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ORIGINAL ARTICLE

Different exogenous sugars affect the hormone signal pathway and sugar metabolism in "Red Globe" (*Vitis vinifera* L.) plantlets grown in vitro as shown by transcriptomic analysis

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Abstract

Main conclusion **Exogenously applied 2% fructose is the most appropriate carbon source that enhances photosynthesis and growth of grape plantlets compared with the same concentrations of sucrose and glucose. The role of the sugars was regulated by the expression of key candidate genes related to hormones, key metabolic enzymes, and sugar metabolism of grape plantlets (*Vitis vinifera* L.) grown in vitro.**

The addition of sugars including sucrose, glucose, and fructose is known to be very helpful for the development of grape (*V. vinifera* L.) plantlets in vitro. However, the mechanisms by which these sugars regulate plant development and sugar metabolism are poorly understood. In grape plantlets, sugar metabolism and hormone synthesis undergo special regulation. In the present study, transcriptomic analyses were performed on grape (*V. vinifera* L., cv. Red Globe) plantlets in an in vitro system, in which the plantlets were grown in 2% each of sucrose (S20), glucose (G20), and fructose (F20). The sugar metabolism and hormone synthesis of the plantlets were analyzed. In addition, 95.72–97.29% high-quality 125 bp reads were further analyzed out of which 52.65–60.80% were mapped to exonic regions, 13.13–28.38% to intronic regions, and 11.59–28.99% to intergenic regions. The F20, G20, and S20 displayed elevated sucrose synthase (SS) activities; relative chlorophyll contents; Rubisco activity; and IAA and zeatin (ZT) contents. We found F20 improved the growth and development of the plantlets better than G20 and S20. Sugar metabolism was a complex process, which depended on the balanced expression of key potential candidate genes related to hormones (*TCP15*, *LOG3*, *IPT3*, *ETR1*, *HK2*, *HK3*, *CKX7*, *SPY*, *GH3s*, *MYBH*, *AGB1*, *MKK2*, *PP2C*, *PYL*, *ABF*, *SnRK*, etc.), key metabolic enzymes (*SUS*, *SPS*, *A/V-INV*, and *G6PDH*), and sugar metabolism (*BETAFRUCT4* and *AMY*). Moreover, sugar and starch metabolism controls the generation of plant hormone transduction pathway signaling molecules. Our dataset advances our knowledge of the genes involved in sugar metabolism and improves the understanding of complex regulatory networks involved in signal transduction in grape plantlets.

Keywords Enzyme activity · Exogenous sugar · Grape · Hormone signal pathway · Plantlet · Sugar metabolism · Transcriptomic analysis

Abbreviations

DEGs	Differentially expressed genes
G6PDH	Glucose-6-phosphate dehydrogenase
GO	Gene ontology
INV	Invertase
JA	Jasmonic acid
NI	Neutral invertase

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SPS	Sucrose phosphate synthase
SS	Sucrose synthase
ZT	Zeatin

Introduction

Grape (*Vitis vinifera* L.) is one of the important fruit trees in the world and has a long evolutionary history. It can be multiplied rapidly through plant tissue culture technology (Thomas 1999), which is an effective and fast propagation strategy for plant seedling production. Photosynthesis leads to the fixation of carbon in leaves and accumulation of carbohydrates. Assimilated carbon must be transported from the leaves (source tissues) to nonphotosynthetic organs (sink tissues), such as roots and seeds (Chenu and Scholes 2015). Sugar metabolism is not only essential to this process, but the sugar also functions as a kind of signal molecule (Yue et al. 2015). Sugar receptors perceive the presence of sugar and then initiate downstream signaling events, and these functions are comparable to the signaling functions of hormones (Fang et al. 2015; Yoneyama et al. 2015). However, the factors involved in the photosynthesis of plantlets in vitro and how photosynthate is translocated from the leaves are poorly understood.

In the past few years, plant-regulatory and control systems have been extensively studied, particularly those involved in sugar input and anabolism. Sugar can directly serve as a substrate-adjusting metabolism and effective signaling molecule (Smeekens et al. 2010). Sugar utilization is, therefore, the key factor that promotes plant growth and development in vitro (Rolland et al. 2006; Meyer et al. 2007). Sugar metabolism plays pivotal roles in plant development, stress response, and yield formation. It mainly contributes to the production of sugars that fuel growth and the synthesis of essential compounds, such as protein, cellulose, and starch (Ruan 2014). Sugar metabolism is tightly coupled with sugar signaling. This coupling is achieved by the generation of sugar signaling molecules, such as sucrose, glucose, fructose, and trehalose-6-phosphate, or perhaps by the signaling role of the metabolic process itself (Ruan et al. 2010, 2012; O'Hara et al. 2013). These signals modulate plant development and their response to stress directly and indirectly through their interactions with other signaling pathways that regulate the expression of microRNAs, transcription factors, and the genes involved in hormone and redox-mediated processes.

Previous researches on sugar metabolism have focused on the high sugar content of fruits or some particular plants mainly grown in fields (Ruan et al. 2012; Jia et al. 2013). Most of the earlier studies, however, focused on the role of sucrose and neglected those of glucose and fructose. Moreover, few studies provided a systematic analysis and comparison of the carbon metabolism between autotrophic photosynthesis and heterotrophic photosynthesis (Mousseau 1986; Wiessner et al. 1991). In addition, the use of bioinformatics to explore the differences and relations in the molecular regulation mechanism of grape has not been reported. In the present research, 2% each of sucrose, glucose, and fructose were used as exogenous sugars. High-throughput RNA-Seq technology was employed to explore the mechanisms of different metabolic pathways and comprehensively explain the regulation of plant carbon metabolism and sugar signaling.

Materials and methods

Plant materials and treatments

The "Red Globe" (*V. vinifera* L.) grape plantlets were used in an in vitro study. The cultivar was kept in the Fruit Tree Physiology and Biotechnology Laboratory of Gansu Agricultural University. The plantlets were propagated in advance and were vigorous in growth and without contamination. Each nodal segment (1.5–2.0 cm long) with a single bud was inoculated on an MS medium. The treatments were: sucrose (2%), glucose (2%), fructose (2%), and control (without sugar), which were designated as S20, G20, F20, and G0, respectively. The growing medium contained compounded MS (4.74 g L^{-1}) + IAA (0.20 mg mL^{-1}) + agar (6.00 g L^{-1}) + NaOH (500 μL L^{-1}), had a pH of 6.0 and was subpackaged in 150 mL triangle bottles. Each treatment was inoculated in 40 bottles and placed on the culture shelf at 27 °C and 16 h day/8 h night conditions. The samples of the four treatments were collected simultaneously at 37 days after inoculation. The leaf samples were transferred immediately to liquid nitrogen and stored at −80 °C until RNA extraction, enzyme activity determination, and hormone assay. Apart from RNA extraction, different treatments were performed on the leaf samples collected from three comparable plants. The leaf samples were then used as three biological replicates. The leaf samples were used to construct four libraries designated as G0, S20, F20, and G20.

Determination of sugar metabolism-related physiological indicators

The content of chlorophyll was determined as described by Coste et al. (2010), whereas the activity of Rubisco was measured with an ELISA kit (Real-Times, Beijing, China).

The measurement of neutral invertase (NI) activity was carried out in accordance with the procedure of Jiang et al. (2014). The soluble NI activity was assayed by adding

50 μL of reaction buffer (0.1 M pH 7.5 phosphate buffer and 1% sucrose) to 50 μL of crude enzyme. The resulting mixture was incubated at 34 °C for 1 h. The controlled trial with 50 μL crude enzyme was incubated at 100 °C for 10 min. The reaction was stopped by boiling the mixture for 5 min and adding 1.5 mL of 3,5-dinitrosalicylic acid. The mixture was then incubated in a water bath at 100 °C for 5 min and adjusted to 25 mL with distilled water. The soluble NI activity was assayed from the obtained light absorption value at 540 nm.

The leaf samples were sliced and then ground and 5 mL of 200 mM Hepes–NaOH (pH 7.5) was added to the ground samples. The relative centrifugal force (RCF) was 9168*g*. The supernatant was the crude enzyme. The extraction and SS assays were carried out as described by Tsai et al. (1985) and Douglas et al. (1988). The SS was assayed by adding 50 μL of 50 mM Hepes–NaOH (pH 7.5), 20 μL of 50 mM $MgCl_2$, 15 μL of 100 mM UDPG, 10 μL of distilled water, and 15 μL of 100 mM fructose. The mixture was incubated in a water bath at 30 °C for 30 min. The reaction was stopped by adding 200 μL of 2 M NaOH. Then, 1.5 mL of concentrated hydrochloric acid and 0.5 mL of 0.1% hydroquinone were added to the mixture, which was then incubated in a water bath at 30 °C for 10 min. SS activity was assayed from the obtained light absorption value at 480 nm. The activity of sucrose phosphate synthase (SPS) was measured in accordance with the procedure of Yu (1985) and Wardlaw and Willenbrink (1994). The assay for SPS activity was similar to that of SS except that the fructose was changed to 100 mM fructose 6-phosphate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was measured as described by Stitt et al. (1989).

