Physiological and Biochemical Responses to Cyanamide-Induced Grape Dormancy Breaking and Cloning and Expression Analysis of Related Genes: Postprint

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Abstract

To investigate the physiological and biochemical responses and molecular regulatory mechanisms underlying cyanamide-induced dormancy release in grapevines, this study utilized 'Shuijing' grape (Vitis vinifera × V. labrusca 'Shuijing') as experimental material. The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), and the production rate of oxygen free radicals in buds were measured following cyanamide treatment. Additionally, full-length cDNA sequences of two FT (Flowering locus T) genes (VvFT1 and VvFT2) and one CBF (C-repeat binding factor) gene (VvCBF) were cloned from grape buds via RT-PCR. Their physicochemical properties, phylogenetic relationships, conserved motifs, structural domains, and differential expression levels in grape buds after cyanamide treatment were analyzed. The results demonstrated: (1) Physiological and biochemical analyses revealed that cyanamide treatment significantly increased the activities of SOD, POD, and CAT, contents of MDA and H_2O_2, and the oxygen free radical production rate in grape buds. (2) The full-length cDNA of VvFT1 and VvFT2 in 'Shuijing' grape was 525 bp, encoding 174 amino acids (aa); the full-length cDNA of VvCBF was 714 bp, encoding 237 aa. (3) Homology analysis indicated that VvFT1 of 'Shuijing' grape exhibited the highest amino acid homology with lychee (Litchi chinensis, LcFT: AEU08960.1) and longan (Dimocarpus longan, DlFT2: ALA55998.1), while VvFT2 showed the highest amino acid homology with LcFT (AEU08961.1) and DlFT2 (AHF27444.1). Phylogenetic analysis revealed that VvFT1, VvFT2, LcFT (AEU08960.1; AEU08961.1), and DlFT2 (ALA55998.1; AHF27444.1) clustered into a single clade, indicating the closest phylogenetic relationship. VvCBF displayed the highest amino acid homology with wild almond (Prunus

ledebouriana, PlCBF: AEB69782.1), and phylogenetic analysis showed that VvCBF and PlCBF clustered into a single clade, reflecting the closest phylogenetic relationship. (4) qRT-PCR analysis demonstrated that cyanamide treatment significantly upregulated the expression of VvFT1 and VvFT2 genes in grape buds, while significantly downregulating VvCBF gene expression. In conclusion, this study comprehensively analyzed the phylogenetic relationships of VvFT1, VvFT2, and VvCBF genes, as well as their expression patterns and associated physiological and biochemical changes in grape buds following cyanamide treatment, thereby establishing a theoretical foundation for research on cyanamide-mediated dormancy release in grapevines.

Full Text

Preamble

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Physiological and Biochemical Responses of Grape Dormancy Breaking with Cyanamide and Cloning and Expression Analysis of Related Genes

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Abstract

To investigate the physiological and biochemical responses and molecular regulatory mechanisms underlying grape dormancy breaking by cyanamide, this study used 'Shuijing' grape (Vitis vinifera×V. labrusca 'Shuijing') as experimental material. We determined changes in the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), and the rate of oxygen free radical production in grape buds following cyanamide treatment. Additionally, we cloned the full-length cDNA sequences of two Flowering locus T (FT) genes (VvFT1 and VvFT2) and one C-repeat binding factor (CBF) gene (VvCBF) from grape buds via RT-PCR technology, and analyzed their physicochemical properties, phylogenetic evolution, conserved motifs and domains, and differential expression levels in grape buds after cyanamide treatment. The results were as follows: (1) Physiological and biochemical analyses revealed that SOD, POD, and CAT activities, MDA and H_2O_2 contents, and oxygen free radical production rate in grape buds were all significantly increased after cyanamide treatment. (2) The full-length cDNA sequences of VvFT1 and VvFT2 from 'Shuijing' grape were 525 bp, encoding 174 amino acids (aa); the VvCBF cDNA was 714 bp, encoding 237 aa. (3) Homology analysis showed that 'Shuijing' grape VvFT1 had the highest amino acid homology with litchi (Litchi chinensis, LcFT: AEU08960.1) and longan (Dimocarpus longan, DlFT2: ALA55998.1), while VvFT2 showed highest

