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Comparative Analysis of SUS Gene Family between *Saccharum officinarum* and *Saccharum spontaneum*

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Abstract

Sugarcane is a major sugar-producing crop, which contributed 80% of the world's sugar in 2010. *Saccarhum officinarum* is a domestic species with high sugar content, while, *Saccarhum spontaneum* is a wild species with stress tolerance. The highly complex polyploid genome of modern sugarcane cultivars arose from the interspecific hybridization between *S. officinarum* and *S. spontaneum*. Sucrose synthase (SUS) is a key enzyme for sucrose metabolism in plants, where activity is bidirectional: both synthetic and separate. In this study, nine genomic sequences of *S. officinarum* and eight genomic sequences of *S. spontaneum* for five *SUS* genes were identified. Phylogenetic analysis showed that the *Saccharum SUS3* and *SUS5* genes were generated from ρ duplication, *SUS1* and *SUS2* were duplicated after the split of dicot and monocot species, and *SUS4* was retained from the last common ancestor before the origination of *Angiospermae*. The gene structure and Ka/Ks analysis suggested the functional constraint of *SUS* genes in the two *Saccharum* species. Gene expression based on RNA-seq analysis revealed that *SUS1*was dominantly expressed in source tissues including the internodes and the basal zone of the leaves, *SUS2* was detectable in all tissues examined, and the remaining three *SUS* genes were expressed at low levels in the examined tissues, indicating *SUS1* is the key member involved in sucrose accumulation. In addition, *SUS* genes were observed to be present at higher expression levels in *S. officinarum* than in *S. spontaneum*, while *SUS2* presented different expression patterns during the circadian rhythm in *S. spontaneum* and *S. officinarum*, suggesting the two *SUS* genes contribute to the differential sugar levels in these species. Our comprehensive study in *Saccharum* provides the foundations for further functional studies of the *SUS* gene family.

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Introduction

Sucrose synthase (SUS) is a key enzyme involved in regulating the distribution and storage of carbohydrates in plant cells (Lutfiyya et al. 2007). During transportation from source leaves to the sink organs, sucrose is frequently broken down by the *SUS* enzyme which is capable of catalyzing a reversible reaction, and preferentially converts sucrose and uridine diphosphate glucose (UDP) into fructose and UDP-glucose (Geigenberger and Stitt 1993; Kleczkowski et al. 2010; Schmalstig and Hitz 1987). Various studies have shown that the enzyme activity is closely related to many physiological processes in plants. For example, *SUS* is responsible for the process of starch storing in potato tubers, carrot roots, maize kernels and pea endosperm (Barratt et al. 2001; Chourey et al. 1998; Fu and Park 1995; Tang and Sturm 1999; Zrenner et al. 1995). Cellulose synthesis is also suggested to require UDP-glucose as a substrate, this being provided by *SUS* cleavage of sucrose (Fujii et al. 2010). In addition, *SUS* also plays an important role in other crucial metabolic processes like environmental stress responses (Harada et al. 2005), sugar import (Klotz et al. 2003; Sun et al. 1992) and nitrogen fixation as well as arbuscular maturation and maintenance in mycorrhizal roots of legumes (Baier et al. 2010; Hohnjec et al. 2003).

A small multi-gene family has been proposed to encode several *SUS* isoforms and a different number of members in plants. There are six *SUS* genes in both *Arabidopsis* and rice (Baud et al. 2004; Hirose et al. 2008), while only three *SUS* genes exist in pea and maize (Barratt et al. 2001; Duncan et al. 2006). There are seven *SUS* genes in poplar (An et al. 2014). The tetraploid cotton genome contains fifteen *SUS* genes and is possibly the largest gene family in plants (Zou et al. 2013). Members of the *SUS* gene family differ not only in number but are also divergent in function and expression levels at different developmental stages in some plants. For instance, pea has at least three genes encoding *SUS1*, *SUS2*, and *SUS3* which are expressed mainly in developing seeds, leaves and flowers, respectively (Barratt et al. 2001). The high expression rate of *SUS1*is specifically required for seed development. However, *SUS2* and *SUS3* cannot compensate for *SUS1* deficiency in mutant seeds and root nodules. Three distinctive genes, *Sh1*, *SUS1* and *SUS3*, were identified in the maize *SUS* gene family. The *Sh1*gene plays a role in cell wall synthesis and is largely expressed in the growing endosperm (Duncan et al. 2006).

Sugarcane (*Saccharum spp.*) serves as the major crop responsible for sugar production and biofuel generation, and accounts for approximately 80% of global sugar output (FAOSTAT, 2010) and roughly 40% of ethanol produced worldwide (Lam et al. 2009). Thus, increasing the biomass of sugarcane via breeding techniques is essential. Modern sugarcane cultivars are complex homologous polyploids and aneuploids acquired by interspecific hybridization between *S. spontaneum and S. officinarum*. The majority of *S. officinarum* are octoploids with normal chromosome numbers 2n = 8× = 80, whereas the *S. spontaneum* chromosome number varies from 2n = 36 to 128 (Irvine 1999), with most polyploidy levels being 8 (Zhang et al. 2012). As a result, the modern sugarcane hybrid is allopolyploid which ranges from octoploid (x = 8) to dodecaploidy (x = 12). Previous *SUS* studies in sugarcane indicated that *SUS* activity was greater in immature internodes than in mature internodes of all cultivars. The outcome of the investigation of expression and localization of *SUS* by immunohistochemistry indicated that SUS activity was ubiquitous. Furthermore, it was also demonstrated that at least one form of *SUS* gene was absent from young tissue or at least present below detectable levels. Comparative genomics showed that there are five *SUS* genes in sugarcane (Zhang et al. 2013), and previous research found that the *SUS4* gene may be involved in the regulation of sucrose distribution at dissimilar stages (Chen et al. 2015). In spite of the haplotype fragments *SUS* genes have been studied thoroughly in *Saccharum* (Zhang et al. 2013), although their gene families and evolution remain unknown.

SUS genes are particularly significant in sugarcane and in this study we used the recently published sugarcane *S. spontaneum* genome and the BAC resources of *S. officinarum* to identify the SUS gene families in *S. officinarum* and *S. spontaneum*, to analyze their evolution and examine their expression patterns. Our results will provide a foundation and framework to understand the physiological functions of the sugarcane *SUS* gene family members in regulating sugarcane plant development, particularly with regard to growth and with a view to improving sugar yields from sugarcane.