Determination of IAA, ABA, and ZT contents

Extraction method: Frozen leaf samples were brought out from storage, and 1 g of each sample was ground quickly after liquid nitrogen was added. Each sample was then combined with 10 mL of 80% chromatographic pure methanol (preparation with DNase/RNase-free double-distilled water). Each sample was washed thrice with solvent, transferred into a test tube, and stored in a refrigerator at 4 °C overnight in the dark. Then, the samples were centrifuged for 20 min under refrigerated conditions at 4 °C. The supernatant fluid was transferred into a new centrifuge tube. The extract was concentrated, and the methanol was volatilized under 40 °C by rotary evaporation to obtain 2 mL of concentrate. The evaporation bottle wall was then washed continuously with 50% methanol, and the volume was raised to 10 mL with 50% chromatographic pure methanol. The fluid for testing was filtered through a 0.22 μm organic membrane. This fluid was then transferred to a 2 mL centrifuge tube and placed in an ice box. The hormone contents from the treatments were identified in the Instrumental Researches and Analysis Center of Gansu Agricultural University.

The determination method was performed with different concentrations of IAA, ABA, and zeatin (ZT), standard samples, which were used to construct a standard curve. The standard samples were purchased from Sigma Company, and the external standard curve and quantitative methods were performed for the measurements. The type of LC–MS apparatus used was the Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). The detector was vwd and the chromatographic column was Extend-C18 (4.6 mm × 250 mm, 5 μm). The mobile phase was chromatographic methanol and 0.6% iced acetic acid previously subjected to ultrasonication (0 min, methanol:acetic acid = 40:60; 11.9 min, methanol:acetic acid = 40:60; 12 min, methanol:acetic acid = 50:50). The flow velocity was 1.0 mL min^{-1}, the wavelength 254 nm, and the column temperature 25 °C.

RNA isolation, library construction, and sequencing

RNA isolation, library construction, and sequencing were conducted in accordance with the method of Yang et al. (2015). The total RNA was extracted with RNA plant reagent (Real-Times Biotechnology, Beijing, China) in accordance with the manufacturer's instructions and treated with RNase-free DNase I (Takara, Dalian, China) to remove DNA contamination. RNA integrity was evaluated with a 1% agarose gel stained with GoldView, and the extract was tested with Agilent 2100. The RNA quality and quantity were assessed in a Nanodrop spectrophotometer and Agilent 2100 spectrophotometer (Agilent Technologies, Sangta Clara, CA, USA). The RNA integrity number (RIN) was greater than 7.6 for all the samples. For each treatment of the "Red Globe" in vitro, RNA samples from three randomly sampled individuals were pooled together in equal amounts to generate one mixed sample. These mixed samples were used for cDNA library construction and Illumina sequencing, which was completed by Beijing Biomarker Bioinformatics Technology Co., Ltd.

A total amount of 3 μg RNA per sample was used to construct a cDNA library. The library was generated using NEBNext Ultra™ RNA library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following the manufacturer's recommendations. Poly(A) mRNA was purified from total RNA using oligo(dT)-attached magnetic beads. The mRNA was then cleaved into small fragments by adding fragmentation buffers. These fragments were used to synthesize first-strand cDNA using random hexamer primer and M-MuLV reverse transcriptase (RNase H^-). Second-strand cDNA synthesis was subsequently performed using

DNA polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3′ ends of DNA fragments, an NEBNext adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then, 3 μL USER Enzyme (NEB) was combined with size-selected and adaptor-ligated cDNA at 37 °C for 15 min, followed by 5 min at 95 °C before PCR. The PCR was performed with Phusion high-fidelity DNA polymerase, universal PCR primers, and index (X) primer. Finally, the PCR products were purified (AMPureXP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) in accordance with the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina HiSeq 2000 platform, and 125 bp paired-end reads were generated.

Sequence analysis results: mapping and differential expression

Raw reads were cleaned by removing the adapter sequences, reads containing poly-N, and low-quality sequences ($Q < 20$). Clean reads were then aligned to the reference genome sequence (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) using the program Tophat v2.0.943 (Trapnell et al. 2012). The default settings of the program were used to allow mismatches of no more than two bases. New transcripts were identified from TopHat alignment results through the Cufflinks v2.1.1 reference-based transcript (RABT) assembly method. For annotations, all novel genes were searched against the Nr, Swiss-Prot, Gene Ontology (GO), COG, KOG, Pfam, Kyoto Encyclopedia of Genes and Genomes (KEGG) databases by BLASTx with 10^{-5} as the E value cutoff point. The sequences with the highest similarities were retrieved. Afterward, the amino acid sequences of the new genes predicted were searched against the Pfam database using the HMMER software to obtain the annotation information of the new genes.

The EBSeq package was used to detect differentially expressed genes (DEGs) between two samples. During the detection process of DEGs, the false discovery rate (FDR) was used to determine the P value threshold in multiple tests (Leng et al. 2013). FDR <0.01 and fold change ≥ 2 were adopted as screening criteria. That is, the absolute value of the $\log_2$ (fold change) with Reads per Kilobase per Million Reads (RPKM) ≥ 1 were used as the threshold to determine the significantly different gene expression in this research. The RPKM eliminates the influence of different gene lengths and sequencing discrepancies on the quantification of gene expression to enable direct comparison of gene expression between samples (Mortazavi et al. 2008).

Functional analysis of DEGs

Functional enrichment analysis by GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms or metabolic pathways. GO enrichment analysis of DEGs was implemented by the GOseq R package, in which gene length bias was corrected. GO terms with corrected P value of less than 0.05 were considered significantly enriched by DEGs. The GO annotations were functionally classified by WEGO software for gene functions. To obtain the detailed functional categorization of the DEGs in different sugar treatments, the KOBAS software was used to test the statistical enrichment of the DEGs in the KEGG pathways. The pathways with an FDR value ≤ 0.05 were defined as those with genes that display significant levels of differential expression (Kanehisa et al. 2004; Xie et al. 2011).

Quantitative real-time PCR validation of RNA-Seq data

The 15 DEGs related to ABA and jasmonic acid (JA) involved in sugar metabolism of the in vitro development process of "Red Globe" were selected for validation through quantitative real-time PCR (qRT-PCR). The primers were designed with the Primer 5.0 software (http://biotools.Umassmed.Edu/bioapps/primer3-www.cgi) for qRT-PCR. The qRT-PCR reactions were analyzed in the ABI StepOne™ Plus Real-Time PCR System with the SYBR Green PCR Master Mix (Takara).

Data processing and analysis

Data on the growth parameters, including plant height, leaf number, and internode length, were analyzed using ANOVA. Treatment means were separated by the Duncan multiple range test at $P < 0.05$. The SPSS statistical software was used for the statistical analyses.

Results

Effect of different sugars on plant growth indices

The sugars significantly promoted plant growth and development at 37 days after treatments (Fig. 1a). The F20, G20, and S20 significantly increased plant height by 50.14, 50.57, and 50.69% compared with that of G0, respectively (Fig. 1b). The internode lengths of F20, G20, and S20 significantly

