BpCBF1* (GenBank acc. no. ADZ23479), *BpCBF2* (GenBank acc. no. ADZ23480); *Citrus trifoliata*: *CtCBF1* (GenBank acc. no. ABH08746); *Capsicum frutescens*: *CfCBF3* (GenBank acc. no. ADM73296); *Solanum lycopersicum*: *SlCBF3* (GenBank acc. no. AAS77819); *Malus baccata*: *MbCBF* (GenBank acc. no. ABQ59086); *Juglans regia*: *JrCBF* (GenBank acc. no. AFV93473); *Solanum tuberosum*: *StCBF* (GenBank acc. no. ACJ26757).

wide (Wei et al., 2014). Within the genus, the closest relatives to apples are *Malus sieversii* (Chen et al., 2007; Yan, 1997; Forte et al., 2002). Vavilov, Ponomarenko, and Janick believed that apples originated in Central Asia and suggested that *M. sieversii* is the ancestor of cultivated apples (Richards et al., 2009; Zhang et al., 2009). *M. sieversii* is a rare wild tertiary relict species (Zhang et al., 2009) that is mainly distributed in the Tianshan Mountains of Central Asia, including Xinjiang China, the State Alma Ata and Tarde Kurgan of Kazakhstan, and the State Issyk Kyrgyzstan (Chen et al., 2007). In Xinjiang, China, *M. sieversii* is mainly distributed in the Yili River Valley and the western part of the Junggar basin. These two regions are far apart and their geographical environments are distinct. In China, the most commonly used apple rootstocks are *Malus baccata*, *M. sieversii,* and *M. micromalus*. *M. baccata* represents poor salt resistance, while *M. micromalus* has a good affinity with apples, but has poor stress resistance compared with *M. sieversii*. *M. sieversii* has a good affinity and an excellent stress resistance (Wang et al., 2016), including resistance to cold, drought, and disease (Wang et al., 1989). It can tolerate extreme temperatures of -35 to -30°C and is not sensitive to higher temperatures. These observations indicate that *M. sieversii* stocks are the most useful resistant gene donors that are introduced in germplasm resources (Li et al., 2011; Jia, 2008). *M. sieversii* is an important resource in western China (Xu et al., 2014). It provides germplasm resources for breeding and cultivating apples and is a high-quality germplasm resource for the genetic breeding of fruit trees. The long-term growth of *M. sieversii* in different environments and its resistance to cold is usually affected by environmental changes. Studying the differences in cold resistance of *M. sieversii* in different habitats will help identify cold-resistant germplasm resources, thereby obtaining new apple varieties with strong cold resistance by hybrid or transgene methods. This represents an important approach for apple breeding in the future. Therefore, studying the adaptability of *M. sieversii* to low-temperature stress in different habitats is necessary. Such studies will provide a theoretical basis for cold-resistant breeding and selection of excellent cold-resistant germplasm resources (Zhang et al., 2014).

Cold resistance of the *CBF* genes has been well studied in *Vitis vinifera* (Karimi et al., 2015), *A. thaliana* (Miura et al., 2007), *Zea mays* (Liu et al., 2015), blueberry (Polashock et al., 2010), bamboo (Zhang, 2012), *Eucalyptus robusta* (Navarro et al., 2009), *Oryza sativa* (Ito et al., 2006), peach (Liang et al., 2013), *Amygdalus communis* (Zhang et al., 2015), and *Juglans regia* (Xu et al., 2014). However, the mechanism and transcript levels of *CBF* genes in *M. sieversii* has not been reported. In this study, we used our data regarding *CBF* expression, to speculate habitats that contained strong or weak strains of *M. sieversii*. Our findings provide a reference for the molecular breeding and selection of cold-resistant germplasm resources of *M. sieversii*.