homology with LcFT (AEU08961.1) and DlFT2 (AHF27444.1). Phylogenetic analysis revealed that VvFT1, VvFT2, LcFT (AEU08960.1; AEU08961.1), and DlFT2 (ALA55998.1; AHF27444.1) clustered into one branch with the closest genetic relationship. VvCBF showed highest amino acid homology with wild almond (Prunus ledebouriana, PlCBF: AEB69782.1), and phylogenetic analysis indicated that VvCBF and PlCBF clustered into one branch with the closest relationship. (4) qRT-PCR analysis demonstrated that VvFT1 and VvFT2 expression levels were significantly upregulated in grape buds after cyanamide treatment, whereas VvCBF expression was significantly downregulated. In summary, this study comprehensively analyzed the phylogenetic evolution of VvFT1, VvFT2, and VvCBF genes, as well as the expression patterns of these genes and changes in physiological and biochemical indicators in grape buds following cyanamide treatment, thereby establishing a theoretical foundation for understanding the mechanisms of grape dormancy breaking by cyanamide.

Keywords: cyanamide, grape, dormancy, transcription factors, VvCBF, VvFTs

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Grape is one of the world's most important fruit crops and the fourth flowering plant to have its genome fully sequenced, following Arabidopsis, rice, and poplar (Yang et al., 2010). Grape exhibits strong seasonality with distinct growing and dormant periods (Liu, 2018). Dormancy is a pause in plant growth and development, representing a beneficial biological characteristic and an adaptive trait acquired through long-term evolution to cope with environmental conditions and seasonal changes, with bud dormancy being the most common form (Min and Fang, 2016). As a typical deciduous fruit tree, dormancy is an essential and unavoidable stage in grape development. With the continuous expansion of protected grape cultivation in northern China, insufficient chilling accumulation has led to prolonged dormancy, delayed maturity, uneven budbreak, and reduced yield. Consequently, promoting dormancy release and early budbreak has become a critical technical challenge in grape production (Tan et al., 2021). Cyanamide is a cyanamide-class chemical agent that has been widely used to break grape dormancy, promoting early and uniform bud germination, particularly in regions with insufficient winter chilling and high humidity (Sudawan et al., 2016; Wang et al., 2016; Niu et al., 2018). However, the underlying mechanisms by which cyanamide breaks grape dormancy require further investigation.

CBF (C-repeat binding factor) and FT (Flowering locus T) genes are widely present in diverse plants and participate in multiple stages of plant growth, development, and abiotic stress responses. FT belongs to the phosphatidylethanolamine-binding protein (PEBP) family and contains a conserved PEBP domain (Hanano et al., 2011). It is a key gene determining flowering time in the photoperiod pathway (Yang et al., 2010), exhibits seasonal variation (Wang et al., 2022), and plays crucial roles in floral transition and reproductive organ development (Chen et al., 2011). Additionally, FT genes are important for storage organ development (Navarro et al., 2015), axillary bud formation (Hiraoka et al., 2013), stomatal opening (Kinoshita et al.,

2011), and plant and seed dormancy (Carmona et al., 2007; Vergara et al., 2016). CBF belongs to the AP2/ERF (APETALA2/ethylene response protein) family and regulates transcription of target genes by binding to core motifs in promoters. CBF has been identified as a major hub in plant cold response pathways, enhancing cold tolerance by regulating downstream cold-responsive genes (Lü et al., 2011; Zhao et al., 2020). CBF also plays important roles in drought, high salinity, and extreme temperature stresses (Chen et al., 2016), as well as in plant growth (Zhao, 2013) and dormancy (Wisniewski et al., 2011), though its role in plant dormancy remains understudied. In 2011, Wisniewski et al. (2011) reported that ectopic expression of peach CBF in apple induced short-day-mediated dormancy. Saito et al. (2015) found that CBF could regulate Dormancy-associated MADS-box (DAM) genes to affect plant dormancy, with FT genes also participating in this process. In summary, both CBF and FT genes play important roles in plant dormancy (Carmona et al., 2007; Wisniewski et al., 2011; Saito et al., 2015; Vergara et al., 2016) and are closely linked, but whether they are involved in cyanamide-mediated grape dormancy breaking warrants in-depth investigation.