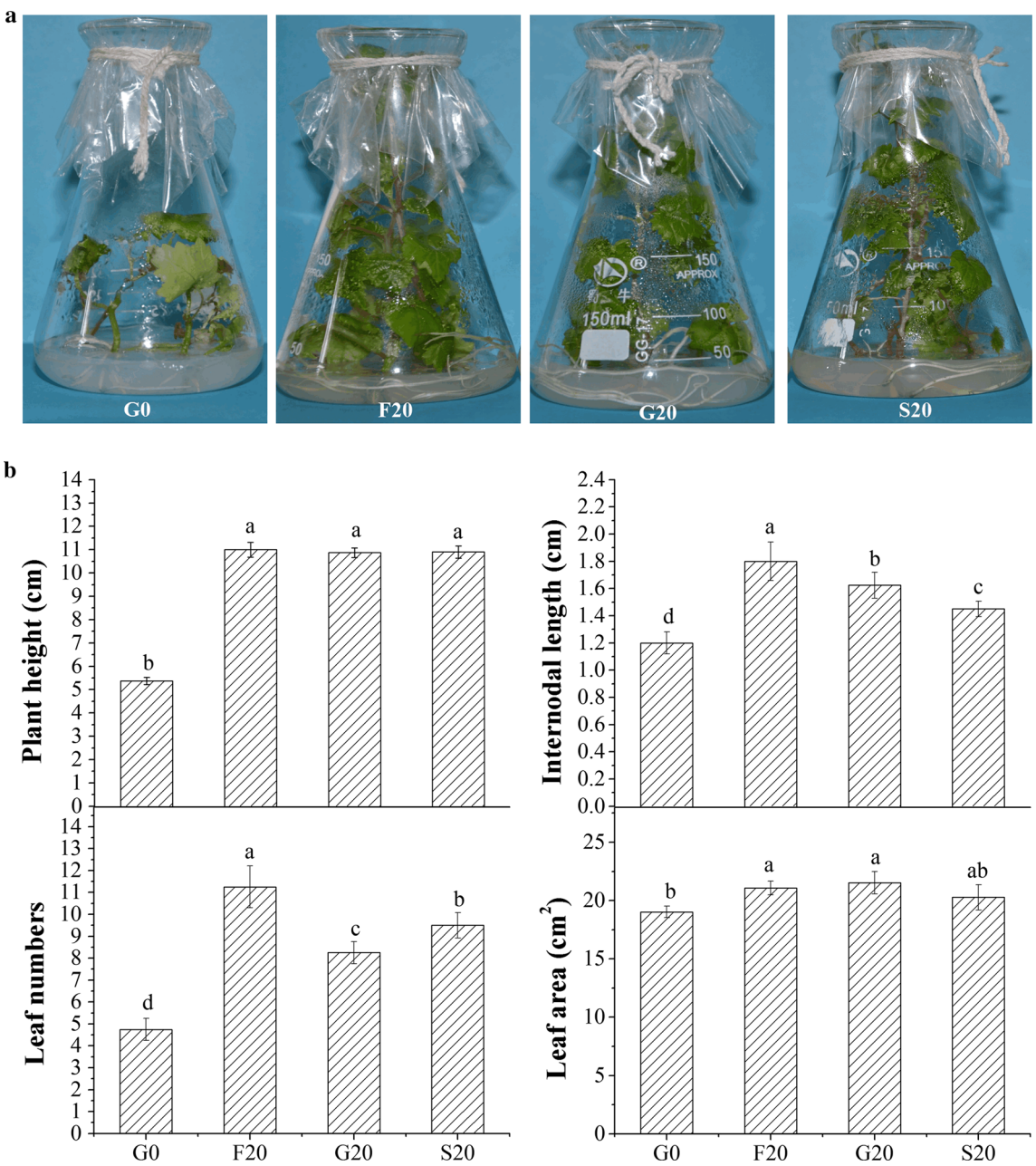


Fig. 1 Phenotypical characteristics change of 'Red Globe' plantlet in vitro caused by sugars on the 37th day. **a** Phenotypical changes of 'Red Globe' plantlet in vitro. **b** Effect of different sugars on plant height, leaf number, internode length, and leaf area of 'Red Globe' plantlets in vitro. Data are mean ± SE of three individual experiments, each performed in quadruplicate. Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

increased by 33.33, 26.15, and 17.24%, respectively, compared with G0. The leaf numbers of F20, G20, and S20 increased by 57.78, 42.42, and 50% while the leaf areas also increased by 9.68, 11.63, and 6.17%, respectively.

Illumina HiSeq mRNA sequencing

After the sequencing quality control, 21.41 Gb clean bases were generated from the four libraries, and the Q30 base percentage of each sample was not less than 90.26% (Table S1). Moreover, 95.72–97.29% high-quality 125 bp reads were selected for further analysis (Table 1). Clean reads of the various samples were aligned against the specified grape reference genome. Mapped reads were from 68.31 to 73.71%. Uniquely mapped reads and multiple-mapped reads were from 66.11 to 72.15% and 1.56 to 2.37%, respectively. Reads mapped to '+' and '−' were derived from 33.76 to 36.62% and 33.59 to 36.54%,

Table 1 Summary of transcriptome sequencing data

	G0	F20	G20	S20
Total reads	39,680,278	41,159,270	49,847,950	39,239,440
High-quality reads (%)	96.16	95.72	96.83	97.29
Mapped reads	27,104,822 (68.31%)	30,088,810 (73.10%)	36,740,734 (73.71%)	26,870,423 (68.48%)
Uniq mapped reads	26,271,962 (66.21%)	29,311,294 (71.21%)	35,963,521 (72.15%)	25,941,113 (66.11%)
Multiple map reads	832,860 (2.10%)	777,516 (1.89%)	777,213 (1.56%)	929,310 (2.37%)
Reads map to '+'	13,398,055 (33.77%)	14,892,371 (36.18%)	18,251,930 (36.62%)	13,246,548 (33.76%)
Reads map to '−'	13,339,115 (33.62%)	14,866,037 (36.12%)	18,215,092 (36.54%)	13,182,441 (33.59%)
Exon (%)	54.05	57.87	60.8	52.65
Intron (%)	28.61	13.13	27.62	28.38
Intergenic (%)	17.33	28.99	11.59	18.97

respectively. By comparing the efficiency, we assessed whether the reference genome assembly could meet the needs of information analysis. Accordingly, 52.65–60.80% of the reads were mapped to exonic regions, 13.13–28.38% to intronic regions, and 11.59–28.99% to intergenic regions.

DEGs after sugar treatment

The differences in gene expression due to the use of three kinds of sugars in the development process of the "Red Globe" plantlets were examined. The changes in gene expression were determined by comparing each of the sugar treatment with the control, that is, G0 versus F20, G0 versus S20, and G0 versus G20 (Fig. 2). A total of 999 up-regulated DEGs and 1698 down-regulated DEGs were identified in our RNA-seq data. Of these DEGs, 249, 410, and 519 genes were up-regulated and 827, 638, and 896 were down-regulated in G0 versus F20, G0 versus S20, and G0 versus G20, respectively.

Effects of exogenous sugar on leaf chlorophyll content and RuBp activity

The relative chlorophyll content of the four treatments was in the following order: F20 = S20 > G20 > G0 (Fig. 3a), whereas the RuBp activities were in the following order: F20 > G20 > S20 > G0 (Fig. 3b). A close link between photosynthesis and carbohydrate metabolism was observed, indicating that the rate of photosynthesis is directly related to the rate of sugar metabolism. In our research, the DEGs related to photosynthesis were selected and divided into two groups on the basis of their functions (Fig. 4). From the first group, the expression of the RNA helicase (*RH39*) of G20 was significantly up-regulated compared to the control (G0). The expression of the Semi dwarf gene (*SDG40*) of F20 was significantly up-regulated compared with that in the control (G0). The second group consisted of 53 DEGs, and their functions were associated with photosynthesis, light reaction, and light harvesting. Among these functions, only four genes (Act domain

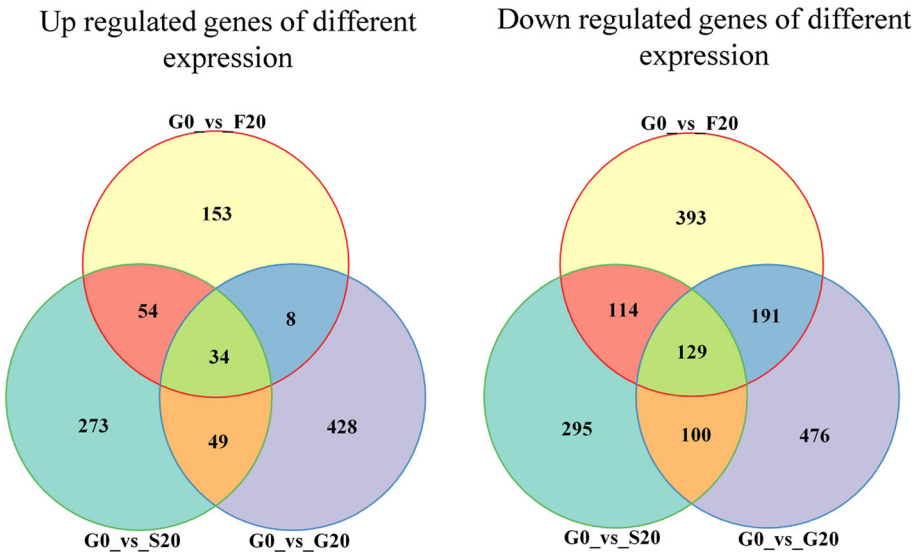


Fig. 2 Venn diagram of numbers of DEGs in four different sugars: G0_vs_F20 (*yellow*), G0_vs_S20 (*blue*), G0_vs_G20 (*purple*)

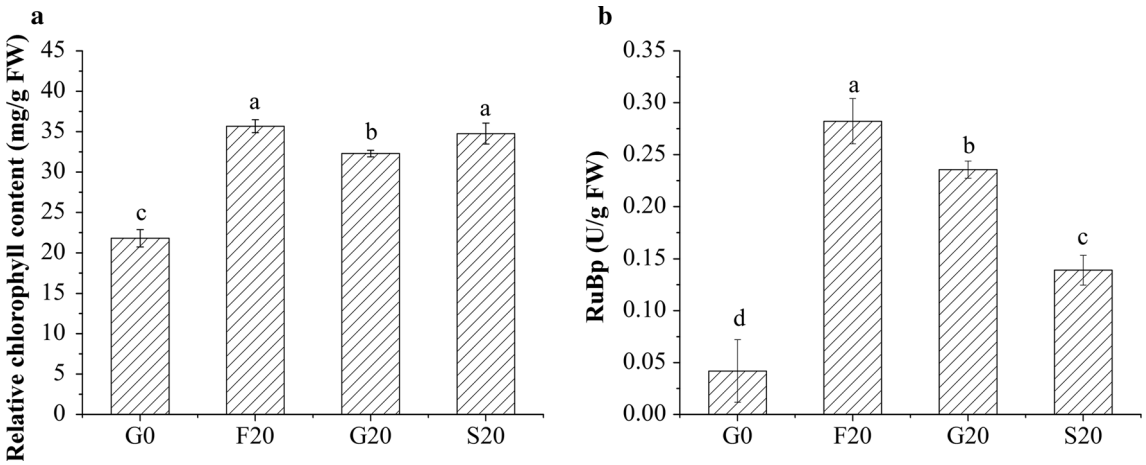


Fig. 3 Chlorophyll content (**a**) and Rubisco activity (**b**) in 'Red Globe' plantlet in vitro due to different sugars. Data are mean ± SE of three individual experiments, each performed in triplicate. Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

repeats 11, *ACR11*; aconitase 1, *ACO1*, map kinase kinase 1, *MKK1*; and necessary for the achievement of Rubisco accumulation 5, *NARA5*) were significantly up-regulated in F20 and G20 compared with those in G0. One gene (NAD(P)H dehydrogenase subunit H, *NDHH*) was significantly up-regulated under S20 with respect to that under G0.

