



Sugar accumulation and characterization of metabolizing enzyme genes in leafy head of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*)

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Received: 15 July 2020 / Revised: 25 August 2020 / Accepted: 28 August 2020 / Published online: 5 November 2020
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Abstract

Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) is a widely cultivated vegetable crop in eastern Asia. The flavor of Chinese cabbage was mostly affected by soluble sugar content. To make clear its accumulation and regulation mechanism, the content of different sugars in the internal blade (IB), the internal midrib (IM), the external blade (EB) and the external midrib (EM) of the leafy head were determined during the leafy head formation. The results showed that fructose was the major sugar accumulated in the internal tissues, followed by glucose. IM was the main tissue of sugar accumulation with the highest contents of total soluble sugar and hexose at harvest. RNA sequencing data of the four tissues at 90 DAS showed that the number of differentially expressed genes (DEGs) in IB and IM was the least (2408), while the number of DEGs between the external and internal tissues ranged from 6037 to 8674. The enzyme genes differentially expressed in ‘starch and sucrose metabolism’ pathway was detected during the leafy head development. The expression of two sucrose synthase (SUS) genes, *SUS1a* (Bra002332) and *SUS1b* (Bra006587), were always higher in IM than in the other tissues, and positively correlated with the content of total soluble sugar and hexose, respectively. *SUS1* was considered to play an important role in leafy head development and sugar accumulation in Chinese cabbage.

Keywords Chinese cabbage · Gene expression · Leafy head · Sugar accumulation · RNA-seq

Communicated by Sung Kyeom Kim.

Qianqian Liu and Junqing Li have contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13580-020-00294-y>) contains supplementary material, which is available to authorized users.

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1 Introduction

Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) is a Cruciferous vegetable with huge consumption and high nutritional value in East Asia. The leafy head comprising a large number of incurved leaves is the main edible organ (Mao et al. 2014). Head shape, flavor, and leaf color are three important quality indices of leafy head (Huang et al. 2016). In recent years, many studies of the heading characteristics and leaf color have been reported. Factors like air temperature, carbohydrate level, light intensity and endogenous hormones could affect the heading traits of Chinese cabbage. While at the gene level, *BrpTCP* and *BrpSPL9*, two target genes of microRNA319 and microRNA156, respectively, have been proved to regulate the shape of leaf head (Mao et al. 2014; Wang et al. 2014). For the orange and yellow color of the inner leaves of the leafy head, genes involved in the β -carotene biosynthetic pathway were thoroughly studied (Li et al. 2019), and *BrCRTISO* (Bra031539) gene was identified as the *Br-or* candidate gene (the gene that controlling orange color) in several studies (Feng et al.

2012; Li et al. 2017). Even though it is an important factor affecting taste, and has a great influence on consumers' choices (Nookaraju et al. 2010), only few studies on sweetness of heading leaves were reported yet. Sweetness of fruits and vegetables depends mainly on type and composition of sugars (Desnoues et al. 2014). In a study on path analysis of indices related to Chinese cabbage flavor, Qiao et al. (1991) reported that the soluble sugar had the greatest influence on the sensory quality of Chinese cabbage. Soluble sugar was formerly regarded as a quality or adversity indicator (Kim et al. 2014; Ma et al. 2014; Ren et al. 2016). Furthermore, starch and sucrose were considered as the temporary storage forms of photosynthate in blade and midrib, respectively (Lee et al. 2020).

Most of the soluble sugars in edible organs of horticultural crops come from the photosynthate of source leaves, and sucrose is the main form of photosynthate output, which can be decomposed into fructose and glucose in sink cells (Park et al. 2008). The distribution and composition of carbohydrate in sink tissues depend on the relative gene expression of enzymes in carbohydrate metabolism (Nookaraju et al. 2010; Zhu et al. 2018). Invertase (INV, EC 3.2.1.26) catalyzes the irreversible hydrolysis of sucrose to fructose and glucose (Nguyen-Quoc and Foyer 2001). In plant cells, INV has three isozyme types with different biochemical properties and subcellular localizations. Cell wall invertase (CWIN), vacuole invertase (VIN) and neutral invertase (NIN) can hydrolyze sucrose in extracellular matrix, vacuole and cytoplasm, respectively. Sucrose synthase (SUS, EC 2.4.1.13) reversibly degrades sucrose into fructose and uridine diphosphate glucose (UDPG) in the presence of uridine diphosphate (UDP). Sucrose phosphate synthase (SPS, EC 2.3.1.14) catalyzes the synthesis of sucrose from UDPG and fructose-6-phosphate (Fru-6-P). (Nielsen et al. 1991; Li et al. 2012). INV, SUS and SPS are recognized as the three main enzymes affecting the accumulation and metabolism of soluble sugar (Nookaraju et al. 2010; Matsukura 2016).

Sucrose and hexose are the two major forms of sugar accumulated in plant sink organs (Fischer et al. 1998; Kim et al. 2014). Previous studies have shown that the effects of different enzymes on sugar accumulation in different species are great different. Verma et al. (2011) found that the activity of SPS and the expression of *SPS* gene in sugarcane cultivars with high sugar content were higher than those with low sugar content throughout the growth period. SPS was also the main determinant of sucrose accumulation in sugar beet, sucrose-accumulating tomato and cantaloupe fruit (Hoffmann 2010; Wen et al. 2010; Matsukura 2016). These studies indicated that SPS played an important role in sucrose-accumulating sinks. While in many hexose-accumulating fruits, INV or SUS had a greater effect on sugar accumulation. During fruit development of strawberry cultivars 'Festival' and 'Ventana', sugar accumulation was

dominated by glucose, and the activity of SUS was difficult to detect, while NIN maintained a high activity, which was the main sucrose hydrolase, and played a major role in the sugar accumulation in sink organs (Basson et al. 2010). During mango ripening, VIN was the main enzyme affecting sugar accumulation, while SPS activity decreased gradually (Wongmetha et al. 2012). Similar results were obtained in hexose-accumulating tomatoes (Levin et al. 2006).

Furthermore, UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9), hexokinase (HK, EC 2.7.1.1) and fructokinase (FK, EC 2.7.1.4) (Moscatello et al. 2011; Hu et al. 2016) were involved in further utilization of sucrose degradation products, including glucose, fructose and UDPG. ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) is also necessary when hexose phosphate is used for the biosynthesis of starch (Zhang et al. 2015). These enzymes affect the accumulation of fructose and glucose to varying degrees (Moscatello et al. 2011). Li et al. (2019) reported the down-regulation of *SPS* gene and up-regulation of *SUS* gene in yellow leaves of Chinese cabbage, and sucrose biosynthesis were thought to be regulated by 6-phosphofructokinase (*PFK6*) and *SUS1* instead of *SPS* gene. However, the effects of sugar-metabolizing enzyme genes on carbohydrate accumulation in the leafy head of Chinese cabbage were not fully understood.

