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Whole genome identification of *CBF* gene families and expression analysis in *Vitis vinifera* L.

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Abstract: The *CBF* (*C-repeat binding factors*) genes play important roles in response to abiotic stress and environmental changes. In the present study, a total of 18 *CBF* genes were identified from a grapevine. Their domains, phylogenetics, and collinearity were analysed. The results revealed, that 18 *VviCBF* genes were distributed on 10 chromosomes unevenly in the grape genome. Promoter data analysis showed that the *CBF* gene has many *cis*-acting elements related to plant growth and development, light response, hormone, and abiotic stress response. We found that six *VviCBF* genes including, *VviCBF5*, *VviCBF13*, *VviCBF14*, *VviCBF15*, *VviCBF16*, and *VviCBF18* differentially expressed during fruit developmental stages. Furthermore, four *VviCBF* genes including, *VviCBF1*, *VviCBF3*, *VviCBF6*, and *VviCBF11* were expressed at the early stage of bud dormancy, whereas, nine *VviCBF* genes were expressed at the bud dormancy-breaking stage. Additionally, various *VviCBFs* genes respond to different abiotic and biotic stress. These findings will lay a foundation for further study of the *CBF* genes in bud dormancy, downy mildew, and abiotic and biotic stresses.

Keywords: biotic and abiotic stresses; bud dormancy; *CBF*; fruit development; grape

The C-repeat binding factors (CBFs)/dehydration-responsive element (DRE) binding proteins (CBF/DREB 1) are unique to plants and belong to APETALA2 (AP2) family transcription factors (TFs). CBF as an activator of transcription is able to recognize C-repeat response and low-temperature-responsive *cis*-acting elements (CRT/DRE) in the promoter region of many stress response genes. CBF TFs show multiple roles in plant growth and development including flowering regulation, biological and abiotic stress response (Akhtar et al. 2012; Artlip et al. 2013). The first *CBF* TF was identified in *Arabidopsis thaliana*, *CBF1*, which binds to the C-repeat/DRE DNA regulatory element and enhances the freezing tolerance of non-acclimated *Arabidopsis* plants (Stockinger et al. 1997; Jaglo-Ottosen et al. 1998). To date, the *CBF* TFs have been isolated and characterized in several plants, including rice (Ito et al. 2006), maize (Qin et al. 2004), soybean (Kidokoro et al. 2015), peach (Wisniewski et al. 2015), tomato (Zhang et al. 2004) and apple (Xie et al. 2018).

It has been well established that CBFs play a critical role in improving the biological and abiotic stress tolerance of plants. In *Arabidopsis*, *CBF1*, *CBF2*, and

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CBF3 were up-regulated by cold stress, whereas *CBF4* was induced by drought stress, but not by low temperature, and its overexpression enhanced both cold and drought tolerance in *Arabidopsis* (Haake et al. 2002). In rice, overexpression of *OsDREB1* increased the content of free proline and soluble sugars, thus improving tolerance to high-salt, low-temperature, and drought stress (Ito et al. 2006). In grapes, three *CBF* genes (*VvCBF1*, *VvCBF2*, and *VvCBF3*) respond to low temperature, drought, and abscisic acid (ABA) (Xiao et al. 2006). The overexpression of *VvCBF4* in grapevine improves the survival rate of plants under freezing conditions (Tillett et al. 2012). Besides response to abiotic stress, it is reported that *CBFs* are also involved in biotic stress. Wu et al. (2017) reported that ectopic expression of *MrCBF2* in *Arabidopsis* enhanced plant resistance against downy mildew disease. Moreover, CBFs have been reported in response to hormones, such as abscisic acid (Xiao et al. 2006), gibberellins (GAs) (Shan et al. 2007), and ethylene (Shi et al. 2012).

The perennial dormancy in woody crops is an ecological growth regulating factor in plants to survive during the winter season (Rohde et al. 2000). The grape bud dormancy is influenced by internal and external environmental factors. Short-day and low temperatures were two main environmental factors that induced bud dormancy of grapes. However, *CBF* genes have been shown to play integral roles in bud dormancy and induction of freezing tolerance. Ectopic overexpression of peach *CBF* TFs in apples significantly improves low-temperature tolerance and induces dormancy under short day lengths (Wisniewski et al. 2011). The *PpCBF* was induced under short-term chilling in the autumn season, and then *PpCBF* activated the expression of *Dormancy-associated MADS-box* (*DAM*) resulting in endo-dormancy in pear (Niu et al. 2016).

Grapevine is one of the most important economic fruit crops cultivated around the world and is widely used for winemaking and raisins (Xu et al. 2014). *Rosario Bianco* is a famous grape cultivar, that is native to Japan. Abiotic stresses such as drought, flood, extreme temperature, and high salt reduce grape productivity by up to 50% (Erpen et al. 2018). In addition, downy mildew is the most common and most serious oomycete disease in grapes. Although some studies have shown that members of the *CBF* gene family are involved in the response to regulate biotic or abiotic stress in grapes, comprehensive identification, characterization and evolutionary analysis of the *CBF* genes in grapes have not yet been carried out. Theretofore, in the present study, we used bioinformatics containing online web tools to identify CBFs gene family members. The gene structural domain, cis-regulatory elements, synonymous/nonsynonymous mutations (Ka/Ks) replication events, and collinearity of grape *CBF* genes were also analysed. We further analysed expression profiles of grape *CBF* genes under various abiotic and biotic stresses, during berry ripening and bud dormancy, through mining publicly available RNA-seq datasets. The results obtained from our study provided a foundation for the evolutionary and functional characterization of *CBF* gene families in grape and other plant species.

MATERIAL AND METHODS

Plant materials. The experimental plant materials were collected from the three-year-old Rosario Bianco grapevine, were grown in a greenhouse at Jiangsu Agricultural Exposition Park, Jiangsu Province, China (32°0'41.99''N 119°15'7.11''E), under the standard cultivation conditions. Target materials were divided into three groups based on the varied growth locations of grape buds, and the vine height; three categorized groups are (1) basal portion/part, (2) middle portion/part, and (3) top portion/part of the bud components. In addition, basal portion (3rd, 4th, and 5th buds), middle portion (8th, 9th and 10th buds,) and top portion (14th, 15th, and 16th buds). The experimental materials were collected from November 2017 to April 2018, for experimental purposes, 30 buds from each group were collected on the 20th of each month, respectively. The samples were immediately frozen in liquid nitrogen and stored at –80 °C for further analysis.