Materials and methods

Materials and treatment

Fruits were collected from *M. sieversii* in five regions of Xinjiang, China: Huocheng county (HC), Gongliu county (GL), Xinyuan county (XY), Emin county (EM), and Toli county (TL). The collected fruits were transferred to the laboratory, and the seeds were planted in nutrition bags after 4°C stratification treatment (80 days). After 40 days, the plants were transferred to a phytotron under 4°C. At defined times (0, 1, 2, 4, 8, 12, and 24 h), leaves were sampled. All samples were frozen in liquid nitrogen and stored at -80°C for RNA extracted. (Tian et al., 2015).

Experimental methods

1. Total RNA extraction and cDNA synthesis. The leaves of *Malus sieversii* were rapidly ground to powder in liquid nitrogen, and RNA was extracted using a Biospin plant total RNA extraction kit. RNA was reverse transcribed using the TIANScript RT kit, and cDNA was stored at -20°C for use in experiments.

2. Cloning and sequencing of *CBF* genes from *Malus sieversii*. Primers were designed for gene cloning using the Primer Premier 5.0 software according to the *MdCBF1*, *MdCBF2*, *MdCBF3*, and *MdCBF4* sequences of *Malus domestica*, which are available in the NCBI database (Table 1). For PCR amplification, the TransStart Taq DNA polymerase was used. Products were introduced into the pMD-19T vectors after detection using 1% agarose gel electrophoresis and recovery of the amplified fragment. The PCR cycle included an initial denaturation step at 94°C for 5 min and 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a 10 min extension at 72°C. Three independent clones for each gene were selected and sequenced (Zheng et al., 2015). Sequencing results were analyzed using the BLAST program to deduce the protein amino acid sequence. Multiple alignments of the deduced amino acid sequences were performed using the DNAMAN software. Phylogenetic trees were analyzed using the MEGA software. Physical and chemical properties of the amino acid sequences were calculated using the online Expasy ProtParam (http://web.expasy.org/protparam/) (Awasthi et al., 2016).

3. Quantitative RT-PCR and expression analysis of *CBF* genes from *Malus sieversii*. PCR primers complementary to the four *MsCBF* genes were designed using the Premier Primer 5 software (Table 2). Actin was used as the reference gene. The qRT-PCR system was tested by BIO-RAD CFX Connect, and the reactions were performed using the Power SYBR

Table 1. PCR primer sequences.

Primer name	Primer sequence	Primer size (bp)	Annealing temperature (°C)
MsCBF1 forward	5'-GAGCTCTTAAATGGAAAAACTCCATAAG-3'	28	57
MsCBF1 reverse	5'-GGATCCATGGATGGTTGCTCTAATTAC-3'	27	57
MsCBF2 forward	5'-CTAATTTGAGAAGCTCCACAGAC-3'	23	57
MsCBF2 reverse	5'-ATGAATACGATCTTCAGTACTCAACTC-3'	27	57
MsCBF3 forward	5'-TTATGAGAAGCTCCACAGACTGA-3'	23	57
MsCBF3 reverse	5'-GATCTTCAGTCAAATCTCCGATTC-3'	24	57
MsCBF4 forward	5'-TTACTTTGAGAAGCTCCACAGATT-3'	24	57
MsCBF4 reverse	5'-ATGAACACAATCTCCAGACAACTC-3'	24	57

Table 2. Primer sequences for qRT-PCR.

Primer	Primer sequence	Primer size (bp)	Annealing temperature (°C)
MsCBF1 forward	5'-CATCGGAGCAGCAACAAGTG-3'	20	55
MsCBF1 reverse	5'-TGTAATGGGGTGGAGGCAAT-3'	20	55
MsCBF2 forward	5'-ATGAATACGATCTTCAGTACT-3'	21	55
MsCBF2 reverse	5'-CGGCTGGACGCCAAAATGACCT-3'	22	55
MsCBF3 forward	5'-ATGAATACGATCTTCAGTCAAA-3'	22	55
MsCBF3 reverse	5'-TGTTGGACGCCAAAATGACC-3'	20	55
MsCBF4 forward	5'-GATTGTGGATGTGCGGAGCA-3'	20	55
MsCBF4 reverse	5'-CCGCCATACTATCAAGCAACC-3'	21	55
Actin forward	5'-CGACTCTGGAGATGGTGTCA-3'	20	55
Actin reverse	5'-AGCTGTGGTGGTGAAGGAAT-3'	20	55