'Shuijing' grape (Vitis vinifera×V. labrusca 'Shuijing') originates from the Americas and exhibits yellow-green color at maturity, with characteristics including thick flesh, high juice content, high sugar content, good palatability, and high soluble solids, offering considerable nutritional value (Tian et al., 2021). Cyanamide is commonly used as a dormancy-breaking agent for 'Shuijing' grape to promote early bud germination. However, the gene sequences and protein functions of CBF and FT, which serve as important regulatory factors in plant dormancy release, have not been reported in 'Shuijing' grape. Therefore, this study used 'Shuijing' grape as material to clone the full-length cDNA sequences of CBF and FT genes involved in bud dormancy via RT-PCR after cyanamide treatment, and analyzed their expression using quantitative real-time RT-PCR (qRT-PCR). Combined with measurements of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities, malondialdehyde (MDA) and H_2O_2 contents, and oxygen free radical production rate in grape buds after cyanamide spraying, we explored the molecular, physiological, and biochemical mechanisms underlying cyanamide-induced dormancy breaking in 'Shuijing' grape to provide a theoretical foundation for elucidating these mechanisms and guiding cyanamide application. This study aimed to address: (1) the physicochemical properties, structures, and evolutionary relationships of VvFT1, VvFT2, and VvCBF proteins; (2) expression pattern changes of VvFT1, VvFT2, and VvCBF genes during cyanamide-mediated dormancy breaking; and (3) changes in CAT, POD, and SOD activities, MDA and H_2O_2 contents, and oxygen free radical production rate during cyanamide-mediated dormancy breaking.

1.1 Materials and Reagents

Healthy and uniform winter-pruned 'Shuijing' grape canes were obtained from Dongfeng Farm Administration in Mile City, Yunnan Province. The canes were propagated in water and sprayed with 2.5% cyanamide once every three days (Liu, 2018; Qiu et al., 2022), with water spray serving as the control (CK). 'Shuijing' grape buds were collected on days 0, 7, 14, 21, and 28, immediately frozen in liquid nitrogen, and stored at -80°C. TPIzol reagent and DL 2000 DNA Marker were purchased from Takara Biotechnology (Beijing) Co., Ltd.

1.2 Determination of Physiological Indices

MDA and H_2O_2 contents were determined using the double-beam spectrophotometry method and titanium sulfate-concentrated ammonia colorimetric method, respectively. Oxygen free radical production rate was measured by the hydroxylamine hydrochloride method. SOD, POD, and CAT activities were determined using the nitroblue tetrazolium method, guaiacol method, and ultraviolet absorption method, respectively (Zou, 2000; Gao, 2006; Zhang et al., 2009).

1.3 Total RNA Extraction and cDNA Synthesis from Grape Buds

'Shuijing' grape buds were ground into powder in a mortar with liquid nitrogen. The powder was quickly transferred to a 2.0 mL sterile centrifuge tube, mixed gently with 1 mL TPIzol reagent, then 200 L chloroform was added and incubated at room temperature for 5 min. After centrifugation at 12,000 $r \cdot min^{-1}$ for 10 min at 4°C, the supernatant was transferred to a 1.5 mL sterile tube, mixed with 500 L isopropanol, and centrifuged again at 12,000 $r \cdot min^{-1}$ for 10 min at 4°C. The supernatant was discarded, and the pellet was washed twice with 1 mL 75% ethanol, gently pipetted several times each time. After air-drying the pellet for 2-3 min at room temperature, 100 L DEPC water was added to dissolve the RNA, gently pipetted to mix, and stored at -80°C. First-strand cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. The obtained cDNA was immediately used for subsequent PCR amplification or stored at -80°C.