Effect of different sugars on the key enzymes

The rate of sugar metabolism is reflected by the activities of related enzymes. The glucose-6-phosphate dehydrogenase (G6PDH) activities of G20, F20, and S20 significantly decreased compared to the G0 (Fig. 5a). The SS and NI activities of G20, S20, and F20 significantly increased with respect to those of the control (G0), and the activity of F20 was the highest (Fig. 5b, c). The SPS activity of F20 significantly increased relative to that of G0 (Fig. 5d). During the enzyme activity determination, each processing setup was repeated three times, and the repeatability was found favorable.

To identify the corresponding genes associated with the growth and development of "Red Globe" carbohydrate metabolism, transcriptome-wide gene expression profiles were compared between the libraries of different kinds of sugar treatments. We selected the DEGs related to key enzymes in the metabolic pathway on the basis of their functions and then divided the genes into four groups (Fig. 6): two of the sucrose phosphate synthase (*SPS*) genes (GSVIVG01034793001 and GSVIVG01012823001) and one of the alkaline/neutral invertase (*A/N-INV*) gene (GSVIVG01034944001) were significantly up-regulated in F20 relative to that in G0. Two *SUS* genes (GSVIVG01015018001 and GSVIVG01028043001), two *SPS* genes (GSVIVG01012825001 and GSVIVG01037179001), two *A/N-INV* genes (GSVIVG01031267001 and GSVIVG01034944001), and one *G6PDH* gene (GSVIVG01000913001) were significantly up-regulated in G20 relative to that in G0. Two other *SUS* genes (GSVIVG01035106001 and GSVIVG01035210001), three *SPS* genes (GSVIVG01034793001, GSVIVG01037186001, and GSVIVG01037179001), one *A/N-INV* gene (GSVIVG01024105001), and one *G6PDH* gene (GSVIVG01031295001) were significantly up-regulated in S20 relative to that in G0.

Effect of different sugars on the endogenous hormones of "Red Globe" plantlets

There was a close link between plant hormone content and glucose metabolism. The IAA and ZT contents of the treatments followed the order F20 > G20 > S20 > G0 (Fig. 7a, b). Meanwhile, the ABA content followed the order G0 > S20 ≥ F20 > G20 (Fig. 7c). The order of the ZT + IAA/ABA of the four treatments was G20 ≥ F20 > S20 > G0 (Fig. 7d). During the process of determination, each processing setup was repeated thrice, and the repeatability was good.

To explore the gene expression conditions in G20, F20, S20, and G0, the DEGs associated with hormone metabolism pathways were selected (Fig. 8). Five DEGs from ZT, including UDP-glycosyltransferase, were involved in the ZT transduction pathway. All of these DEGs were significantly up-regulated in F20 and S20 relative to that in G0. Most of the CTK-related DEGs were significantly up-regulated in G20 and showed higher expression levels in G20 and S20 than in G0. Twelve DEGs, including teosinte branched1/cycloidea/pcf 15 (*TCP15*, GSVIVG01008023

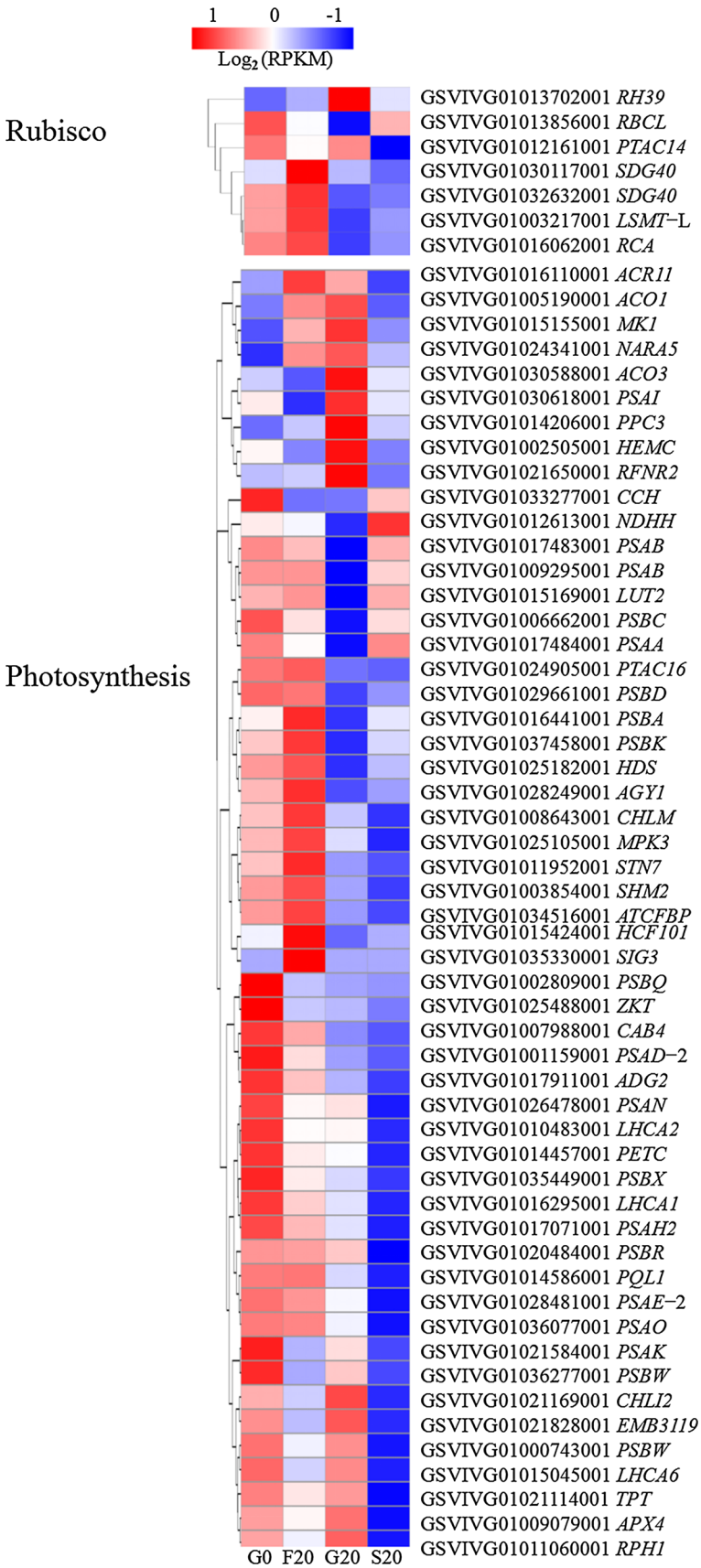


Fig. 4 Heat map of the expressed genes assigned to photosynthesis in the four grape transcriptomes, G0, F20, G20, and S20. *Colors* indicate the expression values of genes. Expression values of four libraries are presented as reads per kilobase per million reads (RPKM) normalized log2-transformed counts

