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Sugar Accumulation Patterns and Transcriptome Analysis during the Developmental Stage of Small-Fruit Watermelon (*Citrullus lanatus* L.)

Xuelian Zhang [1], Binghua Xu [1], Dexu Luo [1], Wenzhao Xu [1], Lian Yin [1], Changwei Zhang [2], Yan Gu [1], Jianfeng Zhao [1], Weiwei Wang [1], Lu Liu [1], Tian Bai [1] and Yudong Sun [1,*]

[1] Facilities Vegetable Centre, Huaiyin Institute of Agricultural Science in Xuhuai Area of Jiangsu Province, Huaian 223001, China; xuelianzhang2022@163.com (X.Z.); xubinghua@jaas.ac.cn (B.X.); luodexu2002@163.com (D.L.); xwz0514@163.com (W.X.); yinlian1996@163.com (L.Y.); guyansczx@163.com (Y.G.); zhaold1977@163.com (J.Z.); heromfjack@163.com (W.W.); lululiu0820@163.com (L.L.); baitian12012024@163.com (T.B.)

[2] State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China; changweizh@njau.edu.cn

* Correspondence: sunyudong@jaas.ac.cn

Abstract: The sugar content in watermelon significantly influences its flavor. To understand the sugar accumulation pattern in small-fruited watermelon and identify candidate genes involved in sugar synthesis and conversion, we measured the sugar content (sucrose, fructose, and glucose) at five developmental stages: 10d, 16d, 22d, 28d, and 34d post-pollination. Two watermelon varieties with the largest sugar content difference at 28d post-pollination (high-sugar G38-28 and low-sugar 482276-28) were selected for transcriptome sequencing. Differentially expressed genes (DEGs) were validated using RT-qPCR. Additionally, the sugar contents of three commercial varieties ('Su Meng NO.5', 'Su Meng NO.6', 'Su Meng NO.7') and their parents were compared at five stages. Results showed glucose and fructose levels peaked between 22d and 28d, followed by a decrease, while sucrose content continuously increased. F1 hybrids exhibited glucose and sucrose trends similar to their paternal parent and fructose trends similar to their maternal parent. Transcriptome sequencing identified 9337 DEGs (5072 upregulated and 4265 downregulated). Gene Ontology analysis highlighted overrepresentation in categories such as pectinase and oxidoreductase activity. KEGG analysis identified 12 DEGs involved in sugar synthesis and conversion pathways, including phenylpropanoid biosynthesis and pentose and glucuronate interconversions. RT-qPCR validation corroborated the transcriptome data. These findings explain the distinct sugar accumulation patterns in G38-28 and 482276-28 at the transcriptional level, offering insights for genetic breeding and regulation of key sugar-related genes in watermelon.

Keywords: watermelon; sucrose; fructose; glucose; transcriptome; phenylpropanoid biosynthesis; pentose and glucuronate interconversion

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1. Introduction

Watermelon (*Citrullus lanatus* L.) is a member of the Cucurbitaceae family [1]. It is a globally cultivated horticultural crop with significant international importance, ranking fifth in the FAO's global agriculture and food statistics for fruit production [2]. The soluble solids content serves as a crucial determinant of watermelon quality and flavor, rendering it an essential indicator for evaluating the quality of the product [2,3]. The soluble solids in watermelon fruit include sucrose, glucose, and fructose [4]. The composition, content variation, and distribution characteristics of these sugar components influence the sensory properties of watermelon [5]. Research has shown that during the early stages of development, all types of watermelon fruits primarily accumulate fructose and glucose, while in the

later stages of development, they start to accumulate a significant amount of sucrose [6,7]. Some scholars believe that during the ripening period of watermelon, early-maturing varieties mainly contain fructose and glucose as the main soluble sugars, while mid-to-late-maturing varieties primarily contain sucrose [8]. However, some middle-ripening, middle-late-ripening, or late-ripening varieties did not contain sucrose at maturity, which was also confirmed in wild-type watermelon [9]. In the study of the sugar accumulation relationship between the parents and the hybrid offspring, it was found that the high-sugar cultivar '04-12' belonged to the sucrose accumulation type, the low-sugar cultivar 'Jingyuan Osaka' belonged to the fructose accumulation type, their hybrid offspring F1 was between the two parents, and the fructose accumulation type was also more biased [10].

The process of sugar accumulation is a key determinant of fruit ripening quality. To elucidate the molecular pathways underlying sugar synthesis and conversion, researchers have used gene fine mapping and transcriptome analysis to reveal the genetic mechanisms regulating sugar content. The intricate interplay of various transporters orchestrates sugar transport in fruits, encompassing light- and cell-mediated sucrose transport, and phloem loading and unloading processes. Notably, a portion of the hexose pool derived from sucrose is sequestered into the vacuole upon fruit entry, contributing to fruit structural integrity [7,11,12]. These transporters are divided into three categories: SUT, MST, and SWEET [13,14]. SUT and SWEET proteins participate in the phloem loading process, and under a concentration gradient promote sugar SWEET protein transport across the membrane [15,16]. The function of SUT in plant cells is to transport sucrose together with protons, utilizing stored energy to facilitate the transmembrane transport of sucrose [17]. Guo annotated the watermelon genome and identified 62 genes related to glucose metabolism and 76 glycosporin genes. Among these, 13 glucose-metabolizing enzyme genes and 14 glycotransporter genes exhibited differential expression during fruit development and across various tissues [18]. After pollination of 34d, 10d, 18d, and 26d, Gong analyzed the watermelon fruit metabolic group and transcription; 517 metabolites were detected, including sugars, organic acids, and volatile organic compounds [4]. Li et al. identified candidate genes related to sugar content in apples, finding higher expression levels for genes involved in sugar synthesis and transport during early fruit maturation. These findings suggest that sugar accumulation during ripening is primarily attributed to the activation of the sugar synthesis pathway [19]. Transcriptome analysis of near-isogenic lines has proven to be an invaluable tool for elucidating the genetic underpinnings of soluble sugar and organic acid accumulation and metabolism. This approach enables the identification of differentially expressed genes (DEGs) that are uniquely expressed in near-isogenic lines compared to their parental lines. These DEGs encompass genes encoding enzymes such as oligosaccharide synthase, sucrose synthase, sucrose-phosphate synthase, and insoluble acid invertase, as well as genes encoding proteins like NAD-dependent malate dehydrogenase, aluminum-activated malate transporter, and citrate synthase [4]. In watermelon, the upregulation of *ClTST2* during fruit maturation is positively correlated with sugar accumulation. Mashilo, J., et al. have verified that the gene *Cla013902* could potentially have a significant impact on the metabolic process of sugar in watermelon [2].

The sugar content of watermelon is an intricate quantitative characteristic regulated by numerous genes, with significant interactions with the environment. Sugar serves as an important energy source, pathway mediator, and precursor for structural substances. Previous reports have focused on the study of individual sugars in self-rooted watermelons. To better reflect reality and guide farmers' production practices, this study used 14 watermelon varieties (F1 generation, advanced lines, and commercial varieties) as research materials. Aiming to unravel the molecular mechanisms underpinning the changes in sucrose, fructose, and glucose levels across five growth stages in watermelon, the analysis aimed to uncover differentially expressed genes and their interconnected networks involved in sugar metabolism and transport. By overcoming the constraints imposed by traditional physiological and genetic research techniques, this study facilitated the elucidation of the genetic and metabolic characteristics of watermelons. It reveals the

sugar accumulation patterns in the parental lines and hybrid F1 generation, addresses the issue of low breeding efficiency, and promotes the development of in-depth improvement techniques for watermelon quality. Additionally, it lays the foundation of transcriptomics data for studying mechanisms related to sugar content and provides relevant research content and evidence.

2. Materials and Methods

2.1. Germplasm Materials

2.1.1. Evaluation Materials for Sugar Content in Watermelon Fruit

This experiment selected 14 small-fruited watermelon varieties, including 7 high generation backbone, 5 commercial varieties, and 1 wild-type material. The 7 high generation backbone are 'G31', 'G33', 'G38', 'G42', 'G48', 'G35', and 'G37'. The commercial varieties are 'Su Meng NO.5', 'Su Meng NO.6', 'Su Meng NO.7', 'Early Spring Red Jade', 'Bangbang', and 'Xiao Lan'. The wild-type material is '482276'. All the trial materials were provided by the Facilities Vegetable Centre of Huaiyin Institute of Agricultural Science.

2.1.2. Transcriptome Sequencing Materials

The specific characteristics of the two watermelon materials used for transcriptome sequencing can be found in Table 1.

Table 1. Watermelon materials used for transcriptome sequencing.

