Sucrose synthase gene family in *Brassica juncea*: genomic organization, evolutionary comparisons, and expression regulation

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**ABSTRACT**

Sucrose synthase (SUS) plays an important role in sucrose metabolism and plant development. The SUS gene family has been identified in many plants, however, there is no definitive study of SUS gene in *Brassica juncea*. In this study, 14 SUS family genes were identified and comprehensively analyzed using bioinformatics tools. The analyzed parameters included their family member characteristics, chromosomal locations, gene structures and phylogenetic as well as transcript expression profiles. Phylogenetic analysis revealed that the 14 members could be allocated into three groups: SUS I, SUS II and SUS III. Comparisons of the exon/intron structure of the mustard SUS gene indicated that its structure is highly conserved. The conserved structure is attributed to purification selection during evolution. Expansion of the SUS gene family is associated with fragment and tandem duplications of the mustard SUS gene family. Collinearity analysis among species revealed that the SUS gene family could be lost or mutated to varying degrees after the genome was doubled, or when *Brassica rapa* and *Brassica nigra* hybridized to form *Brassica juncea*. The expression patterns of *BjuSUSs* vary among different stages of mustard stem swelling. Transcriptomics revealed that the *BjuSUS01-04* expression levels were the most elevated. It has been hypothesized that they play an important role in sucrose metabolism during stem development. The expression levels of some *BjuSUSs* were significantly up-regulated when they were treated with plant hormones. However, when subjected to abiotic stress factors, their expression levels were suppressed. This study establishes SUS gene functions during mustard stem development and stress.

**INTRODUCTION**

Sucrose synthase (Sucrose Synthase, EC2.4.1.13, SUS) is a tetramer composed of subunits with a molecular weight of approximately 83 kD to 100 kD. It is involved in sucrose metabolism (*Tanase & Yamaki, 2000; Chalivendra et al., 2007*). It catalyzes sucrose
synthesis and decomposition. Although sucrose synthase is a reversible enzyme, it converts sucrose and UDP into UDP-glucose and fructose (Nguyen-Quoc & Foyer, 2001). During different stages of plant growth and development, sucrose, fructose and glucose levels are constantly altered. High glucose levels during the early stages are a source of carbon that is required during the rapid growth of plant tissues. This allows plant cells to maintain a high osmotic pressure to ensure adequate water absorption. The gradual increase in sucrose levels after water absorption enhances sugar accumulation in the plant tissues (Dai et al., 2011). Sucrose synthase is involved in sucrose synthesis and decomposition.

Therefore, the biological activities and gene expression profiles of SUS vary among different stages of plant growth and development. Studies have reported that SUS is also important in the synthesis of starch (Heim et al., 1993) and fiber cell wall (Ruan, Llewellyn & Furbank, 2003), fruit development (Wang, Smith & Brenner, 1994) as well as in plant stress (Jha & Dubey, 2004).

Since the identification of the first SUS gene in wheat germs, it has been cloned in various crops such as potato, carrot, corn, citrus and Arabidopsis. The SUS gene family has been identified and reported in several plants. Five SUS genes have been identified in six in Arabidopsis, six in rice, three in maize, 15 in Populus and 14 in tobacco (An et al., 2014; Baud, Vaultier & Rochat, 2004; Duncan, Hardin & Huber, 2006; Hirose, Scofield & Terao, 2008; Wang et al., 2015). The SUS gene family is involved in the regulation of multiple pathways throughout the growth cycle of the plant. It has been revealed that when the SUS4 gene is highly expressed during starch synthesis in potato tubers and the activity of SUS enzyme is significantly elevated, there is an increase in ADPG and UDPG levels and a nearly double starch yield (Baroja-Fernandez et al., 2009). Several SUS genes (GhSUS1A-GhSUS8) are involved in cellulose accumulation and secondary wall thickening of the tetraploid cotton xylem. They are important during fiber elongation at different stages of cotton growth (Ruan, Llewellyn & Furbank, 2003).

The tuber mustard is a stem variety of the mustard in which enlarged fleshy stems are widely consumed while fresh and used for mustard processing. During the growth and development of the tumorous stem, the base of the petiole is metamorphosed to form 1–5 tubercular bulges that laterally expand with stem swelling. The genetic analysis of the shape of the swollen stem showed that its shape was mainly controlled by two major genes and polygenes, and there were obvious additive, dominant and epistatic genetic effects (Liu et al., 2014). The tumor-like swelling of the tuber mustard is closely correlated with its yield and quality. Sugar production and transportation in the mustard accumulate in the stem, enhancing its expansion. Sucrose synthase gene functions during stem swelling have not been established. It is, therefore, necessary to determine the structure, expression patterns and evolutionary relationship of SUS gene family in tuber mustard.

Based on the published sequencing results of the mustard genome, we identified 14 SUS genes in the allotetraploid mustard. Bioinformatic tools were then used to identify and characterize the mustard SUS gene in the whole genome of the mustard. In addition, the expression patterns of each member of the mustard SUS gene family in the process of stem swelling and their potential roles in response to various hormones and abiotic stresses.

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were determined. These results provide a basis for further research on the potential functions of each mustard SUS gene, especially during the development of mustard stems.

