THE SUCEST-FUN PROJECT: IDENTIFYING GENES THAT REGULATE SUCROSE CONTENT IN SUGARCANE PLANTS

By

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Abstract

MODERN sugarcane cultivars are complex hybrids resulting from crosses among several species of the *Saccharum* genus. Traditional breeding methods have been extensively employed in different countries over past decades to develop varieties with increased sucrose yield, and resistance to pests and diseases. Conventional variety improvement, however, may be limited by the narrow pool of suitable genes. In this sense, molecular genetics is seen as a promising tool to assist in the process of developing improved varieties. The SUCEST-FUN Project (http://sucest-fun.org) aims to associate function to sugarcane genes using a variety of tools, in particular through the study of the sugarcane transcriptome. We identified 238 genes associated with sucrose content in sugarcane plants. The genes were found to be differentially expressed when high sucrose and low sucrose plants or populations were compared and when high and low sucrose internodes were compared. Gene expression data were obtained using cDNA microarray and quantitative PCR technologies. The genes identified can potentially be useful in the identification, characterisation and development of plants with increased sucrose content.

Introduction

Sugarcane is an important tropical crop that has served as a main source of sugar for hundreds of years. Sucrose synthesised in sugarcane leaves is translocated from the leaves and stored in the culms. The striking ability of sugarcane to accumulate high levels of sucrose, which can reach 0.7 M in mature internodes (Moore, 1995) makes sugarcane an interesting model for studies on sugar synthesis, transport and accumulation.

The large sugarcane genome, in which every gene may be represented by an average of ten alleles, represents a challenge for genetic analysis. 'Expressed Sequence Tags' (EST) collections may therefore contribute significantly in the identification of candidate genes associated with

agronomical traits of interest (eg, tolerance to abiotic and biotic stresses, mineral nutrition, sugar content, among others).

Within this perspective, an EST collection was developed by the Brazilian sugarcane EST project (SUCEST; http://sucest.lbi.ic.unicamp.br/public/; Vettore *et al*., 2003). A total of 237 954 high quality ESTs were obtained from 27 cDNA libraries representing several organs, developmental stages, temperature stress and endophytic nitrogen fixing bacteria interactions for four cultivars. This collection of ESTs was assembled into 43 141 putative, unique sugarcane transcripts that are referred to as the Sugarcane Assembled Sequences (SAS).

Following sequence identification, a functional genomics project (The SUCEST-FUN Project) was implemented to associate putative biological functions to the sugarcane genes. cDNA microarrays containing sugarcane ESTs have been used to determine temporal and spatial gene expression data. Determining the distribution of gene transcripts in sugarcane tissues has helped in defining tissue-specific activities and ubiquitous genes. Using cDNA microarrays containing 1280 distinct elements, individual gene expression variation of plants grown in the field and transcript abundance in six plant organs (flowers, roots, leaves, lateral buds, 1st (immature) and 4th (mature) internodes were analysed (Papini-Terzi *et al*., 2005).

The expression of 217 genes was found to be tissue-enriched while 153 genes had expression levels highly similar in all tissues analysed. A virtual profile matrix was constructed where tissue expression levels were compared among 24 tissue samples. cDNA microarrays were also used to profile expression of 1545 genes in plants subjected to drought, phosphate starvation, herbivory, N_2-fixing endophytic bacteria and phytohormones (abscisic acid and methyl jasmonate). Adopting an outliers searching method, 179 genes with strikingly different expression levels were identified as differentially expressed in at least one of the treatments analysed (Rocha *et al*., 2007). Most of the genes characterised code for signal transduction components, hormone biosynthesis, transcription factors, and stress and pathogen response-related genes.

A catalogue of sugarcane signal transduction and other regulatory genes can be found at the SUCAST Database (http://sucest-fun.org). The data consolidated in the SUCAST database resource can guide further studies and be useful for the development of improved sugarcane varieties.

In recent years, sugarcane has become a very popular crop because its products ethanol and bagasse are important renewable biofuels. Sugarcane cultivars with increased sucrose content are highly desirable, and knowledge of the gene functions that may regulate sucrose synthesis and accumulation can advance research in this area.

Using cDNA arrays described above, we identified genes associated with sucrose content. The genes identified can be used to distinguish and characterise superior varieties or develop new varieties with increased sucrose content.

Methods

Progenies

Progeny 1 was derived from two intra-specific polycrosses, one among *Saccharum officinarum* genotypes and the other combining *Saccharum spontaneum* genotypes. For each generation, 500 individuals were sampled for brix content and gene expression. The extreme segregants were selected for further analysis.