The objective of this paper was to study the accumulation of different sugar components in various parts of the heading leaves of Chinese cabbage during the leafy head formation, and to investigate the expression patterns of key enzyme genes affecting the sugar content and composition by transcriptome and quantitative real-time PCR (qPCR) methods. These comprehensive results would provide basic information for further functional analysis of the related enzyme genes during leafy head development.

2 Materials and methods

2.1 Plant material and sample collection

Experiments in 2016: 'Xibai no. 4', a Chinese cabbage commercial cultivar with relatively high soluble sugar content (about 3–4% of fresh weight) in inner folding leaves, was chosen as materials in this study. The seeds were supplied by Shandong Denghai Seed Industry, Ltd., West Seed Branch and were sown in the research farm (37° 21' N, 119° 59' E, Laizhou city, China) with a plant spacing of 35 cm × 70 cm. The materials were grown under normal irrigation, fertilization and pest-control conditions and were harvested on 90 days after sowing (DAS) (November 14, 2016). There were around 55 heading leaves (more than 2 cm in length) in the leafy head of 'Xibai no. 4' at harvest. The samples for RNA sequencing (RNA-seq) were collected from four

different tissues of the harvested leafy head (90 DAS) as follows: samples of external blade (EB) and external midrib (EM) were collected from the 1st to 3rd heading leaf from outside to inside; and the 4th–6th internal heading leaf having a length of more than 4 cm from inside to outside were chosen as samples of internal blade (IB) and internal midrib (IM) (Fig. 1a), because it was difficult to obtain enough internal midrib tissues from the heading leaves which were too small. At 42–45 DAS, the plants were in a stage with 20–24 expanded leaves and with the inner leaves beginning to fold. The stage of leaf incurvature is basically consistent with Wang's report (Wang et al. 2014). Later, the leafy head which were composed of heading leaves gradually enlarged and compacted to form a tight head (Fig. 1b). Therefore, the above four types of samples were collected at 45, 60, 75 and 90 DAS respectively for quantitative real-time PCR (qRT-PCR) and the content analysis of soluble sugar (sucrose, fructose and glucose) and starch. The sampling method at 60 DAS and 75 DAS was the same as that at 90 DAS. While at 45 DAS, the samples of EB and EM were obtained from the erect leaves close to the inner folding leaves, because only a few inner leaves incurvated at the initial stage of heading. The sampling times were all around 10:00 am. Three sets of samples as biological replicates were collected from five different plants at each time point. All samples were frozen immediately in liquid nitrogen and stored at -80°C for further processing.

Experiments in 2017: Field grown of cv. 'Xibai no.4' was the same as in 2016. In the autumn of 2017 (from August 16 to November 14), the contents of soluble sugar and starch in four tissues of 'Xibai no.4' were measured according to the aforesaid sampling methods.

2.2 RNA extraction and cDNA library construction

Total RNA of all samples was extracted using the EASYspin Plant RNA Rapid Extraction Kit (Yuanpinghao, Tianjing, China), following the manufacturer's instructions. The RNA samples were treated with RNase-free DNase I (Takara, Tokyo, Japan). The quality of RNA was checked using 1.0% agarose gel electrophoresis and an Agilent 2100 RNA Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RIN (RNA integrity number) values of these samples were 8.6–10.0. Total RNA samples were sent to Biomarker Biotechnology Corporation (Beijing, China) for library construction and transcriptome sequencing.

Twelve sequencing libraries (four types of samples on 90 DAS with three biological replicates) were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). Briefly, mRNA was purified followed by sheared into short fragments. Reverse transcriptase and random hexamer primers were used to synthesize the first-strand cDNA, and the second strand cDNA

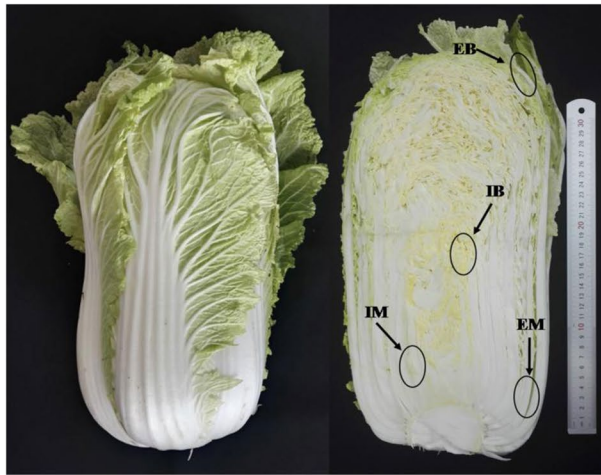
was synthesized using DNA Polymerase I and RNase H. The library fragments were purified for preferentially selecting suitable cDNA fragments (about 200 bp in length). After adenylation of 3' ends and adapter ligation, the suitable fragments were enriched by PCR amplification. The PCR products as sequencing templates were purified via AMPure XP Kit (Beckman Coulter, Brea, CA, USA). At last, library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.3 RNA-seq reads mapping, detection and differentially expressed genes (DEGs)

The constructed libraries were sequenced on an Illumina HiSeq™ 2500 system with the pair-end sequencing protocol (BioMarker Technologies Co Ltd, Beijing, China). Clean reads were obtained by removing adaptor sequences and low quality sequences (reads containing ploy-N more than 10% or more than half of their bases with a quality score ≤ 10). The clean reads were aligned to the *Brassica rapa* genome (Version 1.5) (<https://brassicadb.org/brad/>) using Tophat2 (Kim et al. 2013), and two mismatches were allowed. The quantification of gene expression levels were normalized by the FPKM (fragments per kilobase of transcript per million fragments mapped) using Cufflinks (Trapnell et al., 2012). Differential expression analysis of samples with biological replicates was performed using DEseq R package (Anders and Huber 2010). Genes with a false discovery rate (FDR) < 0.01 and \log_2 ratio ≥ 1.0 (fold change ≥ 2) were identified as differentially expressed genes.

