RESEARCH ARTICLE

EXPRESSION PROFILING OF GENES INVOLVED IN SUCROSE METABOLISM IN DIFFERENT *SACCHARUM* SPP. AND COMMERCIAL SUGARCANE HYBRIDS

P. T. Prathima*, G. S. Suresha and A. Selvi

Abstract

A comprehensive analysis of the transcript levels of genes involved in sucrose accumulation is fundamental for the assessment of the function of each enzyme and regulatory mechanism of sucrose metabolism in sugarcane. The expression profiles of genes encoding sucrose phosphate synthase (SPS), sucrose phosphate phosphatase (SPP), soluble acid invertase (SAI), cell wall invertase (CWI), pyrophosphate fructose-6-phosphate phosphotransferase (PFP), fructokinase (FK), hexokinase (HK) and ADP-glucose pyrophosphorylase (ADP-G) in sugarcane was examined using RT-PCR. Different modes of gene expression were observed for each gene revealing the complexity of sucrose metabolizing pathway in sugarcane. Transcript levels of SPS, SPP, SAI, CWI, Sucrose transporter (SUT1 and SUT4) were found to be differentially regulated between high and low sugar commercial sugarcane hybrids. But no definite pattern of expression of these genes was observed in different Saccharum spp. Similarly PFP, FK and HK were also differentially expressed in commercial sugarcane hybrids as compared to different Saccharum spp suggesting the role of these enzymes in the regulation of sucrose metabolism in sugarcane. Expression of Sucrose synthase (SuSy) was uniform in both high and low sugar genotypes of Saccharum spp. and commercial sugarcane hybrids whereas expression of ADP-G was found only in high sugar Saccharum officinarum clone.

Prathima, P. T., G. S. Suresha and A. Selvi
Sugarcane Breeding Institute
Coimbatore - 641007, Tamil Nadu, India
email: prathimasambandam@gmail.com

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Introduction

Sucrose metabolism is the most studied physiological process in sugarcane. A remarkable feature of sugarcane is its capacity to store sucrose to about 25% of its fresh weight. The ability to accumulate sucrose in storage parenchyma is the net result of sucrose synthesis and breakdown. The important enzymes involved in sucrose metabolism include sucrose phosphate synthase (SPS), sucrose synthase (SuSy) and invertases. SPS, SuSy and invertases have been proposed as key regulators for the accumulation of sucrose in sugarcane stem storage parenchyma (Zhu et al. 1997; Sacher et al. 1963; Slack 1965). Later research, however, discounted the importance of only these three enzymes and several other enzymes such as sucrose phosphate phosphatase (SPP), pyrophosphate fructose-6-phosphate phosphotransferase (PFP), phosphofructokinase (PFK), fructokinase (FK), etc. or the balance of activities of several enzymes appeared to be more important in sucrose metabolism.

Despite much scientific interest in sucrose metabolism, not much has been studied on the regulatory aspects of these pathways in sugarcane (Moore 1995; Lingle 1999). Exact rate limiting steps or control points were not yet identified. Results obtained so far clearly demonstrate that this major pathway is complex and inter-linked with every primary metabolic activity of the cell and thus it is difficult to modify any gene or its product without affecting its growth and development of the crop. There are several alternative pathways and enzymes forming a network of reactions for the

interconversion of sucrose and other metabolites. The roles of these alternative and parallel pathways are highly flexible and influenced by environmental and developmental changes. Hence, sucrose metabolism in sugarcane is highly dynamic as the sugar-responsive genes and rapid turnover of sucrose between cell compartments contribute to adaptive changes in the crop (Whittaker and Botha 1997).

