Sucrose Synthase Expressions in Sugarcane and Their Relations with Sucrose Accumulation

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ABSTRACT

Expression sequence tags and tentative consensus sequences of sucrose synthase (SUS) genes in sugarcane database were blasted based on well-characterized SUS genes from other fully genome sequenced plant species. SUS expression profiles from RT-qPCR on an elite cultivar Q117 grown in glasshouse showed distinctive but overlapped patterns of SUS members over different tissues and developmental stages. Further characterization on field grown high- vs. low-sugar lines demonstrated relatively tight correlations between sucrose contents in whole cane juice and transcript levels of two SUS members at different developmental stages, as well as their enzyme activities in sucrose cleavage direction. Prospects of the experiment results on enhancement of sucrose accumulation in sugarcane molecular breeding by manipulating SUS genes were discussed.

Keywords: *Saccharum* sp., Sucrose synthase expression, Sucrose accumulation

Abbreviations: SUS: Sucrose Synthase; CCS: Commercial Cane Sugar; TVD: Top Visual Dewlap

INTRODUCTION

Sucrose accumulation is particularly interest in sugarcane (*Saccharum* sp.) as it produces about 75% of world sucrose. It is a dynamic process of a continuous cleavage and synthesis in sugar storage parenchyma tissue [1,2]. About 22% of stored sucrose is cleaved and re-synthesized [3] in a process called a 'futile cycling'. Futile cycling is energy wasteful since ATP is required for sucrose resynthesis, which might be an important plant response to some specific environmental stresses. However, under favourable agronomic conditions, it could be minimized to enhance sucrose accumulation.

Sucrose synthase (E2.4.1.13) transfers the glucose moiety from sucrose to form uridine 5'-diphosphate glucose (UDPG) and leaves the fructose part behind. Though sucrose synthase catalyses a reversible reaction, it is widely believed that the digestion direction is the main reaction in mature sugarcane stem tissues [4,5]. In sucrose isomerise transformed sugarcane suspension cell lines, sucrose synthase activity showed the most consistent and strongest down-regulation among all sucrose hydrolysing enzymes, along with highly accumulated sucrose content [6]. Down-regulation of a sucrose synthase gene may enhance sucrose accumulation. The technique of down-regulating a specific gene is applicable in sugarcane [7,8].

Sucrose synthase is encoded by multiple genes in plant species, playing individual roles but having expression patterns overlapped [9,10]. In polyploidy sugarcane, several sucrose synthase genes have been cloned with full lengths [11-14] three forms sucrose synthase proteins have been partially purified from sugarcane tissues [15] and five genes have been identified based on the comparison with other fully genome-sequenced plant species such as sorghum [16].

However, the sucrose synthase expression patterns and their correlations with sucrose accumulation have not been reported yet in sugarcane.

In this study, we further classified the expressed sugarcane sucrose synthase genes available in sugarcane database and demonstrated their expression patterns in sugarcane. We revealed associations between expression levels of specific member(s) of sucrose synthases and sucrose contents in high- vs. low-sucrose lines derived from conventional breeding.

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MATERIALS AND METHODS

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Plant materials

Sugarcane from glasshouse

Sugarcane cultivar Q117 plants were grown in a containment glasshouse under natural light intensity at $28 \pm 2°C$ with watering twice a day. Each plant was grown as a single stalk in a pot of 20 cm diameter (4 L volume) and sampled as a 9-month old ratoon. Leaves were numbered as one for the top visual dewlap (TVD), with higher numbers for older leaves. Internodes were numbered according to the leaf attached to the node immediately above. The sampled tissues include non-photosynthetic (spindle)-3 and (spindle)-2 leaves, mature leaf blades (+3) and sections from the middle of internodes 3, 7 and 15. These internodes represent different physiological status of stalk that were elongating internodes, sucrose loading and matured, respectively. The roots were sampled by carefully selecting the white tender ones. Stem samples were rapidly cored by a hole-borer. All samples were immediately frozen in liquid nitrogen, then transported in liquid nitrogen to the laboratory and temporarily stored in -80°C for late analyses of sugars, or extractions of RNA and enzymes.

Sugarcane from conventional breeding

Eight lines with similar growth and stalk biomass from two bi-parental crosses (KQ97 from Q117 x MQ77-340, n=237; KQ04 from ROC1 × Q142, n=300) were selected for the experiment. Four lines with high commercial cane sugar (CCS) (KQ97-5080, KQ04-6493, KQ97-6677, KQ04-6498) and four with low CCS (KQ04-6461, KQ04-6641, KQ97-6765, KQ97-2599) were planted in a field trial with three replicates (10 m row a replicate), at Kalamia, North Queensland (19°32′S, 147°24′E). Normal commercial agronomic practices were applied. Samples were taken on the first ratoon crop, when the plants were 9 months old with around 22 internodes. In all samplings, materials were pooled from three plants per replicate. The numbering on internodes was the same as glasshouse sampling. Stem samples were rapidly cored by a hole-borer and frozen in liquid nitrogen in the field, then transported on dry ice to the laboratory for analyses of sugars, enzymes and RNA. The remainder of the culm from the sampled stalks was crushed using a small mill for juice extraction. Brix was measured on a 300 µl sample of this 'whole-stalk' juice using a pocket refractometer (PAL-1, Atago Co. Ltd, Japan) zeroed using Milli Q water prior to each sample.

RNA extraction and cDNA synthesis

Frozen plant tissues were ground into fine powder with liquid nitrogen by ball milling (Retsch MM301, Germany). Total RNA was extracted using Trizol following the kit protocol (Invitrogen). Each tissue was extracted with 3 replicates. RNA concentration was determined using a Nanodrop ND-1000 (Biolab).

Complementary DNA was prepared from 1 µg total RNA, following the protocol described in the Superscript III first strand synthesis kit (Invitrogen).