2.4 Functional annotation and classification of DEGs

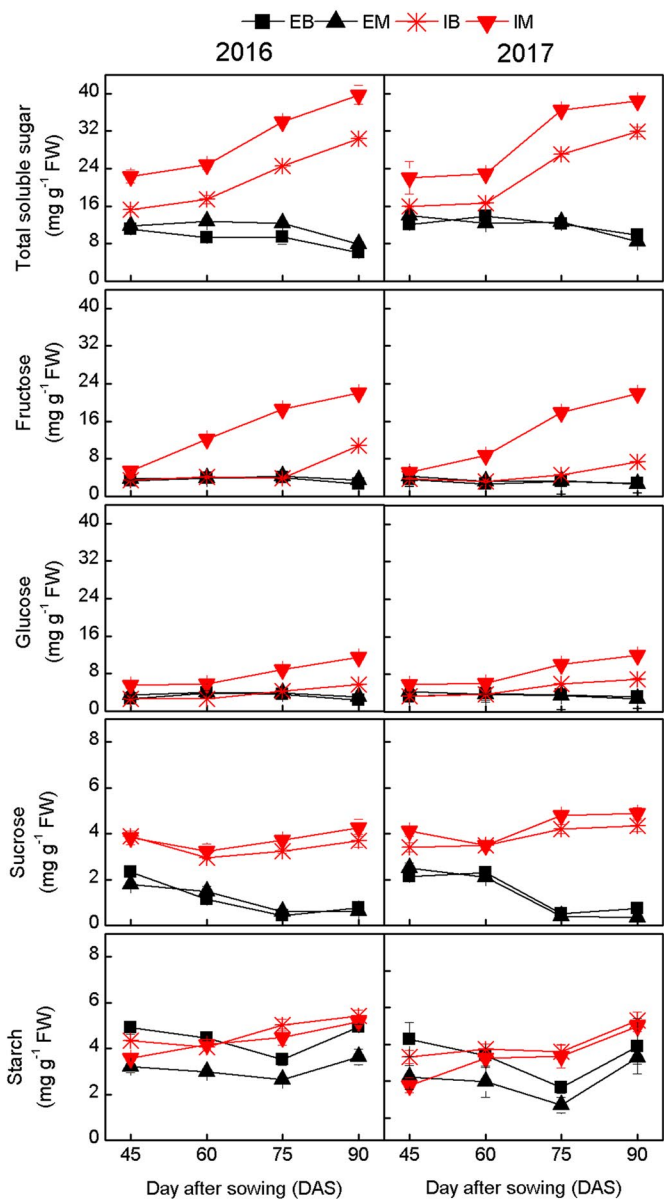
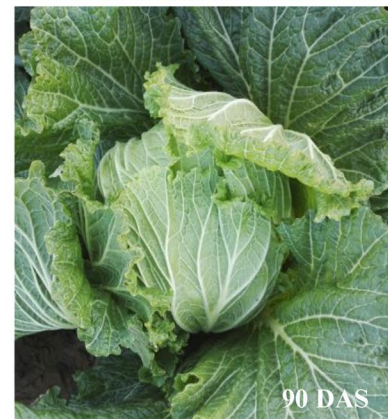
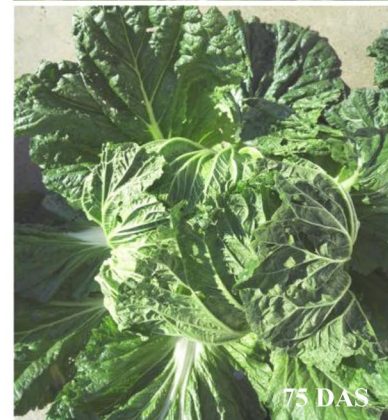
All the resulting DEGs were annotated via alignment-based search against the National Center for Biotechnology Information (NCBI) non-redundant protein (Nr) (<https://www.ncbi.nlm.nih.gov/>), Swiss-Prot (<https://www.expasy.ch/sprot>) (Apweiler et al. 2004), Gene Ontology (GO) (<https://www.geneontology.org/>) (Ashburner et al. 2000), Clusters of Orthologous Groups (COG) (<https://www.ncbi.nlm.nih.gov/COG>) (Tatusov et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) (Kanehisa et al. 2008) using BLAST (E-value $< 10^{-5}$). The Nr BLAST results were imported into the Blast2 GO program for obtaining the GO annotations (Conesa et al. 2005). GO functional classification and assignment of KEGG pathways in DEGs were performed using Perl script (Porollo 2014). The significantly enriched GO terms and KEGG pathways were identified with hypergeometric tests, taking the Bonferroni-corrected p value ≤ 0.05 as a threshold (Abdi 2007).



A



B



C

Fig. 1 Changes of morphology and carbohydrate levels in different parts of ‘Xibai no. 4’ Chinese cabbage during leafy head formation. **a** four types of tissue samples (90 DAS) for RNA Sequencing: external blade (EB), external midrib (EM), internal blade (IB) and internal midrib (IM). The maximum range of the ruler in the figure is 30 cm. **b** leafy head development on different days after sowing (DAS). **c** the contents of soluble sugar and starch in heading leaves at four stages in 2016 and 2017. Error bars represent standard error (\pm SE) for three replicates (LSD test at $p < 0.05$). EB, external blade; EM, external midrib; IB, internal blade; IM, internal midrib

2.5 qRT-PCR analysis

Fifteen unigenes involved in sugar metabolism were selected for qRT-PCR to verify the RNA-seq results. Elongation-factor-1- α (*EF-1- α*) (Accession number: GO479260) was selected as the reference gene (Qi et al. 2010). Specific primer sequences were designed using the software Primer 6.0 (Premier Biosoft Inc., Palo Alto, CA, USA) (Table S1). RNA extraction from the samples collected at 45, 60, 75 and 90 DAS was performed using the same procedure for RNA-seq. cDNAs were synthesized from 1 μ g total DNA-free RNA using Prime ScriptTM RT reagent kit (TaKaRa, Tokyo, Japan). qRT-PCR was performed using the Roche 480 real-time PCR system (Basil, Switzerland) at the standard mode. The reactions were carried out for three times using independent samples. The total mixture volume for each reaction was 20 μ L, which contained 10 μ L of 2 \times SYBR Green I Master (Roche, Indianapolis, IN, USA), 2 μ L of 1:10 diluted cDNA, 0.4 μ L of each primer, and 7.2 μ L of double-distilled water. The qRT-PCR was conducted as follows: 95 $^{\circ}$ C for 5 min, 45 cycles at 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

2.6 Measurement of soluble sugar and starch contents

Total soluble sugar content was determined by the colorimetric Anthrone method (Spiro 1966). Starch content was determined in ethanol extracts following Hendriks’s report (Hendriks et al. 2003). Sucrose, glucose and fructose were measured by high-performance liquid chromatography (HPLC) with some modifications on the basis of Bhandari’s method (Bhandari et al. 2012). Briefly, 5.0 g fresh sample was homogenized in 30 ml distilled water. The homogenate was shaking 20 min in a water bath at 80 $^{\circ}$ C followed by sonication for 20 min. After centrifugation (8000 g, 15 min), the supernatant was filtered through a hydrophilic 0.45 μ m filter. Forty microliters of the filtrate was injected into Agilent 1100 HPLC system (Agilent, San Jose, CA, USA) with a refractive index detector (G1362A, Agilent, San Jose, CA, USA) and a NH₂ column (250 mm \times 4.6 mm, Phenomenex,

Torrance, CA, USA). The mobile phase was acetonitrile: water (80:20) with a flow rate of 1.5 ml min⁻¹, and the column temperature was 30 $^{\circ}$ C.