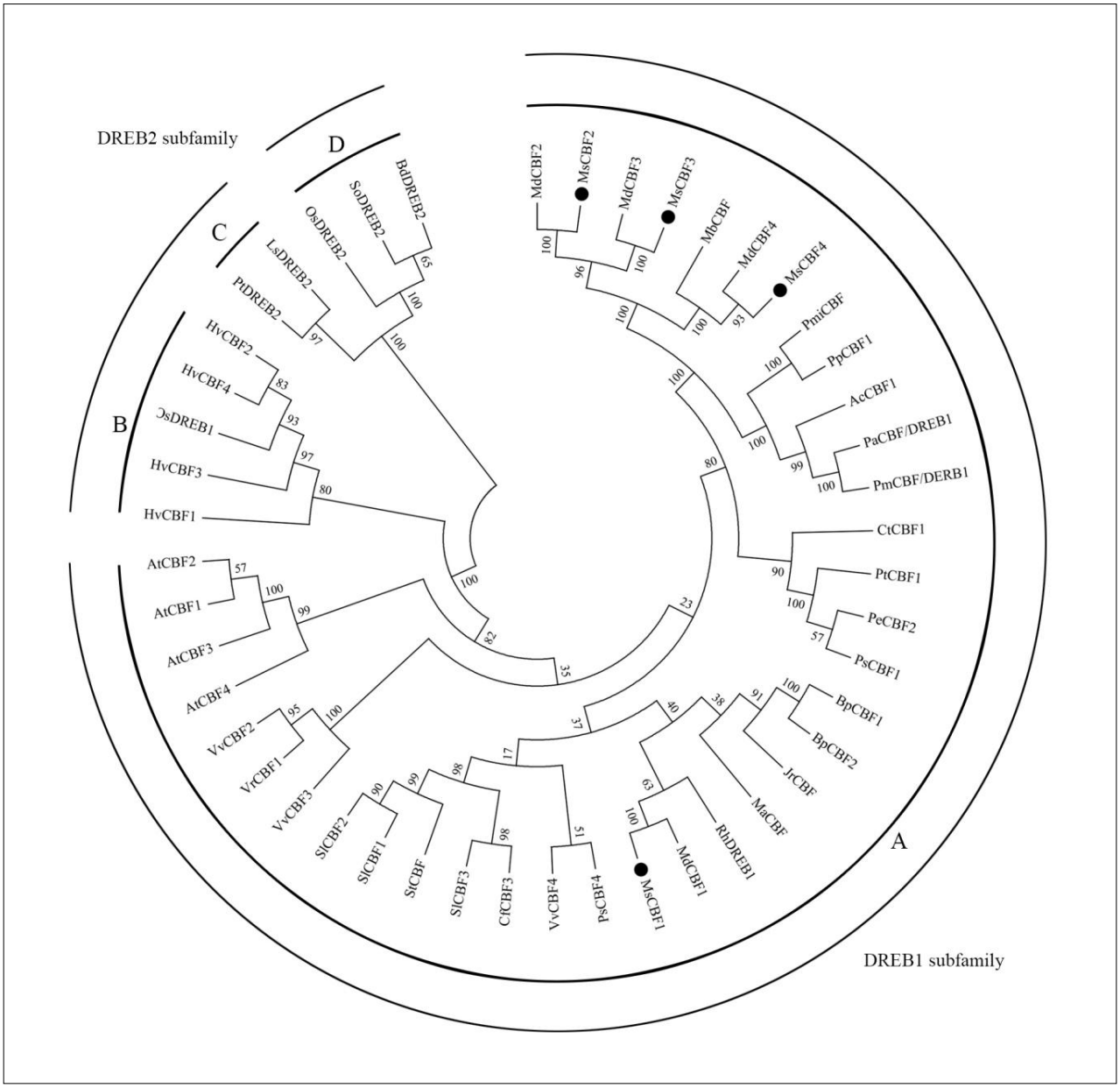


Figure 2. Phylogenetic tree of the amino acid sequence of *CBF* genes from *Malus sieversii* and other plants. Notes: A and C are dicotyledons; B and D are monocotyledons.

Green PCR Master Mix kit (Applied Biosystems). Each reaction was repeated three times. PCR amplification was performed with an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 20 s, and 75°C for 5 s. The relative expression of *CBF* genes was quantified using the $2^{-\Delta\Delta Ct}$ method (Han et al., 2016). Analysis of variance of the *CBF* gene expression was performed using the SPSS 19.0 and Excel 2010 software.

Results and analysis

Characterization and sequence analysis of *MsCBF* genes

The ORF sequences for *MsCBF1*, *MsCBF2*, *MsCBF3*, and *MsCBF4* were cloned from the leaves of *M. sieversii* using RT-PCR. cDNA sequences have been deposited in the NCBI nucleotide sequence database under accession numbers KX532174 (*MsCBF1*), KX532175 (*MsCBF2*), KX532176 (*MsCBF3*), and KX532177 (*MsCBF4*). The ORF sequences are 660 bp, 711 bp, 702 bp, and 756 bp, respectively. Theoretical translation of the four *MsCBF* gene nucleotide sequences encoded proteins of 219 amino acids, 236 amino acids, 233 amino acids, and 251 amino acids, respectively. The molecular weights were 23.94 kDa, 26.29 kDa, 26.17 kDa, and 27.74 kDa, respectively. The isoelectric points were 5.21, 4.96, 5.05, and 5.00, respectively. They were all hydrophilic proteins.