Results

Identification of *SUS* Genes in *S. officinarum* and *S. spontaneum*

Nine BAC sequences were screened in *S. officinarum* (Supplemental Table 1). Meanwhile, the sorghum gene sequences were aligned against *S. spontaneum* genome and 8 *SUS* genes were identified (include alleles) (Zhang et al. 2018). Annotation revealed that the 17 sequences from two sugarcane species can code five *SUS* genes referred to as *SUS1- SUS5*. These five *SUS* genes are referred to with the prefix "So" for *S. officinarum* and "Ss" for *S. spontaneum*. In

the *S. officinarum*, *SoSUS1* and *SoSUS5* had two allelic haplotypes, *SoSUS2* had three allelic haplotypes. Each *SUS* allele haplotype is represented by an additional "a" to "c" at the end of gene name. *SsSUS1* had two allelic haplotypes and *SsSUS5* had three allelic haplotypes. The *S. spontaneum* haplotype naming method is the same as above (Table 1). Both of *SUS* sequences of *S. officinarum* and *S. spontaneum* are available in Supplemental Table 2.

Further analysis of the translated protein sequences revealed that the putative proteins contain 794–898 amino acids with molecular weights ranging from 9.06 to 10.55 kDa and the isoelectric points ranging from 5.91 to 8.42 (Table 1). Investigating the divergence of sorghum/*Saccharum* showed that *SoSUS3* and *SsSUS3* genes share low identities (<=90%) with their orthologous *SbSUS3* (Table 1). Furthermore, all the sugarcane *SUS* genes of the two species were predicted, using the Interproscan algorithm (http://www.ebi.ac.uk/interpro/), to share two domains: conserved sucrose synthase and glucosyltransferase (Supplemental Fig. 1).

Homologs and Allelic Analysis of *SUS*

Genomic sequence comparison within the allelic haplotypes from five *SUSs* of two sugarcane species showed that these allelic haplotypes shared very high identities of above 97%, except *SoSUS1* (95%) and *SsSUS1* (91%) (Tables 2 and 3). In order to display and analyze the exon/intron structure of the sugarcane *SUS* genes, putative full coding regions and genomic sequences, 17 *SUS* sequences from two species, are presented using the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) (Fig. 1). The figure shows that the gene alleles have similar structures, for example three alleles (*SsSUS5.a, SsSUS5.b and SsSUS5.c*) in *S. spontaneum*. Exon fusions and intron extensions were observed in the *Saccharum* species, for instance, the 4th introns of the gene allele *SoSUS1.a/b* were greater than *SsSUS1.a/b*, and the last exon of *SsSUS1.b* gene originated through exon fusion. Further analysis of gene structure revealed that *SsSUS2* had the last exon and the last intron deleted in contrast to the three gene alleles of *SoSUS2*. In addition, there is the significant difference in gene structure between the exons of *SsSUS3* and *SoSUS3* which is mainly caused by the first exon fusion/split.

In addition to the differences between the two species, there are also subtle changes in alleles. We observed key differences in the exon splitting in different gene alleles. We found for example differences in the sixth exon of *SsSUS1* alleles, the ninth exon of *SoSUS2* alleles, the sixth of *SoSUS5* alleles and the seventh of *SsSUS5*. However, the fourteenth exon of the haplotype of *SoSUS5*.a was deleted.

The Ka, Ks and Ka/Ks of the homologous *SUS* genes of *S. officinarum* and *S. spontaneum* were calculated (Fig. 2). Ka/Ks values were less than 0.5, indicating that after differentiation this gene family of two species underwent purifying selection. However, *SUS5* has Ka/Ks values above 1 for the pairwise comparison among the alleles of *S. spontaneum* and *S. officinarum*, indicating *SUS5* neofunctionalization after the split of *S. spontaneum* and *S. officinarum*. All of *SUS* genes showed that Ka/Ks values were low 1 in *S. spontaneum* and

Table 1 Sequence similarity of SUS gene fragments between Saccharum and *Sorghum bicolor*

	Sorghum		Saccharum					
Gene name	Chromosome position	Protein size	Gene name	Chromosome position	Protein size	Identity	Pl	Mw
SbSUS1 Sb01g033060	1	816	SoSUS1.a	–	816	97.31	5.95	9.24
			SoSUS1.b	–	797	97.43	5.96	9.06
			SsSUS1.a	Chr1B	801	97.18	6.11	10
			SsSUS1.b	Chr1D	845	93.72	6.05	10.06
SbSUS2	10	837	SoSUS2.a	–	874	97.35	6.02	10
			SoSUS2.b	–	881	97.48	8.42	10.16
			SoSUS2.c	–	876	96.85	6.94	10.02
			SsSUS2	Chr8D	795	96.80	7.57	10.55
SbSUS3 Sb04g038410	4	838	SoSUS3	–	898	87.26	6.78	9.49
			SsSUS3	Chr4D	883	90.21	6.49	9.66
SbSUS4 Sb01g035890	1	809	SoSUS4	–	839	95.80	5.91	9.58
			SsSUS4	Chr1B	794	96.91	7.34	9.99
SbSUS5	10	892	SoSUS5.a	–	854	94.32	6.77	9.5
			SoSUS5.b	–	846	93.56	6.26	9.07
			SsSUS5.a	Chr8A	861	90.95	6.16	9.83
			SsSUS5.b	Chr8C	856	90.17	6	9.77
			SsSUS5.c	Chr8B	850	88.16	6.48	9.66

*Blast output of SUS genes comparison between Saccharum and *Sorghum bicolor* at amino acid level

Table 2 Comparison within the allelic haplotypes in haplotypes from orthologous SUSs of two sugarcane species

	SoSUS1.a	SoSUS1.b	SoSUS2.a	SoSUS2.b	SoSUS2.c	SoSUS3	SoSUS4	SoSUS5.a	SoSUS5.b
SsSUS1.a	94.74	94.85	74.72	74.84	74.35	46.40	64.33	54.34	54.45
SsSUS1.b	91.37	91.47	71.54	71.66	71.19	45.48	61.55	53.93	53.03
SsSUS2	74.35	74.56	97.39	97.51	96.89	51.53	64.16	54.99	54.23
SsSUS3	49.32	49.63	52.67	52.67	52.29	91.48	51.69	74.35	75.23
SsSUS4	67.37	67.08	67.67	67.67	67.17	49.57	91.66	55.93	55.04
SsSUS5.a	54.43	54.74	55.34	55.34	54.97	71.46	54.72	96.04	98.93
SsSUS5.b	53.69	54.01	54.79	54.79	54.42	70.63	54.01	95.23	98.10
SsSUS5.c	52.38	52.69	53.67	53.67	53.30	68.72	53.01	92.79	95.60

S. officinarum, indicating that purifying selection was the dominant force driving the evolution of *SUS* genes after the speciation of two *Saccharum* species.