Primer design and RT-qPCR

Primers of the sucrose synthase genes for sugarcane were designed as subfamily-specific but universal within each subfamily. Mismatched base pairs for each subfamily were generally designed to be located at the 5' end of the primer and the total was minimized to less than 3% of the total base pairs involved (Table 1). Primer designing principles from the software package Primer Express (Applied Biosystems) were also considered for the five sucrose synthase gene members in sugarcane.

Table 1: Sugarcane *SUS* member specific primers used for RT-qPCR

Oligo Name	Primer Sequence	ESTs[1]	bps[2]	Mismatch[3] (%)
ScSUS1 F	TGGTCCGGCTGAGATCATC	35	665	1.8
ScSUS1 R	TCCAGTGGCTCGAATCTGTCTG	30	660	1.4
ScSUS2 F	GTGCGGTTTGCCAACAATT	40	800	3.0
ScSUS2 R	AAATATCTGCAGCCTTGTCACTGT	40	1000	1.9
ScSUS4 F	CATAACAGGACTGGTTGAAGCTTT	8	200	0.5
ScSUS4 R	CCTTGGACTTCTTGACATCATTGTA	9	234	0.4
ScSUS5 F	CACATATTCATTCCATTGAGACC	6	138	0.0
ScSUS5 R	TGTAACCATGTACACTTTCAGTC	6	138	0.0
ScSUS6 F	ATGTACTGGAACAGAATGTCC	5	105	0.0
ScSUS6 R	TGAAGGTTGTAGAACATTTGT	5	105	1.8
GAPDH F	CACGGCCACTGGAAGCA			
GAPDH R	TCCTCAGGGTTCCTGATGCC			

Available ESTS on the web sides in each subfamily
bps: total base pairs involved; bps=primer length × available ESTs;
Mismatch (%)=mismatched base pairs/(primer length in base pairs × EST number)

RT-qPCR was run on an ABI PRISM® 7900HT Sequence Detection System after preparation on an Eppendorf epMotion™ 5075 Workstation. Each 10 μL reaction contained 1x SYBR® Green PCR Master Mix (Applied Biosystems), 200 nM primers and 1:25 dilution of cDNA (from 40 μL cDNA synthesis). The RT-qPCR program was run at 95°C for 10 min, 45 cycles of 95°C for 15 s and 59°C for 1 min, then dissociation analysis at 95°C for 2 min and 60°C for 15 s ramping to 95°C for 15 s. Means from three sub-samples were used for each analysed cDNA sample.

Amplicons were cloned into pCR®2.1-TOPO® vector (Invitrogen) and multiple products were sequenced to confirm sucrose synthase member specificity.

The reference gene for quantitative PCR was the cytosolic isoform of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) that exhibited stable levels of expression in a broad range of sugarcane tissues [17].

Crude enzyme extraction

Enzymes were extracted by grinding the frozen powder (as for RNA extraction) in a chilled mortar using 3 volumes of extraction buffer that contained 0.1 M Hepes-KOH buffer, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 5 mM DTT, 2% PVP and 1x complete protease inhibitor (Roche) as detailed [18]. The homogenate was centrifuged at 10,000x *g* for 15 min at 4°C. The supernatant was immediately desalted on a PD-10 column (GE Healthcare) that was pre-equilibrated and eluted using an extraction buffer without glycerol. This desalted extract was used for enzyme assays. Protein concentration was assayed by the Bradford reaction using a Bio-Rad kit with bovine serum albumin standards.

Sucrose synthase assays

Sucrose synthase activity (breakage) was assayed in a reaction mixture comprising 100 mM Tris-HCl buffer pH 7.0, 2 mM $MgCl_2$, 160 mM sucrose and 2 mM UDP. Blank reactions without UDP were included as an additional negative control. After 30 min at 30°C, the assay was terminated by boiling for 10 min. The fructose product was measured using a BioLC as described below and further confirmed according to UDPG levels as described [18].

Sugar determination

To measure intracellular glucose, fructose and sucrose, the frozen powder was diluted in 1:20 water (w:w) and then heated for 10 min at 96°C to inactivate enzymes, centrifuged at 16,795x *g* for 10 min at 4°C to remove particulates and analysed by HPAEC [19].

BLAST searches

All sucrose synthase ESTs were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov) and all tentative consensus (TC) sequences were from the Computational Biology and Functional Genomics Laboratory (http://occams.dfci.harvard.edu/pub/bio/tgi/data/). Sorghum and rice genomes were blasted on the Phytozome database (http://www.phytozome.net/search.php).

Statistical analyses

Non-parametric t test and correlation analyses were performed using GraphPad Prism 6.0 software (San Diego, California, USA).

RESULTS

The nomenclature for the identification of sucrose synthase is inconsistent in previous publications. 'SuSy' is frequently used in the articles related to sugarcane. The most commonly used name is '*SUS*' for sucrose synthase genes in other model plants including rice (*Oryza sativa*) [20], Arabidopsis [9], cotton (*Gossypium* sp.) [21], Durum wheat (*Triticum durum,* cvs Ciccio, Svevo and Primadur) [22] and *Lotus japonicas* [23]. In this study we also use the serial gene names *ScSUS1* to *ScSUS6* for sugarcane corresponding to the rice *OsSUS1* to *OsSUS6* that have been clearly described [20].

ESTs and TCs related to sugarcane *SUS* genes in database were classified into five groups

Sugarcane genome has not sequenced yet. However, there are 282,683 ESTs with 42,377 TC sequences from 28 cDNA libraries in the sugarcane database. These libraries cover different organ/tissues (root, stem, leaf, inflorescence and seeds) and various developmental stages. The sugarcane EST database was searched by using each of the 6 transcript sequences of the rice *SUS* genes, resulting in 5 groups expressed genes because rice *OsSUS1* and *OsSUS3* fished out the same group of sugarcane genes (Table 2). The *ScSUS1* members accounted for two thirds of the total *ScSUS* ESTs or TCs and the *ScSUS2* members for 27.6%.