Identification of the *CBF* genes in grape. To identify members of the *CBF* gene family in grapevine, genome sequences, mRNA, and protein annotation files concerning *Vitis vinifera* L. and *Arabidopsis thaliana* (TAIR10) were downloaded from the Ensembl Plants database (http://plants.ensembl.org). The *CBF* sequences of *A. thaliana* (AT4G25470, AT4G25490, AT5G51990, AT4G25480, AT1G12610, and AT1G63030) according to Nakano et al. (2006), were used as a reference targeted protein sequences, and the CBF protein sequences of grapevine were retrieved by using localized Blastp, with E-value of e^{-5}. Finally, the protein sequences of candidate CBF were verified using National Centre for Biotechnology Information (NCBI), Conserved domain database (CDD) online tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

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Bioinformatics analysis. In order to predict the isoelectric point (pI), subcellular localization, and protein molecular weight (MW) of each putative *CBF* gene were analyzed by using ProtParam tool on the ExPASy (http://web.expasy.org/protparam/ and http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). For chromosomal location and gene, distribution maps were constructed using the MapGene2Chro online tool (http://mg2c.iask.in/mg2c_v2.0/). The clustal W software (Ver. 1.8.1) was used for protein sequence alignment. The phylogenetic tree was constructed with IQ-TREE software (http://www.iqtree.org/) (Minh et al. 2020). The CD-Search Tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was used for protein sequence domain analysis. The MEME website (http://meme.nbcr.net/meme) (Bailey et al. 2015) was used to identify conserved motifs and set the motif number as 10.

Collinearity and Ka/Ks analysis of grapevine *CBF* genes. Whole proteins obtained from *V. vinifera* PN40024 v3 and *Arabidopsis* TAIR10 releases were compared against each other using local blast alignment, and results were filtered using an E-value cut off less than $1e^{-5}$.

The blast outputs of all protein-coding genes were imported into MCScanX. MCScanX software was used for collinearity analysis among CBF, and all the duplicate gene pairs, except tandem gene pairs, were visualized using TBtools. The ratio of the gene frequency of synonymous (Ks) and non-synonymous (Ka) values, Ka/Ks were calculated and analyzed by ParaAT.pl and Ka/Ks-Calculator.

Analysis of cis-elements of grapevine *CBF* genes. For the cis-regulatory elements analysis of *CBF* genes, we obtained the 2 000 bp sequence upstream of the *VviCBF* genes as a promoter region and put it forward to the PlantCARE online website (http://bioinformatics.psb.ugent.be/webtools/PlantCARE/html/) (Lescot et al. 2002). Therefore, Cis-acting elements on the promoter sequences of the *VviCBF* genes were visualized.

RNA isolation and quantitative real-time PCR (qRT-PCR). Total RNA was extracted from buds by the SDS-phenol method, and the RNA quality and yield were evaluated by using Nano-Drop (Thermo Fisher Scientific Inc., USA). The first strand of cDNA was synthesized using the Revert Aid TM first-strand cDNA synthesis kit (Transgen Biotech, China). The cDNA was diluted 30 times in the double distilled water. Then the cDNA template was combined with EvaGreen 2× qPCR master mix-ROX, and RT-qPCR was performed using a real-time fluorescent quantitative PCR instrument. The housekeeping gene *Actin* (AB073011, PCR primers: GGAAGCTGCGGGAATTCATGAG, CCTTGATCTTCATGCTGCTGGG) was used as an internal reference gene to quantify mRNA levels. Primer sequences used in this study are given in Table S1 in Electronic Supplementary Material (ESM). The total volume of PCR was 20 µL, which included 2 µL containing 500 ng of cDNA template, 10 µL of 2× EvaGreen qPCR Master Mix, 0.6 µL of forward primer, 0.6 µL of reverse primer, and 6.8 µL of nuclease-free H_2O. PCR reaction conditions were as follows: pre-denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 15 s and annealing at 62 °C for 1 min. Data are expressed as mean ± standard deviation (SD) and subjected to a one-way analysis of variance (ANOVA). All analysis was carried out in at least three replicates for each sample. Results were analysed statistically using SPSS (Ver. 15.0). A value of $P < 0.05$ was considered statistically significant.

Transcript analysis of *VviCBF* genes. The grapevine transcriptome data of fruit development were downloaded from Gene Expression Omnibus (htt://www.ncbi.nlm.nih.gov/geo) along with (GEO accession No. GSE77218), drought stress (SRA accession No. SRP074162), waterlogging (SRA accession No. SRP070475), and salt (SRA accession No. SRP070475) (Leng et al. 2015; Haider et al. 2017; Shangguan et al. 2017; Zhu et al. 2018). Transcriptome data for expression profiles in response to copper (Cu) and downy mildew were retrieved from published data sets by Guan et al. (2018) and Gong et al. (2022). Genes having an false discovery rate (FDR) of less than 0.001 and at least a 1-fold difference between two samples were only characterized as differentially expressed. Transcripts with $|\log2FC| < 1$ were assumed to have no change in expression levels. Expression data was mapped by TBtools and presented in the heat map format (Chen et al. 2020).

RESULTS

Identification and characterization of *VviCBF* genes. In the present study, we identified 18 CBF gene family members from the grapevine genome and named them *VviCBF1* to *VviCBF18* based on their physical location (Table 1). These *VviCBF* genes were 147–239 amino acids (AA) in size, with pIs from 4.75–9.81 (*VviCBF16-VviCBF7*), and predicted MWs 17.03–26.50 (*VviCBF12-VviCBF5*) kDa

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as shown in Table 1. According to the molecular analysis of full-length sequences and web-based predictions of *VvCBFs* location in the cells, different constituents of the *VviCBF* gene family were found in the nucleus and cytoplasm.

