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Isolation and expression characterization of CBF2 in *vitis amurensis* with stress

Chang Dong[1,2], Jianmin Tao[1], Meng Zhang[1], Yang Qin[2], Zhiying Yu[1], Bailin Wang[2], Binhua Cai[1], Zhen Zhang[1*]

[1]College of Horticulture, Nanjing Agricultural University, Nanjing, China; [*]Corresponding Author: zzhang@njau.edu.cn, dongchanggy@126.com, tjm266@sina.com
[2]Department of Horticulture, Heilongjiang Academy of Agricultural Science, Harbin, China

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ABSTRACT

The transcription factor VaCBF2, which interacts with C-repeat/DRE and its promoter, was isolated from *Vitis amurensis.* The VaCBF2 amino acid sequence contained a conserved AP2 domain of 56 amino acids and a potential nuclear localization sequence. The sequence of VaCBF2 showed a high level of homology with other CBF2 family members. Phylogenetic analysis showed that the amino acid sequences may be CBF2 proteins with evolutionary relationship. Quantitative reverse-transcription polymerase chain reaction analysis indicated that the expression of VaCBF2 gene in tissues (roots, stems, leaves, and petioles) was induced by low temperature, high salinity, and application of abscisic acid and salicylic acid in a time-dependent manner but to different extents in the cold-hardy *V. amurensis* and the less cold-hardy *Vitis vinifera*. The presence of *cis*-elements such as MYC and ABRE in VaCBF2 promoter further confirmed that this promoter was a component of the CBF transduction pathway involved in plant response to multiple stresses.

Keywords: *Vitis amurensis*; Stress; CBF2; Expression

1. INTRODUCTION

Abiotic stresses such as low temperature, drought, and salinity can adversely affect crop growth and development. Thus, improving stress tolerance in breeding is important. Plants respond and acclimate to environmental stresses by activating network events from stress perception to effector gene expression [1]. Over the past years, the C-repeat element-binding factor (CBF)/dehydration responsive element binding (DREB) cold response pathway is the most recognized freezing tolerance pathway in plants [2]. All CBF proteins specifically bind to the C-repeat element (CRT)/dehydration responsive element (DRE) in the promoter region of downstream genes [3,4]. These proteins regulate their downstream gene expression and enhance the tolerance of plants to low temperature, drought, and high salinity. Thus, CBF regulation has a fundamental function in cold acclimation [5].

Arabidopsis CBF1, CBF2, and CBF3 are major transcriptional factors affecting cold-inducible gene expression [6,7]. CBF4 from *Arabidopsis* is weakly induced by cold stress [8], whereas DREB2A is induced by drought and salinity [9]. These transcript profiles suggest that CBFs have different expression levels in plants and that a degree of cross-talk exists between these pathways [10]. Furthermore, CBFs widely exist in herbaceous plants that are responsive to cold [11]. Puhakainen *et al.* [12] demonstrated that the CBF pathway also exists in woody trees. Since then, CBF orthologs have been reported in various woody plants, including *Vitaceae* [13], poplar [14], blueberry [15], peach [5], and apple [4]. Among woody species, a positive correlation also exists between the cold tolerance and CBF transcript level of woody species [4,5,14,16]. Therefore, the CBF cold pathway appears to be conserved in woody plants.

Vitis amurensis is one of the most widely used species for freeze-tolerant rootstock and winemaking in grape cultivation. In this study, the VaCBF2 gene was isolated from *V. amurensis*. We also compared CBF2 gene expression in the leaves of cold-tolerant wild *V. amurensis* and cold-sensitive *Vitis vinifera* "Manicure Finger" at different times of cold treatment. Results confirmed the *V. amurensis* transcript patterns in roots, stems, leaves, and petioles at different times of low temperature, high salinity,

and abscisic acid (ABA) and salicylic acid (SA) treatments.

2. MATERIAL AND TREATMENTS

Sixty-day-old *in vitro* samples of wild *V. amurensis* and *V. vinifera* "Manicure Finger" grown in Murashige and Skoog (MS) medium under a 16 h light/8 h dark regime at 25˚C were subjected to the following treatments for stress-responsive gene expression: cold stress by transferring to 4˚C, salinity stress by supplying 200 mM NaCl, and hormonal treatments by directly supplying ABA (10 μM) or SA (5 μM) to plants at different times.

