Sains Malaysiana 55(7)(2026): 1132-1147
http://doi.org/10.17576/jsm-2026-5507-03

Analysis of Sugar Accumulation Patterns and Candidate Gene Mining during Onion Bulb Enlargement

(Analisis Corak Pengumpulan Gula dan Perlombongan Gen Calon semasa Pembesaran Umbi Bawang)

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Received: 2 July 2025/Accepted: 17 June 2026

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ABSTRACT

Sugar content is a critical quality determinant in onion (*Allium cepa* L.), yet the spatio-temporal dynamics and molecular regulation of its accumulation during bulb expansion remain poorly understood. This study showed that soluble sugars in onion bulbs follow a distinct pattern of rapid accumulation followed by a gradual decline, peaking at the rapid expansion stage. This pattern was strongly correlated with the activities of key sucrose-metabolizing enzymes. Through integrated transcriptomic and physiological analyses, we identified three candidate genes namely *Cluster-29851.189607* (INV), *Cluster-29851.38447* (INV), and *Cluster-29851.183626* (SuSy), where their expression patterns (qRT-PCR) were consistent with the observed sugar dynamics. Moreover, virus-induced gene silencing (VIGS) technique showed that *Cluster-29851.183626* (SuSy) altered sugar accumulation patterns, supporting its involvement in bulb sugar metabolism. For the two *INV* genes, the current evidence indicates a strong association with hexose accumulation, and further functional validation is required. Overall, our results provide new spatio-temporal insights into sucrose and fructan-related sugar metabolism in onion bulbs and offer candidate targets for future functional studies and quality-oriented breeding.

Keywords: Bulb enlargement; candidate genes; enzyme activity; onion; sink strength

ABSTRAK

Kandungan gula merupakan penentu kualiti utama dalam bawang (*Allium cepa* L.), namun dinamik ruang-masa dan pengawalaturan molekul pengumpulan gula semasa pengembangan anak umbi masih kurang difahami. Kajian ini menunjukkan bahawa gula larut dalam anak umbi bawang mengikut corak pengumpulan pantas yang diikuti oleh penurunan beransur-ansur, mencapai kemuncaknya pada peringkat pengembangan pesat. Corak ini berkait rapat dengan aktiviti enzim utama yang terlibat dalam metabolisme sukrosa. Melalui analisis transkriptomik dan fisiologi secara bersepadu, kami mengenal pasti tiga gen calon iaitu *Cluster-29851.189607* (INV), *Cluster-29851.38447* (INV) dan *Cluster-29851.183626* (SuSy) yang corak ekspresinya (qRT-PCR) selaras dengan dinamik gula yang diperhatikan. Tambahan pula, teknik penyenyapan gen teraruh virus (VIGS) menunjukkan bahawa *Cluster-29851.183626* (SuSy) mengubah corak pengumpulan gula, menyokong penglibatannya dalam metabolisme gula anak umbi. Bagi dua gen *INV*, bukti semasa menunjukkan perkaitan kuat dengan pengumpulan heksosa dan pengesahan fungsi lanjut diperlukan. Secara keseluruhan, hasil kajian kami memberikan pandangan baharu mengenai metabolisme gula berkaitan sukrosa dan fruktosa dalam anak umbi bawang serta menawarkan calon sasaran untuk kajian fungsi dan pembiakan berorientasi kualiti pada masa akan datang.

Kata kunci: Aktiviti enzim; bawang; calon gen; kekuatan sink; pembesaran anak umbi

INTRODUCTION

Onion (*Allium cepa* L.) is a globally important horticultural crop, valued for its bulb. The bulb's quality for both fresh consumption and processing is directly influenced by its content of soluble sugars, such as fructose, glucose, and sucrose (Devi, Santoshand & Avik 2024; Galsurker et al. 2020; Martin et al. 2005). Soluble sugars are the main forms of carbohydrate metabolism and storage, and serve as the primary energy and nutritional substances for plant growth and development (Kleman, Rosberg & Mogren 2024; Nguyen & Foyer 2001; Zhao et al. 2006). Crucially, the development of onion bulbs is closely

related to sucrose metabolism, as sucrose from the leaves is the primary substrate for carbohydrate synthesis within the bulb (Mallor et al. 2011; Oku et al. 2019; Vandeputte & Delcour 2004).

The key enzymes in plant sucrose metabolism include sucrose synthase (SuSy), sucrose phosphate synthase (SPS), and invertase (INV), which collectively regulate the levels of sucrose, fructose, and glucose (Gan & Wu 2007). SPS is the key rate-limiting enzyme in sucrose synthesis, while SuSy catalyses a reversible reaction between sucrose and UDP-glucose, and INV irreversibly hydrolyses sucrose into glucose and fructose. Previous studies in onion have highlighted the importance of these pathways. For example, key genes for sugar synthesis and transport, such as SuSy and SPS, have been identified (Sanusi et al. 2024). Furthermore, transcriptional profiling has shown that genes controlling soluble sugar metabolism are differentially expressed during bulb development, and factors like AcFT1 are known to regulate sugar transport and accumulation (Lee et al. 2013; Yi et al. 2016). These findings confirm that sugar accumulation in onion bulbs is a tightly regulated process at the molecular level (Maloney et al. 2015; Roitsch & González 2004; Yao et al. 2012).

While studies have shown that the accumulation of sugar during onion bulb expansion is a complex process (Koch 2004), critical gaps in our understanding remain. Although the general changes in sugar content during onion bulb development and storage have been described, the detailed spatio-temporal dynamics of this process are still unclear. Specifically, there are no reports that have systematically evaluated the distribution of soluble sugars in different layers and sections of the onion bulb during its expansion phase. Furthermore, while many genes are known to be involved, the key candidate genes most strongly associated with the dynamic changes in sugar accumulation still need to be identified and functionally prioritized (Banerjee et al. 2022; Patrick, Botha & Birch 2013; Wang et al. 2021; Zhao et al. 2020). Therefore, this study was designed to explore the accumulation pattern and spatial distribution of sugar during the bulb expansion process, correlate these with key enzyme activities, and screen for primary candidate genes related to these metabolic changes. This work aimed to lay a foundation for further exploration of the molecular mechanism of sugar accumulation in onion.

MATERIALS AND METHODS

EXPERIMENTAL MATERIALS

The plant materials used in this study were two purple onion inbred lines (HCH0801 and HCH0803) and two yellow onion inbred lines (SA1 and L17136), provided by the Onion Breeding Research Group of Northeast Agricultural University (Harbin, China). These four inbred lines, SA1 represents a high-sugar, yellow-skinned line, whereas L17136, HCH0801 and HCH0803 differ in their bulb enlargement dynamics and sugar profiles, making them suitable for dissecting sugar accumulation patterns during bulb development.

Based on the bulb expansion period of onions, nine sampling time points were defined after transplanting to the field: 20, 30, 40, 47, 54, 61, 68, 75, and 82 days after transplanting (DAT), designated as S1-S9, respectively. At S1-S9, plants typically had 4, 5, 6, 7, 8, 9, 10, and 11 leaves, and were at early enlargement to maturation stages. For detailed physiological and molecular analyses, three key developmental stages were selected from this timeline: Bulb-A (early expansion, S1, 20 DAT), Bulb-B (rapid expansion, S5, 54 DAT), and Bulb-C (late expansion/maturation, S9, 82 DAT).

For whole-bulb and spatial analyses of soluble sugars, bulbs were harvested and either used intact or cut longitudinally and transversely to separate upper/middle/lower regions and concentric scale layers. For transcriptome sequencing and qRT-PCR, to ensure tissue homogeneity, only the inner scales from the middle portion of the bulb were collected at Bulb-A, Bulb-B, and Bulb-C, immediately frozen in liquid nitrogen, and stored at -80 °C. These inner middle scales represent the most active sink tissues for sugar import and storage, and therefore are expected to best reflect the core sugar metabolism underlying bulb enlargement. All plants were grown under uniform field conditions with consistent irrigation, fertilisation, and pest management. Each biological replicate consisted of a pool of five uniformly sized plants, and three biological replicates were collected per treatment.