Variety Name	Sample Name	The Content of Sucrose	The Content of Fructose	The Content of Glucose	Flesh Color	Picture of Fruit
G38-28	HS	11.4 mg/g	32.82 mg/g	567.29 umol/g	red	
482276-28	LS	2.76 mg/g	8.16 mg/g	154.85 umol/g	Light green	

2.2. Experimental Design

2.2.1. Field Trial of Watermelon Population Materials

The experiment was conducted from February to June 2022 at the Chengnan Base of the Huai'an Agricultural Science Institute in Huai'an City. Plump, insect-free watermelon seeds were selected and disinfected in water at 55 °C for 15 min. Then, they were soaked for 6 h and germinated in a constant temperature incubator at 30 °C until they sprouted. After the cotyledons emerged, they were sown in a 50-hole tray. The seedling substrate used was the residue of Sanhe Purple Mountain biological shiitake mushroom. The seedlings were grown in a sunlit greenhouse with plastic film covering, and the film was removed after the seeds emerged. When the watermelon seedlings grew to three leaves, they were transplanted into substrate troughs. In this experiment, a randomized block design was used. The design involved 14 different materials, which were considered the treatments. There were 3 replications of the experiment, and within each replication, 10 plants were

planted. Each cultivation trough had 2 rows, with a row spacing of 20 cm and a plant spacing of 45 to 50 cm. Plants were pruned, leaving only two watermelon vine branches. Artificial pollination was performed during the flowering period, and the pollination date was recorded; only one fruit was left per plant. According to the maturity characteristics of the watermelon materials, 3 fruits from each material were sampled for experimental data determination. All materials used in this experiment were early-maturing watermelon varieties, samples were collected at 10, 16, 22, 28, and 34 days after pollination. The harvested watermelon was cut in half longitudinally, and fruit samples were collected from the central region of the watermelon for the determination of sucrose, fructose, and glucose content, as well as comparative transcriptome analysis, Upon collection, samples were promptly frozen in liquid nitrogen and maintained at −80 °C.

2.2.2. Determination of Sucrose, Fructose, and Glucose Content

During the watermelon growth period, the testing of sucrose, fructose, and glucose in the fruit was conducted using the Solarbio reagent kits with the following product codes: BC2465, BC2455, and BC2505, and specifications of 100T/96S, 100T/96S, and 100T/96S, respectively. The sample processing method refers to the instructions of the reagent kit. The absorbance of the processed samples was measured using TECAN Infinite F50 and the sugar content was determined using the sample quality formula.

2.2.3. Transcriptome Analysis

RNA extraction, library preparation, and transcriptome sequencing were outsourced to NovogeneCo., Ltd. Utilizing the watermelon 97103 genome as the reference, we constructed a reference genome index using Hisat2 (v2.0.5) and aligned paired-end clean reads to the reference genome using Hisat2 (v2.0.5). Subsequently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to elucidate the functional roles of differentially expressed genes (DEGs) in watermelon.

RNA Quantification and Library Preparation

The self-rooted G38-28 sweet fruit material and the self-rooted 482276-28 non-sweet fruit material were utilized as transcriptome analysis samples. Following RNA quality control measures, total RNA was extracted from watermelon peel using the DP441 Polysaccharide-Polyphenol Plant Total RNA Extraction Kit. The integrity and quantity of the RNA were subsequently assessed using the Agilent 2100 bioanalyzer (Agilent, Beijing, China). To enrich for mRNA molecules with polyA tails, Oligo (dT) magnetic beads were employed. The enriched mRNA was then fragmented using divalent cations in a fragmentation buffer, serving as a template for first-strand cDNA synthesis, which was accomplished using random oligonucleotides as primers in the presence of M-MuLV reverse transcriptase. Subsequently, the RNA strand was degraded by RNaseH, and the second-strand cDNA was synthesized utilizing DNA polymerase I and dNTPs as substrates. The purified double-stranded cDNA underwent end repair, A-tailing, and ligation of sequencing adapters. cDNA fragments ranging from approximately 370 to 420 base pairs were selectively captured using AMPure XP beads. PCR amplification was then performed, followed by purification of the PCR products using AMPure XP beads, yielding the final library.

Transcriptome Sequencing

After passing quality control checks, the constructed libraries were combined according to their effective concentrations and the desired sequencing depth on the Illumina NovaSeq 6000 platform. Sequencing was performed using a paired-end approach, yielding 150 base pair reads. The sequencing workflow included loading a flow cell with fluorescently labeled dNTPs, DNA polymerase, and adapters. As complementary strands were synthesized within each cluster, the incorporation of labeled dNTPs emitted fluorescent signals that were detected by the instrument. These signals were subsequently computa-

tionally translated into sequence information, revealing the nucleotide composition of the target fragments.

Analysis of Sequencing Results

To obtain high-quality sequencing data (clean reads), researchers first pre-processed the raw sequencing data by removing adapter sequences, undetermined base calls, and low-quality reads. Next, they performed reference-based transcriptome alignment analysis using the watermelon 97103 genome as a reference. The number of reads mapped to each gene was then calculated using the featureCounts software (1.5.0-p3). Differential gene expression analysis was subsequently conducted using DESeq2 software (1.20.0). Genes with a padj (adjusted *p*-value) less than 0.05 and an absolute log2 fold change greater than 1 are considered significantly differentially expressed. Finally, researchers leveraged ClusterProfiler software (version 3.8.1) to investigate the functional enrichment and biological context of these differentially expressed genes.

2.2.4. Validation of Gene Expression by Fluorescence Quantitative Real-Time PCR (RT-qPCR)

In this study, the expression of 12 genes involved in sugar synthesis and metabolism was validated in high-sugar HS (G38-28) and low-sugar LS (482276-28) watermelon fruits at 28d after pollination (DAP) using RT-qPCR. Total RNA was extracted from watermelon pulp using the TianGen DP441 Polysaccharide Polyphenol Plant Total RNA Extraction Kit (G38-28 and 482276-28). Following extraction, the RNA was reverse transcribed into cDNA with the HiFiScript gDNA Removal cDNA Synthesis Kit. ChemoHS qPCRMix reagent facilitated RT-qPCR analysis, with the watermelon *Actin* gene as the reference. The Roche LightCycler 96 Instrument (Roche Diagnostics (Shanghai) Ltd., Shanghai, China) was used for real-time PCR, with each gene analyzed in triplicate using the following program: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. Relative quantification of fluorescence data was performed using the $2^{-\Delta\Delta Ct}$ method. Primer sequences for each gene are listed in Table S1.

2.3. Statistical Analysis

Data management, descriptive statistics, and initial data exploration were conducted using Microsoft Excel 2017. Subsequently, GraphPad Prism 8.0 was employed for the generation of graphs, charts, and the performance of advanced statistical analyses, including ANOVA and regression analyses.

3. Results

3.1. Investigating Sugar Content Dynamics in Small-Fruited Watermelon across Growth Stages

During the growing period of watermelon fruit, changes in soluble content determine the flavor quality and ripening time of watermelons. Therefore, we tested six popular watermelon varieties and some of their parent varieties, focusing on the changing trends of sucrose, fructose, and glucose contents at different developmental stages. Unlike previous studies that measured four stages, we shortened the measurement interval from 8d to 6d per cycle for most early-maturing small watermelon varieties. This aimed to obtain more accurate and detailed measurements of the numerical changes. In mature watermelon fruits, the main sources of sugar are sucrose, fructose, and glucose. Figure 1 illustrates the fluctuations in the trends of these three sugars. According to the glucose test results, 'Xiao Lan' exhibited a gradual upward trend in glucose content from 10d to 16d, whereas the wild type '482276' maintained a consistent level throughout the observation period from 10d to 22d. Other watermelon varieties showed a steep increase from 16d to 22d, reaching a peak around 22d. Interestingly, the glucose content decreased and then rebounded from 22d to 28d, with some varieties showing a slow recovery after a rapid decline, while others continued to decline. Based on the sucrose testing results, the wild type '482276' showed a gradual decline from 22d to 34d, while 'G37' exhibited a gradual decrease from 28d to 34d. In contrast, other watermelon varieties showed an upward trend throughout the entire

growth stage, especially from 22d to 34d, reaching a peak around 34d. Notably, the sucrose content of 'Bonbon' surged remarkably during the later stages of development. Fructose, known as the sugar most perceptible to human taste buds, is a key factor determining the flavor of watermelon. The fructose content of most watermelon varieties reaches its peak between 22d and 28d. 'G35', 'G33', and 'G31' can even exceed 60 mg/g at 28d. In contrast, the wild type '482276' gradually declines after reaching its peak at 22d, which may be one reason for its lower sweetness. We found that 28d after pollination is a significant turning point: varieties that initially decreased in fructose content will increase, while varieties that initially increased will decrease, with the same magnitude of change.

