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Dynamics of starch degradation and expression of related genes during chilling stress in grapevine

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Abstract

Frost in late spring is one form of environmental stress that severely damages grapevines. Starch is a major product of photosynthesis that plays essential roles in many biological processes in plants. The dynamics of starch metabolism and related gene expression in the leaves of grapevine during chilling stress have remained unclear. Here, starch metabolism in the leaves of *Vitis vinifera* cv. Cabernet Sauvignon was characterized under normal (25℃) and late-spring frost mimic (4℃) conditions. The results from anthracenone colorimetry, iodine staining and ultrathin sectioning are consistent with a low temperature during the night inhibiting the degradation of starch. Four *α-amylases* (*AMY*) and ten *β-amylases* (*BAM*) genes were identified in the *V. vinifera* genome (PN40024). Low nighttime temperatures downregulated the expression of genes that encode amylases relative to optimal nighttime temperatures. The expression of other genes with functions related to starch degradation, such as *like starch excess four 1* (*LSF1*) and *isoamylase 3* (*ISA3*), was induced by low temperature. The glucose, sucrose, maltose, and fructose contents increased in plants grown under low-temperature conditions with less consumption of starch, indicating complex regulation of soluble sugars. The findings here provide clues that will lead to enhanced frost tolerance of grapevine leaves by modifying the starch degradation pathway.

Keywords Grapevine, Frost in late spring, Starch, *α-amylases* (*AMY*), *β-amylases* (*BAM*)

Introduction

Frost in late spring is a climate phenomenon characterized by the temperature suddenly dropping below zero degrees centigrade. This phenomenon often causes freezing damage to newly germinated seedlings or buds on plants and thus decreases the yield and quality of crops (Rodrigo et al. 2000). Plants have evolved several strategies to avoid damage from chilling or freezing temperatures, such as enhanced reactive oxygen species (ROS) scavenging and osmotic regulation by accumulating soluble sugars and proteins (Koster and Lynch 1992; Renaut et al. 2004; Patton et al. 2007; Theocharis et al. 2012; Saddhe et al. 2020). Uncovering the regulatory mechanisms underpinning the low-temperature response will provide key information for improving the chilling tolerance of plants by genetic engineering.

Starch is one of the main end products of photosynthesis, accumulates in plastids and is the most widely distributed nonstructural carbohydrate in plants (Sulpice 2009; Stitt and Zeeman 2012; Streb and Zeeman 2012). As the

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main energy storage carbohydrate, starch plays an important role in different developmental stages and multiple stress responses in plants (Smith et al. 1993; Thalmann, et al. 2017). Starch usually accumulates as a major end product of photosynthesis during the day and is almost completely degraded into hexose and transported into sink tissues during the night (Lloyd et al. 2005). ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthetase (SSS), starch branching enzyme (SBE) and debranching enzyme (DBE) are key enzymes responsible for starch biosynthesis (Orzechowski 2008; Yu et al. 2015; Smith 2012). Genes that encode α-amylase (AMY), β-amylase (BAM) and debranching enzymes are responsible for the degradation of starch (Fulton et al. 2008; Kötting et al. 2005; Streb and Zeeman, 2012).

The processes of starch accumulation and degradation are modified under stress conditions to enhance tolerance to harsh environments (Thalmann et al. 2016; Zanella et al. 2016; Zhu et al. 2021). Previous studies have demonstrated essential roles for starch in the supply of metabolites and energy required to tolerate cold stress in plants. Maltose, the main product of starch breakdown, increased rapidly when *Arabidopsis thaliana* was subjected to low temperature stress (Kaplan and Guy et al. 2004). Starch is hydrolyzed to osmotic substances, such as hexose, which increases cell fluid concentrations to help cope with low-temperature stress (Savitch, 1997; Sicher et al. 2011; Sitnicka et al. 2014; Dong et al. 2019). Compared with *AMY* genes, *BAM* genes were found to be more closely correlated with starch degradation during stress responses in plants (Yu et al. 2005; Peng et al. 2014; Monroe et al. 2014; Purdy et al. 2013). For example, the overexpression of *BAM3* in *Nicotiana tabacum* and *Pyrus betulaefolia* promoted the degradation of starch into soluble sugars as a penetrant or antioxidant to improve cold tolerance (Zhao et al. 2019). Overexpression of cold-inducible *AaBAM3.1* improved the cold tolerance of kiwifruit by increasing the soluble sugar content (Sun et al. 2021). These results indicate the potential utilization of *BAM* genes as key regulators for improving the cold tolerance of plants by genetic engineering.

Grapevine is cultivated in many countries. Newly germinated leaves, branches and inflorescences of grapevine often suffer from freezing stress during late-spring frost, which causes severe damage to grapevines and the vine industry. Previous studies have indicated that the expression of genes associated with starch pathways changes in response to chilling stress in grapevine. For example, the expression of an *AMY* gene was induced after chilling treatment in the daytime in leaves of *Vitis vinifera* (*V. vinifera*) and *V. amurensis* (Xin et al. 2013; Xu et al.

2014). The expression of five *BAM* genes was increased in grapevine leaves after 24 h and 72 h of chilling treatment at 4℃ (Chai et al. 2019). Overexpression of *VvBAM1* improved the cold hardiness of transgenic tomato by increasing the soluble sugar content and enhancing the ROS scavenging capacity (Liang et al. 2021). Nonetheless, the dynamics of starch metabolism and the expression of associated pathway genes in grapevine leaves in response to sudden exposure to low temperatures at night are still unknown.

In this study, the starch content of leaves from potted *V. vinifera* cv. Cabernet Sauvignon seedlings that were grown in optimal and frost-mimic conditions were quantified using different methods. The *AMY* genes in the grapevine (*V. vinifera* cv. Pinot noir PN40024) genome were identified, and *AMY* expression under chilling stress conditions was quantified. The dynamics of starch degradation products, such as glucose and maltose, and the expression of genes that encode enzymes related to these processes were also quantified. The results provide new clues for strategies that might enhance the cold tolerance of grapevine leaves during late spring frosts by regulating starch degradation.

Materials and methods

Plant materials and chilling treatment

One-year-old grafted seedlings of *V. vinifera* cv. Cabernet Sauvignon were grown in 5-L pots containing peat soil:vermiculite (1:1) at 26°C with 12 h of light (from 6:00 to 18:00) and 12 h of dark (18:00 to 6:00) in a greenhouse. Seedlings with 5–6 well-developed leaves were placed in an illuminated incubation chamber with a photoperiod containing 12 h of light. After 3 d, the third to fourth fully developed leaves were collected at 6:00, 12:00 and 18:00 at 25℃. Then, the temperature was reduced to 4°C to mimic frost conditions in the dark within 30 min, and the leaves were collected at 18:00, 21:00, 24:00, 3:00 and 6:00. Leaf samples grown under optimal conditions (25℃) were collected at the same time and served as controls. Three biological replicates were collected at each time point. Each biological replicate came from three plantlets. The collected samples were fixed with 2.5% glutaraldehyde suitable for electron microscopy. For iodine staining experiments and observation of ultrastructure and for gene expression, starch and sugar content analysis, tissue was suddenly frozen in liquid nitrogen and then stored at -80°C until it was analyzed.

Identification of the *AMY* gene families in grapevine

The amino acid sequences of AMY and BAM proteins from *Arabidopsis thaliana* (*A. thaliana*) and *Oryza sativa* were obtained from the TAIR database (https://www.

arabidopsis.org/) and RAP-DB (https://rapdb.dna.affrc.go.jp/), respectively. These amino acid sequences were used as queries in blastp searches for candidate *AMY* genes in the grape genome (https://phytozome-next.jgi.doe.gov/) with a cutoff e-value < 10^{-5}. To further identify all *AMY* and *BAM* genes in grapevine, NCBI CD searches and Pfam were used to identify the conserved domains in AMY (PF07821 and PF00128) and BAM (PF01373). Proteins containing conserved domains were retained. The amylase genes in grapevine were named based on the established amylase gene nomenclature in *A. thaliana*. Expasy-ProtParam (https://web.expasy.org/protparam/) was used to predict the chemical and physical properties of the amylase proteins. The location of these genes was obtained from genome annotation files (http://genomes.cribi.utnipd.it/DATA/). TargetP-2.0 (https://services.healthtech.dtu.dk/service.php?TargetP-2.0), Chlorop1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and WoLF PSORT (https://wolfpsort.hgc.jp/) were used to predict the subcellular localization of the amylase proteins.