The genus *Saccharum* consists of three cultivated spp, namely *S. officinarum, S. barberi* and *S. sinense,* and two wild spp. of *Saccharum robustum* and *S. spontaneum.* There is wide variation in sugar concentration (Brix) in the *Saccharum* species ranging from 2 in a *Saccharum robustum c*lone (Rao et al. 1985) to 24.5 in a *S. officinarum* clone (Sreenivasan and Nair 1991). *Saccharum officinarum* or the 'noble canes' accumulate very high levels of sucrose in the stem but have poor disease resistance. It has undergone prolonged directional selection for sucrose content, better cane thickness and low fiber. *Saccharum officinarum* accumulates no sucrose but is a highly polymorphic species with much higher levels of disease resistance, adaptability and stress tolerance (Sreenivasan et al. 1987). *Saccharum officinarum* and *S. robustum* genotypes are considered to be closely related groups (Nair et al. 1998). *Saccharum robustum* is a wild species and is considered to be an intermediate step in the evolutionary pathway between *S. spontaneum* and *S. officinarum.* The species *S. sinense, S. barberi* and *S. robustum* also provided minor contributions toward the development of some modern sugarcane varieties. Sugarcane varieties presently under cultivation have been derived through artificial crosses between two or more of these *Saccharum* spp. as the source of genetic materials (Roach 1995; Amalraj et al. 2006).

Although sucrose content as a trait is of relatively high heritability, progress in its improvement in the varieties has been slow. Understanding differences in the expression of genes related directly or indirectly to sucrose accumulation in different *Saccharum* spp. is an important step for improvement of sucrose content (Casu et al. 2003). Most of the studies are related to single genes which are directly associated with sucrose metabolism. Because several inter-connected pathways can utilize the same substrates, and since sucrose has more functions in a cell, it is difficult to understand how sucrose synthesis and accumulation are regulated. The question as to what limits sucrose accumulation and which enzymes are the most important in the regulation of sucrose synthesis therefore remains unresolved. Therefore, in the present study we have selected SPS, SPP, SuSy, SAI, CWI, VI, NI, SUT4, SUT1, PFP, PFK, FK, HK and ADP-G genes which regulate sucrose metabolism. We attempted to analyze the transcript expression pattern of these genes related to sucrose metabolism in sugarcane through reverse-transcription polymerase chain reaction (RT-PCR). We performed these validations in genotypes (*Saccharum* spp. and commercial sugarcane hybrids) differing in levels of sucrose accumulation to further strengthen the correlation.

Materials and methods

Plant materials

Commercial sugarcane hybrids were grown during February 2010 at SBI Coimbatore. The *Saccharum* spp. were selected based on the percent Brix reported by various workers from Sugarcane Breeding Institute (SBI), Coimbatore (Sreenivasan and Nair 1991; Kandasami et al. 1983; Rao et al. 1985). Cane samples from different Saccharum spp. were collected from SBI Research Center, Agali, and molecular studies were conducted at SBI, Coimbatore. Details of the plant materials from different Saccharum spp. and commercial sugarcane hybrids used in the study are listed in Table 1. Different internodal regions of the stem tissues (top, middle and bottom) from 10 month old plants were pooled for the analysis of gene expression. Samples were collected at around 10:00 hours and frozen at -80^{0}C for 2-3 h. All the samples were ground to a fine powder with pestle and mortar using liquid nitrogen and utilized for the extraction of total RNA.

RNA extraction and first strand cDNA synthesis

Total RNA was isolated using a Tri reagent (Sigma, St.Louis, MO, USA) as per the manufacturer's protocol. Isolated RNA was treated with RNase free DNase to remove genomic DNA contamination

Table 1. Plant materials used in the study

Plant material	Name of the genotype	
	High sugar (Brix)	Low sugar (Brix)
Saccharum spontaneum	SES 32 A (16.5)*	SES 606 (6.3)*
Saccharum robustum	Mol 4972 (9.43)*	51 NG 27 (2.92)*
Saccharum officinarum	57 NG 174 (20.30)*	Laukona 15 (4.90)*
Saccharum sinense	Uba-Naquin (15.84)*	Takcha chung Tseng (3.89)*
Saccharum barberi	Pathri (17.79)*	Dhaur Aligarh (5.92)*
Commercial sugarcane hybrids	CoC 671 (23.9)** Co 86032 (22.5)** Co 99006 (21.8)**	MS 68 / 47(18.5)** Co 62175 (16.9)**

* Values as reported in the catalogues (Sreenivasan and Nair 1991; Kandasami et al. 1983; Ramana Rao et al. 1985);
**Observed Brix values for the commercial sugarcane hybrids

(DNA free, Promega). Integrity and normalization of RNA content was analyzed through 1.2% agarose gel electrophoresis. The purified RNA was stored at -20^{0}C until use. cDNA was made from total RNA with RevertAid H reverse transcriptase at 42^{0}C (Fermentas Inc, USA) as per supplier's instructions.

