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Genome Wide Analysis of *GH* Gene Family Reveals *Vvgh9* Positively Regulates Sugar Accumulation under Low Sugar Content in Grape

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Abstract: Sugar content directly affects grape (*Vitis vinifera* L.) berry quality and the resulting wine. Therefore, it is of great importance to study and explore novel genes that affect sugar accumulation in grapes. Glycosyl hydrolases (GHs) are key enzymes hydrolyzing polysaccharides into monosaccharides and play important roles in the regulation of carbohydrate metabolism. Nevertheless, the impact of GHs on the regulation of sugar accumulation in plants has rarely been investigated. In this study, we identified 11 putative *GH* genes in grapevines by phylogeny analysis. RNA-seq and quantitative real-time PCR results demonstrated that the expression level of *VvGH9* was higher during the fruit set stage, which had lower sugar content than the véraison and ripe stages. Treatment of grape berries with exogenous sugar two weeks before véraison revealed that *VvGH9* was rapidly induced by sucrose, fructose, and glucose. When '41B' calli was treated with different concentrations of glucose, *VvGH9* expression increased at first and then decreased with the increase of glucose concentration. Overexpression of *VvGH9* in grape calli and tomatoes also confirmed that this gene could contribute to sugar accumulation. All the above results demonstrated that *VvGH9* promotes sugar accumulation under low sugar content in plants.

Keywords: grape; sugar; *VvGH9*; transcriptomic analysis; overexpression

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1. Introduction

As one of the fruit crops with the most extensively cultivated area and the longest industrial chain in the world [1], grapes (*Vitis vinifera*) substantially contribute to the global economy. Besides the water content, sugars are also the primary substances present in mature grape berries, accounting for 15–25% of the fresh berries [2]. The type and content of sugars not only affect the sensory quality of grape berries, but also affect the abundance and flavor of the wine. The predominant sugars in grape berries are glucose and fructose, while sucrose is present but in low concentrations. Studies have shown that sugar not only acts as a carbon and energy source, but also as a regulatory signal of genes expression, plant growth, and development [3]. Therefore, it is important to analyze the molecular mechanisms underlying the direct and indirect regulation in sugar accumulation of plants.

GHs (glycosyl hydrolases) are key enzymes that hydrolyze glycosidic bonds of carbohydrates and a noncarbohydrate moiety in all living organisms [4]. McCarter et al. [5] investigated the catalytic mechanism of polysaccharide hydrolysis through structural studies of retaining β-glycosyl hydrolases. Heritable deficiencies of glycosyl hydrolases in human may result in lactose intolerance [6] and lysosomal storage diseases [7]. Bauer

et al. [8] summarized the developments and potential functions of GH proteins in hyperthermophilic microorganisms. In 1999, it was first reported that glycosyl hydrolases, which recognize different glycofuranoside residues, share a common sequence motif [9]. It was reported that the activity of O-glycosyl hydrolases was increased by the presence of 1→3-β-D-glucanase inhibitors [10]. Moreover, various GH enzymes are also widely applied in the food industry [11]. It was also assumed that GH may be effectively employed as synthetic enzymes in the near future based on the enzyme catalytic scheme and the development of nonaqueous enzymology [12]. Meanwhile, some GH family 1 members were reported to be involved in response to hormone treatment and abiotic stresses [13,14].

Additionally, GHs are widely used as a natural hydrolytic agent to make raw complex mixtures into value-added simple materials [15]. Since the end of the last century, engineered GHs have become a very helpful biomaterial to improve the efficiency and yields of glycosylation reactions [16]. In 2015, Trincone summed up two possible experimental protocols, the reverse hydrolysis procedure and the kinetic approach, for the use of GHs in synthesis [17]. In many fruits, such as apples [18] and tomatoes [19], starch mainly accumulates at early fruit developmental stages and is degraded into simple sugars during fruit development. It was reported that a gene encoding an α-glucosidase, *AdAGL3*, was significantly induced by ethylene and promoted starch degradation [20]. As GH family members, β-glucosidases can hydrolyze the β-D-glycosidic bonds of glucosides and oligosaccharides non-reducing end to release glucose [21]. A recent study reported that *MaGlu1A*, a new gene encoding β-glucosidase MaGlu1A, strongly responded to the stimulation of supplemental glucose and the active center of MaGlu1A was identified by site-directed mutagenesis [22]. To date, the function of GH genes in sugar accumulation, particularly in grapevines, is still rarely studied.

In this study, we carried out a genome-wide analysis of *GH* genes in *V. vinifera* and measured *GH* genes expression levels at three berry developmental stages belonging to 22 varieties. We also explored their function and regulation mechanism in sugar accumulation. This study is an insight into the function of the *GH* family and provides new molecular markers for the high-sugar-content breeding of grapes.

2. Materials and Methods

2.1. Plants and Growth Conditions

Five different stages (FS: fruit set; T: touching; V: véraison; M: mid-ripening; R: ripe) of *V. vinifera* cv. 'Muscat Hamburg' berries were collected to measure sugar and *VvGH9* gene expression levels. For this purpose, berries of *V. vinifera* cv. 'Chardonnay' were used for exogenous sugar treatment two weeks before véraison. Seedlings of 'Muscat Hamburg' and 'Chardonnay' were planted in the vineyard at the Institute of Botany, Chinese Academy of Sciences, Beijing, all these plants were well planted and were in good condition. '41B' (*V. vinifera* × *V. berlandier*) calli were cultured according to the protocol described by Wang et al. [23].

2.2. Identification and Phylogenetic Analysis of GH Genes

Hidden Markov Model (HMM) (PF01055.26) was used to identify GH proteins. The obtained proteins were also searched against the whole proteins (IGGP V2.1) of the grape by BLAST. Then the candidate members from HMM and BLAST were confirmed by NCBI-Conserved Domain Data (CDD) search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 21 November 2020).

Protein sequences of GH family members were used to construct the phylogenetic tree. These sequences were aligned by MUSCLE first, then the construction of the phylogenetic tree was performed by MEGA 7.0 software (https://www.megasoftware.net/, accessed on 17 July 2021) [23] with the method of neighbor-joining and bootstrap of 1000 [24].