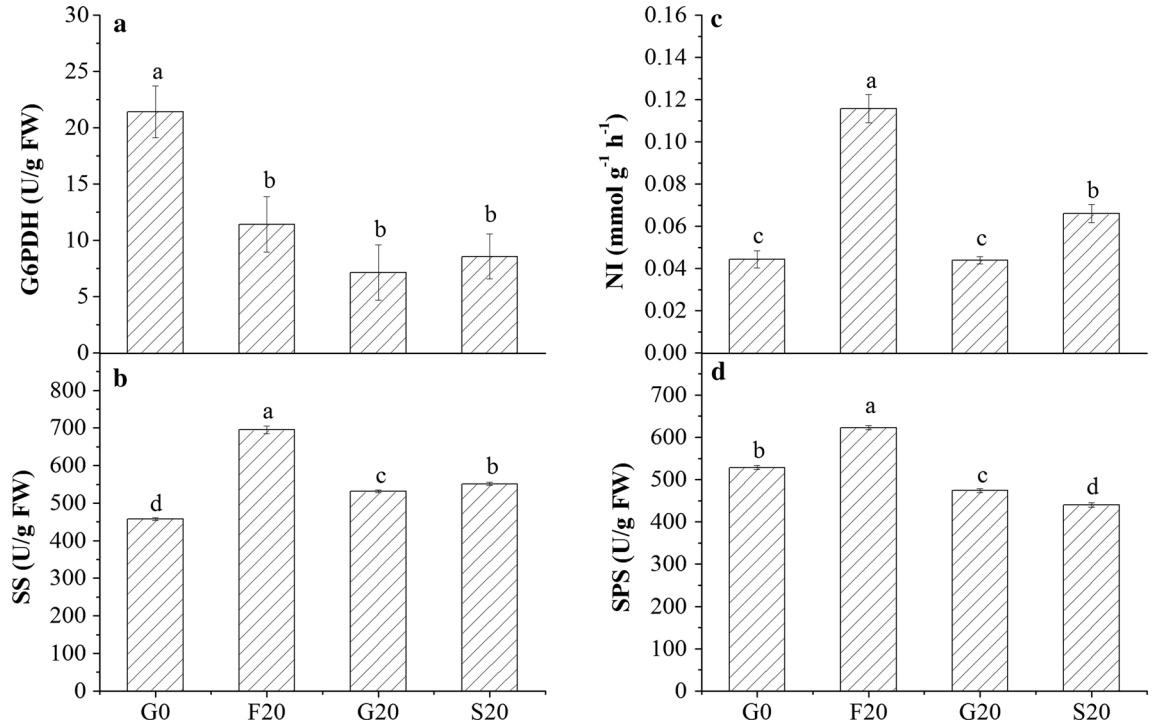


Fig. 5 Effects of different exogenous sugars on sugar metabolism and enzyme activity. **a** Glucose-6-phosphate dehydrogenase activity changes of plantlets. **b** Sucrose synthase activity changes of plantlets. **c** Neutral invertase activity changes of plantlets. **d** Sucrose phosphate synthase activity changes of plantlets. Data are mean ± SE of three individual experiments, each performed in triplicate. Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

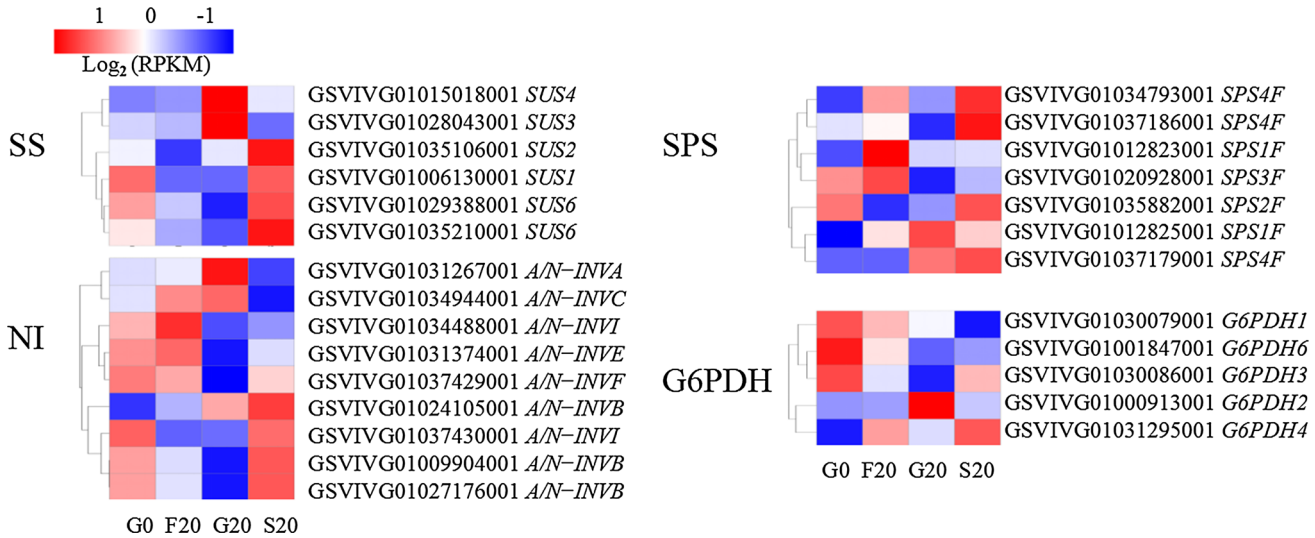


Fig. 6 Heat map of the expressed genes assigned to the key enzyme pathway in the four grape transcriptomes. *Colors* indicate the expression values of genes. The expression values of four libraries are presented as RPKM normalized log2-transformed counts

001), lonely guy 3 (*LOG3*, GSVIVG01009880001), post-meiotic segregation 1 (*PMS1*, GSVIVG01027985001), isopentenyltransferase 3 (*IPT3*, GSVIVG01034295001 and GSVIVG01011107001), ethylene response 1 (*ETR1*, GSVIVG01038085001), glycosyl hydrolase 9A1 (*GH9A1*, GSVIVG01023102001), histidine kinase 2 (*HK2*, GSVIVG01030058001), histidine kinase 3 (*HK3*, GSVIVG01010502001), cytokinin oxidase 5 (*CKX7*, GSVIVG01035468001), *RECA1* (GSVIVG01020482001), and spindly (*SPY*, GSVIVG01033891001) were significantly up-regulated in F20, G20, and S20 relative to that in G0. Twelve DEGs were specifically associated with the

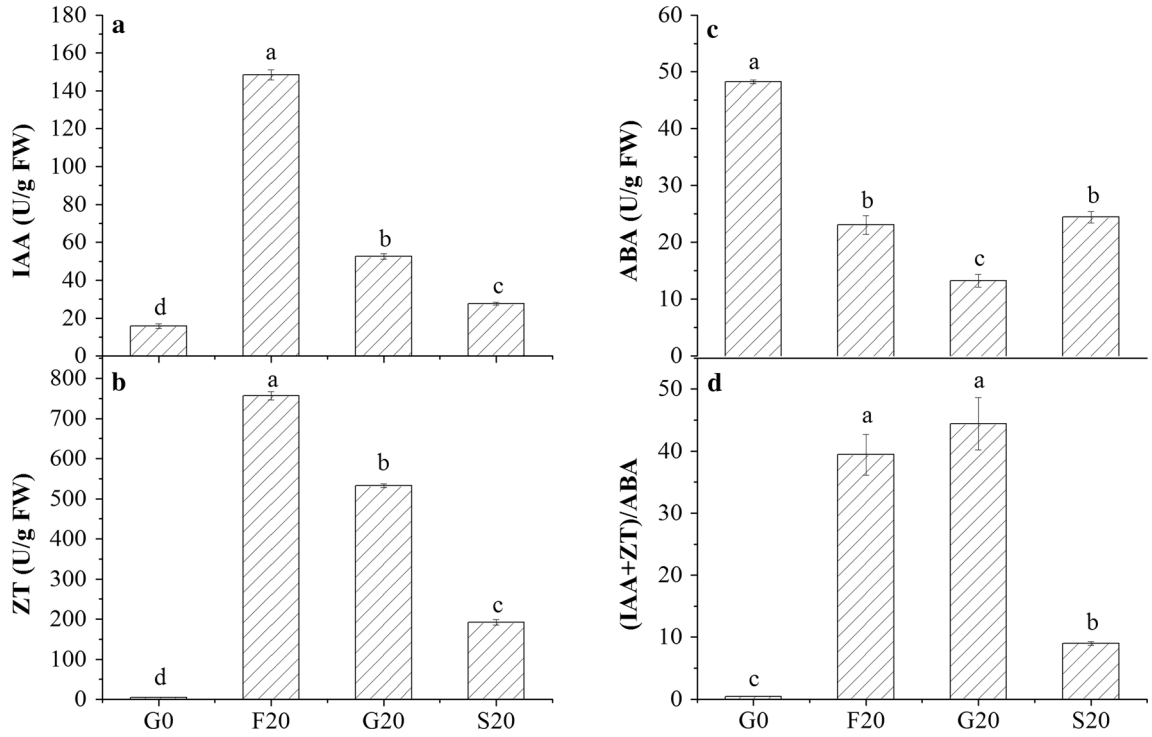


Fig. 7 Effect of different sugars on IAA content (**a**), ZT content (**b**), ABA content (**c**), and ZT + IAA/ABA (**d**). Data are mean ± SE of three individual experiments, each performed in triplicate. Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

auxin-activated signaling pathway and indole-3-acetic acid amidosynthetase activity. Five of Gretchen Hagen 3 family genes (*GH3s*; GSVIVG01030558001, GSVIVG01001112001, GSVIVG01033354001, and GSVIVG01035789001) were significantly up-regulated in S20 relative to those in G0. Two of *GH3s* (GSVIVG01011052001 and GSVIVG01014545001) were significantly up-regulated in G20 with respect to those in G0. Other two of *GH3s* (GSVIVG01023050001 and GSVIVG01027057001) were significantly up-regulated in F20 with respect to those in G0. A total of 33 DEGs were related to JA, and half of these DEGs were up-regulated in F20 and G20 relative to those in G0. Only three genes (oxophytodienoate-reductase 3, *OPR3*; indole-3-acetic acid inducible 14, *IAA14*; SNF1-related protein kinase 3.22, *SNRK3.22*) were significantly up-regulated in S20 relative to those in G0. A total of 32 DEGs participated in the regulation of ABA signal pathways, and most of these DEGs were up-regulated in F20 and G20 with respect to those in G0.