All amylase amino acid sequences from grapevine, rice, and *A. thaliana* were aligned using ClustalW with the default settings. The neighbor-joining method in MEGA7.0 software was used to construct an unrooted phylogenetic tree with a bootstrap value of 1,000. All sequences containing the core functional domains of AMY and BAM were aligned using the ClustalX program with default settings followed by visualization with CLC Sequence Viewer6.0. The conserved motifs in amylase proteins were identified using Multiple Em for Motif Elicitation (MEME) (https://meme-suite.org/meme/tools/meme), a program available online.

Promoter *cis*-regulatory element analysis for *VvAMY* and *VvBAM* genes

Promoter regions were defined as 2,000 bp upstream of the translation start codon for each gene and were downloaded from the grapevine genome sequence (https://phytozome-next.jgi.doe.gov/). The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict conserved *cis* elements in the promoter sequences.

RNA isolation and quantitative real-time PCR (qRT–PCR)

The RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China) was used to extract total RNA from the samples according to the manufacturer's instructions. The concentration and integrity of the isolated total RNAs were determined using a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.2% agarose gel electrophoresis. cDNA was synthesized using TransScript One-step gDNA Removal and cDNA

Synthesis SuperMix (TransGen Biotech, Beijing, China). qRT–PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) with StepOne Plus (ABI, USA). The primers for each gene (Table S1) were designed using Primer Preimer 5. The *β-actin* gene (GenBank accession NO: EC969944) was chosen as a reference gene. The relative expression of each target gene was quantified relative to the reference gene using the $2^{-\Delta\Delta CT}$ method.

Iodine staining and transmission electron microscopy (TEM)

Samples collected at each time point were stained with iodine using published protocols (Eimert et al. 1995; Wang, et al. 2014). Briefly, leaves were depigmented in 95% ethanol for 24 h and then washed twice with deionized water. Rehydrated leaves were stained in an I_2-KI solution containing 8 g of iodine and 1 g of potassium iodide in 100 mL of distilled water for 10 min in the dark and then incubated in water until a clear background was obtained.

For TEM, leaves were fixed in electron microscopy fixation fluid (2.5% glutaraldehyde) overnight at 4℃. Leaves were rinsed in 0.1 M phosphate buffer, pH 7.2, three times. Each rinse was for 15 min. Samples were then fixed with 0.1 M phosphate buffer, pH 7.4, 1% osmic acid for 7 h at room temperature, followed by rinsing in 0.1 M phosphate buffer, pH 7.2, three times. Each rinse was for 15 min. Leaves were dehydrated in a series of 30%, 50%, 70%, 80%, 95%, 100%, 100% (1 h each). Tissues were penetrated in ethanol-acetone mixtures (3:1, 0.5 h; 1:1; 0.5 h; 1:3, 0.5 h (v:v)) and in acetone for 1 h. Leaves were embedded in acetone-EMBed 812 mixtures (3:1, 4 h; 1:1, overnight; 1:3, 4 h (v:v)) and in pure EMBed 812 for 8 h at 37℃. Finally, leaves were embedded in blocks that were incubated at 37°C overnight. Subsequently, samples were allowed to polymerize in a 65℃ oven for more than 48 h. Ultrathin Sects. (70 nm) were stained with uranyl acetate and lead citrate. TEM observations were performed on an HT7800 transmission electron microscope (Hitachi, Japan) with an accelerating voltage of 80 kV (Yandeau-Nelson et al. 2011).

Determination of starch content

Starch content was determined using the anthrone-sulfuric acid method (Magné et al. 2006). First, 0.2 g of leaves was ground with liquid nitrogen and added to 2 mL of 80% ethanol. Then, the samples were incubated in an 80°C water bath for 30 min followed by centrifugation at 3,000 *g* for 10 min. The supernatant was removed. Second, 2 mL of ultrapure water was added to the precipitate to facilitate gelatinization (15 min). Third, 2 mL

of 9.2 mol/L $HClO_4$ was added to the precipitate with stirring for 15 min. Then, 4 mL of ultrapure water was added to the mixture and mixed well. After centrifugation at 3,000 g for 10 min, the supernatant was transferred to a new tube. Fourth, 4.6 mol/L $HClO_4$ was added to the precipitate with stirring for 15 min, and then 5 mL of ultrapure water was added to the mixture and mixed well. After centrifugation at 3,000 g for 10 min, the supernatant was mixed with the previous supernatant. Finally, the precipitate was washed twice with ultrapure water, and the supernatants were mixed with the previous supernatant. Then, 2 mL of the supernatant was mixed with 5 mL of an anthrone-sulfuric acid solution containing 0.2 g of anthrone and 1.0 g of thiourea dissolved in 100 mL concentrated sulfuric acid and incubated in 100℃ water baths for 10 min. The sample was rinsed with running water at room temperature to promote cooling. The absorbance at 620 nm was measured with a spectrophotometer (TECAN, M200 PR, Austria). The standard curve was constructed with the absorbance values of solutions containing different concentrations of glucose (0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1 mg/mL) and used to calculate the concentration of starch in each sample.

Determination of soluble sugar content

The soluble sugar content was measured using high-performance liquid chromatography (HPLC, Zhao et al. 2018). A total of 0.2 g leaves was ground with a mortar and pestle and then transferred to tubes containing 2 mL of 80% ethanol. The tubes were incubated in an 80°C water bath for 30 min followed by centrifugation at 3000 g for 10 min to collect the supernatant. The precipitate was then washed twice with 80% ethanol and mixed with the previous supernatant. Then, the solution was evaporated under a stream of nitrogen and redissolved in ultrapure water. The solution was filtered through a PTFE 0.2 μm filter. HPLC was carried out using an Agilent 1290 Infinity II system (Germany) with an Agilent Polaris NH2 chromatographic column (250×4.6 mm, 5 μm). The flow rate was set at 0.8 mL/min, and the column temperature was 40°C. The mobile phase was acetonitrile–water (75:25, v/v). A refractive index detector was used and set at 40°C. The injection volume was 5 μL. Standard curves for glucose, fructose, sucrose, and maltose were prepared in the concentration range of 0.5–2 mg/mL with three replicates. The standard curve was plotted on the basis of sugar concentration and peak area. The slope, intercept and correlation coefficient of each curve were calculated using linear regression analysis. The concentration of each soluble sugar in the sample was calculated using the standard curve.

Results

Inhibition of starch degradation in grapevine leaves at a low temperature

In general, the rapid cooling of late-spring frosts occurs at night. To mimic late-spring frost conditions, we used a nighttime temperature of 4℃. The changes in starch content during the day (25℃) and night (both 25℃ and 4℃) were compared using both qualitative and quantitative methods. First, iodine staining was performed to observe starch in leaves. The blue color from the staining procedure increased gradually in leaves from 6:00 to 18:00, which indicated that the synthesis and accumulation of starch from photosynthesis increased during the day (Fig. 1a). The intensity of the stain diminished in the leaves that were collected during the night at 25℃, which is consistent with the almost complete degradation of starch in the leaves at night at 25℃. However, there was no obvious difference in the iodine staining of the leaves collected during the night at 4℃ relative to the leaves collected during the day at 25℃ (Fig. 1a), which indicates that the degradation of starch that occurs at 25℃ was blocked by the low temperature treatment. The ultramicroscopic structure of leaves was also examined to observe the starch grains in chloroplasts. As shown in Fig. 1b, the size of the starch grains increased dramatically during the day and then almost disappeared during the night at 25℃. In contrast, starch grains were still observable in the chloroplasts of the 4℃-treated leaves (Fig. 1b). Furthermore, we quantified the starch content using the anthrone-sulfuric acid method and found that the starch content in grapevine leaves accumulated at 25℃ during the day (6:00–18:00), reached a daily peak of 40 mg/g at 18:00 (Fig. 1c), and then declined to approximately 5 mg/g by 6:00 the following day. However, for the leaves subjected to the low temperature (4℃) treatment, the starch content was approximately 25 mg/g at 6:00 the next day. All these results demonstrate the diurnal dynamics of starch metabolism in grapevine leaves and that the frost stress mimic treatment inhibits the starch degradation that occurs during the night.