Phylogenetic Analysis of Sugarcane *SUS* Genes and Other Plants *SUS* Homologs

In order to comprehensively analyze the evolutionary relationships between the *SUS* genes of sugarcane and other plant species, a phylogenetic tree containing 60 *SUS* amino acid sequences from 15 representative species was constructed using the ClustalX with Neighbor-Joining method (Fig. 3). The 15 species were made up of five monocotyledons, six dicotyledons, a gymnosperm, a prokaryote and a basal angiosperm (Supplemental Table 2). As shown in the phylogenetic tree, plant *SUS* genes could be distributed in three groups, referred to as groupI- group III. *SUS* genes from both monocotyledons and dicotyledons are distributed in three groups, suggesting that the three groups originated from a common ancestor before their evolutionary divergence.

Interestingly, we observed that *Amborella trichopoda* was distributed in groups II and III, and the gymnosperms (*Pinustaeda*) were found only in group II. These findings demonstrate that the *SUS* gene already probably existed before the differentiation of the gymnosperms and angiosperms.

In group III, *SUS* proteins in monocotyledonous plants, including pineapple, rice, sorghum and the two species of sugarcane, form an independent clade with dicotyledons and *Amborella trichopoda* being excluded. Interestingly, with the exception of pineapple, other monocotyledon species, contain two *SUS* genes, suggesting that the single *SUS* gene has expanded through common duplication within the graminaceous plants after the ancestor of graminaceous plants separated with pineapple. However, each monocotyledon species in group II includes only one *SUS* gene.

GroupIis the largest group, containing 29 *SUS* genes, and was divided into two subgroups, the monocot-subgroup and dicot-subgroup, as the group shave characteristics distinctive of the monocot-dicot split. In this group, the *SUS* gene is not identifiable in *Amborella trichopoda*, which suggests that these *SUS* genes may have appeared after the divergence of *Amborella trichopoda* and other flowering plants. The monocot-subgroup can be further divided into two subgroups. Both of the subclasses contain *SUS* genes from rice, maize, sorghum, sugarcane, except for pineapple which is only present in one clade. The results indicate that these *SUS* genes from the monocot species may undergo independent evolution within each lineage.

Table 3 Comparison of the protein sequences of allelic SUSs for two sugarcane species

	SoSUS1.b	SoSUS2.b	SoSUS2.c	SoSUS2.c	SoSUS5.b	SsSUS1.b	SsSUS5.b	SsSUS5.c	SsSUS5.c
SoSUS1.a	94.98	–	–	–	–	–	–	–	–
SoSUS2.a	–	98.29	–	–	–	–	–	–	–
SoSUS2.a	–	–	97.84	–	–	–	–	–	–
SoSUS2.b	–	–	–	99.09	–	–	–	–	–
SoSUS5.a	–	–	–	–	99.12	–	–	–	–
SsSUS1.a	–	–	–	–	–	91.24	–	–	–
SsSUS5.a	–	–	–	–	–	–	99.07	–	–
SsSUS5.a	–	–	–	–	–	–	–	96.87	–
SsSUS5.b	–	–	–	–	–	–	–	–	97.66

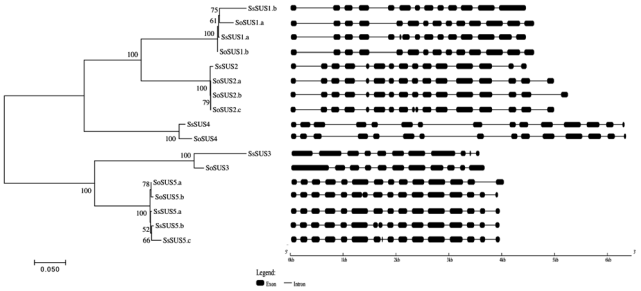


Fig. 1 The allelic gene structures of *SUSs* in *S. spontaneum* and *S. officinarum*

Expression Analysis of the *SUS* Genes

To further understand the possible physiological functions of *SUS* family genes in *S. officinarum* and *S. spontaneum*, we investigated gene expression in different growth processes, such as seedling, pre-mature and mature stage, in two *Saccharum* species using RNA-seq (Fig. 4). As shown in Fig. 4, *SUS1* was the most abundantly expressed gene among the isoforms of *SUS* genes, *SUS3* presented the lowest expression level in the all examined tissues, suggesting that *SUS1* was the primary member of the gene family.

At the seedling stage, the gene expression level of other *SUS* genes was significantly higher compared to the internodes. The expression levels of *SUS1* and *SUS5* in the internode was higher in *S. spontaneum* than in *S. officinarum*. At the pre-mature stage, the *SUS1* and *SUS2* genes were highly expressed in internode 3, 6–9, 9–15 of the two *Saccharum* species. Further analysis was performed and SUS2 was found to have higher expression levels in the *S. officinarum* than in the *S. spontaneum* at the pre-mature stage. At the mature stage, we observed that the most abundantly expressed gene, *SUS1*, is mainly expressed in the internodes. However, *SUS2* and *SUS4* are not only expressed in the internodes, but also in leaves, and their expression levels in the two *Saccharum* species differs greatly, especially in the internode 3 regarding *SUS2* levels. From the above analysis, we determined that expression of the *SUS1* and *SUS2* were very abundant and dominantly expressed in the internodes.