2.7 Statistical Analysis

All statistical analyses were performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA), and all figures were made with OriginPro 8 (Origin Inc., Northampton, MA, USA). Data were analyzed by one-way analysis of variance (ANOVA). The significant differences between means were assessed by the least significant difference (LSD) test at $p < 0.05$.

3 Results

3.1 Carbohydrate levels during leafy head development

In both 2016 and 2017, the contents of total soluble sugar in IM and IB were significantly higher than that in EB and EM during leafy head formation and increased gradually with time (except for the insignificant difference between the soluble sugar content of IB and EM at 45 DAS in 2017). (Fig. 1c; Table S2). In 2016, the fructose content of IM increased rapidly after 45 DAS and was as high as 22.02 mg g⁻¹ FW at 90 DAS. For the other tissues, only the fructose in IB rapidly accumulated after 75 DAS (Fig. 1c; Table S2). The glucose accumulation in IM and IB increased rapidly after 60 DAS, and the glucose content of IM reached 11.53 mg g⁻¹ FW at 90 DAS. The glucose content in external tissues showed similar trend to total soluble sugar and fructose, i.e., it stabilized at a lower level during the early stage and slightly decreased at the harvest time (Fig. 1c; Table S2). During 45–90 DAS, IM and IB had higher sucrose content compared to EB and EM, but the sucrose content of all parts were less than 5 mg g⁻¹ FW. After 75 DAS, this value in external tissue was even below 1 mg g⁻¹ FW (Fig. 1c; Table S2). The starch content of EB and EM decreased from 45 to 75 DAS, and then increased significantly at 90 DAS. The starch content of IM and IB gradually increased to about 5 mg g⁻¹ FW at 90 DAS, which was not significantly different from that in EB (Fig. 1c; Table S2). Figure 1c and Table S2 showed that the tendencies and contents of fructose, glucose and sucrose in 2017 were similar to those in 2016. Although the starch content of EM at each period increased in 2017, it remained within a low level of 3–5 mg g⁻¹ FW. The above results showed that the soluble sugar accumulation in Chinese cabbage is mainly in the internal tissue of the leafy head; fructose is one of the main sugars accumulated in IM and IB, followed by glucose.

3.2 Analysis of RNA-seq and differentially expressed genes

During the formation of the leafy head, the soluble sugar content in different tissues of the leafy head showed the maximum difference at the harvest time (90 DAS) (Fig. 1c). Therefore, EB, EM, IB and IM samples of this period were selected for transcriptome sequencing. RNAs extracted respectively from above samples with three biological replicates were subjected to Illumina sequencing. The sequencing data (accession number: PRJNA505221) were submitted to the National Center for Biotechnology Information Sequence Read Archive (SRA). A total of 227.1 million clean reads were generated from twelve libraries. An average of 95.03% of the clean reads had Phred quality scores at the Q30 level, which ensured the precision of the sequencing. The percentage of the reads that could be mapped to the reference genome ranged from 68.13 to 78.90% in the twelve libraries (Table S3). Under the criteria of \log_2 ratio ≥ 1.0 and $FDR < 0.01$, there was a maximum number of DEGs between EB and IM (8674), while the DEGs between IB and IM from the interior of leafy head was minimal (2408), followed by the EB and EM (4589) (Table S4). A total of 36,324 (99.00%) DEGs successfully obtained the homologous annotation information based on similarities to Nr, Swiss-Port, GO, COG, and KEGG databases (Table S4). DEGs with FPKM values in the pairwise comparison among IB, IM, EB and EM were shown in Table S5.

3.3 Classification and enrichment analysis of DEGs

GO analyses for the DEGs in each comparison (EB vs IM, IB vs IM, EB vs IB, EM vs IM, EB vs EM and EM vs IB) were performed to identify significantly enriched GO terms (Table S6). Within the biological process category, DEGs in EB vs IM were significantly enriched in GO terms related to cell proliferation and growth, such as ‘plant-type cell wall organization’ (GO: 0009664), ‘regulation of meristem growth’ (GO: 0010075) and ‘polysaccharide biosynthetic process’ (GO: 0000271), and the three GO terms are also included in top 20 enriched GO terms in EB vs IB and EM vs IB (according to the Q -value, corrected p value). Within the category of cellular component, ‘plant-type cell wall’ (GO: 0009505) had significant enrichment in comparisons of external and internal tissues (EB vs IM, EB vs IB and EM vs IM). For the category of molecular function, ‘cellulose synthase (UDP-forming) activity’ (GO: 0016760) was found to be significantly enriched in EB vs IM (Q -value < 0.01), but not in IB vs IM.

Pathway analysis was also performed to categorize the biological functions of the DEGs by matching to the KEGG database (Fig. S1; Table S7). ‘Ribosome’ (ko03010) was the most enriched pathway for DEGs in the comparison

of EB vs IM, followed by ‘Photosynthesis—antenna proteins’ (ko00196) and ‘Fructose and mannose metabolism’ (ko00051) (Q -value < 0.01). While for IB vs IM, the most enriched pathway was ‘Photosynthesis’ (ko00195), followed by ‘Photosynthesis—antenna proteins’ (ko00196) and ‘Porphyrin and chlorophyll metabolism’ (ko00860) (Q -value < 0.01). The results of the GO and KEGG analysis suggested that, the gene expression profile of external leaves was quite different from the internal leafy tissues which was undergoing active morphogenesis.

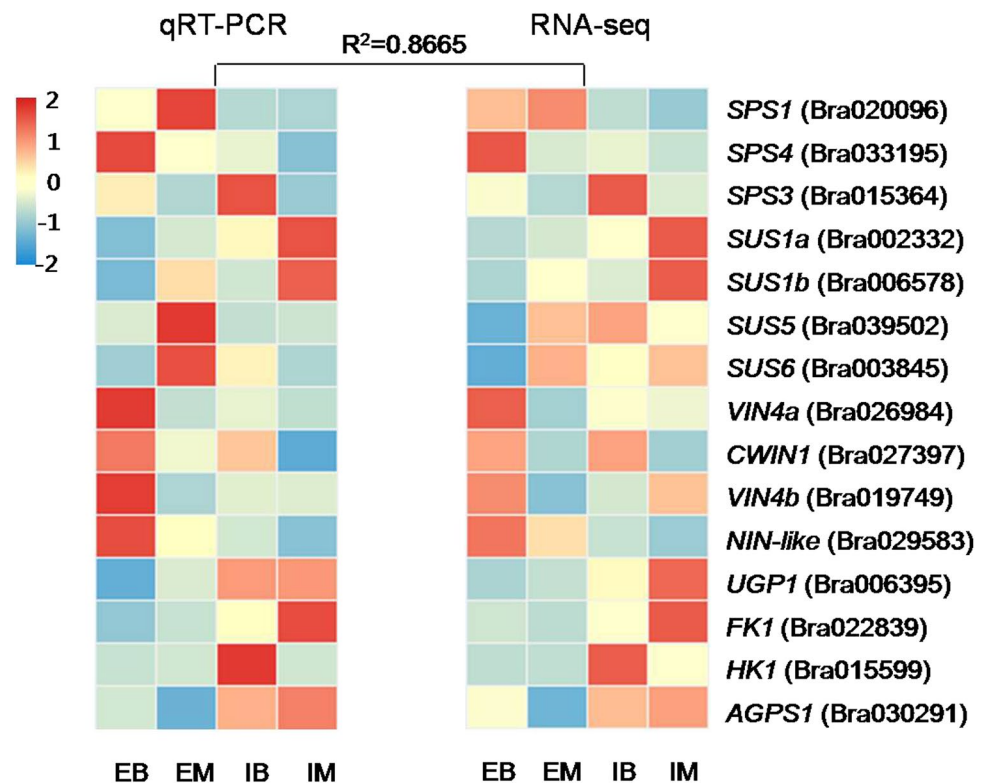
3.4 qRT-PCR analysis of the expression pattern of genes involved in sugar metabolism