MEASUREMENT OF SOLUBLE SUGAR CONTENT

Total soluble sugar content in onions was determined using the anthrone-sulfuric acid colorimetric method (Wu & Gao 2013). Briefly, 0.2 g of fresh bulb tissue was finely chopped and homogenised, then extracted in boiling water. After cooling and clarification, soluble sugars were quantified colorimetrically at 620 nm using an anthrone-sulfuric acid reagent. The specific contents of sucrose, glucose, and fructose were determined using a commercial hexokinase assay kit (Suzhou GRIUS, G0545W) according to the manufacturer's instructions. All sugar contents are expressed on a fresh-weight basis as mg g^{-1} FW. Each treatment included three independent biological replicates, and each biological replicate was measured with technical replicates.

MEASUREMENT OF SuSy, SPS, AND INV ACTIVITIES

The activities of sucrose synthase (SuSy), sucrose phosphate synthase (SPS), and invertase (INV) were determined following the method of Deng (2018) with minor modifications. Frozen onion bulb tissue (1.0 g) from the inner scales was ground in liquid nitrogen and

homogenized in ice-cold extraction buffer containing 5 mM $MgCl_2$, 2 mM EDTA-Na, 2% (v/v) ethylene glycol, 0.2% (w/v) bovine serum albumin (BSA), and 2% (w/v) PEP. The homogenate was centrifuged at 12,000 × g for 15 min at 4 °C, and the resulting supernatant was used as the crude enzyme extract.

Enzyme assays were carried out in reaction mixtures containing appropriate substrates: sucrose and UDP for SuSy, UDP-glucose and fructose-6-phosphate for SPS, and sucrose for INV. After incubation at the optimal temperature and time for each enzyme, the production of reducing sugars was quantified using colorimetric methods. One unit (U) of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of reducing sugar per minute per gram fresh weight (μmol min^{-1} g^{-1} FW) under the assay conditions. Three biological replicates were analysed for each treatment.

qRT-PCR

Total RNA was extracted from onion bulb samples using the Freezol RNA Reagent (Novozymes) and reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Shanghai, China), following the manufacturers' protocols. qRT-PCR was performed on a iQ5 Real-Time PCR Detection System (BIO-RAD, USA) using QPK-201CH (Toyobo®). The reaction program was as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 56 °C for 15 s and 72 °C for 45 s, 95 °C for 10 s, 65 °C for 5 s, 95 °C for 5 min. The onion AcActin gene was used as the internal reference for normalisation (Yuan et al. 2018). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table S1.

SCREENING OF IMPORTANT SUGAR METABOLISM-RELATED GENES IN ONIONS

To identify genes associated with sugar metabolism during onion bulb expansion, we re-analysed a previously published transcriptome dataset of onion bulbs (Qin et al. 2023). Putative sugar metabolism–related unigenes were first screened based on functional annotation. Briefly, Unigene sequences were annotated by DIAMOND BLASTX (e-value ≤ $1e^{-5}$) against public databases, including KEGG, NR, Swiss-Prot, GO, COG/KOG, and TrEMBL. Predicted amino acid sequences were further aligned to the Pfam database using HMMER (e-value ≤ 0.01) to identify conserved protein domains.

Differentially expressed genes (DEGs) between different developmental stages were determined by the following criteria: $|\log_2$ (fold change)$| > 1$ and false discovery rate (FDR) < 0.05. KEGG pathway enrichment analysis was performed on DEGs based on the KEGG orthology annotation system, and pathways with FDR-corrected $p < 0.05$ were considered significantly enriched. To explore expression patterns of sugar metabolism–related genes, K-means clustering was applied to the expression matrix of 188 selected unigenes (including those annotated as INV, SuSy, SPS, and other carbohydrate-metabolism enzymes) across the three developmental stages (Bulb-A, Bulb-B, Bulb-C) in SA1. Prior to clustering, expression values were $\log_2$-transformed and standardised. The number of clusters (K = 9) was chosen based on within-cluster sum-of-squares and biological interpretability. Clustering was performed in R, and the resulting clusters were used to identify genes with similar developmental expression trends.

CONSTRUCTION OF THE ONION VIGS SYSTEM

Healthy, uniform-grown onion bulbs were selected, rinsed under running water, the outer skin removed, and soaked in clean water for ≥3 h or overnight to restore vitality. The rehydrated bulbs were washed, redundant roots were removed, browned parts and neck were cut into two pieces, and holes were pierced in the basal plate and scales with toothpicks. They were then soaked in 0.1% mancozeb for 30 min with continuous stirring, and rinsed with ultrapure water and air-dried slightly.

Agrobacterium GV3101 (with target plasmid) was expanded to OD_{600}=1.0-1.5, centrifuged, resuspended in equal volume of fresh infection solution, and allowed to stand in the dark for 2-3 h. Infection solution (1 L) was prepared with 10 mL 1 mol/L $MgCl_2$ stock, 9.76 mL MES stock, and 400 μL 0.1 g/mL acetosyringone. MES stock was prepared by dissolving 25 g MES in 250 mL sterile water, adjusting pH to 5.6 with NaOH, filter-sterilising (0.22 μm), and storing at -20 °C.

Bulb pieces were immersed in infection solution, vacuum-treated at -0.8 kg/cm² for 20 min, and then air was slowly released for 20 min. The solution was drained, and the pieces were placed on sterilised gauze-lined porcelain plate, covered with two layers of gauze and tin foil, and dark-cultured at 24 °C, 60% RH for 48 h, then continued culturing under 14 h light/10 h dark (4000 lx). After 6 days of infection, the scale outer epidermis was torn and GFP imaging was performed with inverted fluorescence microscope (488 nm excitation, 510 nm detection for optimal results).

STATISTICAL ANALYSIS

All data are presented as means ± standard error (SE) of three biological replicates. Statistical analyses were performed using SPSS. For most measurements (sugar contents and enzyme activities), one-way analysis of variance (ANOVA) was used to test for differences among developmental stages within each genotype or tissue region, because our primary objective was to compare stage-dependent changes in each genetic background or spatial position while keeping the model

structure simple and avoiding over-parameterization with limited replication. When ANOVA indicated significant effects ($p < 0.05$), Fisher's least significant difference (LSD) test was used as a post-hoc test to separate means.