Furthermore, the localized distribution of *VviCBF* genes on the grape chromosomes are shown in Figure 1. A total of 18 *VviCBF* genes were unevenly distributed on 10 chromosomes, a single gene was distributed on chromosome 4, 9, and 14, two genes were distributed on chromosome 2, 6, 8, 11, 15, and 16, and three were located on unknown chromosomes. Multiple sequence alignment showed that the eighteen *VviCBF* genes had a highly conservative DNA-binding AP2 domain (Figure 2). MEME online tool was used to identify ten distinct motifs. The specific information is shown in Figure 2 and Figure S1 in ESM. As illustrated in Figure 2, other than motifs 1, 2, and 3 were widely distributed in all proteins, *VviCBF* members within the same subgroups were

Table 1. The details of C-repeat binding factors (CBFs) captured in the *Vitis vinifera* genomes

Gene name	Gene identifier	Gene identifier (PN40024 v3)	Size (AA)	Genomics position	Theoretical pI	MW (kDa)	Predicted subcellular localization
VviCBF1	VIT_00s0341g00070.t01	Vitvi02g01730.t01	239	Un:24451343-24452062	6.55	25.94045	nucleus
VviCBF2	VIT_00s0341g00080.t01	Vitvi02g01729.t01	225	Un:24460830-24461680	5.99	25.35237	nucleus
VviCBF3	VIT_00s0632g00010.t01	Vitvi00g00859.t01	230	Un:33261288-33261979	6.5	25.06452	nucleus
VviCBF4	VIT_02s0025g04440.t01	Vitvi02g00406.t01	186	2:3903010-3903835	5.19	20.02521	nucleus
VviCBF5	VIT_02s0025g04460.t01	Vitvi02g00407.t01	235	2:3928473-3929345	6.24	26.50371	cytoplasm/ nucleus
VviCBF6	VIT_04s0008g03400.t01	Vitvi04g00300.t01	241	4:2818241-2819492	4.99	26.12993	cytoplasm/ nucleus
VviCBF7	VIT_06s0061g01390.t01	Vitvi06g01411.t01	253	6:19197922-19198617	9.81	27.90246	cytoplasm/ nucleus
VviCBF8	VIT_06s0061g01400.t01	Vitvi06g01414.t01	211	6:19254403-19255122	8.58	22.98067	cytoplasm/ nucleus
VviCBF9	VIT_08s0007g03790.t01	Vitvi08g01501.t01	212	8:17765904-17766542	7.68	23.64844	cytoplasm/ nucleus
VviCBF10	VIT_08s0007g03810.t01	Vitvi08g01503.t01	199	8:17783389-17784006	5.31	20.49676	cytoplasm/ nucleus
VviCBF11	VIT_09s0002g03940.t01	Vitvi09g00323.t01	235	9:3665089-3665967	4.92	25.37681	nucleus
VviCBF12	VIT_11s0016g02140.t01	Vitvi11g01322.t01	147	11:1758493-1761259	9.66	17.03262	nucleus
VviCBF13	VIT_11s0016g03350.t01	Vitvi11g00285.t01	266	11:2729375-2730729	5.18	28.44125	cytoplasm/ nucleus
VviCBF14	VIT_14s0006g02290.t01	Vitvi14g01067.t01	236	14:19671450-19672638	6.31	25.918.02	cytoplasm/ nucleus
VviCBF15	VIT_15s0021g02110.t01	Vitvi15g00601.t01	188	15:12934820-12935386	5.15	21.17246	nucleus
VviCBF16	VIT_15s0046g00310.t01	Vitvi15g00947.t01	207	15:17321737-17322484	4.75	23.15049	cytoplasm/ nucleus
VviCBF17	VIT_16s0100g00380.t01	Vitvi16g00941.t01	218	16:15813825-15814917	5.42	24.21617	nucleus
VviCBF18	VIT_16s0100g00400.t01	Vitvi16g00942.t01	237	16:15837953-15838931	4.91	25.59729	cytoplasm/ nucleus

AA – amino acids; pI – isoelectric point; MW – molecular weight

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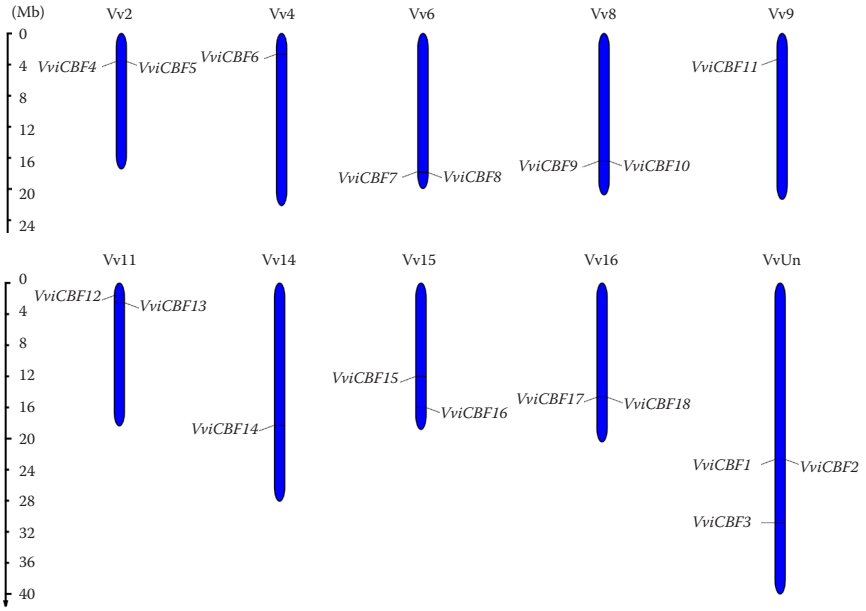

Figure 1. The distribution of identified grapevine *C-repeat binding factors* (*CBFs*) on grapevine chromosomes

The scale on the left represents a chromosomal distance, and the marker line on the chromosome represents the approximate physical position of the gene on the grape chromosome