As shown in Figure 1, the four MsCBF proteins have a conserved AP2/ERF domain, and amino acid sequences PKKR/PAGR and DSAWR are both typical in the *CBF* gene family. The multiple sequence alignment showed that the four MsCBF proteins shared 99% sequence identity with MdCBF proteins. The MsCBF1 protein shared 90%, 89%, and 85% sequence identity with CBF proteins from *BpCBF1*, *JrCBF,* and *CtCBF1*, respectively. The MsCBF2 protein shared 90%, 87%, and 83% sequence identity with CBF proteins from *AcCBF1*, *BpCBF2*, and *AtCBF2*, respectively. The MsCBF3 protein shared 86%, 86%, and 85% sequence identity with CBF proteins from *CfCBF3*, *SlCBF3*, and *AtCBF3*, respectively. The MsCBF4 protein shared 99%, 85%, and 84% sequence identity with CBF proteins from *MbCBF*, *VvCBF4*, and *StCBF*, respectively.

As shown in Figure 2, a phylogenetic tree was constructed using the CBF/DREB protein sequences from different plants. Results showed that all sequences were divided into two branches: DREB1 (A and B) and DREB2 (C and D). The same branch was divided into two branches: monocotyledons (A and C) and dicotyledonous (B and D). In this study, the four *MsCBF* genes were closely related to the DREB1 family of dicotyledonous plants, indicating that they belong to the DREB1 family.

CBF gene expression in *Malus sieversii* in different habitats

MsCBF gene expression was affected under temperature stress. Gene expression increased and then decreased, but the expression levels and their times of increase differed among genes (Figures 3–6). ANOVA revealed significant variation in *MsCBF* gene expressions over time after cold treatment of all samples (Table 3).