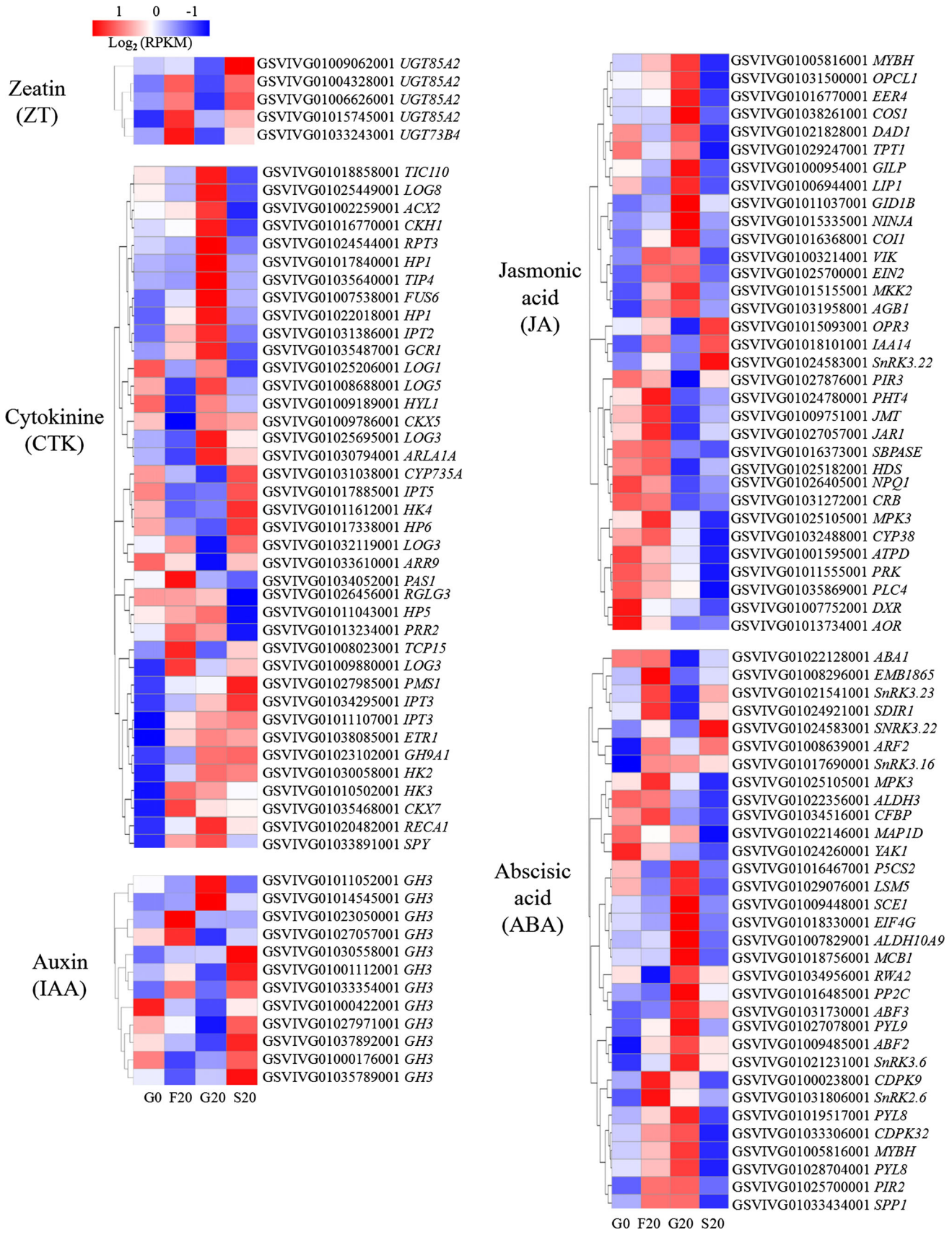
Expression of sugar metabolism-related genes

Sugar metabolism in plantlet in vitro is correlated with the status of the energy supply in the medium. Four pectin methylesterase 17 (*PME17*) genes (GSVIVG01027652001; GSVIVG01037349001; GSVIVG01037352001; GSVIVG01018622001), and one beta-fructofuranosidase (*BETAFRUCT4*) gene (GSVIVG01018625001) identified by DEG analysis were all up-regulated in G20 with respect to those in G0 (Fig. 9a, b). One alpha-amylase-like (*AMY*) (GSVIVG01031747001) gene was up-regulated in F20, G20, and S20 relative to those in G0. By contrast, two *AMY* genes (GSVIVG01031746001; GSVIVG01031740001), and two *BETAFRUCT4* (GSVIVG01035389001; GSVIVG01006154001) were down-regulated in F20, G20, and S20 with respect to those in G0.

Fig. 8 Heat map of the expressed genes assigned to the hormone ▶ signal transduction pathway in the four grape transcriptomes. *Colors* indicate the expression values of genes. Expression values of four libraries are presented as RPKM normalized log2-transformed counts

Verification of the gene expression through qRT-PCR

ABA and JA possess a close relationship with sugar metabolism. Thus, understanding the expression of genes involved in ABA's and JA's functions could provide a theoretical basis for studying the metabolism of different kinds of sugar. We selected 15 DEGs related to ABA and JA by qRT-PCR analysis (Fig. 8). The results indicated that only two genes (13.33%) analyzed by qRT-PCR, i.e.,


1 0 -1
Log2 (RPKM)
Zeatin (ZT)
GSVIVG01009062001 UGT85A2
GSVIVG01004328001 UGT85A2
GSVIVG01006626001 UGT85A2
GSVIVG01015745001 UGT85A2
GSVIVG01033243001 UGT73B4
Cytokinine (CTK)
GSVIVG01018858001 TIC110
GSVIVG01025449001 LOG8
GSVIVG01002259001 ACX2
GSVIVG01016770001 CKH1
GSVIVG01024544001 RPT3
GSVIVG01017840001 HP1
GSVIVG01035640001 TIP4
GSVIVG01007538001 FUS6
GSVIVG01022018001 HP1
GSVIVG01031386001 IPT2
GSVIVG01035487001 GCR1
GSVIVG01025206001 LOG1
GSVIVG01008688001 LOG5
GSVIVG01009189001 HYL1
GSVIVG01009786001 CKX5
GSVIVG01025695001 LOG3
GSVIVG01030794001 ARLA1A
GSVIVG01031038001 CYP735A
GSVIVG01017885001 IPT5
GSVIVG01011612001 HK4
GSVIVG01017338001 HP6
GSVIVG01032119001 LOG3
GSVIVG01033610001 ARR9
GSVIVG01034052001 PAS1
GSVIVG01026456001 RGLG3
GSVIVG01011043001 HP5
GSVIVG01013234001 PRR2
GSVIVG01008023001 TCP15
GSVIVG01009880001 LOG3
GSVIVG01027985001 PMS1
GSVIVG01034295001 IPT3
GSVIVG01011107001 IPT3
GSVIVG01038085001 ETR1
GSVIVG01023102001 GH9A1
GSVIVG01030058001 HK2
GSVIVG01010502001 HK3
GSVIVG01035468001 CKX7
GSVIVG01020482001 RECA1
GSVIVG01033891001 SPY
Auxin (IAA)
GSVIVG01011052001 GH3
GSVIVG01014545001 GH3
GSVIVG01023050001 GH3
GSVIVG01027057001 GH3
GSVIVG01030558001 GH3
GSVIVG01001112001 GH3
GSVIVG01033354001 GH3
GSVIVG01000422001 GH3
GSVIVG01027971001 GH3
GSVIVG01037892001 GH3
GSVIVG01000176001 GH3
GSVIVG01035789001 GH3
G0 F20 G20 S20
Jasmonic acid (JA)
GSVIVG01005816001 MYBH
GSVIVG01031500001 OPCL1
GSVIVG01016770001 EER4
GSVIVG01038261001 COS1
GSVIVG01021828001 DAD1
GSVIVG01029247001 TPT1
GSVIVG01000954001 GILP
GSVIVG01006944001 LIP1
GSVIVG01011037001 GID1B
GSVIVG01015335001 NINJA
GSVIVG01016368001 COI1
GSVIVG01003214001 VIK
GSVIVG01025700001 EIN2
GSVIVG01015155001 MKK2
GSVIVG01031958001 AGB1
GSVIVG01015093001 OPR3
GSVIVG01018101001 IAA14
GSVIVG01024583001 SnRK3.22
GSVIVG01027876001 PIR3
GSVIVG01024780001 PHT4
GSVIVG01009751001 JMT
GSVIVG01027057001 JAR1
GSVIVG01016373001 SBPASE
GSVIVG01025182001 HDS
GSVIVG01026405001 NPQ1
GSVIVG01031272001 CRB
GSVIVG01025105001 MPK3
GSVIVG01032488001 CYP38
GSVIVG01001595001 ATPD
GSVIVG01011555001 PRK
GSVIVG01035869001 PLC4
GSVIVG01007752001 DXR
GSVIVG01013734001 AOR
Abscisic acid (ABA)
GSVIVG01022128001 ABA1
GSVIVG01008296001 EMB1865
GSVIVG01021541001 SnRK3.23
GSVIVG01024921001 SDIR1
GSVIVG01024583001 SNRK3.22
GSVIVG01008639001 ARF2
GSVIVG01017690001 SnRK3.16
GSVIVG01025105001 MPK3
GSVIVG01022356001 ALDH3
GSVIVG01034516001 CFBP
GSVIVG01022146001 MAP1D
GSVIVG01024260001 YAK1
GSVIVG01016467001 P5CS2
GSVIVG01029076001 LSM5
GSVIVG01009448001 SCE1
GSVIVG01018330001 EIF4G
GSVIVG01007829001 ALDH10A9
GSVIVG01018756001 MCB1
GSVIVG01034956001 RWA2
GSVIVG01016485001 PP2C
GSVIVG01031730001 ABF3
GSVIVG01027078001 PYL9
GSVIVG01009485001 ABF2
GSVIVG01021231001 SnRK3.6
GSVIVG01000238001 CDPK9
GSVIVG01031806001 SnRK2.6
GSVIVG01019517001 PYL8
GSVIVG01033306001 CDPK32
GSVIVG01005816001 MYBH
GSVIVG01028704001 PYL8
GSVIVG01025700001 PIR2
GSVIVG01033434001 SPP1
G0 F20 G20 S20