2.3. Gene Location and Duplication Analysis of GH Genes in the Grape Genome

The locations and duplication events analysis of *GH* genes was performed according to Xu et al. [25]. The location of each gene was shown on CIRCOS map [26], and the red curves and the color dots represented segment and tandem duplication events respectively.

2.4. RNA-seq Data Analysis

The expression pattern of *VvGH9* at 3 developmental stages in 22 grape varieties (details are listed in Tables S1 and S2) was obtained from the grape-RNA database (http://www.grapeworld.cn/gt/, accessed on 9 May 2021) [27].

2.5. Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Assays

The total RNA of 'Muscat Hamburg' berries, 'Chardonnay' berries and '41B' calli was extracted by HiPure HP Plant RNA Mini Kit (Magen, Guangzhou, China). HiScript III 1st Strand cDNA Synthesis Kit (R312-02, Vazyme, Nanjing, China) was used to synthesize the first cDNA strand, and cDNA for qRT-PCR was generated by HiScript II Reverse Transcriptase (R201-02, Vazyme, Nanjing, China). The qRT-PCR primers of *VvGH9* and a reference gene *VvACTIN* (Accession: EC969944) are listed in Table S3. The specific methods for qRT-PCR were performed according to Wang et al. [23].

2.6. Exogenous Sugar Treatment of Grape Berry via Injection

About 2 weeks before véraison (V), three bunches of 'Chardonnay' berries with similar numbers and types for each soluble sugar were selected. Sucrose, glucose, and fructose solutions (each 20 g/L) were separately placed into injection bags. A needle with 1 mm diameter was inserted into about 1 cm depth, and the flow rate was about 0.1 mL/min. Subsequently, the expression levels of *VvGH9* were analyzed at 0, 12, 24, and 48 h.

2.7. Treatment of Grape Calli with Different Concentrations of Glucose

The '41B' calli were used for sugar treatment, and the culture method was performed as described by Wang et al. [23]. For different glucose concentrations treatments, the calli were cultured in a corresponding liquid medium with different concentrations of glucose for 7 days. Next, the expression patterns of *VvGH9* in these materials were analyzed.

2.8. Cloning of VvGH9 Gene

The coding sequence of *VvGH9* (VIT_202s0033g01410, also known as alpha-glucosidase 2-like in NCBI) was amplified using the 'Chardonnay' leaves cDNA as template with the primers VvGH9-F/VvGH9-R (Table S3), designed according to the predicted coding sequence of *VvGH9* from *V. vinifera* cv. 'Pinot Noir' (PN40024) [28]. The PCR was conducted with KOD-Plus-Neo (Toyobo, Osaka, Japan) DNA polymerase in a total volume of 50 µL at 95 °C for 2 min; 40 cycles of 98 °C for 10 s, 56 °C for 30 s, 68 °C for 90 s; 68 °C for 10 min. The product of PCR was cloned into pLB-Simple vector (Tiangen, Beijing, China) and analyzed by Sanger sequencing. The verified sequence was then used as the template to amplify the open-reading frame (ORF) of *VvGH9* using the primers GH9-ORF-F/GH9-ORF-R (Table S3), and the PCR was performed as described above. The amplified *VvGH9* ORF fragment was ligated into the *KpnI* (NEB, Ipswich, MA, USA)-digested pCAMBIA2300 vector, which serves as an overexpression vector modified by Wang et al. [23], through the homologous recombination methods using SE Seamless Cloning and Assembly Kit (ZOMANBIO, Beijing, China).

2.9. Transformation of Grapevine Calli and Tomato

The recombinant vector above and the empty vector (EV) were then transformed into '41B' calli as described by Xu et al. [25]. Then, *VvGH9*-transgenic tomatoes were generated to further investigate its functions, and the transformation method was based on Sun et al. [29]. Using gene/kanamycin-specific primers (Table S3) for PCR to confirm the transgenic plants.

2.10. Sugar Determination

The frozen grape berries or calli (0.2 g fresh weight (FW)) were homogenized into 2 mL deionized water for 4 h in an ice bath with intermittent mixing, and then were centrifuged (8000× *g*, 10 min, 4 °C). Specific filtration and analytical methods were performed according to Zhang et al. [30].

3. Results

3.1. Identification and Phylogenetic Analysis of Eleven GH Genes in Grape

In total, 11 *GH* genes named *VvGH1* to *VvGH11* were identified in the grapevine genome (12X, V2.1) (Figure 1). According to the phylogenetic tree of 11 VvGH proteins (Figure 1), VvGH members were classified into four groups (GH_1, GH_2, GH_3, and GH_4). The GH_1 was the largest group, containing 4 members, while the GH_4 was the smallest group, with only one member.

