

THE SUGARCANE METABOLOME

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Abstract

Despite having been studied for many years, there is still a significant lack of understanding of the mechanisms that govern the regulation of sucrose accumulation in sugarcane. Although the expression and activity of all the enzymes directly involved in the system have been described, crucial questions have been left unanswered. In the past few years the study of the regulation of plant metabolism has expanded to include metabolomics. In the same way that genomics studied the differential expression of genes and their potential interactions, metabolomics examines expression profiles on the metabolite level. Changing steady state levels of metabolites could be an indication of points of control in a specific pathway. This work describes the first phase of setting up GC-MS technology that would allow the separation and identification of multiple metabolites. Already single runs allow the identification of approximately 30 metabolites, which exceeds all previous analysis of sugarcane tissue. This method is sensitive enough to allow quantification and identification of metabolites that may previously have been overlooked in similar studies, including less abundant amino and organic acids, as well as sugars which could play a role in signaling (e.g. trehalose). This data would allow comparison of metabolite levels that would lead to the identification of metabolic control points. Current work is focused on comparing metabolite levels between low and high sucrose storing sugarcane varieties.

Keywords: sugarcane, metabolomics, regulation of metabolism, sucrose accumulation

Introduction

Understanding the mechanisms of sucrose accumulation in sugarcane is crucial to streamlining breeding programmes, whether by traditional breeding methods or genetic manipulation. The processes of sucrose metabolism have been studied for decades, focussing mainly on the characterisation of sugar levels and the enzymes directly involved in the production of sucrose, in an attempt to identify the key points of control in the system (see Moore and Maretzki, 1997 for review). The kinetic model of Rohwer and Botha (2001), using parameters gained from previous studies, has taken us a step further to an integrated understanding of sucrose production in the mature sugarcane culm. Although this model has the ability to predict important points of control there are many assumptions that lie in its groundwork, including steady state levels of many metabolites that are not directly linked to sucrose, but rather could potentially affect its levels by regulation of the enzymes involved in carbon partitioning.

To date there is only one published report of metabolite levels other than sucrose, glucose and fructose in sugarcane culm tissues (Whittaker and Botha, 1997).

This publication includes levels of glycolytic intermediates, but makes no reference to organic acids, amino acids, sugar alcohols or other low abundance sugars. There are two chief reasons for the lack of sugarcane metabolite data. Firstly, much of the research investigating the regulation of sugar metabolism has been focussed on differential gene expression. Secondly, the technology available for the determination of metabolites was severely limiting in its sensitivity and labour intensity.

Recently, the value of investigating the metabolome (the full complement of metabolites in a specific tissue) as a tool to understanding metabolic regulation has come to the fore. This has been aided by the advances in technology that allow simultaneous measurement of multiple metabolite levels (up to 160 compounds in potato) (Roessner *et al.*, 2000) in one tissue within an hour. The employed combined method of gas chromatography-mass spectrometry (GCMS) is a powerful tool to separate extracted metabolites of varying types and positively identify them, on the basis of both retention time and mass spectral matching.

In this study, the method for extraction and identification of over 30 metabolites in sugarcane has been optimised. The metabolome description of sugarcane varieties could give an insight into the potential control of differential expression patterns of both the genes and the proteins that lead to their inconsistent abilities to accumulate sucrose.

Materials and Methods

Sugarcane varieties N19 and US6656-15 were grown in pots for 12 months at Stellenbosch, in the Western Cape province of South Africa. Canes were harvested, and internodes three and seven ground in liquid nitrogen and stored at -80°C until extraction.