Expression analysis

Gene specific primers were designed for each gene based on the known plant gene sequences available in the NCBI database. Oligonucleotide sequences of all the genes are given in Table 2. One tenth of cDNA product was used as template for PCR amplification reactions. PCR amplification was performed with the high fidelity *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India).The thermo cycler (PTC-100™ Programmable Thermal Controller, MJ Research, Inc., Watertown, USA) was programmed for 35 cycles of 94 °C for 1 min, 50-58 °C for 1 min (depending on the melting temperature of primers for each gene) and 72°C for 2 min. Transcript expression pattern was observed in 1% agarose gel electrophoresis.

Results and discussion

Differential transcript expression of genes was observed between the high and low sugar genotypes. In general, the *S. officinarum* and *S. robustum*

Table 2. Sugar metabolizing genes used for expression analysis in sugarcane

Gene	Oligonucleotide sequences	Product size (bp)
Sucrose phosphate synthase (SPS)	F5'-TAGCTGTTGCTCGCCCTTAT-3' R5'-TCAGGAACTTCCCGAGTGCTT-3'	241
Sucrose synthase (SuSY)	F5'-ACCGCGTTGTCCACGGCATT-3' R5'-AGCTCCTGCAGCCGCTTGTT-3'	236
Sucrose phosphate phosphatase (SPP)	F5'-GGCTTTGTGCTAACCCACAT-3' R5'-TTACGCACCAAATCCTCTCC-3'	240
Soluble acid invertase (SAI)	F5'-TCCTTGCTTGCCTCTCAAAT-3 'R5'-ACAAATGTAGCCCTGCCTTG-3'	628
Cell wall invertase (CWI)	F5'-CAAGGAAGATCTGGCTCGAC-3' R5'-CTCGAAGGTCACCTCCACAT-3'	176

Vacuolar acid invertase (VAI)	F5'-GACATCGTCAAGAGGGTCGT-3' R5'-GGCGTTGTTGAAGAGGAAGA-3'	189
Neutral invertase (NI)	F5'-ATAAACAGCCGCACCAATTC-3' R5'-GCCTCTGAGGTGGAGTCTTG-3'	250
Sucrose transporter (SUT1)	F5'-CCACAAACAATTGGCACAAG-3' R5'-TTGCTTTTGTTGGGAGGTTC-3'	183
Sucrose transporter (SUT4)	F5'-ACAAAACCCTGGTGCTGAAC-3' R5'-GGGCTTCCTCGGTAGATTTC-3'	177
Pyrophosphate dependent phospho fructokinase (PFP)	F5'-GCATTGGAATGTGCTCTTCA-3' R5'-CACAAGGCCCTCAGGAATAA-3'	171
Phospho fructokinase (PFK)	F5'-TTGCTTGGATACTGTGCTTC-3' R5'-TGTCGTTATCGATGGTCTTG-3'	186
Fructokinase (FK)	F5'-GTCCTGTACGCCATGGTTTT-3' R5'-TCGTATCAAATGCACCCTGA-3'	208
Hexokinase (HK)	F5'-ATCCACTCGCAGCTCAAGAT-3' R5'-GCAGCAGCAATGAAATCAAA-3'	230
ADP-Glucose Pyrophosphorylase (ADP-G-PP-)	F5'-AGAACGCAACCATCAAGGAC-3' R5'-GCTAGCTACTGGACCGATGC-3'	237

clones showed transcript expression for all the genes studied. Interestingly, the transcript level of SPS was higher in *Saccharum* spp. than in the commercial sugarcane hybrids. The expression of SPS was found to be higher in high sugar commercial sugarcane hybrids than in low sugar commercial sugarcane hybrids. In contrast, all the *Saccharum* spp. showed low transcript levels of SPS in the high sugar clones when compared to the low sugar clones. But such a pattern of gene expression was not observed for any other genes in *Saccharum* spp. in our present study.