Gramineae leaves have a unique feature: at the subcellular level the leaf from the base to the tip in the developmental state showed a regular and continuous change. Referring to a study on maize leaves (Li et al. 2010), we investigated the continuous developmental gradient of leaves to profile the transcriptome of the *SUS* genes in the two *Saccharum* species to gain insight into the functional divergence of sucrose metabolism in the sink tissue (Figs. 5 and 6). Using the transcript analysis, *SUS1* was detected at higher levels than the other paralogs in two *Saccharum* species, whereas, *S. spontaneum* displayed higher expression levels in the transitional zone and basal zone than other two zones, suggesting that the *SUS1*gene has an important role in sucrose metabolism. However, the high gene expression was found only in the basal zone in *S. officinarum*. Interestingly, the expression of *SUS2* in both species was found to decrease continuously from basal zone to maturing zone, whereas the Fragments Per Kilo base per Million (FPKM) values in the mature zone were reversed. The expression levels of SUS5 in the two *Saccharum* species also presented the same trend, with levels being higher in the basal zone than in the other three zones. In addition, *SUS3* and *SUS4* were found to have low expression levels in all zones of both species.

Sclerenchyma is the supporting tissue in plants that includes two types of sclerenchyma cells, fibers and sclereids (Buxton and Redfearn 1997). Their cell walls consist of

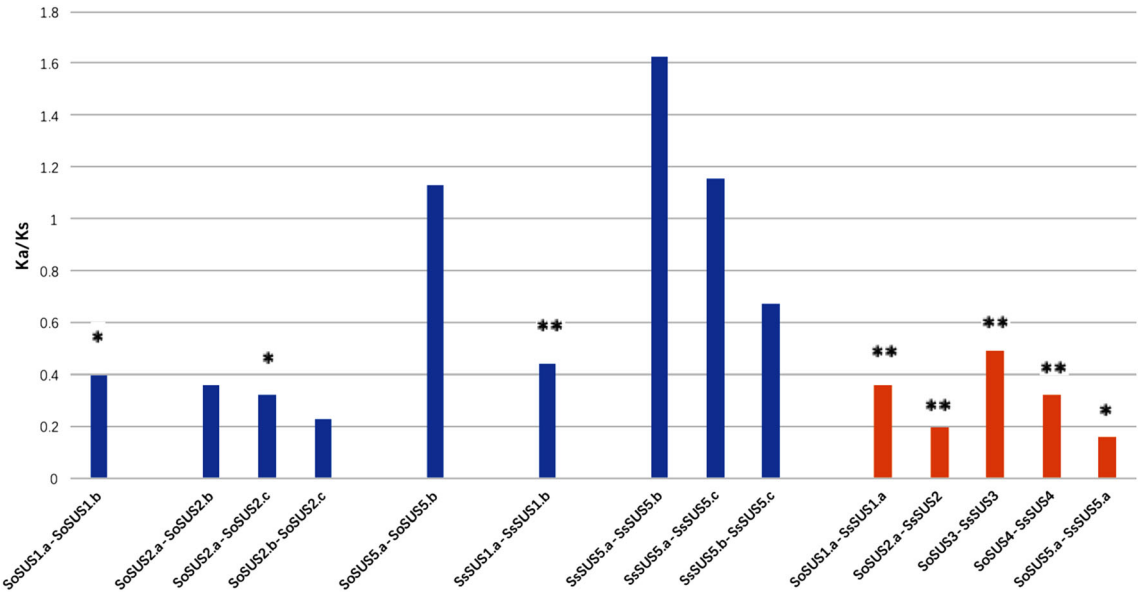
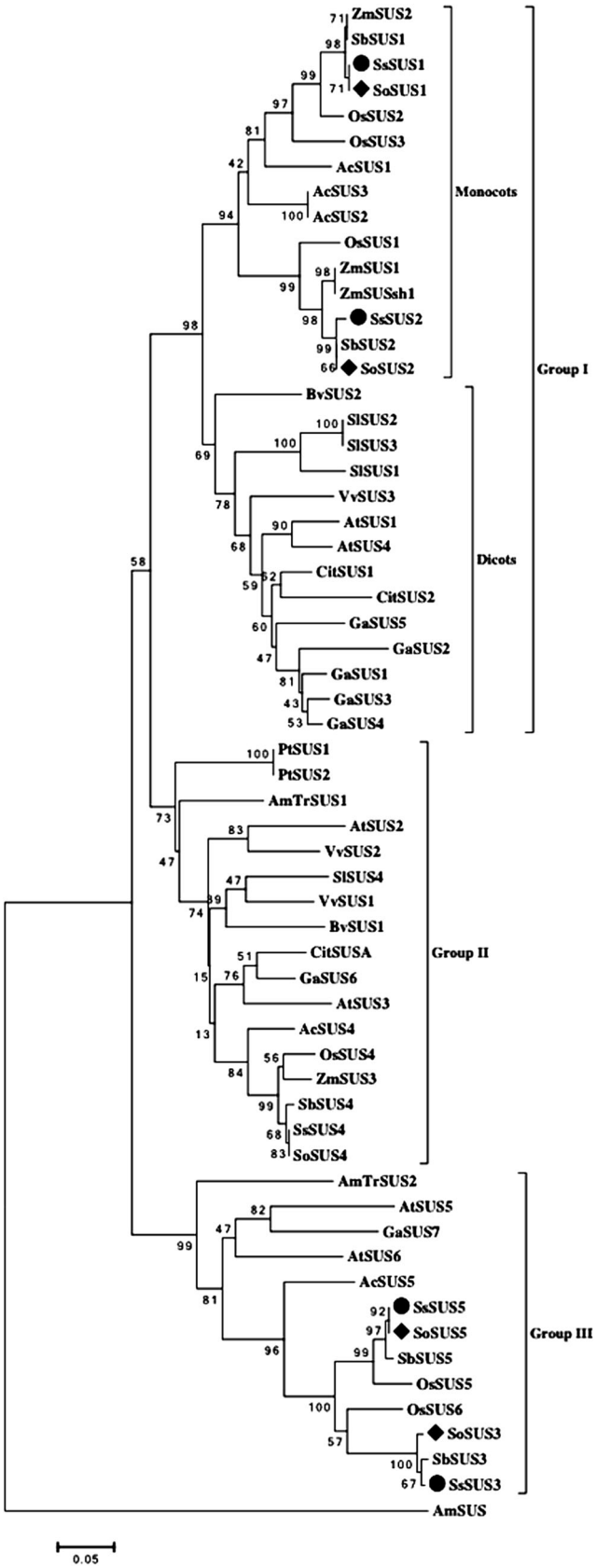