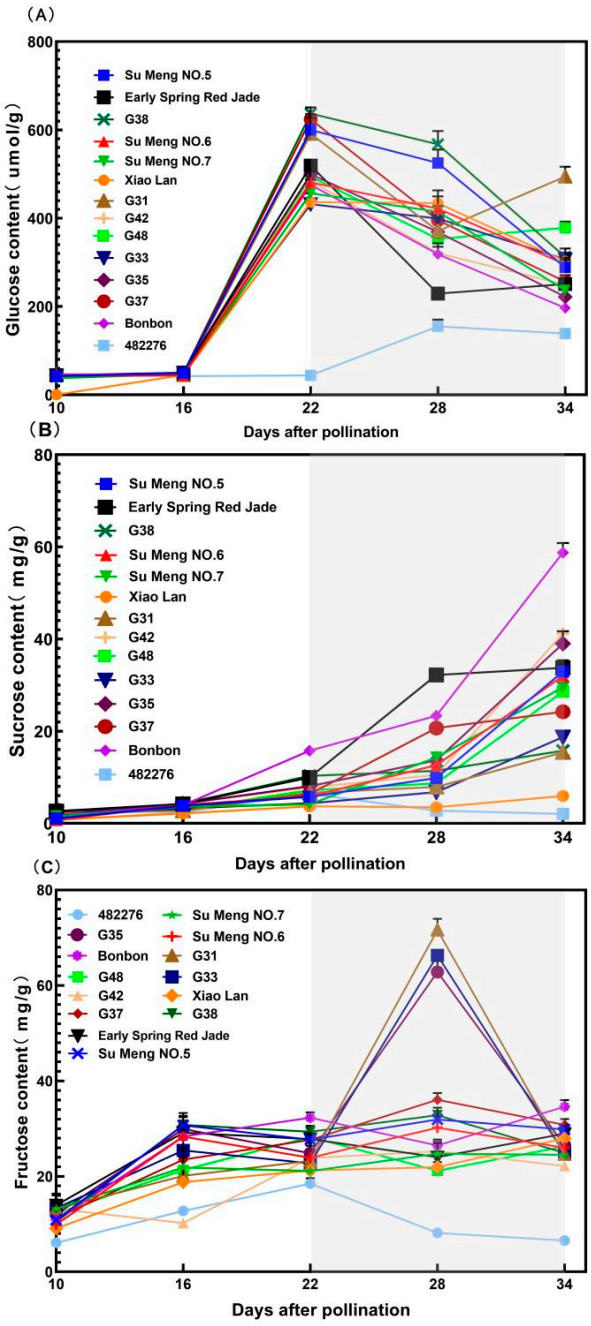


Figure 1. Shows the changes in sucrose, fructose, and glucose content during fruit ripening in different watermelon varieties and their parents. Glucose (**A**), sucrose (**B**), and fructose (**C**) were extracted at 10d, 16d, 22d, 28d, and 34d. Three fruit replicates were taken at each developmental stage to reduce experimental errors. The bar-shaped error lines represent the standard error (SE) (n = 3).

3.2. The Correlation between F1 and the Changes of Glucose, Sucrose, and Fructose of Parents during Fruit Development

The watermelon varieties 'Su Meng NO.5', 'Su Meng NO.6', and 'Su Meng NO.7' are watermelon cultivars developed by the Huai'an Agricultural Science Institute. To minimize genetic background interference, the comparison of glucose, sucrose, and fructose content was conducted between the F1 generation and its parental plants at various stages of fruit development. According to Figure 2, When comparing 'Su Meng NO.5' with its parents, it was found that the F1 generation exhibited a similar trend in glucose changes as the paternal parent. However, significant differences were observed at 22d and 28d compared to the maternal parent, with values falling between those of the parents at each stage. In terms of sucrose comparison, the F1 generation consistently showed higher levels than its parents at the late stage of 34d, with a trend closer to that of the maternal parent. The overall trend of fructose changes was consistent with the paternal parent, with significantly higher levels in the F1 generation at the 34d late stage compared to the parents. Through the comparison between 'Su Meng NO.6' and its parental plants, it was found that the F1 generation exhibited a similar degree of glucose content changes as its maternal parent. The paternal parent consistently had higher glucose levels compared to both the F1 generation and the maternal parent. In comparison to the maternal parent, the F1 generation showed superior glucose levels from 22d to 34d, with significant differences observed at 22d and 28d. Unlike 'Su Meng NO.5', 'Su Meng NO.6' exhibited sucrose accumulation levels between its parental plants. The trend in fructose content changes was consistent with the paternal parent, disregarding the maternal parent's characteristic sudden decrease at 16d of fruit development. When comparing 'Su Meng NO.7' with its parental plants, it was found that the F1 generation exhibited minimal differences in glucose changes compared to the maternal parent. Except for a significant difference at 22d compared to the paternal parent, the F1 generation showed significant differences at 22, 28, and 34d compared to the paternal parent. In terms of sucrose content comparison, the F1 generation consistently had higher levels than its parental plants at 34d. The trend in fructose content changes was similar to the paternal parent, but for the most part, remained lower than the levels observed in the parental plants.

Watermelon primarily contains sucrose, fructose, and glucose. The content and ratio of these sugars vary significantly among watermelon varieties. A higher proportion of sucrose can increase the sweetness of the fruit, while a higher proportion of fructose can enhance both the texture and sweetness. Glucose has a lesser impact on texture and sweetness, possibly due to genetic variations. During the development process of watermelon fruits, there is a regular pattern in the changes of these three important sugars: glucose and fructose show an upward trend followed by a decline, while sucrose continuously increases. It is worth noting that glucose content peaks at 22 days, while fructose content peaks at 28 days. Fructose and glucose are derived from the breakdown of sucrose. Comparative studies have found that the sugar traits of the F1 generation tend to lean towards one parent or fall between the two parents. Overall, in terms of the development stages of the three watermelon varieties, the trend of glucose content tends to lean towards the paternal parent, while the sucrose content leans towards the maternal parent, and the fructose content leans towards the paternal parent.

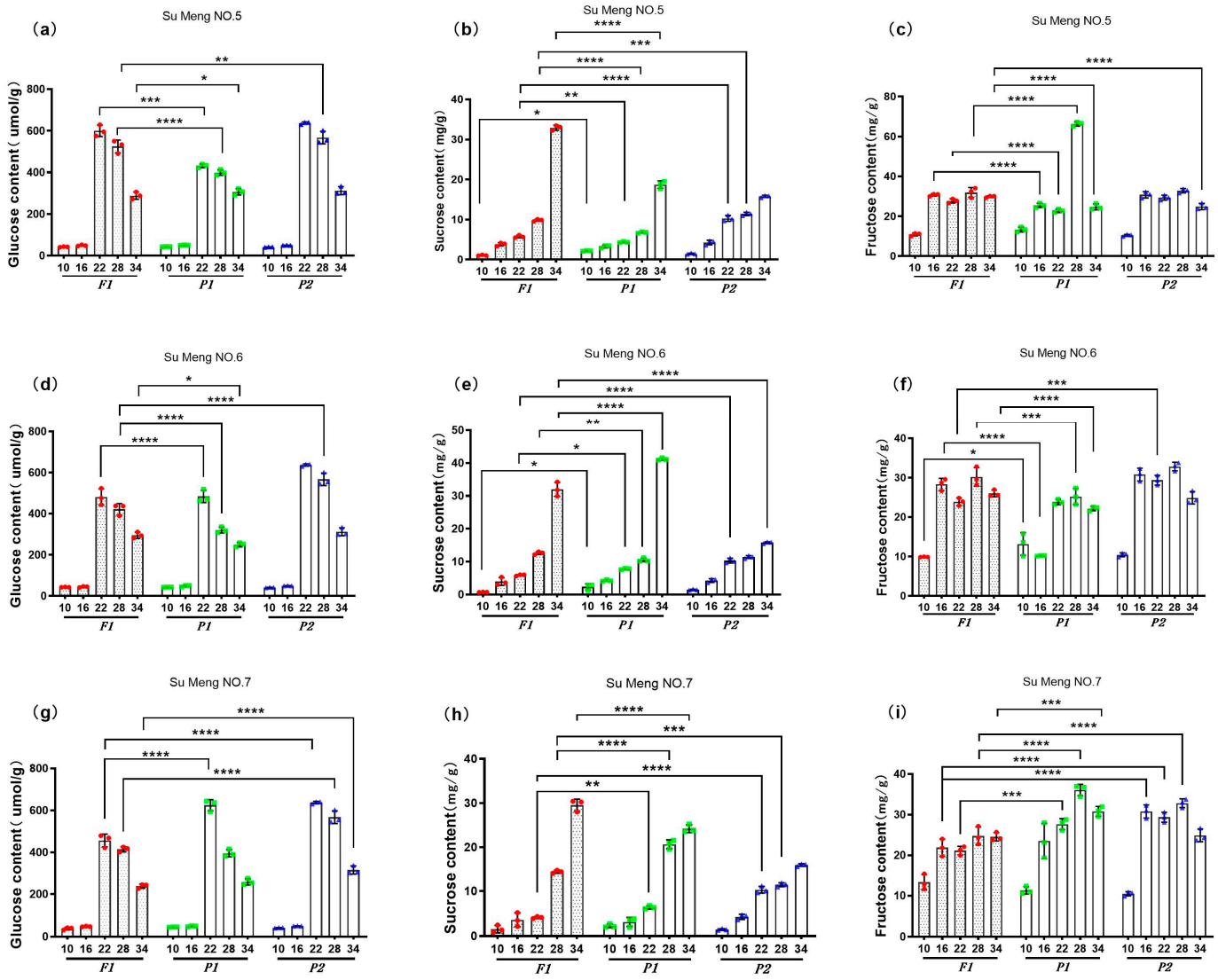


Figure 2. The correlation between the changes in glucose, sucrose, and fructose during the fruit development stage of the F1 generation and its two parents. Correlation between 'Su Meng NO.5' and its parents (**a**–**c**), 'Su Meng NO.6' and its parents (**d**–**f**), and 'Su Meng NO.7' and its parents (**g**–**i**) in glucose, sucrose, and fructose at 10d, 16d, 22d, 28d, and 34d. * represents a significant difference at the $p \leq 0.05$ level, ** represents a significant difference at the $p \leq 0.01$ level, *** represents a significant difference at the $p \leq 0.001$ level, and **** represents a significant difference at the $p \leq 0.0001$ level. Three fruit replicates were taken at each developmental stage to reduce experimental errors. Standard error values are visualized as error bars in the bar chart (SE) (n = 3).