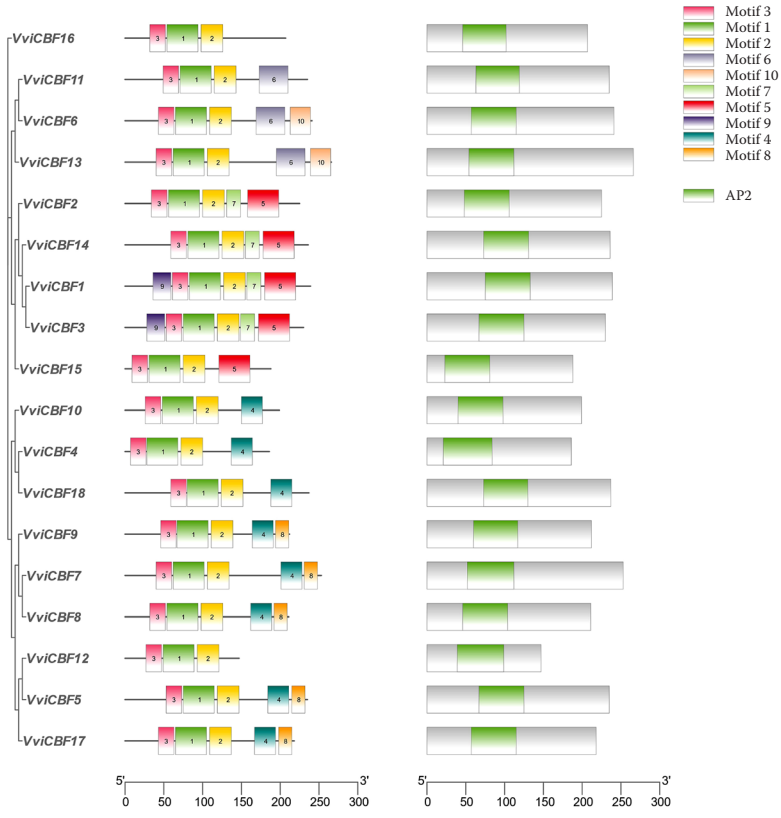

Figure 2. Distribution of conserved motifs and conserved domain in grapevine *C-repeat binding factors* (*CBFs*) genes based on the phylogenetic relationship; ten motifs (motif 1 to motif 10) were identified with MEME tool and representation of each motif was illustrated with different colours; the lengths and positions of the coloured blocks correspond to the lengths and positions of the motifs in the individual protein sequence, respectively; MEME-identified sequence motifs present in the protein sequence is provided in Figure S1 in ESM; conserved domains were identified using CD-Search Tool; APETALA2 (AP2)/Ethylene Responsive Element Binding Factor (EREB) domain consists of 40–70 conserved amino acids involved in DNA binding; CBF protein are known to contain an AP2/ERF domain; they bind a GCC-box-like element found in dehydratation responsive element; binding to this element mediates cold-inducible transcription

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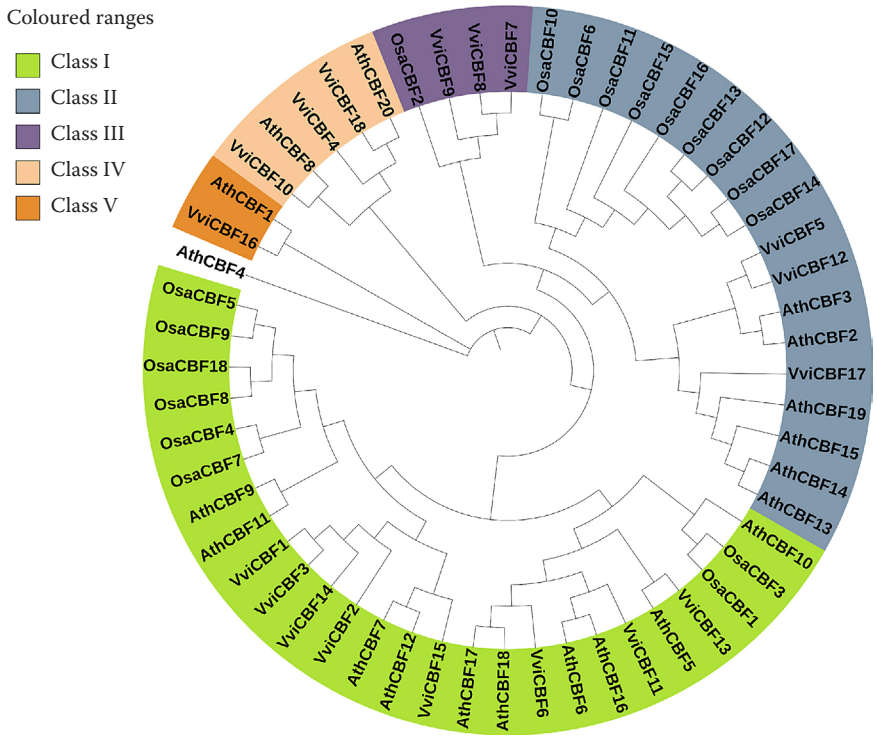

Figure 3. Evolutionary tree analysis; phylogenetic tree constructed using 56 CBF proteins from *Arabidopsis*, rice and grape The phylogenetic tree was constructed using the maximum-likelihood (MJ) method in IQ-TREE software with 5 000 bootstrap replications. The gene names in trees are different coloured to indicate different CBFs classes among different plant species

generally found to share a common motif composition. The results suggest that these members in the same subfamily may have similar functions.

Phylogenetic analysis of the *CBF* gene family. To understand the evolutionary history of the *CBF* gene family, a phylogenetic tree of the *CBF* genes from *Arabidopsis thaliana*, rice, and grape was constructed using IQ-TREE to compare all the protein sequences. As shown in Figure 3, these *CBF* genes were categorized into five classes (class I–V). Among them, class I possessed 26 members which was the largest group, followed by 18 in class II, 4 in class III, 5 in class IV, and 2 in class V. Our results indicated that most of the CBFs of grapevine were close to *Arabidopsis thaliana* but not with rice plant, which may be connected to the fact that grapevine and *Arabidopsis* have more similarity as compared to rice plant (Zhang et al. 2012; Matus et al. 2008) (Figure 3). These results were consistent with the phylogenetic relationship of the species because both grapevine and *Arabidopsis* were dicotyledons, which had diverged more recently from a common ancestor rather than from the lineage between monocots and dicots.

Analysis of the cis-elements in the promoters of *VviCBFs*. To study the possible roles of *VviCBF* genes, the functional characterization, and the transcriptional regulation phenomenon of the *VviCBF* genes, we retrieved 2 000 bp upstream of the initial position of each gene through Grape Genome Database and visualized the cis-elements in the promoter regions. Our results showed that many *cis*-elements related to the hormone signaling pathway, transcriptions factors (TFs) and abiotic stresses, etc. For example, as showed in Table S2 in ESM, 7 members of *VviCBFs* contain salicylic acid (SA) response element (TCA-elements), 15 members of *VviCBFs* had MeJA-response elements (CGTCA motif and TGACG-motif), 17 members of *VviCBFs* contain abscisic acid element ABREs, 6 members of *VviCBFs* with auxin response elements (AuxRR-core, TGA-box, and TGA-element). In addition, there were a large number of *cis*-acting elements related to stress response, such as low temperature, wound, and drought. It should be pointed out a large number of light response elements were observed in the promoters of all *VviCBFs*. (Figure 4 and Table S2 in ESM). The presence of these *cis-elements* in the promoter region of *VviCBFs* indicates their potential roles related to factors such as a hormone, abiotic stress, and light.