2.1. Isolation of the VaCBF2 Gene

Sixty-day-old *in vitro* samples of wild *V. amurensis* were grown in MS medium under a 16 h/8 h dark regime at 25˚C. Total RNA was extracted from non-stressed or cold-stressed plants *in vitro* using the Lagonigro's method [17]. First-strand cDNA was synthesized using Primer Mix according to the instructions of ReverTra Ace RT Kit (TOYOBO, Japan). The complete coding sequence was amplified using the gene-specific primers C2F (5'-TCATCAACATCCTCTCCTTCTCG-3') and C2R (5'-GCTAAAAGTGTGTATGGCAGTGA-3'). The primers were designed based on the genome of *Vitis* (http://www.vitisgenome.it). Amplification was carried out under the following conditions: 94˚C for 4 min; 36 cycles of 94˚C for 30 s, 60˚C for 45 s, and 72˚C for 60 s; and a final extension at 72˚C for 10 min. The polymerase chain reaction (PCR) products were cloned and sequenced by pMD19-T vector. Sequence alignments were conducted using DNAMAN software (Version 5.2.2, Lynnon Biosoft).

2.2. Promoter Isolation of VaCBF2

The isolation of the promoter sequence was carried out using a Universal Genome Walker Kit (Clontech, USA) on 10 ng of *V. amurensis* genomic DNA digested by four blunt-end-generating restriction enzymes (*Eco*RI, *Dra*I, *Sac*I, and *Stu*I). After purification, the restriction fragments were ligated with Genome Walker adaptors. PCR was amplified on each restriction fragment set using the primers C2-R1 (5'-GCTAAAAGTGTGTATGGCAGTGA-3') and C2-R2 (5'-CTTCACTCACCCATTTGTTCTCATT-3') for first- and second-step PCRs, respectively. Finally, the PCR products were cloned into pMD 19-T simple vector and sequenced. The sequences were analyzed online by Plant CARE.

2.3. Expression Analysis of VaCBF2 by Real-Time Quantitative Reverse Transcription (qRT)-PCR

RNA and cDNA were obtained as described above. qRT-PCR was performed in 20 mL volumes containing 10 mL of SYBR Premix Ex Taq mix (TaKaRa), 0.2 mM forward primer (5'-TGAGAACAAATGGGTGAGTG-3'), 0.2 mM reverse primer (5'-TGATGGAGGTTGCTGAAAA-3'), and 1 mL of diluted (1:10 v/v) cDNA. The PCR regime consisted of denaturation at 94˚C for 4 min, followed by 40 cycles of 95˚C for 10 s, 60˚C for 20 s, and 72˚C for 20 s. Melting curve analysis was performed from 80˚C to 95˚C at 0.5˚C intervals. VaCBF2 transcript levels were normalized with grape malate dehydrogenase gene as an internal control using the forward primer 5'-GCATCTGTGGTTCTTGCAGG-3' and reverse primer 5'-CCCTTTGAGTCCACAAGCCAA-3'. The data of 0.5 h of *V. vinifera* in leaves at 4˚C were used as basis for relative comparison. Relative expression was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Two independent replicates were performed per experiment.

3. RESULTS

3.1. Identification of VaCBF2 Gene from *V. amurensis*

The sequence of the *V. amurensis* cDNA clone included one open reading frame (ORF). Compared with the GenBank database, this sequence resulted in an identical coding sequence to the CBF2 cDNA from *V. vinifera* and *V. riparia*. This result clearly indicated that the gene in *V. amurensis* did not have introns interrupting its ORF. The coding regions encoded polypeptides of 250 amino acids with a molecular mass of 27.55 kD and a theoretical isoelectric point of 10.23. The amino acid alignment of the sequence revealed that 97% and 96% of the residues were identical to the CBF2 of *V. riparia* and *V. vinifera*, respectively. Moreover, the degrees of similarity to the CBF2 of *Parthenocissus inserta* and *Eucalyptus grandis* were high (94% and 73%, respectively). Sequence alignment against various CBF proteins suggested that this cDNA encoded a CBF-type protein in *V. amurensis* (**Figure 1**). Therefore, the isolated cDNA was named VaCBF2. In other plants, basic residues similar to CBF2 were included in their N-terminal regions. These residues potentially represented an unclear localization signal (NLS) and putative APETALA 2/ethylene response factor (AP2/ERF) DNA-binding domain. VaCBF2 also contained acidic C-terminal fragments that can act as the transcriptional activation domain, but the LWSY signature was modified from LWSY to LWNHDFL in the grape protein. In addition, potential recognition motifs for CBF protein were detected, such as DSAWR, Domain III, and Domain IV (**Figure 1**). These results indicated that VaCBF2 from *V. amurensis* was a CBF2 or tholog of other plants but with unique features.