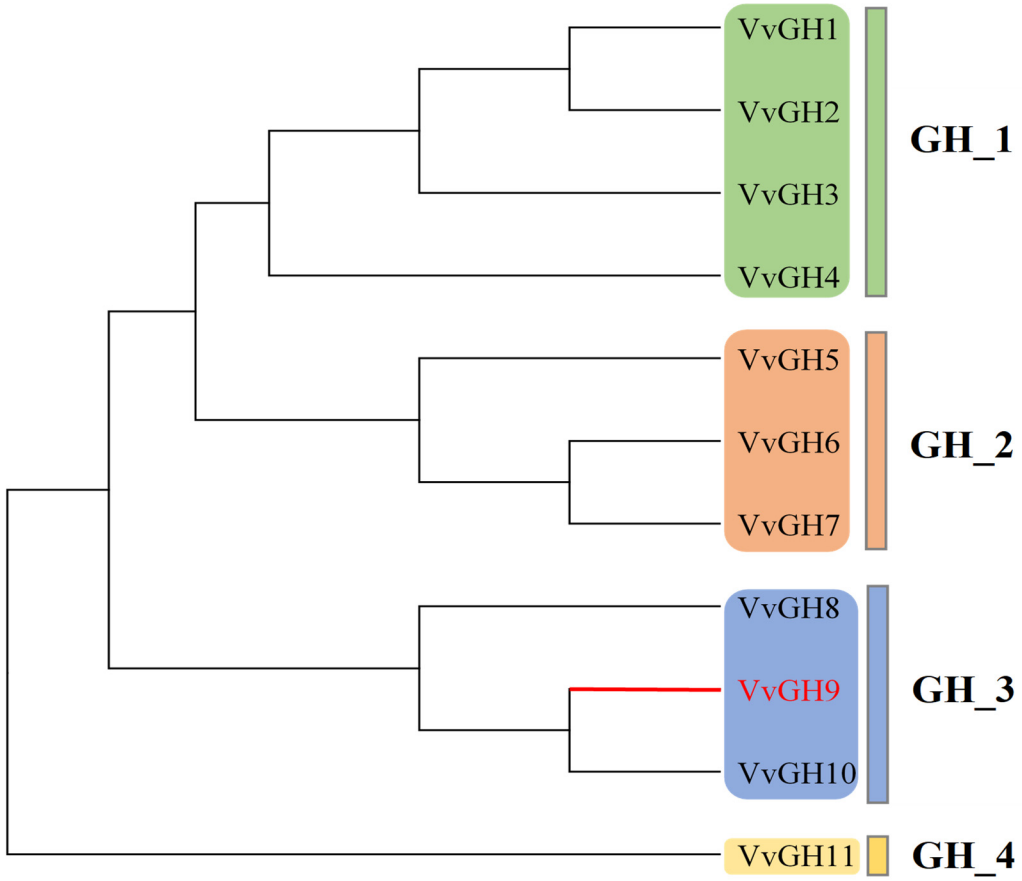

Figure 1. Phylogenetic analysis of *GH* genes in *V. vinifera*. The unrooted tree was generated by ClustalW in MEGA7 using the conserved amino acid sequences of the 11 *V. vinifera* GH proteins.

3.2. Chromosome Localization and Gene Duplication Analysis of Vvgh Genes

The 11 *VvGH* genes were distributed on seven chromosomes including chromosome 1, 2, 5, 8, 10, 13, and 16 (Figure 2). Chromosome 2 contained four *VvGH* genes, chromosome 10 contained two *VvGH* genes, and each of the other chromosomes contained the 1*VvGH* gene respectively. Three segmental duplication events of *GH* genes were identified in *V. vinifera*, and eight members of the *VvGH* genes were related to these events. Moreover, four genes (*VvGH4*, *VvGH10* and *VvGH6*, *VvGH7*) were involved in two tandem duplication events. These four members were involved in both segmental and tandem duplication and three members of *VvGH* genes were not detected in the duplication events.

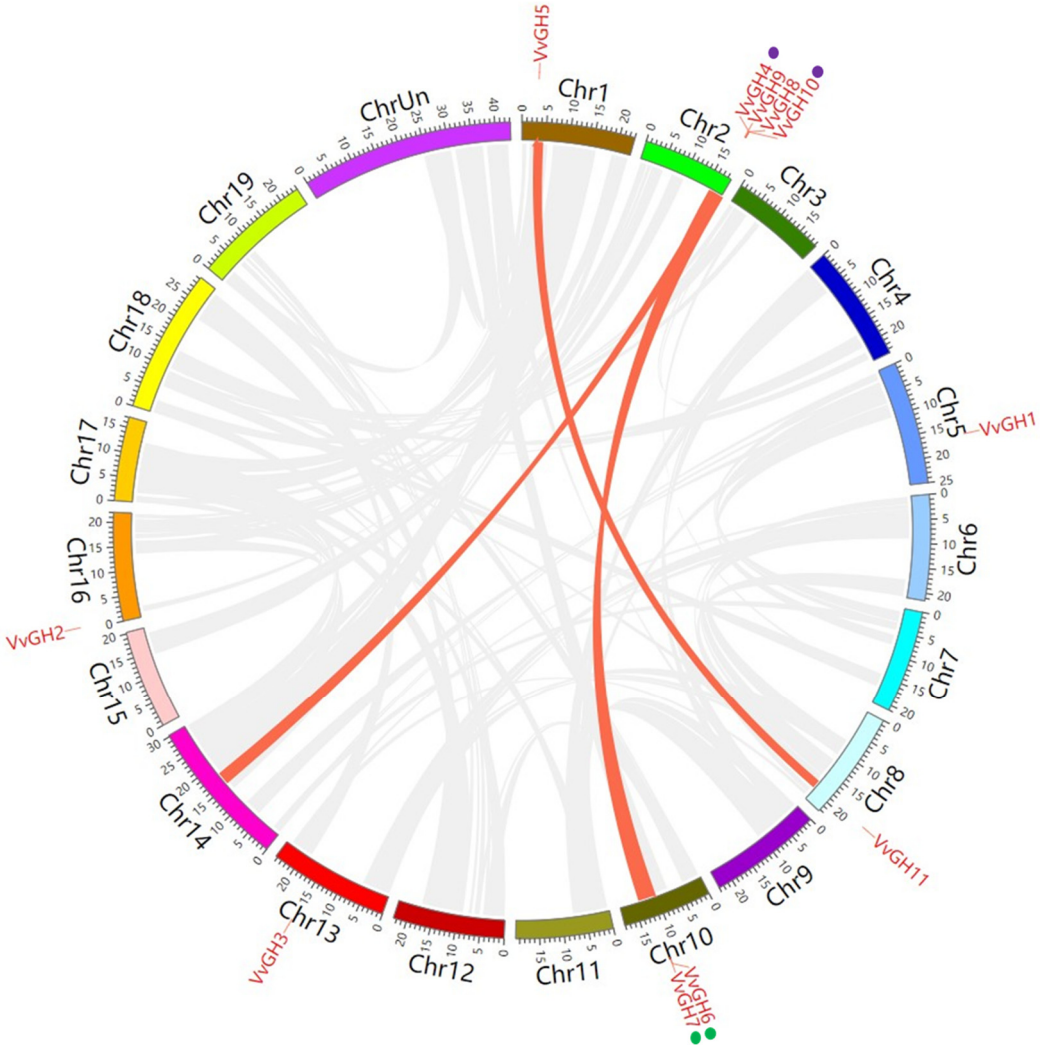

Figure 2. Chromosome distribution and duplication analysis of grapevine *GH* genes. Chromosomes 1–19 are shown in different colors and a circular form. The approximate distribution of each *VvGH* gene is marked with a short red line on the circle. Red curves denote the details of segmental duplication between grape *GH* genes. Different colorful dots denote the details of tandem duplication between grapevine *GH* genes.