Table 2: Sugarcane ESTs and TCs blasted out from DFCI sugarcane gene index by rice *SUS* genes

Corresponding to rice gene	Sugarcane EST (>90% identity)		Number of sugarcane TCs (>90% identity)
	SUS number	% of SUS EST	
OsSUS1 (or OsSUS3)	534	66.42	35
OsSUS2	222	27.61	15
OsSUS4	27	3.36	4
OsSUS5	18	2.24	2
OsSUS6	3	0.37	1

The group of the sugarcane ESTs searched out had higher homology with *OsSUS1* than with *OsSUS3,* for example, the TC123316 (Table 3). We observed the similar results when we used rise *OsSUS1* and *OsSUS3* to search maize ESTs (Table 3). Aligning the rice OsSUS1 or OsSUS3 protein with the putative polypeptide in either sorghum, maize or millet showed SUS1 has higher similarities and identities than that of OsSUS3 (Table 4).

Table 3: Blasting scores on sugarcane or maize EST and tentative consensus database by rice sucrose gene *OsSUS1* and *OsSUS3*

	Rice	
	OsSUS1	*OsSUS3*
Sugarcane TC123316	10164	9208
Maize TC549963	9784	9011

Table 4: Similarity/identity between rice OsSUS1 or OsSUS3 and corresponding putative SUS proteins from Sorghum, maize and millet

	Sorghum	Maize	Millet
OsSUS1	96.9/95.3	97.3/95.2	97.2/95.3
OsSUS3	94.0/89.8	94.0/90.0	93.90/90.0

The above comparisons between rice and sorghum (or maize, or millet) in ESTs and putative proteins suggested that either the *OsSUS3* gene has not expressed or lost in these species. Blasting genome sequences of sorghum, maize and millet with cDNA sequences of the six rice *SUS* genes [20] identified 5 loci (Table 5, top) further indicated *OsSUS3* is lost in all these tested C4 species. In clear contrast, blasting other sequenced C3 plants showed that they have both *OsSUS1* and *OsSUS3* loci located either on the same or different chromosomes (Table 5, bottom). (To be consistent, *SbSUS1* to *SbSUS6* will be used for genes of sorghum sucrose synthases, corresponding to the rice *OsSUS1* to *OsSUS6*; *ZmSUS1* to *ZmSUS6* for corn; *SiSUS1* to *SiSUS6* for millet; *BdSUS1* to *BdSUS6* for purple false brome; *PtSUS1* to *PtSUS6* for poplar).

Table 5: Chromosome distributions of *SUS* genes relative to the corresponding rice genes in C4 plants (sorghum, maize and millet) and C3 plants (purple false brome and poplar putative)

Rice	C4 plants								
	Sorghum bicolor			*Zea mays*			*Setaria italica*		
	Gene	X#[1]	Gene code[2]	Gene	X#[1]	Gene code[2]	Gene	X#[1]	Gene code[2]
OsSUS1	*SbSUS1*	1	sb01g033060	*ZmSUS1*	9	GRMZM2G152908_T01	*SiSUS1*	9	Si034282m.g
OsSUS2	*SbSUS2*	10	sb10g006330	*ZmSUS2*	9	GRMZM2G089713_T04	*SiSUS2*	9	Si005859m.g
OsSUS3	*SbSUS1*	1	sb01g033060	*ZmSUS1*	9	GRMZM2G152908_T01	*SiSUS1*	9	Si034282m.g
OsSUS4	*SbSUS4*	1	sb01g035890	*ZmSUS4*	1	GRMZM2G318780_T02	*SiSUS4*	9	Si034293m.g
OsSUS5	*SbSUS5*	10	sb10g031040	*ZmSUS5*	5	GRMZM2G060659_T02	*SiSUS5*	1	Si020148m.g
OsSUS6	*SbSUS6*	4	sb04g038410	*ZmSUS6*	4	GRMZM2G045171_T01	*SiSUS6*	1	Si005845m.g
OsSUS5							*SiSUS5*	4	Si020148m.g

Rice	C3 plants					
	Brachypodium distachyon			*Populus trichocarpa*		
	Gene	X#[1]	Gene code[2]	Gene	X#[1]	Gene code[2]
OsSUS1	*BdSUS1*	Bd1	Bradi1g60320	*PtSUS1*	18	POPTR_0018s07380
OsSUS2	*BdSUS2*	Bd1	Bradi1g46670	*PtSUS2*	6	POPTR_0006s13900
OsSUS3	*BdSUS3*	Bd1	Bradi1g20890	*PtSUS3*	6	POPTR_0006s13900
OsSUS4	*BdSUS4*	Bd1	Bradi1g62957	*PtSUS4*	2	POPTR_0002s19210
OsSUS5	*BdSUS5*	Bd1	Bradi1g29570	*PtSUS5*	15	POPTR_0015s05540
OsSUS6	*BdSUS6*	Bd3	Bradi3g60687	*PtSUS6*	17	POPTR_0017s02060
OsSUS5				*PtSUS5*	12	POPTR_0012s03420
OsSUS5				*PtSUS5*	4	POPTR_0004s07930

X#: The chromosome number on which each *SUS* gene is located
The gene codes in the Phytozome from Joint Genome Institute (http://www.phytozome.net)

Sugarcane *SUS* genes in database showed their overlapping expression patterns

ESTs or TCs belonging to the same *ScSUS* subfamily were mapped to organs and tissues based on their appearance in different libraries to obtain a general picture of sugarcane *SUS* expression patterns (Table 6). *ScSUS1* expressed in almost all libraries across different organs, tissues and developmental stages, except for developing seeds and mature leaves. Even though *ScSUS2* was less compared to *ScSUS1* (Table 2), it expressed more extensively than *ScSUS1* across all tissues and developmental stages. Overlapping patterns of *SUS* genes is typical except for *ScSUS6*. *ScSUS6* has only one TC and three ESTs, appearing only in the stalk bark cDNA library. It should be pointed out that this analysis has indicated only the overlapping patterns of the *SUS* expressions and the real proportion of each *SUS* member will be analysed in next section.