Fig. 2 The Ka/Ks of *SsSUS* haplotypes, *SoSUS* haplotypes and *SsSUS-SoSUS*. Note: The lower value of Ka/Ks was indicated by stars



◀ **Fig. 3** Phylogenetic tree was constructed of *SsSUS* and other plant *SUS* homologs. *SUS* genes in group I and III are highlighted. Note: refer to Supplemental material Table S2

cellulose, hemicellulose and lignin. We investigated transcriptome profiling between the parenchyma and sclerenchyma cells from mature sugarcane stalks in two *Saccharum* species to study SUS gene functional divergence (Fig. 4). The expression levels of *SUS2* genes were higher in parenchyma and sclerenchyma type cells from the high sugar content species *S. officinarum* than in the stress tolerant species *S. spontaneum*, with the later having other isoforms that were more abundant. It is worthy of note that the expressional levels of *SUS1*, *SUS2* and *SUS4* showed the same differences in both parenchyma and sclerenchyma cells, indicating that the gene expression levels, especially of *SUS2*are higher in the sclerenchyma cells than in parenchymal cells. These findings indicate that the *SUS* genes may be involved in both sucrose and cellulose accumulation in *Saccharum*.

The enzymes for sucrose metabolism have been found to be regulated by the circadian clock, the cell-wall invertases (CWINVs), the vacuolar invertases (VINVs), sucrose phosphate synthase and sucrose synthase (Bläsing and Stitt 2005). Given these findings, we investigated the *SUS* gene expressional pattern under the diurnal cycles. Both *SsSUS2* and *SoSUS2* were observed to respond to the diurnal cycles, *SsSUS2* presented peak expressional levels at the end of day (16:00–18:00), while, *SoSUS2* was highly expressed at the beginning of the day (06:00) and in the middle of night (12:00), suggesting the divergence of the regulated network for the two *Saccharum* species. The *SUS4* gene in both *Saccharum* species responded to the diurnal cycles and generated a similar expressional pattern, with *SUS4* having a higher expression level in *S. officicnarum* than in *S. spontaneum*.

Discussion

A very large proportion of worldwide sugar and ethanol production is generated from sugarcane. *SUS* is one of the key enzymes involved in the decomposition and storage of carbohydrate. Recently, many *SUS* gene families in various plants were analyzed by comparative genome studies, such as Arabidopsis, rice, maize, tobacco, popular and sorghum (Bieniawska et al. 2007; Duncan et al. 2006; Hirose et al. 2008; Wang et al. 2015; Zhang et al. 2013). We identified the complete *SUS* gene family of sorghum using the whole sorghum genome, we designed the probes based on the high synteny of the sorghum and sugarcane genomes. Previous studies on sugarcane revealed the presence of several *SUS* genes that were cloned and their expression analyzed (Lingle and Dyer 2001). However, *SUS* genes are still poorly understood due to the complexity of the sugarcane genome. In

	Seedling		Pre-mature					Mature					Cell wall type	
	seedling-leaf	seedling-stem	pre-m-leaf-roll	pre-m-leaf	pre-m-stem3	pre-m-stem/-9	pre-m-stem9/15	m-leaf-roll	m-leaf	m-internodes3	m-internodes6/9	m-internodes9/15	parenchymal cell	sclerenchyma cell
SsSUS1	11.45	558.39	13.97	4.03	727.56	104.54	75.83	5.03	5.10	789.95	327.53	1017.81	42.55	68.25
SoSUS1	8.25	369.58	3.37	0.00	517.84	269.08	156.92	10.46	3.48	519.44	108.87	39.71	45.25	67.16
SsSUS2	11.30	101.34	18.81	17.13	99.05	75.00	56.53	35.11	21.86	161.69	92.58	125.22	217.52	504.35
SoSUS2	34.00	112.65	27.86	25.22	217.41	168.05	201.67	78.00	79.03	362.09	177.05	182.91	86.65	182.66
SsSUS3	0.08	4.05	0.00	0.00	10.78	0.00	0.00	0.15	0.00	9.37	0.00	0.00	0.06	0.02
SoSUS3	0.14	4.47	0.52	0.00	0.00	0.00	0.43	0.62	0.00	7.60	0.24	0.60	0.29	0.66
SsSUS4	2.00	3.69	0.00	4.40	7.57	8.34	12.04	10.47	9.68	13.95	22.60	10.00	17.38	28.20
SoSUS4	7.49	9.39	23.97	27.06	19.05	15.41	22.67	24.20	19.58	33.24	37.56	65.98	36.31	47.76
SsSUS5	0.89	25.31	0.73	0.00	45.29	0.89	0.52	0.77	1.01	36.44	0.98	0.68	0.47	0.55
SoSUS5	0.08	16.84	0.00	0.00	13.85	0.28	0.00	0.00	0.00	11.34	1.78	1.05	1.57	1.46

FPKM

0 1000

Fig. 4 The expression pattern of *SUSs* based on FPKM in different tissues of different stages in *S. spontaneum* and *S. officinarum*

this study, we identified 9 and 8 *SUS* genes from *S. officinarum* and *S. spontaneum* respectively (Table 1). Further investigation of their evolution history, their exon-intron structure and expression pattern in different growth processes was then carried out.