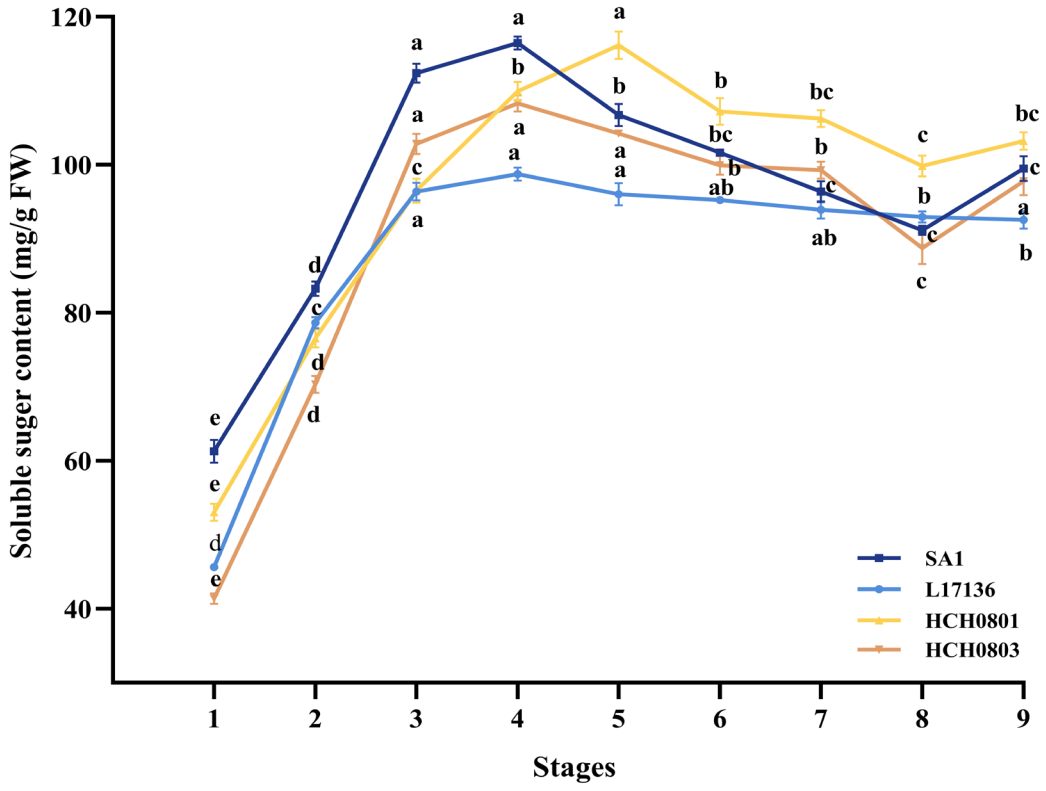
RESULTS

SOLUBLE SUGAR ACCUMULATION DURING ONION BULB EXPANSION

During the bulb expansion process of different onion lines, the accumulation of soluble sugar exhibited similar patterns (Figure 1). From the Bulb-A stage to the Bulb-B stage, the content of soluble sugar increased significantly and reached its peak at the Bulb-B. Afterward, as the bulb size increased, the sugar content slightly decreased, followed by minor fluctuations until the harvest period. At the Bulb-B stage, the three varieties (SA1, L17136 and HCH0803) reached peak accumulation, with values of 116.45, 98.74, and 110.24 mg g^{-1} FW, respectively. No significant differences in soluble sugar content were observed among the four onion lines after the Bulb-C stage. These results indicated that most soluble sugar accumulation in onion bulbs occurred before or around the rapid expansion stage, and that Bulb-B represented a key period for increasing soluble sugar content.

CHANGES IN SOLUBLE SUGAR DISTRIBUTION IN ONION BULBS DURING DEVELOPMENT

The onion bulbs were longitudinally trisected from the base of the leaf sheath to the bulb plate (Figure 2) to explore the soluble sugar accumulation in each part during the Bulb-B stage and the Bulb-C stage. The results showed that at the Bulb-B stage, soluble sugar content was high and relatively evenly distributed across the upper, middle, and lower parts of the bulb. As the bulb matured from the Bulb-B to the Bulb-C stage, the overall sugar content decreased, and its spatial distribution shifted. Specifically, sugar became predominantly concentrated in the middle part of the onion bulbs, followed by the lower part. The upper part, close to the base of the leaf sheath, had the lowest soluble sugar content.



Values are presented as the mean ± SE (n = 3). Different letters at each data point indicate significant differences among different developmental stages for the same variety ($p < 0.05$), according to the LSD test

FIGURE 1. Soluble sugar content of four onion varieties at different developmental stages

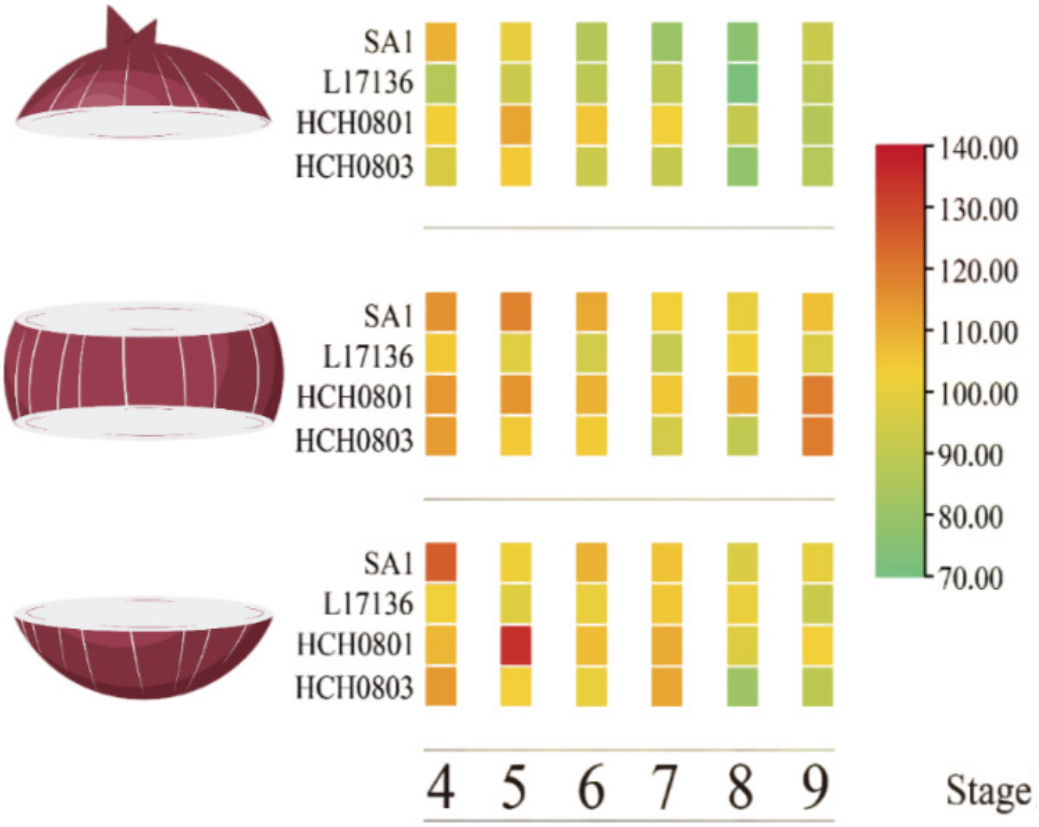


FIGURE 2. Heat map of the soluble sugar content in different parts of onion bulb at different developmental stages

CHANGES IN SOLUBLE SUGAR CONTENT IN DIFFERENT LAYERS OF ONION BULBS DURING ENLARGEMENT

To explore the changes in soluble sugar content in different layers of onion bulb scales, the onion bulbs of the four onion lines from Bulb-B stage to Bulb-C stage were separated layer by layer. The scales of each layer were designated as layers C1 to C10 in the order from the inner to the outer part. There were differences in the soluble sugar content of onion scales in different layers. At the Bulb-B stage, the content of soluble sugar in layers C1 to C6 of SA1 was the highest. From the Bulb-B stage to the Bulb-C stage, the content of soluble sugar in layers C3 to C5 of HCH0801 was relatively high. At the Bulb-C stage, the content of soluble sugar in layer C4 of HCH0801 was the highest. Irrespective of the variety, a common pattern was observed in the vertical sugar distribution: inner scales (C1–C4) accumulated significantly higher soluble sugar than outer scales (C7–C10) at both Bulb-B and Bulb-C ($p < 0.05$). This confirmed that the innermost, younger scales function as stronger sinks for carbohydrate storage compared with the outer, more mature scales. To understand the biochemical basis for these observed spatio-temporal sugar dynamics, we next investigated the activities of key sucrose-metabolising enzymes.

ANALYSIS OF THE CHANGES IN SUCROSE, FRUCTOSE, AND GLUCOSE CONTENT DURING ONION BULB ENLARGEMENT

Sucrose, fructose, and glucose are the main sugar components that affect the quality and taste of onions. In order to conduct a further investigation into the accumulation and metabolism of soluble sugar components, we measured the contents of sucrose, fructose and glucose in four onion lines during the period of onion bulb expansion (Figure 4). During the process of onion bulb expansion, the content variations of glucose and fructose were consistent, both demonstrating a pattern of initial increase, subsequent decrease, and eventual slow increase. However, during the expansion process of the four onion varieties, the variation trends of sucrose content were diverse. The sucrose content of the three lines namely L17136, HCH0801 and HCH0803 exhibited a pattern of initially significant increase, followed by decrease, and eventually a marginal increase and decrease. In contrast, the sucrose content of the SA1 variety rose significantly and reached the peak rapidly at first, and then declined. This showed significant differences compared to those observed in other varieties.