3.3. Transcriptome Sequencing Quality Assessment

After transcriptome sequencing, the filtered sequences, known as clean reads, totaled 272,373,920. All subsequent analyses were conducted using the information obtained from error-free sequences. The Q20 and Q30 values represent the probabilities of base identification errors being 1% and 0.1%, respectively. Both the Q20 and Q30 values exceeded 90%. A GC percentage distribution check was performed to detect any AT or GC separation phenomenon. In this experiment, the GC percentage of watermelon rind was above 43% (Table S2).

Over 70% of the aligned clean reads exhibited perfect alignment to the watermelon reference genome 97103 (Table S3). The lower number of aligned reads in the L group might be attributed to genetic divergence from the wild type. The unique alignment rate was above 66%, while the multiple alignment rate was below 4.6%. The exon alignment

rate was above 68%. Based on these results, the sequencing quality of this experiment is deemed satisfactory.

3.4. Gene Expression Data Analysis

Inter-sample biological reproducibility of gene expression was assessed using a watermelon sample correlation heatmap. The correlation of gene expression levels between samples is a crucial indicator for evaluating experimental reproducibility and the appropriateness of sample selection. A correlation coefficient (R^2) closer to 1 indicates a higher degree of similarity in expression patterns among samples. As is evident in Figure S1, the minimum R^2 value for the six watermelon samples was 0.800, satisfying the experimental requirements for biological reproducibility.

Principal Component Analysis (PCA) is commonly employed to evaluate inter-group differences and intra-group sample reproducibility. PCA utilizes linear algebra computational methods to perform dimensionality reduction and extract principal components from thousands of gene variables. PCA analysis was conducted on the gene expression values (FPKM) of all samples, as shown in Figure S2. The H and L group samples were distinctly separated, while intra-group samples were clustered together with relatively small distances. This indicates a significant biological difference between the two groups, meeting the research technical requirements.

According to Figure 3, the low-sugar (LS) watermelon material (482276-28) and high-sugar (HS) watermelon material (G38-28) have 10,134 genes expressed in common. There are 2474 genes specifically expressed in LS and 1472 genes specifically expressed in HS. Differential gene expression analysis was performed using DESeq2, with genes considered significantly differentially expressed if their *p*-value was less than 0.05 and their absolute log2 (fold change) was greater than 1. According to the volcano plot presented in Figure 3, a total of 9337 genes with differential expression were detected in the flesh of LS and HS watermelons. Among these genes, 5072 were upregulated, while 4265 were downregulated.

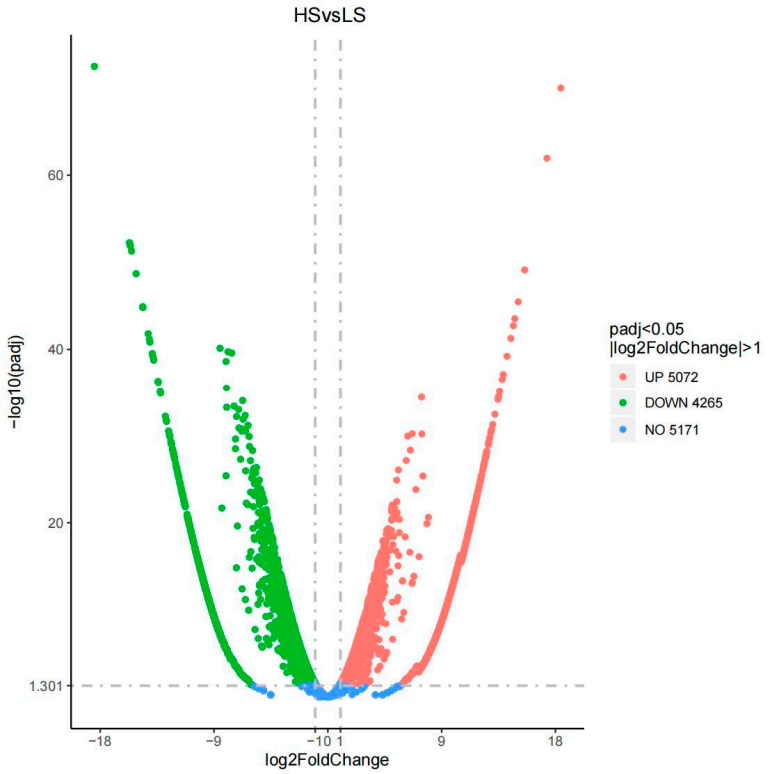


Figure 3. A volcano plot depicting differentially expressed genes.

3.5. GO Enrichment Analysis

After conducting data comparison, we performed a functional enrichment analysis of the 9337 differential genes we obtained using Gene Ontology (GO). The top 30 enriched GO terms for upregulated and downregulated differential genes were categorized and presented in a bar chart (Figure 4). These genes were annotated based on their biological processes (BP), cellular components (CC), and molecular functions (MF). The differential genes were found to be significantly enriched in the following GO terms: pectinesterase activity (GO: 0003824)—21 genes, iron ion binding (GO: 0005506)—104 genes, oxidoreductase activity, acting on NAD(P)H (GO: 0016651)—103 genes, heme binding (GO: 0020037)—128 genes, and tetrapyrrole binding (GO: 0046906)—131 genes, Additionally, the differential genes with the highest expression count were found in the following GO terms: transition metal ion binding (GO: 0046914)—283 genes, response to stress (GO: 0006950)—202 genes, and ribonucleoprotein complex (GO: 1990904)—140 genes.

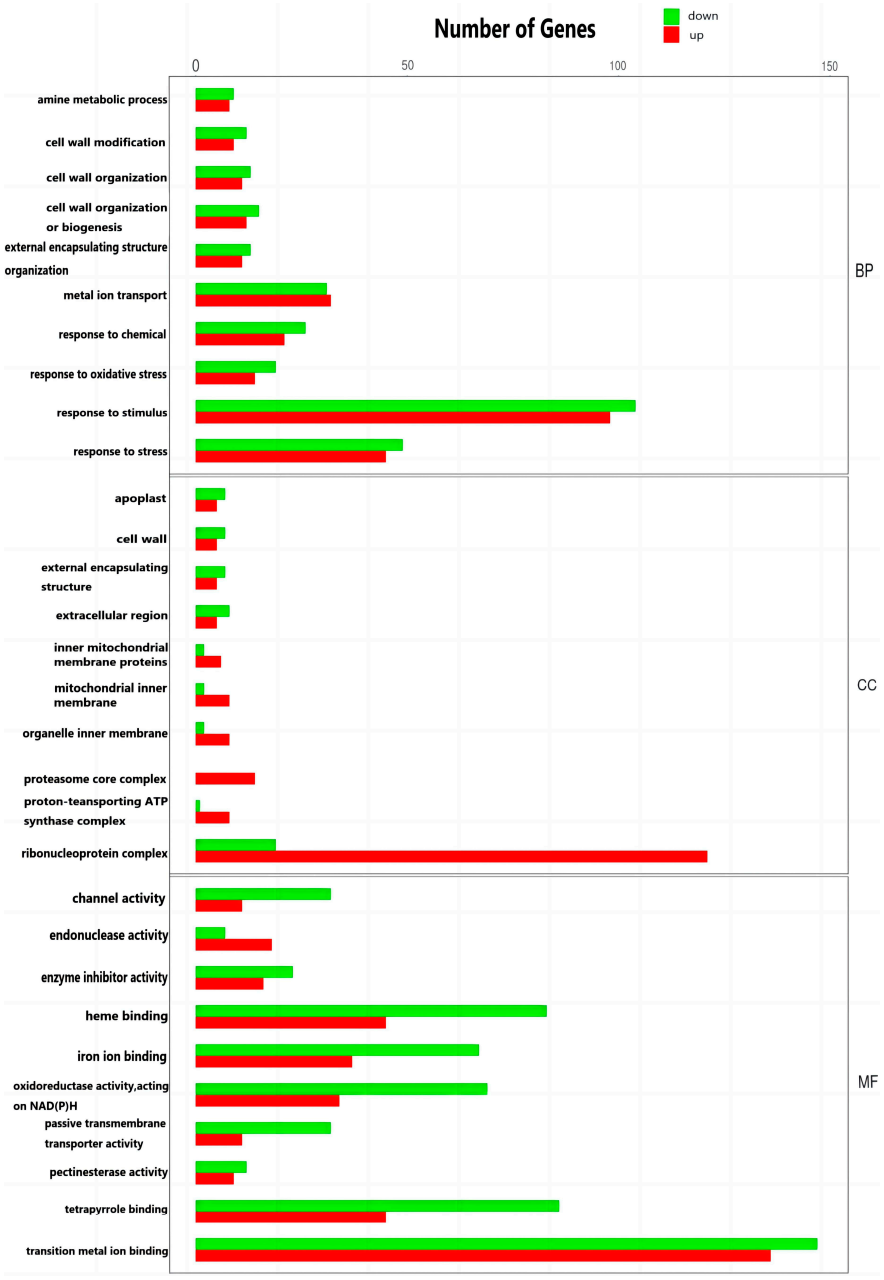


Figure 4. Grouping the top 30 DEGs based on their Gene Ontology (GO) annotations.