Identification and characterization of *VvAMY* and *VvBAM* genes in grapevine

We speculated that the reduced expression of *AMY* genes may explain the inhibition of starch degradation at low temperatures. We used blastp to identify the *AMY* and *BAM* genes in the *V. vinifera* cv. Pinot Nior PN40024 genome (Jaillon et al. 2007). The conserved domains were identified using NCBI CD and Pfam. In a previous study, six members of the *BAM* gene family were identified (Liang et al. 2021). Here, a total of four *VvAMY* genes and ten *VvBAM* genes were identified. Detailed information on these genes, including gene names, ORF
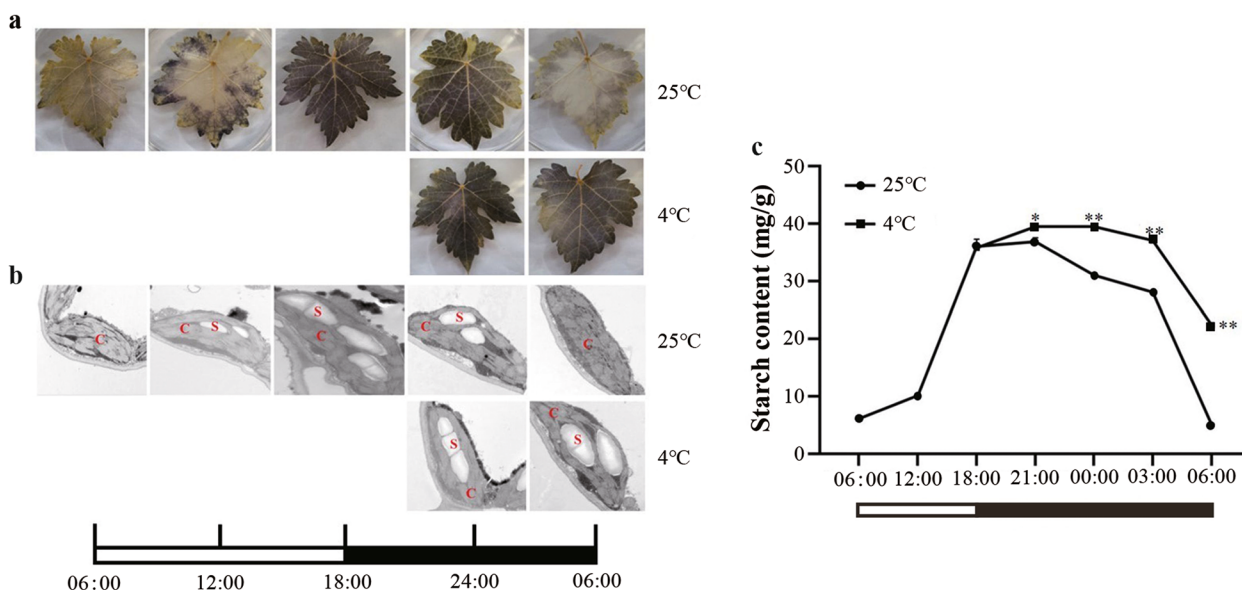
Fig. 1 Diurnal dynamics of starch content in grapevine leaves at optimal and cold temperatures (**a**) Iodine-stained grapevine leaves. (**b**) Ultrastructure of grapevine leaves. C, chloroplast; S, starch grain. (**c**) Starch content of grapevine leaves. The data points represent mean values ± SEs from three replicates. ** and * indicate statistically significant differences determined using Student's t test ($P < 0.01$ and $P < 0.05$, respectively) between the data collected at different temperatures

lengths, molecular weight and subcellular localizations of encoded proteins, is summarized in Table 1. The phylogenetic relationships among *AMY* genes from grapevine, *Arabidopsis* (Stanley et al. 2002; Monroe et al. 2018) and rice (Yue et al. 2019; Koide et al. 2011) were determined (Fig. 2a). Genes from the *AMY* or *BAM* families from different species form two clades. The results from the conserved motif prediction analysis showed that the VvAMY and VvBAM sequences contain similar motifs (Fig. 2b, c). We found structural variation in two VvBAM amino acid sequences, including the absence of motifs 2, 3, and 6 in VvBAM1a and the absence of motif 9 in VvBAM8. For more information on the variation that may influence catalytic activity, the amino acid sequences of VvAMY and VvBAM proteins were clustered (Zhang and Li 2017). The catalytic sites (*i.e.* the Asp-Glu-Asp motif) were highly conserved in VvAMY sequences (Fig. 2d). BAMs that belong to the GH-14 family are characterized by a conserved core glucosyl hydrolase domain (Fig. 2e). In particular, glutamic acid residues Glu186 and Glu380 of soybean BAMs play essential roles as general acid and general base catalysts, respectively (Kang et al. 2004). A multisequence alignment of VvBAM amino acids revealed that Glu186 was highly conserved and that only Glu380 was not conserved in VvBAM2 and VvBAM7. Structural differences in BAM families indicate varied enzyme activities among members.

Expression of *VvAMY* and *VvBAM* genes in grapevine leaves under optimal and low temperatures during the night

We explored the expression patterns of *VvAMY* and *VvBAM* genes in leaves at night at optimal and low temperatures (Fig. 3). The expression of all genes was detectable in grapevine leaves but with different patterns. At the optimal temperature, the expression of three *AMY* genes (*VvAMY1*, *VvAMY3a* and *VaAMY3b*) and six *BAM* genes (*VvBAM1a*, *VvBAM1b*, *VvBAM3*, *VvBAM4*, *VvBAM7* and *VvBAM9*) was induced at night. The expression patterns were negatively correlated with the dynamics of starch content, which provides evidence that the upregulation of *AMY* and *BAM* expression leads to starch degradation in grapevine leaves grown at optimal temperatures at night. In contrast, the increased nighttime expression for some of these genes was more or less repressed in the cold-treated leaves. Reduced *AMY* and *BAM* expression is consistent with reduced amylase catalytic activity and may partially explain the elevated starch content in grapevine leaves grown under chilling conditions.

Expression patterns of genes encoding other enzymes associated with starch degradation

Another explanation for the elevated starch content in grapevine leaves during cold treatment is the reduced expression of other genes responsible for the degradation of starch. Other genes associated with starch degradation