Table 6: Tissue expressions of the sugarcane ESTs, homologous to *SbSUS* isoforms, from different libraries. ESTs attributable to specific organ/tissue libraries are presented in this table. The value inside parenthesis is the number of ESTs attributed to this subfamily; outside the parenthesis is the percentage relative to different tissues to this family

Organ	Stage	*ScSUS1* 100 (412)[a]	*ScSUS2* 100 (146)[b]	*ScSUS4* 100 (19)[c]	*ScSUS5* 100 (19)[d]	*ScSUS6* 100 (3)
Callus		4.2 (18)	6.2 (9)	5.0 (1)		
Root	Young	5.4 (22)	9.6 (14)			
	Mature	7.8 (32)	16.5 (24)	5.0 (1)	5.3 (1)	
Shoot/root transition		6.6 (27)	13.8 (20)		10.5 (2)	
Stem	Meristem+IN#1	15.9 (66)	13.6 (16)		10.6 (2)	
	Young	3.4 (14)	0.7 (1)	5.0 (2)	5.3 (1)	
	Mature	1.7 (7)	4.9 (7)	25.0 (5)	5.3 (1)	
	Bark	7.3 (30)	6.2 (9)	10.0 (2)	21.1 (4)	100 (3)
Leaf	Etiolated	1.5 (6)	2.1 (3)			
	rolls	13.1 (54)	5.6 (8)	15.0 (3)	15.9 (2)	
	mature		0.7 (1)			
Lateral buds		4.1 (17)	4.2 (6)	20.0 (4)		
Seeds			4.8 (7)	10.0 (2)		
Inflorescence and rachis	Young	7.8 (32)	3.5 (3)		15.8 (3)	
	Mature	18.4 (76)	10.2 (15)	5.0 (1)	15.8 (3)	

Organ/tissue un-attributable EST numbers in each subfamily: a. 24 ESTs; b. 49 ESTs; c. 8 ESTs; d. 1 EST

Sucrose synthase isoforms differentially expressed in glasshouse grown sugarcane

Expression profiles of *ScSUS* members were further characterized by RT-qPCR in the elite commercial sugarcane variety Q117 grown under glasshouse conditions. *ScSUS6* was not detected from the selected material for RNA extraction. Figure1 illustrates the expression levels for the rest four SUS members as normalized to the constitutive GAPDH gene transcript level. There were relatively small changes in the mRNA pool sizes of the *ScSUS4* and 5 between different tissues and developmental stages. *ScSUS1* and *ScSUS2* not only accumulated high levels of mRNA but also showed large variations. Sink organs such as elongating internodes, young roots and non-photosynthetic leaf blades presented large pool sizes of *ScSUS2* and especially *ScSUS1* isoforms. The mRNA amount of *ScSUS1* was still high in mature stem tissues.
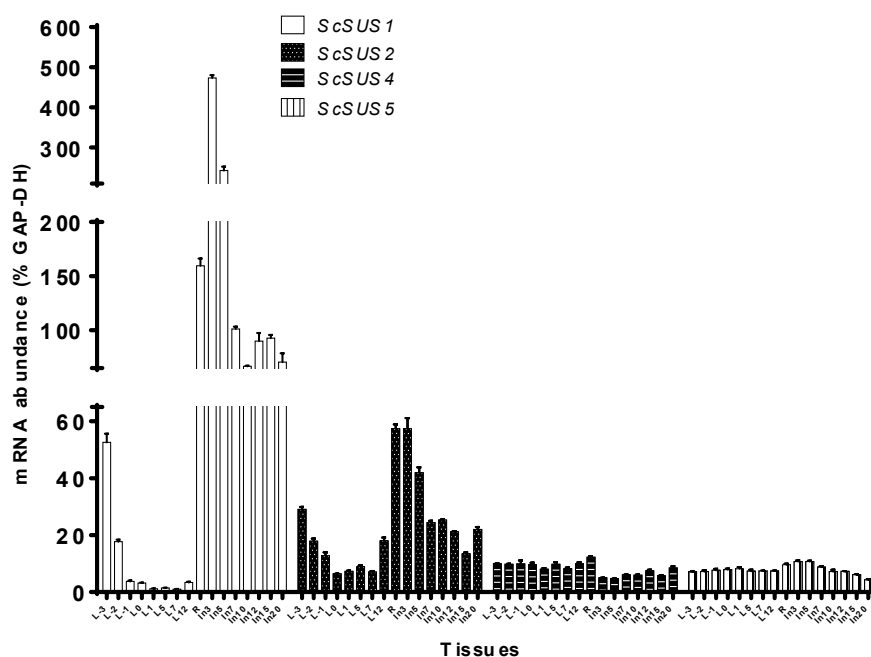

Figure 1: Transcript levels of the *SUS* genes in various sugarcane tissues, The sugarcane plant Q117 was 9 month old ratoons with 22 internodes grown under glasshouse conditions. L: leaf blades; In: Internodes; R: white young roots. The numbers after L or In are numbers from the top visual dewlap (TVD). Values are means (3 replicates) with SE

Expressions of sucrose synthase genes were differentially reduced in the high-CCS stem tissues

SUS mRNA profiles were compared between two populations of sugarcanes with high-CCS vs. low-CCS lines to determine if any relationship exists between sucrose accumulation and *SUS* gene expression. Table 7 illustrates detailed sucrose contents at different developmental stages of the sugarcane stalks. RT-qPCR was performed on the three typical developmental stages along stem (elongating internode #3, peak sucrose loading internode #7 and matured internode #15) and sink/source leaves.