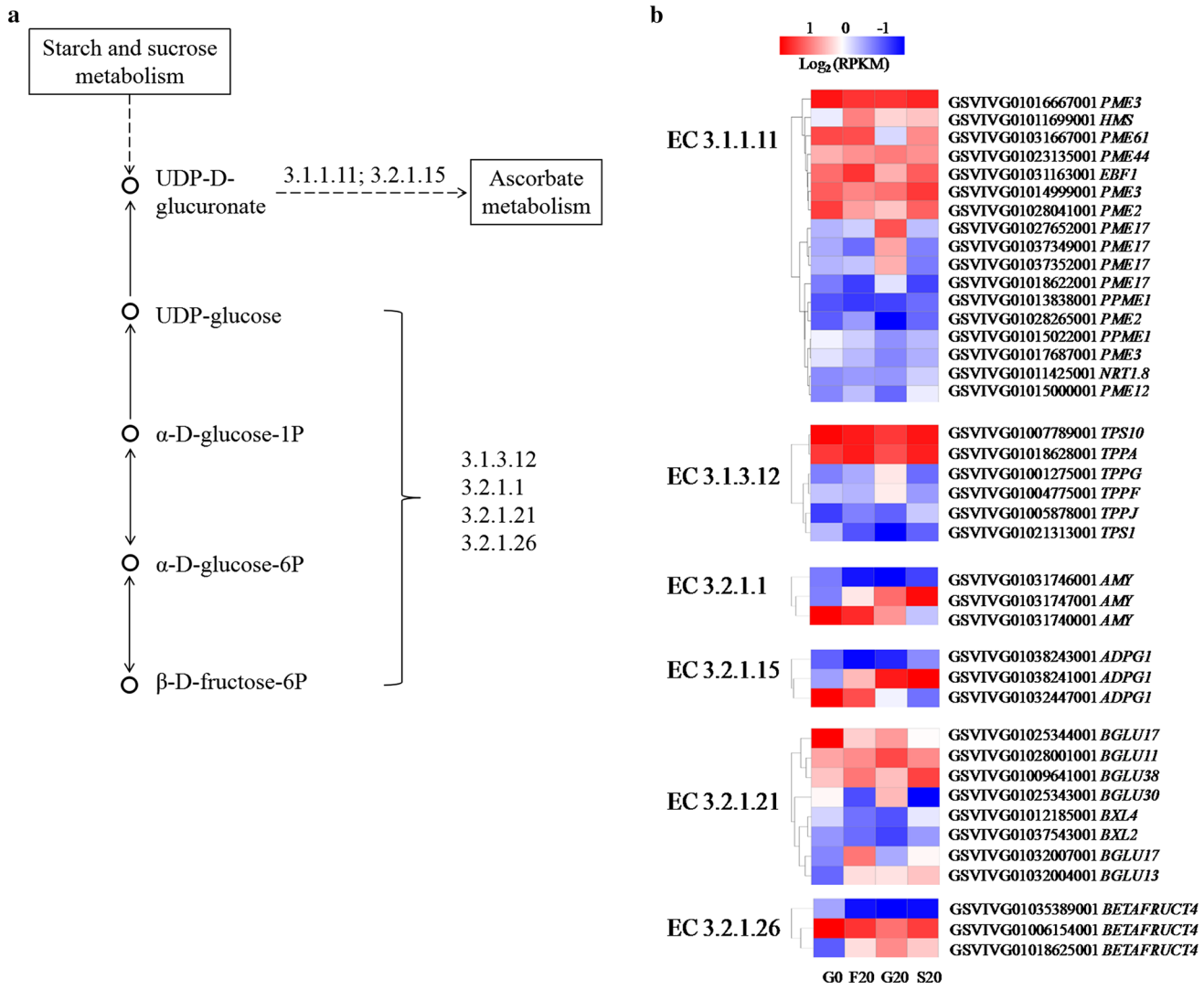


Fig. 9 Selected genes related to sugar metabolism from RNA sequencing data. **a** A schematic diagram of sugar metabolism. Bounding *boxes* represent metabolic processes or metabolite, and *digits* represents regulatory enzyme for specific process. *Straight arrow* indicates transformational direction of metabolite (*solid lines* directly, *dotted lines* indirectly). **b** Selected genes related to starch and sucrose metabolism in 'Red Globe' plantlets in vitro. *Red* and *blue colors* indicate up- and down- regulated transcripts, respectively

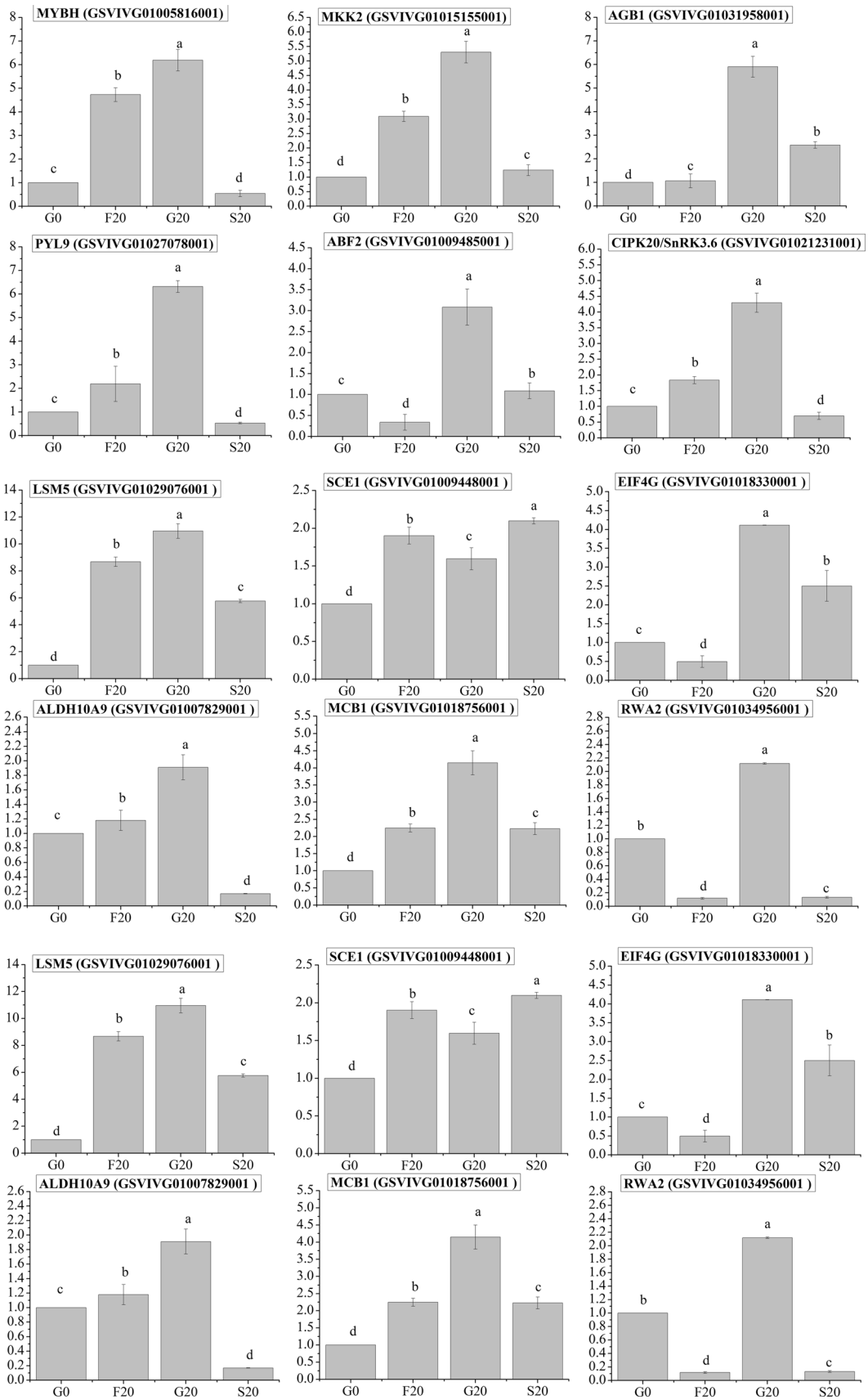
CDPK32 (GSVIVG01033306001) (F20/G20) and *SCE1* (GSVIVG01009448001) (F20/G20), were not consistent with our RNA-seq data (Fig. 10). However, 86.77% (13 genes) of the remaining genes analyzed by qRT-PCR agreed with the RNA-seq data.