3.6. KEGG Enrichment Analysis

After conducting data comparison, we enriched the obtained 9337 differential genes in metabolic pathways and selected the top 20 enriched metabolic pathways for scatter plot visualization (Figure 5). Differential gene enrichment analysis revealed seven primary pathways. Among the identified differentially expressed genes, the following pathways were particularly noteworthy: phenylpropanoid biosynthesis (62 genes); pentose and glucuronate interconversions (37 genes); cutin, suberine, and wax biosynthesis (17 genes); galactose metabolism (32 genes); tropane, piperidine, and pyridine metabolism (15 genes); alanine, aspartate, and glutamate metabolism (31 genes); and cyanoamino acid metabolism (26 genes) were particularly noteworthy, and cutin, suberine, and wax biosynthesis pathways exhibited significant enrichment (padj < 0.05). The metabolic pathway with the highest number of annotated differential genes is the phenylpropanoid biosynthesis pathway, which is significantly enriched. Next is the pentose and glucuronate interconversions; these metabolic pathways have been found to have significant roles in the development of watermelon fruit and the metabolism of sugars.

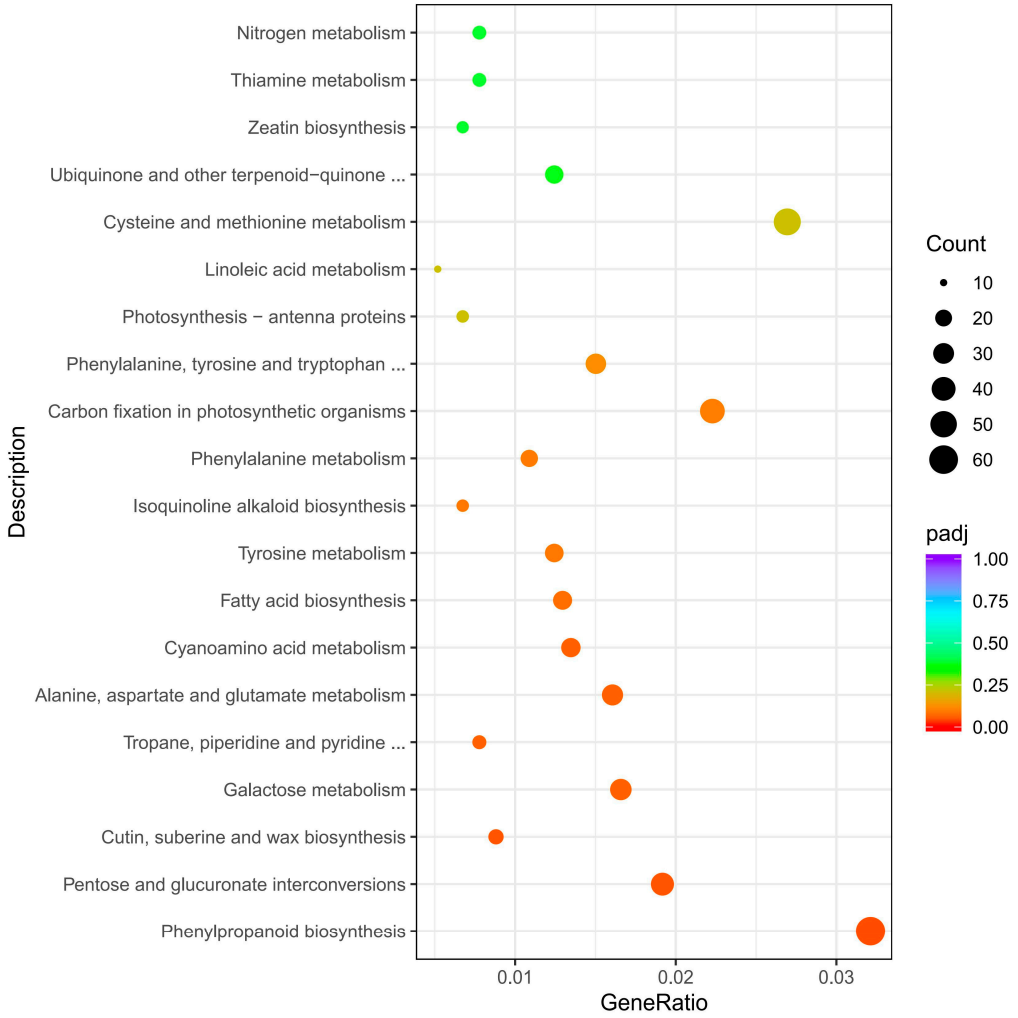


Figure 5. Grouping the top 20 DEGs based on their KEGG pathway annotations.

3.7. Analysis of DEGs in Phenylpropanoid Biosynthesis

After analyzing 9337 different genes, it was found that there are 62 different genes between the high-sugar material HS (G38-28) and the low-sugar material LS (482276-28). Through the analysis of the phenylpropanoid biosynthesis pathway (KO00940), it was discovered that there are 11 beta-glucosidase (*β-Glu*/E3.2.1.21) genes, 4 4-coumarate-

CoA ligase (*4CL*/E6.2.1.12) genes, 3 coniferyl-alcohol glucosyltransferase (*CGT*/E2.4.1.111) genes, 3 cinnamyl-alcohol dehydrogenase (*CAD*/E1.1.1.195) genes, and 3 trans-cinnamate 4-monooxygenase (*C4H*/E1.14.14.91) genes.

As shown in Figure 6, in the *β-Glu* gene, the expression level of *Cla97C08G153160* is highest in HS and is 4.7 times higher than LS. As a sugar-promoting enzyme, beta-glucosidase catalyzes the hydrolysis of *β-D-Glu*, releasing *β-D-Glu*. In the *4-CL* gene, *Cla97C06G122680* has the highest expression level in HS, reaching 28.64, which is 5.8 times higher than LS. The biosynthesis pathway of phenylpropanoids relies on the crucial involvement of this enzyme gene, serving as a precursor for numerous compounds, including sugar-related compounds. In the *CGT* gene, only *Cla97C01G018700* has the highest expression level in HS, being 7.6 times higher than LS. In the *CAD* gene, the other two downstream genes displayed low expression levels and lacked any significant differences in expression. The gene expression level of *Cla97C05G092680* in LS reaches a maximum of 101, which is 6.4 times higher than HS. In the *C4H* gene, the expression difference between HS and LS is not significant.

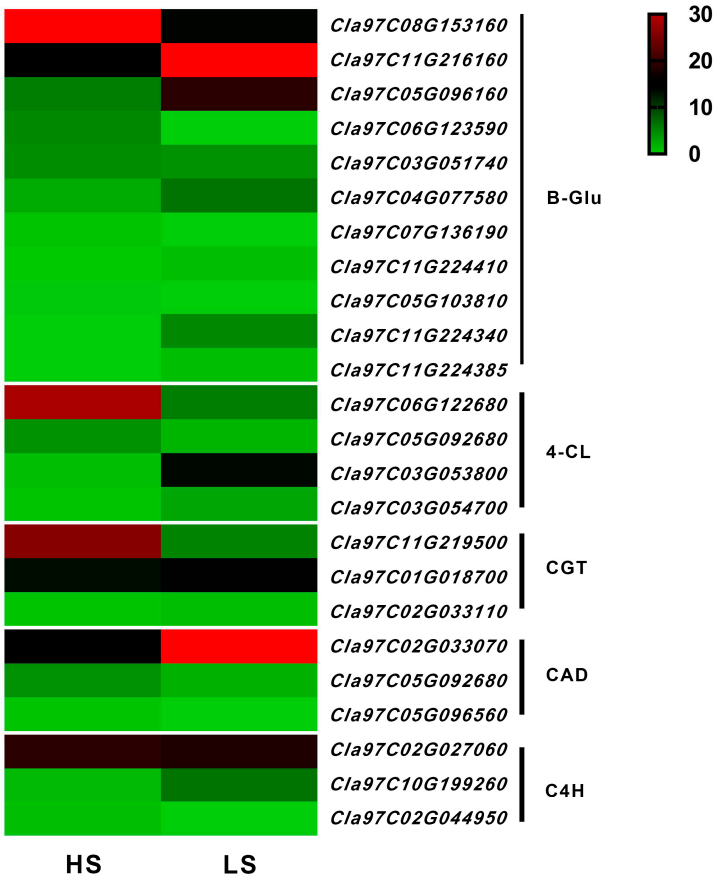


Figure 6. Analyzing the expression of genes involved in carbohydrate synthesis or metabolism within the phenylpropanoid biosynthesis pathway.