3.3. Expression Profiles of Vvgh Genes at Different Developmental Stages in Grape Berries

To obtain insights into the potential roles of the 11 genes in different developmental stages of grape berry, we estimated their expression patterns based on RNA-seq data (http://www.grapeworld.cn/gt/, accessed on 9 May 2021) of 22 grape varieties (Figure 3). Most *VvGH* genes had significant expression differences between different stages. *VvGH5*, *VvGH6*, and *VvGH9* had similar expression patterns in all samples and expressed as significantly different between the first two stages in some varieties. However, only *VvGH9* was observed to be expressed differently in the three stages of almost all the 22 grape varieties.

Moreover, six representative varieties were selected to further analyze the three significant differential expressed genes (*VvGH5*, *VvGH6*, and *VvGH9*), and the results showed that only *VvGH9* was expressed and existed a significant differential expression at all stages of the six selected varieties (Figure 4).

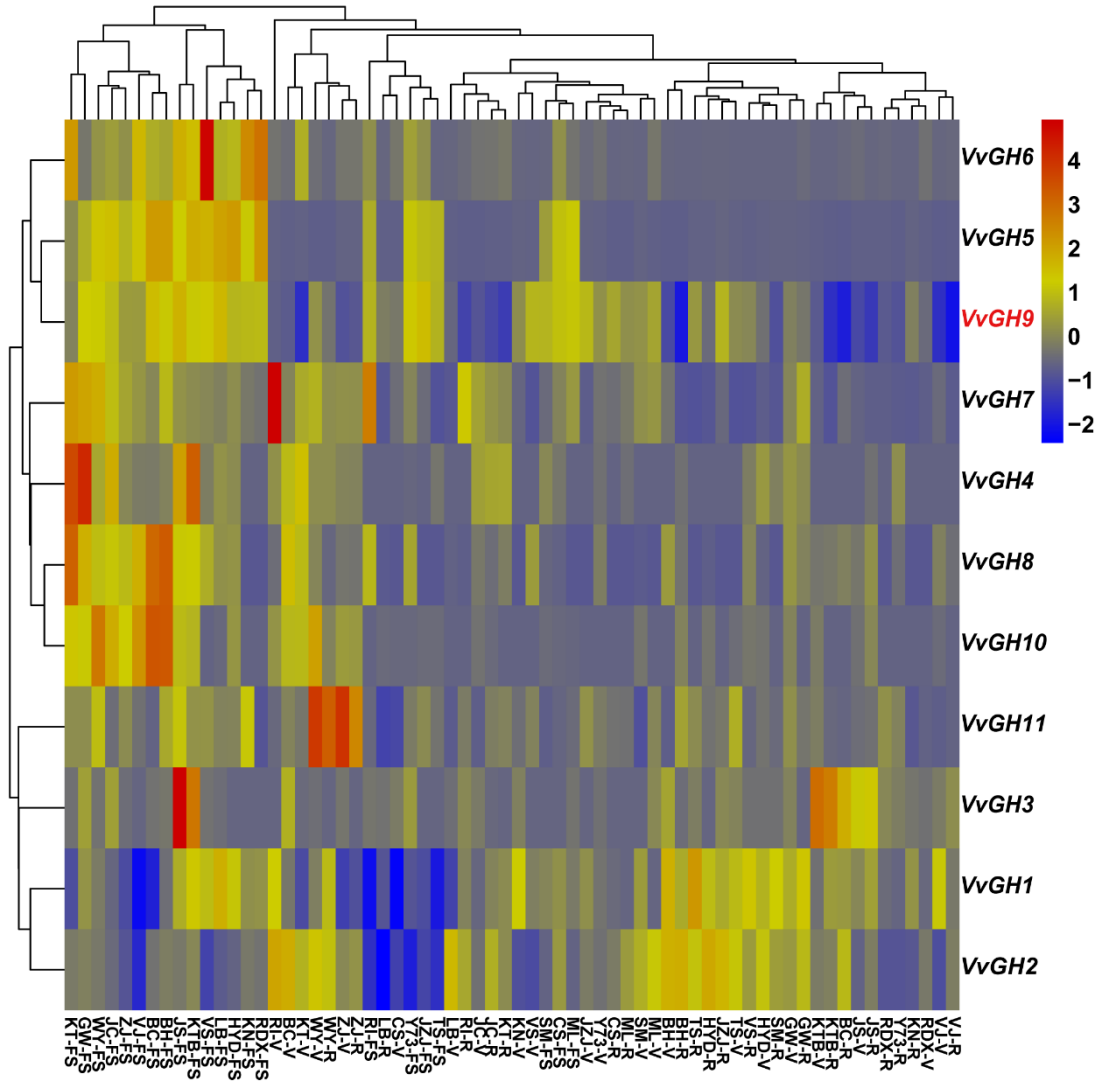

Figure 3. Expression profiles of *VvGH* genes at 3 developmental stages of 22 grape varieties. The expression levels of these genes were downloaded from the grape-RNA database. The color intensity represents relative expression levels, with red as increased transcript abundance and blue as decreased transcript abundance. The acronyms below the heatmap represent grape varieties (Table S2). FS: fruit set. V: véraison. R: ripe.

To further elucidate the expression patterns of *VvGH9* at five developmental stages in the fruits of *V. vinifera* cv. 'Muscat Hamburg' (Figure 5a), we performed an assay of qRT-PCR (Figure 5b). According to the qRT-PCR results, *VvGH9* exhibited a higher expression level at the fruit set (FS, E-L stage 27) and touching (T, E-L stage 32) [31]. The results of the qRT-PCR were basically consistent with the transcriptomic analysis. We also measured the sugar content of five developmental stages of 'Muscat Hamburg'. The measurement results showed that the sucrose content was too low to be detected in all the developmental periods in 'Muscat Hamburg' berry, while the two monosaccharides (glucose and fructose) content were very low (<10 mg/g) at FS and T stages but increased rapidly since véraison (>30 mg/g) (Figure 5c). The general trend of *VvGH9* expression pattern was basically opposite to that of total sugar content.