Duplication events and divergence rates of *VviCBF* gene families. To investigate more information and understand the evolutionary behaviour of *CBF* genes in grapevine and *Arabidopsis thaliana* species genomes, we visualized the synteny analysis respectively (Figure 5 and Table 2). The primary

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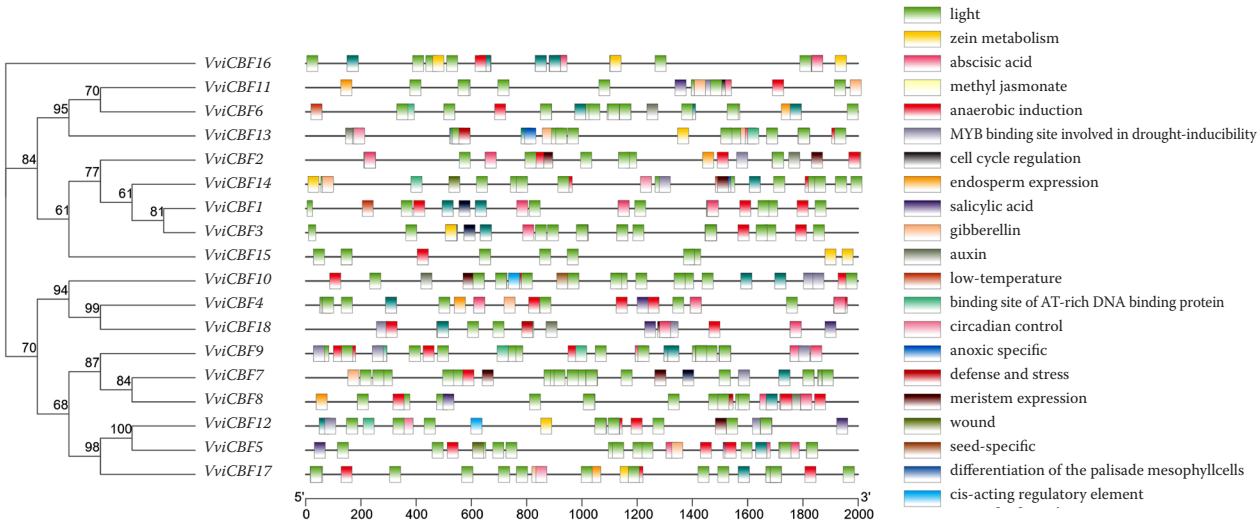

Figure 4. Identification of cis-elements on the promoter region of the *VviCBF* genes
Individual colour demonstrates different cis-elements; names of the *VviCBF* genes are present on the left side, and names of the cis-elements are present on the right side

mechanisms behind gene expansion are tandem and segmental duplication. Synteny analysis indicated that there are two pairs of segmental duplication and one-tandem duplication events of genes of *VviCBFs*. The segmental duplication of *VviCBFs* and *AthCBFs* genes were demonstrated in a circos map, since tandemly duplicated loci are too close to show in the circos map. Additionally, the Ka/Ks ratio of each

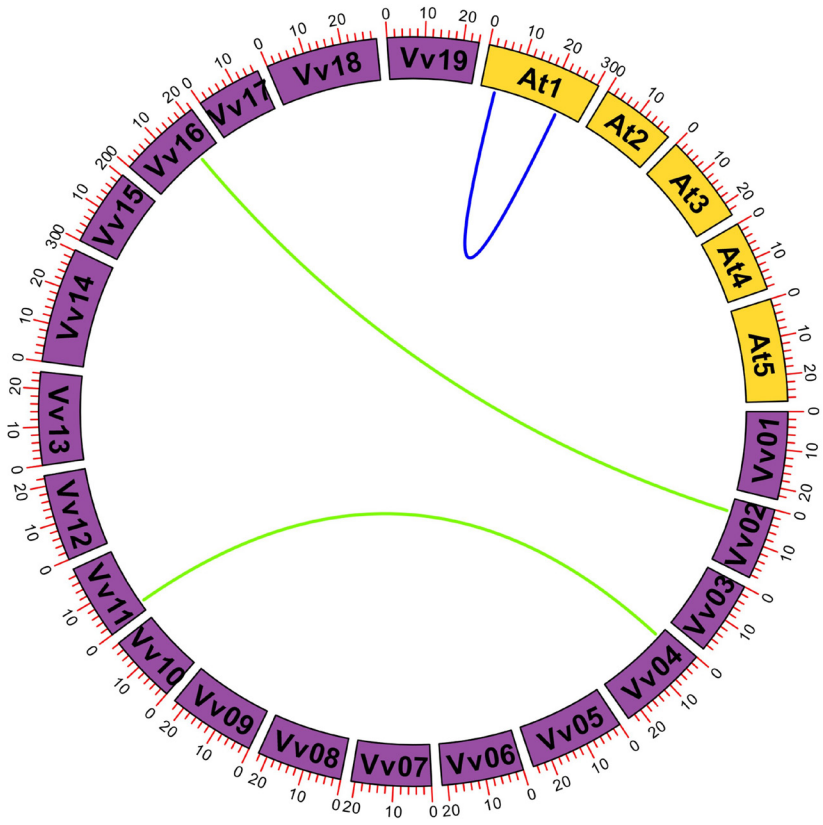

Figure 5. Syntenic analysis of grape and *Arabidopsis CBF* genes
Chromosomes of *V. vinifera* and *Arabidopsis* are shown in different colours and in circular form; the green line represents the collinearity of respective genes in grape, the blue line represents the collinearity within the *CBF* gene family in *Arabidopsis*

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Table 2. Ka/Ks analysis in the *Arabidopsis* and grape *CBF* homologues

Duplicate pair	Location in chromosome (Chr: start-end)	Ka	Ks	Ka/Ks	Duplication type
AthCBF2-AthCBF3	1:4289883-4291017 1: 23367394-23368416	0.136726	0.942184	0.145116	segmental duplication
VviCBF13-VviCBF6	11:2729375-2730729 4:2818241-2819492	0.190177	1.58581	0.119924	segmental duplication
VviCBF18-VviCBF4	16:15837953-15838931 2:3903010-3903835	0.347856	1.85398	0.187626	segmental duplication
VviCBF7-VviCBF8	6:19197922-19198617 6:19254403-19255122	0	2.18131	0	tandem duplication

Ks – synonymous; Ka – non-synonymous values

orthologous gene pair was calculated to determine the selective pressure during the evolution process of respective *VviCBF* genes. As shown in Table 2, The Ka/Ks ratios were determined between 0.1 to 0.18, the divergences for both genes shows the segmental and tandem duplicate. Furthermore, the Ka/Ks value (< 1) of each pairs of genes represented the purifying selective diversity pressure during the process of evolution.