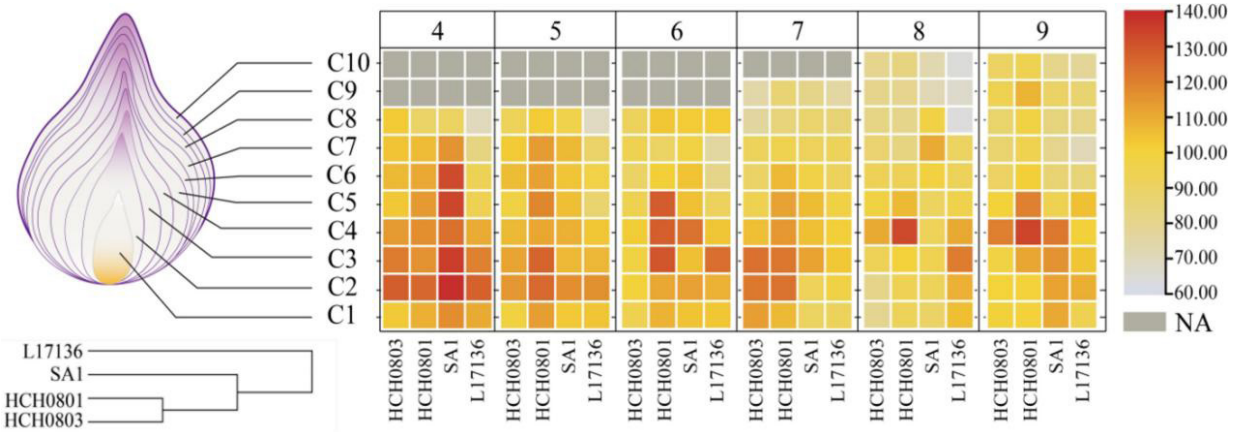
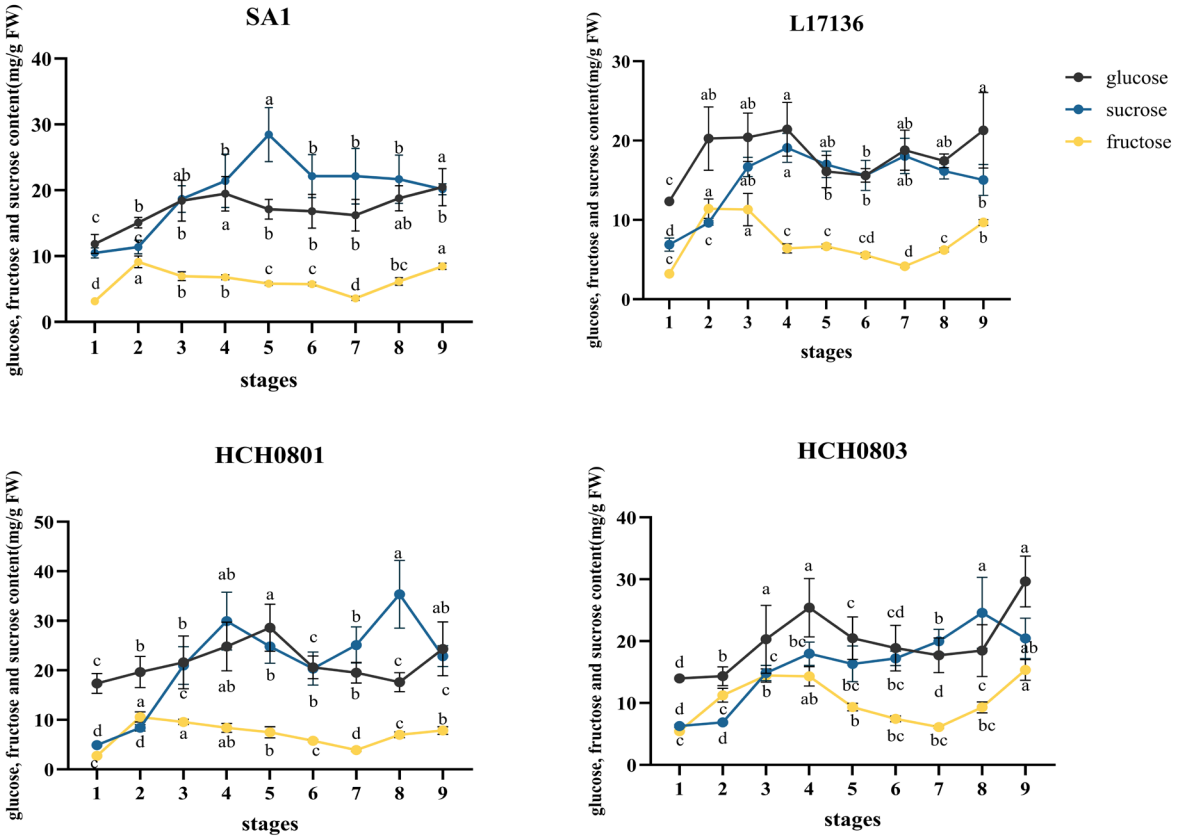


FIGURE 3. Heat map of soluble sugar content in different layers of bulbs of four different onion varieties. The color scale at the right indicates the soluble sugar content, with pale yellow representing lower values and dark red representing the highest values. Grey squares indicate 'Not Applicable' (NA) data points



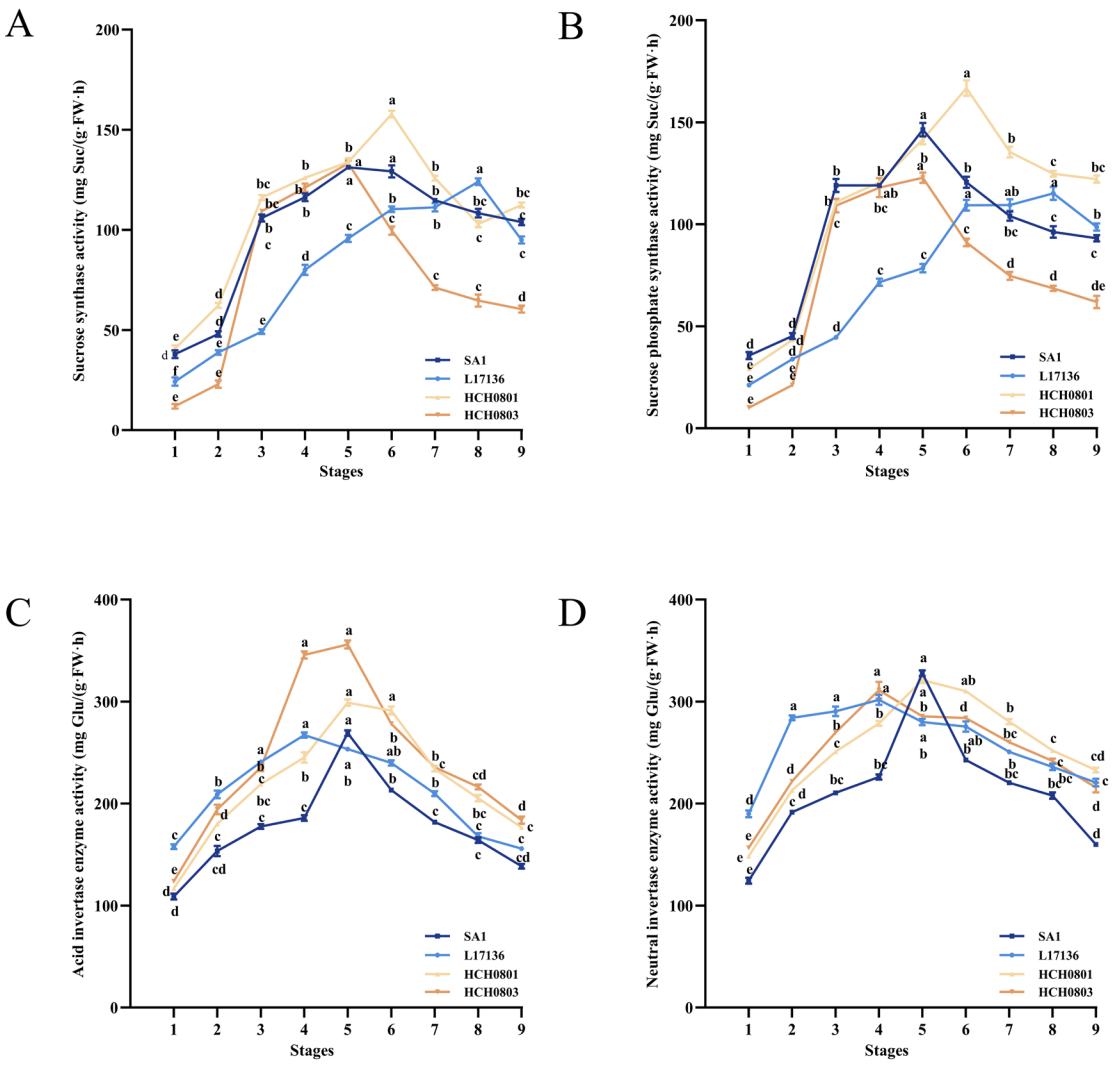
Values are presented as the mean ± SE (n = 3). For each sugar type within a single variety, different letters at each data point indicate significant differences among developmental stages ($p < 0.05$), according to the LSD test