1.4 Full-Length cDNA Cloning of VvFTs and VvCBF

Multiple FT (GenBank: JN214350.1, KP099711.1, JN214351.1, KF881011.1) and CBF (GenBank: HQ908653.1, JX464665.1, XM_{021947969}.1, KU587043.1) homologous gene sequences from GenBank were aligned, and primers were designed using Premier 5.0 and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Primer sequences are listed in Table 1 . PCR amplification was performed using PrimeSTAR HS® DNA Polymerase in a 50 L reaction containing 10 L 5× PrimeSTAR Buffer (Mg^{2+} Plus), 4 L dNTP

Mixture (2.5 mmol · L^{-1} each), 0.5 L PrimeSTAR HS DNA Polymerase (2.5 U · L^{-1}), 1 L each of forward (F) and reverse (R) primers, 2 L cDNA, and 33.5 L sterile distilled water. The PCR conditions were: 98°C for 2 min; 30 cycles of 98°C for 10 s, 58°C for 10 s, and 72°C for 60 s; final extension at 72°C for 2 min. PCR products were electrophoresed on 1.0% agarose gel, and target bands were excised and recovered. An A-tail was added to the 3' end of the target fragment using the Mighty TA-cloning Reagent Set for PrimeSTAR®, then ligated into the pMD20-T vector and transformed into *E. coli* DH5α competent cells. Positive clones were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Two FT genes were obtained and named VvFT1 and VvFT2, and one CBF gene was obtained and named VvCBF.

1.5 Bioinformatics Analysis of VvFT1, VvFT2, and VvCBF Genes

DNAMAN8 was used for nucleotide sequence alignment and protein sequence translation. The ProtParam tool (http://web.expasy.org/protparam/) was used to analyze protein molecular weight and theoretical isoelectric point. Online tools GOR4 (http://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{gor4}.html) and NetSurfP3.0 (https://services.healthtech.dtu.dk/services/NetSurfP-3.0/) were used to predict protein secondary structure. SWISS-MODEL (http://www.swissmodel.expasy.org/interactive) was used for tertiary structure prediction. The CDD database in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to analyze conserved domains. MEGA-X software was used to construct phylogenetic trees using the Neighbor-Joining method with the Poisson model, complete deletion, and 1,000 bootstrap replicates.

1.6 qRT-PCR Detection of VvFT1, VvFT2, and VvCBF Genes

First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit (Perfect Real Time), followed by qRT-PCR using TB Green Premix Ex Taq II (Tli RNaseH Plus) according to the manufacturer's instructions. The UBQ gene was used as an internal reference (Zhang et al., 2022). Expression of VvFT1, VvFT2, and VvCBF genes in cyanamide-induced 'Shuijing' grape buds was detected by qRT-PCR using primers listed in Table 1.

2.1 Changes in MDA, SOD, POD, CAT, H_2O_2, and Oxygen Free Radical Production Rate in 'Shuijing' Grape Buds After Cyanamide Spraying

MDA, SOD, POD, CAT, H_2O_2, and oxygen free radical production rate are important physiological and biochemical indicators of bud germination. We measured these indicators in 'Shuijing' grape buds on days 0, 7, 14, 21, and 28 after cyanamide treatment. The results showed that all these parameters were

enhanced at different stages following cyanamide application. Specifically, MDA content, H_2O_2 content, and oxygen free radical production rate in cyanamide-treated buds showed a trend of initial increase followed by decrease across the sampling time points, whereas these parameters in untreated buds showed a continuous increasing trend (Figure 1 [Figure 1: see original paper]). Under cyanamide treatment, MDA content peaked on day 21, H_2O_2 content peaked on day 7, and oxygen free radical production rate peaked on day 14. Notably, all measured parameters in cyanamide-treated buds were higher than those in the CK on days 7, 14, 21, and 28. Compared with CK, cyanamide treatment increased MDA content by 98.20%, 96.22%, 96.05%, and 47.29% on days 7, 14, 21, and 28, respectively (Figure 1A). SOD and POD can decompose reactive oxygen species to prevent membrane lipid oxidation, and their activities positively correlate with this protective capacity. SOD activity increased by 23.51%, 49.73%, 45.80%, and 52.78% on days 7, 14, 21, and 28, respectively (Figure 1B). POD activity increased by 59.34%, 41.03%, 40.55%, and 43.00% on days 7, 14, 21, and 28, respectively (Figure 1C). CAT activity increased by 80.86%, 136.18%, 115.21%, and 100.65% on days 7, 14, 21, and 28, respectively (Figure 1D). H_2O_2 content increased by 67.51%, 31.31%, 20.88%, and 26.51% on days 7, 14, 21, and 28, respectively (Figure 1E). Oxygen free radical production rate increased by 149.48%, 161.41%, 80.39%, and 6.13% on days 7, 14, 21, and 28, respectively (Figure 1F). These results demonstrate that cyanamide spraying can break grape dormancy and promote early germination by inducing physiological and biochemical responses including changes in MDA, SOD, POD, CAT, H_2O_2, and oxygen free radical production rate. On day 28 after cyanamide treatment, grape bud growth was significantly longer than that in CK (Figure 2 [Figure 2: see original paper]).