Expression of SPP was observed in all the species of *Saccharum* although no differentiation could be derived between the high and low sugar clones. But in the commercial sugarcane hybrids, there was clear differentiation in the transcript levels between the high and low sugar genotypes (Fig. 1). SPP is the last enzyme that pulls the reaction catalyzed by SPS in the direction of net sucrose synthesis. Earlier reports suggest that the enzyme is unlikely to make a significant contribution to the control of sucrose biosynthesis (Hawker and Smith 1984). However, recent studies suggest that SPP could contribute to control of the flux through the pathway of sucrose synthesis. Recent evidence that SPP and SPS could exist as a complex has added further interest to the possible role of SPP in the regulation of sucrose synthesis (Echeverria et al. 1997; Lunn et al. 2000). Similar to SPP, differential expression of SAI, CWI, SUT1 and SUT4 was observed between high and low sugar commercial sugarcane hybrids. Among the *Saccharum* spp., high transcript levels of SAI were observed in low sugar *S. robustum* clone and in both high and low sugar *S. officinarum* clones (Fig.1). Expression of CWI in *Saccharum* spp. was not in accordance with the high and low sugar types. High level of SUT1 transcripts were found both in high and low sugar *S. robustum* clones, low sugar *S. officinarum* and low sugar *S. spontaneum* clones. Expression of SUT1 in *S. sinense* was similar to that of the commercial sugarcane hybrids whereas transcript level was not detected in *S. barberi*. SUT4 expression was uniform in all the *Saccharum* spp. except in high sugar *S. spontaneum* and low sugar *S. barberi* where low expression of SUT4 was observed (Fig.1). There are reports of coordinated expression of a monosaccharide transporter gene along with a concomitant expression of an apoplastic invertase (Sauer and Stadler 1993; Ehness and Roitsch 1997).

We do not know if such a mechanism operates in sugarcane where high level of simultaneous expression of sucrose transporters SUT1, SUT4 and SAI is found in the high sugar genotypes.

Similarly, PFP, FK and HK transcripts were also differentially expressed in commercial sugarcane hybrids; however no definite pattern was observed in *Saccharum* spp. In commercial sugarcane hybrids, these three genes exhibited similar expression patterns, with the highest expression levels of each gene present in high sugar genotypes as compared to low sugar genotypes (Fig. 2). Earlier reports, however, suggest that in sugarcane the amount of PFP activity in internodal tissue is inversely correlated with sucrose (Huber and Akazawa 1986; Black et al. 1987; ap Rees et al. 1988). In contrast, the findings of Wong et al. (1988, 1990) suggested that PFP might favour sucrose accumulation by maintaining substrate levels for sucrose synthesis and our results for PFP expression in the commercial sugarcane hybrids agree with these findings. *Saccharum spontaneum, S. robustum* and commercial sugarcane hybrids showed similar pattern of expression for FK gene while *S. officinarum*, *S. sinense* and *S. barberi* showed a reverse trend in expression pattern. Transcript expression of ADP-G was found only in the high sugar *S. officinarum* clone while rest of the *Saccharum* spp. and commercial sugarcane hybrids showed very low or below detectable level of transcript accumulation (Fig. 2). Expression of SuSy was uniform both in *Saccharum* spp. and commercial sugarcane hybrids. There was very little or no expression of PFK in commercial sugarcane hybrids and in *Saccharum* spp. (data not shown). The low expression of PFK might be due to the high expression of SPS and other sucrose biosynthetic enzymes leading to the diversion of hexose phosphate pool into sucrose biosynthesis. There was also very little or no expression of VAI and NI in both commercial sugarcane hybrids and in *Saccharum* spp. (data not shown).