Table 1 Summary information on *VvAMY* and *VvBAM* genes from grapevine

Name	ID	Location	Amino acids	Molecular weight	Theoretical pI	Subcellular localization prediction		
						TargetP1.1	ChloroP1.1	WoLF PSORT
VvAMY1	VIT 201s0026g01660	Chr1:10,828,879–10,841,717	901	101,425.16	5.64	C	—	cyto: 7, nucl: 4, pero: 2, chlo: 1
VvAMY2	VIT 218s0001g00560	Chr18:1,419,555–1,420,221	411	46,857.52	8.48	—	—	nucl: 6, cysk: 6, plas: 1, pero: 1
VvAMY3a	VIT 203s0063g00400	Chr3:3,981,014–3,983,570	424	47,243.23	5.25	S	—	extr: 7, E.R.: 2, chlo: 1, cyto: 1, mito: 1, plas: 1, vacu: 1
VvAMY3b	VIT 203s0063g00450	Chr3:4,012,122–4,015,556	425	47,779.49	6.33	S	—	cyto: 5, E.R.: 3.5, E.R._plas: 3, mito: 2, plas: 1.5, nucl: 1, extr: 1
VvBAM1a	VIT 205s0051g00010	Chr5:10,138,570–10,141,670	268	30,546.47	8.26	—	—	mito: 9, nucl: 2, cyto: 2, plas: 1
VvBAM1b	VIT 205s0077g00280	Chr5_ran-dom:167,899–171,065	573	63,157.77	5.86	C	—	cyto: 5, chlo: 3, extr: 2, mito_plas: 1.83333, mito: 1.5, cysk_plas: 1.33333, pero: 1
VvBAM2a	VIT 215s0046g02620	Chr15:19,379,203–19,386,567	554	62,635.70	5.66	C	Y	chlo: 14
VvBAM3	VIT 202s0012g00170	Chr2:5,803,876–5,804,363	543	60,524.64	8.71	C	Y	chlo: 6, mito: 4.5, cyto_mito: 3, nucl: 2, cyto: 1
VvBAM4	VIT 219s0015g00500	Chr19:8,567,688–8,585,390	522	59,727.50	9.02	—	—	cyto: 10, nucl: 2, chlo: 1, cysk: 1
VvBAM5	VIT 212s0059g02670	Chr12:7,373,835–7,379,807	596	66,277.03	5.25	C	Y	chlo: 8, cyto: 4, mito: 1, cysk: 1
VvBAM7	VIT 215s0046g02640	Chr15:19,389,359–19,399,836	699	78,783.75	5.58	—	—	nucl: 9, chlo: 2, cyto: 1, plas: 1, vacu: 1
VvBAM8	VIT 202s0087g00430	Chr2:17,760,111–17,774,984	670	75,305.16	5.59	—	Y	nucl: 13, vacu: 1
VvBAM9	VIT 205s0020g01910	Chr5:3,615,647–3,619,532	541	59,819.69	6.23	M	—	nucl: 6, mito: 5, chlo: 1, cyto: 1, cysk: 1
VvBAM10	VIT202s0025g02120	Chr2::1,885,110–1,885,569	542	61,457.06	8.69	C	Y	chlo: 11, nucl: 1, cyto: 1, pero: 1