**MATERIALS AND METHODS**

**Characterization and phylogenetic analysis of SUS genes in the *B. juncea* genome**

The *B. juncea* genome sequence data (V 1.5) used in our study are available within the *Brassica* database (http://brassicadb.org/brad/). Using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the expectation value of $E > 10^{-10}$, the amino acid sequences of SUS genes in *Arabidopsis* were used as query sequences to identify homologous genes. All the non-redundant gene sequences encoding complete amino acid sequences were considered as BjuSUS genes. Sequence information of 14 BjuSUSs were submitted to NCBI website, with accession numbers from MW370524–MW370537.

Amino acid numbers, their molecular weights, predicted theoretical isoelectric points (pI), exon numbers, and subcellular localization were predicted using the ProtParam tool (http://www.expasy.org/tools/protparam.html). Amino acid sequences were aligned using ClustalX (2.1), while MEGA V6.06 was used to construct a phylogenetic tree based on the neighbor joining (N–J) method. A bootstrap value of 1,000 was used to test the reliability of the obtained trees (Kumar et al., 2008). The specific conserved domains in the SUS family were searched on InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/), and the conserved domains were predicted by Pfam (http://pfam.xfam.org/search/sequence) (Sonnhammer, Eddy & Durbin, 1997). The GO (Gene Ontology, http://geneontology.org/) annotations of the BjuSUS proteins were analyzed using the Blast2GO (https://www.blast2go.com/). TBtools (https://github.com/CJ-Chen/TBtools) were used to generate the genetic structures of the SUS family while MEME (http://meme-suite.org/tools/meme) was used to identify conserved motifs (Bailey et al., 2009). The program Clustal W2 was used to calculate the similarity/identity of amino acid sequences. Subcellular localization analysis was performed using WOLF PSORT (https://wolfsort.hgc.jp/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/). Gene pair collinearity was determined using the MCScanX software (http://chibba.pgml.uga.edu/mcscan2/) (Wang et al., 2012) and then plotted using the Circos software. All cis-regulatory elements in the gene promoter are available on the online PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**Plant materials, phytohormone and abiotic treatments**

The experimental samples used to analyze the expression patterns at the developmental stage (2, 4, 6 and 8 cm) were obtained from the four stages of swollen stems of the tuber mustard variety 'Fuza No.2'. All samples were grown in pots in a controlled growth environment. For hormonal and abiotic stress treatment, 4 week old plants with 5–6 true leaves were used in the experiments. The seedlings were separately sprayed with auxin (IAA) (10 µM), abscisic acid (ABA) (75 µM), salicylic acid (SA) (0.5 mM) and gibberellin (GA) (50 µM) and stored for 24 h. Abiotic stress was simulated by low temperature (4 °C), drought treatment (10% PEG8000), NaCl treatment (400 mM) and high temperature
treatment (38 °C). All treatments were set to three biological replicates. After 12 h, the samples were obtained and immediately frozen in liquid nitrogen. They were then stored at −80 °C.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from stems at different growth stages by using the RNA prep Pure Plant kit (Tiangen, Beijing, China). The extracted RNA was spectrophotometrically quantified and electrophoresed on a 1.0% agarose gel to verify its integrity. First-strand cDNA was synthesized from 1.0 µg of total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan). All cDNA samples were diluted 1:10 with RNase-free water.

**Expression analysis and Quantitative real-time PCR verification**

The data obtained from the transcriptome sequencing of the different developmental stages of the stem tumor mustard variety ‘Fuza No.2’ (2, 4, 6 and 8 cm in diameter, respectively) was submitted to NCBI under the accession number SRP151320. The FPKM value was used to determine the expression abundance of the SUS gene during different developmental stages of B. juncea. The gene expression heat map was plotted using TBtools.

Quantitative real-time PCR (qRT-PCR) was performed using the Bio-Rad CFX96™ real-time PCR System at the standard mode with the 2×T5 Fast qPCR Mix (SYBRGreenI) (TsingKe, Beijing, China). All experiments were performed in triplicate. To normalize gene expression, UBC was used as the internal control (Li et al., 2020). The relative expression levels of BjuSUSs were calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Primers were designed using Primer Premier 5.0 software. The primer sequences used in this study are listed in Table S1.

**Functional divergence analysis**

Diverge 3.0 was used to perform type I and type II functional disproportionation analysis by the phylogenetic tree of the SUS proteins. Both type I and type II functional divergence occurred after gene replication. Type I functional divergence leads to specific amino acid selective changes, that is, changes in evolutionary rates, and its coefficient $\theta_I$ fluctuates between 0 and 1. This fluctuation reflects the functional divergence between gene categories from weak to strong. Type II functional divergence indicates that there are site-specific shifts of amino acid properties between subgroups (Gu, 2006). A high Qk (posterior probability) value indicates that the evolution rate or site-level physicochemical amino acid properties between the two clades are highly likely to be different. If Qk > 0.9, it is inferred that the amino acid position may exhibit functional divergence after SUS gene replication (Xun & Kent, 2002).

**Analysis of non-synonymous mutation rate to synonymous mutation rate ratios**

The homologous gene pair of the BjuSUS gene family was identified using the OrthoMCL software (https://orthomcl.org/orthomcl/). The non-synonymous mutation rate (Ka),
synonymous mutation rate (Ks) and Ka/Ks ratio of each pair of homologous genes were calculated using the Ka/Ks Calculator software. If Ka/Ks > 1, the gene is said to have been subjected to positive selection pressure; Ka/Ks = 1 indicates neutral selection; Ka/Ks < 1 is considered to have purifying selection.