The *F3* hybrid individuals selected for molecular studies were planted in a field in single rows of 5 m using standard sugarcane cultivation practices. Brix readings and tissue samples were collected very early in the season, and in March of the following year, when plants were 10 months old. The soluble solids (brix) content of mature internodes of each sugarcane stalk was measured with a portable refractometer (N1 model, ATAGO, Japan). Individuals or pools of eight individuals had their tissues collected and RNA extracted.

In the case of progeny 2, five hundred sugarcane *F1* plants from a cross between two commercial varieties (SP80-180 x SP80-4966) were field-grown. The stem sugar content from the different plants showed a normal distribution, and seven plants with extreme brix values were selected. Mature (In9), intermediate (In5) and immature internodes (In1) were collected from the selected plants 7 and 11 months after planting.

A total of 132 biological replicates were analysed.

Gene expression data

cDNA microarray experiments were conducted and data extracted essentially as previously reported (Papini-Terzi *et al*., 2005). The designed microarray contains 1830 genes which yielded 1545 good-quality PCR fragments. Reverse transcription, labelling and hybridisations were done using the reagents provided with the CyScribe Post-Labeling kit (GE Healthcare). The microarrays were scanned according to the manufacturer's instructions using the Generation III System (Molecular Dynamics). Hybridisations were carried out as depicted in Table 1.

Table 1—cDNA microarray hybridisations used to compare (I) internode expression profiles from high and low brix progenies and (II) high and low brix internode tissues.

	Hybridisations	
	Cy3	Cy5
(1) Comparison of high and low brix plants		
S. spontaneum vs *S. officinarum* progenies 10-month-old plants	In1-high In1-low In5-high In5-low In9-high In9-low	In1-low In1-high In5-low In5-high In9-low In9-high
SP80-180 vs *SP80-4966 progenies* 7-month-old plants	In1-high In1-low In5-high In5-low In9-high In9-low	In1-low In1-high In5-low In5-high In9-low In9-high
SP80-180 vs *SP80-4966 progenies* 11-month-old plants	In1-high In1-low In5-high In5-low In9-high In9-low	In1-low In1-high In5-low In5-high In9-low In9-high
(II) Comparison of high and low brix internodes		
SP80-180 vs *SP80-4966 progenies* 7-month-old plants	In1-high In9-high Inl-low In9-low	In9-high Inl-high In9-low Inl-low
SP80-180 vs *SP80-4966 progenies* 11-month-old plants	In1-high In9-high Inl-low In9-low	In9-high In1-high In9-low Inl-low

Two technical replicates were performed for each microarray experiment. Data were collected using the ArrayVision (Imaging Research Inc.) software. The fluorescence ratios were normalised in the MxS space, where M is the base 2 logarithm of the intensities ratio and S is the

base 2 logarithm of the average intensity of each spot. The M values were normalised to account for systematic errors using the LOWESS fitting. The identification of differentially expressed genes was performed using a local implementation of the HTself method (Vencio and Koide, 2005). The SAS presenting more than 70% of its replicates outside fold-change cut-off curves were defined as differentially expressed.

Validation of microarray results by real-time PCR (RT-PCR)

Real-time PCR reactions were done essentially as described (Papini-Terzi *et al*., 2005). The ratio between the relative amounts of the target gene and the endogenous control gene in the RT-PCR reactions was determined based on the Ct method (Livak and Schmittgen, 2001) with modifications.

The normalised expression level was calculated as $L = 2^{-\Delta Ct}$ and $\Delta C_T = C_{T,\,target} - C_{T,\,reference}$. A polyubiquitin gene (SCCCST2001G02.g) was used as an endogenous reference in the RT-PCR reactions after verification that its mRNA levels were similar in the populations and individuals tissues.

The significance of differential gene expression by RT-PCR was determined considering normal distributions for each tested condition and comparing them to the average result for all samples. For this aim, the probability value P of being greater or smaller than the average was calculated depending on whether the condition was respectively up- or down-regulated according to microarray evidence.

Results

cDNA microarrays were used to identify genes that were differentially expressed in two sugarcane populations contrasting in sugar content. A total of 1545 genes were expression profiled. The lines analysed are derived from multiple crossings among *S. officinarum* and *S. spontaneum* genotypes (progeny 1) and from progenies of crosses between commercial varieties that have been selected for sugar content for over 12–15 years (progeny 2).

Table 2 indicates the genotypes used in the polycrosses to obtain progeny 1. The parents of progeny 2 were the cultivars SP80-180 and SP80-4966.