FIGURE 4. Contents of glucose, fructose, and sucrose in four onion varieties during different stages of bulb development

ANALYSIS OF CHANGES IN SuSy, SPS, AND INV ENZYME ACTIVITY DURING ONION BULB ENLARGEMENT

SuSy, SPS and INV enzymes play indispensable roles in sucrose metabolism, and their activities exert a direct influence on the contents of sucrose, fructose, and glucose. To gain a deeper understanding of the mechanisms associated with accumulation of carbohydrate substances during the bulb expansion, we measured the activities of SuSy, SPS and INV enzymes, respectively (Figure 5). The activities of SuSy, SPS and INV increased at Bulb-B stage and then decreased, which was roughly consistent with the changing trends of glucose and fructose contents. The activities of these enzymes were relatively low in Bulb-A stage, but significantly increased in Bulb-B stage and then rapidly declined until the end of Bulb-C stage.

We conducted a correlation analysis between the changes in enzyme activity and the total sugar-related indicators (Figure 6). Among them, the positive correlation between the enzyme activity and the total sugar-related indicators of the HCH0801 and HCH0803 varieties was more intense and extensive, followed by the SA1 variety, while the correlation intensity and coverage of the L17136 variety were relatively weak. Among the four types of enzymes, the correlation between SPS and SuSy and the total sugar-related indicators was generally higher than that of INV, and all significant correlations were positive correlations. Building on these enzymatic findings, we then sought to identify the key candidate genes responsible for regulating these activities at the transcriptional level through transcriptome analysis.



Values are presented as the mean ± SE (n = 3). For each enzyme within a single variety, different letters at each data point indicate significant differences among developmental stages ($p < 0.05$), according to the LSD test

FIGURE 5. Activities of key enzymes in sucrose metabolism in four onion varieties during bulb development. (A) Sucrose synthase (SuSy), (B) Sucrose phosphate synthase (SPS), (C) Acid invertase, and (D) Neutral invertase

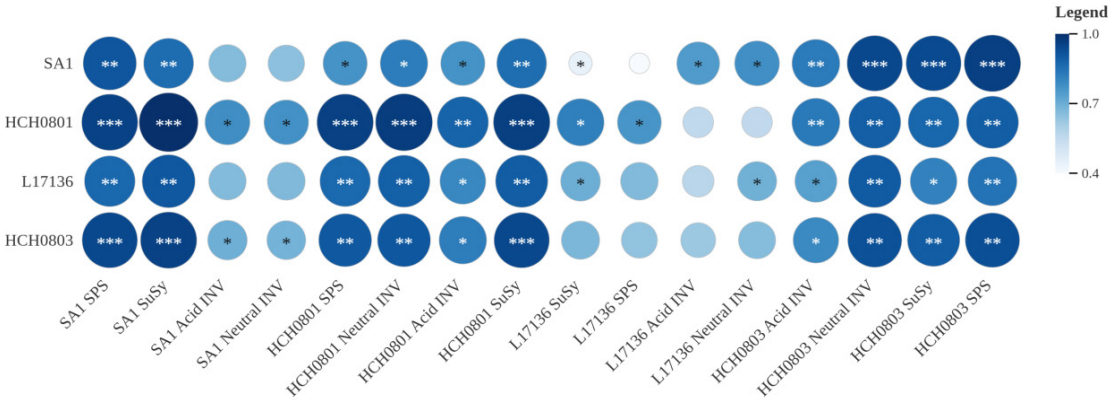


FIGURE 6. Heat map of the correlation between the enzyme activity and total sugar content of the bulbs of four different onion varieties. The color scale on the right indicates the degree of correlation, with light blue representing the lower values and dark blue representing the highest values

SCREENING OF SUGAR METABOLISM RELATED GENES DURING SA1 BULB DEVELOPMENT

To explore the molecular regulation of sugar accumulation during the bulb expansion and development of onion, based on the previous determination of soluble sugar, we selected the yellow-skinned onion variety SA1 and collected the middle part of the bulb at three key developmental stages. According to the annotation results of the RNA-Seq database, we screened for differentially expressed genes related to sugar metabolism and obtained a total of 188 unigenes, among which 25 were related to INV. This indicates that sugar metabolism continuously plays a role throughout the entire growth period of bulb development.

The K-means analysis method was adopted to conduct cluster analysis on the earlier-mentioned unigenes (Figure 6). The K-means analysis results indicated that these unigenes were classified into a total of 9 subcategories. Notably, the unigene numbered *Cluster-29851.183626* was annotated as sucrose synthase (SuSy), and its expression pattern belonged to the continuously rising fourth subcategory. During the early stage of onion bulb development, researchers also found that 11 single genes (*Cluster-29851.125216, Cluster-29851.189607, Cluster-29851.109394, Cluster-29851.63724, Cluster-29851.63725, Cluster-29851.125554, Cluster-29851.48087, Cluster-29851.114709, Cluster-29851.78487, Cluster-29851.78486* and *Cluster-29851.78488*) were annotated as invertase (INV), and all showed varying degrees of up-regulation. This result indicated that these 12 genes are highly likely to be potential functional genes for sugar accumulation in onions during the early stage.

CORRELATION NETWORK ANALYSIS OF SUGAR CONTENT AND DIFFERENTIALLY EXPRESSED STRUCTURAL GENES

To more accurately screen the structural genes related to sugar metabolism, we correlated the sugar content data measured in the previous stage with the differentially expressed structural genes, aiming to further show the correlation between gene expression and sugar content. With $R^2 > 0.9$ and p value < 0.01 as the screening criteria, we conducted Pearson correlation analysis on the 188 unigenes selected based on annotation results and literature data and the data of soluble sugar, sucrose, glucose and fructose at three time points during the bulb expansion of SA1. The results showed that 29 structural genes were strongly correlated with sugar content.

The results (Figure 7) indicated that 29 structural genes were highly correlated with sugar content. Among them, *Cluster-29851.189607*(INV) and *Cluster-29851.183626* (SuSy) were significantly positively correlated with the content of all four sugars; while another gene, *Cluster-29851.38447*(INV) was only positively correlated with the content of glucose. This indicated that during the bulb expansion process of SA1, *Cluster-29851.189607*(INV), *Cluster-29851.38447*(INV), and *Cluster-29851.183626*(SuSy) are strongly associated with sugar accumulation and can be prioritised as candidate genes for further functional validation.

IDENTIFICATION OF CANDIDATE GENES

Based on a comprehensive evaluation of the K-means clustering and the correlation network analysis, three key genes were prioritized as the strongest candidates associated with sugar accumulation during onion bulb expansion: *Cluster-29851.183626* (annotated as

SuSy), *Cluster-29851.189607* (annotated as INV), and *Cluster-29851.38447* (annotated as INV). The rationale for their selection is as follows: First, the SuSy-related gene, *Cluster-29851.183626*, exhibited a continuous upregulation in expression throughout bulb expansion. This expression pattern, combined with its significant positive correlation with all measured sugars, suggests it may play a sustained role in sucrose metabolism. Second, the two INV-related genes, *Cluster-29851.189607* and *Cluster-29851.38447*, showed expression patterns that were initially ascending and subsequently descending. This trend closely mirrored the accumulation patterns of total soluble sugar, fructose, and glucose. Furthermore, *Cluster-29851.189607* was strongly correlated with all four sugar indicators. Together, these results suggest that these INV genes are promising candidates potentially involved in sucrose hydrolysis and hexose accumulation during the rapid expansion stage. However, it should be noted that the evidence here is primarily based on correlation between transcript abundance and sugar levels, and direct functional proof (genetic manipulation and or enzyme characterisation with subcellular localisation) is still needed. These three genes were also among the 12 potential functional genes identified in the early sugar accumulation K-means cluster, further strengthening their candidacy.