were identified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/pathway/map00500). Starch degradation begins with glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD). GWD and PWD catalyzed the phosphorylation of starch grains, which loosened the structure of starch grains. Then, β-amylase, isoamylase (ISA) and disproportionating enzyme (DPE) degraded the starch to yield glucose and maltose. Finally, the glucose transporter (pGlcT) and maltose exporter 1 (MEX1) transport glucose and maltose across the chloroplast envelope and into the cytoplasm. We thus quantified the expression levels of these genes in leaves grown under optimal and chilling conditions. Among the eight genes we studied, the expression of only *like starch excess four 1* (*LSF1*) and *MEX1* increased during the night under optimal conditions. However, the expression of these genes was significantly upregulated in leaves subjected to chilling stress relative to leaves grown at the optimal temperature (Fig. 4). These results indicate that low temperatures induce the expression of most of the genes that encode enzymes that contribute to starch, except genes that encode amylases. Therefore, we speculate that the down-regulated expression of genes that encode amylases may contribute to the reduced degradation of starch in grapevine leaves at low temperature.
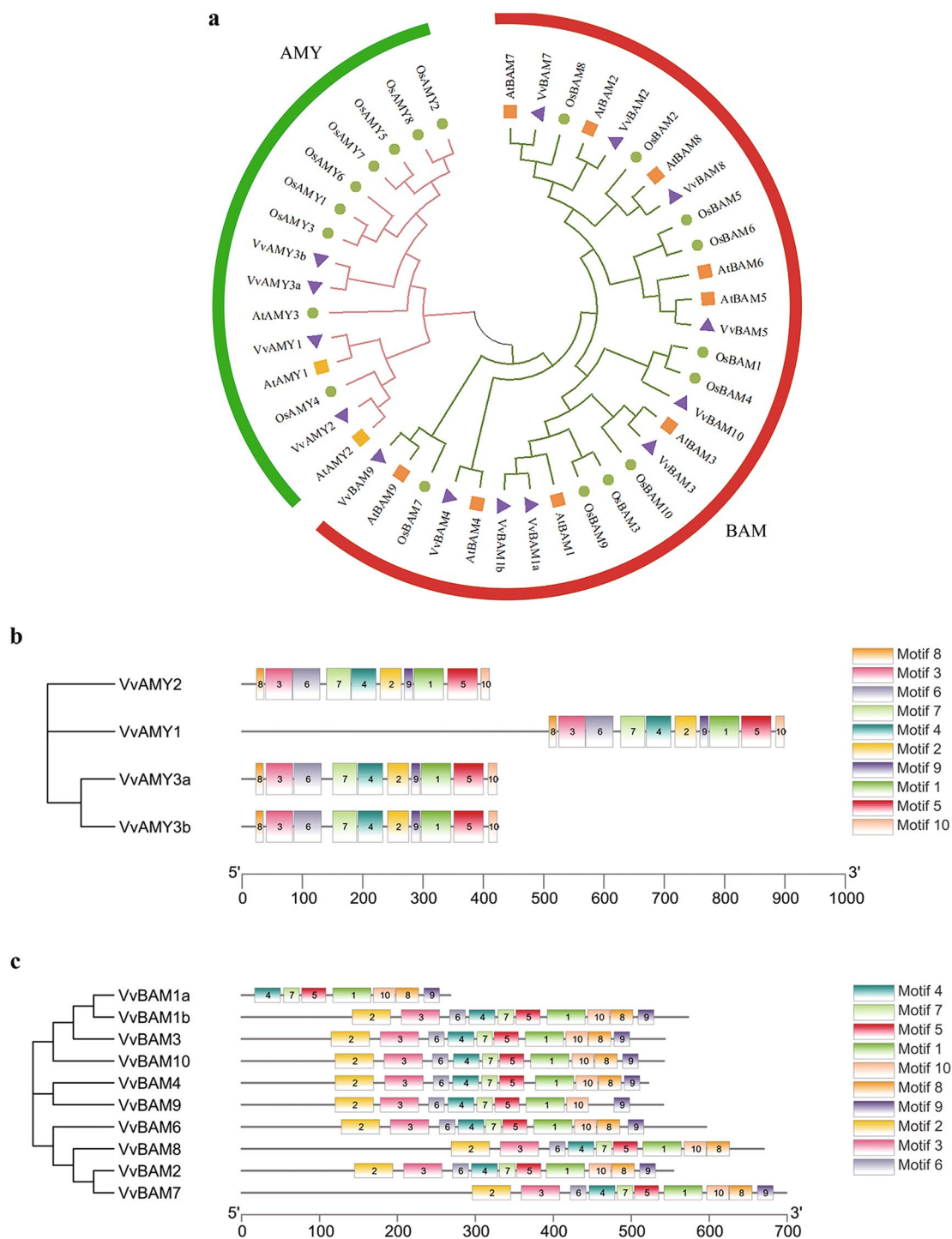
Fig. 2 Phylogenetic and conserved motif analysis of VvAMYs and VvBAMs (**a**) Phylogenetic analysis of amylases from grapevine, *Arabidopsis* and rice. The amino acid sequences from *Arabidopsis*, rice, and grapevine are indicated with yellow squares, green circles and purple triangles, respectively. The accession numbers for the 15 *α-amylases* (*AMY*) and 29 *β-amylases* (*BAM*) used in the phylogenetic tree are provided (Table S1). (**b**) Structural analysis of *VvAMY* genes. (**c**) Structural analysis of *VvBAM* genes. (**d**) Multiple sequence alignment of VvAMY amino acid sequences. Three catalytically important residues and the putative N-glycosylation sites are indicated with open red boxes. Two carbohydrate-binding sites in the consensus sequence are highlighted with green boxes. (**e**) Multiple sequence alignment of VvBAM amino acid sequences. Black arrowheads and black lines indicate substrate-binding residues. Red arrowheads indicate the two catalytic residues (Glu 186 and Glu 380)
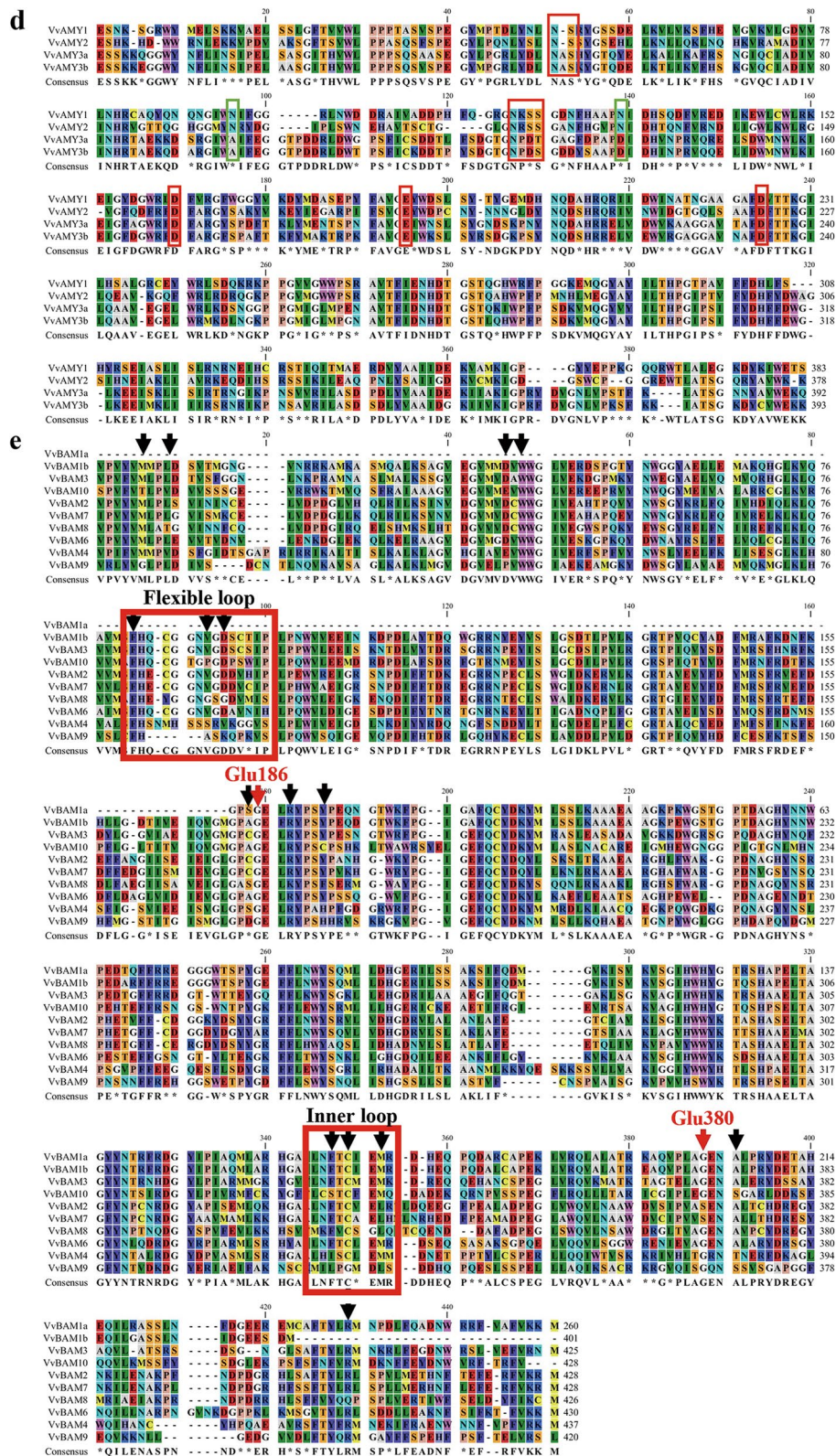
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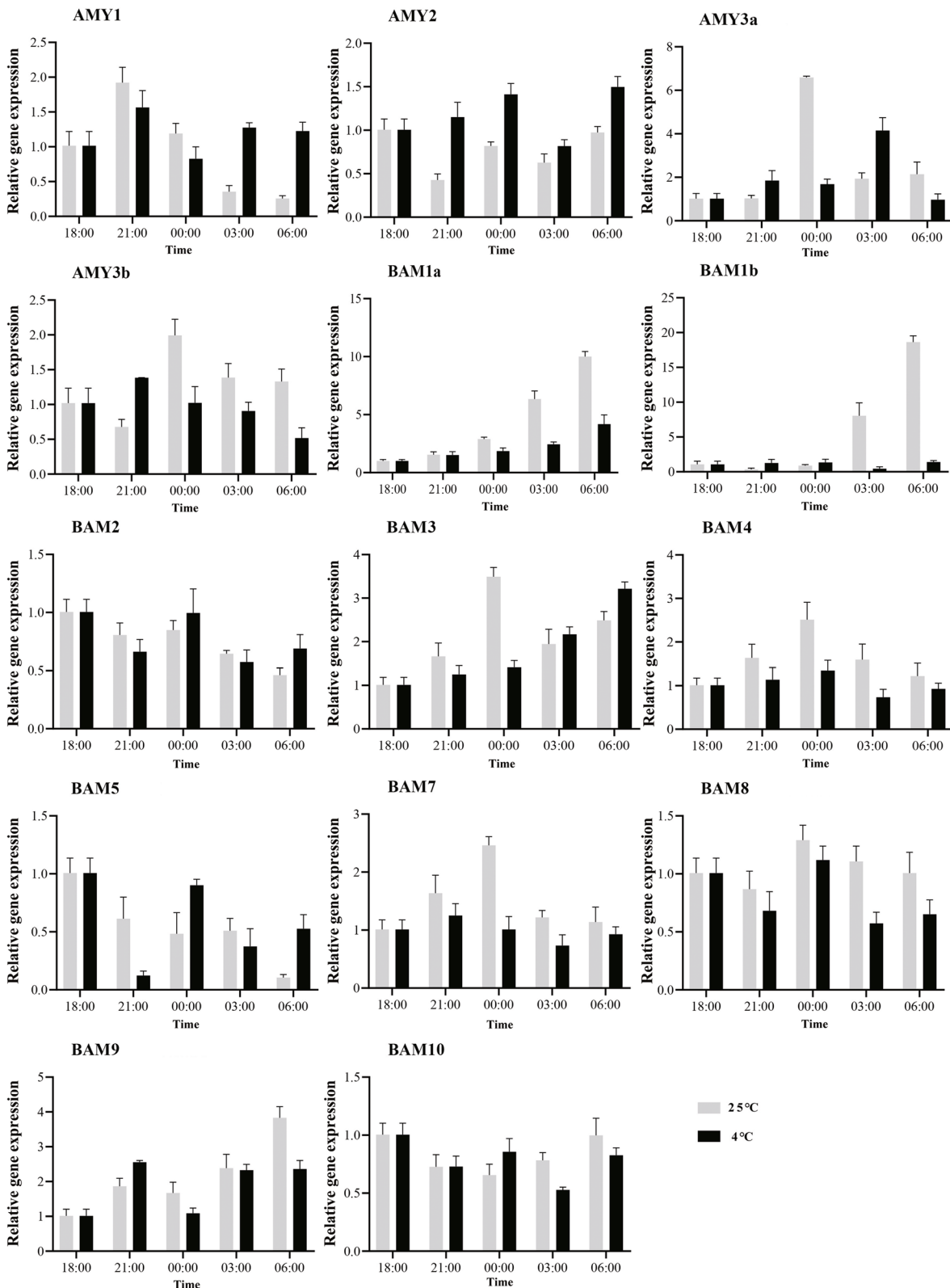Page 8 of 16