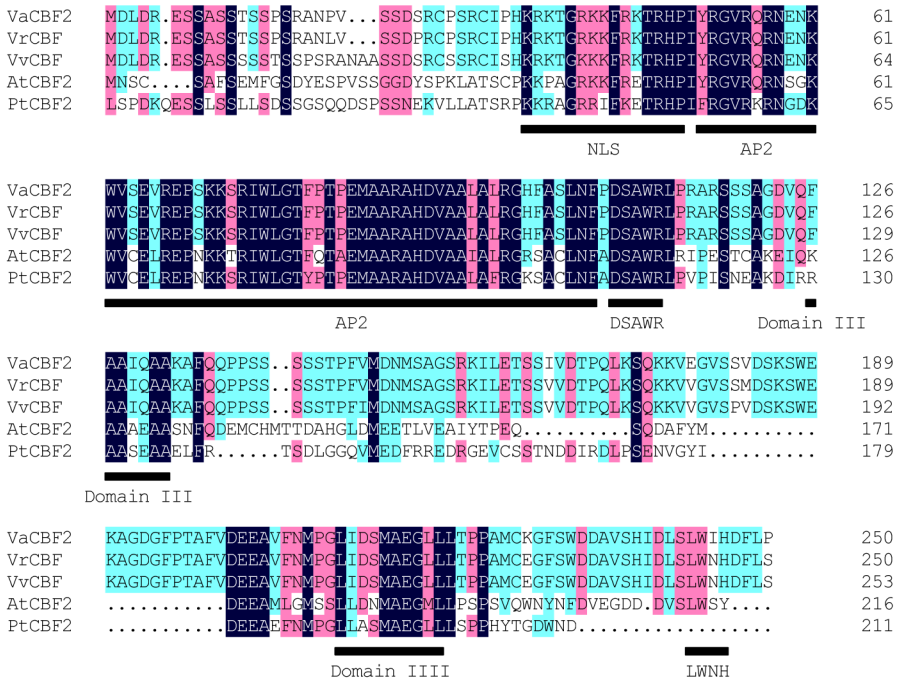

Figure 1. Multiple alignment of VaCBF2 with the CBF2 proteins of Vitaceae, *Parthenocissus*, and *Arabidopsis*. The amino acid sequences are from NCBI [AtCBF2 (ABV27118), PtCBF2 (CBF94893), VrCBF (AAR28674), and VvCBF (AAR28677)]. The nuclear localization signal, AP2, DSAWR, and LWNH domains are indicated by bars.

3.2. Phylogenetic Analysis of VaCBF2

A BLAST search was performed using the deduced amino acid sequence of VaCBF2. Several homologous sequences of VaCBF2 in higher plants were found in the GenBank database. Alignment analysis and domain comparison indicated that VaCBF2 shared high homology with other proteins in the AP2/ERF domain (**Figures 1** and **2**). By systematic phylogenetic analysis, AP2/ERF proteins were classified into 17 plant CBF proteins using the neighbor-joining phylogenetic method (**Figure 2**). The results showed that the sequence from *V. amurensis* belonged to the CBF2 cluster of CBF proteins.

3.3. Sequence Analysis of VaCBF2 Promoter

VaCBF2 promoter was obtained using the UGWK method (**Figure 3**). The result revealed that a typical TATA-box was located at −82 bp upstream of the ATG translational initiation codon. Furthermore, *cis*-acting elements involved in stress-related responses were found in VaCBF2 promoter. These elements including ABRE, MYC (MYC recognition site), TC-rich repeats, and CGTCA-motif were necessary for stress-responsive expression under abiotic stress.