3.8. Analysis of DEGs in Pentose and Glucuronate Interconversions

After analyzing 9337 different genes, it was found that there are 33 different genes between the high-sugar material HS (G38-28) and the low-sugar material LS (482276-28). Through the analysis of the pentose and glucuronate interconversions pathway (KO00040), the following genes were identified: 12 pectinesterase (PE/E3.1.1.11) genes, 7 pectate lyase (PL/E4.2.2.2) genes, 4 polygalacturonase (PG/E3.2.1.15)genes, 2 galacturan 1,4-alpha-galacturonidase (1,4-α-D-galacturonide/E3.2.1.67) genes, 2-UDPglucose-6-dehydrogenase (UGDH/E1.1.1.22) genes, 2-utp-glucose-1-phosphate uridyltransferase (UGPase/K00963) genes, 1 ribulose-phosphate 3-epimerase (PPE/E5.1.3.1) gene, 1 UDP-sugar pyrophos-

phatase (USP/K12447) gene, 1 alcohol dehydrogenase (NADP+) (AKR1A1/K00002) gene, and 1 xylulokinase (xylB/E2.7.1.17) gene.

Figure 7 illustrates the upregulated expression of Cla97C09G179370, Cla97C09G171500, and Cla97C09G168010 genes in the PE gene compared to the HS. Among them, the expression level of Cla97C09G171500 in HS is 93.8 times higher than in LS. On the other hand, Cla97C05G089160 has the highest gene expression level in LS, which is 12.4 times higher than in HS. The next highest expression level is observed in Cla97C09G163490, which is 19 times higher in HS. In the PG gene, Cla97C09G176880 has the highest expression level in HS, which is 5 times higher than in LS. However, this gene is not expressed in LS. In the UGDH gene, Cla97C06G125310 has the highest expression level in LS, which is 3.6 times higher than in HS. In the PPE gene, the expression level is higher in HS compared to LS, but the difference is not significant. In the USP and AKR1A1 genes, the expression levels are higher in HS compared to LS, with fold changes of 7.3 and 10.6, respectively. Furthermore, there are no fold changes observed in the PL and 1,4-α-D-galacturonide genes between the two samples.

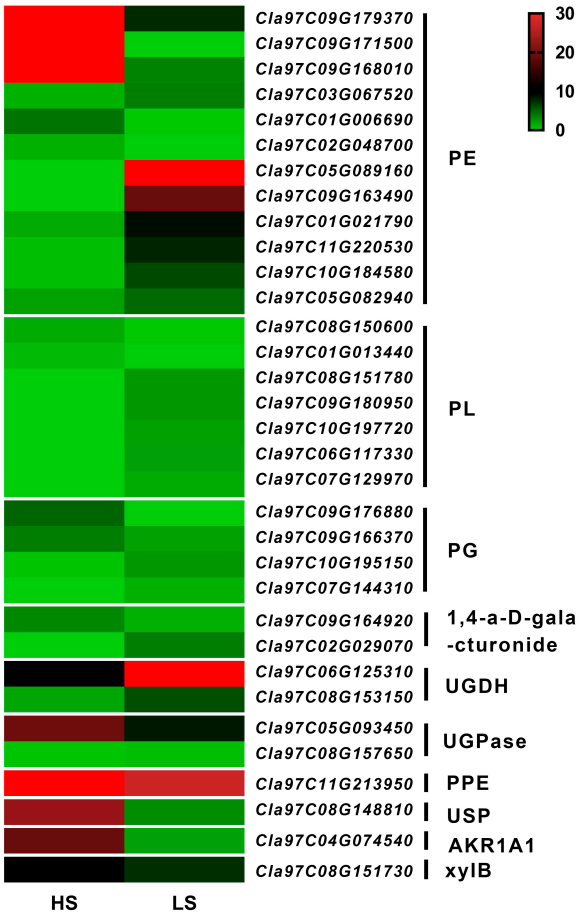
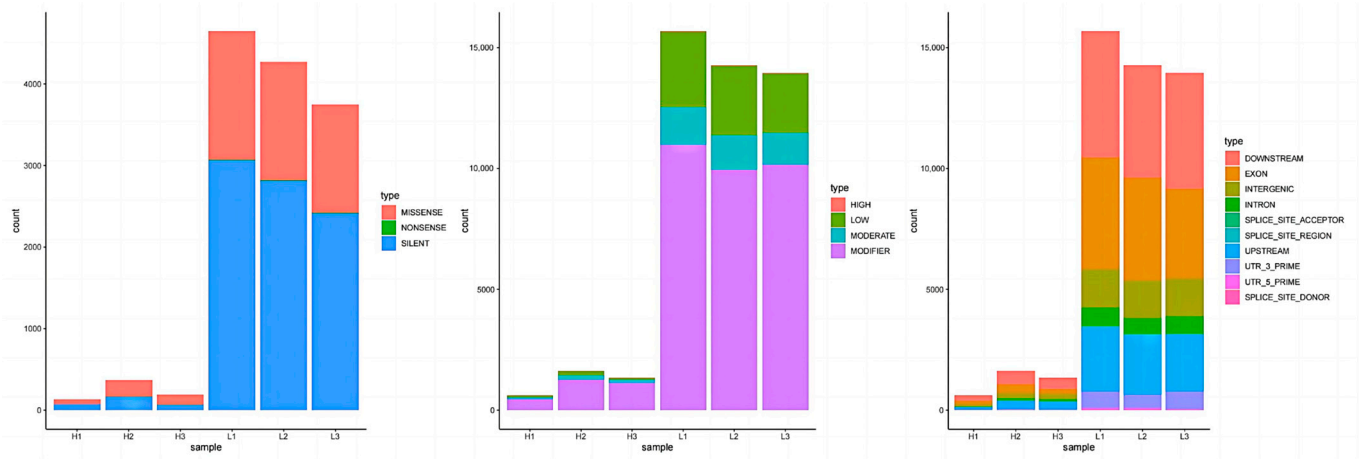


Figure 7. Examining the transcriptional dynamics of carbohydrate metabolism-related genes in the pentose–glucuronate interconversion pathway.

3.9. Analysis of Variant Sites

In this study, the GATK software (3.8) was used to detect variant sites. Each variant site was analyzed and statistically evaluated based on SNP function, SNP impact, and SNP region using SnpEff annotation information. Images were generated to visualize the results. According to Figure 8, the watermelon cultivar 97103 was used as a template for the analysis of mutation sites. In SNP function, most of the mutations were classified as MISSENSE and NONSENSE. Additionally, there was a significant increase in mutation sites in the low-sugar variety (L group), while the high-sugar variety (H group) had

fewer mutation sites (a). This suggests that there is a distant genetic relationship between the two groups. In the context of SNP impact, the vertical sizes of the bars indicate the extent of influence that SNP variants have on gene function. From this experiment, it can be observed that the majority of SNP variants have a MODIFIER impact, meaning they have no phenotypic effect on their own. However, there is a significant impact on the low-sugar variety group (L group) compared to the high-sugar variety group (H group) based on the overall trend (b). This suggests that these SNP variants may have a greater effect on the gene function of the low-sugar variety. In SNP region, the SNP variant sites can be divided into the following regions: DOWNSTREAM, EXON, INTERGENIC, INTRON, SPLICE_SITE_ACCEPTOR, SPLICE_SITE_REGION, UPSTREAM, UTR_3_PRIME, UTR_5_PRIME, and SPLICE_SITE_DONOR regions. Among these regions, SNP variant sites show the strongest association and higher statistical significance with the DOWNSTREAM, EXON, and UPSTREAM regions, while the association is weakest in the SPLICE_SITE_DONOR region (c).



(**a**) (**b**) (**c**)

Figure 8. Variant site statistics. The *x*-axis represents sample names, where H1-3 refers to high-sugar materials G38-28, and L1-3 refers to low-sugar materials 482276-28. The *y*-axis depicts the distribution of variants across genomic regions (EXON, INTRON, INTERGENIC). In this context, (**a**) represents SNP function, (**b**) represents SNP impact, and (**c**) represents SNP region.

3.10. Co-Expression Analysis Related to Sugar Synthesis and Conversion

Day 28 after watermelon fruit pollination is a critical time point for sugar synthesis and metabolism. Transcriptome analysis of high-sugar watermelon G38-28 and low-sugar watermelon 482276-28 revealed five differentially expressed genes involved in the phenylpropanoid biosynthesis pathway: *beta-glucosidase* (*Cla97C08G153160*), *4-coumarate-CoA ligase* (*Cla97C06G122680*), *4-coumarate-CoA ligase-like* (*Cla97C03G054700*), *UDP-glycosyltransferase* (*Cla97C01G018700*), and *cinnamyl alcohol dehydrogenase* (*Cla97C05G092680*). Additionally, seven differentially expressed genes were identified from the pentose and glucuronate interconversions pathway: *beta-glucosidase* (*Cla97C09G179370*), *pectinesterase/pectinesterase inhibitor* (*Cla97C09G171500*, *Cla97C09G168010*), *pectinesterase* (*Cla97C05G089160*, *Cla97C09G163490*), *polygalacturonaseand* (*Cla97C09G171500*), and *UDP-glucose 6-dehydrogenase* (*Cla97C06G125310*).