Table 2—*S. officinarum* and *S. spontaneum* genotypes used for the polycrosses.

S. officinarum	*S. spontaneum*
Caiana Fita	IN8458
IK76108	IN8488
Lahaina	Krakatau
MZ151	SES 147b
MZ151 roxa	US56158
Sabura	US7440
Salangor	US851008
Sinimbu	UM721
NG213	UM691
Fiji 47	SES 194
Hinahina 18	IK7686
Manjri Red	US56193
Muntok Java	US571723
NG77142	
Soff 8268	
SS601	
Sylva	
NG2880	
Vae Vae Ula	
IJ76315	
IN8425	

Table 3 shows the brix values of each of the individuals from progeny 1 and 2 selected for this study.

Table 3—Brix values of 8 individuals with the highest and lowest sucrose content of an F3 population originated from crosses between *S. officinarum and S. spontaneum.*

Progeny I genotypes		Progeny 2 genotypes	
CTC98-241	18.00	C158	18.3
CTC98-242	18.60	C121	18.8
CTC98-243	19.20	C171	16.8
CTC98-244	14.60	C496	17
CTC98-246	18.80	C 11	19.2
CTC98-252	18.00	C6	18.2
CTC98-253	19.60	C1 13	21
CTC98-258	18.00		
Average high brix 18.10 +/- 1.44		Average high brix 18.47 +i- 1.41	

CTC98-261	7.00	C436	13.9
CTC98-262	7.40	C292	15
CTC98-265	6.40	C231	13.9
CTC98-268	4.80	C38	12.9
CTC98-271	6.00	C250	*11.5*
CTC98-272	6.80	C405	15.2
CTC98-277	7.40	C144	13.2
CTC98-279	7.80		
Average low brix 6.70 +/- 0.96		Average low brix 13.65 +I- 1.27	

Sucrose accumulating tissues, also known as sink tissues (herein internodes) were collected from field grown plants. Samples were collected from single individuals and pools of 7 or 8 plant lines after 7 to 11 months after planting. Two designs were used to perform transcriptome comparisons for the identification of genes differentially expressed when High Sugar and Low Sugar plants were directly compared (I) or High Sugar and Low Sugar internodes were compared (II) (Table 1).

Using hybridisation design I, 125 genes were found to be differentially expressed in at least one comparison between high and low brix genotypes (internode 1, 5 or 9), and 10 were found to be differentially expressed in both progenies analysed. Using hybridisation scheme II a total of 171 genes were found to be differentially expressed when mature and immature internodes were compared in high and low brix individuals.

Overall, 238 unique genes were identified as differentially expressed when samples with contrasting sucrose content were compared using either hybridisation design. Table 4 lists the categories and number of unique SAS identified and Figure 1 summarises the number of SAS identified differentially expressed in all experiments. Eight SAS had contrasting expression levels in all the hybridisations made. Among the genes whose expression was found to be associated with brix content, 58 displayed altered expression during culm maturation.

The purpose of performing crosses in breeding programs is to generate genotypic variation among the individuals of each progeny. The parents of each progeny were genotypically quite diverse. Therefore it was not surprising to find that most of the differentially expressed genes were found to be expressed differentially in only one of the progenies.

An interesting finding was that, in both progenies, approximately half of the genes differentially expressed in high and low brix plant comparisons were found to have their expression modulated during internode development. This relationship may be an additional indication of a role for these genes in sucrose accumulation.

Table 4—Number of Unique SAS identified as differentially expressed across all experiments and corresponding categories.

Category	Unique SAS
Adapter	4
Amino acid metabolism	1
Calcium metabolism	10
Carbohydrate metabolism	3
Development	4
DNA metabolism	3
Hormone biosynthesis	11
Hormone related	2
Inositol metabolism	3
Lipid metabolism	2
No match	12
Nucleotide metabolism	1
Other	12
Pathogenicity	13
Protein kinase	29
Protein metabolism	2
Protein phosphatase	4
Receptor	21
RNA metabolism	1
Small gtpase	3
Stress	42
Transcription factor	26
Transport	1
Two component	1
Ubiquitination	11
Unknown protein	16
Total	238

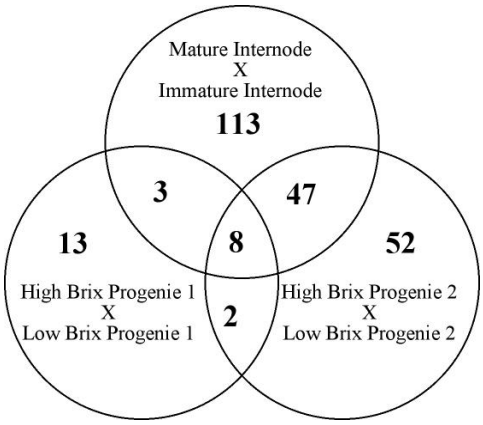

Fig. 1—Venn diagram indicating the number of SAS identified as differentially expressed in comparisons made between progenies and development samples.