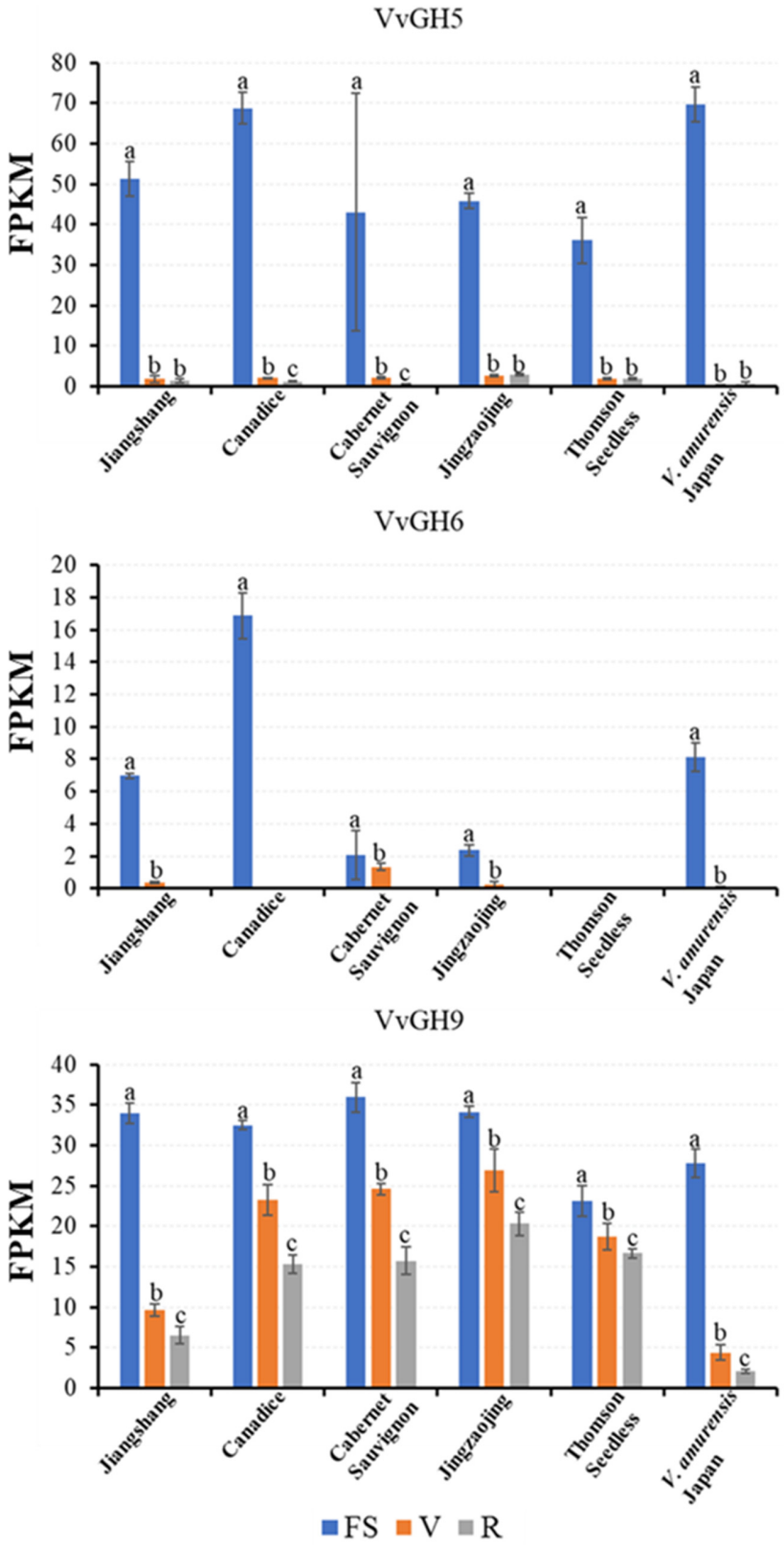

Figure 4. Transcriptomics analysis of *VvGH5*, *VvGH6*, and *VvGH9* at three developmental stages of six representative grape varieties berry. The expression levels of the three genes were downloaded from the grape-RNA database. FS: fruit set; V: véraison; R: ripe. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

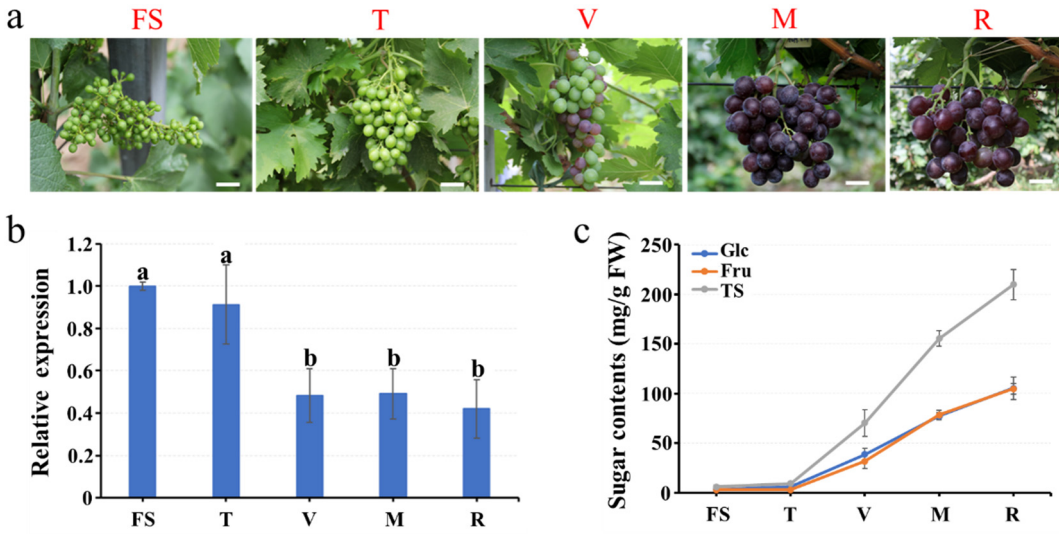

Figure 5. The relative expression level of the *VvGH9* gene and the sugar content of 'Muscat Hamburg' grape fruit at five different developmental stages. (**a**) Fruit growth status in different developmental stages. Scale bars, 2 cm. (**b**) Relative *VvGH* expression in different berry developmental stages. (**c**) Sugar content determination results of grape berries. FS: fruit set; T: touching; V: véraison; M: mid-ripening; R: ripe. FW: fresh weight. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