3.4. Accumulation of CBF2 in Vitaceae under Low-Temperature Stress

CBF2 gene expression in leaves was compared with

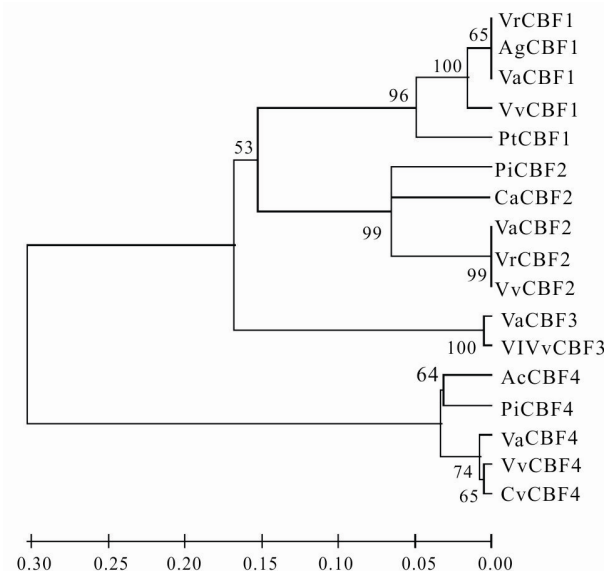

Figure 2. Bootstrap phylogenetic analysis of the selection of plant CBF proteins from NCBI. The amino acid sequences are VrCBF1 (AAR28671), AgCBF1 (ABU55659), VaCBF1 (ADY17818), VvCBF1 (AAR28673), PtCBF1 (ABU55661), PiCBF2 (ABU55670), CaCBF2 (ABU55671), VaCBF2 (ADY17812), VrCBF2 (AAR28674), VvCBF2 (AAR28677), VaCBF3 (ADY17813), VIVvCBF3 (ACT97164), AcCBF4 (ABU55676), PiCBF4 (ABU55674), VaCBF4 (ADY17814), VvCBF4 (XP_002280097), and CvCBF4 (ABU55679).

expression *in vitro* in the relatively cold-hardy wild *V.*

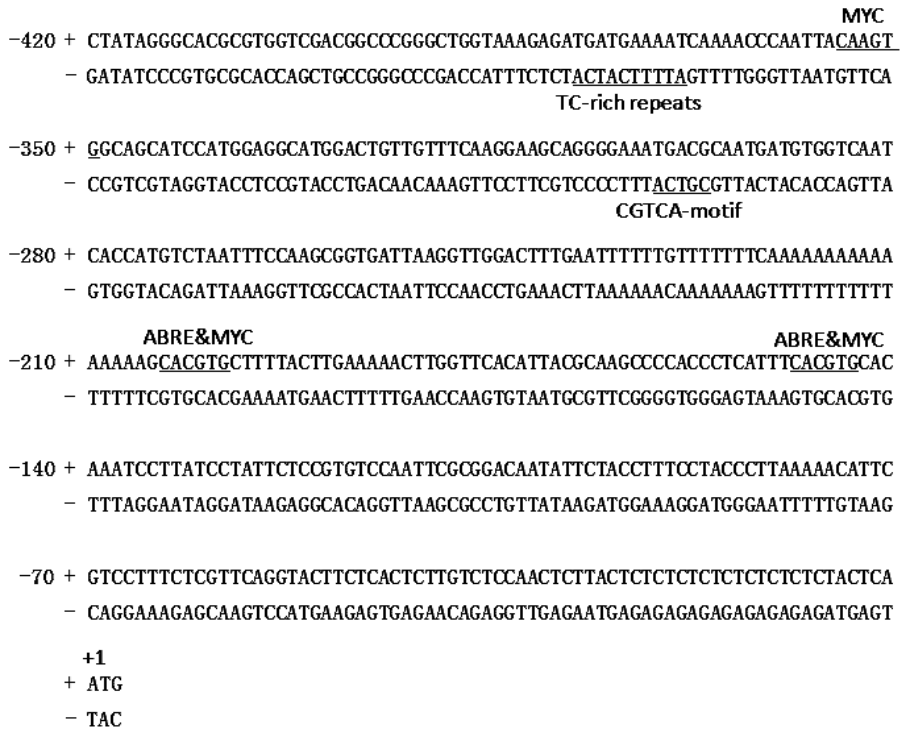

Figure 3. Distribution of putative regulatory elements in VaCBF2 promoter from *V. amurensis*.