Expression analysis of *VviCBF* genes in fruit development. The expression profiles of *VviCBFs* in grapevine were visualized and show different trends at different developmental stages of berries (green fruit, veraison, and ripening stages) (Figure 6 and Table S3 in ESM). Among them, six *VviCBF* genes, *VviCBF15*, *VviCBF13*, *VviCBF16*, *VviCBF14*, *VviCBF5*, and *VviCBF18* have been identified as differentially expressed genes (DEGs) during fruit developmental stages. The *VviCBF5*, *VviCBF13*, *VviCBF14*, *VviCBF15*, and *VviCBF16* were down-regulated with the growth and development of grapes. However, the expressions of *VviCBF18* were gradually up-regulated. In addition, the specific expression of these genes might have various roles rely on the tissues of the grapevine.

Expression analysis of *VviCBF* gene family members during bud dormancy and qRT-PCR analysis. We examined expression of the *VviCBF* during bud dormancy using qRT-PCR analysis. For gene expression quantification in grapevine bud dormancy, some genes were selected by further confirmation (*VviCBF1*, *VviCBF3*, *VviCBF4*, *VviCBF5*, *VviCBF6*, *VviCBF7*, *VviCBF11*, *VviCBF12*, *VviCBF13*, *VviCBF15*, *VviCBF16*, *VviCBF17* and *VviCBF18*) (Figure 7). The expression level of these genes in bud tissues of several genes e.g. (*VviCBF1*, *VviCBF3*, *VviCBF6*, *VviCBF11*, and *VviCBF2*) showed an increasing trend concerning December and March. While (*VviCBF4*, *VviCBF5*, *VviCBF7*, *VviCBF13*, and *VviCBF15*) showed a decreased trend from November to March. Furthermore, the remaining genes have shown an inconsistent expressional level trend in bud tissues of the grapevine concerning November to April (Figure 7 and Table S3 in ESM).

Expression profiles of *VviCBF* gene family members under abiotic stress and biotic stress. The expression patterns of *VviCBF* genes in abiotic stresses namely (a) drought, (b) waterlogging, (c) salt stress, and (d) copper stress, and (e) downy mildew infections were investigated by using transcriptomic data

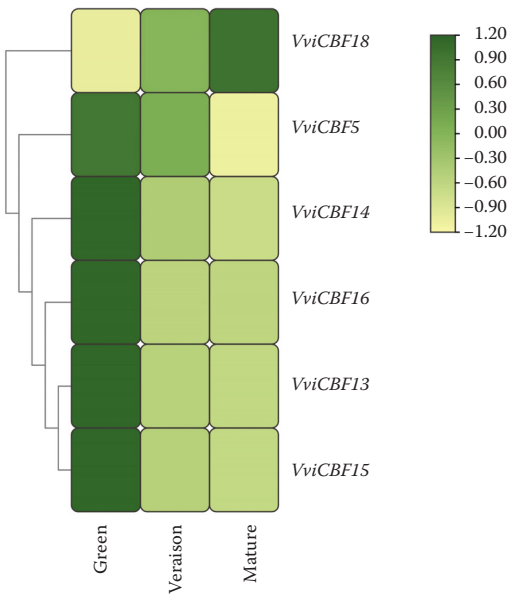

Figure 6. Expression profiles of *VviCBF* genes during grapevine fruit development

The expression value was represented by the colours; colours ranging from yellow to green indicate expression enhancement genes

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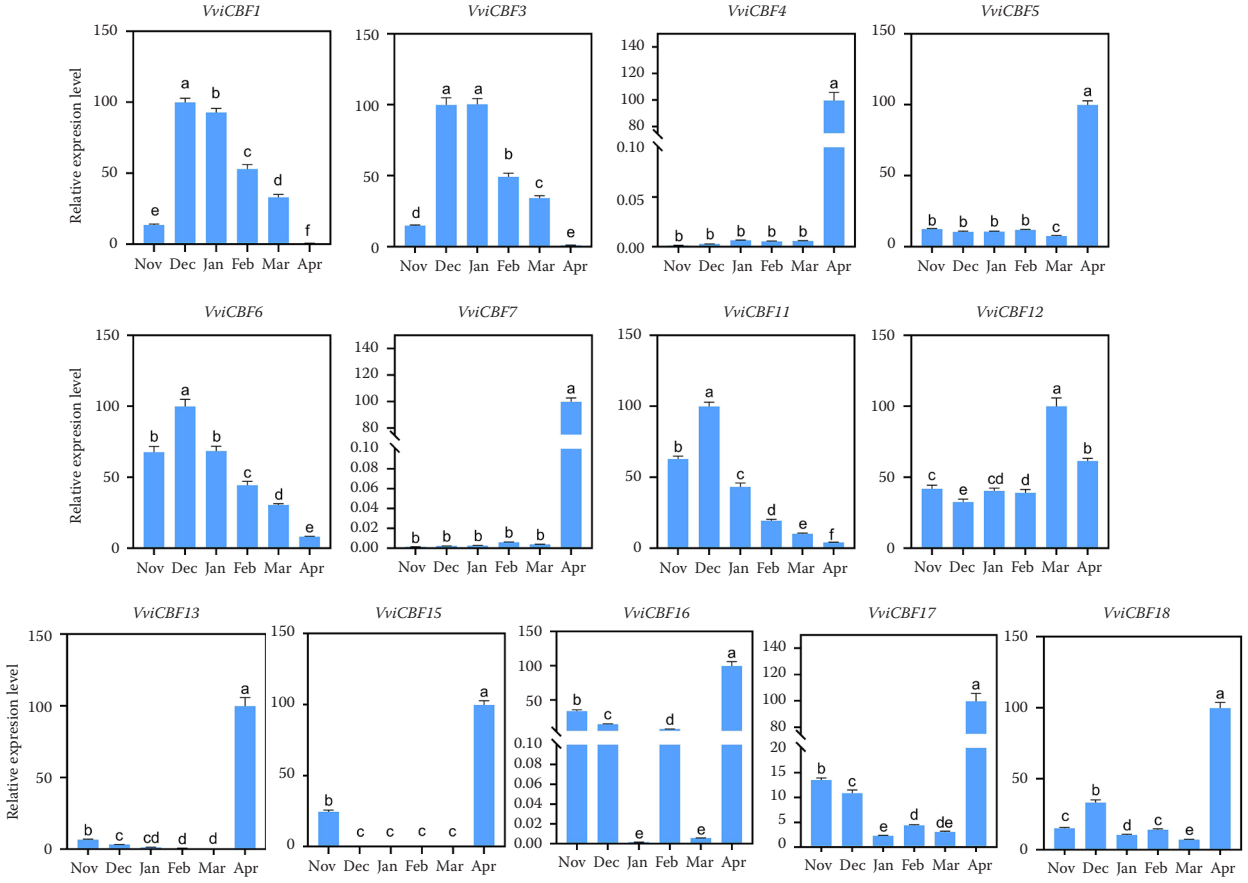