Evolution of the *SUS* Family

SUS is a very ancient family of sucrose metabolic genes which is prevalent in higher plants and is also found in archaea. Interestingly, angiosperm *SUS* families are much more abundant and diverse (Xiao et al. 2014). In previous studies, multiple *SUS* families have been identified both in monocot and dicot species and are divided into at least three major groups on the basis of phylogenetic tree analysis of their sequences (Bieniawska et al. 2007; Chen et al. 2012; Hirose et al. 2008). Using sorghum *SUS* genes as a reference, 9 and 8 orthologous genes in high sugar content *S. officinarum* and stress tolerant *S. spontaneum* were identified respectively (Fig. 3). Since the identified *SUS* genes contain alleles we selected five genes from the two-sugarcane species *S. officinarum* and *S. spontaneum,* for the construction of the phylogenetic tree. Phylogenetic analysis of sugarcane *SUS* genes and other plant homologues confirmed the previous classification that higher plant species have at least one gene for each of the three groups. The evolutionary history or origin of sugarcane *SUS* genes in each group is different. The five *SUS* isoforms are separated into the three groups: *SsSUS1/2* and *SoSUS1/2* in group I, *SsSUS4* and *SoSUS4* in group II, *SsSUS3/5* and *SoSUS3/5* in group III. Based on the cotton evolution data of the *SUS* family, *SsSUS1/2* and *SoSUS1/2* may be evolutionarily younger than the other *SUS* genes.

	basal zone			transitional zone			maturing zone				mature zone				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SoSUS1	134.67	126.76	69.83	43.55	1.64	1.85	0.76	0.00	0.75	0.46	0.22	0.09	0.00	0.17	0.15
SsSUS1	1032.91	960.50	637.35	389.91	249.04	111.20	46.94	11.50	3.46	1.76	1.24	0.85	0.78	0.98	1.21
SoSUS2	97.06	88.24	67.38	45.58	36.48	24.98	22.04	15.76	11.59	9.34	8.49	8.55	10.83	12.43	20.35
SsSUS2	65.16	60.77	47.74	29.73	29.46	20.56	14.98	11.97	6.69	6.63	5.40	6.44	8.62	7.86	14.85
SoSUS3	0.40	1.49	2.51	0.61	0.30	0.35	0.23	0.14	0.00	0.00	0.04	0.06	0.04	0.03	0.08
SsSUS3	1.43	4.11	4.01	2.51	1.13	1.08	0.62	0.28	0.22	0.17	0.19	0.17	0.23	0.10	0.15
SoSUS4	2.91	2.96	3.31	4.20	4.37	4.35	4.11	3.69	3.99	3.80	4.65	4.93	6.29	5.62	6.34
SsSUS4	2.21	2.16	2.30	3.01	3.32	3.33	3.18	2.39	3.00	2.70	3.66	3.70	4.45	4.59	5.25
SoSUS5	13.58	34.83	32.87	13.50	10.08	9.45	5.64	1.34	0.30	0.16	0.14	0.12	0.07	0.18	0.60
SsSUS5	21.16	59.35	57.10	24.24	17.21	17.94	10.46	2.75	0.81	0.51	0.35	0.50	0.34	0.47	1.05

FPKM

0 1000

Fig. 5 The expression patterns of *SUSs* based on FPKM across leaf gradients in *S. spontaneum* and *S. officinarum*

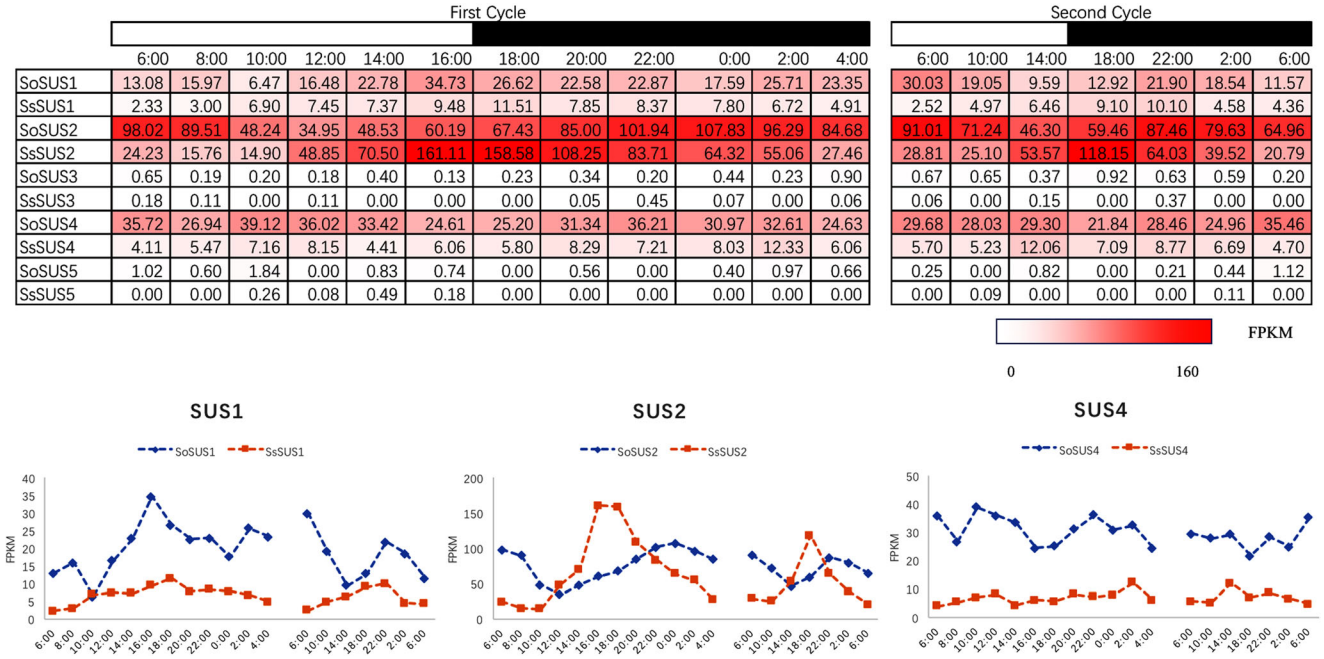


Fig. 6 The expression pattern of *SUSs* based on FPKM under the diurnal cycles changes in both *S. spontaneum* and *S. officinarum*
Upper portion: The RPKM of *SUS* genes under the diurnal cycles in *S. spontaneum* and *S. officinarum*. Lower portion: The *SUS1*, *SUS 2* and *SUS4* profiles under the diurnal cycles in *S. spontaneum* and *S. officinarum*.