Fifteen enzyme genes related to sucrose and hexose metabolism were selected for qRT-PCR to validate the gene expression profile obtained from RNA-seq. These genes were differentially expressed in internal and external leaf tissues (under the criteria of \log_2 ratio ≥ 1.0 and $FDR < 0.01$), and can be stably detected by qRT-PCR. The enzymes involved in these genes included SPS, SUS, VIN, CWIN, NIN, UGPase, HK, FK, and AGPase. Pearson’s correlation coefficient ($R^2 = 0.8665$) after Z-score standardization showed a high correlation between the qRT-PCR results of these genes and the RNA-seq data (Fig. 2; Table S8), which indicates that the transcriptome data here is reliable. To further confirm gene expression patterns of the above genes in heading leaves during leafy head formation, qRT-PCR was performed to investigate their expression at 45, 60, 75 and 90 DAS.

Results of the qRT-PCR analysis showed that these selected genes exhibited different expression characteristics in different leaf tissues during leaf head formation (Fig. 3). The expression level of *SPS1* (Bra020096) in EB was the highest at 75 DAS, and then decreased at 90 DAS. In EM, the *SPS1* expressed increasingly with time, while in IB and IM, it always showed very low expression, especially in IM. The *SPS4* (Bra033195) gene was highly expressed only in the early stage of EB tissue (45 and 60 DAS) and then stabilized at a low level thereafter. The expression profiles of *SPS3* (Bra015364) showed tissue specificity, and it had a higher expression in blade (EB and IB) than that in midrib (EM and IM) during the whole process of leaf formation.

SUS, the bidirectional enzyme of sucrose metabolism, had active expressions in different stages of leafy head development. *SUS1a* (Bra006578) and *SUS1b* (Bra002332) showed significantly high expression in IM compared to other tissues. Especially at 75 DAS, the expression level of *SUS1b* (Bra006578) was the highest in the four developmental stages among all tissues. The expression pattern of *SUS1a* (Bra002332) was highly similar to that of *SUS1b* (Bra006578), while the expression levels in EM and IB were somewhat different at 90 DAS. *SUS5* and *SUS6* had

Fig. 2 Validation of RNA-Seq data by qRT-PCR in Chinese cabbage cv. ‘Xibai no. 4’. Fifteen DEGs related to sugar metabolism were selected to confirm their expression profiles derived from RNA-Seq. R^2 represents the Pearson’s correlation coefficient, which was calculated from z-normalized values. EB, external blade; EM, external midrib; IB, internal blade; IM, internal midrib



significantly higher expression in EM than in other tissues throughout all stages, and their expression in all tissues decreased gradually.

As for INV, the four selected genes all have their own expression patterns. *CWIN1* had an extremely low expression in IM in each period. *CWIN1* in EM was only up-regulated significantly at 75 DAS. The expression of *NIN-like* in EB and EM reached the highest level in 75 DAS, and then decreased at 90 DAS. In IM, *NIN-like* had a low expression level similar to *CWIN1*. From 45 to 60 DAS, *VIN4b* (Bra019749) in EB and EM showed significantly higher expression compared to the internal tissues (IB and IM). For *VIN4a* (Bra026984), its expression level in EB elevated rapidly during leafy head formation; while in EM, it showed an opposite expression trend.

UGP1 (Bra006395), a key gene regulating UDPG synthesis, showed significantly higher expression in EB than in other tissues at 45 and 60 DAS. The expression of *FK1* (Bra022839), which can phosphorylate fructose, was significantly higher in IM than in the other three tissues. Unlike *FK1*, the expression of *HK1* (Bra015599) was dominant in IM. At 90 DAS, the expression level of *HK1* in IM was four times higher than that in other tissues. The expression of the *AGPS1*, which controls starch synthesis, was higher in EB and IB than in midrib tissues (EM and IM) in all stages. At 90 DAS, *AGPS1* had the lowest expression level in all tissues.

3.5 Correlation analysis between sugar accumulation and genes involved in sugar metabolism

Based on the above gene expression results, further correlation analysis was performed to study the relationship between the expression of these enzyme genes and the variation in soluble sugar and starch content at four stages during leafy head formation (Fig. 4 and Table S9). Pearson correlation analysis ($N = 16$) revealed that fructose, glucose and sucrose all exhibited a significantly positive correlation with the accumulation of total soluble sugar ($p < 0.01$). The positive correlation between starch and soluble sugars were not significant. Among the 15 enzyme genes, only *SUS1a* (Bra002332), *SUS1b* (Bra006578) and *FK1* (Bra022839) had significant positive correlation with soluble sugar content. The expression level of *SUS1a* (Bra002332) was significantly correlated with changes in soluble total sugar ($R^2 = 0.730$, $p < 0.01$), fructose ($R^2 = 0.798$, $p < 0.01$) and glucose content ($R^2 = 0.780$, $p < 0.01$), while no significant correlation with sucrose content was found. *SUS1b* (Bra006578) was found to have only significant positive correlation with fructose and glucose levels ($p < 0.05$; $p < 0.01$), respectively. *FK1* (Bra022839) also showed a significant positive correlation with the content of soluble total sugar, fructose and glucose ($R^2 = 0.515$ – 0.558 , $p < 0.05$). *SPS* gene, which is responsible for sucrose synthesis, showed a