Figure 7. Relative expression analysis of thirteen selected *VviCBF* genes demonstrated by RT-qPCR during grapevine bud dormancy

The error bar demonstrates standard error via various letters above the bars demonstrating the significant difference of the data at *P* value < 0.05 (Tukey's test)

(Figure 8 and Table S4 in ESM). The data revealed that all 18 *VviCBF*s have been identified as differentially expressed genes under drought stress. The expression of *VviCBF2*, *VviCBF11* and *VviCBF12* were downregulated, the others were up-regulated under drought stress (Figure 8A). Seven *VviCBF*s including *VviCBF1*, *VviCBF3*, *VviCBF7*, *VviCBF13*, *VviCBF16*, *VviCBF17* and *VviCBF18* were identified as differentially expressed genes under waterlogging stress, however, most of them were inhibited under waterlogged stress except for *VviCBF18* (Figure 8B). Under copper stress, the expression of *VviCBF3*, *VviCBF7* and *VviCBF14* were up-regulated, whereas the expression of *VviCBF13*, *VviCBF17* and *VviCBF18* were down-regulated (Figure 8C). In response to salt stress, 8 *VviCBFs* including *VviCBF1*, *VviCBF3*, *VviCBF5*, *VviCBF7*, *VviCBF13*, *VviCBF14*, *VviCBF17*, and *VviCBF18* have been identified as differentially expressed genes. Among them, the expression level of *VviCBF1*, *VviCBF3*, and *VviCBF14* expressions was increased, and the others were inhibited under salt stress (Figure 8D). Based on gene expression data our results found that *VviCBF17* was highly expressed under different abiotic stresses including drought, waterlogging, copper, and salt stress (Figure 8).

Downy mildew is a common fungal disease of grapes. To elucidate the response of *VviCBF*s to downy mildew pathogen, the RNA-Seq data from the susceptible cultivar Zitian Seedless and the resistant cultivar Kober 5BB was determined (Figure 8E). However, the expression level in most of *VviCBF* did not show any significant variations in both susceptible and resistant cultivar samples. While some *VviCBF*s were shown response to differentially between susceptible and resistant cultivars. Such as *VviCBF5*, which was highly expressed in a susceptible sample in all three-time points, while *VviCBF5* was slightly down-regulated in the resistant sample at 24 h and then

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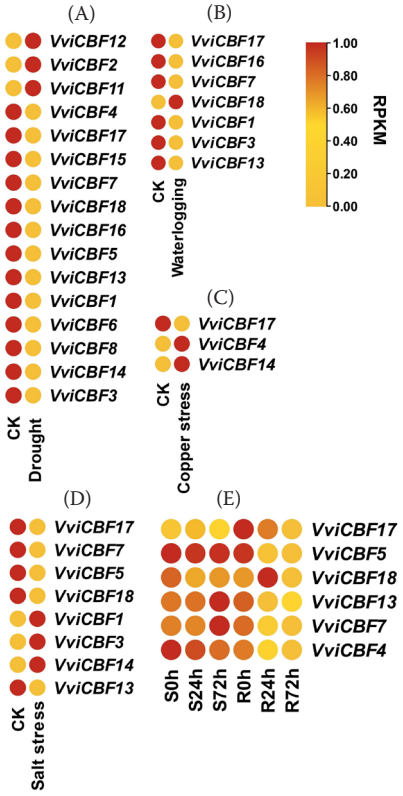

Figure 8. The expression patterns of grapevine *CBF* gene family members under abiotic stress and biotic stress: drought (A), waterlogging (B), copper stress (C), salt stress (D), the expression profiles of *VviCBF* genes under downy mildew (E)

S – susceptible cultivar; R – resistant cultivar; yellow colour represents the up-regulation and red colour represents the down-regulation; RPKM – reads per kilobase per million mapped reads; CK – control group

up-regulated at 72 h. The expression of *VviCBF17* reached to peak at 24 h in susceptible sample, but it was significantly inhibited in resistant cultivar. Furthermore, the transcription of *VviCBF18* was also reached to peak at 24 h and dropped to a minimum at 72 h in resistant cultivar, while decreased gradually with a time of infection in the susceptible sample (Figure 8E). Overall, we predicted that *VviCBF* gene expression between susceptible and resistant cultivar plays a diverse role in the regulation of resistance to downy mildew in grapevine.

DISCUSSION

C-repeat binding factors (CBF) belong to a member of transcription factors (TFs) in plants that control the tolerance mechanisms related to abiotic and biotic stresses. However, no genome-wide identification of the CBFs gene family has been studied in the grapevine until now. Therefore, we studied the C-repeat binding factors (CBF) in grapevine, and in the present study, a total of 18 *CBF* genes were identified and characterized in the grapevine genome (Table 1). Moreover, characteristics of CBF family members, distribution of genes on the chromosomal levels, conserved motifs, conserved domains, phylogenetic analysis, cis-acting elements in the promoter regions, duplicate events and expression analysis of *VviCBF* genes in different developmental stages, were analysed using a bioinformatics approach.

In *Arabidopsis*, six CBF proteins have been identified, i.e., CBF1, CBF2, CBF3, CBF4, CBF5 and CBF6 (Nakano et al. 2006). Meanwhile, 10, 7, 6 and 14 were identified in *Brassica rapa* (Lee et al. 2012), pomegranate (Wan et al. 2022), tea (Hu et al. 2020) and lettuce (Park et al. 2020), respectively. In this study, we identified 18 *CBF* genes in the grape. The phylogenetic analysis comparing the *CBF* genes from the grapevine, *Arabidopsis*, and rice plant, revealed five subgroups, ranging from Class I- Class V (Figure 3). The large number of *CBF* genes in grape is comparable to the number found in barley and wheat but contrasts with the six genes present in *Arabidopsis*. Since grapevines are sensitive to freezing temperatures during the growing season, the amplification of the *CBF* gene family may be due to the adaptation to environmental changes. In addition, there are numerous duplication events in angiosperms (Li et al. 2020). Tandem duplication and segment duplication are the main drivers force for gene expansion. In this study, we found two segmental duplication pairs and one tandem duplication pairs of *VviCBFs* (Figure 5). Tandem duplication has been observed in multiple species, such as *Arabidopsis* (Cao et al. 2015), *Liriodendron chinense* (Guan et al. 2021), lettuce (Park et al. 2020) and apple (Zhao et al. 2012). Our results indicated that the grape *CBF* gene family is a slowly conserved gene family, and fragment duplication is the main driving force for the expansion of members of this gene family.