Metabolite extraction and derivitisation

Metabolites were extracted using a modified method of Roessner *et al.* (2000). Approximately 50 mg of powdered tissue and 39.4 μg internal standard (ribitol) were homogenised in 300 μl ice-cold methanol, 150 μl 150 mM Tris (pH 8.1) and 230 μl chloroform. The homogenate was incubated at 4°C for an hour, after which 350 μl water was added and the mixture vortexed. This was followed by centrifugation at 13 000 rpm for two minutes. The methanol/water phase was transferred to a new tube. The chloroform phase was re-extracted with 220 μl water. The mixture was again centrifuged at 13 000 rpm for two minutes and the water phase added to the previous methanol/water phase. All water soluble metabolites were thus extracted in a volume of 1020 μl . Aliquots were dried down under vacuum for derivitisation. An amount of 15 μl was dried down for high abundance metabolite analysis (e.g. sucrose, glucose and fructose), and 350 μl for metabolites occurring in low abundance (amino and organic acids, sugar phosphates, and low abundance sugars and sugar alcohols).

Samples were derivitised by methoxyamination and trimethylsilylation as in Roessner *et al.* (2000), with the exception that the time standards added were the alkanes dodecane, pentadecane, nonadecane, docosane, octacosane, dotriacontane and hexatriacontane to a final concentration of 23.8 ng/ μl in the derivitisation of each alkane.

GC-MS analysis

Samples were analysed on a ThermoFinnigan TRACE GCMS. One μl was injected in splitless mode. GC was performed in a 30 m Rtx-5Sil column (Restek).

The GC programme and MS analysis were carried out as in Roessner *et al.* (2000). Chromatograms and mass spectra were generated and processed using XCalibur software (ThermoFinnigan).

Results

Sugarcane variety N19 has the ability to accumulate more sucrose in maturing internode seven than variety US6656-15 (Figure 1). Less abundant sugars identified included xylose, trehalose, raffinose, maltose and the sugar alcohol inositol (Figure 1). Trehalose levels were highest in internode three of variety US6656-15. Organic acid concentrations were higher in young than maturing internode in both varieties (Table 1). Amino acids were most abundant in N19 internode three. Other metabolites identified from sugarcane tissues using GC-MS included glycerate3-phosphate, inorganic phosphate, fumarate, threonine, trans-hydroxy-proline, glycine, glutamine, tryptophan, methionine, asparagine and tyrosine (data not shown).