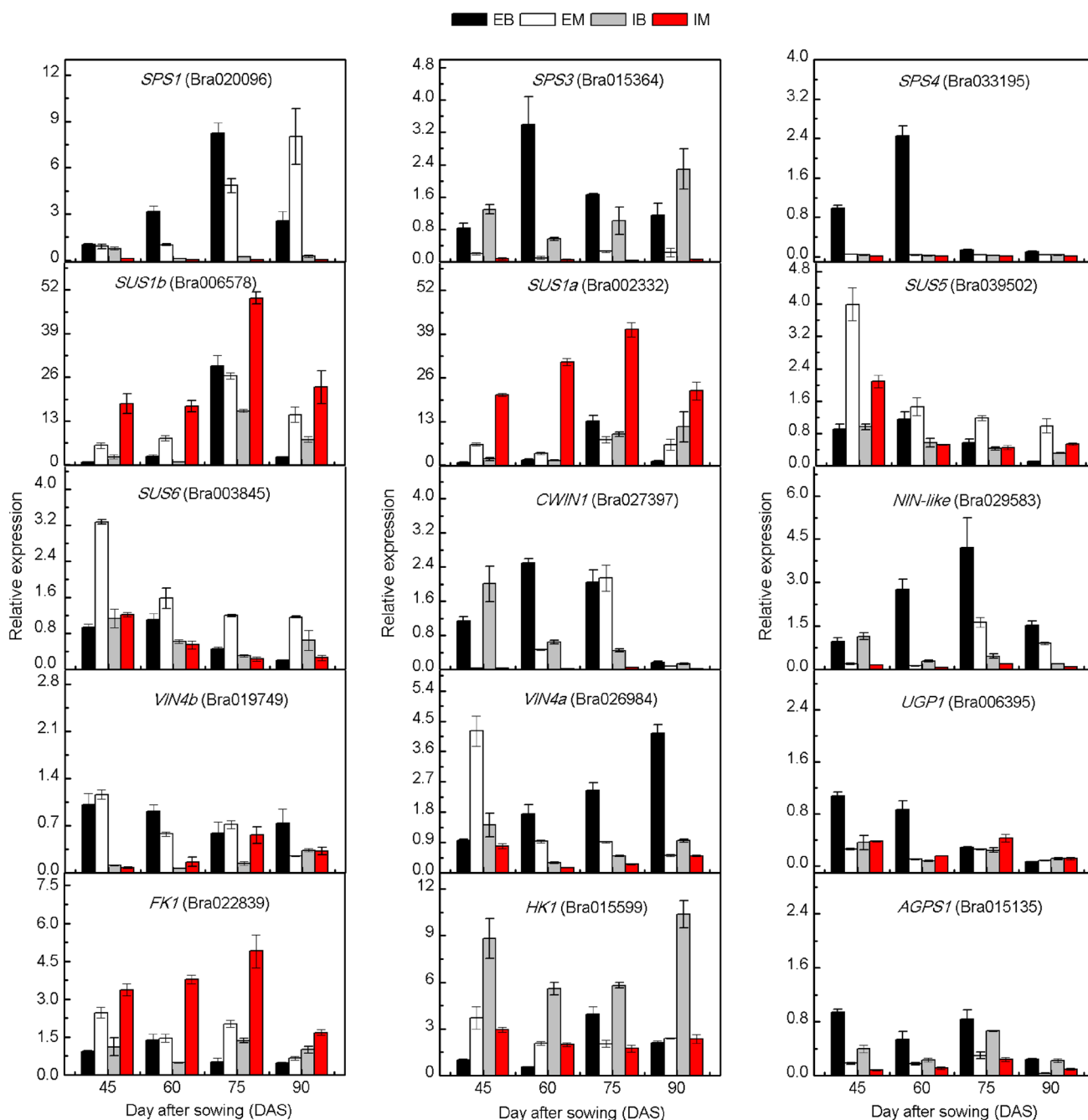


Fig. 3 Relative expression levels of qRT-PCR of 15 enzyme genes involved in sugar metabolism in different tissues of the heading leaves during leafy head development at 45, 60, 75 and 90 DAS of Chinese cabbage cv. 'Xibai no. 4'. The Y-axis represents the relative gene

expression levels obtained using the $2^{-\Delta\Delta CT}$ method. *EF-1- α* was used as the reference gene. Error bars represent the standard error (\pm SE) for three replicates (LSD test at $p < 0.05$). EB, external blade; EM, external midrib; IB, internal blade; IM, internal midrib

negative correlation with sucrose content in this study. Furthermore, *SPS1* was negatively correlated with sucrose and total soluble sugar. The above results suggested that *SUS1a* and *SUS1b* are closely related to soluble sugar accumulation during leaf head formation. Meanwhile, the expression of *FK1* gene which further metabolizes fructose is also more active in tissues with high sugar accumulation.

4 Discussion

4.1 The characterization of sugar metabolism during leafy head development

In 2016 and 2017, the content of all sugars detected in the external tissues of the leafy head (EB and EM) were low at

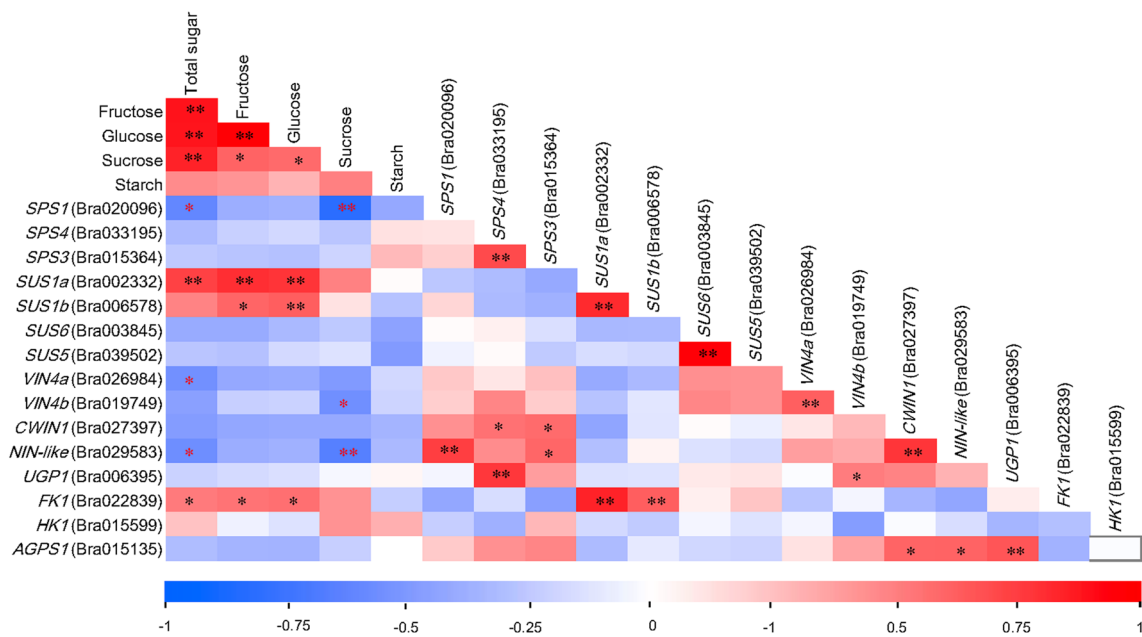


Fig. 4 Visualization of Pearson's correlation analysis between gene expression and soluble sugar content during the leafy head development in Chinese cabbage cv. 'Xibai no. 4'. The color in the square corresponds to the Pearson's correlation value shown in the legend, which is between 1 and -1. The correlation coefficient is calculated