As shown in Figure 3, *MsCBF1* expression significantly increased after 2 h of plant exposure to low temperatures, and the expression was highest after 4 h. At that time, *MsCBF1* expression was significantly higher in TL and EM than in the other counties. The *MsCBF1* expression was significantly higher in HC than in XY. In case of *MdCBF2* (Figure 4), level of the transcript level significantly increased after 2 h of plant exposure to low temperature. The *MsCBF2* expression in samples collected from GL after 4 h of cold-temperature treatment was significantly higher than that from XY, HC, and TL. Regarding the other four counties, *MsCBF2* expression was highest after exposure to low temperatures for 8 h and it decreased in samples collected from GL. At that time, the *MsCBF2* expression was significantly higher in TL than in XY,

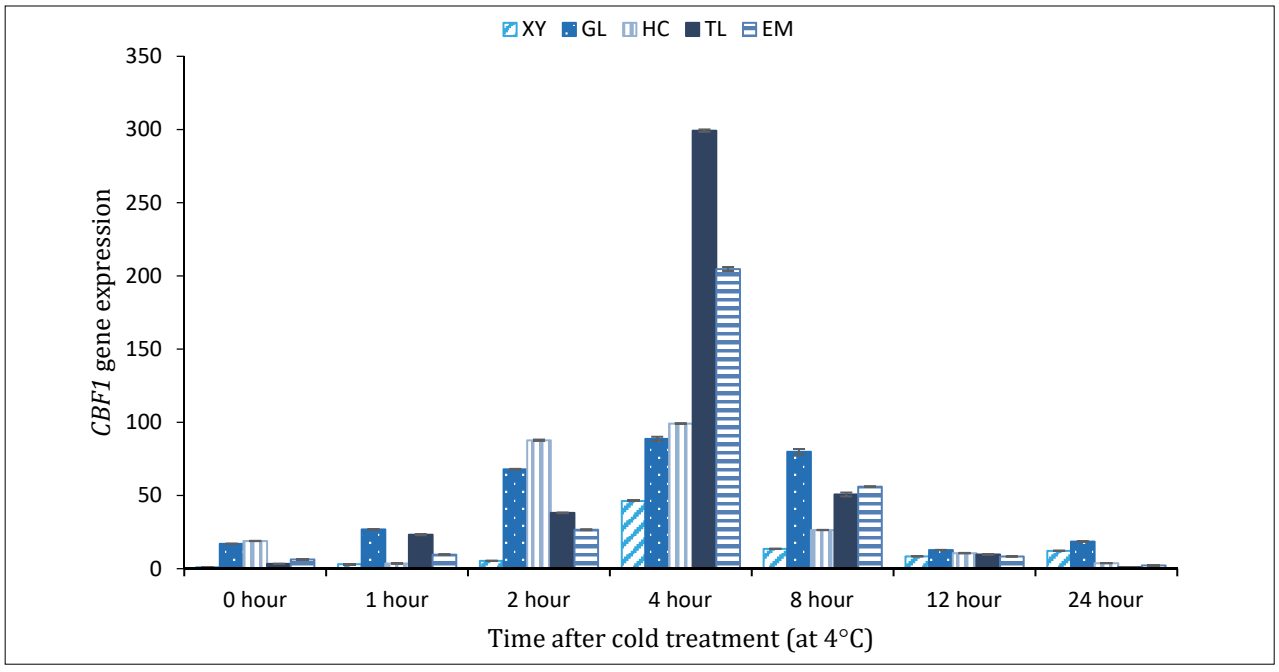


Figure 3. *CBF1* expression in different *Malus sieversii* habitats at different times after cold treatment. Notes: XY, Xinyuan county; GL, Gongliu county; HC, Huocheng county; TL, Toli county; EM, Emin county; the same is applicable for Figures 4–6.