In this study, the visualization of promoter sequences of *VviCBF* genes demonstrated that there are various types of cis-regulatory elements that respond to various stimuli, which might be closely related to the diverse functions of *VviCBF* genes in grapevine (Figure 4). Particularly that *VviCBF* genes may be played significant role in diverse kinds of hormones as well as the hormone mediated stress

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response. Similar to *TkCBF* genes of *Taraxacum kok-saghyz* (Zhang et al. 2022), in our present study, all *VviCBF* gene promoters contain light-responsive elements (Figure 4). Previous studies revealed that light photoperiod and the circadian clock induced the expression of *CBFs* and are involved in the regulation of CBFs (Maibam et al. 2013). Based on these findings it is predicted that *VviCBFs* might have an important role in controlling the growth and development of grapevine through response to light signalling. Additionally, *cis*-regulatory elements related to plant hormones including ABA, methyl jasmonate (MeJA), gibberellin, salicylic acid, and auxin were present extensively in *VviCBF* promoter regions, suggesting their potential roles in the response to these hormones.

The grapevine fruit development is an important process in viticulture, even in harsh climatic conditions based on bud dormancy plants have the potential to survive in a better way. In our present study, we found that *VviCBF* genes in grapevine in different fruiting stages of the tissues help in the growth and development of the plants. There are six *VviCBF* (*VviCBF5*, *VviCBF13*, *VviCBF14*, *VviCBF15*, and *VviCBF16*) genes differentially expressed and up-regulated during grapevine growth expansion, while *VviCBF18* was down-regulated (Figure 6). Further research is necessary to determine whether *VviCBF18* contributes to the accumulation of anthocyanins in grapes. An et al. (2020) ensured that MdbHLH33, is a positive regulator in apple anthocyanin accumulation during cold tolerance, which has been shown to control the transcription of *MdCBF2*. The *CBF* genes have characteristic expression features during bud dormancy to support plantation during harsh environmental conditions. Balogh et al. (2019) found that the expression level of *ParCBF1* was highest in December, when the temperature was the lowest, however, decreased with the increase in temperature. It was observed that 13 of 18 *VviCBF* genes were significantly expressed during bud dormancy, indicating that the *CBF* genes were actively involved in grape bud dormancy driving. Similarly, *CBFs* are necessary for the regulation of dormancy in *Arabidopsis*, the inhibition of *CBF* expression may be a feature allowing cold to promote (Kendall et al. 2011). Wisniewski et al. (2011) found that ectopic overexpression of *PpCBF1* in apples delayed bud break in the spring. The expression of *VviCBF1*, *VviCBF3*, *VviCBF6*, and *VviCBF11* reached a peak in December, and the higher expression of *VviCBF1* and *VviCBF3* continued until January. Such results suggest that *VviCBF1*, *VviCBF3*, *VviCBF6,* and *VviCBF11* are involved in the maintenance of bud dormancy. However, the expression levels of *VviCBF4*, *VviCBF5*, *VviCBF7*, *VviCBF13*, *VviCBF15*, *VviCBF16*, *VviCBF17,* and *VviCBF18* were low in the first 5 months and increased only in April. This result suggests that these genes may be involved in process of dormancy breaking.

The expressional profile of *CBF* genes plays significant roles in terms of coping with different stresses, *CBF* genes have been described to have different functional patterns in plant growth and development. However, in transgenic plants like *Arabidopsis thaliana* the overexpression of CBF1/DREB1 showed strong tolerance under freezing stress (Jaglo-Ottosen et al. 1998), and increased tolerance against drought, high salt, and freezing stress (Liu et al. 1998). In grapes, the *CBF4* was induced by cold treatment, while *CBF1*, *CBF2*, and *CBF3* responded to drought, ABA, and low temperature (Xiao et al. 2006, 2008). These findings suggest that CBF genes from several crops can increase a plant's resistance to drought, excessive salt, and cold stress. Moreover, some studies have shown that CBFs are also involved in heavy metal stress, such as copper stress (Ban et al. 2011), and cadmium stress (Charfeddine et al. 2017). In this study, 16 differentially expressed genes were found in drought stress, 7 differentially expressed genes in waterlogging stress, 6 differentially expressed genes in copper stress, and 8 differentially expressed genes against salt stress were detected. Interestingly, among the differentially expressed genes, *VviCBF3*, *VviCBF7*, *VviCBF13*, *VviCBF17*, and *VviCBF18* response to drought stress, salt stress, waterlogging stress, and copper stress resistance, suggesting their key roles in abiotic stress.

Downy mildew is one of the main diseases of grapes, but the researches on the CBFs function in downy mildew development were limited. Therefore, our analysis shows that CBF genes have the potential role to work against downy mildew (Figure 8E). Our analysis shows that *VviCBF4*, *VviCBF7*, *VviCBF13*, *VviCBF18*, *VviCBF5*, and *VviCBF17* genes were up-regulated in resistant cultivar, on the other hand, *VviCBF4*, *VviCBF7*, *VviCBF13*, *VviCBF18*, and *VviCBF5* were down-regulated except *VviCBF17* in the susceptible cultivar. The inconsistent role of the *CBF* genes against downy mildew resistance has indicated that further research needs to be conducted in the upcoming days.

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CONCLUSION

To conclude, in this study we carried out a systematic way to identify the characterization of *CBF* gene family members in grapevine plants. In this study, 18 *CBF* genes were identified in the grapevine genome. Phylogenetic analysis showed that the *VviCBF* gene family was divided into five subgroups. In addition, chromosomal location, promoter *cis*-acting elements, and gene duplication were performed. Expression analysis showed that 6 *VviCBF* genes including *VviCBF5*, *VviCBF13*, *VviCBF14*, *VviCBF15*, *VviCBF16*, and *VviCBF18* are involved in the regulation of fruit development, four *VviCBF* genes *VviCBF1*, *VviCBF3*, *VviCBF6*, and *VviCBF11* were expressed at bud dormancy stage and 9 *VviCBF* genes including *VviCBF4*, *VviCBF5*, *VviCBF7*, *VviCBF12*, *VviCBF13*, *VviCBF15*, *VviCBF16*, *VviCBF17*, and *VviCBF18* increased their expression at breaking bud dormancy stage. Moreover, many *VvCBFs* were found to be involved in abiotic and biotic stress in this study. Therefore, our results show the preliminary evidence of the *CBF* gene family members in grapevine plants and lay a solid foundation in terms of future studies on molecular, biological, and physiological functions of the *VviCBF* genes in grapes.

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