2.2 Full-Length cDNA Cloning of Grape VvFT1, VvFT2, and VvCBF Genes

To elucidate the molecular mechanism of cyanamide-mediated dormancy breaking in 'Shuijing' grape, we extracted total RNA from grape buds on day 28 after cyanamide treatment, synthesized cDNA, and amplified VvFT1, VvFT2, and VvCBF genes using specific primers. The amplified fragments of VvFT1 and VvFT2 were approximately 500 bp, while VvCBF was approximately 800 bp, all consistent with the expected sizes (Figure 3 [Figure 3: see original paper]). Sequencing of the cloned genes revealed that VvFT1 and VvFT2 were 525 bp, while VvCBF was 826 bp. Analysis using DNAMAN 8.0 showed that VvFT1 and VvFT2 encoded 174 amino acids, while VvCBF encoded 237 amino acids with a stop codon at nucleotides 712-715.

2.3.1 Physicochemical Properties and Protein Structure Prediction of VvFT1 and VvFT2 Amino Acid Sequences

The ProtParam tool was used to predict the polypeptide chain length, molecular formula, isoelectric point, and relative molecular weight of VvFT1 and VvFT2.

VvFT1 encoded 174 aa with a molecular formula of $C_{874}H_{1359}N_{247}O_{256}S_4$, molecular mass of 19.55 kDa, theoretical pI of 8.81, and contained 2,740 atoms. VvFT2 encoded 174 aa with a molecular formula of $C_{856}H_{1335}N_{245}O_{263}S_6$, molecular mass of 19.46 kDa, pI of 6.11, and contained 2,705 atoms. Secondary structure prediction using GOR4 and NetSurfP 3.0 revealed that VvFT1 protein consisted of 62.64% random coil, 9.77% alpha helix, and 27.59% extended strand (Figure 4A), while VvFT2 consisted of 70.11% random coil, 2.30% alpha helix, and 27.59% extended strand (Figure 4B). Tertiary structure prediction using SWISS-MODEL showed that both VvFT1 (Figure 4C) and VvFT2 (Figure 4D) contained random coil, alpha helix, and extended strand structures, with random coil being the most abundant component, consistent with secondary structure predictions. In conclusion, the protein structures encoded by VvFT1 and VvFT2 were dominated by random coil, followed by extended strand and alpha helix. Random coil structures primarily determine protein function, suggesting that VvFT1 and VvFT2 may play important roles in cyanamide-mediated grape dormancy breaking.

2.3.2 Conserved Domain Analysis of VvFT1 and VvFT2 Amino Acids

The NCBI CDD database was used to predict conserved domains in grape VvFT1 and VvFT2 proteins. Both proteins contained a PEBP superfamily domain with a substrate binding site at amino acid positions 70-119 (Figure 5A, B). BLAST analysis identified the two most homologous FT proteins for each: for VvFT1, litchi (Litchi chinensis, LcFT: AEU08960.1) and longan (Dimocarpus longan, DlFT2: ALA55998.1) with 94.83% and 93.68% amino acid identity, respectively; for VvFT2, litchi (LcFT: AEU08961.1) and longan (DlFT2: AHF27444.1) with 95.98% and 93.68% identity, respectively. Multiple sequence alignment using DNAMAN revealed that VvFT1 and VvFT2 contained DPPAP and GIHR motifs characteristic of the PEBP family functional domain, as well as key conserved amino acid residues Tyr84 (Y) and Gln139 (Q) and the 11-residue conserved sequence (RQTxYAPGWRQ) critical for FT activity (Figure 5C). In summary, both NCBI CDD database analysis and homology alignment demonstrated that VvFT1 and VvFT2 possess typical PEBP family functional domains that are highly conserved, indicating their importance for protein function.