RESULTS

Identification and analysis of BjuSUSs

Fourteen genes encoding BjuSUS proteins were identified and renamed BjuSUS01 through BjuSUS14. Detailed information on genomic positions, coding region lengths, exon numbers, subcellular localizations and the corresponding proteins are summarized in Table S2. The open reading frame (ORF) size of BjuSUSs ranged from 3,447 (BjuSUS04) to 9,068 bp (BjuSUS10). They coded for proteins with a length that ranged from 755 to 905. The molecular mass of the fourteen proteins ranged from 85.42 to 102.93 kD, whereas their theoretical PI was between 5.67 (BjuSUS01) and 6.36 (BjuSUS13). These findings show that the BjuSUS protein is a weakly acidic protein. Subcellular localization prediction revealed that, except for BjuSUS07 and BjuSUS08 that are located in the mitochondria, the remaining BjuSUSs are located in the cytoplasm.

Multiple sequence alignment revealed a high level of similarity between the amino acid (52.08–99.02%) sequences (Table S3). The highest percentage of amino acid sequence identity was found between BjuSUS09 and BjuSUS10 (99.02%). A high pairwise identity of amino acid sequences was found in BjuSUS01/02, BjuSUS01/04, BjuSUS07/08 and BjuSUS13/14, ranging from 97.01% to 98.64%. The most of the sequences showed less than 80% amino acid sequence identity. The lowest sequence identity was found between BjuSUS04 and BjuSUS09 with 52.08% amino acid sequences.

These 14 genes were widely dispersed on 10 of 18 B. juncea chromosomes and two contigs (Fig. 1). The number of chromosomal gene distribution was similar in sub-genomes A and B. The BjuSUS13 and BjuSUS14 genes were shown to be accumulated on the same region of the chromosome. This proves the clustering phenomenon. Three BjuSUSs (BjuSUS12, 13 and 14) were located on the chrB03, while the other chromosomes only had one BjuSUS gene.

Phylogenetic relationships of BjuSUS genes

A phylogenetic tree of the mustard and eight other species of SUS proteins was constructed to determine the evolutionary relationships of the SUS gene family in B. juncea. As shown in Fig. 2A, the SUS genes were classified into three groups based on phylogenetic analysis: SUS I, SUS II and SUS III. The SUS I group was branched into the monocot and dicot subgroup while SUS II and SUS III groups were branched into the monocot subgroup only. Phylogenetic analysis revealed that the SUS family members of dicot and monocot plants were distributed in SUS I to SUS III. This implies that amplification of the SUS gene family occurs before differentiation of the dicot and monocot plants. Furthermore, all the SUSs in maize and Triticum aestivum occur in SUS I. Each SUS I and SUS II group contained four BjuSUSs while the SUS III family contained six BjuSUSs (Fig. 2B). The phylogenetic relationship diversity exhibited by the BjuSUSs show the discrete biological role for their
paralogs, although multiple sequence alignments have proved that they have high sequence similarities.

**Structure and motif location analysis of BjuSUSs**

The exon/intron structure analysis showed that the 14 BjuSUSs contained multiple exons and introns that ranged from 11 to 14 and 10 to 13, respectively (Fig. 3A).
The organization of the exons/introns was highly conserved, comparable to the high-level similarity observed by the alignment of the 14 amino acid sequences. The coding sequences of BjuSUSs were interspersed by the introns. The BjuSUS10 gene had a long intron of approximately 5 kb sequence in length. It was the longest untranslated region.

Fifteen conserved motifs were finally identified from the BjuSUS proteins. The 14 members shared at least 12 common conserved motif compositions and exhibited a coincidental order of motif arrangement (Fig. 3B; Table S4). Motifs 6, 4 and 12 were missing in BjuSUS04, 09 and 11, respectively. In addition, the BjuSUS10 lacked motifs 13 and 9, while both BjuSUS13 and 14 genes had motif 15 at the end of the protein sequence. Motifs 1 and 5 were connected in each protein sequence, which was similar to motifs 2 and 4. However, the other motifs were separated.

**Evolutionary analysis of the BjuSUS genes**

Type-I and type-II functional difference analyses were performed among the three SUS groups. As shown in Table 1, the type-I functional divergence coefficient $\theta_I$ was significantly greater than zero. It signified apparent type-I functional differences. This implied that amino acid site-specific selection may constraint the action in the different SUS groups and lead to specific evolution after diversification of different ethnic groups. The results from type-II functional divergence showed that the coefficient $\theta_{II}$ of any two groups was greater than zero. This finding implied that the proteins encoded by the three SUS subtypes exhibited significant type-II functional differentiation. The proteins encoded by the sub-type SUS genes have different physicochemical properties at certain amino acid positions, causing significant disproportionation. In the type-I analysis, 0, 3 and 0 critical amino acid sites (CAASs) that are associated with functional divergence were found in SUS I/SUS II, SUS I/SUS III and SUS II/SUS III groups, respectively. Moreover, in type-II analysis, the corresponding CAASs numbers were 59, 80 and 64, respectively. Variations in critical amino acid numbers between the type-II and type-I groups suggested
the promotion of functional divergence in the evolution of the SUS family is mainly attributed to the site-specific shift of amino acid properties, followed by changes in the evolution rate.