We searched for expression patterns that were up or down regulated in at least two different biological samples to highlight biological replication and take advantage from the fact that we had profiled the transcriptome of 13 pools of biological samples. From 122 SAS that fit this criterion, 21 SAS were differentially expressed in at least two biological samples derived from the high and low brix plant comparisons and 3 in at least three samples. When the mature and immature internode samples were compared, 51 SAS were found differentially expressed in two samples, 13 in three

samples and 11 in four samples. The differentially expressed genes that were identified belong to several functional categories including calcium signalling, stress responses, transcription and ubiquitination. The categories with the highest number of hits include protein kinases from the SNF-related family of kinases, auxin hormone signalling, the Cyp family of cytochrome P450 monoxigenases and other stress-related genes.

Differential expression of more than 30 genes was also investigated by real-time PCR in the samples corresponding to pools of individuals and the individual lines themselves. For the pools, the validation rate was 70% on average while for individuals it was around 65%. This result confirms that a pool of 8 individuals can be made to determine gene expression between progeny populations.

The significance of the gene expression differences was inferred by calculating the *P* value of each expression level against the average of expression levels for the gene in all genotypes analysed. Table 5 lists the *P* values obtained in real-time PCR reactions for 5 SAS identified as differentially expressed when internode 9 tissues from high and low brix plants were compared using cDNA microarrays. The median *P* value was 0.89 across eight genotypes thus confirming the usefulness of these expression patterns as molecular markers of sucrose content. The analysis also indicated several genotypes that consistently presented contrasting levels of expression for the genes (CTC98-241, CTC98-253, CTC98-261 and CTC98-265). Specifically, it is interesting to note that the most significant markers found belong to the same family of aquaporins. Each high brix genotype had reduced expression of at least one of them (with a *P* value 1.0 for three of them). Contrarily, each low brix genotype had higher expression of at least one aquaporin (with a *P* value 1.0 for three of them). This indicates a potential use of one or a combination of these genes as molecular markers of sucrose content for the identification of high sugar progeny in crosses from breeding programs.

Table 5—Validation of cDNA microarray gene expression data in Internode 9 samples by real-time PCR. Normalised gene expression levels were calculated and the *P* values were determined for each expression pattern in reference to the average expression level across all genotypes. The five SAS were expected to be less expressed in high brix (HB) plants in relation to low brix plants (LB). *P* values equal to 1.0 are indicative of a high probability of a gene expression difference. In HB genotypes, a *P*=1 indicates that the expression levels are down regulated relative to the average expression level. In LB, genotypes a *P*=1 indicates that the expression levels are up regulated relative to the average expression level. Lower *P* values are progressively indicative of less reliable differences. We considered a gene to be validated when *P* = 0.9.

Putative Gene Function		Differential expression	p value Down in HB				p value Down in HB				Average
category	sub category	ŻHBIn9Ż	241	243	246	253	261	265	272	279	
NAM	NAC	down	1.00	0.01	0.14	1.00	1.00	1.00	1.00	0.00	0.64
cytochrome P450	CYP74A	down	1.00	1.00	0.00	1.00	1.00	0.00	0.00	nd	0.57
drought and cold response	aquaporin	down	0.92	1.00	0.96	0.98	0.83	1.00	1.00	0.63	0.92
drought and cold response	aquaporin	down	0.00	0.81	1.00	1.00	0.00	1.00	1.00	1.00	0.73
ARC1 (arm repeat protein)	.	down	1.00	0.00	1.00	1.00	1.00	1.00	0.00	0.00	0.63
Median p value in genotype			1.00	0.81	0.96	1.00	1.00	1.00	1.00	0.32	0.89
% SAS validated in genotype			80.0	60.0	60.0	100.0	80.0	80.0	60.0	20.0	67.50

Discussion and conclusions

A strategy introduced by Jansen and Nap (2001), that involves large-scale analysis of gene expression in a segregating population, was used to identify genes associated with sucrose content. In an alternative approach, mature and immature culm samples were compared. Genes identified as developmentally regulated during culm maturation relate to hormone signalling (auxin, ethylene,

jasmonates, salicylic acid), stress responses, sugar transport, lignin biosynthesis and fibre content. Two members of the aquaporin family of transporters were identified as highly associated with sugar content. This large family of proteins is primarily involved in the regulation of water movement between cells and cell compartments, although many of them also facilitate the passage of small solutes (rev. Maurel and Chrispeels, 2001; Chaumont *et al.*, 2005).