3.4. Exogenous Sugar Treatment by Injection Improves the Expression Level of Vvgh9 in Grape Berries

In order to investigate whether *VvGH9* responds to different sugars in fruit, 20 g/L of sucrose, glucose, and fructose were separately injected into spike-stalk of 'Chardonnay' berry about two weeks before véraison (Figure 6a). Expression of *VvGH9* was rapidly induced by treatment with each of the three soluble sugars, with the response to sucrose at 12 h being strongest (Figure 6b–d).

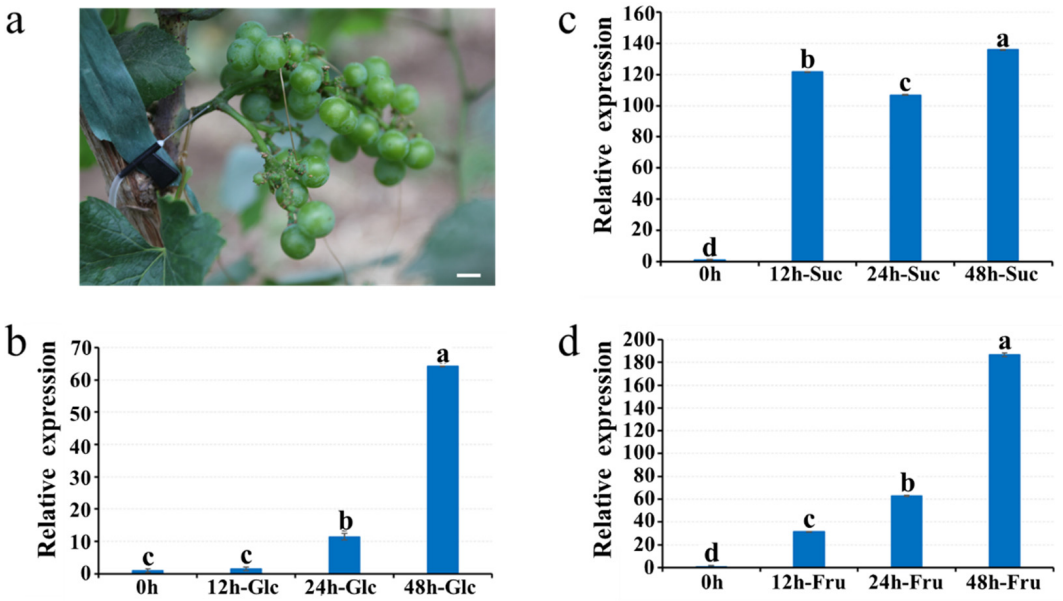

Figure 6. The relative expression profiles of *VvGH9* in grape berry injected with sucrose (**a**), glucose (**b**), and fructose (**c**) and treatment of 'Chardonnay' grape berry before véraison with exogenous sugars via injection (**d**). Scale bars, 1 cm. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

3.5. The Influence of Different Glucose Concentrations on the Expression Pattern of Vvgh9 in '41B' Calli

To explore whether different sugar contents affect the expression pattern of *VvGH9* in grapes, we treated '41B' calli with different glucose concentrations and detected the expression level of *VvGH9*. The results revealed that the *VvGH9* expression level increased remarkably first and then decreased with the increase of glucose concentration. The expression of *VvGH9* reached the highest at 30 mg/g glucose concentration (Figure 7).

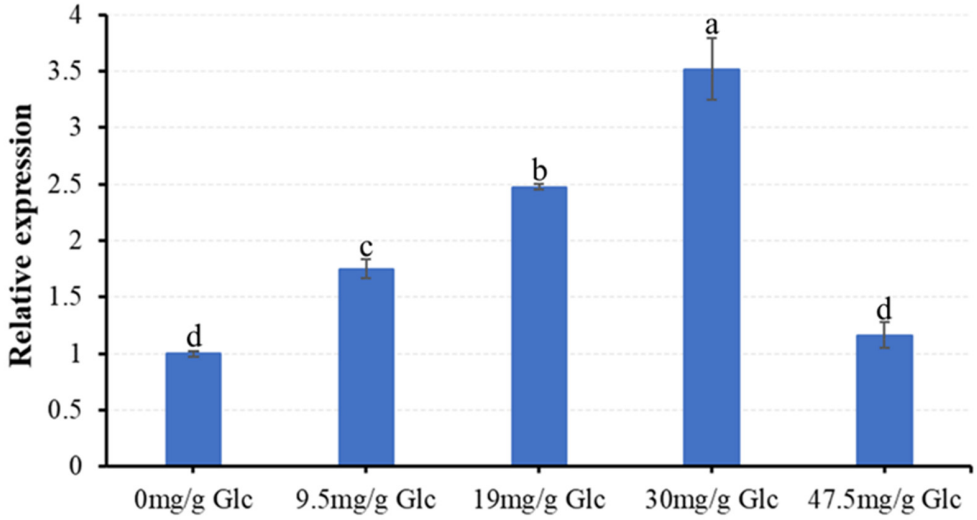

Figure 7. The expression level of *VvGH9* in '41B' wild-type calli treated with different concentrations of glucose. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

3.6. Overexpression of VvGH9 Gene Improved Sugar Content in 41B Calli

In order to investigate whether *VvGH9* expression affects the sugar content of grape cells, a 35S::VvGH9 construct was then stably transformed into '41B' calli via Agrobacteria-mediated transformation. The successful transformation was confirmed by PCR (Figure 8a). *VvGH9* expression in the '41B' calli of overexpressed *VvGH9* gene (GH9-OE) was 39 times higher than that of transformed with 35S::pCAMBIA2300 empty vector (EV) (Figure 8b). Sugar analysis of GH-OE and EV by high performance liquid chromatography (HPLC) showed that the contents of sucrose and fructose in GH9-OE were significantly higher than that of EV, while there was no observable difference in glucose content of GH-OE and EV (Figure 8c). The contents of all three sugars were less than 10 mg/g both in GH-OE and EV.