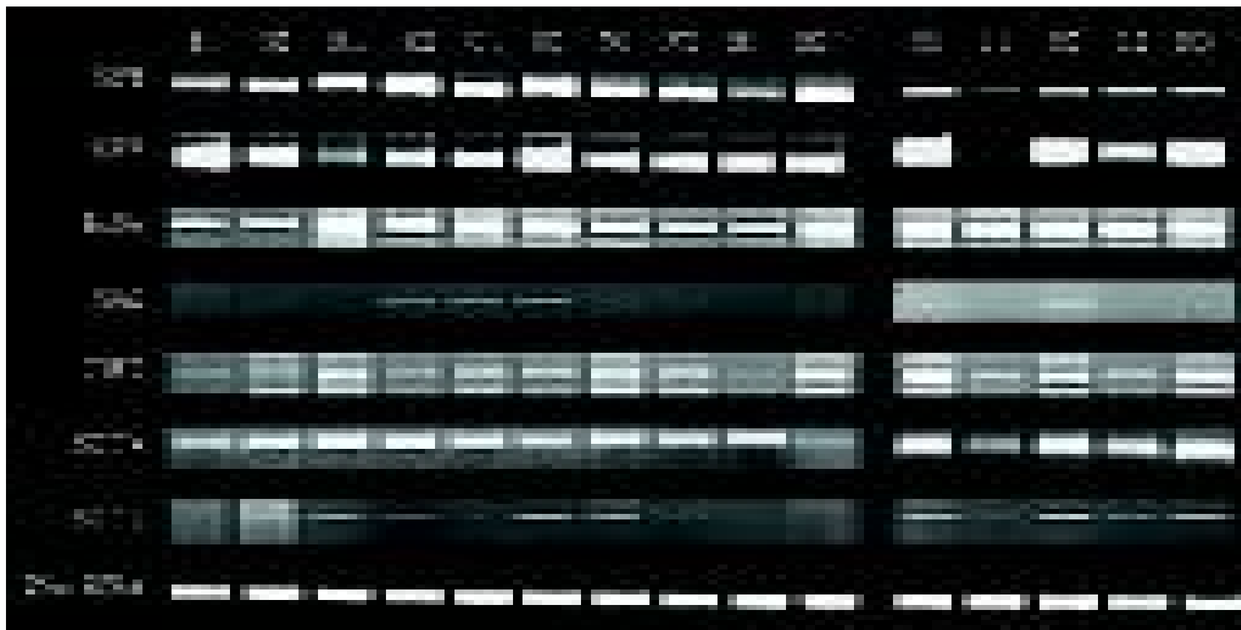

Fig. 1 Transcript expression pattern of sucrose metabolizing genes in different *Saccharum spp* and commercial sugarcane hybrids using RT-PCR. Lane S1. High sugar *S. spontaneum*; Lane S2. Low sugar *S. spontaneum*; lane R1. High sugar *S. robustum*; Lane R2. Low sugar *S. robustum*; Lane O1. High sugar *S. officinarum*; Lane O2. Low sugar *S. officinarum*; Lane N1. High sugar *S. sinense*; Lane N2. Low sugar *S. sinense*; Lane B1. High sugar *S. barberi*; Lane B2. Low sugar *S. barberi*; Lane H1. High sugar CoC 671; Lane L1. Low sugar MS 68/47; Lane H2. High sugar Co 86032; Lane L2. Low sugar Co 62175; Lane H3. High sugar Co 99006. Name of sucrose metabolizing genes along with 25s rRNA gene (Positive control) are indicated in the left.

In general, most of these genes showed strikingly higher expression in high sugar genotypes as compared to the low sugar genotypes of the commercial sugarcane hybrids whereas this pattern was not observed in *Saccharum* spp. Interestingly, SAI transcripts were observed in a *S. robustum*