2.3.3 Phylogenetic Tree Construction of VvFT1 and VvFT2 Proteins

Sequence analysis revealed 90.29% nucleotide identity and 83.3% amino acid identity between VvFT1 and VvFT2. The VvFT1 and VvFT2 protein sequences were BLASTed to download the top 10 homologous plant FT protein sequences plus two additional grape FT proteins. Phylogenetic trees were constructed using MEGA-X. The most homologous FT proteins to VvFT1 were LcFT (AEU08960.1), DlFT2 (ALA55998.1), cork oak (*Quercus*

suber, QsFT: XP_{023899320}.1), Japanese beech (*Fagus crenata*, FcFT: BAP28173.1), jujube (*Ziziphus jujuba*, ZjFT: XP_{015873598}.1), parasponia (*Parasponia andersonii*, PanFT: PON55382.1), cacao (*Theobroma cacao*, TcFT: XP_{007028083}.1), white poplar (*Populus alba*, PaFT: KAJ6891859.1), Chinese white poplar (*Populus tomentosa*, PtFT: AFU08240.1), honeysuckle (*Lonicera japonica*, LjFT: WGO49542.1), and two grape VvFT proteins (ABF56526.1 and NP_{001267907}.1), with identities of 94.83%, 93.68%, 86.78%, 85.06%, 85.63%, 84.48%, 84.48%, 83.91%, 83.91%, 83.33%, 80.46%, and 79.31%, respectively. The most homologous FT proteins to VvFT2 were LcFT (AEU08961.1), DlFT2 (AHF27444.1), cork oak, Japanese beech, papaya (*Carica papaya*, CpFT: XP_{021911503}.1), ZjFT (XP_{015873598}.1), witchweed (*Striga hermonthica*, ShFT: CAA0821245.1), black cottonwood (*Populus trichocarpa*, PtFT: XP_{002311264}.1), rose (*Rosa chinensis*, RcFT: XP_{024189593}.1), loquat (*Rhaphiolepis bibas*, RbFT2: ALA56300.1), and two grape VvFT proteins (ABF56526.1 and NP_{001267907}.1), with identities of 95.98%, 93.68%, 85.63%, 85.06%, 84.48%, 84.48%, 84.48%, 83.33%, 83.33%, 83.33%, 81.03%, and 79.89%, respectively. The phylogenetic analysis revealed complex evolution of FT genes, with VvFT1 and VvFT2 clustering with litchi and longan FT proteins in a distinct branch, while other plant FT proteins formed separate branches, indicating early and substantial divergence of FT among species (Figure 6 [Figure 6: see original paper]).

2.4.1 Physicochemical Properties and Protein Structure Prediction of VvCBF Amino Acid Sequence

The ProtParam tool predicted that VvCBF encoded 237 aa with a molecular formula of $C_{1158}H_{1839}N_{335}O_{354}S_{15}$, molecular mass of 26.60 kDa, pI of 8.31, and contained 3,701 atoms. Secondary structure prediction using GOR4 revealed that VvCBF protein consisted of 32.91% alpha helix, 22.36% extended strand, and 44.73% random coil (Figure 7A). Tertiary structure prediction using SWISS-MODEL showed that random coil and alpha helix were most abundant, with extended strand being the least abundant, consistent with secondary structure predictions (Figure 7B).

2.4.2 Conserved Domain Analysis of VvCBF Amino Acids

The NCBI CDD database predicted that grape VvCBF protein contained an AP2 superfamily domain with a DNA binding site at amino acid positions 58-86 (Figure 8A). BLAST analysis identified the two most homologous CBF proteins: wild almond (*Prunus ledebouriana*, PlCBF: AEB69782.1) and mountain peach (*Prunus davidiana*, PdCBF: AFU54607.1) with 100% and 95.40% amino acid identity, respectively. Multiple sequence alignment revealed that VvCBF contained an AP2 DNA binding domain flanked by the characteristic sequences PKK/RAGRRVFKETRHP and DSAWR, typical of the CBF transcription factor family (Figure 8B).