Whole-genome duplication (WGD), or polyploidization, is an extreme mechanism of gene duplication and a driving force for the evolution of angiosperms (Chalhoub et al. 2014; Jiao et al. 2011). WGD has occurred multiple times over the past 200 million years in angiosperm evolution, such as ε WGD, γ WGD and ρ WGD events. It has recently been reported that the pineapple has one fewer ancient WGD (ρ) event than the other sequenced grass genomes (Jiao and Paterson 2014; Jiao et al. 2011). The ρ duplication is thought to have occurred before the origin of the lineages leading to rice, wheat and maize but after the separation of lineages thought to have led to grasses and pineapple 95–115 million years ago (Ming et al. 2015; Paterson et al. 2004; Wang et al. 2005). In group III, besides pineapple, other monocots (rice, sorghum and sugarcane) underwent gene duplication events (Fig. 3). Hence, we can infer that *SoSUS3/5* and *SsSUS3/5* were generated from ρ duplication. In group II, it contained two *SUS* genes from gymnosperm (*PtSUS1* and *PtSUS2*), indicating *SoSUS4* and *SsSUS4* could have originated from the last common ancestor (LCA) in gymnosperm and are older genes. At the same time, only one *SUS* gene was found in each monocot plant, and we therefore speculate that *SUS* may be more conservative. The group I *SUS* genes could have originated from LCA in angiosperm, with one cluster within monocot genes and another clustered within dicot genes. The younger group contains the most dominant genes (*SUS1/2*), compared to the other two groups, the direct evidence for this being the multiple gene duplication events that occurred after the monocot/dicot divergence both within dicots and monocots.

There are three main mechanisms that lead to the structural differences in the exon/intron of genes during the evolution of the genes: the gain or loss of exons or introns, the exoniation or the exonization, and missing introns (Xu et al. 2012). Therefore, differences in exon/intron structures could be used, to some extent, to estimate the evolutionary history of the gene families. Previous studies of the *SUS* homologs revealed conservation in terms of gene structure in several dicot and monocot plants, containing 14 introns in the conserved positions (Chen et al. 2012). In our research, comparison of *SUS* genes exon andintrons between the two-sugarcane species highlighted a low frequency of structural variation (Fig. 1), indicating that SUS genes are evolutionary more conserved in *S. officinarum* and *S. spontaneum*. The *SUS3* first exon, located in group III, is longer than the other gene of *SUS* in *Saccharum* and we predict that the first exons of other *SUS* genes had two introns inserted into them thus leading to an increased number of exons in the *SUS* genes.

The Expression of *SUS* Families and Potential Function in the Two *Saccharum species*

It is well documented that gene duplication followed by functional diversity has played a crucial role in driving the evolution of the *SUS* gene family (Flagel and Wendel 2009;

Zhenglong et al. 2003). To date, although the expression pattern of the *SUS* gene has been studied in several plants such as Arabidopsis, rice, cotton, poplar and rubber trees (Baud et al. 2004; Bieniawska et al. 2007; Chen et al. 2012; Hirose et al. 2008; Xiao et al. 2014; Zhang et al. 2011), the expression patterns of the *SUS* gene in *Saccharum* has not yet been analyzed in detail. This study aimed to understand the potential function of *SUS* genes in *Saccharum* species based on three sets of experimental designs, five different tissues (including the leaf, leaf roll, mature stalk, maturing stalk and immature stalk), the continuous developmental gradient of leaves and parenchyma cells and sclerenchyma cells.

It is vital for the plant life cycle that sucrose is synthesized in the leaves by photosynthesis and is transported to sink tissues. The gradient leaf sections (Li et al. 2010) were separated to four zones, these being basal zone, transitional zone, maturing zone and mature zone, of which the mature zone belongs to the source organ while basal, transitional and maturing zone belong to sink organs. It has been reported that *SUS* activity is bidirectional: synthesis and decomposition (Schaffer et al. 2010). *SUS1* and *SUS5* genes in the two *Saccharum* species had higher expression levels in the basal and transitional zone than other zones in the gradient leaf sections (Fig. 5). The transcript level of *SUS2* genes of *S. officinarum* and *S. spontaneum* were continuously reduced in the basal to maturing zone while slowly increasing in mature zone. *SUS3* and *SUS4* transcripts were considerably lower or almost undetectable in the four zones. Hence, it is tempting to speculate that *SUS* activity serves in the synthesis of cell wall polysaccharides and the synthesis of sucrose for export. However, the sum of transcript levels of all *SUS* genes was more than 10-times higher in immature zone (basal, transitional and maturing) than in the mature zone. In addition, the *SUS1* gene could be primarily responsible for cellulose synthesis and thus play key role for sugar accumulation.

Interestingly, the *SUS1* gene has similar transcript levels in parenchymatous cells as in sclerenchymatous cells of two sugarcane species whereas the transcript levels of *SUS2* in parenchymatous and sclerenchymatous cells were significantly higher in *S. spontaneum* compared to *S. officinarum*. The rice results generated from the *SUS* gene research indicate that *SUS1* plays a possible role in primary cell-wall synthesis, rather than secondary wall development (Hirose et al. 2008). We therefore speculated that the *SUS1* gene is primarily responsible for primary wall development.

S. officinarum has a high sucrose content, while, *S. spontaneum* accumulates a low sugar content. The *SUS1* genes were dominant in the sink tissues of *Saccharum*, and were observed to have high expression levels in the *S. spontaneum* internodes at the mature internode 9th and internode 15th.Thisphenomenon can be explained by the function of *SUS* which is capable of catalyzing a reversible reaction, converting sucrose and uridine diphosphate glucose (UDP) into fructose and UDP-glucose (Geigenberger and Stitt 1993; Kleczkowski et al. 2010; Schmalstig and Hitz 1987), thus the low level of *SUS* in the mature internodes contributes to the sucrose accumulation. In the leaves, *SUS2* was the dominant gene and presented an expression pattern with diurnal cycles, indicating the *SUS* genes respond to the diurnal cycles in plants, which is consistent with a previous study (Bläsing et al. 2005). It is interesting to note that the *SsSUS* and *SoSUS* genes have different expression patterns for diurnal cycles which may regulate the balance of sucrose accumulation in *Saccharum* and thus give rise to the variation of sugar content seen between the two *Saccharum* species.

This study provides the first comprehensive evaluation of the evolution, gene allelic haplotypes, exon/intron structure and tissue-expression patterns of the entire *SUS* gene family in two *Saccharum* species. The results provide an underlying foundation and framework for future understanding of the potential physiological roles of each sugarcane *SUS* gene and the evolution of *SUS* gene family in response to sucrose metabolism during sugarcane development. We also realize that *SUS* genes, containing allelic haplotypes in sugarcane contain frequent structural variations and mutations. Different *SUS* genes in sugarcane might play different roles in regulating sucrose accumulation and fiber development. However, understanding the specific functions of each Saccharum *SUS* gene and their possible functional interactions, need to be experimentally verified.