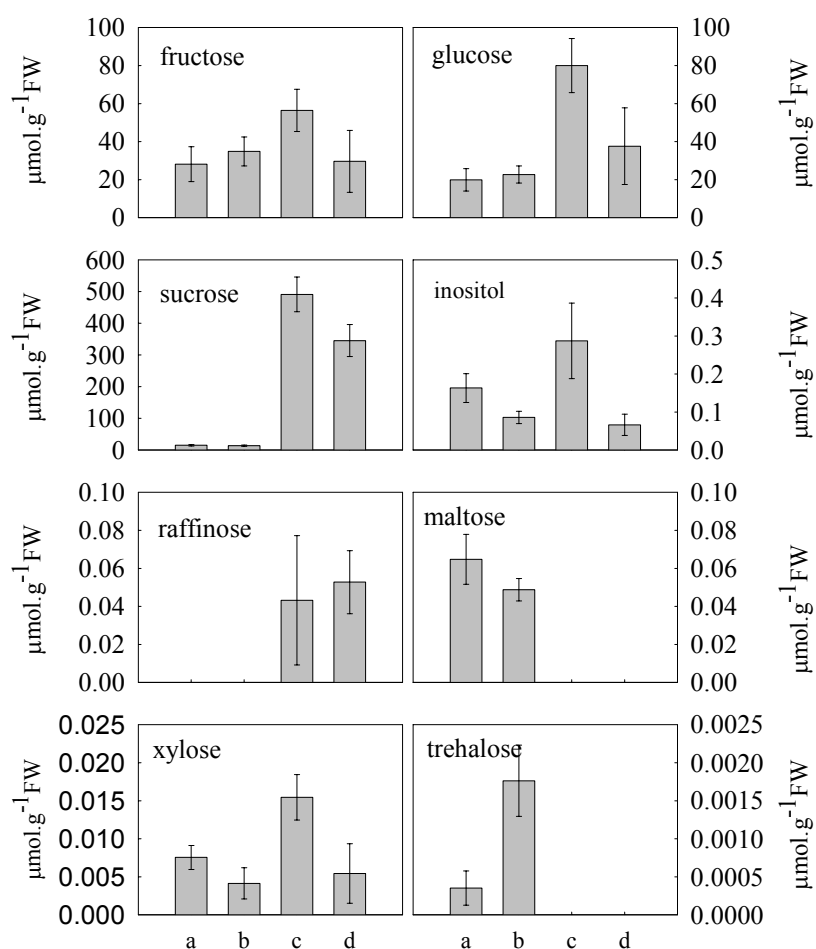


Figure 1. Sugar levels ($\mu\text{mol/g FW}$) in (a) N19 internode 3, (b) US6656-15 internode 3, (c) N19 internode 7, (d) US6656-15 internode 7. Values are the mean of three independent determinations \pm se.

Table 1. Organic acids, sugar phosphates and amino acids in internodes 3 and 7 of sugarcane varieties N19 and US6656-15. Values are the Response Ratio normalised by gFW \pm se (n=3). Compounds not detected in a specific tissue are designated nd.

Class	Compound	Response ratio/g FW			
		Internode 3		Internode 7	
		N19	US6656-15	N19	US6656-15
Organic acids	Aconitic acid	65748.1 \pm 14200.9	26170.9 \pm 2277.3	1521.5 \pm 469.6	172.4 \pm 91.4
	Malic acid	2873.6 \pm 882.7	1319.1 \pm 139.7	133.6 \pm 14.8	217.9 \pm 52.8
	Citric acid	609.9 \pm 229	135.9 \pm 37.4	165.0 \pm 84.3	1283.3 \pm 664.9
	Shikimic acid	590.5 \pm 15.6	1326.9 \pm 246	62.2 \pm 10.1	151.8 \pm 40.5
	Succinic acid	18.2 \pm 4.6	nd	nd	nd
Sugar phosphates	Fructose 6-P	141.9 \pm 17.7	73.2 \pm 21.2	17.6 \pm 2.6	nd
	Glucose 6-P	113.8 \pm 28.3	49.3 \pm 8.5	nd	nd
Amino acids	Glutamic acid	730.3 \pm 364.3	75.2 \pm 30.1	nd	nd
	Serine	192.9 \pm 52.9	81.6 \pm 14.9	39.2 \pm 12.6	nd
	Valine	112.3 \pm 44.7	nd	25.2 \pm 4.0	nd
	Leucine	78.9 \pm 38.8	nd	nd	nd
	Isoleucine	78.6 \pm 30.9	nd	nd	nd
	Phenylalanine	73.5 \pm 36.4	nd	nd	nd
	Alanine	23.9 \pm 11.1	nd	nd	nd

Discussion

From this preliminary comparison of metabolite levels in immature and maturing internodes of sugarcane varieties storing different amounts of sucrose, patterns are already emerging. Higher levels of amino and organic acids in immature internodes (Table 1) are an indication of the investment of metabolism in growth and respiration. Some interesting phenomena have emerged, including the high concentration of aconitic acid. Previously overlooked in sugarcane, this intermediate of the tricarboxylic acid cycle constitutes a significant portion of the organic acid pool. Apart from high abundance metabolites, the sugar trehalose showed higher levels in immature internodes than in more mature internodes. Trehalose and its phosphate derivative trehalose-6-phosphate have recently come under the spotlight as signalling molecules involved in carbon partitioning in plants (Eastmond *et al.*, 2003).

Much more work is required to gain a better understanding of the sugarcane metabolome and how it differs between varieties. The data gained from the application of this technology will significantly increase our understanding of carbon partitioning and sucrose accumulation in sugarcane.

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