To validate the expression patterns of the candidate genes obtained from the RNA-seq data, qRT-PCR was performed on six representative genes (Figure 8). The qRT-PCR results were highly consistent with the transcriptome data. Specifically, the expression of *Cluster-29851.183626* (SuSy), *Cluster-29851.125554* (INV), and *Cluster-29851.109394* (INV) showed a continuous upward trend throughout bulb development (Figure 8(a), 8(c), 8(e)). In contrast, the expression levels of *Cluster-29851.189607* (INV), *Cluster-29851.125216* (INV), and *Cluster-29851.38447* (INV) all increased from Bulb-A to Bulb-B and then decreased at Bulb-C, peaking at the rapid expansion stage (Figure 8(b), 8(d), 8(f)).

Among these, the expression pattern of *Cluster-29851.183626* (SuSy) was strongly and positively correlated with sugar accumulation, while the expression patterns of *Cluster-29851.189607* (INV) and *Cluster-29851.38447* (INV) closely mirrored the dynamic changes in soluble sugar, fructose, and glucose content. This validation supported their prioritisation as key candidate genes associated with sugar metabolism in our study.

CLUSTER-29851.183626 GENE-SILENCED PLANTS WERE CONSTRUCTED VIA VIGS

To explore the function of SuSy (sucrose synthase) in the sugar accumulation process of onion bulbs, this study used virus-induced gene silencing (VIGS) technology to construct *Cluster-29851.183626* gene-silenced plants. Healthy onion bulbs with consistent growth conditions were selected for infection treatment. The TRV1+TRV2 empty vector group was set as the control group, and the TRV1+TRV2-*Cluster-29851.183626* recombinant vector group was set as the experimental group. After infection, the bulbs were dark-cultured for 2 days for phenotypic observation. Under ultraviolet light, obvious GFP fluorescence signals were observed at the tips, the junctions of scales and basal plates, and the infection