For RT-qPCR validation, we conducted a random selection of significant genes associated with the synthesis and conversion of sugars in watermelon. The results (Figure 9) revealed that the expression patterns of the 12 genes in high-sugar HS (G38-28) and low-sugar LS (482276-28) watermelon fruits at 28d after pollination were similar to those observed in the transcriptome analysis; the consistency between the transcriptome sequencing results in this study reinforces the reliability of the transcriptome sequencing data.

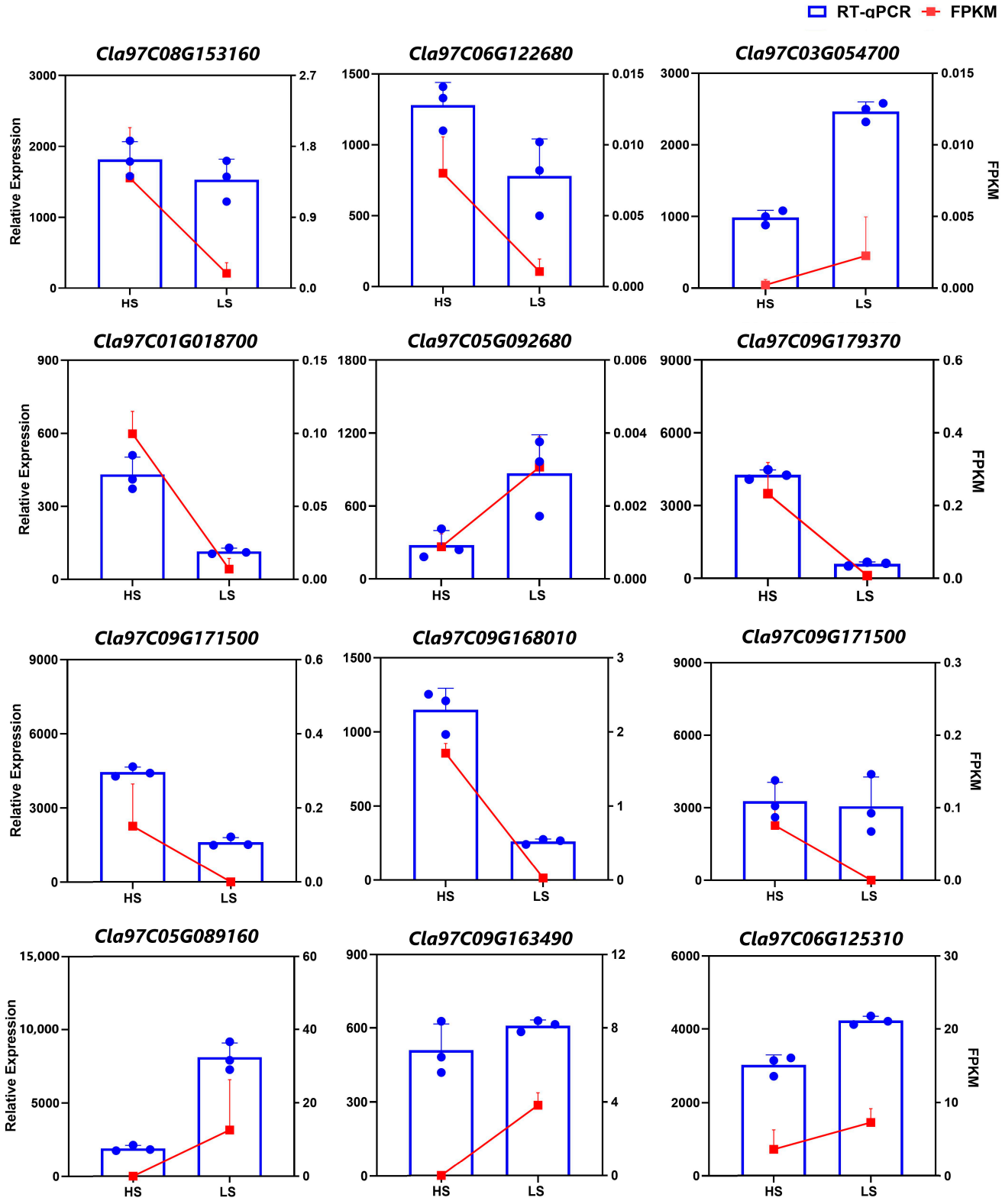


Figure 9. Validating the transcriptional regulation of candidate genes in watermelon sugar synthesis and metabolism pathways by RT-qPCR.

4. Discussion

As watermelon sugar content is a crucial factor influencing fruit quality and flavor, we explored the mechanisms driving sugar accumulation by examining the patterns of sucrose, fructose, and glucose accumulation during fruit development. To identify candidate genes

involved in sugar synthesis and conversion, we used transcriptomic approaches. Fourteen small-fruited watermelon samples, including market-leading varieties and parental materials, were selected as experimental materials. Two samples with extreme differences in sugar content were chosen for transcriptomic sequencing.

In contrast to conventional research methods that focus on four fruit developmental stages, this study selected five stages, which resulted in stronger numerical continuity and facilitated the detection of abrupt changes in sugar content. Additionally, for the first time, a comparison of sugar accumulation at different developmental stages was conducted between three F1 commercial varieties and their parental materials, revealing the genetic variation in sugar accumulation. Based on transcriptomic analysis, a total of 12 candidate genes associated with the synthesis and conversion of sugar were finally identified. To avoid genetic background confusion and ensure the authenticity and validity of the data, The experiment took place in a field where watermelons were cultivated for the first time. This research offers comprehensive data and a strong theoretical basis for comprehending the patterns of sugar content accumulation and genetic breeding in small-fruited watermelons.

Sucrose, fructose, and glucose are important carbohydrates in many plants, playing roles in energy storage, transport within plant cells, and regulating plant growth and development [20]. For example, researchers have found that the levels of fructose and glucose increase as tomatoes ripen, while sucrose remains relatively low, suggesting their significance in the ripening process [21]. Similar trends are observed in apples, where fructose and glucose contribute to sweetness, while sucrose is the primary carbohydrate in sugarcane, with its production regulated by genes encoding sugar synthesis enzymes and transporters [22–24]. In watermelon, a research team has revealed the biosynthetic mechanism of sucrose, including key processes like RFO hydrolysis, sucrose transport, and storage. RFOs transported through vascular bundles are hydrolyzed into sucrose within the rind [25]. This sucrose is then unloaded to intercellular spaces before being absorbed by sugar transport proteins on the flesh cells' cytoplasmic membrane. Finally, the sugar is stored in vacuoles [26,27]. Fructose biosynthesis in watermelon fruit is more complex, involving multiple genes and sugar transport proteins from families like SWEET, SUT, and TMT [28,29]. Research further validated the crucial role of sugar transport proteins in fruit growth and fructose accumulation [30]. The understanding of glucose synthesis and transport in watermelon and grapes is relatively limited. However, studies have identified sugar transport proteins like ClSWEET15 and ClSWEET3 that significantly impact glucose movement and accumulation in watermelon [26]. Additionally, studies reveal significant genetic diversity in sugar composition ratios throughout watermelon development, with some genotypes accumulating more sucrose, fructose, or glucose [27]. This indicates variations in sugar accumulation abilities and enzymatic activity related to sugar metabolism among different genotypes [28–30]. A key significance of this experiment lies in its exploration of sugar accumulation patterns between the F1 generation and their parents. Research has identified specific genes that regulate the production and transformation of sugar compounds in watermelon fruits. For instance, within the gene family responsible for sugar transportation, *ClVST1* is accountable for facilitating the transfer of sucrose from the fruit's outer layer to the cell apoplast, *ClSWEET3* transports sugars across the mesocarp cell plasma membrane into the cell, and *ClTST2* transports and stores sugars in vacuoles. In watermelon fruits, the *ClAGA2* gene is essential for converting oligosaccharides derived from the cottonseed sugar series into sucrose. Its expression is controlled by the transcription factor ClNF-YC2 in wild watermelons, but ClNF-YC2 is incapable of inducing the expression of *ClAGA2*, resulting in the inability to rapidly hydrolyze cottonseed sugars into sucrose [26]. The exploration of key genes that control watermelon sugar content has potential value for watermelon breeding and improvement. By utilizing the variation sites of these genes, molecular marker-assisted breeding techniques can be developed to select watermelon varieties with high sugar content, high yield, excellent quality, and strong disease resistance. Interestingly, a new

perspective has been proposed in the latest research on the new mechanism of sugar accumulation controlled by sugar transporters. It suggests that the highly expressed glucose efflux protein MdERDL6 in apple fruits can cause the efflux of glucose from vacuoles, increasing the concentration of glucose in the cytoplasm. This further stimulates the expression of the sugar influx protein TST, promoting the accumulation of glucose, fructose, and sucrose in vacuoles [31]. This regulatory mode has also been confirmed in tomato fruits, and it could potentially open up a novel avenue for investigating the process of sugar buildup regulated by sugar carriers in watermelon.