The ratio of non-synonymous mutation frequency to synonymous mutation frequency for 15 homologous gene pairs in the SUS gene family was analyzed using the Ka/Ks calculation software. In the 15 paralogous gene pairs, all the Ka/Ks ratios were lower than 1, indicating that the 15 paralogous gene pairs were under a strong purifying selection during evolution (Table 2). Since deleterious mutations are usually eliminated by the purification selection process, SUS genes may share conserved functions in B. juncea species.

**Synteny analysis of the SUS gene in B. juncea and Arabidopsis**

To determine the evolutionary relationship and degree of homology between mustard and Arabidopsis, their comparative synteny at the genome level was analyzed to elucidate the origin of the BjuSUSs (Fig. 4A). There were 2,208 synteny blocks between the B. juncea and Arabidopsis genomes, of which 66.71% were collinear. In the largest linear region,
648 gene pairs were shared between the *B. juncea* chrA02 and *Arabidopsis* chr5. This implied that mustard and *Arabidopsis* have a high homology. Collinearity analysis of *AtSUSs* and *BjuSUSs* was used to identify homologous candidate genes for important traits such as morphological development, yield, quality and metabolism of the mustard (Fig. 5). Collinearity analysis revealed 29 SUS gene pairs in *B. juncea* and *Arabidopsis*. Among them, fifteen *BjuSUSs* pairs showed fragmental duplication. These findings revealed that most SUS genes are orthologous in both species. The results show that some *BjuSUSs* are co-linear with *AtSUSs*. *BjuSUS01*, 02, 03 and 04 were found to be homologous to *AtSUS1*.

The evolutionary relationships of *BjuSUS* genes were further determined based on the genetic relationships between the *Brassica* crops (Fig. 4B). In the collinearity analysis of *B. juncea*, *Brassica rapa* and *Brassica nigra*, it was found that the synteny blocks between *B. juncea* and *B. rapa* was more than 70%. The same finding was established between *B. juncea* and *B. nigra*. The higher homology revealed that *B. juncea* was the offspring of *B. rapa* and *B. nigra*. Twelve *BjuSUSs* were collinear with the genes in *B. rapa* while 10 *BjuSUSs* were collinear with the genes in *B. nigra*. Most of the *BjuSUS* genes were homologous to the two genes in *B. rapa* and *B. nigra*, respectively.

**Analysis of cis-regulatory elements and functional annotation of *BjuSUS* genes**

All cis-regulatory elements in the promoter region of *BjuSUSs* were analyzed using the Plant-CARE database. Elements were classified into three groups based on their functional associations; plant growth and development (13 elements), phytohormone response (8 elements) as well as abiotic and biotic stress (7 elements) (Fig. 6). The results showed that the light-responsive elements (Box-4, G-box, GATA-motif and TCT-motif) appeared
29, 29, 18 and 16 times in the 14 BjuSUSs, respectively. These four cis-regulatory elements were widely distributed in the BjuSUSs promoter region and may be involved in the regulation of mustard growth and development. In particular, G-box appeared eight times in BjuSUS08 while Box 4 appeared six times in BjuSUS05, indicating that they may be significant in light responsiveness and photosynthetic product accumulation. Moreover, 26 abscisic acid responsive elements (ABRE) and 21 anaerobic induction elements (ARE) were detected in BjuSUSs promoters.

GO annotation was performed to predict the functions of 14 BjuSUS genes. A total of 116 annotation results were obtained and further divided into 23 GO terms, which were in turn assigned into three ontology categories: biological process (BP), cellular component (CC), and molecular function (MF) (Table S5). Biological process made up the majority of the GO annotations, followed by cellular component and molecular function.
Among the annotations, all the BjuSUSs were involved in “sucrose metabolic process” and “sucrose synthase activity”.

**Expression patterns of BjuSUS genes**

**Expression levels of BjuSUS genes at different stem developmental stages**

Analysis of the expression abundance of BjuSUSs in the transcriptome data at different developmental stages revealed that the expression of each BjuSUS gene has similarities and differences (Fig. 7A). Among them, BjuSUS01-04 maintained a high transcription level at each stage, especially in the first two stages. Its expression levels were highest in the second stage, with a significant decrease in the last two developmental stages. In addition, the expression of BjuSUS07 and 08 was low in the first stage, but was significantly elevated in the latter stage. BjuSUS05, 06 and 10 showed relatively low expression profiles throughout the stem development stages. As shown in Fig. 7B, most BjuSUSs exhibited a distinct but partially overlapping expression trend, that is, high expression in the early stages of stem swelling, and low expression levels in the latter stages of development. However, there were no detectable expression levels in the latter two stages of BjuSUS05 and 06. BjuSUS07 and 08 were highly expressed in the latter stages of development. These findings were consistent with the results of the transcriptome analysis.