Fig. 10 Real-time quantitative qRT-PCR confirmation of 15 candidate genes related to the JA and ABA from the four treatments, G0, G20, S20, and F20. The *left y*-axes indicate relative gene expression levels determined by qRT-PCR and analyzed by the $2^{-\Delta\Delta C_T}$ method. All qRT-PCRs for each gene used three biological replicates, with three technical replicates per experiment. Data are mean ± SE of three individual experiments, each performed in triplicate. Within each set of experiments, *bars* with *different letters* were significantly different at the 0.05 level

Discussion

Grape plantlets in vitro respond variably to different kinds of externally added sugars

Lim et al. (1992) demonstrated that the test tube seedlings of *Dendrobium cv.* White Fairy I grew in heterotrophic mode during cultivation by measuring the changes during the CO_2 fixation of test tube seedlings. In conventional culture, exogenous sugar is the main carbon source. *Cymbidium roseum* grows better in sucrose than in maltose, glucose, or fructose as carbon source. By contrast, fructose is beneficial to the growth of *Dendrobium nigrum* and *V. mongolica*. The addition of 3% sucrose to the medium


MYBH (GSVIVG01005816001)
MKK2 (GSVIVG01015155001)
AGB1 (GSVIVG01031958001)
PYL9 (GSVIVG01027078001)
ABF2 (GSVIVG01009485001)
CIPK20/SnRK3.6 (GSVIVG01021231001)
LSM5 (GSVIVG01029076001)
SCE1 (GSVIVG01009448001)
EIF4G (GSVIVG01018330001)
ALDH10A9 (GSVIVG01007829001)
MCB1 (GSVIVG01018756001)
RWA2 (GSVIVG01034956001)
LSM5 (GSVIVG01029076001)
SCE1 (GSVIVG01009448001)
EIF4G (GSVIVG01018330001)
ALDH10A9 (GSVIVG01007829001)
MCB1 (GSVIVG01018756001)
RWA2 (GSVIVG01034956001)
G0
F20
G20
S20

resulted in a three times faster growth of *Nicotiana tabacum* (var. Samsun) (Mousseau 1986). The shoot length and the number of leaves of *Dendrobium* were almost equal in the plantlets grown on medium with 2% sucrose or without sucrose and under normal or enhanced (40 g m^{-3}) CO_2 concentration. Fresh and dry masses were higher in the cultures grown in sucrose-containing media or under CO_2 enrichment (Mitra et al. 1998). In this study, the internode length, leaf number, RuBp activity, acid invertase (AI) activity, NI activity, SS activity, SPS activity, and IAA and ZT contents of F20 were all significantly higher than those of G20 and S20. F20 was the most appropriate carbon source for improving the growth, development, and photosynthesis of grape plantlets in vitro, followed by G20 and S20. Thus, we confirmed that "Red Globe" plantlets in vitro exhibited different growth response to different kinds of exogenous sugars.

Candidate genes involved in the complex regulation of sugar and starch metabolism

Studies have shown that the transfer of sucrose to sink organs during photosynthesis often requires sucrose hydrolysis by invertase (INV) into glucose and fructose. Moreover, SS degrades sucrose into UDP glucose and fructose in the presence of UDP (Geigenberger and Stitt 1993; Kleczkowski et al. 2010). SS and SPS are involved in the regulation of the translocation of photosynthates. Thus, sucrose is involved in the metabolic pathways. NI is involved in the regulation of plant growth and development, while G6PDH is involved in the catalysis of 6-phosphogluconate for dehydrogenation and a key enzyme related to the pentose phosphate pathway. In the present study, genes related to SS, SPS, NI, and G6PDH activities were selected and we found that these candidate genes were differentially expressed in the different treatments (Fig. 6). This observation may explain why the processing of different sugars represents different phenotype (Li et al. 2015; Lloyd et al. 2015). Thus, *SUS*, *SPS*, *A/N-INV*, and *G6PDH*, which were related to these enzymes (Fig. 6), were considered to be correlated with the sugar and starch metabolism of grape plantlet in vitro.

The genes in Fig. 9 were involved in the carbohydrate metabolic process and hold different molecular functions. *AMY* is involved in starch mobilization, carbohydrate metabolic process, and response to abscisic acid and gibberellin (Whan et al. 2014). *BETAFRUCT* is involved in the abscisic acid-activated signaling pathway, cellular response to gibberellin stimulus, primary root development, response to bacterium, and sucrose metabolic process (Ryan et al. 2005). One *AMY* (GSVIVG01031747001) gene, which was up-regulated in all the sugar treatments, was considered positively correlated with the carbohydrate metabolism of grape plantlet in vitro. This finding was similar to that of *BETAFRUCT4* (GSVIVG01018625001), which was up-regulated in G20 relative to that in G0. By contrast, two *BETAFRUCT4* genes (GSVIVG01035389001; GSVIVG01006154001) and two *AMY* (GSVIVG01031746001; GSVIVG01031740001) genes down-regulated in all the sugar treatments were considered to be negatively correlated with the carbohydrate metabolism of grape plantlet in vitro.

Sugar and starch metabolism controls the generation of plant hormone transduction pathway signaling molecules

Plant hormones, including ABA, GA, JA, CTK, IAA, ZT, and ETH, are involved in many different plant processes, including growth, development, and senescence (Sarnowska et al. 2016; Huang et al. 2016). Researchers have shown that plant hormone signals participate in regulating plant sugar metabolism (Zhu et al. 2011; Ming et al. 2014; Wang et al. 2015; Xiao et al. 2016). Our transcriptome analysis also revealed that many genes were involved in the plant hormone response to sugar and starch metabolism. The auxin-responsive *GH3* gene family members were significantly up-regulated in all the sugar treatments. Similarly, the DEGs (*TCP15*, *LOG3*, *IPT3*, *ETR1*, *HK2*, *HK3*, *CKX7*, and *SPY*), which were involved in CTK biosynthesis to control cell division and shoot initiation, were also significantly up-regulated in the different sugar treatments (Fig. 8). This suggested that all the genes promoted growth and development in grape plantlets in vitro. The ABA-related DEGs, such as protein phosphatase 2C (*PP2C*), PYR-Like (*PYL*), *SnRK*, and ABA responsive transcription factors (*ABF*), as well as half of the JA-related DEGs, including the MYB hypocotyl-elongation-related gene (*MYBH*, GSVIVG01005816001), the G-protein β-subunit gene (*AGB1*, GSVIVG01031958001), and the MAPK kinase gene (*MKK2*, GSVIVG01015155001), were significantly up-regulated in sugar treatments (Fig. 8), suggesting that they were all involved in the response to sugar and starch metabolism.

In conclusion, the results of our research revealed that F20 is the most appropriate sugar for improving the growth and development of grape plantlets in vitro. Sugar metabolism was a complex process, which depended on the balanced expression of key potential candidate genes related to hormones (*TCP15*, *LOG3*, *IPT3*, *ETR1*, *HK2*, *HK3*, *CKX7*, *SPY*, *GH3s*, *MYBH*, *AGB1*, *MKK2*, *PP2C*, *PYL*, *ABF*, *SnRK*, etc.), key metabolic enzymes (*SUS*, *SPS*, *A/V-INV*, and *G6PDH*), and sugar metabolism (*BETAFRUCT4* and *AMY*). It can be concluded that there was close connection between the sugar signal and hormone signal transduction pathway. The results of the experiment

improved our understanding of the complex regulatory networks of signal transduction.

Author contribution statement BHC conceived and designed the experiments. JM, WFL and BQM conducted experiments and observed the plant growth. WFL performed qRT-PCR and part of the data analysis. ZHM and YMZ participated in the preparation of the plant material and part of the data analysis. JM and BQM wrote the manuscript. MMD and ACU reviewed the manuscript and part of the data analysis. All of the authors in this study read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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