amurensis and the relatively cold-sensitive *V. vinifera* cultivar "Manicure Finger" (**Figure 4**). *V. amurensis* reached the maximum midwinter cold hardiness level at <−40˚C, whereas "Manicure Finger" was cold hardy at approximately −7˚C. The expression levels of CBF2 in the cold-hardy *V. amurensis* and the cold-sensitive *V. vinifera* were not detected under normal conditions (**Figure 4(a)**). In both samples, expression slightly increased and peaked at 48 h under cold treatment. However, the expression level of VaCBF2 in the cold-hardy *V. amurensis* was higher than that of VvCBF2 in the cold-sensitive *V. vinifera* at a low temperature (**Figure 4(b)**). This finding was further confirmed by qRT-PCR.

3.5. Expression Pattern of VaCBF2 at Low Temperature

Accumulations of each transcript in the roots, stems, leaves, and petioles were analyzed by qRT-PCR to further determine the acquisition mechanisms of cold acclimation in relation to VaCBF2 gene expression. Accumulations of VaCBF2 transcript in all organs were activated and exhibited different expression patterns at a low temperature (**Figure 5**). During the subsequent chilling period, the transcript level of VaCBF2 in the leaves slightly increased at the early stage of treatment and then peaked on days 2 or 3. By contrast, the transcript level of VaCBF2 in roots and stems sharply increased, reached the peak at 8 and 48 h, respectively, and then gradually decreased. However, mRNA expression in the petioles was wavy and ebbed at 1 and 24 h of treatment at 4˚C.

3.6. Expression Pattern of VaCBF2 in Salinity Stress

We analyzed the expression of VaCBF2 gene under high salinity stress to obtain more information about its expression under stress. Results showed that VaCBF2 expression was induced by salinity throughout the treatment period (**Figure 6**). However, distinct organs displayed marked differences in gene expression levels throughout the treatment period. The expression of VaCBF2 in petioles, stems and leave speaked at approximately 6 and 8 h of treatment, respectively. The peak transcript accumulation in petioles was observed at 2 h, whereas that in roots was wavy.

3.7. ABA and SA Are Regulatory Factors Affecting VaCBF2

ABA and SA have important functions in physiological and genetic regulation during stress. We hypothesized that the expression pattern of VaCBF2 may be affected by ABA and SA. Therefore, we measured the transcript level of organs treated with ABA and SA and then compared their expression patterns. The expression of VaCBF2 was induced by exogenous ABA and SA treatments (**Figure 7**). However, treatments with ABA and

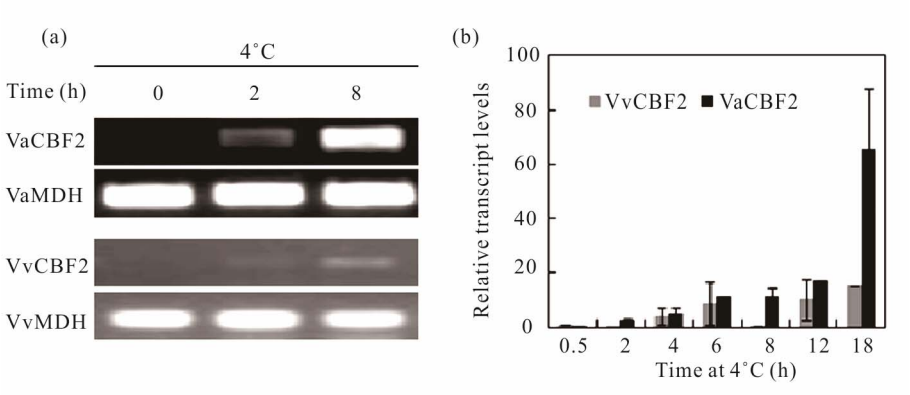

Figure 4. Transcript accumulation of *Vitis* CBF2 under low temperature stresses. (a) An expression difference of CBF2 in the cold-hardy *V. amurensis* (Va) and the less cold-hardy *V. vinifera* "Manicure Finger" (Vv). (b) Expression of organism in *Vitaceae* at low temperature by qRT-PCR.