Fig. 2 continued

Fig. 3 Expression patterns of *VvAMY* and *VvBAM* genes in grapevine leaves under optimal and cold conditions. Relative expression levels were quantified using qRT–PCR and the $2^{-\triangle\triangle ct}$ method. Data are expressed as the mean values ± SEs from three independent replicates
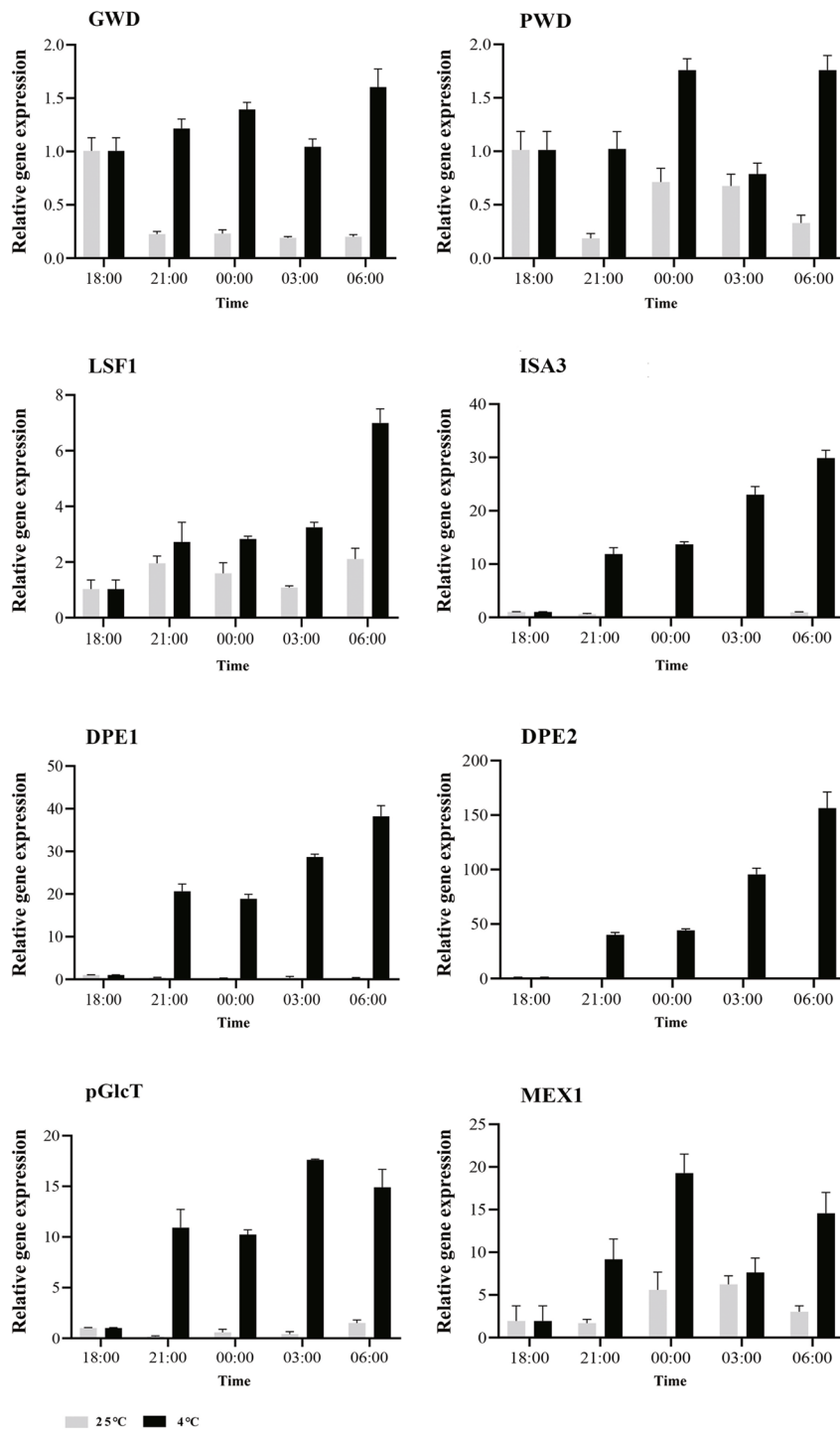
Fig. 4 Expression of genes encoding eight enzymes associated with starch catabolism under optimal and cold conditions at night. Relative expression was quantified using qRT–PCR and calculated using the $2^{-\triangle\triangle Ct}$ method. The relative expression of genes encoding glucan water dikinase (GWD), phosphoglucan water dikinase (PWD), like starch excess four 1 (LSF1), isoamylase 3 (ISA3), disproportionating enzyme 1 (DPE1), disproportionating enzyme 2 (DPE2), glucose transporter (pGlcT) and maltose exporter 1 (MEX1) were quantified from leaves grown under optimal and chilling conditions at night. Data are expressed as the mean values ± SDs, based on three replicates

Changes in soluble sugar content in grapevine leaves subjected to chilling treatment

Starch is one of the sources for soluble sugars that promote abiotic stress tolerance. We tested the influence of starch degradation on fructose, glucose, sucrose and maltose contents in grapevine leaves grown in normal and frost mimic conditions. The fructose and glucose contents decreased gradually under optimal conditions but remained almost unchanged during the chilling treatment (Fig. 5a, b). The sucrose content decreased at the end of the night in leaves grown at the optimal temperature but decreased first and then increased dramatically in leaves subjected to the chilling treatment (Fig. 5c). Although similar fluctuations in maltose content were found at the different temperatures, the maltose content was higher in the middle of the night in leaves grown at the chilling temperature

(Fig. 5d). These results indicate that low temperatures induced the accumulation of soluble sugars, especially fructose, glucose, and sucrose. Considering that chilling treatment inhibited the degradation of starch, these data provide evidence that the regulation of soluble sugar content in grapevine leaves subjected to low-temperature stress may be complex.

Identification of *cis*-regulatory elements in the promoters of *VvAMY* and *VvBAM* genes

The *cis*-regulatory elements in the 2.0 kb region upstream of the *VvAMY* and *VvBAM* genes were analyzed. A large number of core *cis*-elements are present in these genes, such as the CAAT-box and TATA-box (summarized in Fig. S1). Sequences similar to light-responsive elements were found in the promoters of all
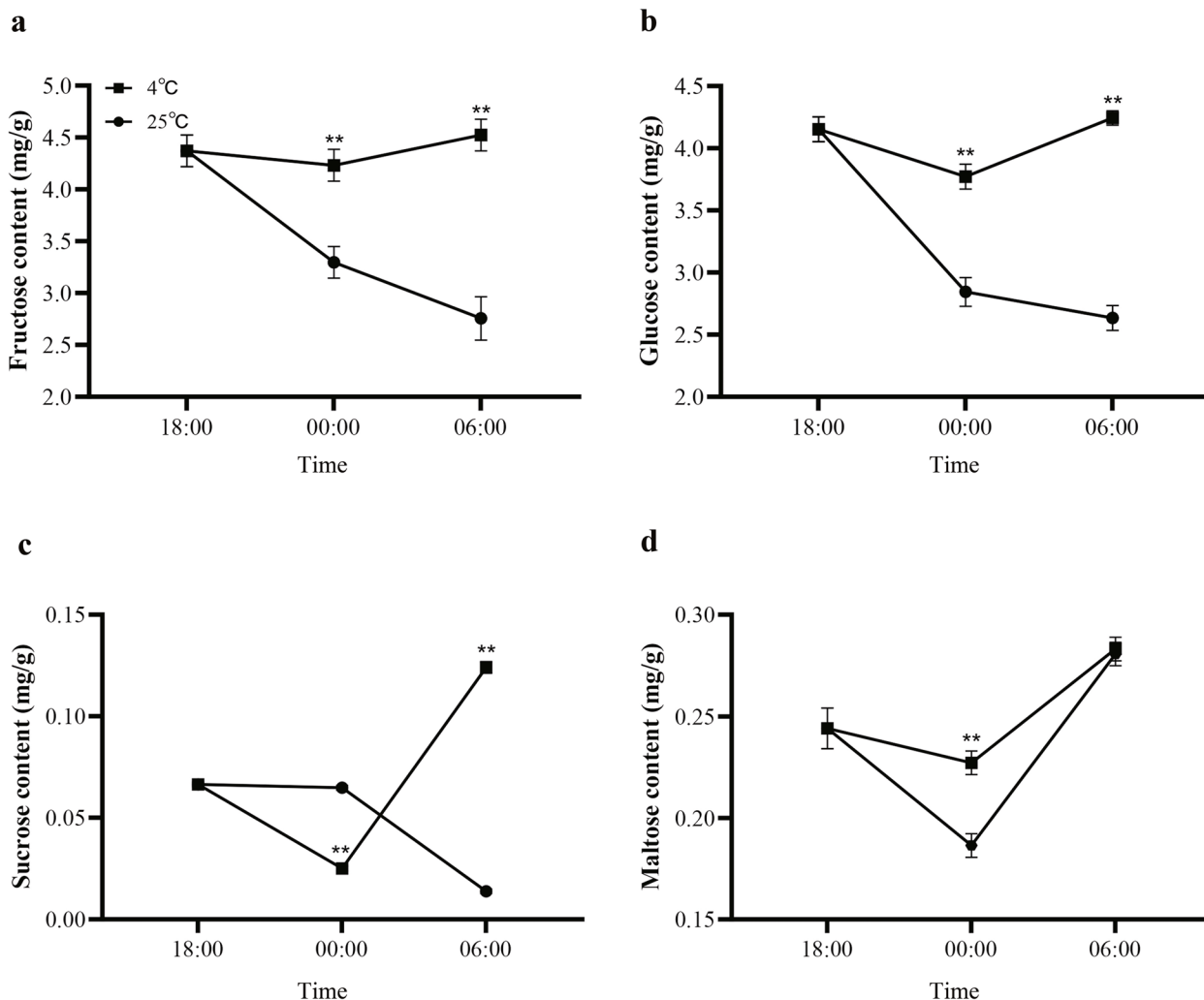