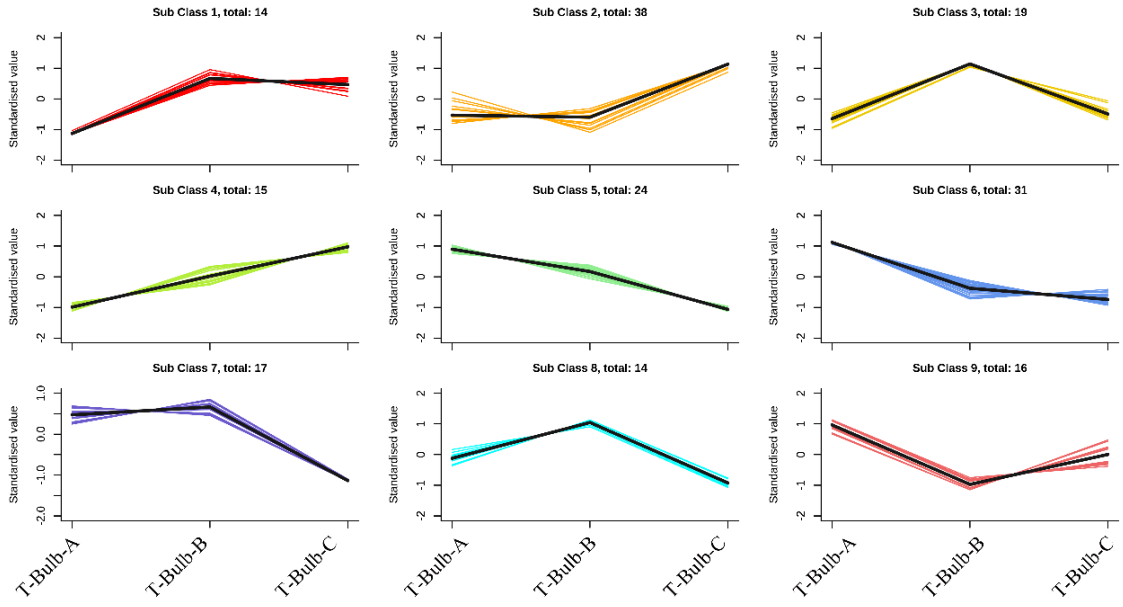


FIGURE 7. K-means clustering of genes related to sugar metabolism

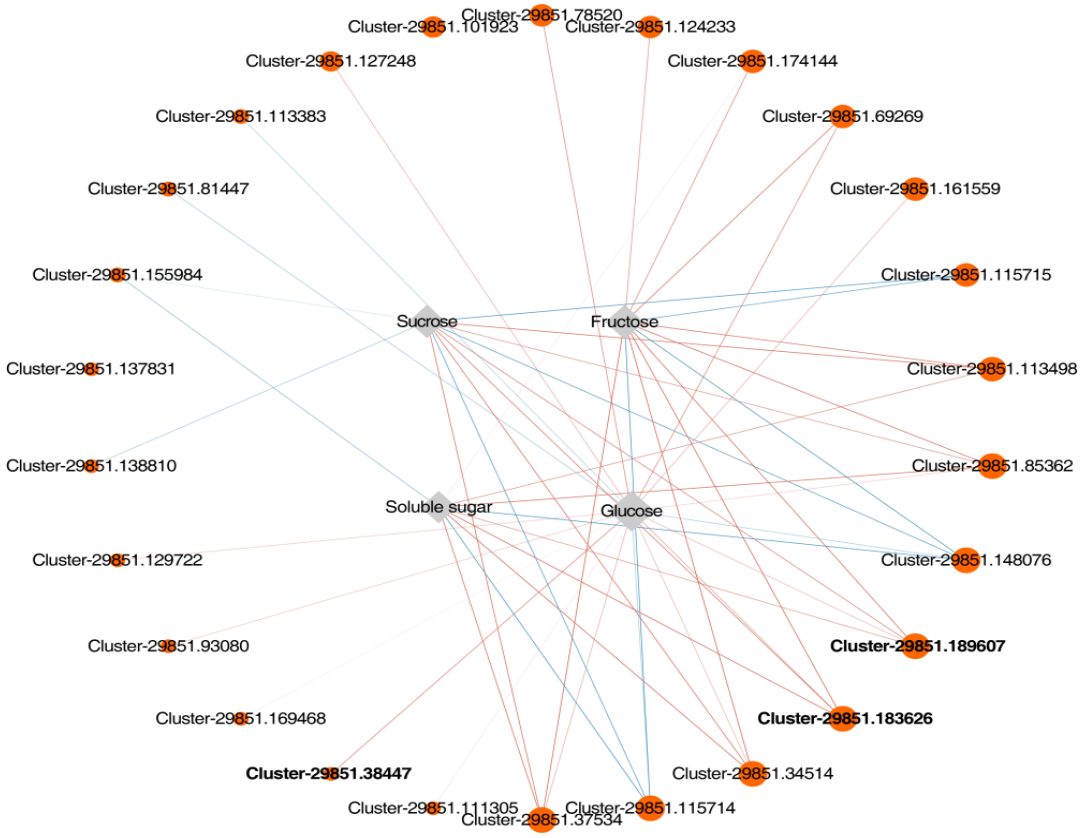


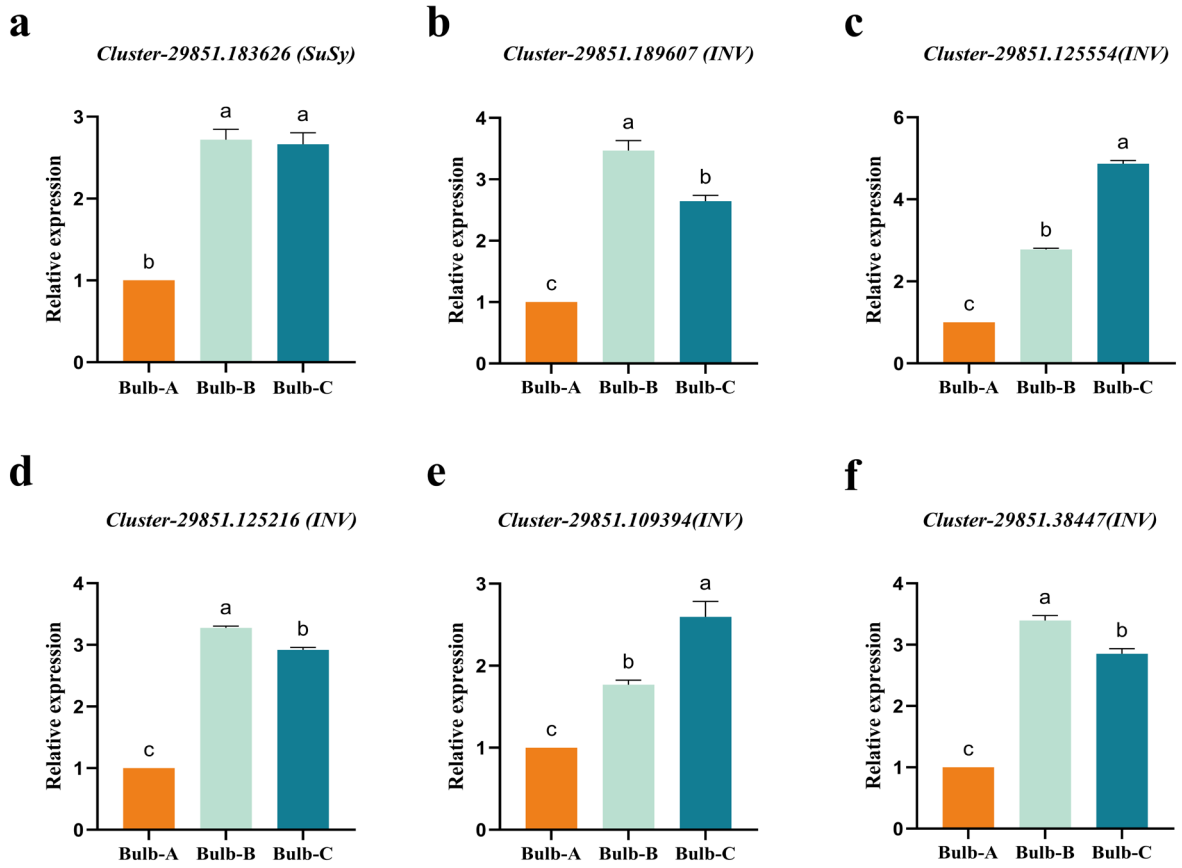
FIGURE 8. The correlation network diagram between 188 differentially expressed genes and soluble sugar content. Red lines indicate positive correlation, and blue lines indicate negative correlation

sites of the bulbs in both the control group (TRV2-GFP empty vector) and the experimental group (gene-silenced vector), indicating that the VIGS vectors had successfully infected the onion bulbs (Figure 10(A)). Further observation of the bulb hand sections on the 6th day after treatment under an inverted fluorescence microscope showed that GFP fluorescence could be clearly observed in the parenchyma cells of the bulbs in the experimental group, while no fluorescence signal was detected in the wild-type control group, confirming that the VIGS vectors had been successfully expressed at the cellular level (Figure 10(B)).

The results of qRT-PCR quantitative detection showed that the expression level of the *Cluster-29851.183626* gene in the experimental group was significantly downregulated compared to the control group ($p < 0.001$) (Figure 11), indicating that the VIGS technology had successfully achieved gene silencing of the target gene in onion bulbs. The content of soluble sugar in the bulbs was determined by the anthrone-sulfuric acid colorimetric method. The results showed that the content of soluble sugar in the gene-silenced lines was significantly lower than that in the control group (Figure 11). Based on the experimental results, the *Cluster-29851.183626* gene may participate in the sugar accumulation process in onion bulbs by regulating the sugar metabolism pathway, providing experimental evidence for further clarification of the biological function of this gene and the mechanism of SuSy in onion sugar metabolism.

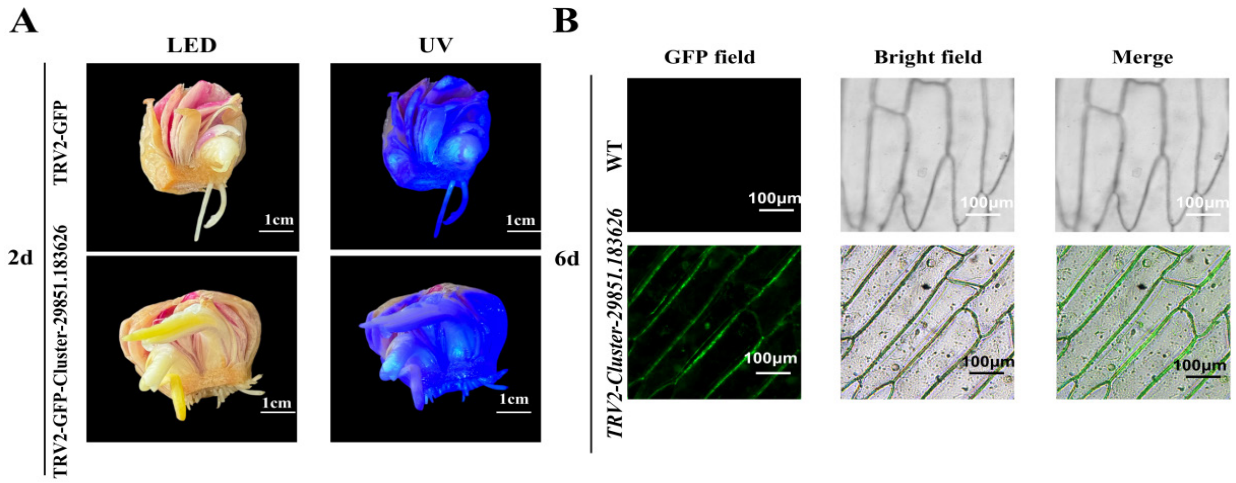
DISCUSSION

To the best of our knowledge, this study provides the first systematic analysis of soluble sugar distribution in onion bulbs that integrates both longitudinal regions (upper, middle, lower) and concentric scale layers (C1–C10) across clearly defined developmental stages. Previous studies in onion mainly focused on total sugar contents at a limited number of time points or during storage (Mallor et al. 2011; Oku et al. 2019; Vandeputte & Delcour 2004), without resolving how sugars are partitioned among individual scales and along the vertical axis of the bulb during active enlargement (Shiomi, Benkeblia & Onodera 2005). By mapping sugar contents in both dimensions, our results bridged this gap and demonstrated that the inner, middle-to-lower scales act as the strongest sinks, which is directly relevant for bulb sweetness, dry matter distribution, and postharvest behaviour (Wu & Gao 2013). This study showed that



Values are presented as the mean ± SE (n = 3). For each gene, different letters above the bars indicate significant differences in relative expression levels among the three developmental stages ($p < 0.05$), according to the LSD test

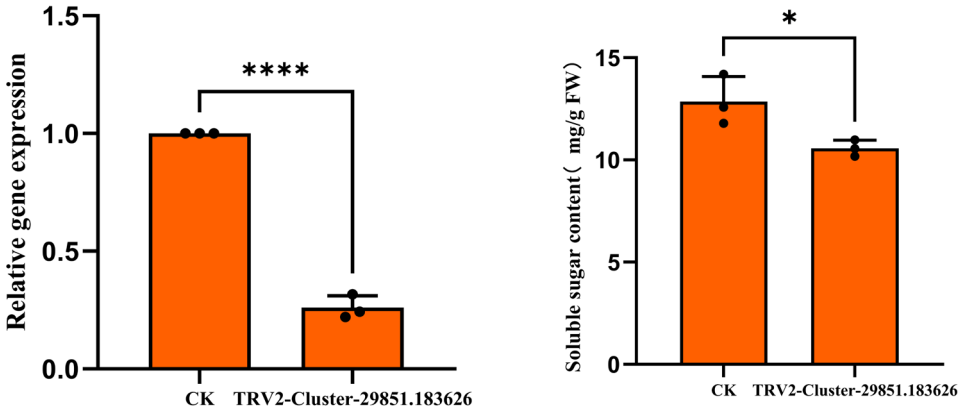
FIGURE 9. qRT-PCR validation of six candidate genes related to sugar metabolism. (a) *Cluster-29851.183626* (SuSy), (b) *Cluster-29851.189607* (INV), (c) *Cluster-29851.125554* (INV), (d) *Cluster-29851.125216* (INV), (e) *Cluster-29851.109394* (INV), and (f) *Cluster-29851.38447* (INV)



A.