3.7. Heterologous Overexpression of Vvgh9 Increased the Sugar Content of Tomato Berries

To further investigate the function of *VvGH9* in fruits, we obtained overexpressed *VvGH9* tomato (*Lycopersicon esculentum*) plants (VvGH9-OE-Le, Figure 9a). The successful transformation was confirmed by PCR (Figure 9b). In addition, we also detected the sugar content of the ripe fruit of VvGH9-OE-Le and wild-type tomatoes (WT-Le). The results showed that *VvGH9* markedly increased the content of sucrose, glucose, and fructose in tomato fruits (Figure 9c). Meanwhile, overexpression of *VvGH9* could promote the production of sucrose, while the sucrose content was too low to be detected in the fruit of wild-type tomatoes.

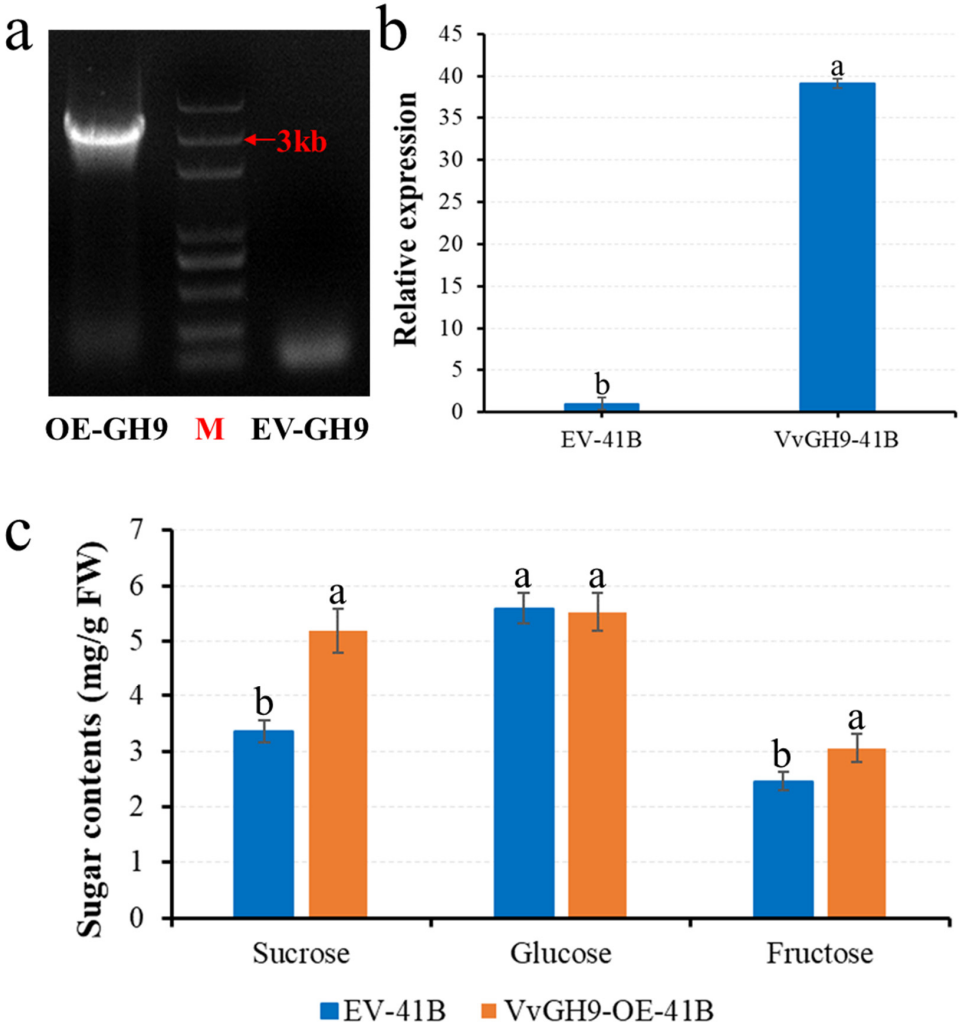

Figure 8. *VvGH9* overexpression and phenotypic identification of grape calli. (**a**) Verification of *VvGH9* insertion in the transgenic 41B calli. (**b**) The upregulation of GH9-OE in 41B calli was determined by qRT-PCR. (**c**) Analysis of sugar levels in EV and GH9-OE calli. FW: fresh weight. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

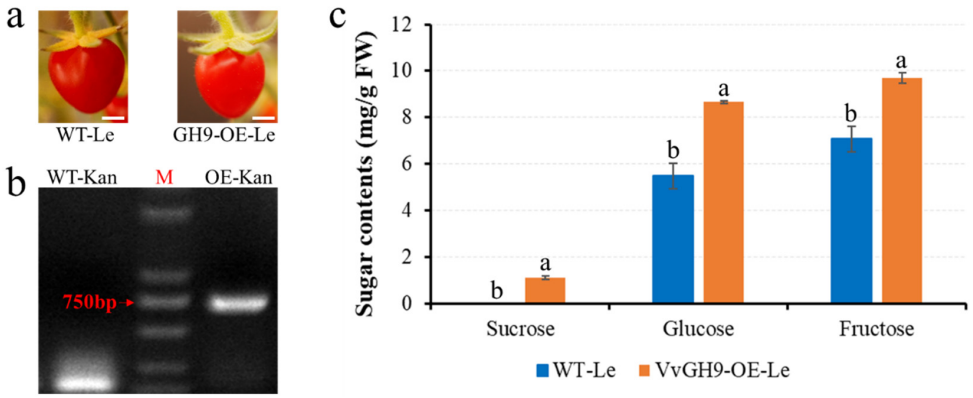

Figure 9. *VvGH9* overexpression and phenotypic identification of tomato (*Lycopersicon esculentum*) fruits. (**a**) The left panel shows the ripe fruit of the wild-type tomato (WT-Le), and the right panel is the ripe fruit of overexpressing *VvGH9* (VvGH9-OE-Le). Scale bars, 1 cm. (**b**) Verification of *VvGH9* insertion in the transgenic tomatoes. Kan: kanamycin. (**c**) Analysis of sugar levels in WT-Le and VvGH9-OE-Le. FW: fresh weight. The letters above the bars indicated the significant differences by student's *t*-test ($p < 0.05$). Three biological replicates were analyzed, and the error bars represented the SD.