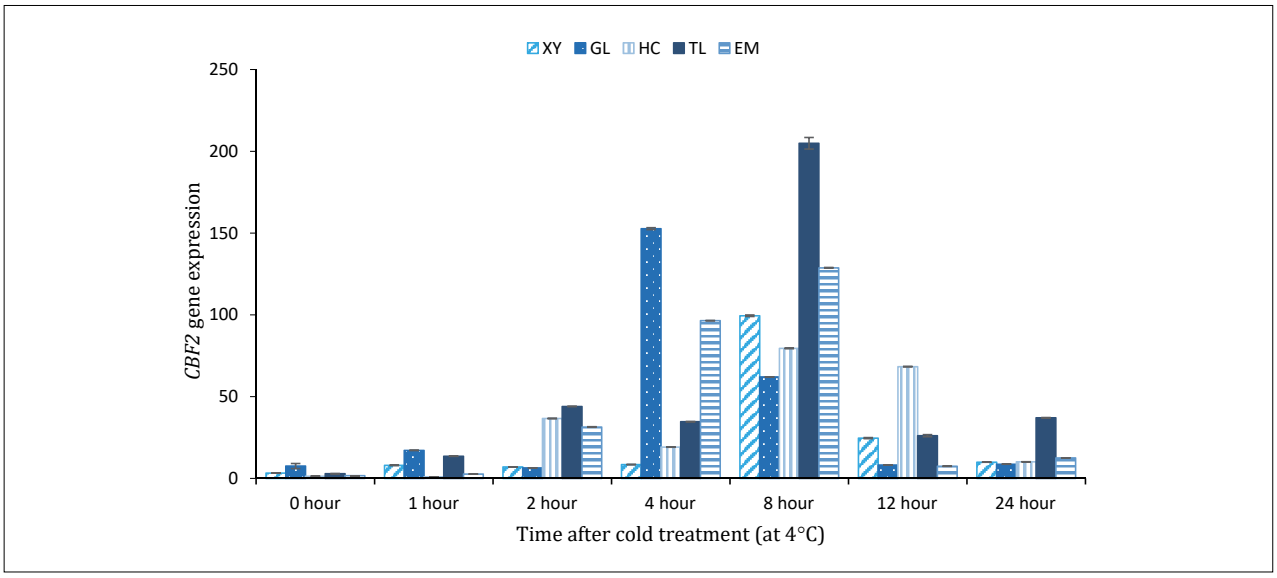


FIGURE 4. *CBF2* expression in different *Malus sieversii* habitats after cold treatment.