2.4.3 Phylogenetic Tree Construction of VvCBF Protein

The VvCBF protein sequence was BLASTed to download the top 10 homologous plant CBF protein sequences plus two additional grape CBF proteins. Phylogenetic analysis revealed that the most homologous CBF proteins to VvCBF all belonged to Rosaceae: PlCBF (AEB69782.1), PdCBF (AFU54607.1), sweet cherry (*Prunus avium*, PaCBF: XP_{021803661}.1), Japanese apricot (*Prunus mume*, PmuCBF: ANW82784.1), Tibetan peach (*Prunus mira*, PmCBF: AFU54606.1), peach (*Prunus persica*, PpeCBF: XP_{007209842}.2), Yoshino cherry (*Prunus yedoensis*, PyCBF: PQQ14279.1), almond (*Prunus dulcis*, PduCBF4: BBH03298.1), apricot (*Prunus armeniaca*, ParCBF: AWR88517.1), cherry (*Prunus pseudocerasus*, PpCBF: AIU39990.1), and two grape VvCBF4 proteins (AFV73334.1 and AIL00786.1), with identities of 100%, 95.40%, 94.94%, 94.94%, 94.14%, 94.14%, 94.09%, 94.09%, 78.01%, 74.69%, 39.66%, and 39.66%, respectively. VvCBF clustered with PlCBF (AEB69782.1) in one branch, while PpCBF (AIU39990.1) and ParCBF (AWR88517.1) each formed separate branches, and the two VvCBF4 proteins each formed individual branches, with other plant CBF proteins clustering separately. This indicates that VvCBF has the highest homology and closest relationship with wild almond CBF protein (Figure 9 [Figure 9: see original paper]).

2.5 Relative Expression Levels of VvFT1, VvFT2, and VvCBF Genes in 'Shuijing' Grape Buds After Cyanamide Spraying

qRT-PCR analysis revealed that after cyanamide spraying, VvFT1 and VvFT2 expression levels were significantly increased on days 7, 14, 21, and 28 compared with CK. VvFT1 expression increased by 120.12% on day 14, 47.13% on day 21, and 57.76% on day 28 (Figure 10A). VvFT2 expression increased by 390.08% on day 7, 142.32% on day 14, 129.20% on day 21, and 64.90% on day 28 (Figure 10B). In contrast, VvCBF expression decreased by 26.45% on day 7, 50.30% on day 14, 39.87% on day 21, and 13.05% on day 28 (Figure 10C). Previous studies have shown that FT and CBF genes play important roles in plant dormancy and are negatively correlated. Our results demonstrate that cyanamide spraying upregulates VvFT1 and VvFT2 expression while downregulating VvCBF expression, suggesting that cyanamide induces dormancy breaking and promotes early germination by modulating the expression of these key regulatory genes.

3 Discussion and Conclusion

'Shuijing' grape has high nutritional value and is popular among consumers (Tian et al., 2021). Cyanamide is commonly used as a dormancy-breaking agent for 'Shuijing' grape to promote early bud germination, but the mechanisms of FT and CBF in grape dormancy breaking remain poorly understood (Vergara et al., 2016). This study first measured changes in SOD, POD, and CAT activities, MDA and H_2O_2 contents, and oxygen free radical production rate in 'Shui-

jing' grape buds after cyanamide treatment. We then cloned VvFT1, VvFT2, and VvCBF genes involved in this process, performed bioinformatics analysis, and analyzed expression patterns, establishing a foundation for understanding the physiological and molecular mechanisms of cyanamide-mediated grape dormancy breaking.

Shao et al. (2004) reported that physiological and biochemical parameters including CAT, POD, SOD, MDA, H_2O_2, and oxygen free radical production rate are important indicators of dormancy release. In pear, oxygen free radical production rate and H_2O_2 content showed a trend of initial increase followed by decrease during dormancy release, while SOD and POD activities increased, consistent with our results in cyanamide-treated 'Shuijing' grape. However, CAT activity decreased in pear, contrary to our findings. POD and CAT are important H_2O_2-scavenging enzymes that decompose H_2O_2 into O_2 and H_2O, constituting the antioxidant enzyme defense system (Mittler, 2002; Tan et al., 2021). In our study, cyanamide treatment increased both POD and CAT activities, which were higher than CK on days 7, 14, 21, and 28. H_2O_2 content peaked on day 7 and subsequently decreased, indicating that elevated POD and CAT activities effectively promoted H_2O_2 decomposition, reducing H_2O_2 content and facilitating bud germination.