GFP under LED light source and UV light source; B. Expression of reporter gene GFP under confocal microscopy

FIGURE 10. Expression of the GFP gene in onion bulbs



CK: The control plant with empty vector, and the same as below. Four asterisks (****) indicate significant differences at the 0.001 probability level; n = 3.

FIGURE 11. The expression level of *Cluster-29851.183626* gene was detected by qRT-PCR and the content of soluble sugar in silenced plants was measured

the accumulation of soluble sugars during onion bulb enlargement follows a distinct pattern of rapid increase followed by a gradual decrease, peaking around the rapid expansion stage (Bulb-B). This dynamic is crucial for bulb quality (Kleman, Rosberg & Mogren 2024; Sanusi et al. 2024). The peak of sucrose accumulation in the early-to-mid developmental stages observed in our work differed from the report by Shannon et al. (2003), where sucrose content remained stable. However, the accumulation patterns of fructose and glucose in later stages were consistent, suggesting that while the timing of sucrose metabolism may vary among genotypes, the subsequent hexose dynamics are more conserved (Jin et al. 2017; Kawakami, Yoshida & Van den Ende 2005). This study further provided novel insights into the spatial distribution of these sugars. We found that soluble sugars predominantly accumulated in the inner scales and the middle-to-lower parts of the bulb. This spatial pattern likely reflected the distinct physiological roles of the scales: the inner, younger scales function as active metabolic sinks, vigorously importing and converting sugars for storage, while the outermost scales gradually transition to a more structural and protective role as they mature and senesce. The particularly active sugar accumulation in the early stages, especially noted in the high-sugar line SA1 which showed a sharper and earlier accumulation peak, indicated a period of rapid sugar conversion and storage that is fundamental to the final size and sweetness of the onion bulb.

The observed patterns of sugar accumulation were likely shaped by the coordinated action of key metabolic enzymes and their corresponding genes (Maloney et al. 2015; Roitsch & González 2004; Yao et al. 2012). Our results showed that the activities of SuSy, SPS, and INV peaked during the rapid expansion stage, and these changes were positively correlated with the changes in glucose and fructose content. At the molecular level, our study identified specific candidate genes that may contribute to these enzymatic patterns (Qin et al. 2023; Yi et al. 2016; Yuan et al. 2018). The expression of two *INV* genes, *Cluster-29851.189607* and *Cluster-29851.38447*, peaked at the Bulb-B stage, consistent with the accumulation of hexoses. Combined with the increase in INV activity, these observations suggested that INV-mediated sucrose hydrolysis may contribute to hexose formation and sink strength during the critical bulb-filling stage. Nevertheless, our transcriptome and correlation analyses did not directly demonstrate causal roles for these INV genes, and the proposed mechanistic interpretation should be regarded as a working hypothesis that requires further functional validation (Shannon et al. 2003).

Interestingly, while studies in other major storage organs like tomato fruits and potato tubers report SuSy as the main enzyme for sucrose breakdown (Ma et al. 2017; Nguyen & Foyer 2001; Zhang et al. 2023), our results suggested a prominent role for INV in onions, which is consistent with some fruit ripening models (Fu 1995; Goren et al. 2017). Concurrently, the continuous upregulation of a *SuSy* gene (*Cluster-29851.183626*) suggested that it may play a different, yet sustained role. Instead of primarily cleaving sucrose for storage, this SuSy isoform might be important for providing UDP-glucose, a vital precursor for the biosynthesis of cell wall components and other polysaccharides required to support the rapid cell expansion during bulb enlargement (Schäfer, Rohwer & Botha 2004; Stein & Granot 2019; Uys et al. 2007).

From a breeding standpoint, the *INV* and *SuSy* genes identified here, particularly *Cluster-29851.189607*, *Cluster-29851.38447*, and *Cluster-29851.183626*, represent promising candidate targets for improving bulb sweetness and quality. Their expression patterns were tightly associated with sugar accumulation and key developmental transitions, suggesting that they could be exploited as markers in selection schemes for high-sugar lines, or as candidates for precise manipulation via gene editing. However, especially for the two *INV* genes, functional validation will be essential before firm conclusions about their roles or application potential can be drawn.

This study employed virus-induced gene silencing (VIGS) to provide functional evidence for the involvement of the candidate gene *Cluster-29851.183626* in sugar metabolism and bulb sweetness. In this experiment, researchers used an improved viral vector to selectively silence the target gene in onion plants, thereby observing phenotypic changes associated with reduced gene activity. After successfully achieving gene silencing, researchers conducted physiological and biochemical analyses of soluble sugars in bulb tissues. The results indicated that compared with the control group, the sugar accumulation pattern in silenced plants changed significantly, supporting that *Cluster-29851.183626* is involved in metabolic pathways affecting sugar accumulation. Future studies should use methods such as VIGS, transient overexpression, or stable transformation in onion or heterologous systems to verify the roles of the two candidate *INV* genes, *Cluster-29851.189607* and *Cluster-29851.38447*, in sugar metabolism and bulb sweetness.

CONCLUSIONS

In conclusion, this study provides a comprehensive view of sugar metabolism during onion bulb enlargement by integrating spatio-temporal sugar profiling, key enzyme activities, and transcriptome-based candidate gene mining. We showed that soluble sugars peak during the rapid expansion phase and are preferentially localised to the inner scales and the middle-to-lower portions of the bulb, highlighting these tissues as the strongest sinks. These physiological patterns were closely associated with coordinated changes in SuSy, SPS, and INV activities, and with the expression of three key candidate genes namely *Cluster-29851.189607* (INV), *Cluster-29851.38447* (INV), and *Cluster-29851.183626* (SuSy). VIGS results supported that the candidate gene *Cluster-29851.183626* (SuSy) is involved in pathways affecting sugar accumulation. For the two *INV* genes, our results indicated strong associations with sugar dynamics, but further functional validation is required. Together, our findings clarified how sucrose metabolism may contribute to bulb sweetness and sink strength in onion, provided a clearer differentiation from previous work that lacked spatial resolution, and offered specific candidate targets for future functional studies and breeding programs aimed at improving onion quality and processing traits.

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TABLE S1. List of primers

Primer Name	Primer sequence (5'to 3')	Base number	Application of primer
AcActin-F	ACACGGCCTGGATAGCAACAT	21	Onion reference gene
AcActin-R	AGAGCAGTATTCCCAAGCATT	21	
Cluster-29851.183626-F	CCTGCGTAGGCTATAAGAA	19	*SuSy* Fluorescent Quantitative PCR Primer
Cluster-29851.183626-R	CTGTAGAGGTTTATTTGGAATTG	24	
Cluster-29851.38447-F	AGCACTTCCTACACTGAG	18	*INV* Fluorescent Quantitative PCR Primer
Cluster-29851.38447-R	ACCTCCAGAACATTCCAA	18	
Cluster-29851.189607-F	CGATATGGACCTACGACAT	19	
Cluster-29851.189607-R	ATTCTGGACCTGGATGAG	18	
Cluster-29851.109394-F	GATTAGTCCACGGATACGA	19	
Cluster-29851.109394-R	CGCTCTTATGCTTCTGATG	19	
Cluster-29851.125554-F	ATACCTACGCTGTTGACTT	19	
Cluster-29851.125554-R	TTATTGCCTCGGTTGGATA	19	
Cluster-29851.125216-F	TTCTGACATTGGCGATGCCT	20	
Cluster-29851.125216-R	TGGAGTCCGTAACACAGTGC	20	