Fig. 5 Fructose **a**, glucose **b**, sucrose **c** and maltose **d** contents in grapevine leaves grown under optimal and low temperature conditions. Data are presented as the mean values ± SDs from three replicates. ****** and *indicate statistically significant differences relative to 0 h determined with Student's t test ($P < 0.01$ and $P < 0.05$, respectively)

VvAMY and *VvBAM* genes analyzed, including Box 4, the G-box, and the GT1 motif (Fig. S1). Sequences similar to *cis*-elements that respond to phytohormones, including abscisic acid, auxin, salicylic acid, MeJA and gibberellin, were also identified (Fig. 6, 7). Promoter elements associated with abiotic stress, such as ABREs and STREs, were ubiquitously distributed among all *VvAMY* and *VvBAM* genes (Fig. S1). Low-temperature-responsive (LTR) elements were present mainly in *VvAMY2*, *VvAMY3b*, *VvBAM1b*, *VvBAM7*, *VvBAM9* and *VvBAM10*. Sequences similar to promoter elements that confer cold and osmotic stress responsiveness (*e.g.* TC-rich repeats and the DRE core) were rarely found in these promoter regions (Fig. S1). Circadian response elements were found only in the promoter region of *VvBAM8*. These results provide evidence that the transcription of *VvAMY* and *VvBAM* genes is tightly related to light, phytohormone and stress responses.

Discussion

Frost in late spring usually occurs at night, which causes severe injuries to newly geminated leaves, flowers and even the branches of fruit trees (Lamichhane 2021). Several strategies are used to reduce the damage of this meteorological disaster on fruit production, such as heaters, wind machines, overvine sprinklers and foggers (Evans 2000; Perry 2001; Tong et al. 2019). All these interventions are costly. The unpredictability of frost in late spring also limits the use of these methods. In recent years, increased knowledge of cold stress responses in plants has led to new strategies for improving the cold tolerance of plants and thus helping to overcome the damage caused by frost.

Starch is a fundamental substance in plants (Yelenosky and Guy 1977). Consistent with previous reports, we found that starch accumulates during the day and at optimal temperatures and degrades during the night in grapevine leaves (Santelia 2015; Skeffington 2014) (Fig. 1). Generally, plants remobilize starch to provide energy and carbon
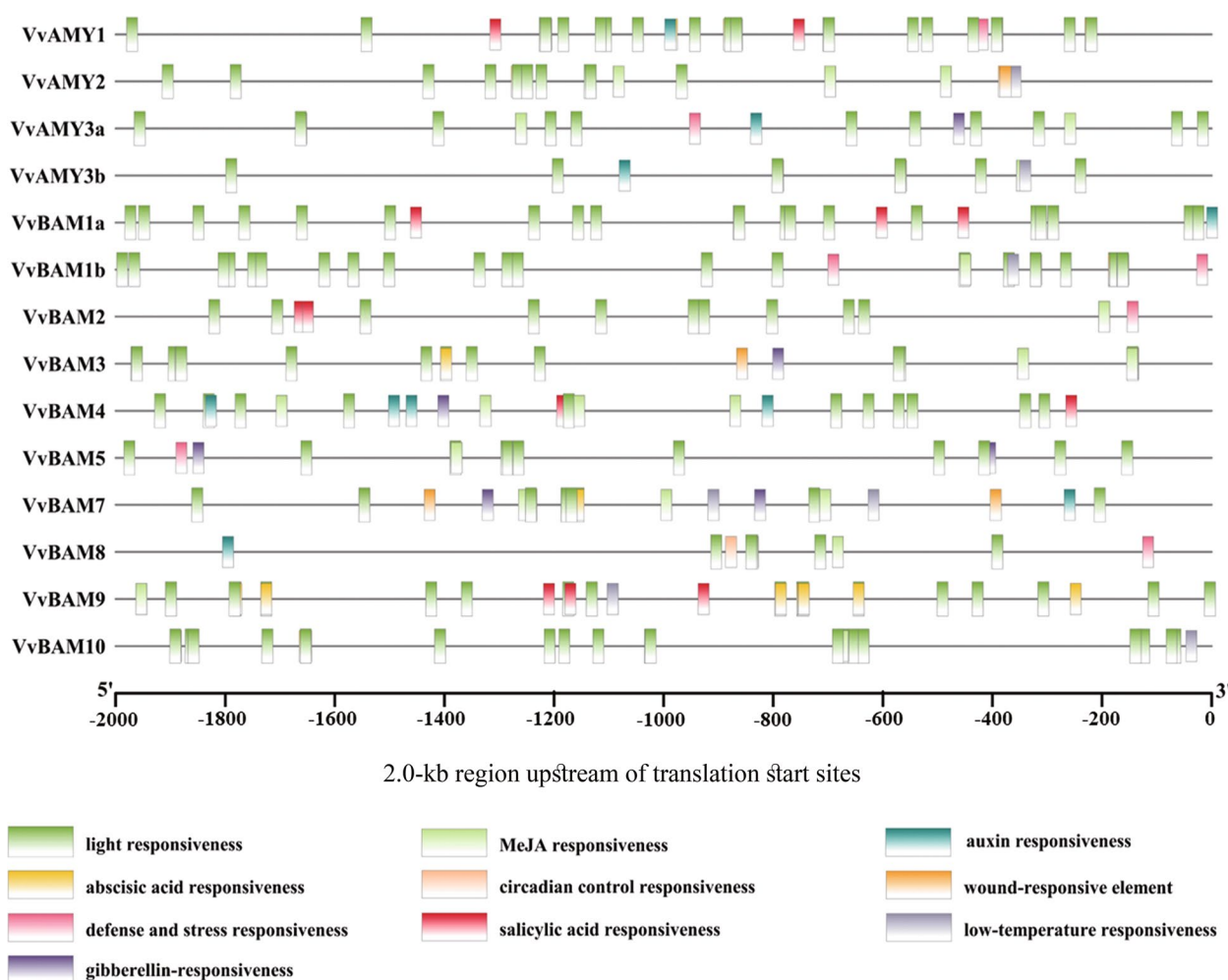