Currently, small-sized watermelons dominate the watermelon sales market. For this experiment, we specifically chose representative small-sized watermelons as the experimental materials. These watermelons were selected based on the availability of abundant germplasm resources and our extensive breeding experience. This study revealed changes in sucrose, fructose, and glucose levels throughout watermelon fruit development, providing a theoretical basis for understanding the fruit's growth and ripening processes. Notably, glucose content increased from 10d to 22d after fruit set, followed by a decrease from 22d to 28d, followed by a slow increase. The 22d was an important turning point for most of the changes in glucose content. In contrast, the sucrose content exhibited a progressive pattern throughout the entire developmental phase reaching its peak at 34d of maturity. The trend of fructose content was similar to that of glucose, reaching its peak between 22d and 28d and then decreasing. As fructose is the most perceptible sugar for humans, early conversion of fructose may be one of the reasons why some watermelon varieties are not sweet. There is research evidence that there is a mutual conversion mechanism between sucrose, fructose, and glucose in watermelon fruits. Sucrose, a disaccharide composed of glucose and fructose, can be interconverted under the action of specific enzymes [32]. Additionally, this implies that enzymatic activity during the latter part of watermelon fruit maturation (after 28d) contributes to the conversion of glucose and fructose into sucrose. This study validates this assertion, which is additionally the primary factor behind the gradual reduction in glucose and fructose levels and the ongoing elevation in sucrose levels during the later phase of growth. For most early-maturing small-sized watermelons, the 28d after pollination is a critical time point for determining the sugar content and flavor quality of the variety. Investigations into sugar concentrations during plant development are ongoing in various species. For instance, in tomato fruit development, sucrose and fructose levels gradually increase while glucose remains relatively low, Similarly, in maize plants, sucrose and fructose concentrations are minimal in early seedlings but rise as the plant matures, while glucose levels remain stable [33]. In the growth process of Arabidopsis thaliana, the sucrose and fructose concentrations are minimal during the seedling phase and progressively rise as the plant matures, while the level of glucose remains relatively stable [34]. The impact of parental genetics on watermelon sugar composition was investigated by comparing sucrose, fructose, and glucose levels in three F1 hybrid cultivars and their parental lines at various stages of fruit maturation. The experimental results showed that sugar content is greatly influenced by genetic genotype factors. By comparing, it was found that the sugar characteristics of the F1 generation often tend to favor one parent or lie between the parents. Based on the performance of the three commercial varieties, the development trend of glucose and sucrose content tends to favor the paternal parent, while the development trend of fructose content tends to favor the maternal parent. This is based on the condition that the parental backgrounds of the commercial varieties are similar, as they are cultivated varieties. These findings offer valuable insights for genetic and combination breeding strategies in watermelon. Previous studies have utilized cultivated cultivars and wild varieties for crossbreeding to investigate the genetic diversity and metabolic processes influencing sugar content in watermelon fruits. Remarkable genetic variability in sugar content and the distribution of sucrose, glucose, and fructose was revealed in mature fruits through these studies [35]. Additionally, researchers used Osaka Black Seed melon and high-sugar watermelon with edible flesh, as well as their hybrid offspring, as materials. The findings indicated that the enhancement in sucrose levels in the self-fertilized fruit of

the high-sugar watermelon is contingent upon the extent of the sucrose augmentation. In the hybrid offspring, the levels of fructose and glucose displayed a continuous distribution, while the variability in sucrose levels demonstrated a normal continuous distribution in the offspring resulting from the backcross with the parents [36,37]. The traits of the parents have a close relationship with the distribution of sugar trait inheritance and metabolism, as indicated by the above results. However, research in this area is limited, and the viewpoints presented in this article can serve as a reference.

Through measuring the sugar content at different growth stages of watermelon, two watermelon materials with significant differences in sugar traits were identified: high-sugar watermelon material G38-28 (HS) and low-sugar watermelon material 482276-28 (LS). Comparative transcriptome analysis of HS and LS identified 9337 differentially expressed genes (5072 upregulated and 4265 downregulated). GO enrichment analysis revealed significant enrichment of functional categories including pectinesterase activity, differentially expressed genes associated with diverse molecular functions, including iron ion binding, NAD(P)H-dependent oxidoreductase activity, heme binding, and tetrapyrrole binding, were identified through KEGG enrichment analysis. Twelve of these genes were found to be significantly enriched in two pathways: phenylpropanoid biosynthesis and pentose and glucuronate interconversions. Within the phenylpropanoid biosynthesis pathway, the *β-Glu* gene *Cla97C08G153160* exhibited the highest expression level in HS, indicating its potential involvement in the regulation of this pathway. β-glucosidase, as a glucosidase, is responsible for catalyzing the hydrolysis of β-D-glucoside. This enzymatic reaction breaks the glycosidic bond in β-D-glucoside, resulting in the release of β-D-glucose, as one of the substrates, can hydrolyze the glucosidic bond in the substrate, releasing glucose molecules [38,39]. In the *4-CL* gene, *Cla97C06G122680* exhibits the highest expression level in HS, and this gene is a precursor of compounds related to sugars. The enzyme encoded by the *4-CL* gene catalyzes the formation of 4-coumaroyl-CoA by combining 4-hydroxycinnamic acid with coenzyme A in the phenylpropane metabolic pathway [40,41]. Phenylpropanoid biosynthesis, a critical regulatory process, exerts a profound influence on plant development throughout its lifecycle, particularly during fruit ripening. It is involved in processes such as fruit enlargement, formation of fruit color, and texture [42,43]. In pentose and glucuronate interconversions, among the *PE* gene, the expression level of *Cla97C09G171500* in HS is 93.8 times higher than in LS, showing the largest difference. PME is an enzyme that participates in both the formation and breakdown of pectin, and sugar is one of the main components of pectin. Research has shown that PME has a promoting effect on sugar. It achieves this by degrading the methyl ester groups in pectin, facilitating the release and utilization of sugars. However, the activity of PME is regulated by the degree of pectin methylesterification and other factors [44,45]. Among the differentially expressed genes, *Clar97C06G125310*, encoding UDP-glucose dehydrogenase (UGDH), exhibited the highest expression in LS, at levels 3.6-fold higher than in HS. Glucose-6-dehydrogenase (G6PD) and glucose-6-phosphate dehydrogenase (G6PDH) are critical enzymes in carbohydrate metabolism, and G6PDH catalyzes the conversion of glucose-6-phosphate (G-6-P) to 6-phosphogluconate, which is an essential step in the glycolytic pathway, thereby promoting energy production. An increase in the activity of G6PD may potentially inhibit the process of carbohydrate synthesis [1,46]. Pentose and glucuronate interconversions are two pathways related to glucose metabolism. The pentose phosphate pathway occurs in the cytoplasm and converts glucose into 5-phosphoribose, while generating NADPH. On the other hand, in the glucuronate pathway, glucuronate is one of the products of glucose metabolism and can be reduced back to glucose. This process is catalyzed by glucuronate reductase [47].

5. Conclusions

To unravel the temporal dynamics of sucrose, fructose, and glucose content during watermelon fruit development, this study employed 14 diverse small-fruited watermelon germplasm resources. Key time points that influence the changes in sugar content in

watermelon were identified. This study pioneers the comparative analysis of sucrose, fructose, and glucose content trends across five developmental stages in three watermelon varieties and their parental cultivars. It was found that the hybrid offspring exhibited genetic patterns in sugar traits that were similar to one parent or intermediate between the parents. This result provides new breeding strategies for exploring the sugar accumulation patterns and genetic breeding of small-fruited watermelons. Additionally, transcriptome analysis was conducted using high-sugar G38-28 and low-sugar 482276-28 watermelon varieties, revealing metabolic pathways closely related to watermelon growth, development, and sugar content, including phenylpropanoid biosynthesis and pentose and glucuronate interconversion. Twelve differentially expressed genes enriched in these two pathways were identified, including genes such as *Cla97C08G153160*, *Cla97C06G122680*, *Cla97C03G054700*, *Cla97C01G018700*, *Cla97C05G092680*, and *Cla97C09G179370*, etc. This study's systematic analysis of sugar content trends and genetic variations establishes a theoretical foundation for identifying key genes regulating watermelon sugar content. Moreover, this study sheds light on the molecular mechanisms responsible for the transcriptional-level variations in sugar content observed between G38-28 and 482276-28.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071544/s1, Figure S1: The correlation analysis among 6 watermelon samples. Figure S2: Principal component analysis of 6 watermelon samples. Supplemental Table S1: The primer sequence of qRT-PCR. Supplemental Table S2: The sequencing quality of 6 sample transcriptome data. *H group is G38-28, L group is 482276-28, with three replicates within each group, and the table below is the same. Supplemental Table S3: Sequence comparison of samples with reference genome and genes.

Author Contributions: Conceptualization and experimental design were spearheaded by Y.S. and X.Z., while X.Z., B.X., D.L., W.X., L.Y. and C.Z. meticulously conducted the experiments. Data analysis was expertly handled by X.Z., W.W., J.Z. and Y.G., while X.Z. crafted the manuscript. Y.S. provided valuable contributions and editing to the paper, which was further refined by L.L. and T.B. Language polishing was the expertise of W.X. and D.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original data of the sugar test transcriptome has been uploaded to the NCBI website with the query login number in parentheses.

Conflicts of Interest: Transcriptome sequencing raw data were uploaded to NCBI (PRJNA1132570): https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1132570 (accessed on 7 July 2024). The authors declare no conflicts of interest.

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