Table 7: Sucrose contents in sugarcane stem tissues of the 4 high-CCS and 4 low-CCS lines. The samples were collected from 9 month old ratoons grown in the field. Values are means of 3 reps ± SE

Internodes	Sucrose content in lines (mM)							
	High-CCS				Low-CCS			
	5080	6493	6677	6498	2599	6461	6641	6765
3	126 ± 13	83 ± 12	362 ± 16	74 ± 21	116 ± 15	111 ± 12	53 ± 9	71 ± 10
7	126 ± 17	419 ± 14	561 ± 14	391 ± 10	256 ± 22	394 ± 18	483 ± 14	263 ± 20
15**	494 ± 27	576 ± 15	677 ± 15	528 ± 11	339 ± 12	454 ± 31	441 ± 21	430 ± 28

**There is a significant difference ($P<0.01$) in the means of sucrose contents (pooled 4 lines) between high-CCS and low-CCS lines in internode15 by nonparametric t test

Similar to the data observed from the glasshouse grown sugarcanes, the *ScSUS1* accumulated the highest level of transcripts among all the tested *ScSUS* members in stem and young leaf tissues (Figure 2) of the field samples. More importantly, significant differences were observed between the high-CCS and the low-CCS lines in *ScSUS1* transcripts in mature sugarcane internode 15 and in *ScSUS2* transcripts in peak sucrose loading internode 7 ($P=0.0006$) (Figure 2).
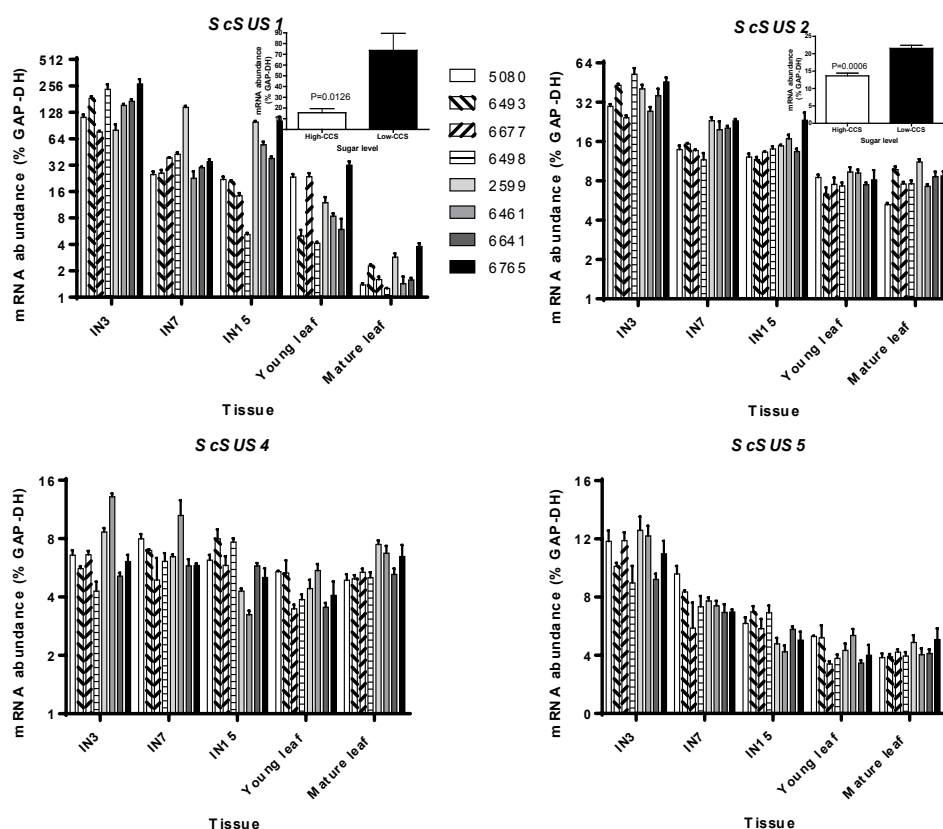
33

Figure 2: Relative expressions of *ScSUS1* (top left), *ScSUS2* (top right), *ScSUS4* (bottom left) or *ScSUS5* (bottom right) in stem and leaf tissues of the 4 high-CCS (the left 4 bars in each group) and 4 low-CCS (the right 4 bars in each group) lines The samples were from 9 month old ratoons grown in the field. Value of each bar is mean of 3 reps ± SE. IN: internode #. Young leaf: sink leaf; Mature leaf: source leaf. Note the significant difference in comparisons in the insets showing a nonparametric t test on average values of the 4 lines in internode15 (top left) and in internode 7 (top right) in the corresponding high- or low-CCS lines

Significant reduction (P<0.01) in *ScSUS1* transcripts was observed from internode #7 to internode #15 in high-CCS group but not in low-CCS group (Figure 2). In contrast, significant reductions were observed in *ScSUS1* and *ScSUS2* transcripts from internode #3 to internode #7 in both high-CCS and low-CCS canes (Figure 2), which is in agreement with the data from glasshouse grown cane (Figure 1) and also with the young tissue richness of *ScSUS1* and *ScSUS2* ESTs in database (Tables 2 and 6).

ScSUS1, ScSUS2 and *ScSUS5* genes were expressed less in leaves than that in stem tissues; especially *ScSUS1*.There was no significant difference in *ScSUS4* between different organs and developmental stages (Figure 2).