4. Discussion

In the present study, we identified 11 *GH* genes divided into four groups in the grapevine genome, and examined the expression patterns across the different developmental stages of 22 grape varieties. Finally, *VvGH9* was found to contribute to increased sugar content in plant and its function was verified by the transgene overexpression approach. The obtained results provide the foundation for future functional analysis of individual *VvGH* gene.

Gene duplication events are important reasons for the expansion of the gene family, which include segmental and tandem duplication [32]. According to the whole genome duplication and tandem repeat analysis, eight *VvGHs* were involved in three segmental duplication events and four *VvGHs* were involved in two tandem duplication events, which implied that the segment duplication and tandem duplication all contribute to the *VvGH* family expansion.

Recent studies of GH proteins mainly focused on their enzyme structures and involvement in sugar metabolism. The first enzyme structure of a glycosyl hydrolase, which was a lysozyme of hen egg white, was cracked more than 56 years ago [33]. Henrissat et al. [34] proposed that more than 45 families of glycosyl hydrolases were classified by their similarities of amino acid sequence. Nomenclature for sugar-binding subsites of glycosyl hydrolases was first proposed by Davies et al. [35]. White et al. [36] elucidated the mechanism of polysaccharide hydrolysis by retaining β-glycosyl hydrolases. In one recent report, the loss-of-function mutants of GH43 in *Arabidopsis thaliana* exhibited expansion defection for root cells [37]. In addition, alternative splicing event formed a shorter isoform of β-D-glucosidase, a GH1 family member, leading to a completely lack its activity in *Catharanthus roseus* [38]. However, there was almost no report that *GH* genes involved in sugar accumulation in plants.

Since gene expression patterns can provide important clues for gene function, we then investigated the expression level of *VvGHs* in different developmental stages of 22 grape varieties [27]. The results revealed that most *VvGH* genes mainly played vital roles at the early stages of grape berry development. More so, the expression level of *VvGH9* was detected to be markedly different at three developmental stages among almost all the 22 grape varieties, and its expression was higher in early development stages (FS) with low sugar contents of the grape berry. In addition, *VvGH5*, *VvGH6*, and *VvGH9* had similar expression patterns, while only *VvGH9* was expressed and existed marked differential expression at three stages of all the six selected cultivars. Meanwhile, five different developmental stages of 'Muscat Hamburg' berries were chosen to measure sugar contents and to further verify *VvGH9* expression patterns via a qRT-PCR assay, which also implied that *VvGH9* might be involved in the sugar metabolism and accumulation of grape berries. Further experiments on grape fruits treated with exogenous sugar showed that *VvGH9* responded to the supplemental sucrose, glucose, and fructose, and responded most quickly and strongly to sucrose, which suggested that it may be involved in the hydrolysis of sucrose into monosaccharides. On the other hand, treatment of '41B' with different glucose concentrations indicated that the expression level of *VvGH9* was promoted by the increasing sugar concentrations lower than 30 mg/g and its expression was inhibited by higher sugar concentrations. Combined with the analysis of *VvGH9* expressions and sugar content evidence, we concluded that *VvGH9* may play an important role in promoting sugar metabolism and accumulation of fruits under low sugar content.

Overexpression of *VvGH9* both in grape calli and tomato fruit resulted in a marked increase of sucrose, fructose, and total sugar (all the three soluble sugars detected in this study) content. In addition, sucrose was produced in VvGH9-OE-Le, while the wild-type tomato contained almost no sucrose, suggesting that *VvGH* may play a vital role in the hydrolysis of polysaccharide to disaccharide. However, the contents of all three sugars were less than 10 mg/g in all the transgenic materials, which means that *VvGH9* may work in low sugar content.

Based on all of these results above, we draw a preliminary conclusion that *VvGH9* in grapevine might participate in sugar metabolism and accumulation under low sugar content. The different expression patterns of the different *GH* genes indicated that each might play a specific and unique role. This study may provide some theoretical basis and experimental methods for exploring the physiological functions and molecular mechanisms of other *GH* genes.

5. Conclusions

The *GH* gene family of the grape contains 11 members divided into four groups and is unevenly distributed on seven chromosomes. Most *VvGH* members exhibited significant expression difference between different stages, while only *VvGH9* had observable differences in the three stages of almost all the 22 grape varieties. The expression patterns of *VvGH9* in different stages of grape fruit and in the materials treated with exogenous sugar revealed that *VvGH9* was induced by the increase of sugar with low content and was inhibited by high sugar content. Overexpressed *VvGH9* in grape calli and tomatoes further proved that it could promote sugar accumulation under low sugar content. This study is a new perspective on an important grape gene family for the molecular breeding of grape sugar in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7110453/s1, Table S1: Names and backgrounds of different grape varieties corresponding to different acronyms, Table S2: RNA-Seq data of the 11 VvGH members in 22 grape cultivars, Table S3: List of primers used in this study.

Author Contributions: Conceptualization, Z.L.; methodology, G.X.; software, Y.W. (Yi Wang); validation, G.X., C.R. and P.F.; formal analysis, G.X. and Y.W. (Yi Wang); investigation, Y.K. and Y.W. (Yue Wang); resources, P.F. and Z.L.; data curation, G.X. and Y.W. (Yi Wang); writing—original draft preparation, G.X. and Y.W. (Yi Wang); writing—review and editing, G.X., Y.W. (Yi Wang), C.R. and Z.L.; visualization, G.X. and Y.W. (Yi Wang); supervision, P.F. and Z.L.; project administration, Z.L.; funding acquisition, Z.L. All authors have read and agreed to the published version of the manuscript.

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