Methods

Plant Materials and RNA Isolation

One wild type *Saccharum* species SES208 (*S. spontaneum*, Ss, 2n = 8 × = 64) and one cultivated species LA-purple (*S. officinarum*, So, 2n = 8 × = 80) were used in this study. Seedling sugarcane plants were grown in plastic pots under greenhouse conditions and standard growth conditions (14 h light 30 °C/10 h darkness 22 °C, under humid conditions with 60% RH). Pre-mature and mature sugarcane were grown in the field on the campus of Fujian Agricultural and Forestry University (Fuzhou, China) in the February of 2015. Tissue samples were obtained from leaf roll, leaf, top immature internode (i.e. internode number 3), premature internode (i.e. internode number 9 for 'LA-purple' and internode number 6 for SES208) and mature internode (i.e. internode number 15 for 'LA-purple' and internode number 9 for SES208) at 9-month-old premature and 12-month-old mature developmental stages. The mature internode (i.e. internode number 13) of LA-purple and SES208 was then collected to isolate parenchyma and sclerenchyma cells

using scalpel with the aid of a stereoscopic microscope to obtain the material for RNA isolation. In addition, we also collected leaf and internodes material from seedlings from these two species at 35 days after planting. The plants of *Saccharum* were grown in a growth chamber at 30 °C/22 °C L/D, 60% RH, a 14 h:10 h photoperiod and light intensity of 350 μ mol/m2/s. The first true leaf was also collected from 4 individual plants of two sugarcane species respectively as three biological replicates. 15 different leaves segments of about 1 cm were collected from each the first true leaf. The internodes were numbered from top to bottom according to the method of Moore (Moore 1987). Leaves from the mature plants of LA Purple and SES208 were used for investigating the gene expression under circadian rhythms (Wang et al. 2019). The tissues were immediately frozen using liquid nitrogen and stored at −80°Cprior to RNA isolation.

BAC Libraries

A series BAC libraries was constructed for the *S. officinarum* (LA Purple, 2n = 8× = 80). Nuclei were isolated from the young leaf tissues following the previously described method. In brief, the high molecular weight DNA embedded in agarose was partially digested into fragments using Hind III. The fragments of approximately 100 kb were recovered and inserted into pSMART BAC vector (Lucigen, LA). The BAC library of LA Purple consists of 74,880 clones in 195,384-well plates with average insert size of 150 kb, resulting in 1.5× coverage of the octoploid genome and 12× coverage of the monoploid genome.

Identification and Sequencing of *SUS* Families from BAC Library

The BAC library screening was implemented using the probe amplified with the primers of *SUS* (Zhang et al. 2013) on the basis of that described by *Wang* et al (Wang et al. 2010). The BAC clones belonging to different haplotypes were selected. The BAC DNAs were isolated using the Phase Prep ™TMBAC DNA kit (Sigma-Aldrich, NA0100-1KT) and the insert size of BAC clones estimated using standard size markers and CHEEL gel electrophoresis equipment. The sequencing libraries provided separate unique barcodes for each clone. The DNA-seq libraries which contained potential *SUS* genes were then pooled and sequenced with 150 bp, pair-end reads on Illumina Hiseq2500 at Center for Genomics and Biotechnology in Fujian Agriculture and Forestry University. The BAC sequences were then assembled using SPAdes Genome Assemblerv.3.1.1 (http://bioinf.spbau.ru/en/spades).

Genomic Sequence Annotation and Functional Prediction

Firstly, the genomic sequences of *SUS* genes were annotated by DNA subway (http://dnasubway.iplantcollaborative.org/). Sorghum and sugarcane ESTs from GenBank were taken as references, and the CDS sequences of *SUS* genes were translated into protein sequences using the EXPASy-translate tool (http://web.expasy.org/translate/). Subsequently, the conserved putatively domains of *SUS* protein were detected using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and InterPro (http://www.ebi.ac.uk/interpro/scan.html). Both the isoelectric point and relative molecular mass of the protein were graphed using ExPASy (http://web.expasy.org/compute_pi/). The exon-intron structures for the *SUS* genes annotated was displayed by executing GSDS (http://gsds.cbi.pku.edu.cn/).

Database Search and Phylogenetic Analysis

For phylogenetic analysis, the protein sequences of *SUS* family members from 15 plants, including 6 monocotyledons (*Zea mays, Sorghum bicolor, Oryza sativa, Ananas comosus, Saccharum spontaneum, Saccharum officinarum*), 6 dicotyledons (*Arabidopsis thaliana, Solanum lycopersicum, Vitis vinifera, Citrus sinensis, Gossypium spp, Beta vulgaris*), gymnosperm (*Pinusteada L.*), *Amborella trichopoda*as basal angiosperms, and one protista(*Acaryochloris marina*) as an outgroup from public databases available at various resources were primarily aligned using ClustalX 2.0(Thompson et al. 2002) with default parameters. The phylogenetic trees were constructed using the MEGA5.2.1(Tamura et al. 2011) program. We calculated pair distances between *SUS* alleles of *S. spontaneum* and *S. officinarum* respectively, as well as between these two subspecies at synonymous sites (Ks) and non-synonymous sites (Ka) by using the KaKs_Calculator (Zhang et al. 2006).

Expression Analysis Using RNA-Seq Data

Paired-end sequencing (100 bp reads length) was implemented with the HiSeq 2500 platform. To obtain clean reads, adapter sequences, empty reads, low-quality sequences, and reads with more than 10% bases with Q value <20 were removed from raw data by using Trimommatic. Subsequently, RNA-seq from different species were de novo assembled using Trinity v2.2.0, and the Trinity program was performed to map clean reads to assembled transcript s to calculate gene expression level with default parameters, FPKM.

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Authors' Contributions JZ designed the experiments. JZ and YS conceived the study. YS, HX, QS, JL,YW, XH, WY, QYand, RM performed the experiments and analyzed the data. JZ and YS wrote the manuscript. All authors read and approved the final article.

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