with $n=16$. Blue color indicates a negative correlation while red color indicates a positive correlation. "*" and "**" indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively. Black and red asterisks indicate significant positive or negative correlation, respectively

all stages, which is in sharp contrast with the rapid accumulation of soluble sugar in the internal tissues, especially in IM. For Chinese cabbage, the leafy head is the storage organ (Wang et al. 2012). For the developing inter young leaves, the external green heading tissues (EB) are also a source of carbon (Chang et al. 2017). Previous study on *Arabidopsis* and *Hevea brasiliensis* showed that there was more carbohydrate input in the vigorously growing tissues (Durand et al. 2018; Zhu et al. 2018). The distribution of soluble sugar in cv. 'Xibai no. 4' was similar to the above studies.

In the present study, the content of hexoses (fructose and glucose) was higher than that of sucrose in internal tissues. This result is in accordance with that of Lee et al. (2020). The average fructose content in mature rosette leaves was less than 1%, and higher fructose level was found in leaf blade than in midrib (Lee et al. 2020). With the leafy head formation, fructose accumulation in internal midrib was significantly higher than that in internal blade and external tissues. This difference may be related to the developmental stage and function of leaves in different parts of Chinese cabbage. The accumulation of glucose and fructose in the leafy head also showed divergence, it was supposed to be related to the difference of their own metabolic patterns (Desnoues et al. 2014), and may also be affected by other external factors. It is certain that fructose accumulation at maturity plays an important role in sugar sweetness (Shen et al. 2017).

Starch is a primary energy storage substance in plants, which is closely related to the metabolism of sucrose and hexose (Teixeira et al. 2005; Zhang et al. 2015). During the whole developmental process of leafy head, the starch content of the four tissues ranged from 3.2–5.65 mg g⁻¹ FW (Table S2). Relative carbohydrate content in rosette leaf was measured in Lee's report (Lee et al. 2020), and the Chinese cabbage was considered as a starch accumulator, given that the absolute starch content (%) was higher than that of sucrose. However, the hexose content in the internal leaf tissues always dominated at the later stage of leafy head formation. The present results may further help understanding of the carbohydrate accumulation in Chinese cabbage.

4.2 The distinction of gene expression profiles in different tissues of leafy head

The differences in the number and enrichment (GO and KEGG) of DEG genes between the external and internal tissues suggested that spatial position and developmental stage had greater influence than tissue morphology for the gene expression differences in leafy head (Table S4). Also in blueberries, the two sink tissues, e.g. fruits and flower buds shared more unigenes than fruits and leaves, as well as flower and leaves (Rowland et al. 2012). In this study, GO terms related to cell wall formation and meristem growth were significantly enriched in EB vs IM and EB vs

IB (Table S6). Compared with the mature rosette leaves, the developing inner leaves are the main sites of primary cell wall synthesis (Verbančič et al. 2018). Cellulose, as a major constituent of cell walls, is synthesized with UDPG as substrate (McFarlane et al. 2014). In sink tissues, SUS are responsible for the UDPG producing activities (Leszek et al. 2010). The active expression of *SUS1a* and *SUS1b* in IM with high fructose accumulation also reflected the decomposition and utilization of imported sucrose in young tissue via SUS enzyme.

4.3 Differences in the roles of enzymes involved in soluble sugar metabolism in the leafy head

SPS genes was found to have a high expression level in ripening fruits of pineapple, citrus, apple, etc. with the sucrose accumulation (Komatsu et al. 1996; Li et al. 2012; Zhang et al. 2012). While the expression of three *SPS* genes here were inconsistent with the content of sucrose (Fig. 4), and their expression in IM were always at a low level. In transgenic tobacco overexpressing *AtSPS*, the sucrose content significantly increased in the stem rather than in the leaf tissue, and it was believed that sucrose was transferred rapidly and continuously from source leaves to sink tissue (Park et al. 2008). In addition, we also found that many sucrose transport-related genes, such as *SUC1*, *SUC2*, *SWEET11* and *SWEET12*, which are involved in phloem loading and long-distance transport (Kühn et al. 1996; Durand et al. 2018), were also higher expressed in EB than in internal tissues (Table S10). Therefore, *SPS* may not be primarily responsible for sugar accumulation in the leafy head.

In IM with rapid accumulation of fructose, the *SUS1* gene showed more active expression than in other tissues. Expression of the *SUS1a* (*Bra002332*) and *SUS1b* (*Bra006578*) were highly correlated with the content of fructose and glucose (Fig. 4). The transcript levels of the two *SUS1* genes were also up-regulated inside the yellow leafy head during the heading stage, and the authors speculated that they may play a major role in regulating sucrose synthesis in Chinese cabbage (Li et al. 2019). While the results in the present study supported that the *SUS1* gene may be more responsible for the sucrose decomposition and utilization during leafy head development. In cotton overexpressing the potato *SUS* gene, the expansion of young leaves was accelerated with a significant increase in fructose content and a slight decrease in sucrose (Xu et al. 2012). The transgenic tobacco overexpressing *AtSUS1* showed an increase in total soluble sugar, sucrose, fructose and starch (Nguyen et al. 2016). Previous studies also found that *SUS1* primarily decomposed sucrose in the immature fruit of pears, citrus and grapes and provided a substrate for cell wall construction or glycolysis (Tanase and Yamaki 2000; Komatsu et al. 2002; Zhu et al. 2017). Like the immature fruits, internal leaf tissues also

had a strong need for sucrose decomposition, which was also reflected in aforesaid gene enrichment results ('Classification and enrichment analysis of DEGs' section). *SUS* is considered to play an important role in controlling the direction of carbon partitioning, regulating cell division and growth as well as the accumulation of storage reserves (Fig. 5) (Moscatello et al. 2011; Zhang et al. 2012; Tong et al. 2018). The active expression of *SUS1a* and *SUS1b* in IM during the leafy head formation partly reflected the close association between *SUS* and growing sink tissues.