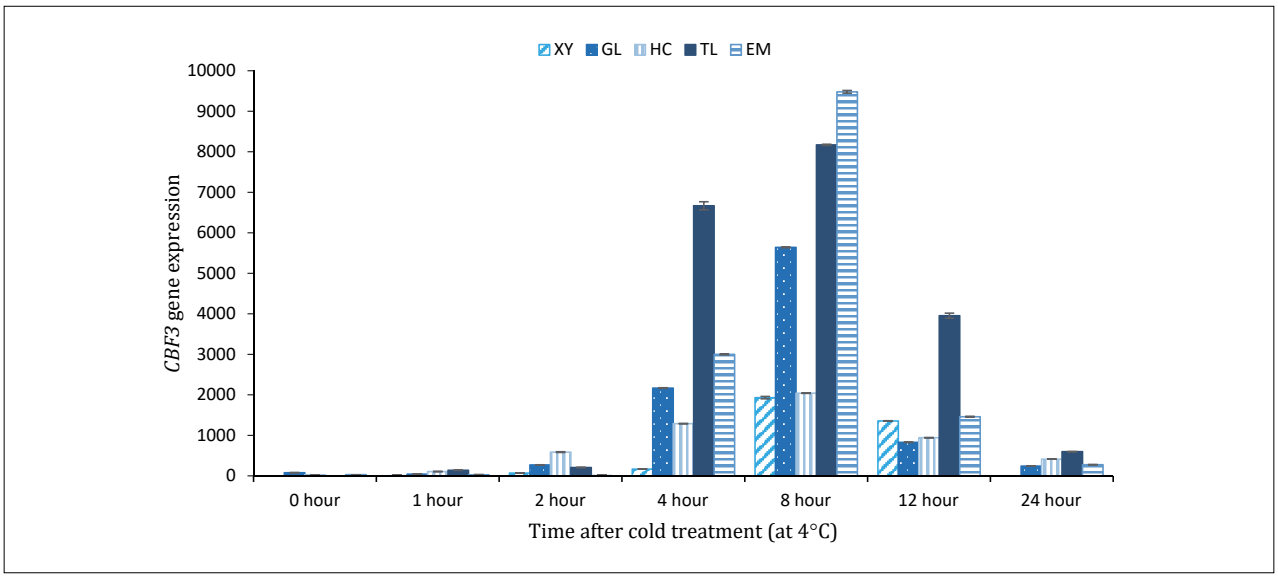


FIGURE 5. *CBF3* expression in different *Malus sieversii* habitats after cold treatment.

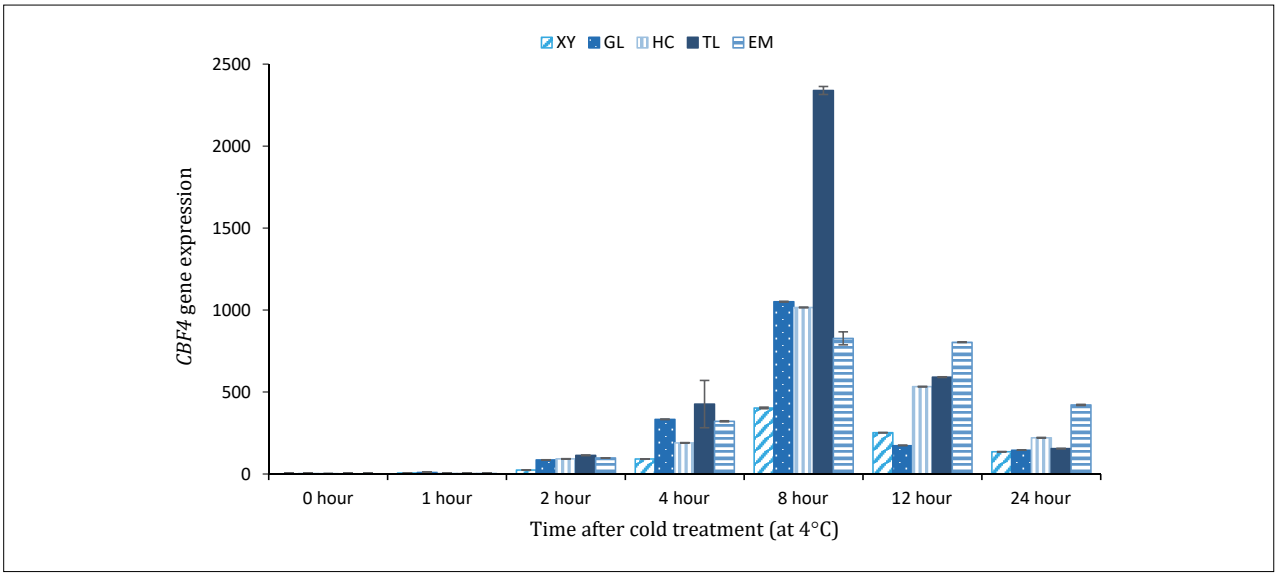


FIGURE 6. *CBF4* expression in different *Malus sieversii* habitats after cold treatment.

Table 3. Effect of habitat and cold treatment on *CBF* expression in *Malus sieversii*.

Source	Degree of freedom	*CBF1*	*CBF2*	*CBF3*	*CBF4*
Genotype	4	6482.81*	2451.27*	18808141.72*	450651.80*
Sampling time	6	38106.81*	23357.86*	60694088.17*	2375003.62*
Genotype × Sampling time	24	5215.23*	3578.56*	6763617.29*	239477.02*
Error	70	0.30	0.47	477.99	660.19
CV (%)	–	153.61	131.17	162.80	148.34

* Significant at 1% level of probability.