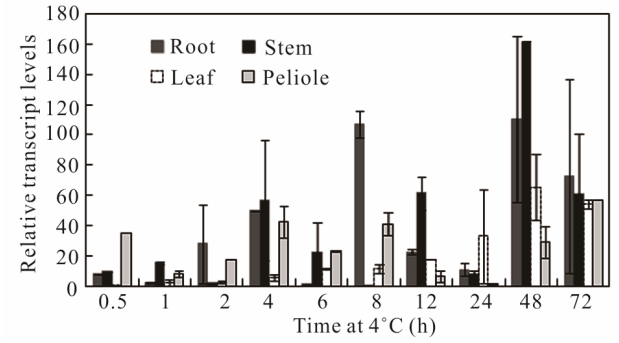

Figure 5. Changes in the mRNA accumulation of VaCBF2 in roots, stems, leaves, and petioles under 4˚C treatment.

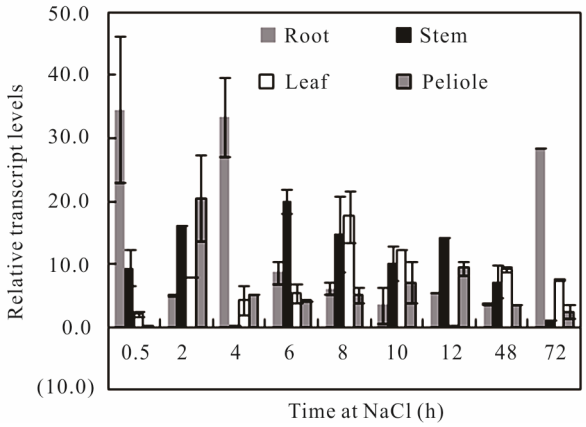

Figure 6. Changes in the mRNA accumulation of VaCBF2 in roots, stems, leaves, and petioles under 200 mM NaCl treatment.

SA exerted significantly different effects on gene expression. Under ABA treatment, the peak pattern was found at 6 h of treatment in stems, at the early and late stages in roots, and at 48 h in leaves. Under SA treatment, the transcript levels of VaCBF2 were higher. The relative transcript levels in organs under SA were several times higher than those under ABA, and this high level was maintained in all tissues throughout entire treatment stages.

4. DISCUSSION

We isolated and characterized a complete cDNA sequence of CBF2 from *V. amurensis*. The N-terminal region of VaCBF2 was found to share the conserved CBF2 domains with that of other known CBF2 (**Figure 1**). *Vitis* CBFs have been proven to be a nuclear protein [18], and VaCBF2 was found to be mapped in CBF/DREB subfamily of the CBF2 group through phylogenetic analysis (**Figure 2**). The conservation of these sequences in evolutionarily diverse plant species suggested that they played an important functional role. These data indicated that VaCBF2 was a CBF protein that may function as a transcription factor in *V. amurensis*.

As one of the important environmental factors affecting abiotic stress signal pathway, low temperature induced and regulated expression of many genes, and this regulation occurred at the transcript level. Cold reportedly induces the expression of most reported CBF genes, which is important for the expression of cold acclimation and cold tolerance [6,16]. In addition, the expression of *cor* 6.6 and *kin* 1 depended on the induction of CBFs. In the present study, we demonstrated that VaCBF2 transcription was induced by cold.

CBF2 transcripts were detectable in *V. amurensis* and *V. vinifera* in different tissues (roots, stems, leaves, and petioles) under low temperature (**Figures 4** and **5**). These transcript accumulations remained for a long period, with lower expression in leaves than in roots and stems. In agreement with the high identity between their amino acid domains, the expression patterns showed almost the same changes between *V. amurensis* and *V. vinifera*