The accumulation of sucrose in such high concentrations as seen in sugarcane cells certainly represents an osmotic challenge that demands efficient control of solute compartmentalisation. As key players in the equilibration of water potentials via regulation of membrane permeability, aquaporins may have a fundamental role in the process of sugar storage in sugarcane vacuoles.

It has been observed in *Arabidopsis* that loss of the aquaporin TIP1;1 severely affects carbohydrate metabolism and transport (Ma *et al.,* 2004), and the authors postulate that this aquaporin could be involved in a vesicle-based routing of carbohydrates towards the central vacuole.

Due to the diversity of roles described for the identified genes, additional experiments will be necessary to elucidate their possible roles in the sugarcane sucrose accumulation process. Our group is currently generating transgenic plants with modified expression levels for these genes to confirm the hypothesis raised about the function of those genes.

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LE PROJET SUCEST-FUN: IDENTIFICATION DES GÈNES REGULATEURS POUR LA TENEUR EN SACCHAROSE CHEZ LA CANNE À SUCRE

Par

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MOTS CLÉS: Canne à Sucre, Génomiques Fonctionelles, Analyse du Transcriptome, Saccharose.

Résumé

LES VARIÉTÉS modernes de canne à sucre sont des hybrides complexes provenant des croisements parmi plusieurs espèces du genre *Saccharum*. L'amélioration variétale traditionnelle a été largement employée pendants les dernières décennies dans différents pays pour développer des variétés plus riches en saccharose et résistantes aux ravageurs et aux maladies. L'amélioration conventionnelle de la canne à sucre peut être cependant limitée par le patrimoine restreint de gènes appropriés. Cependant, la génétique moléculaire est vue comme un outil prometteur pour le développement de nouvelles variétés. Le projet SUCEST-FUN (http://sucest-fun.org) vise à associer des fonctions à des gènes de canne à sucre en utilisant une variété d'outils, mais principalement par l'analyse du transcriptome de la canne à sucre. Nous avons identifié 238 gènes liés à la teneur en saccharose chez la canne. Ces gènes s'expriment différemment chez les plantes ou dans les populations avec une teneur en saccharose soit forte ou faible et aussi dans les entre-nœuds avec une forte ou faible teneur en saccharose. Les données d'expression du gène ont été obtenues par des analyses microarray d'ADNc et la PCR quantitative. Les gènes identifiés peuvent potentiellement être utiles pour l'identification, la caractérisation et le développement des variétés avec un meilleur rendement en saccharose.

EL PROYECTO SUCEST-FUN: IDENTIFICANDO GENES QUE REGULAN EL CONTENIDO DE SACAROSA EN PLANTAS DE CAÑA DE AZÚCAR

Por

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PALABRAS CLAVE: Caña de Azúcar, Genómica Funcional, Transcriptoma, Sacarosa.

LOS CULTIVARES modernos de caña de azúcar son híbridos complejos producto de cruzamientos entre varias especies del género *Saccharum.* En varios países, se han empleado métodos tradicionales de mejoramiento durante décadas, con el fin de desarrollar variedades con incremento en su producción de sacarosa, y variedades resistentes a plagas y enfermedades. El mejoramiento varietal tradicional, puede ser limitado debido a la estrechez de la base genética y al poco número de genes deseables disponibles. En este sentido, la genética molecular es vista como una herramienta prometedora para asistir y colaborar en el desarrollo de nuevas variedades mejoradas. El Proyecto SUCEST-FUN (http://sucest-fun.org) busca asociar los genes de caña de azúcar con su función usando diversas herramientas, especialmente a través del estudio del transcriptoma de la caña de azúcar. Hemos identificado 238 genes asociados con contenido de sacarosa en plantas de caña de azúcar. Estos genes se expresan diferencialmente cuando se comparan poblaciones de alta y baja sacarosa. Igualmente sucede cuando se comparan entrenudos con alta y baja sacarosa. Los datos de expresión de estos genes se obtuvieron mediante microarreglos de ADNc y PCR cuantitativo. Los genes identificados pueden ser útiles en la identificación, caracterización y desarrollo de plantas con alto contenido de sacarosa.