To find out if there is a coarse regulation on sucrose accumulation by *SUS* transcripts, *SUS* mRNA levels and sucrose contents in whole cane juice of the sugarcanes grown in the field were further analysed. There was a strong correlation (P<0.0001) between *ScSUS1* mRNA pool size in internode 15 and sucrose content in whole cane juice (Figure 3c). The inverse relationship (P<0.0001) was also observed between *ScSUS2* mRNA pool size in internode 7 and sucrose content in whole cane juice (Figure 3e).

A strong correlation between *ScSUS1* transcripts in internode15 and *ScSUS2* ones in internode 7 was found, implying a coordination between different *SUS* genes in different developmental stages. To find out whether the regulation on sucrose accumulation by transcripts of internode 15 *ScSUS1* and of internode 7 *ScSUS2* is via their enzymes, we further measured SUS activities in breakage direction (SUS(b)) on the same plant materials (Table7). The SUS(b) activity was significantly higher in low-CCS lines than that in high-CCS lines in internodes 7 (P=0.0378) and 15 (P=0.0021). Inverse relationships between sucrose contents in whole cane juice and SUS(b) activities in internode 7 (Figure 5b) or 15 (Figure 5c). The strength order of *in vitro* activity was #7, #15 then #3 (Table 8) different from the patterns at *ScSUS* transcripts (Figure 2).

Table 8: SUS breakage (SUS(b)) activities in stem tissues of the 4 high-CCS and 4 low-CCS lines The samples were from 9 month old ratoons grown in the field. Values are means of 3 reps ± SE

Internode	Enzyme activities (n mol mg^{-1} protein min^{-1})							
	High-CCS				Low-CCS			
	5080	6493	6677	6498	2599	6461	6641	6765
3	19.8 ± 1.2	9.8 ± 0.8	9.0 ± 0.6	15.8 ± 0.7	8.1 ± 1.4	6.0 ± 0.6	9.5 ± 0.6	10.0 ± 0.4
7*	43.7 ± 1.5	53.5 ± 3.1	46.0 ± 0.8	29.9 ± 1.9	121.9 ± 2.9	67.8 ± 2.3	64.9 ± 10.7	71.4 ± 2.4
15**	17.8 ± 1.1	18.9 ± 15.3	20.5 ± 1.0	22.1 ± 1.4	44.2 ± 2.7	29.4 ± 0.9	35.2 ± 1.8	41.5 ± 2.5

There is a significant difference in enzyme activities between high-CCS and low-CCS lines in internode7 (*P<0.05) and internode 15 (**P<0.01) by nonparametric t tests
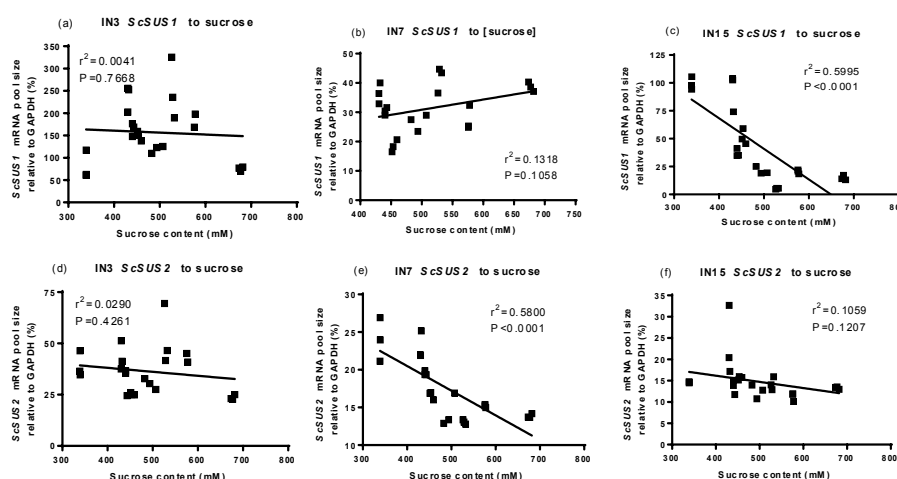
Figure 3: The relationships between sucrose contents in whole cane juice and *ScSUS1* mRNA pool sizes (a, b, c) or ScSUS2 mRNA pool sizes (d, e, f) in internode 3 (a, d), internode 7 (b, e) and internode 15 (c, f) of the 4 high-CCS and 4 low-CCS lines shown in Table 7
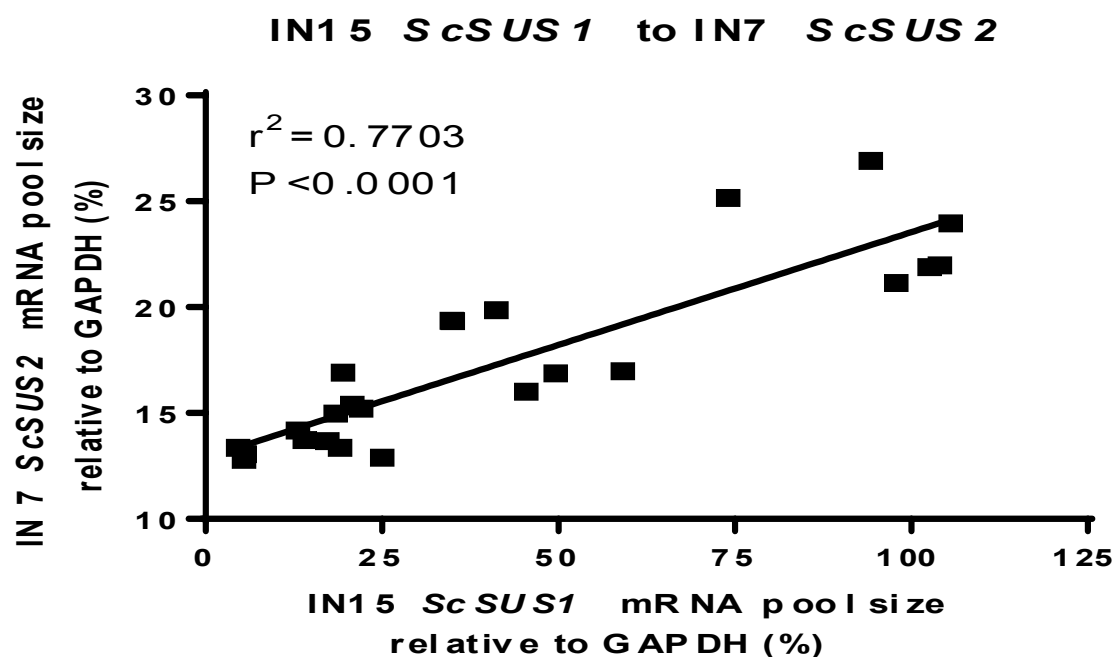
Figure 4: Correlation between internode 15 *ScSUS1* mRNA amounts and internode 7 *ScSUS2* mRNA levels of the 4 high-CCS and 4 low-CCS lines shown in Table 7