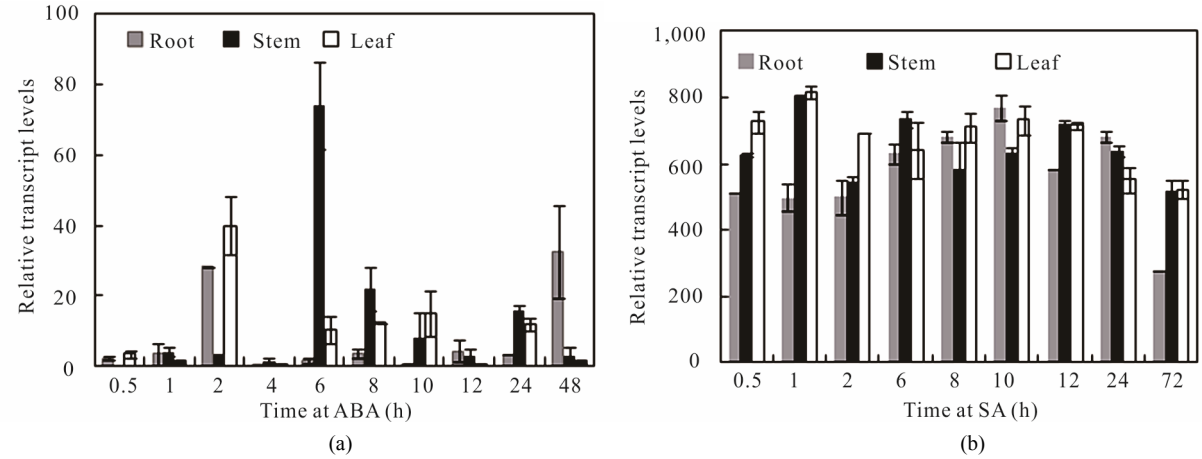

Figure 7. Changes in the mRNA accumulation of VaCBF2 in roots, stems, and leaves under ABA and SA treatments.

"Manicure Finger" under low-temperature treatment, except in *V. amurensis* in which the level was high. Interestingly, CBF2 expression in *V. vinifera* "Chardonnay" was visible in tissues (leaves, tips, buds, and stems) under natural conditions [18], whereas CBF2 expression in *V. amurensis* was not detectable in leaves, stems, roots, and petioles. The same results were observed in leaves of *V. vinifera* "Manicure Finger". This finding was further confirmed by qRT-PCR. The important role of CBF2 proteins in the freezing tolerance of *V. amurensis* can be attributed to the difference in regulon size between *V. vinifera* and *V. amurensis* because of the high similarity between VaCBF2 and VvCBF2 (**Figure 1**). Liu *et al.* [19] showed that rice CBF2 expression is induced by $CaCl_2$ and MeJA, cold, dry, and NaCl treatments but not by ABA and SA under experimental conditions. At the same time, rice CBF2 was expressed in shoots and seeds but not in roots and leaves. Novillo *et al.* [7,20] also reported that CBF2 is induced later than CBF1 and CBF3 during cold acclimation. Novillo *et al.* [20] confirmed that non-acclimated and cold-acclimated CBF2 mutants have higher capacity for tolerating freezing and dehydration than the corresponding WT plants. Over expression of *Arabidopsis* CBF2 increases plant freezing tolerance through the proline and sugar pathways and by inducing the expression of similar gene sets. All these results suggest that the three CBFs may not have an equivalent function with other CBFs.

Thomashow [1] suggested that a low-temperature signal recipient element, inducer of CBF expression (ICE), can induce the expression of CBF at normal temperature. However, ICE exists in the non-activated status at normal temperature. Chinnusamy *et al.* [21] found that the transcription factor ICE1 is expressed at a base level in *Arabidopsis*. ICE is a MYC-like bHLH transcription factor which specially binds to the MYC recognition site in the CBF promoter region and then activates CBF generating a low temperature. After analyzing promoter of VaCBF2 gene, we identified several MYC sites upstream of the initiation transcription site to which MYC transcription factor can specifically bind (**Figure 3**). The promoter sequences of this gene were also found to contain several *cis*-regulatory elements, such as ABRE, TC-rich repeat, and CGTCA motif. These elements were related to stress response, specifically to cold/dehydration. These *cis*-regulatory elements were conserved in several plant species. The presence of these conserved motifs confirmed that VaCBF2 gene was regulated by cold, high salinity, and application of hormones of ABA and SA. The multiple stress response of the VaCBF2 gene also suggested that *V. amurensis* can be used for further research on molecular mechanism and stress breeding.

5. ACKNOWLEDGEMENTS

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