Studies have shown that FT transcription factors play important roles in regulating dormancy in narcissus and kiwifruit (Varkonyi-Gasic et al., 2013; Feng et al., 2015). Our results also demonstrate that FT transcription factors are important in cyanamide-mediated grape bud dormancy breaking. The AP2/ERF superfamily is a large plant-specific transcription factor family involved in growth, development, and stress responses. Based on the number of AP2 domains and other DNA-binding domains, the AP2/ERF superfamily is divided into four families: AP2, ERF, RAV, and Soloist. The ERF family is further divided into ERF and DREB subfamilies, with CBF belonging to the DREB subfamily (Mizoi et al., 2012; Shu et al., 2015). A 2018 study showed that ERF transcription factors from the AP2/ERF superfamily are involved in cyanamide-mediated grape dormancy breaking (Wu, 2018), but whether DREB subfamily transcription factors participate in this process had not been reported. Our study demonstrates that the CBF transcription factor VvCBF from 'Shuijing' grape is also involved in cyanamide-mediated dormancy breaking. Future research should investigate whether other AP2/ERF superfamily members, such as AP2 and RAV transcription factors, are also involved.

CBF is reportedly an upstream trans-acting factor of DAM (Dormancy-associated MADS-box) genes during cold stress and dormancy, while DAM can directly regulate FT transcription factor expression, indicating that CBF and FT can influence each other through DAM in a negative correlation (Horvath et al., 2008; Saito et al., 2015), consistent with our results. During cyanamide-mediated dormancy breaking in 'Shuijing' grape buds, VvFT1 and VvFT2 expression was upregulated over time, while VvCBF expression was downregulated, showing a negative correlation between VvFTs and VvCBF

expression.

Through BLAST analysis, we downloaded the top 10 proteins with highest amino acid homology to VvFT1, VvFT2, and VvCBF. Only two proteins showed >90% homology with VvFT1 [LcFT (AEU08960.1) and DlFT2 (ALA55998.1)], and only two showed >90% homology with VvFT2 [LcFT (AEU08961.1) and DlFT2 (AHF27444.1)]. These four proteins belong to litchi and longan FT genes, and phylogenetic analysis showed that VvFT1, VvFT2, LcFT (AEU08960.1), DlFT2 (ALA55998.1), LcFT (AEU08961.1), and DlFT2 (AHF27444.1) clustered together, indicating close evolutionary relationship among FT transcription factors from 'Shuijing' grape, litchi, and longan, though functional complementation requires further investigation. Eight proteins showed >94% homology with VvCBF, all belonging to Rosaceae. Except for PlCBF (AEB69782.1), which is currently classified in the genus *Prunus* of Rosaceae (Orazov et al., 2022), the other nine CBF transcription factors belong to the genus *Prunus*, indicating important evolutionary connections between 'Shuijing' grape CBF and Rosaceae (especially *Prunus*) CBF transcription factors.

In this study, we obtained VvFT1, VvFT2, and VvCBF genes from 'Shuijing' grape through cDNA cloning and performed detailed bioinformatics functional prediction analysis of their encoded proteins, providing a reference for functional studies of these genes. Using qRT-PCR, we analyzed the transcriptional expression of VvFT1, VvFT2, and VvCBF and found that VvFT1 and VvFT2 expression was inversely correlated with VvCBF expression, and all were affected by cyanamide treatment, indicating that these genes play important roles in cyanamide-mediated grape bud dormancy breaking. Additionally, we measured multiple physiological and biochemical indicators in grape buds after cyanamide treatment, further explaining the mechanism of cyanamide-mediated dormancy breaking from a physiological perspective and providing theoretical guidance for grape production.

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Note: Figure translations are in progress. See original paper for figures.