Relationship between transcript levels of a specific *ScSUS* member and SUS(b) activities could also be established in some cases: *ScSUS1* transcripts correlated with SUS(b) activity in internode 15 and *ScSUS2* with SUS(b) in internode 7 (Figure 6).
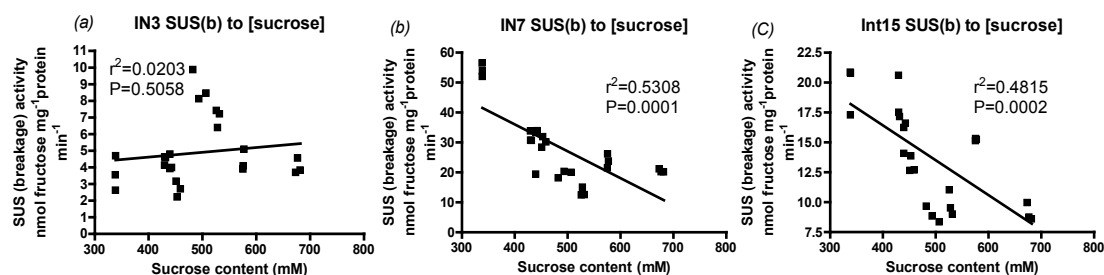
Figure 5: The relationships between sucrose contents in whole cane juice and SUS breakage activities (SUS(b)) in internodes 3 (a), 7 (b) and 15 (c) of the 4 high-CCS and 4 low-CCS lines shown in Table 7
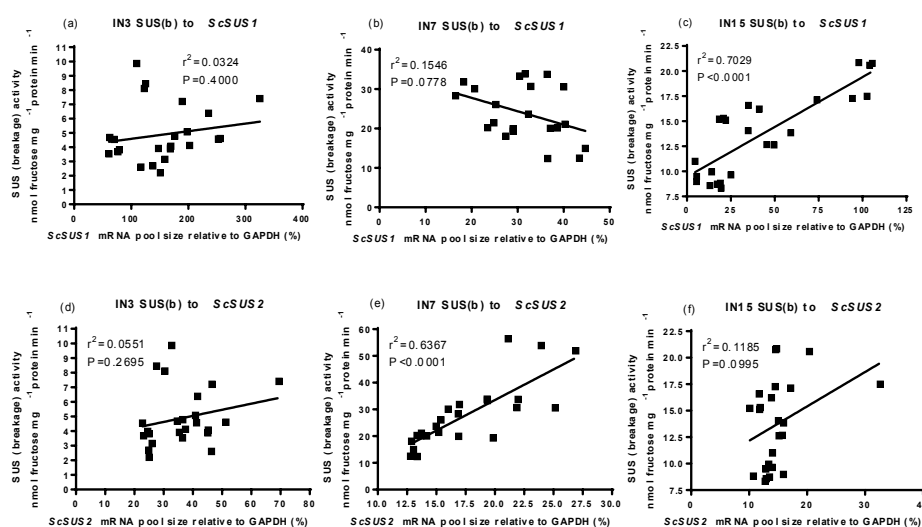

Figure 6: The relationships between SUS breakage (SUS(b)) activities and *ScSUS1* mRNA pool sizes (a, b, c) or *ScSUS2* mRNA pool sizes (d, e, f) in internodes 3 (a, d), 7 (b, e) and 15 (c, f) of the 4 high-CCS and 4 low-CCS lines shown in shown in Table 7

DISCUSSION

In this study, through extensive sugarcane cDNA database searches by using rice and sorghum *SUS* genes, we have classified the expressed sugarcane *SUS* genes into 5 subfamilies. Expression profiles in different sugarcane tissues were characterized using RT-qPCR technique based on the classification. Comparison of expression patterns between high- and low-CCS lines revealed that sucrose accumulation in stem was, at least partially, regulated by specific *SUS* gene transcription at specific developmental stages. After searching some available plant genome sequences by using well-described six rice *SUS* genes, we found sorghum, along with other C4 plant species, has 5 loci of *SUS* genes with the *SUS3* missing compared to C3 plants.