**Expression patterns of BjuSUS genes in response to phytohormones**

The expression patterns of BjuSUSs in response to abscisic acid (ABA), auxin (IAA), gibberellin (GA) and salicylic acid (SA) were determined (Fig. 8). The expression levels of BjuSUS04, 11, 13 and 14 were up-regulated after treatment with four phytohormones. The expression levels of BjuSUS13 were significantly elevated. In addition, most genes,
especially BjuSUS03 and 07, exhibited elevated expression levels after ABA treatment. BjuSUS02 and 07 exhibited up-regulated expression levels after IAA treatment, while the expression levels of BjuSUS03 and 07 were also up-regulated after SA treatment. Furthermore, the BjuSUS06, 08, 09 and 12 were all down-regulated with hormones.

Expression patterns of BjuSUS genes in response to abiotic stresses
To determine whether BjuSUSs are involved in abiotic stress, qRT-PCR was used to measure their expression levels under salt, drought, and high and low temperature stress. As shown in Fig. 9, the expression level of most BjuSUSs decreased under stress treatment compared to the control group, however, the expression patterns were different when subjected to different stresses. Most of the genes were down-regulated, with some expressions not being detected. The expression levels of BjuSUS03 were significantly
up-regulated under salt, drought heat and cold stress, while the expression levels of BjuSUS08 were significantly up-regulated under cold stress. In response to abiotic stress, BjuSUS01 and 02 exhibited similar expression patterns.

**DISCUSSION**

Comparative genomics approaches have been used to analyze SUS gene families in various plant species such as *Arabidopsis*, rice, maize, cotton, grape, peach and poplar (Xu et al., 2019). In this study, 14 SUS genes from *B. juncea* were identified. Based on their molecular structures, evolutionary models and expression profiles, the BjuSUSs play an important role in the development of *B. juncea* stems.

**Evolutionary conservation among BjuSUS genes**

Phylogenetic, structural and evolutionary pattern analyses are fundamental in predicting the possible genetic and evolutionary relationships as well as potential functions of gene families. Phylogenetic tree analysis showed that the mustard SUS genes were associated with dicotyledonous plants such as *Arabidopsis*. The BjuSUSs were found to be closely clustered in the subgroups. This emphasizes the diversity of SUS genes in monocot and dicot plants. Studies have documented that the protein structure, the length and positional characteristics of introns and exons of the SUS family genes are highly conserved among different species. This protein structure provides important information regarding evolutionary relationships between genes (Lecharny et al., 2003). We found that BjuSUSs have 10–13 introns, and most of the exons between SUS I, SUS II and SUS III groups are highly similar, which slightly differs from the 11–13 introns in the peach (Zhang et al., 2015). In addition, the number of introns in PtrSUS in the *Populus* and MdSUS in the apple is between 10 and 14 (Tong et al., 2018; Zhang et al., 2011). These results indicate that the number of introns in the SUS gene family among different species is highly conserved.
To determine the evolutionary relationships of the BjuSUS gene family and explore their possible functions, gene duplication events and homology analysis were performed. Tandem duplications (BjuSUS13 and BjuSUS14) were found in chromosomal mapping. Collinearity analysis of mustard and Arabidopsis revealed that there were more fragment duplications between BjuSUSs. Duplicated gene pairs were all from the same subgroup, with highly similar exon/intron structures and protein characteristics. This implies that the amplification methods of mustard SUS gene family include tandem and fragment duplication, which may increase copy numbers in the gene family, resulting in functional redundancy (Cannon et al., 2004). Furthermore, the Ka/Ks results showed that during evolution, BjuSUSs eliminated harmful mutations through purification and selection in order to maintain a conservative structure and functions. Homological analysis provides a basis for the evolutionary and functional characterization of the SUS gene family in mustard and Arabidopsis. Homology analysis revealed that 14 BjuSUSs exhibited a homology with 5 AtSUSs in Arabidopsis. The BjuSUS genes and their corresponding AtSUS genes have a close phylogenetic relationship, which strongly implies a certain degree of functional similarity. Brassica and Arabidopsis belong to the cruciferous family, but diverged about 20 million years ago (Vision, Brown & Tanksley, 2000). After being separated from the Arabidopsis, the Brassica species such as B. rapa and mustard have tripled at the genome level. Moreover, in the “U-triangle” theory, there are three basic subgenomes (A subgenome, B subgenome and C subgenome), and B. juncea (AABB, 2n = 36) is a heterotetraploid formed by interspecific hybridization of B. rapa (AA, 2n = 20) and B. nigra (BB, 2n = 16) (Nagaharu, 1935). Chromosomal rearrangements, translocations, duplications, and gene deletions during doubling greatly promoted the evolution of Brassica. Some SUS genes in mustard have paralogs, and they all exhibited a homology with certain genes (BjuSUS05/06 and BjuSUS01/02/03) in B. rapa and B. nigra. These findings confirm an evolutionary relationship. The mustard did not undergo a larger chromosomal doubling process after the triploid event (Song et al., 2020). However, some BjuSUS genes did not find their corresponding genes, indicating that the SUS family members may have been lost or mutated to varying degrees during evolution.