Fig. 6 *Cis*-acting elements in the promoters of *VvAMY* and *VvBAM* genes. Promoter regions are defined as 2.0 kb regions upstream of the translation start codon
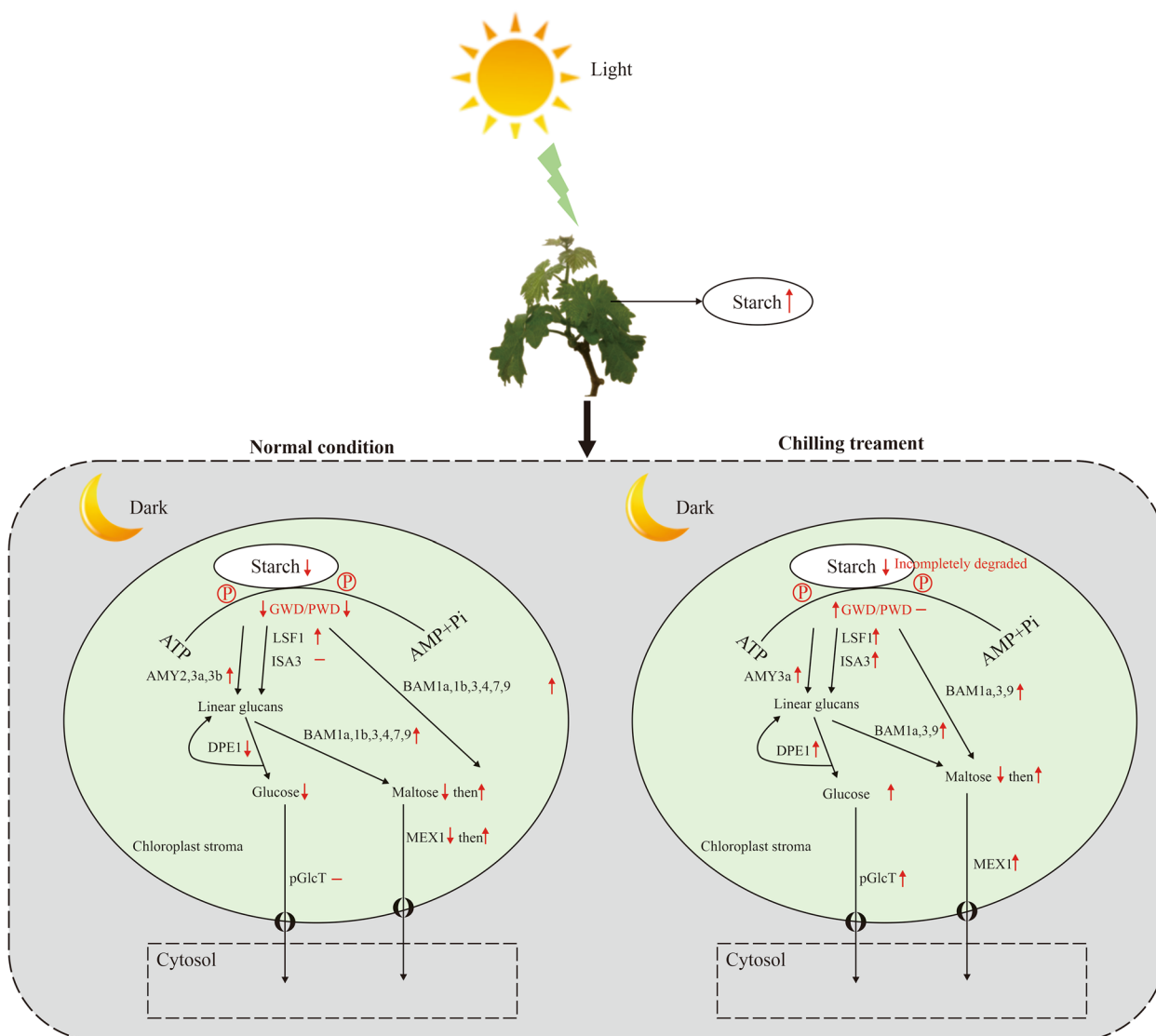
Fig. 7 Dynamics of starch degradation and related gene expression in the leaves of grapevine during chilling stress. GWD, glucan water dikinase; PWD, phosphoglucan water dikinase; *LSF 1, like starch excess four 1*; *ISA3, isoamylase 3*; *AMY, a-amylases*; *BAM, β-amylases*; DPE, disproportionating enzyme; MEX1, exporter 1; pGlct, glucose transporter

during stressful conditions (Thalmann 2017). Since frost in late spring always occurs at night, we designed experimental conditions that mimic this stress condition. We thought that chilling stress might accelerate the degradation of starch and therefore induce increases in the accumulation of the products of starch degradation, such as soluble sugar, to increase the energy supply and osmotic potential and thus to promote chilling tolerance. Instead, we found that the degradation of starch was dramatically blocked within a 12 h chilling treatment in the dark. Several reports have mentioned that the overexpression of *amylase* increases the content of soluble sugar and chilling tolerance in plants. For example, kiwifruit lines that overexpress *AaBAM3.1* are more freezing tolerant than control lines (Sun et al.

2021). Thus, enhancing starch degradation should improve the chilling tolerance of grapevine leaves.

There are several *AMY* and *BAM* genes in the grapevine genome. Thus, choosing a suitable candidate for the genetic improvement of grapevine is a challenge. Among the *AMY* and *BAM* genes in grapevine, the induced expression of *VvAMY* genes in the dark or in response to chilling stress was weaker than we observed for the *VvBAM* genes. Previous work in other species indicates that the expression of *AMY* genes is induced by heat, drought and biotic stress and mainly affects bud dormancy, leaf senescence and seed germination (Satoru et al. 2005; Doyle et al. 2007; Rubio et al. 2014; Fan et al. 2018). *BAM* genes were previously found to play major roles in stress responses in plants (Peng

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et al. 2004; Zhao et al. 2019; Zhu et al. 2021). We found that the expression of *VvBAM3* was gradually induced by chilling treatment, which is consistent with its role in starch degradation at low temperatures, and thus, *VvBAM3* can be considered a candidate for the genetic improvement of grapevine. Glu380 serves a critical function as a general base catalyst and was changed in the amino acid sequences derived from *VvBAM2* and *VvBAM7*, but the effect on enzyme activity is unknown. More work is needed to evaluate the potential of each *VvBAM* gene for the enhancement of cold tolerance in grapevine leaves.

Promoters are also potential targets for modifying gene expression by genetic engineering (Mao et al. 2019). Light-responsive elements are abundant in *VvAMY* and *VvBAM* gene promoter regions, which is consistent with the dark-induced expression patterns for most *VvAMY* and *VvBAM* genes. Although *cis*-elements associated with abiotic stress, such as LTR, ABRE, TC-rich repeats, DRE core and STRE, were identified in the promoters of *VvAMY* and *VvBAM* genes, large increases in the expression of these genes were not observed within 12 h of chilling stress. The timely response of *amylase* genes to cold stress might be a strategy for enhancing the soluble sugar content and thus for increasing cold tolerance when temperatures suddenly drop (Steponkus 1984). In recent years, advanced technology for gene editing has allowed for the introduction of point mutations in nucleic acids, and thus, in theory, the modification of regulated gene expression by the limited modification of promoter elements is now possible (Dietrich et al. 1996; Jang et al. 2003; Jeong et al. 2010). Further studies on the core *cis*-elements required for the cold-inducible expression of *VvAMY* and *VvBAM* genes may facilitate the discovery of new cultivars for grapevine and for other crops with enhanced cold tolerance without influencing growth and development (Fig. 7).

Conclusions

In conclusion, the findings of this study indicate that low temperature inhibits the degradation of starch in grapevine leaves. The expression of *VvAMY* and *VvBAM* genes at night at the optimal temperature was also inhibited by low temperature. However, the expression of other genes related to starch degradation, such as *LSF1* and *ISA3*, was induced by low-temperature treatment. Therefore, we speculate that transcriptional regulation of genes that encode amylase may be the main factor promoting starch degradation in grapevine leaves at low temperatures. The findings presented here provide new clues that will be useful for developing strategies for enhancing the frost tolerance of grapevine leaves by modifying the starch degradation pathway.

Abbreviations

AMY	*α*-Amylases
BAM	*β*-Amylases
LSF1	Like starch excess four 1
ISA3	Isoamylase 3
ROS	Reactive oxygen species
AGPase	ADP-glucose pyrophosphorylase
GBSS	Granule-bound starch synthase
SSS	Soluble starch synthetase
SBE	Starch branching enzyme
DBE	Debranching enzyme
TEM	Transmission electron microscope
GWD	Glucan water dikinase
PWD	Phosphoglucan water dikinase
pGlcT	Glucose transporter
MEX1	Maltose exporter 1
LTR	Low temperature responsiveness

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44281-023-00004-5.

Additional file 1:

Availability of data and materials

Data will be made available on request. All plant materials were provided free of charge. The laboratory experiments were conducted under permissions of regulations and laws.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. The authors declare that they have no financial or nonfinancial interests.

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