Results in the current study indicate that *ScSUS1*, as the largest mRNA pool size among all *ScSUS* members, was predominately expressed in sugarcane stem and root tissues, though also in leaves. This is in agreement with the expression pattern of rice *OsSUS1* (20). Consistently, more than 66% of the total *ScSUS* ESTs appeared as *ScSUS1* in the searched sugarcane database. Several cDNAs with full lengths cloned by different research groups [11-14] drop into different *ScSUS* subfamilies. Increasing evidence suggests one of its main functions is to channel UDP-glucose for the cellulose synthase (CesA) in cell wall thickening [24]. However this hypothesis is also challenged by the fact that the SUS1 proteins are not always parallel to the CesA [25]. Deficient sucrose synthase activity in developing wood does not specifically affect cellulose biosynthesis, but causes an overall decrease in cell wall polymers [26]. High expression level of *ScSUS1* along whole sugarcane stem implies other functions are involved. The high-CCS lines grew normally with significantly reduced *ScSUS1*expression levels in this study indicates sugar content could be improved by manipulating *ScSUS1* expression in matured sugarcane stem tissues (Figure 2a).

ScSUS2, as the second largest mRNA pool sizes in stem, leaf and root tissue, showed less difference between leaf and stem tissues than *ScSUS1* did in the current study. The cDNA database indicates *ScSUS2* expressed in a wide

range of tissues, which is agreement with those reported in rice [20,27]. Of the high-CCS lines, in contrast to the *ScSUS1* which showed significant difference in expression levels at matured internode 15, *ScSUS2* transcript levels showed significant reduction in sucrose loading internode 7 (Figure 2b). Rice *OsSUS2* genes could be induced under submerging conditions [20,28], whilst *At SUS1* and *4* in Arabidopsis could be induced by hypoxia [10,28,29]. It is not known if *ScSUS2* can be induced by stress yet, but it (also *ScSUS4*, no other members) could be found in the cDNA libraries from developing seeds (under desiccate stress) (Table 6).

Gene expression and metabolism could be directly regulated at the transcript level [28,29]. Experimental results in this study imply a transcriptional coarse control on sucrose accumulation via SUS(b) enzyme activities, at least partially. Three pieces of experimental data support this argument:

1) Tight associations of sucrose content in whole cane juice with SUS enzyme activity (breakage) in the maturing and matured internodes (Figure 5).

2) Strong correlations between *ScSUS1* expression level in matured internodes (and between *ScSUS2* expression level in maturing sucrose loading internodes) and sucrose contents (Figure 3).

3) Coincidence of the significant reductions in mRNA pool sizes of *ScSUS1* and *ScSUS2* genes in matured and sucrose loading internodes, respectively (Figure 4).

However, the mRNA pool sizes and even *in vitro* enzyme activities are not measures of flux. Detailed investigation on the roles of the different SUS isoforms encoded by these *SUS* members on sucrose accumulation will be required.

Sucrose stored in sugarcane storage tissue is not static in that sucrose is soluble and ready to be metabolized. Digestion and re-synthesis of sucrose is a continuous process in sugarcane stem, a process called 'futile cycling', which metabolizes 22% of stored sucrose (3). In response to a particular stress environment; futile cycling may be important for plant. On the other hand, under favourable agronomic conditions, it might be decreased to improve sucrose building up. SUS(b) activities was down-regulated in the sucrose isomerase transformed suspension cell lines characterized as high-sucrose content (6). High-CCS lines showed expression of *ScSUS1* and *ScSUS2* genes were down-regulated in this study. Sugar crops are well below the theoretical physiological limits to sugar accumulation. Further improvement on sucrose accumulation might be achieved by down-regulating the expression of genes that encode sucrose-breakdown enzymes, like *ScSUS*.

There was a typical overlapping of expression patterns for all sugarcane SUS genes except ScSUS6, which is consistent with other characterized species such as Arabidopsis and rice. Since sucrose synthase plays an important role in wide variety of processes including storage [30,31], nitrogen fixation [32], cellulose synthesis [33], xylogenesis [34], starch synthesis [35], phloem transport [36], fruit ripening [37], and auxin signalling [38], it is necessary to down-regulate a specific *SUS* member in appropriate tissue and developmental stages for enhancement of sucrose accumulation.

ScSUS4 and *ScSUS5* expressed relatively lower levels than *ScSUS1* and *ScSUS2* did in all tested tissues at all different developmental stages (Figures 1 and 2). Consistently, the ESTs from these two *ScSUS* genes together only accounted for 5.6% of the total *SUS* genes (Table 6). These two members did not show any difference between the high- and low-CCS lines (Figure 2).

The RXXS consensus element recognized by Ser/Thr protein kinase in the N-terminal region of SUS proteins [39] appeared in all ScSUS proteins. According to the method described by Komatsu et al. [40] and Hirose et al. [20], the deduced proteins from the longest sugarcane TCs and the cloned full length cDNAs [11-14] could be classified into four isoforms. High similarity (87.3%), comparable molecular weights and isoelectrical points of the deduced polypeptides between *ScSUS1* and *ScSUS2* indicate they might belong to same protein isoform. ScSUS4, ScSUS5 and ScSUS6 could each be individual protein isoforms. ScSUS6 was found only in the stalk bark library and it was not detected in this study, probably due to the limited sample tissues. Consistently, three SUS isoforms (SuSyA, B, C) were identified and purified in sugarcane [15]. The immunochemistry data indicated that the presence of ScSUS proteins throughout young and mature tissue [5] is agreement with the SUS expression patterns at transcriptional and enzyme activity levels in this study. It requires more studies to match the expressed *ScSUS* genes in this study to the purified SUS proteins of SuSyA, B, C [15]. Duncan et al. [41] demonstrated three maize SUS isoforms differed in tissue distribution, intracellular localization and enzyme phosphorylation, which are important for cytosolic and membrane–associated sucrose degradation. Duncan and Huber [42] further pointed out sucrose concentration and

hyper-phosphorylation regulate sucrose synthase oligomerization, resulting in association with plasma membrane and actin. More study is required to understand how these finely tune the regulation of SUS enzymes related to sucrose accumulation in sugarcane.

COMPETING INTERESTS

The authors have no conflict of interests.

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