Divergence in BjuSUS gene expression patterns

BjuSUS genes involved in mustard growth and development

Plant growth and development is a complex process. A majority of the cis-regulatory elements associated with plant growth and development are found in the promoter regions. In this study, four genes (BjuSUS01, 02, 03 and 04) were highly expressed during growth and development. The expression levels exhibited an overall decreasing trend, implying that these genes are more important in the early stages of stem development. However, their high expression levels in stems indicate that they are involved in the entire developmental stage of the stem and sucrose metabolism. Similar results have also been reported in other studies. Overexpressing the bamboo BeSUS5 gene increased the stem cellulose content, cell wall thickness and fiber quality in transgenic poplar (Huang et al., 2020). Also, the TcSUS3 gene in cacao pods exhibited a downward expression trend in the developmental stage of the pods (Li et al., 2015). Similar findings were observed...
in the expression of the SBSS2 gene in sugarbeet root tissues (Haagenson, Klotz & McGrath, 2006).

The four BjuSUSs were found to be structurally similar and belonged to the SUS I group. This implied that they exhibit the same functions during stem development. Studies have shown that the transcription level of the AtSUS1 gene in A. thaliana is highest in the stem while the expression levels of OsSUS1, OsSUS2 and OsSUS3 genes in rice are highest during the early caryopsis development stages (Bieniawska et al., 2007; Hirose, Scofield & Terao, 2008). These genes belong to the SUS I group and exhibit a high level of redundancy, where some genes with similar expression patterns have similar functions. The six AtSUS genes also exhibit distinct but partially overlapping expression patterns (Baud, Vaultier & Rochat, 2004). Except for BjuSUS01-04, the expression levels of BjuSUS genes was low or not expressed, while BjuSUS07 and 08 were shown to be significantly up-regulated in the middle and late stages of stem swelling. During cucumber fruit development, the expression of CsSUS4 in the latter stages has been shown to be significantly high when compared to the early stages. This indicates that they may play an important role in mustard development, thereby, affecting the size and weight of the stem (Fan et al., 2019). Understanding the structure, phylogeny, and homology of the SUS gene is essential in the functional analysis of the SUS gene family. Nucleotide and amino acid sequence similarities of the four BjuSUSs were all found to be above 90%, indicating that they have a high sequence homology. This finding lays credence to their structural and functional similarities.

**Phytohormones induce BjuSUSs expression in mustard**

When subjected to phytohormonal pressure, the expression levels of some genes in the mustard SUS family were altered. After being subjected to ABA, the expression levels of most genes were shown to be up-regulated. It has been previously reported that ABA elevates the expression levels of grape SUS gene in grape berry calli (Wang et al., 2017). BjuSUSs are up-regulated when exposed to GA while GhSusA1 was shown to be upregulated in fiber culture after exogenous administration of gibberellin (Bai et al., 2014). Furthermore, the expression levels of BjuSUS03, 07, 11 and 13 after phytohormonal treatment were found to be significantly different from those of the control group. Therefore, we hypothesized that BjuSUSs are involved in responses to a variety of phytohormone signals and regulate the associated physiological processes.

**Abiotic stress-induced BjuSUSs expression in mustard**

During plant growth and development, many abiotic stresses are encountered. The SUS gene family has been shown to be involved in regulating stress responses. For example, it can induce AtSSI gene expression in A. thaliana when the plant is exposed to cold, drought or hypoxic stress factors (Baud, Vaultier & Rochat, 2004). Members of the mustard SUS gene family showed different expression patterns in leaves when exposed to different abiotic stresses. When exposed to salt, drought, heat, and cold stress, the expression levels of BjuSUS03 were significantly up-regulated, indicating that abiotic stress induces its overexpression to enhance the ability of the mustard to resist stress.
The overexpression of OsSUS3, which belongs to the SUS I group, suppresses the damage associated with high temperatures to rice yield and quality (Takehara et al., 2018). Furthermore, the expression of BjuSUS08 was found to be significantly up-regulated in cold stress conditions and down-regulated in the presence of other abiotic stressors. The expression level of tobacco Sus5 was also shown to be significantly elevated after low-temperature treatment (Wang et al., 2015). The expression levels of the other BjuSUSs were significantly down-regulated after exposure to the different abiotic stress factors. These findings are in tandem with those documented in grapes (Zhu et al., 2017) and rubber trees (Xiao et al., 2014).

CONCLUSIONS

Fourteen SUS genes were identified in mustard. Collinearity analysis revealed that the SUS gene family may be lost or mutated to varying degrees after genome doubling or hybridization of B. rapa and B. nigra to form mustard. The expression patterns of BjuSUSs in different stages of mustard stem development indicate that BjuSUS01-04 have an important role in the biological processes associated with development. Plant hormones and abiotic stress induced the expression of BjuSUSs, indicating that this gene family is involved in their regulation.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests
The authors declare that they have no competing interests.

Author Contributions
• Mengyao Li conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
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Data Availability
The following information was supplied regarding data availability:
  RNA-Seq data are available at the NCBI Sequence Read Archive: SRP151320
  The genome sequence data of Brassica juncea are also available at NCBI: PRJNA285130
  Raw data are available as a Supplemental File.