GL, and HC. As shown in Figure 5, the *MsCBF3* expression was higher than *MsCBF1*, *MsCBF2*, and *MsCBF4* expression. The *MsCBF3* expression significantly increased after exposure to low temperature for 4 h, and the expression was highest after 8 h. At that time, the *MsCBF3* expression was significantly higher in TL, EM, and GL than in XY and HC. As shown in Figure 6, the *MsCBF4* expression significantly increased after 4 h of plant exposure to low temperature and expression was highest after 8 h. At that time, the *MsCBF4* expression was significantly higher in TL and significantly lower in XY than in the other four counties.

Discussion

In this study, we characterized four *CBF* genes from *Malus sieversii* and deduced their amino acid sequences. CBF proteins from *M. sieversii* had the same highly conserved regions as those from plants, indicating that *MsCBF* proteins play an important role in the transcriptional regulation of cold resistant genes. Homology analysis revealed that *CBF* genes have high sequence similarity among *M. sieversii* and some other plants, hence, they are homologous and may have similar functions.

CBF expression is very low in plants under normal temperatures and rapidly increases after exposure to low temperatures (Lee et al., 2012). *CBF* expression significantly increases in grapes (Karimi et al., 2015), *Arabidopsis thaliana* (Novillo et al., 2012), and *Poncirus trifoliata* (He et al., 2012) after low temperature stress. In this study, we showed that *CBF* expression was induced in *M. sieversii* by low temperature and that the expression of the different genes increased at different time points.

MsCBF1 is expressed much earlier than the other three *MsCBF* genes, which is similar to previous findings in grapes (Xiao et al., 2006). *CBF1* is the first cold-responsive gene to be expressed in plants, triggering cold response pathways and helping the plant resist low-temperature stress (Karimi et al., 2015). The *MsCBF2* gene was abundantly expressed at low temperature for 4–8 h, followed by *MsCBF1* gene overexpression. The first group of cold-responsive transcription factors to be expressed is not sufficient for cold stress acclimation. Therefore, a second group of transcription factors is expressed (Karimi et al., 2015). Some studies have shown that *CBF2* indirectly activates other cold-responsive genes in *A. thaliana* (Novillo et al., 2007). Transgenic expression of grape *CBF2* in *A. thaliana* significantly improved the cold resistance of *A. thaliana* (Kobayashi et al., 2012). The *MsCBF3* expression increased after exposure to cold for 4 h but not 2 h. This may be explained by the regulation of *CBF3* expression by ICE1 transcription factors, which bind the promoter sequence of *CBF3*. The *MsCBF3* gene was abundantly expressed after the plant suffered damage to a certain extent, thus improving cold resistance of plants (Buskirk et al., 2006). The increase in the *MsCBF4* gene expression in response to cold temperatures was later than that in the other *CBF* gene expressions. This was similar to previous findings of *CBF4* expression in grapes (Karimi et al., 2015) and *Brassica napus* (Gao et al., 2002).

Previous studies have shown that *CBF* genes can transmit low temperature signals and activate the expression of other cold-resistant genes (Zhong et al., 2006), therefore, the high *CBF* gene expression level implies that the activation of the cold resistance genes may be more, which makes the plants more freeze-tolerant. The *CBF* expression in *Malus sieversii* was influenced by the habitat. The *MsCBF* expression was high in TL and low in XY. *MsCBF3* expressions were higher than the other *CBF* gene expressions, indicating that it may influence the cold resistance of *M. sieversii*. *MsCBF3* expression was highest in TL, followed by EM, GL, HC, and XY. In summary, the quantification of four *MsCBF* expressions in *M. sieversii* from different habitats revealed that cold resistance was strongest in TL, followed by EM, GL, HC, and XY. Our finding that cold resistance was stronger in GL than in XY is consistent with that results of previously reported (Zhang et al., 2001).

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