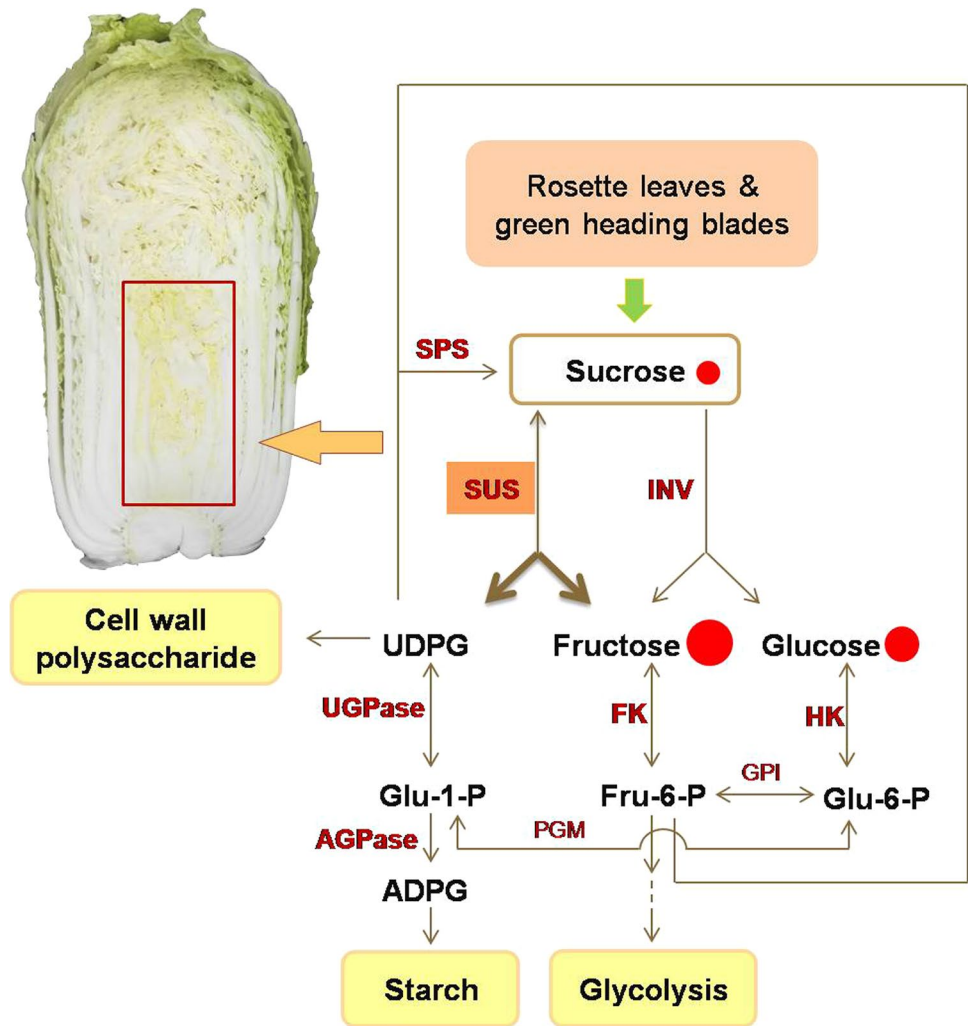
While providing UDPG, fructose is another product of sucrose decomposition by *SUS*. Invertase also hydrolyzes sucrose to fructose and glucose. Hexose monomers must first be phosphorylated by *FK* or *HK* respectively for further metabolism, such as glycolysis (Yao et al. 2018) (Fig. 5). The expression of *FK1* displayed significantly positive correlations with that of *SUS1a* and *SUS1b*, which had high transcription levels in IM. It suggested that *FK1* may play an important role in fructose phosphorylation in IM. The utilization of fructose further promoted the sucrose decomposition catalyzed by *SUS*. The expression of other genes such as *SUS5*, *SUS6* and *AGPS1* in IM had no remarkable up-regulation, and even showed higher level in external tissues. *AtSUS5* and *AtSUS6* were reported to be phloem specific and related to callose deposition (Stein and Granot 2019). So we speculated that these differentially expressed enzyme genes may play a special role in the energy production and the balance of sucrose and its graduation products.

Another way of unloading sucrose in sinking cells is hydrolyzing sucrose by *INV*. Interestingly, the four *INV* genes here had relative low expression in the inner leading leaves, especially in IM (Fig. 3). Three *INV* genes were also significantly down-regulated in inner leaves of the yellow-head Chinese cabbage, which was speculated that it may contribute to the sucrose accumulation in leaves (Li et al. 2019). We presumed that, the *SUS* and its production of UDPG may be important for directing carbon to the development of vascular tissue and cellulose synthesis, and it is necessary for the development of young leaves inside the leafy head. During the development of kiwifruit, *SUS* also acted a pivotal part in sucrose degradation than *INV* (Moscatello et al. 2011). Therefore, the role of invertase in the development of leafy head needs further study.

5 Conclusion

To conclude, this study presented the dynamic changes of sugar content in different tissues of Chinese cabbage during the leafy head formation. The dominant role of fructose in the soluble sugar composition of the leafy head was clarified, and the internal petiole was the primary tissue for fructose accumulation. Two *SUS1* genes were found

Fig. 5 A schematic illustration of sugar metabolism in the leafy head of Chinese cabbage cv. ‘Xibai no. 4’. Sucrose in the internal tissues of the leafy head mainly comes from the rosette leaves and the external heading leaves that are capable of photosynthesis. The decomposition of sucrose in the internal tissues of the leafy head may be actively catalyzed by SUS (indicated by bold arrows). Decomposition products are used for cell wall construction, energy storage and respiratory metabolism. The size of the red dots represents the distinction in the content of fructose, glucose and sucrose in the internal tissues of the leafy head. SUS, sucrose synthase; SPS, sucrose phosphate synthase; INV, invertase; FK, fructokinase; HK, hexokinase; UGPase, UDP-glucose pyrophosphorylase; AGPase, ADP-glucose pyrophosphorylase; PGM, phosphoglucosmutase; GPI, glucose phosphate isomerase



to be highly correlated with the accumulation of total soluble sugar and hexose. These findings here would help to understand the regulation of soluble sugar metabolism in the heading leaves of Chinese cabbage, and provide a framework for future understanding of the roles of *SUS1* genes and other *SUS* gene members in the development and quality formation of the leafy head.

Acknowledgements The authors thank Yonglin Deng at the Shandong Denghai Seed Industry, Ltd., West Seed Branch for assistance with sample collection. This work was supported by the National Natural Science Foundation of China (Grant No. 31401864), the National Science Foundation of Shandong Province (Grant No. ZR2016CM15), the Key Research and Development Program of Shandong Province (Grant No. 2018GNC110022), the Project of Shandong Province Higher Educational Science and Technology Program (Grant No. J18KA161) and the Higher-level Talents Research Fund of Qingdao Agricultural University (Grant No. 6631113319).

Author contributions LQQ performed the majority of the experiment and data analysis, and wrote the manuscript. LJQ was responsible for

part of the experiment and revision of the manuscript. LWX designed and revised the manuscript.

Data availability The datasets generated and/or analyzed during the study can be obtained from the corresponding author on request.

Compliance with ethical standards

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

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