Supplemental Information
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REFERENCES
An X, Chen Z, Wang J, Ye M, Ji L, Wang J, Liao W, Ma H. 2014. Identification and characterization of the Populus sucrose synthase gene family. Gene 539(1):58–67 DOI 10.1016/j.gene.2014.01.062.
Bai WQ, Xiao YH, Zhao J, Song SQ, Hu L, Zeng JY, Li XB, Hou L, Luo M, Li DM, Pei Y. 2014. Gibberellin overproduction promotes sucrose synthase expression and secondary cell wall deposition in cotton fibers. PLOS ONE 9(5):e96537 DOI 10.1371/journal.pone.0096537.
Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi I, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37S:W202–W208 DOI 10.1093/nar/gkp335.
Baroja-Fernandez E, Jose Munoz F, Montero M, Etxeberria E, Teresa Sesma M, Ovecka M, Bahaji A, Ezquer I, Li J, Prat S, Pozueta-Romero J. 2009. Enhancing sucrose synthase activity in transgenic potato (Solanum tuberosum L.) tubers results in increased levels of starch, ADPglucose and UDPglucose and total yield. Plant and Cell Physiology 50(9):1651–1662 DOI 10.1093/pcp/pcp108.
Baud S, Vaultier M, Rochat C. 2004. Structure and expression profile of the sucrose synthase multigene family in Arabidopsis. Journal of Experimental Botany 55(396):397–409 DOI 10.1093/jxb/erh047.

Bieniawska Z, Barratt DHP, Garlick AP, Thole V, Kruger NJ, Martin C, Zrenner R, Smith AM. 2007. Analysis of the sucrose synthase gene family in Arabidopsis. Plant Journal 49(5):810–828 DOI 10.1111/j.1365-313X.2006.03011.x.

Cannon SB, Mitra A, Baumgarten A, Young ND, May G. 2004. The roles of segmental and tandem gene duplication in the evolution of large gene families in Arabidopsis thaliana. BMC Plant Biology 4(1):10 DOI 10.1186/1471-2229-4-10.

Chalivendra S, Huber SC, Sachs MM, Rhoads D. 2007. Sucrose synthase. Plant Signaling & Behavior 2(1):28–29 DOI 10.4161/psb.2.1.3646.

Dai N, Cohen S, Portnoy V, Tzuri G, Harel-Beja R, Pompan-Lotan M, Carmi N, Zhang G, Diber A, Pollock S. 2011. Metabolism of soluble sugars in developing melon fruit: a global transcriptional view of the metabolic transition to sucrose accumulation. Plant Molecular Biology 76(1–2):1–18 DOI 10.1007/s11103-011-9757-1.

Duncan KA, Hardin SC, Huber SC. 2006. The three maize sucrose synthase isoforms differ in distribution, localization, and phosphorylation. Plant and Cell Physiology 47(7):959–971 DOI 10.1093/pcp/pcj068.

Fan J, Wang H, Li X, Sui X, Zhang Z. 2019. Down-regulating cucumber sucrose synthase 4 (CsSUS4) suppresses the growth and development of flowers and fruits. Plant and Cell Physiology 60(4):752–764 DOI 10.1093/pcp/pcy239.

Gu X. 2006. A simple statistical method for estimating type-II (cluster-specific) functional divergence of protein sequences. Molecular Biology and Evolution 23(10):1937–1945 DOI 10.1093/molbev/msl056.

Haagenson DM, Klotz KL, McGrath JM. 2006. Sugarbeet sucrose synthase genes differ in organ-specific and developmental expression. Journal of Plant Physiology 163(1):102–106 DOI 10.1016/j.jplph.2005.05.006.

Heim U, Weber H, Baumlein H, Wobus U. 1993. A sucrose-synthase gene of Vicia faba L.: expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta 191(3):394–401 DOI 10.1007/BF00195698.

Hirose T, Scofield GN, Terao T. 2008. An expression analysis profile for the entire sucrose synthase gene family in rice. Plant Science 174(5):534–543 DOI 10.1016/j.plantsci.2008.02.009.

Huang Y, Wang L, Hu S, Luo X, Cao Y. 2020. Overexpression of the bamboo sucrose synthase gene (BeSUS5) improves cellulose production, cell wall thickness and fiber quality in transgenic poplar. Tree Genetics & Genomes 16:75 DOI 10.1007/s11295-020-01464-w.

Jha AB, Dubey RS. 2004. Carbohydrate metabolism in growing rice seedlings under arsenic toxicity. Journal of Plant Physiology 161(7):867–872 DOI 10.1016/j.jplph.2004.01.004.

Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9(4):299–306 DOI 10.1093/bib/bbn017.

Lecharny A, Boudet N, Gy I, Aubourg S, Kreis M. 2003. Introns in, introns out in plant gene families: a genomic approach of the dynamics of gene structure. Journal of Structural and Functional Genomics 3(1–4):111–116 DOI 10.1023/A:1022614001371.

Li F, Hao C, Yan L, Wu B, Qin X, Lai J, Song Y. 2015. Gene structure, phylogeny and expression profile of the sucrose synthase gene family in cacao (Theobroma cacao L.). Journal of Genetics 94(3):461–472 DOI 10.1007/s12041-015-0558-1.
Li M, Xie F, He Q, Li J, Liu J, Sun B, Luo Y, Zhang Y, Chen Q, Zhang F, Gong R, Wang Y, Wang X, Tang H. 2020. Expression analysis of XTH in stem swelling of stem mustard and selection of reference genes. *Genes* **11**(1):113 DOI 10.3390/genes11010113.

Liu Y, Zhang Z, Leng R, Li J, Xiao L. 2014. Study on the inheritance of tumorous stem shape in tumorous stem mustard. *Plant Science Journal* **32**(1):34–39 DOI 10.3724/SP.J.1142.2014.10034.