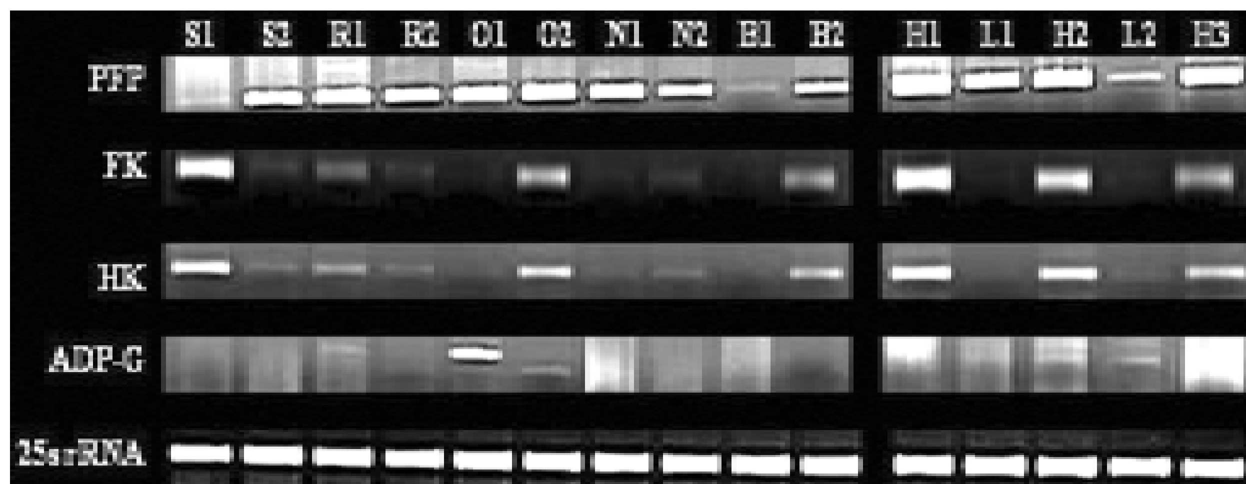

Fig. 2 Transcript expression pattern of sucrose metabolizing genes in different *Saccharum spp* and commercial sugarcane hybrids using RT-PCR. Lane S1. High sugar *S. spontaneum*; Lane S2. Low sugar *S. spontaneum*; laner R1. High sugar *S. robustum*; Lane R2. Low sugar *S. robustum*; Lane O1. High sugar *S. officinarum*; Lane O2. Low sugar *S. officinarum*; Lane N1. High sugar *S. sinense*; Lane N2. Low sugar *S. sinense*; Lane B1. High sugar *S. barberi*; Lane B2. Low sugar *S. barberi*; Lane H1. High sugar CoC 671; Lane L1. Low sugar MS 6847; Lane H2. High sugar Co 86032; Lane L2. Low sugar Co 62175; Lane H3. High sugar Co 99006. Name of sucrose metabolizing genes along with 25s rRNA gene (Positive control) are indicated in the left.

clone and in both *S. officinarum* clones whereas in commercial sugarcane hybrids SAI was highly expressed in high sugar genotypes compared to the low sugar genotypes. As reported in other studies, SAI did not show gradual decline in the expression of transcript as the cane matured (Verma et al. 2010). SAI activity is required for internode elongation and increased source activity. The authors wish to add that this result was further confirmed through additional experiments where SAI expression was studied at various developmental stages (data unpublished). The possible physiological roles of acid invertase and the function of this enzyme in sink organ development must now be reconsidered. It could be assumed that other genes like PFP, HK and FK also regulate sucrose metabolism and are likely to be involved in the sugar sensing mechanism in plants (Wong et al. 1990; Pego and Smeekens 2000). However, there could be regulation of these genes at the protein level through reduced translation of these gene transcripts that needs detailed studies.

Conclusion

In summary, we have described the expression pattern of several genes related to sucrose metabolism in sugarcane. This is the first report which involves simultaneous studies on differential expression of all the key genes involved in sucrose metabolism of high and low sugar *Saccharum* spp. and commercial sugarcane hybrids. The occurrence of multiple genes that are differentially regulated between the high and low sugar genotypes, apart from the previously well established genes like SPS, is shown in this study. Transcript levels of SPS, SPP and invertases (SAI and CWI), were found to be differentially regulated between high and low sugar commercial sugarcane hybrids. Similarly, genes like PFP, FK, HK, SUT1 and SUT4, whose roles are not clearly defined in the sucrose synthesis pathway, were also found to be differentially regulated between high and low sugar commercial sugarcane hybrids. However, there was no definite pattern of expression of all these genes in *Saccharum* spp. revealing the allelic diversity of sucrose metabolizing genes in the complex sugarcane genome. Therefore, expression profiling of genes could help in the identification of genes involved in the regulation of sucrose metabolism and provide valuable target genes for increasing the sucrose content of sugarcane through genetic manipulation.

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