Nagaharu U. 1935. Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany* 7:389–452.

Nguyen-Quoc B, Foyer CH. 2001. A role for ‘futile cycles’ involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. *Journal of Experimental Botany* **52**(358):881–889 DOI 10.1093/jexbot/52.358.881.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**(9):e45 DOI 10.1093/nar/29.9.e45.

Ruan Y, Llewellyn DJ, Furbank RT. 2003. Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. *Plant Cell* **15**(4):952–964 DOI 10.1105/tpc.010108.

Song X, Wang J, Sun P, Ma X, Yang Q, Hu J, Sun S, Li Y, Yu J, Feng S, Pei Q, Yu T, Yang N, Liu Y, Li X, Paterson AH, Wang X. 2020. Preferential gene retention increases the robustness of cold regulation in *Brassicaceae* and other plants after polyploidization. *Horticulture Research* 7:20 DOI 10.1038/s41438-020-0253-0.

Sonnhammer EL, Eddy SR, Durbin R. 1997. Pfam: a comprehensive database of protein domain families based on seed alignments. *Proteins-Structure Function and Bioinformatics* **28**(3):405–420 DOI 10.1002/(SICI)1097-0134(199707)28:3<405::AID-PROT10>3.0.CO;2-L.

Takehara K, Murata K, Yamaguchi T, Yamaguchi K, Chaya G, Kido S, Iwasaki Y, Ogiwara H, Ebitani T, Miura K. 2018. Thermo-responsive allele of sucrose synthase 3 (*Sus3*) provides high-temperature tolerance during the ripening stage in rice (*Oryza sativa* L.). *Breeding Science* **68**(3):336–342 DOI 10.1270/jsbbs.18007.

Tanase K, Yamaki S. 2000. Purification and characterization of two sucrose synthase isoforms from Japanese pear fruit. *Plant & Cell Physiology* **41**(4):408–414 DOI 10.1093/pcp/41.4.408.

Tong X, Wang Z, Ma B, Zhang C, Zhu L, Ma F, Li M. 2018. Structure and expression analysis of the sucrose synthase gene family in apple. *Journal of Integrative Agriculture* **17**(4):847–856 DOI 10.1016/S2095-3119(17)61755-6.

Vision TJ, Brown DG, Tanksley SD. 2000. The origins of genomic duplications in *Arabidopsis*. *Science* **290**(5499):2114–2117 DOI 10.1126/science.290.5499.2114.

Wang F, Smith AG, Brenner ML. 1994. Temporal and spatial expression pattern of sucrose synthase during tomato fruit development. *Plant Physiology* **104**(2):535–540 DOI 10.1104/pp.104.2.535.

Wang XQ, Zheng LL, Lin H, Yu F, Sun LH, Li LM. 2017. Grape hexokinases are involved in the expression regulation of sucrose synthase- and cell wall invertase-encoding genes by glucose and ABA. *Plant Molecular Biology* **94**(1–2):61–78 DOI 10.1007/s11103-017-0593-9.

Wang Y, Tang H, DeBarry JD, Tan X, Li J, Wang X, Lee T, Jin H, Marler B, Guo H, Kissinger JC, Paterson AH. 2012. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Research* **40**(4):e49 DOI 10.1093/nar/gkr1293.

Wang Z, Wei P, Wu M, Xu Y, Li F, Luo Z, Zhang J, Chen A, Xie X, Cao P, Lin F, Yang J. 2015. Analysis of the sucrose synthase gene family in tobacco: structure, phylogeny, and expression patterns. *Planta* **242**(1):153–166 DOI 10.1007/s00425-015-2297-1.
Xiao X, Tang C, Fang Y, Yang M, Zhou B, Qi J, Zhang Y. 2014. Structure and expression profile of the sucrose synthase gene family in the rubber tree: indicative of roles in stress response and sucrose utilization in the laticifers. *FEBS Journal* **281**(1):291–305 DOI 10.1111/febs.12595.

Xu X, Yang Y, Liu C, Sun Y, Zhang T, Hou M, Huang S, Yuan H. 2019. The evolutionary history of the sucrose synthase gene family in higher plants. *BMC Plant Biology* **19**(1):5661 DOI 10.1186/s12870-019-2181-4.

Xun G, Kent VV. 2002. DIVERGE: phylogeny-based analysis for functional-structural divergence of a protein family. *Bioinformatics* **18**(3):500–501 DOI 10.1093/bioinformatics/18.3.500.

Zhang C, Yu M, Ma R, Shen Z, Zhang B, Korir NK. 2015. Structure, expression profile, and evolution of the sucrose synthase gene family in peach (*Prunus persica*). *Acta Physiologiae Plantarum* **37**(4):81 DOI 10.1007/s11738.

Zhang D, Xu B, Yang X, Zhang Z, Li B. 2011. The sucrose synthase gene family in *Populus*: structure, expression, and evolution. *Tree Genetics & Genomes* **7**(3):443–456 DOI 10.1007/s11295-010-0346-2.

Zhu X, Wang M, Li X, Jiu S, Wang C, Fang J. 2017. Genome-wide analysis of the sucrose synthase gene family in grape (*Vitis vinifera*): structure, evolution, and expression profiles. *Genes* **8**(4):111 DOI 10.3390/genes8040111.